SHEEP LIVER PHOSPHOFRUCTOKINASE:

A COMPARISON OF THE PRIMARY STRUCTURE WITH THOSE OF OTHER MAMMALIAN ISOZYMES

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ABSTRACT

Phosphofructokinase (PFK) is the key regulatory enzyme of glycolysis, catalysing the synthesis of fructose 1,6-bisphosphate from fructose 6-phosphate and ATP.

Several PFK isozymes have been identified from different tissues, including muscle, liver and platelet. Each isozyme is under the control of a different structural locus in humans the muscle isozyme gene is carried on chromosome 1, liver on chromosome 21, and platelet on chromosome 10.

The tetramer of PFK is the lowest active form, and there are both species and tissue differences in the composition of the tetramer. Some, such as human muscle PFK consist of four identical subunits (M_4), whereas others, such as human erythrocyte PFK comprise a five membered isozyme system, made up of both muscle and liver subunits.

The activity of PFK is modulated by a variety of effectors such as; ATP, fructose 6-phosphate, ADP, AMP and fructose 2,6-bisphosphate, as well as covalent modification, and hormonal regulation. Each PFK isozyme exhibits its own characteristic kinetic properties in response to changes in effector concentrations. This project aims to compare the primary structures of two PFK isozymes; liver and muscle, from a single species (sheep), in order to explain their different kinetic properties in terms of differences in their primary structures.

A purification procedure for sheep liver PFK was developed, and a 48% yield was obtained, with a final specific activity of 100Units/mg of protein.

Sodium dodecyl sulphate-gel electrophoresis indicated a protomer molecular weight of 84 000 for sheep heart muscle PFK, and 81 000 for sheep liver PFK, indicating that liver PFK is approximately 30 residues shorter than the muscle enzyme.

The comparison of the amino acid compositions showed a significantly lower arginine and lysine content in liver PFK compared to muscle PFK. There was also a lower threonine content in liver PFK, accompanied by an increase in the number of serine residues, compared to muscle PFK. So overall, the number of residues with

hydroxy-alkyl sidechains remained the same. The tyrosine content of liver PFK was also higher than that of muscle PFK.

The comparison of HPLC and FPLC peptide maps of liver and muscle PFK provided by digestion with CNBr and trypsin, showed a number of interesting differences between the two isozymes. Generally, the CNBr peptides of liver PFK appeared to be smaller than those of muscle PFK, while the radioactively labelled cysteine containing peptides from the tryptic digest of muscle PFK, were more hydrophobic than those from liver PFK.

Sequence information was obtained from purified peptides produced by digestion of liver PFK with CNBr, trypsin, and trypsin following maleylation of the sheep liver PFK with maleic anhydride to block the lysine residues. 87% of the amino acid sequence of sheep liver PFK was determined, and aligned with the sequences from rabbit muscle and sheep heart PFK. Approximately 70% sequence identity was observed between the liver and muscle isozymes, particularly in the regions where contacts to substrates and effectors are thought to be made. There are two regions exhibiting major sequence changes between the muscle and liver isozymes, both are thought to lie on the exterior of the molecule, and therefore would not disrupt the tertiary structure. One of these regions however, is thought to contain the ATP inhibtory site; and the large variation in sequence at this site may explain the greater susceptibility of the liver enzyme to ATP inhibition. Other, more subtle changes in the sequence may account for the additional differences in the kinetic properties displayed by muscle and liver PFK.

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LIST OF ABBREVIATIONS

ADP	adenosine 5'-diphosphate
Am Bic	ammonium bicarbonate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
βME	β-mercaptoethanol
Bs	Bacillus stearothermophilus
BSA	bovine serum albumin
Ca^{2+}-CaM	Ca^{2+}-calmodulin complex
CaM	calmodulin
cAMP	cyclic-AMP
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DTT	dithiothreitol
Ec	E. coli
EDTA	ethylenediamine tetraacetic acid
F6P	fructose 6-phosphate
F16BP	fructose 1,6-bisphosphate

F26BP	fructose 2,6-bisphosphate
FBPase	fructose bisphosphatase
F16BPase	fructose 1,6-bisphosphatase
F26BPase	fructose 2,6-bisphosphatase
FDNB	1-fluoro-2,4-dinitrobenzene
FPLC	fast protein liquid chromatography
α-GPD	α-glycerophosphate dehydrogenase
G1P	glucose 1-phosphate
G6P	glucose 6-phosphate
G16BP	glucose 1,6-bisphosphate
HPLC	high performance liquid chromatography
MLCK	myosin light chain kinase
NADH	nicotinamide adenine dinucleotide, reduced form
PEG	polyethylene glycol-6000
PEP	phosphoenolpyruvate
PFK	phosphofructokinase
PFK-2	phosphofructokinase-2
Pi	inorganic phosphate
PMSF	phenylmethylsulphonyl fluoride

POPOP	1,4-bis[2(5-phenyloxazolyl)]benzene
PPO	2,5-diphenyloxazole
RM	rabbit muscle
SDS	sodium dodecyl sulphate
SH	sheep heart
SL	sheep liver
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCA	tricarboxylic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tes	N-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid
TPCK	L-1-tosylamide-2-phenylethyl chloromethyl ketone
TPI	triose phosphate isomerase
Tris	tris (hydroxymethyl) aminomethane

The single letter amino acid code is used in accordance with the IUPAC recommendations.

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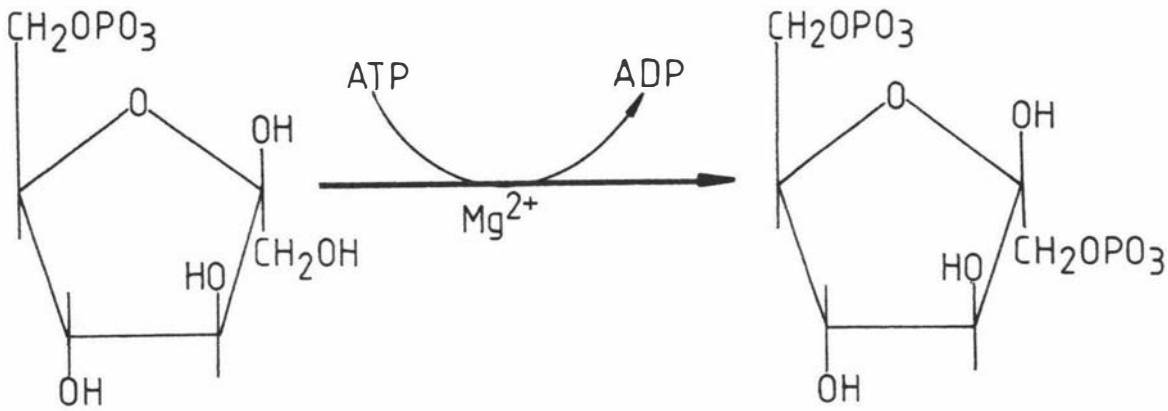
CHAPTER 1

INTRODUCTION

1.1 GENERAL BACKGROUND

Phosphofructokinase (ATP: D-fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11) catalyses the transfer of the terminal phosphate of ATP to the C-1 hydroxyl of fructose 6-phosphate (F6P), to produce fructose 1,6-bisphosphate (F16BP) and ADP, as shown below.



Fructose 6-phosphate Fructose 1,6-bisphosphate

After it was shown that the phosphorylation of F6P was an essential reaction of glycolysis in yeast (Harden and Young, 1908; Young, 1909; Harden, 1927) this reaction was discovered in red blood cells (Dische, 1935), and in muscle (Ostern et al., 1936). The first report of phosphofructokinase (PFK) as the possible regulatory enzyme of glycolysis occurred that same year (Negelein, 1936). In 1943, Engel'hardt and Sakov proposed that the "Pasteur effect" (the inhibition of glycolysis by oxygen) could be due to inactivation of PFK by oxygen. Aisenberg et al. in 1957 suggested that PFK was inhibited by an intermediate of oxidative phosphorylation, resulting in a

decreased level of F16BP when glucose was metabolised aerobically compared with anaerobically. The observation that ATP strongly inhibits muscle PFK, and that this inhibition may play an important regulatory role in carbohydrate metabolism was made by Lardy and Parks in 1956. Since this discovery, an enormous number of studies on PFK isolated from a variety of sources, have expanded the information available on the structure, function, catalytic and regulatory properties of PFK.

1.2 STRUCTURAL ASPECTS OF PROKARYOTE AND EUKARYOTE PHOSPHOFRUCTOKINASES

PFK has been isolated and studied from a variety of sources, including bacteria, yeast, plants, and animals. A common characteristic of the PFKs of different biological origin is their tetrameric structure. Although the molecular weights of the subunits differ widely, each individual subunit species is capable of undergoing self-association to a tetramer formation. While the subunit molecular weight of the bacterial PFK is about 35 000 (Blangy, 1968; Hengartner and Harris, 1975; Uyeda and Kurooka, 1970), the molecular weight of PFK subunits is about 100 000 in yeast (Kopperschläger et al., 1977), 80 000 in plants (Goldhammer and Paradies, 1979), and about 85 000 in mammals (Uyeda, 1979). While the smallest active form of PFK in mammals is the tetramer (Paetkau and Lardy, 1967; Hesterberg et al., 1981), the enzyme is capable of undergoing further aggregation to form active higher molecular weight oligomeric forms (Paetkau and Lardy, 1967; Uyeda, 1979). This is in contrast to the prokaryotic enzyme, which does not aggregate to forms higher than the tetramer (Goldhammer and Paradies, 1979). Yeast PFK has the structural peculiarity of two tetramers being paired in such a way that a stable octameric assembly, with a molecular weight of about 800 000 is formed (Kopperschläger et al., 1976, 1977). These octamers of the yeast enzyme do not associate further.

The reaction catalysed by PFK is regarded as the first unique step of the glycolytic pathway. It is not surprising therefore that the enzyme is stringently regulated by various metabolites in a manner that controls the rate of glycolysis in accord with the cell's need for energy, or glycolytic intermediates. In general, prokaryotic PFKs are controlled by a smaller number of effectors than PFKs from higher organisms.

Due to the ability of mammalian PFK to polymerise to higher molecular weights, to undergo covalent modification, and to be regulated by a number of metabolites and hormones, the study of mammalian PFK becomes a very complex and challenging

project. The understanding of PFK is further complicated by the expression of PFK isozymes in different tissues, which have distinct kinetic and regulatory properties.

1.3 PHOSPHOFRUCTOKINASE ISOZYMES IN MAMMALS

Lowry and Passonneau (1964), first speculated on the existence of isozymes of mammalian PFK on the basis of their kinetic studies of crude extracts from the various organs of the rat. The existence of an isozyme system for PFK in man was first suggested in 1965 based on the observation of a recessively inherited muscle disease associated with PFK deficency (Tarui et al., 1965). In affected individuals, PFK activity was entirely absent in muscle, but the activity in the erythrocytes was half that of normal erythrocytes. This differential tissue involvement led to the hypothesis that the erythrocyte isozyme was composed of two types of subunits, one of which was the sole subunit present in the muscle PFK (Layzer et al., 1967; Layzer and Conway, 1970). The proposed structural heterogeneity of erythrocyte PFK was supported by the work of a number of groups (Layzer et al., 1967, 1969; Tarui et al., 1969; Layzer and Conway, 1970; Lee, 1972; Karadsheh et al., 1977). Since then, evidence supporting the existence of further types of PFK and multiple molecular forms of the enzyme from a number of species, has been presented. The existence of hybrid structures composed of two or more different subunit types in some tissues has also been shown.

The most studied forms of mammalian PFKs are from the rat, rabbit and human, although some information on PFK isozymes from the mouse and guinea pig has also been obtained. Thus far, the evidence suggests the existence of at least three different subunit types of mammalian PFK; muscle type (M), liver type (L), and platelet type (P). These subunit types are under the control of three structural loci (Vora, 1981; Vora et al., 1985). In the human, the genes coding for the M, L and P subunits have been assigned to chromosomes 1 (Vora et al., 1982), 21 (Vora and Francke, 1981), and 10 respectively (Weil et al., 1980; Vora et al., 1983). These different forms of PFK are expressed to different degrees in the various organs, and differences in the distribution of the subunits between the tissues of different species have also been found to occur.

1.3.1 TISSUE DISTRIBUTION OF PHOSPHOFRUCTOKINASE ISOZYMES

Skeletal muscle PFK has been found to consist of a distinct homotetramer of M_4 in the rat (Kurata et al., 1972; Vora et al., 1985; Taylor and Bew, 1970), rabbit (Tsai and

Kemp, 1972; 1973), mouse and guinea pig (González et al., 1975) and human (Cottreau et al., 1979; Kahn et al., 1979; Vora et al., 1980). The M subunit also appears to be the sole constituent of heart muscle PFK in the rat (González et al., 1975), rabbit (Tsai and Kemp, 1973; González et al., 1975) and human (Cottreau et al., 1979). In the mouse and guinea pig however, heart PFK appears to be composed of small amounts of the L form, as well as the predominant M form (González et al., 1975).

Rabbit liver has been found to consist of the homotetramer L_4 (Tsai and Kemp, 1972; 1973; González et al., 1975). Human liver PFK however, contains not only the L_4 homotetramer, but also the P_4 homoteramer (Davidson et al., 1983). Rat liver PFK has been variably reported to exhibit one (Kurata et al., 1972), two (Taylor and Bew, 1970; Dunaway and Weber, 1974a; Hosey et al., 1980), three (Vora et al., 1985) or five isozymes (Kirby and Taylor, 1974), with the L_4 homotetramer always being the predominant species. The subunit composition of the other isozymes is still unclear, having been reported as consisting of P and L subunits (Vora et al.,1985) and M and L hybrids (Taylor and Bew, 1970; Dunaway, 1983). It is noteworthy that the guinea pig and mouse, closely related rodents, also exhibit two or three hybrid species in addition to the major L_4 isozyme in the liver (González et al., 1975).

Erythrocyte PFK in the rabbit and mouse were found to consist of a single isozyme species, L_4 in the case of rabbit erythrocyte PFK (Tsai and Kemp, 1973; González et al., 1975), and an undetermined species in the mouse (González et al., 1975). Human erythrocyte PFK has been shown to consist of a five membered isozyme system, resulting from the random polymerization of two non-identical subunits types; the M and L type, to form all possible tetramers i.e. M_4, M_3L, M_2L_2, ML_3, L_4 (Layzer et al., 1967; Layzer and Rasmussen, 1974; Karadsheh et al., 1977; Kaur and Layzer, 1977; Kahn et al., 1979; Vora et al., 1980). Hybrids were also found to exist in the guinea pig and mouse erythrocyte PFK, with the L_4 species being the predominant isozyme (González et al., 1975).

Brain PFK in the rabbit and human has been found to consist of all three subunit types. In the rabbit the P subunit is the predominant form, with the M present in slightly lesser amounts, and a small amount of L subunit (Foe and Kemp, 1984; 1985), however, in human brain the M subunit is predominant, with small amounts of the L and P subunits present (Cottreau et al., 1979; Kahn et al., 1979; Davidson et al., 1983). Rat brain PFK has been variably reported as consisting of solely the P_4 isozyme (Kurata et al., 1972; Vora et al., 1985), a hybrid with M the predominant subunit type (Taylor and

Bew, 1970; Kirby and Taylor, 1974; Dunaway and Kasten, 1985), or hybrids containing all three types of subunit, where the L type subunit predominates (Dunaway et al., 1984). It has been suggested that these differences in isozyme composition in the brain are the result of differences in the strains of rats studied (Vora et al., 1985).

Human platelet PFK is made up of P and L subunits, of which only the P_4, P_3L and P_2L_2 forms have been found to exist (Kahn et al., 1980; Vora, 1981). The isozyme distribution patterns for various other tissues such as lung, adipose tissue, placenta, kidney, stomach and malignant tissues have also been determined. Each of these consist of hybrids of the M, L and P subunit types (Tsai and Kemp, 1972; 1973; Kahn et al., 1979).

Khoja and Kellett (1983), have reported the purification of rat mucosal PFK, and have postulated that it is composed of another PFK isozyme, distinct from those of muscle, liver and platelet type subunits (Khoja, 1986).

1.3.2 DEVELOPMENTAL CHANGES

Several reports of the PFK levels in fetal tissues being greater than in adult tissues have been made for liver (Burch et al., 1963; Sydow, 1969), skeletal muscle and heart muscle (Thrasher et al., 1981). Futhermore, differences in the isozyme composition between fetal and adult tissues have been detected. In the rat, fetal liver has been found to contain both M and L subunits. Both isozymes decreased rapidly within 24hr after birth, and within a week the M subunit was no longer detectable, while the levels of the L subunit decreased to the adult values within two weeks (Dunaway, 1983). In rat fetal heart, L subunits have been found to be the predominant species, with a small amount of the M subunit present, while in adult heart the M:L ratio is 70:30. This has been found to be due to an increase in the levels of the M subunit during maturation (Thrasher et al., 1981) and leads to a subsequent increase in PFK activity. It has been suggested that the presence of the L subunit in fetal heart as well as other fetal tissues may be associated with the increased tolerance to anoxia exhibited by most fetal tissues, which promotes survival at parturition (Dunaway, 1983).

The above tissue distribution patterns show that although the same three basic PFK subunits appear to exist in each mammalian species studied, the extent to which each is expressed within the organs is dependent not only on the species but the strain and maturity of the tissue as well. The existence of further PFK isozymes can not be ruled out as yet.

1.3.3 SEPARATION OF PHOSPHOFRUCTOKINASE ISOZYMES

The isozymes of PFK can be separated by DEAE ion-exchange chromatography. In each species tested the M_4 isozyme is eluted first, as a single species, P_4 elutes shortly after M_4, followed much later by L_4 (Davidson et al., 1983; Oskam et al., 1985; Vora et al., 1985; Dunaway and Kasten, 1985). If hybrids of M and L, M and P, or P and L are present in the tissue extract, then the elution profile becomes more complicated. M and L, and P and L hybrids are eluted between the respective homotetramers, while hybrids of M and P subunits are not separately resolved, but are co-eluted as a single species between the M_4 and P_4 tetramers. When all three subunits are expressed by any cell or organ 8-10 hybrid species out of the 12 possible can be resolved (Vora et al., 1985).

Human PFK isozymes are generally less acidic than those from the rat, as consistantly higher salt concentrations were required to elute each of the three rat PFKs compared to their human counterparts (Oskam et al., 1985; Davidson et al., 1983; Vora et al., 1985).

PFK isozymes can also be separated by cellulose acetate electrophoresis. The L_4 isozyme migrates the fastest towards the anode, M_4 the slowest, and P_4 in between the M_4 and L_4 subunits. As with ion-exchange chromatography, hybrids run in between their respective homotetramers (Kemp, 1971; Kurata et al., 1972; Tsai and Kemp, 1972, 1973). Clearly some species differences in the skeletal muscle PFK subunits do exist, because when four different animal skeletal muscle PFKs were run on cellulose acetate under identical conditions the distance migrated decreased in the order rat>mouse>guinea pig>rabbit (González et al., 1975).

1.3.4 IMMUNOLOGY OF PHOSPHOFRUCTOKINASE ISOZYMES

Since a given antibody reacts with the respective subunit, whether contained within a homo or heterotetramer, evidence obtained from immunological studies on the existence of common structural features between the different PFK subunits is only valid when the PFK being tested, as well as the PFK against which the antibody was raised, have been conclusively shown to consist of homotetramers.

1.3.4.1 CROSS-REACTIVITY WITH HOMOTETRAMERS

As expected, antibodies raised against a homotetramer, completely precipitate the respective PFK isozyme (González et al., 1975; Vora et al., 1985). This holds true across species boundaries as well. For example, rabbit antibodies raised against human muscle PFK, will fully precipitate muscle PFK from the rat (Vora et al.,1985), likewise anti-liver PFK raised in guinea pigs against rabbit liver PFK will precipitate liver PFK from the rat and mouse (González et al., 1975).

Virtually no cross-reactivity exists between anti-muscle PFK and liver PFK (Tsai and Kemp, 1973), although some cross reaction occurs with platelet PFK (Vora et al., 1985), reflecting partial structural homology between the muscle and platelet type subunits (Vora et al., 1985).

Anti-liver antibodies are highly monospecific for liver PFK; no cross-reactivity occurs with either M_4 or P_4 PFKs (Oskam et al., 1985; Vora et al., 1985). These results indicate some degree of structural homology exists between the muscle and platelet subunits, but none is found between muscle and liver or liver and platelet subunits.

1.3.4.2 CROSS-REACTIVITY WITH HETEROTETRAMERS

Based on the above information on the cross-reactivity between subunits, it becomes possible to identify the constituent subunits of hybrid PFKs. Vora et al. (1985), have shown that the hybrid species (minor peaks on DEAE ion-exchange) in the rat liver most probably represent PL_3 and P_2L_2 hybrids, since anti-muscle antibody did not precipitate the rat liver enzyme. No cross reaction with the platelet subunits was detected, probably because the amount of precipitate was below the level of detection. The minor hepatic isozyme described by Dunaway et al (1974; 1978a) has been interpreted as being P_4 or hybrids of platelet and liver subunits by Vora et al. (1985) since the enzyme was only partially precipitated by anti-muscle and anti-liver antisera.

The use of antisera has also proved useful in showing isozyme distribution within different cells of a organ. Dunaway et al. (1978a) have shown that the major rat liver species (L_4) is found only in parenchymal cells, whereas the minor (P_4) species originates from the Kupffer (sinusoidal) cells.

1.3.5 STRUCTURAL PROPERTIES OF PHOSPHOFRUCTOKINASE ISOZYMES

1.3.5.1 SUBUNIT MOLECULAR WEIGHT

The molecular weights of the subunits of PFK isozymes from various species have been determined (Table I). The molecular weight values show that Platelet PFK has the highest molecular weight with a value of 86-87 500 . Muscle PFK is slightly smaller, at 84-85 000 , and Liver PFK is smaller still at 80 000.

The low value for liver PFK (Table I) reported by Dunaway and Weber (1974a) was possibly the result of proteolytic cleavage, since no protease inhibitor was used during the purification procedure.

Electron microscopy of rabbit muscle and pig liver PFK showed that the tetramer was composed of four individual subunits, 4 x 6 x 6nm in size, arranged in D_2 symmetry and forming a tetrameric structure 9nm in diameter by 14nm in length (Foe and Trujillo, 1980; Hesterberg et al.,1981). Dimers of PFK are constructed of two monomers lying side by side, and tetramers are composed of two dimers lying side by side in a square planar array. These tetramers are then capable of undergoing end to end association to form the long chains observed by Foe and Trujillo (1980).

1.3.5.2 AGGREGATION STATE

A general property of mammalian PFK is the tendency to self-associate to oligomeric forms higher than the active tetramer (Paetkau and Lardy, 1967; Layzer et al.,1969; Kemp, 1971; Tarui et al.,1972; Massey and Deal, 1973; Dunaway and Weber, 1974a; Brand and Söling, 1974; Trujillo and Deal, 1977; Reinhart and Lardy, 1980b; Foe and Kemp, 1985). This ability of PFK to aggregate has been exploited by several workers by using gel filtration in the purification of PFK (Brand and Söling, 1974; Reinhart and Lardy, 1980a). It is generally found that the liver enzyme self-associates to a greater extent than muscle PFK under comparable conditions (Kemp, 1971; Trujillo and Deal, 1977; Reinhart and Lardy, 1980b). Pig liver PFK has been shown to associate to a state larger than any previously studied PFK; 104S (Trujillo and Deal, 1977) compared to human erythrocyte 80S (Tarui et al.,1972), and sheep heart 54S (Mansour, 1966,1972), 7S, 30S (Brennan et al.,1974). The self-association properties of the rabbit brain isozyme (predominantly P_4) are quite different from those of the

TABLE I

SUBUNIT MOLECULAR WEIGHTS OF THE MUSCLE, LIVER, AND PLATELET ISOZYMES OF HUMAN, RAT AND RABBIT PHOSPHOFRUCTOKINASES.

	Muscle	Liver	Platelet	Ref
Human	85 000	80 000		(i)
	80 000			(ii)
	85 000	80 000		(iii)
			85 000	(iv)
Rat	85 000	80 000	87 500	(v)
	82 000	85 000		(vi)
	82 500	80 000	86 000	(vii)
		82 000		(viii)
		65 000		(ix)
Rabbit	84 000	80 000	86 000	(x)
	84 000			(xi)
		90 000		(xii)

Molecular weights of purified muscle, liver and platelet PFK isozymes from different species, as determined by SDS-PAGE.

(i) Karadsheh et al., 1977
(ii) Kaur and Layzer, 1977
(iii) Cottreau et al., 1979
(iv) Kahn et al., 1980
(v) Dunaway and Kasten, 1985
(vi) Kasten et al., 1983
(vii) Heesbeen et al., 1987
(viii) Brand and Söling, 1974
(ix) Dunaway and Weber, 1974a
(x) Foe and Kemp, 1984
(xi) Hesterberg et al., 1981
(xii) Tarui et al., 1972

muscle and liver enzymes, in that, at pH 8.0, the brain enzyme did not form oligomers larger than a tetramer, under conditions where the other two isozymes did (Foe and Kemp, 1985).

The aggregation of PFK appears to be an equilibrium process influenced by a variety of factors, including the enzyme concentration, presence or absence of metabolic effectors, and pH (Paetkau and Lardy, 1967; Layzer et al., 1969; Brand and Söling, 1974; Reinhart and Lardy, 1980b).

The protein concentration at which significant association occurs is subject to some controversy however. Several groups have concluded that aggregation does not occur until the protein concentration exceeds 0.5-1.0mg/ml (Aaronson and Frieden, 1972; Leonard and Walker, 1972; Pavelich and Hammes, 1973), while others report aggregation at protein concentrations of 1μg/ml or less (Reinhart and Lardy, 1980b).

At low protein concentrations, PFK has been shown to dissociate into low molecular weight, inactive forms (Hulme and Tipton, 1971; Underwood and Newsholme, 1965; Reinhart and Lardy, 1980b; Layzer et al., 1969) with at most 2% of the activity of the associated enzyme (Hulme and Tipton, 1971).This inactivation is fully reversible, and can be prevented by the presence of various ligands (Reinhart and Lardy, 1980b). Activators prevent dissociation of the enzyme, presumably by binding to the associated form of the enzyme, while PFK inhibitors increase the dissociation effect of dilution (Hofer, 1971; Hulme and Tipton, 1971). The process of reassociation appears to be rapid, since addition of AMP, or an additional aliquot of enzyme to an inhibited sample causes an immediate activation (Hulme and Tipton, 1971). The transition from active to inactive forms however is rather slow, taking approximately 2min (Ramaiah and Tejwani, 1970; 1973). In the absence of either substrate, PFK dissociates beyond the tetrameric form with concomitant loss of enzyme activity. The associated and dissociated forms of the enzymes have different affinities for the substrates F6P and ATP. The associated enzyme has a higher affinity for F6P than for MgATP, whereas the dissociated enzyme has a very low affinity for F6P (Ramaiah and Tejwani, 1970; 1973; Reinhart and Lardy, 1980a; 1980b; Reinhart, 1980). The addition of MgATP to the stock enzyme solution favours dissociation, while addition of the other substrate F6P, exerts an opposing effect, tending to favour the associated form of the enzyme. The mean PFK content of rabbit muscle is approximately 10^{-6}M. The dissociation constant for PFK has been estimated to be 10^{-6}M from computer simulations, under cellular conditions. This suggests that at low substrate concentrations, such as those present in resting muscle, and at physiological pH, PFK is at least partially present in

an inactive dissociated form. In addition to the allosteric effects exerted by substrates (F6P and ATP), the rising concentration of the substrates and F16BP lead to a decrease in the dissociation constant by several orders of magnitude, thus leading to an increase in the amount of active enzyme. Since under cellular conditions the half-time of the molecular weight transition is between 10-30sec, which is similar to the time requirement of interconversion reactions, it is possible that the association-dependent changes in the concentration of active PFK may be responsible for establishing a certain functional state of this regulatory enzyme (Hofer and Krystek, 1975).

Dissociation and inactivation of PFK can also be brought about by lowering the pH from 8.0 to 5.8 (Mansour, 1965; Paetkau and Lardy, 1967; Hofer and Pette, 1968). This process appears to involve dissociation to the dimeric form, and is co-operative with respect to the H^+ concentration (Hofer and Pette, 1968; Pavelich and Hammes, 1973; Aaronson and Frieden, 1972). Dissociation can be reversed by raising the pH (Hofer and Pette, 1968) and by the addition of ligands (Alpers et al., 1971; Lad et al., 1973), resulting in an increase in activity and concomitant formation of active enzyme of higher molecular weight.

1.3.6 PHYSICAL PROPERTIES OF MAMMALIAN PHOSPHOFRUCTOKINASE

The pH optima for mammalian PFKs from a variety of sources have been reported. Although most appear to have an optimum activity at slightly alkaline pH (Layzer et al., 1969; Staal et al., 1972; Massey and Deal, 1973; Balinsky et al., 1979; Dunaway et al., 1972) the PFK from Erlich ascites tumor has an unusually low pH optimum of 7.1 (Sumi and Ui, 1972).

The isoelectric points of human PFK isozymes have been reported as pH 6.6 for muscle, pH 5.0 for normal erythrocyte (M and L subunits), and pH 4.6 for erythrocyte PFK obtained from patients with Taruis disease (L subunits only)(Kaur and Layzer, 1977).

1.3.7 STABILITY OF PHOSPHOFRUCTOKINASE

PFKs from most mammalian sources have been found to be very labile at all stages of purification, particularly at high dilutions.

The addition of DTT was found to be effective in slowing the normally rapid disappearance of activity in sheep liver PFK (Brock, 1969). Phosphate has also been found to stabilize the enzyme from rabbit muscle (Leonard and Walker, 1972; Pavelich and Hammes, 1973; Bloxham and Lardy, 1973; Paradies and Vetterman, 1976; Pettigrew and Frieden, 1979b; Liou and Anderson, 1980). In addition ATP and $(NH_4)_2SO_4$ have been suggested as necessary for the full protection of both muscle and liver PFK activity during purification (Kemp, 1971; Dunaway and Weber, 1974a). NaF also increases the stability of PFK (Ling et al., 1965; Karadsheh et al., 1974) by protecting the levels of ATP during extraction, presumably by the inhibition of ATPase activity (Dunaway and Weber, 1974a).

A common feature of the enzyme that is used in purification is its stability to heat. PFK from many sources withstands heating to 40-66°C for 30min without loss of activity provided suitable stabilizers are present.

1.4 KINETIC PROPERTIES OF MUSCLE AND LIVER PHOSPHOFRUCTOKINASE

The principal compounds affecting PFK activity are the substrates, ATP and F6P, and its reaction products: F16BP and ADP. The complexity of the regulation of PFK lies in the fact that PFK is also allosterically regulated by a multiplicity of ligands; the physiological significance of which is dependent upon the tissue being studied.

1.4.1 EFFECTS OF ATP ON PHOSPHOFRUCTOKINASE

ATP is the major product of the glycolytic pathway. As well as acting as a substrate, ATP at high concentrations can exert an inhibitory effect on PFK (Underwood and Newsholme, 1965). This inhibition is caused by the binding of either ATP or MgATP to an inhibitory site, distinct from the catalytic site (Colombo et al., 1975). When the ATP concentration rises above a particular level PFK is inhibited, thus slowing the glycolytic pathway and hence the rate of ATP production. The ATP concentration at which inhibition arises varies somewhat for the muscle and liver isozymes. The degree of inhibition by a fixed ATP concentration, as well as the concentration at which inhibition is first observed is a function of the pH and F6P concentration (Ui, 1966; Brock, 1969; Dunaway and Weber, 1974a; Pettigrew and Frieden, 1979b).

1.4.1.1 EFFECTS OF pH ON ATP INHIBITION

When assayed at pH values close to 7.0, PFK displays regulatory kinetic behavior in the form of sigmoidicity with respect to F6P, and inhibition by ATP, as well as allosteric interaction from a number of effectors. However at pH 8.0, the kinetic behaviour is substantially non-regulatory, and is not altered by allosteric effectors (Lowry and Passonneau, 1966; Pettigrew and Frieden, 1979b). The decreased sensitivity of PFK to ATP inhibition at alkaline pH is well documented (Mansour, 1972; Bloxham and Lardy, 1973), and although both muscle and liver isozymes display this trait, the muscle enzyme is the most strongly affected (Tsai and Kemp, 1974). At pH 8.2, and 2mM ATP, substrate inhibition of rabbit liver PFK was observed, whereas muscle PFK was not affected. At pH 7.0, ATP inhibition of both muscle and liver PFK became apparent (Table II). However the liver enzyme was inhibited at ATP concentrations that were low in comparison with the amount required to inhibit the muscle enzyme to the same extent (Kemp, 1971; Tsai and Kemp, 1974; Dunaway and Kasten, 1985). At pH 7.4 the concentrations of ATP required to reduce the velocity to half that at the optimum ATP concentration, were 3.1mM and 0.7mM for the muscle and liver enzymes respectively. However at low ATP concentrations (< 0.5mM), the velocity of the reaction catalysed by the liver enzyme was greater than that of the muscle enzyme.

1.4.1.2 EFFECT OF F6P CONCENTRATION ON ATP INHIBITION

ATP inhibition of PFK can be relieved by increasing the concentration of the substrate F6P (Underwood and Newsholme, 1965). At any given level of ATP, the F6P concentration required to give half the maximum velocity was higher for liver PFK than muscle PFK, by a factor of two or more (Table III)(Kemp, 1971; Tsai and Kemp, 1974). At low F6P concentrations (0.4mM) the liver enzyme was almost inactive, even at ATP concentrations less than 0.1mM (Tsai and Kemp, 1974).

ATP inhibition is thought to occur by lowering the affinity of the enzyme for the second substrate, F6P (Underwood and Newsholme, 1965). The liver enzyme with its susceptibility to ATP inhibition occurring at lower concentrations than for the muscle enzyme, must therefore have a greater affinity for ATP (Kemp, 1971).

TABLE II

Ki ATP VALUES OF RABBIT MUSCLE AND LIVER PHOSPHOFRUCTOKINASE.

	[F6P] mM	pH	Muscle	Liver
Ki ATP	0.4	7.1	0.4	0.1
mM	4.0	7.1	1.0	0.4
	1.0	7.4	3.1	0.7

ATP concentrations required to give 50% inhibition of rabbit muscle and liver PFK, at pH 7.1 and 7.4, in 50mM Tes, 150mM KCl, 1mM EDTA, 1mM DTT at different F6P concentrations.(Tsai and Kemp, 1974).

TABLE III

MICHAELIS CONSTANTS FOR F6P AND ATP FOR RABBIT MUSCLE AND LIVER PHOSPHOFRUCTOKINASE.

	[ATP] mM	[F6P] mM	pH	Muscle	Liver
Km F6P mM	0.5	-	7.0	0.06	0.13
	1.0	-	7.0	0.10	0.19
	2.0	-	7.0	0.16	0.38
	0.75	-	8.2	0.06	0.05
Km ATP mM	-	1.0	8.2	0.05	0.04

Apparent affinity constants for F6P at pH 7.0 at varying ATP concentrations, determined in 25mM glycylglycine, 1mM EDTA, 6mM $MgCl_2$, 3mM $(NH_4)_2SO_4$, 0.1mM DTT. The Kms for both ATP and F6P at pH 8.2, were also determined in the above buffer system (Kemp, 1971).

1.4.2 EFFECTS OF ADENINE NUCLEOTIDES ON PHOSPHOFRUCTOKINASE ACTIVITY

AMP and ADP are activators of PFK, and their presence can relieve ATP inhibition (Underwood and Newsholme, 1965; Brock, 1969; Kemp, 1971; Tsai and Kemp, 1974). Liver PFK required 6-8 times higher concentrations of AMP and ADP to achieve comparable rate increases to those elicited on muscle PFK (Table IV).

The differences in sensitivity of the liver enzyme towards AMP and ADP is indicative of the higher affinity of liver PFK for ATP at the inhibitory site, rather than a lesser affinity for the activators. The relative insensitivity of liver PFK to these effectors suggests that it is less suited for anaerobic energy production than the muscle enzyme. Since in muscle, ATP inhibition can be overcome by small increases in the concentrations of ADP, AMP, and Pi, all of which increase in the cell whenever the use of ATP exceeds the rate of its production (Lowry and Passonneau, 1966).

Like ADP and AMP, cAMP is an activator of PFK, however cAMP is effective at lower concentrations than either AMP or ADP (Kemp, 1971; Tsai and Kemp, 1974). A greater concentration of cAMP is necessary for the liver enzyme to achieve the same level of activation as the muscle enzyme (Table IV)(Kemp, 1971).

The relief of ATP inhibition brought about by adenine nucleotides is primarily due to a decrease in the affinity for binding ATP at the inhibitory site (Pettigrew and Frieden, 1978; Wolfman et al., 1978), which in turn lowers the apparent Km for F6P (Dunaway et al., 1972; Dunaway and Weber, 1974a; Tornheim and Lowenstein, 1976), thus leading to an increase in F6P binding, and deinhibition of the enzyme.

1.4.3 EFFECTS OF FRUCTOSE 1,6-BISPHOSPHATE ON PHOSPHOFRUCTOKINASE ACTIVITY

F16BP, like the other product of the reaction, ADP, is also an activator of PFK. The mechanism by which activation is achieved is the same as that of ADP. F16BP acts by decreasing the apparent affinity of the inhibitory site for ATP or MgATP (Pettigrew and Frieden, 1979a), thus resulting in the loss of ATP inhibition (Bloxham and Lardy, 1973).

TABLE IV

ACTIVATION OF RABBIT MUSCLE AND LIVER PHOSPHOFRUCTOKINASE BY ADENINE NUCLEOTIDES.

Adenine nucleotide	[ ] $^1/_2$ max activity mM	
	Muscle	Liver
ADP	0.040	0.310
AMP	0.035	0.210
cAMP	0.015	0.075

The concentrations of ADP, AMP and cAMP required to give half maximal activation of rabbit muscle and liver PFK at pH 7.0, determined in 25mM glycylglycine, 1mM EDTA, 6mM $MgCl_2$, 3mM $(NH_4)_2SO_4$, 0.1mM DTT, 0.3mM F6P and 3.2 mM ATP (Kemp, 1971).

1.4.4 ACTIVATION OF PHOSPHOFRUCTOKINASE BY NH_4^+ AND K^+

Ions such as NH_4^+ and K^+ which are not directly involved in the enzymic reaction are also capable of altering PFK activity. NH_4^+ and $(NH_4)_2SO_4$ have been found to both increase PFK activity (Table V), and also relieve ATP inhibition (Underwood and Newsholme, 1965; Brock, 1969; Kemp, 1971). The effect of NH_4^+ is not to change the apparent affinity of the enzyme for ATP or MgATP (Pettigrew and Frieden, 1979a), but may arise from the increased affinity of the enzyme for F6P, which has been observed in the presence of NH_4^+ (Kemp and Krebs, 1967; Dunaway and Weber, 1974a). The levels of NH_4^+ have been found to rise during anoxia in certain tissues (Lowry and Passonneau, 1966), so the effects of NH_4^+ may be of physiological significance in these tissues.

K^+ is essential for PFK enzymic activity (Uyeda and Racker, 1965; Lowry and Passonneau, 1966; Paetkau and Lardy, 1967; Kemp, 1971), and has been found to decrease the apparent affinity of the inhibitory site for ATP (Sumi and Ui, 1972).

1.4.5 INHIBITORS OF PHOSPHOFRUCTOKINASE ACTIVITY

It is difficult to readily assess the relative effect that other inhibitors besides ATP have on PFK isozymes because of the differing sensitivity of the isozymes to ATP inhibition, and because ATP acts synergistically with other inhibitors (Mathias and Kemp, 1972).

1.4.5.1 TRICARBOXYLIC ACID CYCLE INTERMEDIATES

Intermediates of the tricarboxylic acid cycle (TCA), α-oxo-glutarate, succinate, malate and citrate have all been shown to inhibit PFK (Underwood and Newsholme, 1965). Inhibition by citrate is the most potent (Table VI)(Underwood and Newsholme, 1965).

Muscle PFK is much more sensitive to citrate inhibition than liver PFK (Table VII)(Kemp, 1971; Tsai and Kemp, 1974). The inhibitory effect of citrate is synergistic with ATP (Underwood and Newsholme, 1965; Brock, 1969), hence, relief of ATP inhibition by activators such as AMP and F16BP, is less marked in the presence of citrate (Underwood and Newsholme, 1965). AMP is more effective at easing citrate inhibition of liver PFK than of the muscle enzyme (Dunaway and Kasten,

TABLE V

EFFECT OF NH_4^+ AND K^+ ON THE ACTIVITY OF RABBIT MUSCLE AND LIVER PHOSPHOFRUCTOKINASE.

	Muscle	Liver
Ka NH_4^+ (mM)	0.20	0.35
Ka K^+ (mM)	17-18	17-18

Affinity constants for potassium and ammonium ions measured at pH 7.0, in 25mM glycylglycine, 1mM EDTA, 6mM $MgCl_2$, 0.1mM DTT, 1mM F6P and 0.7mM ATP (Kemp, 1971).

TABLE VI

INHIBITION OF RAT LIVER PHOSPHOFRUCTOKINASE BY TCA CYCLE INTERMEDIATES.

	Concentration of intermediate (mM)	% Inhibition
Control	-	-
Citrate	0.5	50
Succinate	2.5	46
α-oxo-glutarate	2.5	44
Malate	2.5	33

Inhibitory effects of intermediates of the tricarboxylic acid cycle on partially purified rat liver PFK, at pH 7.0 measured in 20mM imidazole, 5mM $MgCl_2$, 67mM KCl, 0.01mM ATP and 5mM G6P with addition of the appropriate intermediate (Underwood and Newsholme, 1965).

TABLE VII

INHIBITION OF RABBIT MUSCLE AND LIVER PHOSPHOFRUCTOKINASE BY CITRATE

	Muscle	Liver
[citrate] to achieve 50% inhibition	0.25mM	2mM

Concentrations of citrate required to inhibit rabbit muscle and liver PFK by 50% at pH 7.0, measured in 25mM glycylglycine, 1mM EDTA, 6mM $MgCl_2$, 3mM $(NH_4)_2SO_4$, 0.1mM DTT, 0.3mM F6P and 1mM ATP (Kemp, 1971)

1985). Citrate/ATP inhibition can also be relieved by increasing the concentration of F6P, $(NH_4)_2SO_4$, $MgCl_2$, and Pi (Brock, 1969; Layzer et al., 1969; Dunaway and Weber, 1974a; Dunaway and Kasten, 1985). Increasing the F6P concentration however, relieves the inhibition more strongly for the muscle PFK than liver PFK, this is probably associated with the greater affinity of the muscle enzyme for F6P than the liver enzyme.

Inhibition by citrate occurs by decreasing the affinity of the enzyme for F6P at the active site (Pettigrew and Frieden, 1979a), and is in contrast to earlier views of citrate increasing the affinity of the enzyme for MgATP at the inhibitory site (Randle et al., 1968; Wolfman et al., 1978; Colombo et al., 1975).

Citrate is formed from the condensation of oxaloacetate and acetyl-CoA, a major end product of aerobic glycolysis. Thus an increased level of citrate results from a raised acetyl-CoA level, which may arise from increased activity of the glycolytic pathway. Hence citrate acts as a negative feedback mechanism in controlling glycolysis by inhibiting PFK (Passonneau and Lowry, 1963), and enhancing the intracellular coordination of glycolysis and ATP production.

1.4.5.2 PHOSPHATE ESTERS

Phosphoenolpyruvate and phosphocreatine both inhibit muscle PFK, but show little or no inhibitory properties towards liver PFK (Table VIII).

3-Phosphoglycerate is a more potent inhibitor towards muscle PFK than 2-phosphoglycerate, which is in turn more potent than 2,3-diphosphoglycerate. In contrast to this, 2,3-diphosphoglycerate is a more potent inhibitor towards liver PFK than either 2 or 3-phosphoglycerate (Table VIII). Only in the erythrocytes however, where the liver subunit constitutes the predominant isozyme, does the 2,3-diphosphoglycerate concentration reach levels high enough to play a significant role in PFK regulation (Tsai and Kemp, 1974). Indeed it has been suggested that inhibition by 2,3-diphosphoglycerate may be of more significance in the erythrocytes, than ATP, because the former is found at higher, and more variable concentrations than ATP (Lenfant et al., 1968).

Early reports on the kinetics of liver PFK (Reinhart and Lardy, 1980a), suggested that at physiological pH and temperature, and at in vivo concentrations of F6P, ATP, AMP

TABLE VIII

INHIBITION OF RABBIT MUSCLE AND LIVER PHOSPHOFRUCTOKINASE BY PHOSPHATE ESTERS.

	Muscle	Liver	Ref
2.5mM PEP	50%	0%	(i)
1.9mM Phosphocreatine	50%	0%	(ii)
0.8mM 3-P-glycerate	50%	7%	(i)
2mM 2-P-glycerate	40%	35%	(i)
0.5mM 2,3-diP-glycerate	20%	50%	(i)

Inhibitory effects of various concentrations of phosphate esters on rabbit muscle and liver PFK, measured at:
(i) pH 7.0 in 25mM glycylglycine, 1mM EDTA, 6mM $MgCl_2$, 0.1mM DTT, 3mM $(NH_4)_2SO_4$, 0.3mM F6P and 1mM ATP (Kemp, 1971).
(ii) pH 7.1, in 50mM Tes, 150mM KCl, 1mM EDTA, 1mM DTT, 6mM $MgCl_2$, 0.4mM F6P and 0.2mM ATP (Tsai and Kemp,1974).

and F16BP the kinetic activity of liver PFK was insufficient to account for the necessary cellular activity. Even when the combined effects of several activators; F6P, AMP, and Pi, were considered, the PFK activity realized was a small percentage of the total available.

Isotopic investigations performed with rat liver in vivo (Van Schaftingen et al., 1980a) and with isolated hepatocytes (Van Schaftingen et al., 1980b) led the authors to conclude that PFK is completely inactive in the liver of starved animals, whereas it displays a rather large activity in the fed condition, or after the administration of glucose to starved rats.

The discovery of F26BP and its effects on PFK provided a mechanism by which PFK activity could be controlled, since at physiological F6P concentrations rat liver PFK is inactive unless activators such as F26BP and AMP are present (Pilkis et al., 1981a).

1.4.6 FRUCTOSE 2,6-BISPHOSPHATE

Fructose 2,6-bisphosphate (F26BP) was discovered in 1980 as a potent regulator of liver PFK (Van Schaftingen et al., 1980b, 1980c). Since then its presence has also been detected in brain, heart, skeletal muscle, lung, kidney, epididymal fat, pancreatic islets and in hepatoma tumor cells, but it is not detectable in human erythrocytes (Heylin et al., 1982). F26BP has also been found in yeast, bacteria and fungi as well as in higher plants, including mung beans (Sabularse and Anderson, 1981), and Jerusalem artichoke tubers following incubation to break dormancy (Van Schaftingen and Hers, 1983b).

Despite the presence of F26BP in plants and bacteria, as yet no effect of F26BP has been demonstrated on PFK from these sources (Sabularse and Anderson, 1981; Cséke et al., 1982; Van Schaftingen and Hers, 1983b; Ashihara and Stupavska,1984, Hers and Van Schaftingen, 1982), although nanomolar concentrations have been found to stimulate all animal PFKs tested.

F26BP is synthesized from F6P and ATP by the enzyme phosphofructokinase-2 (PFK-2)(Furuya and Uyeda, 1981; El-Maghrabi et al., 1981; Van Schaftingen and Hers, 1981b), and it is hydrolyzed to F6P and Pi by a specific fructose 2,6-bisphosphatase (FBPase-2)(El-Maghrabi et al., 1982b). PFK-2 and FBPase-2 have been co-purified in a number of cases, and evidence has suggested that the two catalytic properties belong to a single multi-functional protein (Van Schaftingen et al.,

1981a; 1982; El-Maghrabi et al., 1982a). The cellular concentration of F26BP is controlled by the relative activities of PFK-2 and FBPase-2. It has now been conclusively shown that the PFK-2/FBPase is a single protein (Pilkis et al., 1987; Tauler et al., 1987).

Rabbit muscle and liver PFK bind one mol of F26BP/mol of monomer, at the allosteric site for F16BP. ATP inhibits the binding of F26BP (Kitajima and Uyeda, 1983), possible explanations for this effect are that either ATP causes sufficient conformational changes on the enzyme to weaken the affinity of F26BP, or that F26BP competes for the inhibitory site of ATP (Kemp and Krebs, 1967).

Studies using a mixture of analogues of F26BP (D-manno and D-gluco-2,5-anhydro-1-deoxy-1-phosphohexitol 6-phosphate), which act in a similar manner, but are 4-20 times less potent than the natural effector, demonstrated the importance of the CH_2OH group at position 1 on F26BP in the recognition of this metabolite by PFK and F16BPase (McClard et al., 1986).

The level of F26BP in muscle has been reported as 10-fold lower than in the liver (10^{-5}M)(Hue et al., 1982; Kuwajima and Uyeda, 1982). The activity of PFK-2 in muscle is also 10-20-fold lower than in the liver. However, muscle is more sensitive to activation by F26BP compared to the liver enzyme (Uyeda et al., 1981). The $K_{0.5}$ for activation of muscle PFK was 2.5-4-fold lower than those observed for liver PFK. Data suggest that F26BP could render muscle PFK in a partly activated state in vivo. At present however, no data providing the mechanisms of regulation of F26BP levels in muscle in co-ordination with the functional state, are available.

1.4.6.1 EFFECT OF FRUCTOSE 2,6-BISPHOSPHATE ON PHOSPHOFRUCTOKINASE ACTIVITY

Fructose 2,6-bisphosphate is a potent allosteric activator of liver PFK, with a Ka of 0.05μM, and is 50-100 times more effective than fructose 1,6-bisphosphate in activating PFK (Pilkis et al., 1981a). In the absence of any activators, PFK exhibits a low affinity ($S_{0.5}$=2.5mM) and a high degree of positive co-operativity (n_H=3.3) towards its substrate, F6P. The concentration of F6P has a positive influence on F26BP (Hue et al., 1981). The apparent affinities of PFK and F16BPase for F26BP depend on the concentration of substrates or effectors such as F16BP and AMP. F26BP increases the affinity of the enzyme for F6P, but has no effect on the maximum activity of the enzyme (Pilkis et al., 1981a; Van Schaftingen et al., 1981b; Uyeda et al., 1981). F26BP is highly efficacious at counteracting inhibition caused by high concentrations of ATP and citrate (Söling et al., 1981). Half maximal inhibition by

ATP was observed with 1.5mM ATP in the absence of F26BP, while 8mM ATP was required for half-maximal inhibition in the presence of 1μM F26BP. F26BP has also been shown to act synergistically with AMP to release ATP inhibition (Uyeda et al., 1981; Van Schaftingen et al., 1981b). The effects of 6-phosphogluconate, an activator of PFK from liver, adipose tissue, kidney, and skeletal muscle, are synergistic with those of F26BP under most conditions (Sommercorn et al., 1984). F26BP is able to stabilize liver PFK against the rapid spontaneous inactivation which occurs in its absence at 37°C (Söling et al., 1981) and 50°C (Uyeda et al., 1981) as well as inactivation by PFK-phosphatase, and low pH.

F26BP has significant effects on the association state of PFK, by dramatically slowing the dissociation of high molecular weight aggregate forms of the enzyme when the enzyme is diluted to concentrations as low as $4x10^{-8}$M. F26BP also strongly promotes the reassociation to the tetramer and larger forms of the enzyme which have previously been allowed to dissociate to the dimer in its absence. F26BP is also able to overcome the tendency of MgATP to promote dissociation to the tetrameric form, and instead promotes a very high degree of high MW aggregate formation (Reinhart, 1983). Influencing the aggregation state of rat liver PFK, may be one mechanism by which F26BP achieves its activating effects on PFK in vivo, since aggregation increases the activity of the enzyme under physiological MgATP concentrations.

1.4.6.2 EFFECTS OF FASTING ON FRUCTOSE 2,6-BISPHOSPHATE LEVELS

Recovery of PFK from the livers of fasted rats can be greatly impaired due to the 10-20-fold decrease in the level of F26BP that occurs after fasting (Claus et al., 1982; Hers and Van Schaftingen, 1982). A 60-70% decrease in the PFK activity of rat hepatocytes has been reported by Dunaway and Weber (1974b), after a 6-day fast. This can be attributed to two factors: a) a decrease by approximately 15% of the activity of PFK, and the increase by 16 % of aldolase and lactate dehydrogenase, as well as the content of soluble protein, resulting in a 27% decrease in the specific activity of PFK. b) the decrease in the weight of the livers to 45% of the values observed in the fed state. The combination of these two effects would account for a 62% decrease in PFK per liver, per cell (Van Schaftingen and Hers, 1983a), since it is known that the number of cells per liver, remains essentially constant during starvation (Allard et al., 1957).

1.4.6.3 CHANGES IN PHOSPHOFRUCTOKINASE ACTIVITY IN RESPONSE TO GLUCAGON ADMINISTRATION

The effect of low concentrations of glucagon in stimulating gluconeogenesis, urea production, and ketogenesis in perfused liver (Williamson et al., 1969), mediated by cAMP (Clark et al., 1974; Castaño et al., 1979), has been well documented.

The addition of glucagon to isolated hepatocytes results in an inhibition of PFK (Taunton et al., 1972; 1974; Pilkis et al., 1979; Castaño et al., 1979; Kagimoto and Uyeda, 1979; Claus et al., 1980; Nieto and Castaño, 1980; Van Schaftingen et al., 1980b), characterised by an apparent decrease in the enzymes affinity for F6P (Castaño et al., 1979; Pilkis et al., 1979) and an increased sensitivity to inhibition by ATP (Nieto and Castaño, 1980). The glucagon concentration causing half-maximum inhibition of PFK has been reported as 0.2nM (Pilkis et al., 1979) and 0.1nM (Castaño et al., 1979). These concentrations are similar to that necessary for half-maximum stimulation of gluconeogenesis, and half-maximum inactivation of liver pyruvate kinase (Felíu et al., 1976; Riou et al., 1976; Castaño et al., 1979).

Glucagon has no effect on the intracellular levels of various adenine nucleotide effectors of the enzyme (Clark et al., 1974), but has been reported to depress the levels of the inhibitor citrate (McGarry et al., 1978). Glucagon also lowers the level of F16BP (Pilkis et al., 1976; Blair et al., 1973; Claus et al., 1979). In 1980 it was suggested that the decrease in PFK activity following glucagon administration was due to changes in the level of an allosteric effector, rather than changes in the kinetic properties of PFK (Claus et al., 1980). This effector which increased the affinity of PFK for F6P (Van Schaftingen et al., 1980a), was later identified as F26BP. A diagramatic representation of the regulation of F26BP levels in the liver by glucagon and other metabolites is shown in Fig. 1.

1.4.6.4 CHANGES IN FRUCTOSE 2,6-BISPHOSPHATE CONCENTRATION IN RESPONSE TO GLUCOSE AND GLUCAGON ADMINISTRATION

The concentration of F26BP in hepatocytes is increased at least 15-fold, within 8 min of the administration of glucose (Richards and Uyeda, 1980). Upon administration of glucagon (10^{-7}M) to isolated hepatocytes, the level of F26BP decreased rapidly,

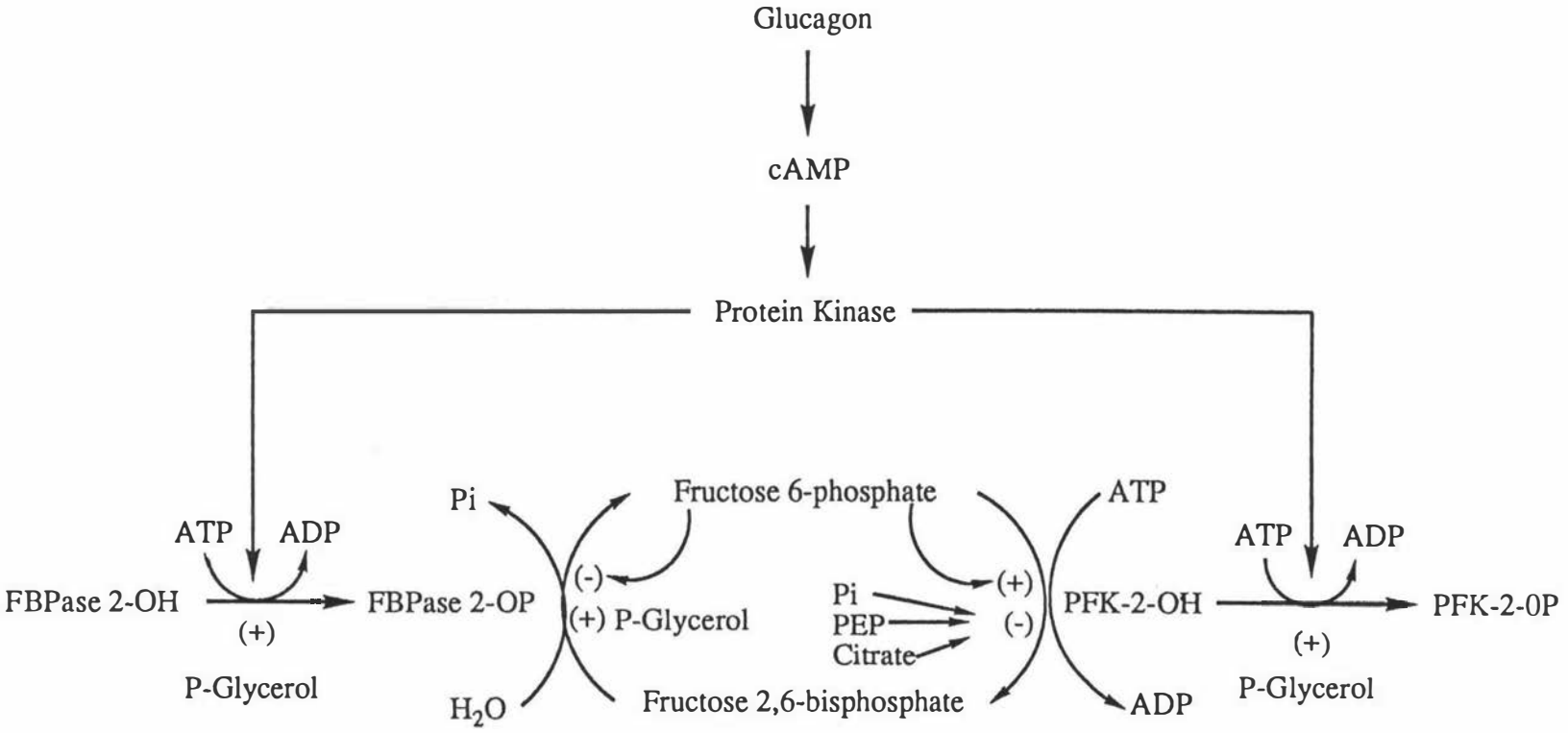


Figure 1: Biosynthesis and Degradation of Fructose 2,6-Bisphosphate in the Liver.

The control of the synthesis and degradation of F26BP in the liver by glucagon and other metabolites (Hers and Van Schaftingen, 1982).

and within 2 min was $^1/_{13}$ of its original concentration (Richards and Uyeda, 1980). This decrease in F26BP concentration is due to the decreased rate of synthesis, and the increased rate of degradation of F26BP. Glucagon causes the phosphorylation, via cAMP-dependent protein kinase, of the bifunctional PFK-2/F26BPase enzyme, responsible for the synthesis and degradation of F26BP. Phosphorylation leads to the inactivation of the PFK-2 moiety (Hue et al., 1981; El-Maghrabi et al., 1982a; 1982b; 1982c; Richards et al., 1981), and the activation of F26BPase (El-Maghrabi et al., 1982b), resulting in a net decrease in the F26BP levels.

1.4.6.5 EFFECT OF FRUCTOSE 2,6-BISPHOSPHATE ON FRUCTOSE 1,6-BISPHOSPHATASE ACTIVITY

F26BP, at micromolar concentrations, is a potent inhibitor of liver, skeletal muscle, yeast and plant F16BPase (Pilkis et al., 1981b; Van Schaftingen and Hers, 1981a; Mörikofer-Zwez et al., 1981). The concentration of F26BP required for half-maximal activation of PFK (Van Schaftingen et al., 1980a; 1980b; Pilkis et al., 1981a), is similar to that required for half-maximal inhibition of F16BPase, and is similar to the concentration of this effector in isolated hepatocytes (Van Schaftingen et al., 1980a; 1980b). The main characteristics of F26BP inhibition of F16BPase are that: a) the inhibition is much stronger at low substrate concentrations; b) the effect of F26BP is markedly synergistic with the action of AMP (Van Schaftingen et al., 1981b), a strictly non-competitive inhibitor; and c) F26BP changes the substrate saturation curve from hyperbolic to sigmoidal. However, there is controversy as to whether F26BP binds to the catalytic site of F16BPase (Pontremoli et al., 1982), or to an allosteric site which is different to the AMP site (François et al., 1983).

1.4.6.6 CONTROL OF GLYCOLYSIS AND GLUCONEOGENESIS BY FRUCTOSE 2,6-BISPHOSPHATE

Due to its action on both PFK and F16BPase, the most obvious role of F26BP is to control glycolysis and gluconeogenesis. This role is of particular importance in the liver, in which both pathways can operate. Gluconeogenesis is stimulated by glucagon and predominates during fasting and diabetes, whereas glycolysis becomes operative in plethoric conditions as well as in anoxia.

All the available evidence indicates that F26BP plays a major role in the hormonal and nutritional (i.e. differences between fed/starved) control of PFK and F16BPase activity.

The rate of glycolytic flux can be correlated with the concentration of F26BP in the liver under plethoric conditions, but not with those of ATP, AMP, or citrate.

Increases in glycolysis can occur in the liver and other tissues, with no concomitant increase in F26BP levels. This was observed in hepatocytes during anoxia, in muscle during electrical stimulation, and in hearts treated with epinephrine (Hue et al., 1982). Obviously, mechanisms other than changes in F26BP concentration must then be involved in the regulation of glycolysis under these conditions. A major consideration is whether or not changes in F26BP concentrations have physiological significance for metabolic processes other than hepatic glycolysis and gluconeogenesis.

1.4.7 COVALENT MODIFICATION OF PHOSPHOFRUCTOKINASE BY PHOSPHORYLATION

Mouse muscle PFK (Hofer and Fürst, 1976; Riquelme et al., 1978a), rabbit skeletal muscle PFK (Hussey et al., 1977; Uyeda et al., 1978), rat skeletal muscle PFK (Uyeda et al., 1978), and rat liver PFK (Kagimoto and Uyeda, 1979), are all partially phosphorylated in vivo, and can be phosphorylated in vitro by the catalytic subunit of cAMP-dependent protein kinase (Riquelme et al., 1978b; Sørensen-Ziganke and Hofer, 1979; Riquelme and Kemp, 1980; Claus et al., 1982; Pilkis et al., 1982). Skeletal muscle PFK has also been phosphorylated in vitro by Ca^{2+} and phospholipid-dependent protein kinase C (Hofer et al., 1985). Rat liver, but not rat skeletal muscle PFK is an excellent substrate for the multi-functional Ca^{2+}-calmodulin-dependent protein kinase (Mieskes et al., 1987).

1.4.7.1 EXTENT OF PHOSPHORYLATION OF PHOSPHOFRUCTOKINASE

As can be seen in Table IX, the reported number of phosphate groups per PFK tetramer differs widely. These discrepancies probably result from different techniques used for the purification of the PFK, and for the determination of protein bound phosphate.

That the degree of phosphorylation of skeletal muscle PFK can be influenced by the metabolic state of the muscle was shown in trials by Hofer and Sørensen-Ziganke (1979), and Hofer (1983). Although the extent of phosphorylation differs between the

TABLE IX

PHOSPHATE CONTENT IN MUSCLE

Source		Phosphate content moles Pi/tetramer	Ref
Rat :	Fresh muscle	0.32-0.81	(i)
Rabbit :	Fresh muscle	0.60-1.26	(ii)
	Fresh muscle	1.12-1.36	(iii)
	Frozen muscle	0.80-0.96	(iii)
Mouse:	Fresh muscle	2.08	(iv)
	Fresh muscle	0.48-0.60	(v)

Amounts of covalently bound phosphate found in rat rabbit and mouse skeletal muscle PFK purified in different laboratories.

(i) Uyeda et al., 1978
(ii) Hussey et al., 1977
(iiii) Riquelme et al., 1978b
(iv) Hofer and Fürst, 1976
(v) Riquelme et al., 1978a

two trials, the trend of increased phosphorylation of contracting muscle compared to resting muscle is conserved (Table X).

Muscle PFK has been separated into low and high phosphate forms (Hussey et al., 1977; Uyeda et al., 1978), with phosphate content values reported for rabbit muscle PFK of 0.15 and 0.29, 0.20 and 0.34moles of Pi/mol monomer (Hussey et al., 1977; Riquelme et al., 1978b), and 0.3 and 0.8mol Pi/mol tetramer for the low and high phosphate forms respectively (Uyeda et al., 1978). Values of 0.12 and 0.15mol Pi/mol monomer have been reported for the low and high phosphate forms of mouse muscle PFK (Riquelme et al., 1978a).

Liver PFK has also been separated into low and high phosphate forms (Furuya and Uyeda, 1980; Sakakibara and Uyeda, 1983). The phosphate content of liver PFK has been reported as being much higher than that of muscle PFK, with values of 0.3 and 0.8mol Pi/mol monomer respectively for the low and high phosphate forms of rat liver PFK (Furuya and Uyeda, 1980). In contrast to this, a recent report (Domenech et al., 1988), indicates that rat liver PFK cannot be phosphorylated to more than 2mol Pi/mol of tetramer. The phosphorylation of liver PFK by Ca^{2+}-calmodulin-dependent protein kinase was also found to never exceed a stoichiometry of 2mol Pi/mol of tetramer (Mieskes et al., 1987).

1.4.7.2 THE EFFECTS OF PHOSHORYLATION ON PHOSPHOFRUCTOKINASE

The extent of phosphorylation of both muscle and liver PFK does not influence the maximum catalytic activities of the enzymes, or their electrophoretic mobilities on SDS-PAGE (Sakakibara and Uyeda, 1983).

Differences in the responses of the phosphorylated and dephosphorylated PFK to allosteric effectors have been detected however. Phosphorylated muscle PFK is less sensitive to activation by AMP, than the dephosphorylated enzyme (Foe and Kemp, 1982), and exhibits an increased affinity for ATP at the inhibitory site, with decreased affinity for F6P at the active site (Foe and Kemp, 1982; Kitajima et al., 1983). Therefore, the high phosphate form is more strongly inhibited by ATP than the low phosphate or dephosphorylated form.

TABLE X

INFLUENCE OF METABOLIC STATE ON THE DEGREE OF PHOSPHORYLATION OF MUSCLE PFK.

Metabolic state	Phosphate content moles Pi/tetramer	
Resting, post mortem	1.93	(i)
Resting, in vivo	4.78	(i)
Contracting, in vivo	7.92	(i)
Resting+epinephrine	1.67	(ii)
Resting	2.16	(ii)
Contracting	3.85	(ii)

Amounts of covalently bound phosphate found in resting rabbit skeletal muscle PFK, and in muscle which prior to excision from the anesthetized animal, had been rhythmically contracting for 10min as a result of nerve stimulation.

(i) Hofer and Sørensen-Ziganke, 1979
(ii) Hofer, 1983

Similar differences in the kinetic properties of the low and high phosphate forms of liver PFK have also been reported (Sakakibara and Uyeda, 1983). In addition, the high phosphate form of liver PFK exhibited a slightly higher (1.6 times) $K_{0.5}$ for F6P than the low phosphate form, and the difference in the $K_{0.5}$ values became greater at lower pH's (Sakakibara and Uyeda, 1983).

In contrast to the above effects on PFK phosphorylated using cAMP-dependent protein kinase, rabbit muscle PFK phosphorylated using protein kinase C appears to increase PFK activity, whereas with protein kinase A, a slight decrease in activity was observed (Hofer et al., 1985). This has led the author to suggest that phosphorylation of PFK by protein kinase C may lead to a change in the conformation of the PFK, favouring a form with high affinity for F6P, and that such a change in the regulatory properties of PFK by protein kinase C could be of physiological significance.

Reports on the effect of F26BP on the high and low phosphate forms of both muscle and liver PFK are contradictory, with some reports indicating that the high phosphate forms are less sensitive to activation by F26BP (Furuya and Uyeda, 1980; Foe and Kemp, 1982; Sakakibara and Uyeda, 1983), while others indicate that there is no difference in sensitivity between the two forms (Pilkis et al., 1982; Kitajima et al., 1983).

1.4.7.3 SITE OF PHOSPHORYLATION

The site of in vitro phosphorylation by cAMP-dependent protein kinase in rabbit muscle PFK, is a serine, the sixth residue from the carboxyl terminus (Riquelme and Kemp, 1980; Kemp et al., 1981). This site, which also appears to be the site of phosphorylation in vivo (Kemp et al., 1981), has been isolated and sequenced (Kemp et al., 1981).

The phosphorylation sites of both muscle and liver PFK are susceptible to limited proteolysis by trypsin (Sakakibara and Uyeda, 1983), and subtilisin (Riquelme and Kemp, 1980; Kemp et al., 1981; Kitajima et al., 1983). The phosphorylation site is labile to these proteases under conditions which leave the catalytic properties of the enzyme intact, but reduce the molecular weight by about 2 000 (Riquelme and Kemp, 1980; Krystek and Hofer, 1981). Reports of a second phosphorylation site in muscle PFK (Hofer and Sørensen-Ziganke, 1979; Sørensen-Ziganke and Hofer, 1979), have thus far not been confirmed. The Ca^{2+} and phospholipid-dependent protein kinase C,

and Ca^{2+}-calmodulin-dependent protein kinase also phosphorylate PFK at the same trypsin labile site as cAMP-dependent protein kinase (Hofer et al., 1985; Domenech et al., 1988). However, protein kinase C also effectively phosphorylates one or more separate sites of PFK (Hofer et al., 1985).

Based on their finding that rat liver PFK cannot be phosphorylated to more than 2moles Pi/mol of tetramer, Domenech et al. (1988), have proposed a "half of the sites phosphorylation" hypothesis. Where a phosphorylated protomer would immediately interact with an unphosphorylated monomer in such a way that the phosphorylation of the second monomer was inhibited. Two of these mixed dimers could associate to form a tetramer with no more than 2moles Pi/mol of tetramer. Another possibility could be that PFK in the phosphorylation assay exists mainly in the inactive dimeric form, and that the interaction between the two subunits is such that the phosphorylation of one subunit inhibits the phosphorylation of the second.

1.4.7.4 FACTORS AFFECTING PHOSPHORYLATION

Phosphorylation of both muscle and liver PFK can be influenced by allosteric effectors. The addition of AMP, or elevation of pH increases the rate of phosphorylation of both the muscle and liver enzymes (Kemp et al., 1981; Pilkis et al., 1982; Foe and Kemp, 1984; Domenech et al., 1988). F26BP enhanced the rate of phosphorylation of the rat liver enzyme (Pilkis et al., 1982; Foe and Kemp, 1984; Domenech et al., 1988), while F16BP had no effect on the rat liver enzyme, but increased the rate of phosphorylation of the muscle enzyme (Kemp et al., 1981). Phosphorylation of both enzymes was inhibited by ATP and citrate (Kemp et al., 1981; Pilkis et al., 1982; Foe and Kemp, 1984; Domenech et al., 1988). Thus, both rat liver and rabbit muscle PFK appear to be better substrates for the cAMP-dependent protein kinase when they are in the active form rather than the inactive form. However, since the effect of phosphorylation is to increase the inhibition of the enzyme, stimulation of the phosphorylation rate by activators would seem to be counterproductive. It is conceivable that the kinetic effects of PFK phosphorylation could be ancillary to some other, more subtle effect that is significant in vivo yet difficult to demonstrate in vitro.

1.4.7.4.1 EFFECTS OF GLUCAGON AND GLUCOSE ON THE PHOSPHORYLATION OF PHOSPHOFRUCTOKINASE

Glucagon induced changes in PFK activity (Taunton et al., 1972; 1974; Pilkis et al., 1979; Castaño et al., 1979; Kagimoto and Uyeda, 1979; Claus et al., 1980; Nieto and

Castaño, 1980; Van Schaftingen et al., 1980a), can be attributed to hormone-induced changes in the levels of F26BP. However, glucagon also stimulates ^{32}P-incorporation into liver PFK in vivo (Kagimoto and Uyeda, 1979), as well as in isolated hepatocytes (Claus et al., 1980), and in isolated perfused livers (Kagimoto and Uyeda, 1980), leading to a phosphorylated enzyme which exhibits considerably lower activity than the unphosphorylated enzyme (Kagimoto and Uyeda, 1979). This is consistent with the effects of glucagon in decreasing F26BP levels, and therefore decreasing PFK activity (see Section 1.4.6.4).

Glucose is also able to increase the phosphorylation of rat liver PFK (Brand and Söling, 1982; Brand et al., 1983; Claus et al., 1982). Brand et al. (1983), found that the extent of phosphorylation was dependent on the metabolic state of the animal. Liver cells from fed animals required both glucose and glucagon to achieve maximum phosphorylation, whereas in starved liver cells, glucagon or glucose alone increased ^{32}P-incorporation to a limited extent, but both glucose and glucagon increased the phosphorylation to a maximum value (Brand and Söling, 1982; Claus et al., 1982). The addition of L-alanine inhibits almost completely the effects of glucose or glucagon on PFK phosphorylation (Brand and Söling, 1982).

1.4.7.5 EFFECT OF PHOSPHORYLATION IN VIVO

The biochemical mechanism by which the effect of phosphorylation of PFK induces changes in its allosteric behaviour is not known. Results from rat liver (Sakakibara and Uyeda, 1983), and muscle PFK (Foe and Kemp, 1982), suggest that phosphorylation results in an enzyme conformation which binds the negative effectors more effectively than the positive effectors.

1.4.7.6 SIGNIFICANCE OF PHOSPHORYLATION

The question as to whether the regulation of PFK by effectors other than F26BP plays any role in the liver is a very important one. Since the concentrations of metabolites such as F6P, AMP and ATP do not change significantly in vivo (Veech et al., 1979; Guynn et al., 1972), control by these metabolites appears to be unimportant. However, because of this very reason, the conversion of PFK from the dephosphorylated to the phosphorylated form may become an important alternative control mechanism over the changes in the metabolite concentration, in order to inhibit the activity of PFK. Furthermore, the phosphorylation of the enzyme may be an auxiliary means to inhibit PFK in addition to the control by F26BP concentration.

The question as to which is more important in the control of PFK; the F26BP concentration, or the phosphorylation state of the enzyme may depend on the physiological state of the liver. At very low concentrations of glucagon (10^{-11}M), the F26BP concentration is decreased rapidly, (Richards et al., 1981), while a higher concentration of glucagon (10^{-10}M) is required to promote sufficient phosphorylation of PFK to cause half-maximum inactivation of PFK (Kagimoto and Uyeda, 1980). These results suggest that the primary controlling factor is the level of F26BP, and that phosphorylation may be of secondary importance. Under conditions of starvation however, where F26BP concentrations remain at basal levels and do not change, the activity of PFK may be regulated by the phosphorylation state. Similarly, under some conditions in which the F26BP level does not change, the activity of PFK in the liver may also be controlled by the relative concentration of phosphorylated to dephosphorylated forms of the enzyme in vivo.

1.4.7.7 PHOSPHORYLATION AND ACTIN

Upon stimulation of muscle, PFK is phosphorylated to a greater extent than in the previously unstimulated muscle (Hofer and Sørensen-Ziganke, 1979; Luther and Lee, 1986). Phosphorylated PFK has a higher affinity for F-actin than does the dephosphorylated enzyme. F-actin does not significantly affect the basic kinetic properties of the dephosphorylated form, but acts as a positive effector of the phosphorylated form (Luther and Lee, 1986). Based on the findings that PFK is found in greater abundance in the particulate fraction of stimulated muscle and in the cytosol of unstimulated muscle (Walsh et al., 1980; 1981; Clarke et al., 1980), Luther and Lee (1986), have proposed a mechanism by which phosphorylation of PFK, triggered by muscle stimulation serves as a means to regulate the compartmentalization of the enzyme in order to provide energy to the cellular components where it is needed. The phosphorylated PFK with its increased affinity for F-actin, binds F-actin and its activity is increased, as well as being localised to the muscle matrix. There may be a lag time between phosphorylation and the formation of the PFK-actin complex, hence to conserve energy resources the kinetic activity of the phosphorylated enzyme is lower than that of the PFK-actin complex (Luther and Lee, 1986). In resting muscle, the need for energy is lower, so the PFK is dephosphorylated, and less is associated with the muscle matrix. This is however, only an hypothesis and further experimental evidence is needed, so as yet the role of phosphorylation of muscle and liver PFK remains open to debate.

1.4.8 INTERACTION OF CALMODULIN WITH PHOSPHOFRUCTOKINASE

Recently muscle PFK has been shown to be a calmodulin binding protein (Mayr and Heilmeyer, 1983). Calmodulin, a multifunctional Ca^{2+}-binding protein ubiquitous in eukaryotes, mediates the effect of Ca^{2+} on numerous enzymes and intracellular processes.

Each PFK subunit is capable of binding two calmodulin molecules in a reaction which is Ca^{2+}-dependent. Each binding site has a different affinity, with Kd values of 3mM and 1μM (Buschmeier et al., 1987). Binding at the high affinity site occurs with the dimeric enzyme and with monomers, but not with tetramers (Mayr, 1984b). Obviously, the high affinity sites present in the tetrameric enzyme are inaccessible or attenuated in their binding affinities. The two calmodulin binding sites of rabbit skeletal muscle PFK have been isolated and sequenced (Buschmeier et al., 1987). The sequence revealed that the high affinity site is located in a region of the subunit where two associating dimers have been proposed to make contact (Poorman et al., 1984). The low affinity site corresponds to the C-terminal region of the polypeptide, and contains the site which is phosphorylated by cAMP-dependent protein kinase (Kemp et al., 1981).

PFK is active in the absence of calmodulin, and is regulated by effectors both by allosteric mechanisms, and by changing the association state of the enzyme. Under certain conditions Ca^{2+}-calmodulin (Ca^{2+}-CaM), dependently interferes with regulatory effectors, at one or both levels of regulation (Mayr, 1984a; 1984b), accelerating the transition of the enzyme between an active state and an inactive form, differing essentially in association state and conformation. Whenever the concentration of the enzyme and other effectors of PFK present allow for a significant dissociation of tetramers, Ca^{2+}-CaM strongly binds to the dimers and monomers, and, by stabilizing these states, shifts the equilibrium towards the dissociated inactive forms. Ca^{2+}-CaM binding accelerates a conformational change in the dimers, which makes the inactivation hysteretic, i.e. not simply reversible after its dissociation (Mayr, 1984a). Therefore, the dimers changed in conformation can no longer reconstitute active tetramers if CaM dissociates upon the lowering of Ca^{2+} concentration. Instead, large inactive polymers are instantaneously formed (Mayr and Heilmeyer, 1983; Mayr, 1984a). In the presence of excess Ca^{2+}-CaM, polymer formation from dimers is suppressed, and a slow depolymerisation to inactive oligomers can occur. Reactivation of this CaM inactivated enzyme requires changes in effector concentrations to allow for a back isomerization,

and reassociation of dimers. The isomerization process is promoted by MgATP, while the reassociation reaction is most effectively promoted by F26BP, to completely regain the catalytic activity however, an ongoing complexation with CaM is required (Mayr, 1984a). However, high concentrations of Ca^{2+}-CaM can prevent an inactivation of tetrameric PFK. In this case, a stabilization of tetramers is mediated by the binding of CaM to low affinity sites, which are accessible in the tetrameric state (Mayr, 1984b). The previously described inhibitory sites of high affinity may be buried in the tetramer interior, and may only become available if tetramers dissociate. The hypothesis for the action of calmodulin on living muscle is summarized in Fig. 2.

The structural basis of how CaM can bind and selectively activate or modulate so many different proteins, is far from clear. Studies of a number of naturally occurring peptides and proteins which bind Ca^{2+}-CaM with high affinity have shown a number of common structural features (Malencik and Anderson, 1982; 1983). Notably, an excess of basic amino acids clustered together with hydrophobic residues.

The CaM binding sites sequenced from chicken gizzard myosin light chain kinase (MLCK)(Lukas et al., 1986; Guerrierro et al., 1986), rabbit skeletal muscle MLCK (Buschmeier et al., 1987), can each be divided into two segments; "A" and "B", which share common properties and are separated by a variable number of amino acids.

Segment "A" has a tryptophan residue, which has been shown to have an essential involvement in the binding of CaM to MLCK (Blumenthal et al., 1985; Lukas et al., 1986), and PFK (Buschmeier et al., 1987). Another feature in common in segment "A" is the occurrence of two or three aromatic residues amongst several hydrophobic ones.

Segment "B" contains a three residue cluster of one to three serine residues, neighboured on the N-terminal side by basic residues. This pattern is similar to the recognition sequence for cAMP-dependent protein kinase. It is known that binding of CaM can prevent phosphorylation and vice versa, phosphorylation reduces the binding affinity for CaM (Conti and Adelstein, 1981). Based on such findings it has been suggested that CaM and protein kinase act on common sequences in proteins, subject to dual control by Ca^{2+} and cAMP, and that modification of at least certain CaM binding sites is one of the functions of the cAMP-dependent protein kinase (Malencik and Anderson, 1982; 1983).

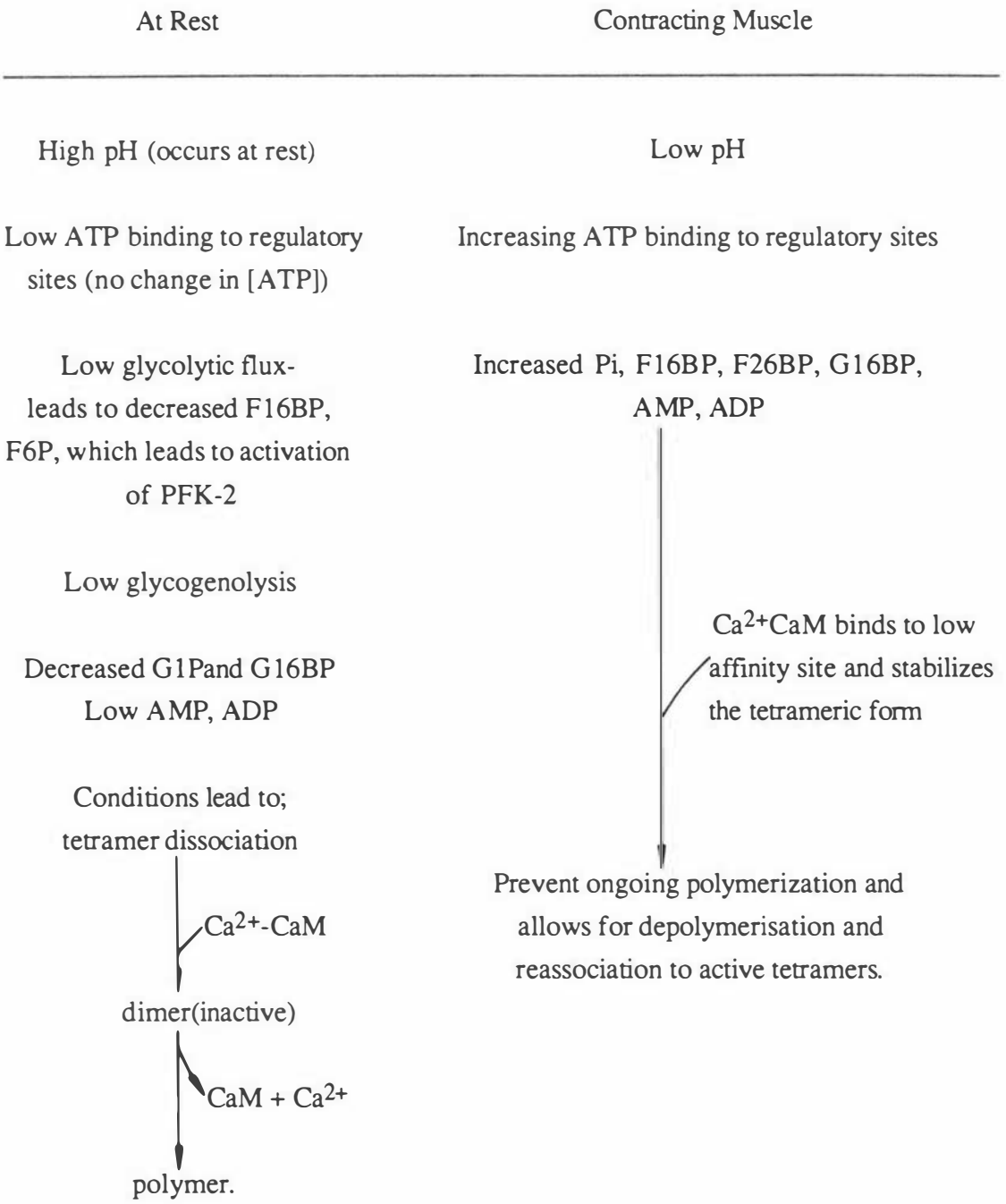


Figure 2: Hypothesis of Calmodulin Action in Living Muscle.

Summary of calmodulin action in living muscle. Under conditions which do not strongly stabilize the PFK tetramer, such as at rest, Ca^{2+}-CaM accelerates the inactivation of PFK due to binding at the high affinity (inhibitory site) Reactivation can occur with a decrease in pH such as occurs in contracting muscle. Excess Ca^{2+}-CaM, can then bind to the low affinity site, and stabilize the tetramer.

Chicken gizzard MLCK can be phosphorylated (Blumenthal et al., 1985), at a site in segment "B". The low affinity CaM binding site of PFK represents the established phosphorylation site of PFK by cAMP-dependent protein kinase (Kemp et al., 1981). No such phosphorylation site has been shown for the high-affinity PFK site despite the presence of the "recognition" sequence of cAMP-dependent protein kinase.

Several peptides which bind CaM can form basic, amphiphilic α-helices. Cox et al. (1985), have gone so far as to suggest that the minimal requirement for a high affinity CaM binding peptide is a basic amphiphilic α-helix of at least 3 turns in length. The CaM binding sites from chicken and muscle MLCK have been calculated as having a high probability for adopting an amphipathic α-helix (Cox et al., 1985; Blumenthal et al., 1985; Lukas et al., 1986). Likewise, segment "A" of both CaM binding domains of PFK may adopt such a structure. But in segment "B", helix destabilizing residues are found. Secondary structure predictions for sequences phosphorylated by cAMP-dependent protein kinase, suggest that most adopt a β-turn structure around the phosphorylation site (Small et al., 1977). It may in fact be, that the requirement for the amphiphilic α-helix postulated by Cox is satisfied by the adoption of such a structure in segment "A", while segment "B" is free to form the predicted β-turn.

1.4.9 EFFECT OF HORMONES ON PHOSPHOFRUCTOKINASE

Epinephrine (adrenalin) administration has been found to increase glycolysis, by causing an increase in PFK activity. This increase is due to a decrease in sensitivity to ATP inhibition, and a higher affinity for F6P (Mansour, 1972). Epinephrine also causes a stimulation of gluconeogenesis, and it has been suggested that the effects of epinephrine are mediated by changes in the F26BP levels in a similar manner to glucagon (Kuwajima and Uyeda, 1982), since the levels of F26BP have been shown to increase in rat skeletal muscle, following epinephrine administration (Hue et al., 1982; Boscá et al., 1985).

In hepatocytes from fed rats, and starved rats, and hepatocytes incubated with glucose, vasopressin stimulates PFK activity, and therefore glycolysis . This increase in activity is due to the accumulation of F26BP (Hue et al., 1981).

The short-term regulation of PFK by insulin, which results in the activation of PFK (i.e. opposite to glucagon), is also thought to be mediated through F26BP. Insulin is thought to act through affecting the levels of F26BP by mediating its tendency to be degraded (Dunaway and Weber, 1974b; Dunaway et al., 1978b).

1.4.10 PHOSPHOFRUCTOKINASE ASSOCIATION WITH STRUCTURAL ELEMENTS OF THE CELL

The contribution of structural proteins to cellular function has long been recognised in muscle , where the precise orientation of structural proteins is essential of translating microscopic movements to macroscopic forces. However, the implicationsfor such organisation for the cellular distribution of enzymes has not often been addressed. Several enzymes exhibit a reversible intracellular partitioning between soluble and particulate forms of the cell. The association of glycolytic enzymes with the structural proteins of muscle has been widely studied (Masters, 1978). Histochemical experiments have shown that the glycolytic and glycogenolytic enzymes are located within the I band of the muscle fibre, corresponding to the site of the thin filaments in the relaxed myofibril (Sigel and Pette, 1969; Arnold et al., 1969). Experiments in vitro have demonstrated the adsorption of several glycolytic enzymes to actin, with PFK being the most strongly adsorbed (Clarke and Masters, 1975).

Myofibrils, F-actin and reconstituted thin filament exert striking effects on the catalytic properties of rabbit muscle PFK (Liou and Anderson, 1980; Choate et al.,1985). The addition of any of these components, partially reverses the inhibition of PFK seen at high ATP concentrations, and increases the apparent affinity of the enzyme for F6P with a slight change in V_{max}. F-actin is more effective than the reconstituted thin filament in activating PFK.

The extent of binding of the glycolytic enzymes to actin in live anesthetized animals has been found to depend on the functional state of the muscle (Walsh et al., 1981). Electrical stimulation led to significant increases in the amount of PFK, aldolase and glyceraldehyde-3-phosphate dehydrogenase bound to the particulate fraction. The enhanced binding appeared to be the result of a decrease in pH caused by glycolysis. The increase in enzyme binding was rapidly reversible, as it was shown that the amount of enzyme bound, quickly returned to control values when the muscles were allowed to recover under aerobic conditions following cessation of stimulation.

The N-terminal segment of Band 3 protein is suggested to bind to the adenine nucleotide activation site. This would cause the activation of the PFK tetramer demonstrated by several workers, following incubation of PFK with erythrocyte membranes (Karadsheh and Uyeda, 1977), due to the relief of allosteric inhibition by ATP and 2,3 diphosphoglycerate. However, a slow inactivation of the enzyme also

results, with Band 3 shifting the equilibrium from the active tetramer to the inactive dimeric form. This is thought to be brought about by binding to the dimer with higher affinity than the tetramer, presumably because of interactions with the dimer that are hindered within the tetramer (Jenkins et al., 1985).

As described already (Section 1.4.7.7), phosphorylated forms of PFK have a higher affinity for actin than dephosphorylated forms (Luther and Lee, 1986; Kuo et al., 1986). PFK and filamentous actin from rabbit skeletal muscle form a specific association as demonstrated by the distinct cross-striation shown by electron microscopy of the negatively stained proteins. The periodicity of these striations, 37 ± 1nm, corresponds to the cross-over spacing of the actin helix 36 ± 1nm (Roberts and Somero, 1987). The reversible association of PFK with actin may play a role in regulating PFK activity, and therefore, glycolysis during periods of metabolic acidosis.

1.5 pH AND PROTONATION

Low pH has been found to decrease the affinity of PFK for F6P (Trivedi and Danforth, 1966; Reinhart, 1985), as well as to cause inactivation due to dissociation (Paetkau and Lardy, 1967; Hofer and Pette, 1968; Mansour, 1965). This process can be reversed by raising the pH (Hofer and Pette, 1968), and by the addition of ligands (Alpers et al., 1971; Lad et al., 1973), with a corresponding increase in activity and the formation of active enzyme of higher molecular weight.

Changes in pH do occur within muscle cells (Caldwell, 1956), and do affect glycolysis (Cori, 1956; Ronzoni and Kerly, 1933). In a series of contractions, muscle first becomes alkaline, because of hydrolysis of creatine phosphate (Dubuisson, 1939), and at this stage glycolysis may well be promoted by increased activity of PFK. Later, as glycolysis proceeds, the pH decreases due to the accumulation of lactic acid (Dubuisson, 1939; Karpatkin et al., 1964). The decrease in apparent affinity of PFK for F6P brought about by this fall in pH probably explains the progressive rise of hexose-phosphates in contracting frog sartorii which occurs as lactic acid accumulates (Karpatkin et al., 1964). It may also account for the fact that lactic acid production stops before the pH falls low enough to damage the cell (Danforth, 1965).

1.5.1 MECHANISM FOR THE INACTIVATION OF PHOSPHOFRUCTOKINASE BY DECREASING pH

Bock and Frieden (1976a, 1976b)have proposed a mechanism for the inactivation of PFK by decreasing the pH. This involves a protonation step followed by an isomerization to an inactive form, and then dissociation to a species of one half the original molecular weight. Reactivation of the inactive enzyme, occurs by a kinetically different pathway, involving deprotonation of an inactive dissociated form, to a form which may either isomerize to another inactive form, or dimerise to the active enzyme (Bock and Frieden, 1976a).

The inactivation of PFK at low pH is due to the increased ratio of protonated to unprotonated groups at the ATP binding site, which inhibits PFK by facilitating ATP binding and by simultaneously reducing the affinity for the substrate F6P. Bock and Frieden (1976a, 1976b) postulated that the residues responsible for the pH-dependent loss of activity were histidines, which underwent specific ionization (Bock et al., 1975). This was supported by the loss of 3 H^+ during the formation of the tetramer from monomers between pH 6.0-8.55, since the ionizable residues has an apparent pKa of 6.9 (Luther et al., 1986).

Following studies into the effects of ligands, including substrates and allosteric effectors on the activity of PFK, Bock and Frieden (1976b), have suggested that the ligands exert their effect by binding preferentially to either the protonated or unprotonated forms of the enzyme, thus shifting the apparent pKa of the ionizable group involved in the inactivation or reactivation process. ATP and F6P influence the apparent pKa to different extents, and in different directions, with ATP binding preferentially to the protonated form, and F6P to the unprotonated form. Enzyme which has been inactivated by ATP can be reactivated by the addition of F6P. Inactivation and reactivation in the presence of these ligands can occur by kinetically different pathways, as has been found for these processes in the absence of ligands. Citrate appears to bind preferentially to protonated forms of the enzyme, while F16BP and AMP bind preferentially to unprotonated forms. ATP and citrate, shift the pKa to a higher pH, while AMP and F6P shift the pKa to a lower pH.

It has also been suggested that the phosphorylation of PFK may alter the ionization of the residues involved in protonation, with the resulting conformational change causing the altered affinity of the ligands (Sakakibara and Uyeda, 1983; Kitajima et al., 1983). At a given pH the phosphorylated enzyme (pKa=6.86) would exist as the protonated

form as opposed to the dephosphorylated (pKa=6.78) unprotonated form (Kitajima et al., 1983). Consequently the phosphorylated enzyme could bind F6P less effectively than the dephosphorylated enzyme, but would bind ATP more effectively. However this explanation is highly speculative (Sakakibara and Uyeda, 1983; Kitajima et al., 1983).

During high rates of rest-to-work transition where the glycolytic flux in muscle may be activated by three orders of magnitude (Newsholme and Crabtree, 1978), the intracellular pH often falls below the pH reported to be inhibitory to PFK. After electrical stimulation of cat biceps the internal pH falls to 6.4, but glycolysis and muscle work can be reactivated during the initial phase of recovery before the pH returns to the pre-exercise levels of between pH 7.0-7.2 (Meyer et al., 1982). Moreover there are extreme cases in the literature where the internal pH of the myocardium has been reported to fall well below pH 6.4 before there is any curtailment of muscle glycolysis (Bailey and Seymour, 1983). The conclusion to be drawn from these studies is that low pH does not necessarily limit glycolysis. By implication this means that mechanisms must exist to reverse or preclude pH inhibition of muscle PFK. Dobson et al. (1986), have concluded that the modulator, or combination of modulators, that increase the ratio of unprotonated to protonated forms of the enzyme could in effect provide muscle cells with a means of avoiding ATP inhibition in the face of falling pH. Not only do the positive modulators such as F26BP, G16BP and AMP counteract the pH-mediated ATP effect, but they also stabilize the unprotonated form of the enzyme against citrate inhibition. Both these kinetic and regulatory features of PFK catalysis are considered to be of importance in the co-ordinated control of glycolytic flux during altered work states.

In rat liver (Reinhart, 1985), decreasing pH lowers the affinity of the enzyme for F6P by a different mechanism to that which occurs in muscle. In liver lowering the pH directly affects the intrinsic affinity of the enzyme for F6P, the pKa for this effect is pH 8.1. This difference probably reflects a very real difference in the molecular mechanism of the allosteric regulation of these two enzymes, which is consistent with earlier observations that unlike the muscle enzyme, liver PFK continues to exhibit co-operativity and MgATP inhibition even at high pH (Reinhart and Lardy, 1980a).

1.6 GLYCOLYSIS AND GLUCONEOGENESIS

Although glycolysis is a universal metabolic pathway, the relative importance of the breakdown of glucose or glycogen to provide energy, and of the reversal of these

processes varies greatly from one tissue to another. Compare for example brain, muscle and liver, the brain has a fairly constant demand for glucose that does not vary greatly at any level of mental activity. Glycolysis in skeletal muscle on the other hand varies greatly. In resting muscle, glycolysis is almost completely shut down, but a surge in glycolytic flux occurs during, and immediately following exercise. The liver passes a very modest amount of glucose through this pathway and, in fact usually runs the pathway in reverse to synthesize glucose, since glycogen serves as a store for the generation of blood glucose. In addition, connections between carbohydrate metabolism and the metabolism of amino acids and lipids are particularly important in the liver in contrast to other tissues.

Despite the common occurrence of the enzymes of the glycolytic sequence in all tissues, there does not appear to be a single mode of regulation of the pathway in all tissues. Almost all of the glycolytic enzymes exist as isozymes, and the occurrence of typically "liver" or "skeletal muscle" isozyme distributions is particularly noteworthy. In general it is observed that specific isozymes are present in various tissues that are particularly sensitive to those metabolites that reflect the energy state of the cell of that particular tissue. Differences in the levels of metabolites among tissues can also provide diversity in the regulation observed.

1.7 REGULATION OF PFK UNDER PHYSIOLOGICAL CONDITIONS

1.7.1 REGULATION OF MUSCLE PHOSPHOFRUCTOKINASE UNDER PHYSIOLOGICAL CONDITIONS

Skeletal muscle achieves the transition from prolonged rest to the attainment of maximal activity in a fraction of a second. At rest, the majority of the cell's energy requirements are provided via the TCA cycle. During contraction however, to meet the sudden increase in the requirement for ATP, much of the energy is derived from glycolysis, using the store of muscle glycogen as well as blood glucose. Creatine phosphate is an important regulator because it is the first energy reservoir to be exhausted in stressed muscle (Hearse and Chain, 1972). The large excess of creatine phosphate compared to ATP, and the equilibrium catalysed by creatine kinase assures a high ratio of ATP to ADP and AMP under most conditions. Helmreich and Cori (1965), have shown that the levels of ATP do not vary greatly under conditions where contractions induced by electrical stimulation have caused a profound increase in glycolytic flux. This increase in flux could be the result of falling creatine phosphate levels, which would lead to deinhibition of PFK, pyruvate kinase (Kemp, 1973), and glyceraldehyde-3-phosphate

dehydrogenase (Oguchi et al., 1973). Regulation of PFK by changing levels of ATP, ADP and AMP may only be significant under conditions of extreme stress where energy reserves are severely depleted.

Muscle contraction has also been shown to increase the phosphorylation state of muscle PFK, this in turn leads to a higher affinity of PFK for actin, which leads to further activation of PFK. This increased affinity of PFK for actin ensures the compartmentalization of PFK and perhaps the other glycolytic enzymes to the region where ATP is required.

Fatigued muscle derives its energy largely from the anaerobic transformation of glucose or glycogen to lactic acid. This use of anaerobic pathways can be self-limiting, because in spite of the rapid diffusion of lactic acid into the blood stream, enough accumulates in the muscles to eventually exceed the capacity of the tissue buffers, thus producing a decrease in pH, which would inhibit the glycolytic enzymes. A mechanism by which PFK could remain active at these decreased pH values has been described in Section 1.5.1. Citrate, which is at high levels when fatty acids are being metabolised in muscle and heart, is a potent inhibitor of PFK. At high levels it reduces the glycolytic contribution to energy metabolism as well as lactate formation under conditions when ATP is being actively formed by oxidative phosphorylation.

1.7.2 REGULATION OF LIVER PHOSPHOFRUCTOKINASE UNDER PHYSIOLOGICAL CONDITIONS

In the liver, glycolysis is of minor importance in comparison to gluconeogenesis. Gluconeogenesis is a vital function which provides an adaptive mechanism for mammalian systems to produce glucose from non-carbohydrate precursors. The reaction cycle formed from PFK and F16BPase is of significance to the regulation of glucose metabolism (Hers, 1976; Weber et al., 1967). When PFK and F16BPase are simultaneously active the capability of cell metabolism to maintain ATP homeostasis is diminished and the net flow in either the glycolytic or gluconeogenic direction is decreased (Hers and Hue, 1983). For efficient glycolysis, F16BPase activity must be suppressed, while for efficient gluconeogenesis a high ratio of F16BPase to PFK activity must be maintained.

The greater sensitivity of liver PFK to ATP inhibition should be related to the observations that lower levels of ATP have been reported for liver (Williamson et al., 1969), as compared to frog muscle (Helmreich and Cori, 1965), and rat heart

(Williamson, 1965). The relative insensitivity of the liver enzyme to most of the other activators and inhibitors makes one question their role as control factors in the liver. Indeed control by AMP and ADP may only be significant in muscle, which in some circumstances must produce energy under partially anaerobic conditions.

Despite the fact that F26BP is a potent inhibitor of F16BPase (Van Schaftingen and Hers, 1981a; Pilkis et al., 1981b; Ganson and Fromm, 1982), there is good experimental evidence that this inhibition is not complete. Indeed, in the livers of fed rats (Van Schaftingen et al., 1980a), and in isolated hepatocytes (Van Schaftingen et al., 1980b), there is recycling of metabolites between F6P and F16BP, indicating that PFK and F16BPase operate simultaneously. High rates of cycling are observed only at the transition between glycolysis and gluconeogenesis. Glucose present in fed livers causes an increase in the concentration of F26BP, this results in the activation of PFK. F16BPase although inhibited by F26BP remains partially active due to the high concentration of its substrate F16BP, which is nearly 10-fold greater than in the fasting state. The advantage of this mechanism seems to be the prevention of an excessive accumulation of F16BP in the liver, because F16BP is a potent activator of pyruvate kinase, it seems probable that the activity of the latter enzyme is also increased under these circumstances, also leading to the reduction in the levels of F16BP. Under conditions of starvation, and in conditions when F26BP levels do not change, the effect of phosphorylation/dephosphorylation, along with other effectors may regulate PFK activity and therefore glycolysis.

1.8 ROLE OF SPECIFIC RESIDUES IN ENZYMIC ACTIVITY

1.8.1 THIOL GROUPS

The susceptibility of PFK to oxidizing agents such as ferricyanide, H_2O_2, I_2, quinones, and alloxan was recognised at an early stage (Engel'hardt and Sakov, 1943). That certain thiol groups were essential for PFK was shown by the reversible loss of activity following sulfhydryl oxidation with oxidised glutathione (Paetkau and Lardy, 1967).

Skeletal muscle PFK contains approximately 16 thiol groups per monomer (Kemp and Forest, 1968). These can be classified into 5 groups based on their reactivity towards 5,5' dithio 2-nitrobenzoic acid (DTNB), as follows:

(1) The class I group consists of a single, highly reactive thiol group, which can be completely protected by MgATP (Kemp and Forest, 1968; Kemp, 1969a).

Modification of this group with DTNB results in the decrease of enzyme activity by approximately two-thirds (Kemp, 1969b).

(2) The class II thiols consist of two less reactive thiols, which can be protected by adenine nucleotides or F6P. Following the reaction of these thiol groups, greater than 90% of the enzyme activity was lost.

(3) One thiol which reacts at 0.4 of the rate of the second group comprises the class III group.

(4) Five thiols make up the class IV group. These as well as the class III thiol, are pH-dependent, and only become available for rapid reaction as the pH is increased to 9.0. Two of these thiols can be protected by F16BP.

(5) The remaining thiols make up the class V thiol group. These react very slowly, and can only be made available for rapid reaction if the tertiary structure of the enzyme is destroyed.

The apparently unique reactivity of the thiol in class I has been extensively studied by Kemp and his co-workers (Kemp and Forest, 1968; Kemp, 1969a; 1969b). This thiol has a reactivity 2 x 10^4 times greater in the native than in denatured PFK, suggesting that this thiol is activated by virtue of its location in the native enzyme. The major effect of reaction of DTNB with this class I thiol is to cause a decrease in V_{max}, indicating that this activated thiol may be affecting the active site of the enzyme (Kemp, 1969b). Modification of the class I thiol with [^{14}C]-fluorodinitrobenzene (FDNB), which reacts more slowly with the class I thiol than does DTNB, was carried out by Mathias and Kemp (1972), their results showed that activators such as cAMP, AMP, ADP, F6P and F16BP were able to increase the reactivity of the thiol group when present at low concentrations. Inhibitors MgATP, phosphocreatine, citrate and phosphoenolpyruvate lead to a decrease in the reactivity. These changes in reactivity were interpreted as being due to different conformational changes at the active site brought about by the binding of different effectors. Mathias and Kemp (1972), suggested that the class I thiol was not directly involved in the binding of ATP, but its accessibility is reduced by the conformational change that results from MgATP binding. The class I thiol has been isolated as a fragment of a tryptic digest, and sequenced (Simpson et al., 1977). This highly reactive cysteine lies in the N-terminal half of the protein (Ogilvie, 1980), at position number 88 in comparison with the rabbit muscle sequence (Poorman et al., 1984). Comparison of this region of the sequence from rabbit muscle PFK with that

from E. coli shows that the cysteine, and surrounding residues are highly conserved (Hellinga and Evans, 1985). The highly reactive cysteine is analogous to a cysteine in the Bacillus stearothermophilus (Bs) PFK which has been found by x-ray crystallography to form H-bonds between the main chain amide and carbonyl to the ribose hydroxyl groups of ATP (Hellinga and Evans, 1985), indicating that this cysteine may be important in the binding of ATP in mammalian PFK also.

"Essential" protein sulfhydryl groups are ubiquitous, and it is a common feature of a large number of enzymes to be inactivated by reagents such as iodoacetamide and to be stabilized by reducing agents such as β-mercaptoethanol (βME), and dithiothreitol (DTT). This common occurrence of reactive and essential sulfhydryl groups could just be chance, or it could reflect a more subtle and useful purpose for the existence of these groups.

The oxidation states of the sulfhydryl groups of PFK have been found to influence the physical properties of the enzyme. The modulation of PFK activity is linked to a subunit association-dissociation and changes in the qua ternary structure. Glutathione, inactivates PFK and results in a lower S_{20w} value than the native enzyme (at pH 7.0, 100μg/ml of protein, S_{20w}=12 for inactive, and 13 for active enzyme). This can be reversed by the addition of DTT (Luther et al., 1983). The authors have proposed the existence of an inactive tetramer which is capable of undergoing association-dissociation, but cannot undergo the conversion to the active tetrameric form. Activators and inhibitors perturb the equilibria among the inactive species, while only substrates can favour the formation of the active form.

Recently it has been suggested that the modulation of PFK activity by thiol/disulfide exchange, in vivo is feasible (Gilbert, 1982). The oxidation/reduction of PFK by biological disulfides is an equilibrium process. The equilibrium constant for the reaction E_{red} + GSSG ⇌ E_{ox} + GSH at pH 8.0 is 7.1 in the absence of substrates, while in the presence of 0.1mM ATP the K_{eq} shifts to 2.5. This suggests that ATP binds more tightly to the reduced enzyme than to the oxidised form, since the addition of ATP shifts the equilibrium towards the reduced form of the enzyme.

The ratio of [GSH]:[GSSG] has been found to vary between 5-20 depending on the metabolic state of the animal. The redox equilibrium for PFK lies within this range, therefore the enzyme activity could fluctuate between 40-80% if the enzyme were maintained in redox equilibrium. PFK and F16BPase are affected in reciprocal fashion by thiol/disulfide exchange i.e. PFK is inhibited by GSSG, while F16BPase is

activated (Nakashima et al., 1969; Pontremoli and Horecker, 1970). Therefore it is possible that disulphide exchange with GSSG as the third messenger, and cAMP as the second messenger provides some degree of regulation of PFK in mammals.

1.8.2 IDENTIFICATION OF REACTIVE CYSTEINE RESIDUES

A tryptic peptide, containing a class II thiol which was protected by F6P and AMP, was isolated and sequenced (Latshaw et al., 1987), after labelling with [^{14}C]-iodoacetic acid.

```
                          !
Bs  114 EHGFPCVGVPGTIDNDIPGTDFTIGFDTALNT 145
RM  152 SSYLNIVGLVGSIDNDFCGTDMTIGTDSALHR 183
                          *   *
```

* Residues labelled with [^{14}C]-iodoacetic acid

! Catalytic residue at the active site of Bs PFK (127).

Residue 127 of the bacterial enzyme has been implicated in the enzyme's interaction with F6P (Hellinga and Evans, 1985). If indeed the binding site of mammalian enzyme is similar, the modified cysteine at position 169 (cf 131 bacterial enzyme), could be close to the F6P site, and its modification might be expected to reduce activity. The proximity of the F6P site would also help to explain the protection provided by the substrate, against reaction of the thiol with DTNB.

Another peptide which also contained a labelled cysteine was isolated and sequenced following a sub-digestion of a tryptic peptide with SV8 protease (Latshaw et al., 1987).

```
Bs  172 HAGDIALWSGLAGGAETILIPEADYDMNDVIARLK 206
RM  210 HCGYLALVTSLSCGADWVFIPECPPDDNWEDHLCR 244
                          *
```

* Residue labelled with [^{14}C]-iodoacetic acid.

This tryptic peptide was identified as residues 210-244 of the mammalian sequence, and is homologous to residues 172-206 of the Bs enzyme. The labelled cysteine was localised from the subdigestion as being at residue 232. Due to the large number of cysteine residues in this peptide, it is possible that the partial protection of several of

these thiols by F6P may occur so together these cysteines could comprise the second class II thiol.

1.8.3 REACTIVE METHIONINE RESIDUES

Two rapidly reacting methionine residues were labelled with [^{14}C]-iodoacetic acid, and the tryptic peptides containing these residues isolated and sequenced (Latshaw et al.,1987). One was present in the same fragment as the highly reactive class II cysteine residue. Like the cysteine, methionine 173 was protected by the presence of F6P and cAMP. The sequence adjacent to the second of the two rapidly reactive methionine residues is located at residues 66-87 of the mammalian enzyme, which corresponds to and is homologous to residues to 51-72 of the bacterial enzyme.

```
Bs  51 KLEVGDVGDIIHRGGTILYTAR 72
RM  66 EATWESVSMMLQLGGTVIGSAR 87
                  *
```

* Residue labelled with [^{14}C]-iodoacetic acid.

This suggests that the methionine residue corresponds to an aspartic acid residue at position 59 of Bs and E. coli PFK. Since position 59 has been suggested to be involved in the binding of ADP to the allosteric site in the bacterial enzymes, it is possible that the ADP-activating site in the mammalian enzyme has evolved from this site. Results to test these two possibilities showed that methionine 74 had no effect on the interaction of the adenine nucleotides at any of the potential binding sites: the catalytic site, ATP inhibitory site, or the AMP-activating site (Latshaw et al., 1987). The authors suggested two possibilities: that either the inference of the bacterial enzyme having both the side chain, and main chain amide of residue 59 involved in the ligand-binding site is incorrect; or that the structures of the two enzymes in this region are not entirely homologous. With regard to the former possibility, an examination of the published crystal structure of the bacterial enzyme (Evans and Hudson, 1979), suggests clear involvement of the main chain amide, but the role of the side chain is less convincing. Involvement of the main chain amide in ligand binding may not be disrupted by modification of the side chain. That the structures may not be totally homologous in this particular region can be seen by examining the area immediately around position 59 (bacterial). The sequence has greater homology with residues at the C-terminal side of residue 63 (bacterial).

A region of very strong homology is also found towards the N-terminus, from residues 6-45 of the bacterial enzyme. Residues 46-63 of the bacterial enzyme, including the ligand binding residues in question, show little homology with the mammalian enzyme, and undoubtedly indicate divergence to provide a site of somewhat different specificity. The facile reaction of iodoacetic acid in this region of the molecule clearly suggests a unique microenvironment (Latshaw et al., 1987).

One possibility not investigated by the authors is that methionine 74 may be part of the citrate binding site.

1.8.4 REACTIVE HISTIDINE RESIDUES

Modification of histidine residues by photo-oxidation (Ahlfors and Mansour, 1969), or ethoxyformylation (Setlow and Mansour, 1970), results in the desensitization of the enzyme to inhibition by ATP or citrate, and to activation by AMP or cAMP. The catalytic activity is not destroyed to any significant degree by either modification procedure. These results seem to provide a basis for the involvement of histidine in the regulatory binding site of PFK.

1.9 METABOLITE BINDING STUDIES

The reported values for the number of metabolite binding sites vary widely (Table XI). Concensus of opinion would indicate that the mammalian PFK monomer contains three ATP binding sites, one or two F6P binding sites, one adenine binding site, one citrate binding site, and one FBP binding site.

Of the three binding sites for ATP, one is identical to the AMP binding site (Kemp and Krebs, 1967), one represents the catalytic site (Kemp and Krebs, 1967), and one is the ATP inhibitory site. The three sites differ from each other with respect to their ligand specificities and biological actions. The catalytic site has a high affinity for ATP (Kd 1μM), while the inhibitory site has a lower affinity (Kd 100μM)(Wolfman et al., 1978; Pettigrew and Frieden, 1979a).

The binding of ATP appears to be biphasic (Roberts and Kellett, 1980). The binding of ATP to PFK has been shown to cause a decrease in the reactivity of the class I thiol (Kemp, 1969a; Mathias and Kemp, 1972). This suggests that a change in conformation of the enzyme occurs leading to a decrease in the local mobility of the

TABLE XI

PROPOSED NUMBER OF METABOLITE BINDING SITES IN PFK

	Binding sites/monomer	Ref
ATP	3	(i)
	3	(ii)
	3	(iii)
	3	(iv)
	2	(v)
	3	(vi)
F6P	2	(i)
	1	(ii)
	1	(vii)
	1.5	(iii)
	2	(viii)
Adenine	3	(i)
	1	(ii)
	1	(viii)
	1	(ix)
Citrate	1	(i)
	1	(x)
Sugar Bisphosphates	1	(xi)

Number of different metabolite binding sites proposed for mammalian PFK by different laboratories.

(i) Garfinkel, 1966
(ii) Kemp and Krebs, 1967
(iii) Lorenson and Mansour, 1969
(iv) Ogawa and Atkinson, 1985
(v) Wolfman et al., 1978
(vi) Foe et al., 1983
(vii) Hill and Hammes, 1975
(viii) Setlow and Mansour, 1972
(ix) Pettigrew and Frieden, 1978
(x) Colombo et al., 1975
(xi) Liou and Anderson, 1978

class I thiol due to either immobilization or burial of the group (Jones et al., 1972; 1973), resulting in a decrease in its reactivity.

Citrate, 3-phosphoglycerate, phosphoenolpyruvate, and creatine phosphate each increase the affinity of PFK for ATP (Kemp and Krebs, 1967; Colombo et al., 1975), while AMP, cAMP and Pi decrease the affinity for ATP (Kemp, 1969a).

Muscle PFK treated with subtilisin resulted in an inactive enzyme of molecular weight 74 000 which still retained its tetrameric structure (Riquelme and Kemp, 1980). Binding studies revealed that only one adenine binding site remained in contrast to the three present in the native enzyme. Both the ATP and F6P binding sites were lost along with the ATP inhibitory site. cAMP bound to the proteolysed enzyme with the same affinity as the native enzyme. Binding sites for F16BP and AMP were retained (Gottschalk et al., 1983). These data suggests a relatively discrete domain essential to the catalytic activity, but structurally distinct from several allosteric regulatory sites.

ADP, cAMP and AMP competively bind to the same site with dissociation constants of 0.5, 0.6, and 1.8μM respectively. The dissociation constant for cAMP was found to decrease in the presence of F6P and F16BP (Kemp and Krebs, 1967). Structural mapping studies of rabbit muscle PFK have shown that the distance between the cAMP binding site and the most reactive sulfhydryl group of PFK is 28 ± 6Å (Craig and Hammes, 1980).

A tryptic peptide from sheep heart PFK labelled with the affinity label p-fluorosulfonyl [^{14}C]-benzoyl-5'-adenosine, which binds specifically to the allosteric activator site has been isolated and sequenced (Weng et al., 1980).

Ec	262	ILASRMGAYAIDLLLAGY	279
Bs		VLSARLGARAVELLLEGG	
SH	673	NFATKMGAK	681
		*	
RM		MGAKAMNWMAGK	689
		*	

* Residue labelled with p-fluorosulfonyl [^{14}C]-benzoyl-5'-adenosine

It was suggested (Weng et al., 1980), that the side chain of the labelled lysine (677) was involved in the binding of the phosphate moiety of the allosteric activators, AMP,

cAMP and ADP. They also suggested that the C-terminal lysine residue in this sequence, or the lysine or arginine residue preceding this sequence may also be involved in this binding. Evidence in support of this came from the work of Kemp et al. (1987), who isolated a tryptic peptide from rabbit muscle PFK which contained a labelled lysine, corresponding to that at position 681 in the sheep heart. Thereby suggesting that both the lysine residues at positions 677 and 681 of the rabbit enzyme are involved in a nucleoside phosphate binding site. Since the adenine moiety in NAD^+ has been found to bind to a hydrophobic pocket in several enzymes (Rossman et al., 1975), the aromatic ring of the phenylalanine at position 674 may play a role in the binding of the adenine moiety if it is properly oriented (Weng et al., 1980).

At the time of the publication of the sheep heart PFK adenine nucleotide binding site sequence, the complete amino acid sequence of mammalian PFK was unavailable. Therefore the authors compared their sequence with that of the Bacillus stearothermophilus enzyme, and suggested it was homologous to the region 17-25 of the Bs sequence, which was known to contain two arginine residues which bound to the phosphate groups of allosteric effectors (Evans and Hudson, 1979). Since then, the complete amino acid sequence of rabbit muscle PFK has become available (Lee et al., 1987), and a comparison of the complete sequence with that of the bacterial enzyme shows that the adenine binding site region is not homologous to the region suggested by Weng et al. (1980). The region of the adenine site corresponds to a region of the bacterial enzyme where no contacts to effectors are proposed at all (Hellinga and Evans, 1985).

Mammalian PFK is thought to have evolved from the bacterial enzyme, of molecular weight 35 000, by gene duplication, fusion, and mutation of duplicated catalytic and regulatory sites to generate additional allosteric sites (Poorman et al., 1984). The "hinge" region joining the two halves of the molecule is therefore an additional structure which must be fitted into the molecule. Since this hinge is located around the region of the adenine binding site, its presence may have necessitated the movement of the site in mammalian PFK to a site distinct from that of the bacterial enzyme. Thereby explaining the lack of complementarity between the binding sites of the mammalian and bacterial enzymes.

One mole of citrate is bound per monomer of PFK. For tight binding MgATP is required, in the absence of either Mg^{2+} or ATP very weak binding is observed. Both phosphoenolpyruvate and 3-phosphoglycerate compete with the binding of citrate, indicating a common binding site on the enzyme for these three inhibitors. Creatine

phosphate does not compete with citrate binding, and an additional site for the binding of creatine phosphate, distinct from the ATP site is indicated (Colombo et al., 1975).

The amino acid sequence at the allosteric site for citrate has been determined for rabbit muscle PFK (Kemp et al., 1987). Since the citrate binding site is likely to have multiple positive charges, pyridoxal phosphate was used to label the site. The sequence of the peptide isolated from a tryptic digest matches the sequence at positions 555-563 of rabbit muscle PFK (Poorman et al.,1984), which is homologous to the Bs sequence 153-161.

```
Bs  153 IRDTATSHE 161
RM  555 IKQSAAGTK 563
          *
```

* Residue labelled with [^{3}H]-pyridoxal phosphate

The arginine in position 154 of the bacterial enzyme has been implicated by crystallography to be important in the allosteric binding site (Hellinga and Evans, 1985). This residue is homologous to the phosphopyridoxalated lysine, which suggests, as predicted by Poorman et al. (1984), that this allosteric site of the bacterial enzyme has evolved into one of the allosteric binding sites of rabbit skeletal muscle PFK.

Sheep heart PFK appears to have two types of binding site for F6P, a low affinity site (Kd 11μM) and a high affinity site (Kd 0.2μM). The binding of F6P to both these sites is influenced by effectors. Fructose bisphosphates appear to bind to a single site on the enzyme. F26BP binds to the enzyme with greater affinity than F16BP, which in turn binds with greater affinity than G16BP (Foe et al., 1983). The authors have suggested that very subtle differences in the conformations of PFKs exist when bound by F26BP, and when bound with F16BP, with F26BP inducing a "less inhibitable" subconformation due to a better fit at the sugar bisphosphate binding site. Poorman et al. (1984) have proposed that the sugar bisphosphate binding site has evolved from a mutated F6P binding site.

1.10 THE EVOLUTION OF PROTEINS

Since the mammalian PFKs are thought to have evolved from the bacterial enzyme it is useful to discuss the manner by which this can occur. The two most critical genetic

phenomena leading to changes in protein structure are: a) single base substitutions, b) gene duplication. The majority of amino acid replacements occurring during protein evolution are the result of single base substitutions. Most of these tend to be relatively conservative, eg valine to leucine, and would therefore be tolerable under a variety of circumstances. However a neutral change in one position may lead to a relaxation of the protein structure, and allow the acceptance of more radical amino acid replacements at other important regions of the structure. Hence conservative changes in one position can affect the acceptance rate of a substitution, which previously would have been rejected.

About one-third of all single base substitutions are likely to result in a change in the charge of the residue. Such changes could lead to alterations in the conformation of the protein, resulting in different ionizable residues to those in the unmodified protein, being exposed to the surrounding medium.

Many enzymes in their active form are composed of several polypeptide subunits. The regions of the polypeptides involved in the contacts between subunits are highly conserved (Klotz, 1970), this is to be expected since any mutations which prevented subunit association would abolish the catalytic activity, and would therefore be selected against. In a protein structure such as PFK, where each monomer contacts two other subunits, each monomer is predicted to lose approximately 29% of its accessible surface in tetramer formation (Teller, 1976). This represents a considerable portion of the protein which must remain essentially unchanged. Evidence that these regions have been conserved in muscle and liver PFK comes from the fact that a complete set of hybrid isozymes structures can be formed following the dissociation of muscle (M_4) and liver (L_4) PFK subunits, and their subsequent reassociation (Tsai and Kemp, 1972).

The catalytic sites of enzymes are usually highly conserved, and it has been suggested that the outsides of proteins are often better able to adjust to amino acid replacements than the interior, where the packing demands are more severe (Dickerson, 1971). In support of this, the "surface residues" of mammalian haemoglobin have been found to be experiencing ten times more evolutionary change than the residues around the haem pocket (Kimura and Ohta, 1973).

If mutations were limited to individual amino acid replacements evolution could probably not have progressed very far. Gene duplication, which leads to part, or all of a polypeptide chain being present twice in a genome, results in twice as much of the

polypeptide chain being produced. If there is no advantage in this over-production, then the selection pressure on one of the duplicated sequences should be relaxed, thus allowing more radical types of amino acid substitutions. Hence during a period of adaptive selection a "new" protein can be fashioned from an "old" protein.

1.10.1 EVOLUTION OF GLYCOLYSIS AND PHOSPHOFRUCTOKINASE

Comparison of the primary, tertiary and quarternary structures of enzymes is particularly informative regarding their evolution and function. There are three main theories describing how enzymes could have evolved:

(1) Enzymes which bind similar ligands have evolved from a common ancestor i.e. divergent evolution.

(2) Enzymes which catalyse similar reactions have developed independently to exhibit common sequences i.e. convergent evolution.

(3) Consecutive enzymes in biochemical pathways, which interact with the same ligand have evolved by a series of gene duplication events. Hence all the members of a pathway such as glycolysis would be related to one another.

There is more known about the detailed structures of the 15 enzymes involved in the glycolytic sequence than any other comparable group of enzymes. Each has been found to consist of a core of mostly parallel β-strands surrounded by α-helices (Fothergill-Gilmore, 1986). Another striking property of the glycolytic enzymes is that their amino acid sequences are strongly conserved. The glycolytic enzymes appear to be evolving at a rate of 4-6 accepted point mutations/100 million years which is much slower than other proteins, eg ribonuclease, which is evolving ten times faster, and trypsin three times faster (Dayhoff, 1978).

Glycolytic enzymes can be categorised by the type of reaction they catalyse: kinase, mutase, dehydrogenase etc. Crystallographic and sequence information indicates that the enzymes within each group are unlikely to have diverged from a common primitive ancestor. There is one example of divergence of consecutive enzymes from a common precursor in the glycolytic pathway: monophosphoglycerate mutase and diphosphoglycerate mutase. Limited divergence following relatively recent gene duplication has been suggested for these two enzymes (Haggarty et al., 1983).

The development of enzymes due to convergent evolution is difficult to prove, since it can always be argued that divergence has occurred long ago, or so rapidly as to be no longer observable. One example of convergent evolution is the symmetrical arrangement of eight β-strands and eight α-helices in a cylindrical barrel structure described for muscle triose phosphate isomerase (Banner et al., 1975). This extensive structure is also present in one domain of muscle pyruvate kinase (Stuart et al., 1979).

Both tissue-specific and species-specific isozymes are common amongst the glycolytic enzymes. Isozymes are undoubtedly the most convincing examples of divergent evolution in glycolysis. A comparison of the sequences of bacterial and mammalian PFKs shows an example of the duplication of one gene to yield another gene approximately twice the size, with each half being homologous to the original gene (Poorman et al.,1984). Rabbit muscle PFK is thought to have diverged from the bacterial enzyme 1 500 million years ago (Fothergill-Gilmore, 1986). The N-terminal half of rabbit muscle PFK is considerably more like the Bs enzyme than the C-terminal half, and has therefore tolerated fewer mutations. It is likely that the N-terminal portion has been constrained to retain the catalytic properties of PFK, whereas the C-terminal portion has been able to evolve more rapidly, and has thus acquired the additional allosteric properties of the mammalian enzyme. Comparison of the amino acid residues at the ligand binding sites adds further support to this supposition (Hellinga and Evans, 1985). The catalytic aspartic acid at the F6P binding site is conserved in the two bacterial enzymes, the N-terminal half of the rabbit muscle enzyme, but not the C-terminal half.

The 3 dimensional structure of the bacterial (Bacillus stearothermophilus) PFK has been determined, but the crystals of mammalian PFK do not diffract sufficiently to enable the crystal structure to be determined. Mammalian PFKs are thought to have evolved from the bacterial enzyme by gene duplication and fusion followed by mutation. Since the structures are therefore related, comparison of two monomers of the bacterial enzyme with a single PFK monomer is possible. Based on such a comparison, positions of active sites and effector sites can be postulated for mammalian PFK, and a comparison of the residues at the important regulatory sites can be made.

1.11 AIMS OF THIS PROJECT

The ultimate goal in the study of a protein is to understand its biological function in terms of its unique molecular structure. The structure of a protein is determined by the

sequential order of hundreds of amino acids, along the polypeptide chain, and this dictates the folding of the chains and the function of the protein molecule.

Interest in understanding the evolution and mechanisms of regulation has motivated efforts in this laboratory to characterise two mammalian PFKs and compare them with other eukaryotic and prokaryotic PFKs.

The well documented differences in kinetic properties of the liver and muscle isozymes of mammalian PFKs are reflected in their different roles in the regulation of carbohydrate metabolism in their respective tissues. Based on the three dimensional structure of Bs PFK, it is possible to compare the residues at the catalytic and regulatory sites of both the mammalian and bacterial enzymes. This should indicate whether major changes in the residues at these sites has occurred, or if the different kinetic properties are more likely due to more subtle changes. The aim of this study was therefore to investigate the primary structure of sheep liver PFK, and to compare this with other mammalian PFK sequences (rabbit, human and sheep heart), in an effort to explain their different kinetic properties in terms of differences in their amino acid sequences.

The development of the purification procedure for sheep liver PFK is described in Chapter Three. Chapter Five describes the characterization of liver PFK, including comparisons of the molecular weight, amino acid analyses and peptide maps with that of sheep heart muscle PFK. The amino acid sequences of the sheep liver PFK peptides, which were isolated and sequenced from CNBr, tryptic and maleyl-tryptic digests are shown in Chapter Six. These peptides have been aligned with the sequences from rabbit muscle and sheep heart muscle PFK, and amino acid residues which are different have been highlighted. In Chapter Seven the implications of the amino acid differences between muscle and liver PFK in terms of differences in their kinetic properties are discussed.

CHAPTER TWO

MATERIALS

The following chemicals were obtained from Sigma Chemical Company, St Louis: ADP, ATP, ammonium sulphate, βME, BSA, CNBr, Coomassie Brilliant Blue G-250, Coomassie Brilliant Blue R-250, 2,5-diphenyloxazole (PPO), F26BP, glycerol, guanidine-HCl, iodoacetic acid, magnesium chloride, phenylmethylsulphonyl fluoride (PMSF), polyethylene glycol-6000 (PEG), TEMED and Triton X-100.

British Drug Houses (BDH) Ltd., Poole, England supplied acetic acid, acetonitrile (HiPerSolv), ammonium persulphate, Fructose 1,6-bisphosphate (F16BP), glycine (chromatographically homogeneous), HCl (Analar), isopropanol (HiPerSolv), magnesium sulphate and sodium dodecyl sulphate (specially pure).

Tris (Analar grade), Fructose 6-phosphate (F6P), and NADH were obtained from United States Biochemical Corporation, Cleveland, Ohio.

EDTA, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and ethanol were from Ajax, Sydney, Australia.

Acrylamide and bis-acrylamide (>99.9%) were from Bio-Rad Laboratories, Richmond, U.S.A.

DTT was purchased from Aldrich-Chemie, Steinheim, West Germany.

1,4-Di[2-(5-phenyloxazolyl)]benzene (POPOP) from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England.

Formic acid (Pronalysis) and H_3PO_4 were obtained from May and Baker Ltd., Dagenham, England.

Ammonium bicarbonate and NaF were from Riedel-De Haën AG, Seelze, Hannover.

DEAE-cellulose (DE52) and Sepharose 4B-CL were purchased from Whatman, Kent, England.

Triose phosphate isomerase (EC 5.3.1.1) α-glycerophosphate dehydrogenase (EC 1.2.1.12), Aldolase (EC 4.1.2.13) and Trypsin (TPCK treated)(EC 3.4.4.4) were also obtained from the Sigma Chemical Co., St Louis.

[^{14}C]-Iodoacetic acid was obtained from Amersham International PLC., Amersham.

CHAPTER THREE

METHODS , RESULTS AND DISCUSSION OF THE PURIFICATION OF SHEEP HEART AND SHEEP LIVER PFK.

3.1 DEVELOPMENT OF A PURIFICATION PROCEDURE FOR SHEEP LIVER PHOSPHOFRUCTOKINASE

In the literature, the only purification procedure reported for sheep liver PFK was that described by Brock (1969), which despite the use of an extensive procedure achieved only a low degree of purity. An initial attempt to purify sheep liver PFK using the method described for the purification of rabbit liver PFK (Kemp, 1971), resulted in a very low yield, with large losses in activity occurring during most steps. It was therefore decided to perform a series of method trials, in order to develop an effective procedure for the purification of sheep liver PFK.

3.1.1 HOMOGENIZATION BUFFER TRIAL

Many different homogenization systems have been reported for the purification of liver PFK. These include a range of pHs and concentrations as well as constituent buffers. The inclusion of various components such as NaF, $MgSO_4$ and EDTA have also been reported. None of the reported procedures appeared to use a buffer system that produced a markedly high specific activity (Table XII), so an homogenization buffer trial, which was designed to encompass as many different buffer systems as possible, was performed.

Studies by Brock (1969) suggested that the low molarity buffers previously used (Wallace and Newsholme, 1967; Mansour et al.,1966) were inadequate to cope with the strong acidity of sheep liver. In view of the known susceptibility of PFK to inactivation at low pH, it is necessary to maintain a pH of 7.0 or higher following homogenization of the liver. High-molarity buffers were found to be the most effective in terms of both extracting the enzyme and maintaining its stability (Brock, 1969), therefore 0.1M Phosphate buffers were predominantly used for the homogenization buffer trial (Table XIII).

TABLE XII

HOMOGENIZATION BUFFERS USED FOR THE PURIFICATION OF LIVER PFK.

Source	pH	Buffer	Other compounds	Specific Activity Units/mg	Ref
Sheep	8.0	0.1M PO_4	-	0.0386	(i)
Rabbit	7.5	none	30mM KF 10mM EDTA 0.1M ATP	0.018	(ii)
Rat	7.5	0.1M PO_4	3mM $MgSO_4$ 5mM βME 50mM NaF 1mM F6P	0.0054	(iii)
Rat	8.5	50mM Tris-PO_4	50mM NaF 10mM DTT 1mM ATP	0.046	(iv)
Rat	8.0	0.15M PO_4	50mM NaF 1mM EDTA 2.5mM DTT 1mM ATP	0.017	(v)
Rat	8.0	50mM Tris-HCl	50mM NaF 5mM DTT 1mM ATP	0.02	(vi)
Rat	8.0	50mM Tris-HCl	50mM NaF 10mM DTT 1mM ATP	0.006	(vii)
Pig	8.0	50mM Tris-HCl	50mM βME 5mM EDTA	0.014	(viii)

Specific activity of crude homogenates using different homogenization buffer systems reported in the literature for the purification of liver PFK from different species.

(i) Brock, 1969
(i) Kemp, 1971
(ii) Brand and Söling, 1974
(iv) Kasten et al., 1983
(v) Kagimoto and Uyeda, 1979
(vi) Reinhart and Lardy, 1980a
(vii) Dunaway and Weber, 1974a
(viii) Massey and Deal, 1973

TABLE XIII

COMPONENTS OF THE TEN BUFFER SYSTEMS USED IN THE HOMOGENIZATION BUFFER TRIAL.

Buffer System	Components	Buffer system	Components
1) Control	0.1M KH_2PO_4 pH 8 50mM NaF 1mM DTT 1mM ATP	2) No ATP	0.1M KH_2PO_4 pH 8 50mM NaF 1mM DTT
3) $MgSO_4$	0.1M KH_2PO_4 pH 8 50mM NaF 1mM DTT 3mM $MgSO_4$	4) $MgSO_4$ + F6P	0.1M KH_2PO_4 pH 8 50mM NaF 1mM DTT 1mM F6P 3mM $MgSO_4$
5) F6P	0.1M KH_2PO_4 pH 8 50mM NaF 1mM DTT 1mM F6P	6) Tris-PO_4	50mM Tris-PO_4 pH 8.5 50mM NaF 1mM DTT 1mM ATP
7) Tris-HCl 1mM DTT	50mM Tris-HCl pH 8.0 50mM NaF 1mM DTT 1mM ATP	8) Tris-PO_4 10mM DTT	50mM Tris-PO_4 pH 8.5 50mM NaF 1mM ATP 10mM DTT
9) F26BP	0.1M KH_2PO_4 pH 8 50mM NaF 1mM DTT 1mg F26BP	10) Thawing	0.1M KH_2PO_4 pH 8 50mM NaF 1mM DTT 1mM ATP

Components of the 10 homogenization buffers, based on the systems in Table XII, which were used to examine the conditions which provide the greatest yield and specific activity of sheep liver PFK.

Since NaF has been described as being essential for stabilizing PFK (Section 1.3.7), it was therefore included in each of the buffer systems tested. DTT was also included since it also has been shown to be effective in the stabilization of liver PFK (Brock, 1969; Tsai and Kemp, 1973).

3.1.1.1 PROCEDURE FOR THE HOMOGENIZATION BUFFER TRIAL

Since buffer system 1 (Table XIII), had components in common with all the other buffers it was used as the basis (control) for comparison of the specific activities of PFK in the other buffer systems. Ten different trials were carried out using 150g of frozen sheep liver. Except for one batch which was allowed to thaw overnight (10), in the homogenization buffer at 0°C, 150g of the frozen liver was minced into 300ml of the appropriate cold homogenization buffer, homogenized for 30s at high speed in a stainless steel Waring blender, and then centrifuged at 9 000rpm for 25min at 0-5°C. The supernatants were decanted and placed on ice for protein determinations, enzyme assays and pH readings.

The results from the homogenization trial are shown in Table XIV. These show that the omission of ATP from the buffer system (2) resulted in the lowest yield and specific activity of liver PFK, at 95Units and 0.023Units/mg respectively. This yield was approximately one third that achieved using control conditions (1), while the specific activity was only half that of the control. The inclusion of $MgSO_4$ (3) and $MgSO_4$ and F6P (4) in the buffer system resulted in slightly better yields than system 2, but were still less effective than the control. System 5 containing F6P only resulted in a slightly higher yield and specific activity than 3 and 4, but was still a less effective extraction buffer system than the control. The use of buffer systems 6 (Tris-PO_4 pH 8.5) and 7 (Tris-HCl pH 8.0), resulted in similar specific activities to the control although the yield was slightly lower in both cases. Buffer system 8 which contained 10mM DTT resulted in a 20% increase in specific activity over the control although the yield was slightly lower. Thawing the liver prior to homogenization (10) resulted in a below average yield of only 185Units, although the specific activity was significantly higher than the control, due to the low protein concentration in the supernatant. The inclusion of the activator F26BP in the buffer system (9) resulted in both the greatest yield and specific activity, of all the systems tested.

This trial emphasized the necessity of having ATP and a high concentration of DTT in the homogenization buffer. Since the greatest yield was obtained in the buffer system

TABLE XIV

RESULTS FROM THE HOMOGENIZATION BUFFER TRIAL.

Buffer System	pH after homogenization	Total Activity Units	Total Protein mg	Specific Activity Units/mg
1) Control	7.10	311	6220	0.050
2) No ATP	7.20	95	4087	0.023
3) $MgSO_4$	7.10	181	5817	0.031
4) $MgSO_4$ + F6P	7.20	189	5944	0.032
5) F6P	7.20	272	6369	0.043
6) Tris-PO_4 1mM DTT	7.80	264	5494	0.048
7) Tris-HCl 1mM DTT	7.10	253	5014	0.050
8) Tris-PO_4 10mM DTT	7.80	288	4837	0.060
9) F26BP	7.10	313	4721	0.066
10) Thawing	7.05	185	3233	0.057

150g of frozen sheep liver (except for system 10, where the liver was allowed to thaw) was minced into 300ml of each of the 10 buffer systems, homogenized and centrifuged as described in Section 3.1.1.1. PFK enzyme activity, and the protein concentration of the supernatant were measured as described in Sections 3.3 and 3.4, the pH was also measured.

containing F26BP, this along with 1mM ATP and 10mM DTT were included in the homogenization buffer for future trials. However, when the liver PFK enzyme purifications were scaled up to 4kg liver over 2 days, the F26BP was omitted, and the 10mM DTT reduced to 1mM, and 20mM βME included, due to the high cost of these items. It was also apparent that the Tris-PO_4 buffer system pH 8.5 (6 and 8) were marginally better at stabilizing the pH after the homogenization of the liver, than the other buffer systems, since only a 0.7 shift in pH occurred using this buffer compared to the 0.8-0.95 shift in pH experienced using the other buffer systems. Tris-PO_4 pH 8.5 was therefore chosen as the buffer for future use.because of its ability to maintain a high pH even after the addition of the acidic sheep liver.

3.1.2 HOMOGENIZATION EXPERIMENT

In an experiment to determine the optimum homogenization time, 1kg of frozen sheep liver was minced into 1.5litre of cold homogenization buffer (50mM Tris-PO_4 pH 8.5, 50mM NaF, 10mM DTT, 2mg F26BP, 1mM ATP, 174mg PMSF). The minced material was homogenized in a stainless steel Waring blender on low speed for 90sec with samples being removed after 5, 10, 30, 45, 60 and 90sec. The samples were immediately placed on ice, and following the conclusion of homogenization, were centrifuged at 9 000rpm for 25min. The supernatants were decanted and assayed for PFK enzyme activity and the protein concentration determined.

The results shown in Table XV suggest that blending for 45sec or longer leads to a decreased specific activity due to an increase in the protein concentration. Homogenization for 30sec at low speed appeared to be the optimum homogenization time for obtaining the highest liver PFK specific activity, and was therefore adopted for future use.

3.1.3 DETERMINATION OF THE EFFECT OF FRUCTOSE 2,6-BISPHOSPHATE ON THE THERMAL STABILITY OF LIVER PHOSPHOFRUCTOKINASE

F26BP is a well known activator and stabilizer of liver PFK. It is unstable at slightly acid pH (Dunaway and Segal, 1976), and has a remarkable ability to protect PFK against thermal or enzymatic inactivation (Söling et al., 1981; Uyeda et al., 1981; Claus et al., 1982). Reports of investigations of thermal stabilization in the literature are limited however. Söling et al. (1981), demonstrated that at pH 6.5 and 37°C, the normally rapid spontaneous inactivation of rat liver PFK could be abolished by the

TABLE XV

RESULTS FROM THE HOMOGENIZATION EXPERIMENT.

Homogenization Time (sec)	PFK Activity Units/ml	Protein mg/ml	Specific Activity Units/mg
5	0.73	49.5	0.015
10	0.75	48.5	0.015
30	0.80	48.0	0.017
45	0.65	48.5	0.013
60	0.80	56.5	0.014
90	0.58	55.5	0.010

1kg of frozen sheep liver was minced into 1.5litre of homogenization buffer. Following homogenization on low speed for 5, 10, 30, 45, 60 and 90sec, samples were removed and centrifuged as described in Section 3.1.2. PFK enzyme activity and the protein concentration of the supernatants were measured as described in Sections 3.3 and 3.4.

addition of F26BP. A similar result was demonstrated at pH 7.1 and 37°C (Van Schaftingen and Hers, 1983a). At 50°C and pH 8.0 rat liver PFK was rapidly inactivated, with half the activity being lost within 3min. The addition of F26BP at a ratio of 1Unit F26BP[1]/200Units PFK resulted in the full protection of PFK activity over the 15min period assayed (Uyeda et al., 1981).

The F26BP heat stabilization trial was therefore carried out to investigate the stabilizing effect of F26BP on PFK activity at high temperatures, in order to ascertain whether an effective heat treatment step could be used for the purification of sheep liver PFK.

3.1.3.1 METHOD, AND RESULTS FOR THE FRUCTOSE 2,6-BISPHOSPHATE HEAT STABILIZATION TRIAL

500g of frozen sheep liver was minced into 1.5litre of cold homogenization buffer (as in Section 3.1.2 except F26BP was omitted), homogenized for 30sec at low speed in a stainless steel Waring blender, and centrifuged at 9 000rpm for 25min at 0°C. The supernatant was retained for the F26BP trial. 2mg of F26BP was added per 1.2litre of supernatant, and the material heated with constant stirring in an 80°C water bath. The temperature of the solution was measured every 30sec, while 15-20ml samples were removed at 0, 3, 4 and 5min and every 30sec thereafter, up to 11min, at which time the temperature of the material had reached 66°C. Each sample was immediately placed on ice until the completion of the heating, when they were centrifuged at 9 000rpm for 25min at 0°C. The supernatants were retained for protein and PFK enzyme activity determinations.

The plot of temperature and protein concentration vs time (Fig. 3), shows a slow decrease in the protein level in the supernatants to 86% of its original value between 0-55°C (the first 6min of heating), following this, a sharp decline in the protein concentration could be seen over the next minute, with a decrease of 5.8mg protein/ml, to 42% of the original protein concentration. Over the next 4min (59-65°C), the protein concentration continued to decline but at a slower rate, to give 12% of the original protein concentration at 11min.

No significant loss of PFK activity occurred with heating to 65°C (Fig. 3).

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[1] 1Unit is the amount of F26BP which increases PFK activity by 1Unit under the assay conditions used.

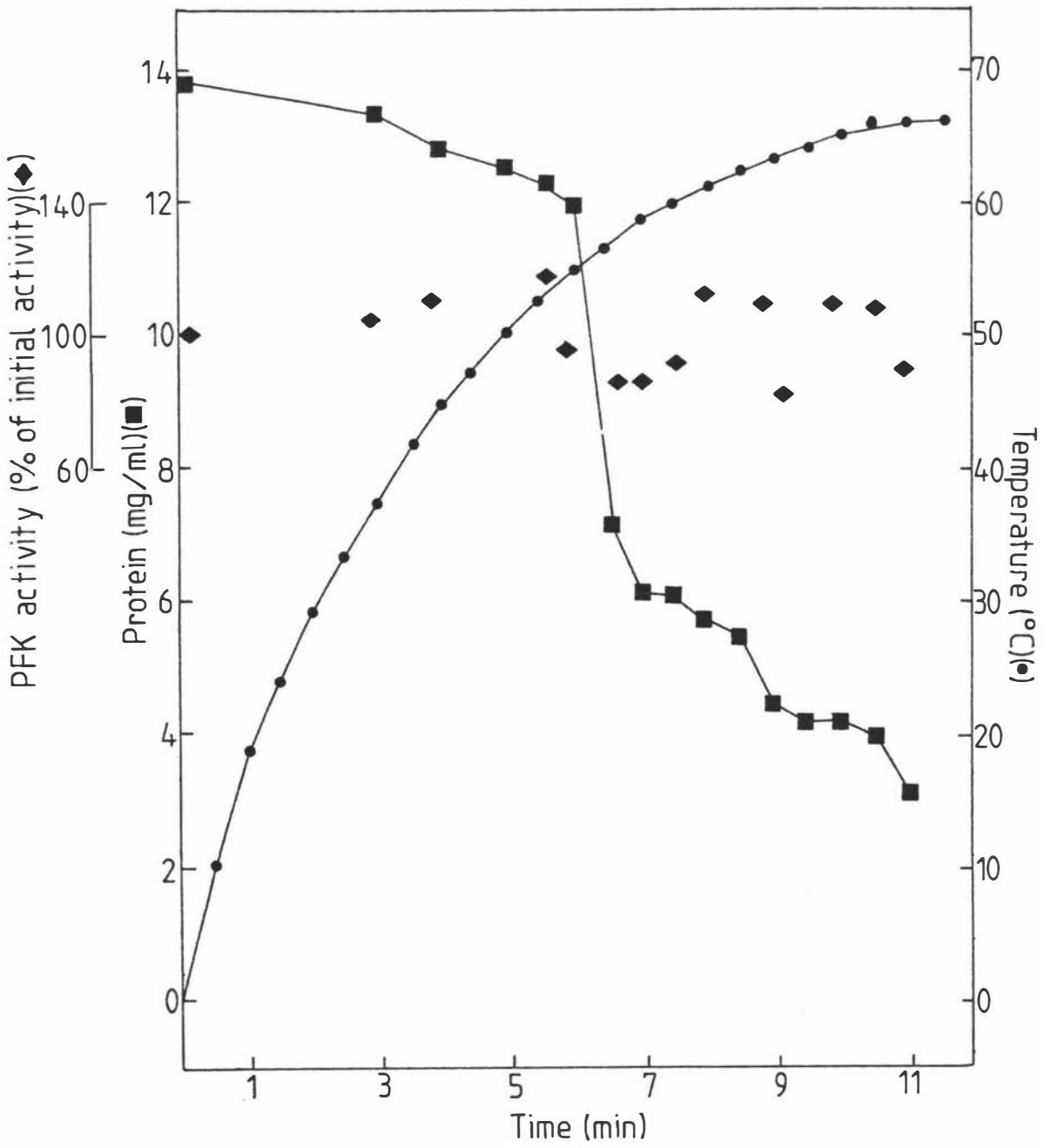


Figure 3: The Effect of Fructose 2,6-Bisphosphate on the Heat Stabilization of Sheep Liver PFK.

500g of frozen sheep liver was minced into 1.5litre of homogenization buffer, homogenized and centrifuged as described in Section 3.1.3.1. 2mg F26BP/1.2litre of supernatant was added, and the material heated to 66°C in an 80°C water bath. Temperature readings were taken at 30sec intervals, samples were removed at 0, 3, 4, and 5min, and every 30sec thereafter up to 11min. Following centrifugation as described in Section 3.1.3.1 the PFK enzyme activity and protein concentrations of the supernatants were determined as described in Sections 3.3 and 3.4.

As a result of the heating process a 4-fold purification was achieved, with the specific activity increasing from 0.05Units/mg at 0 time to 0.23Units/mg at 11min. This increase in specific activity eventuated because of the thermal denaturation and removal of a large amount of the protein (88%) present in the original supernatant, while liver PFK activity remained relatively constant due to the addition of F26BP. These results showed that the addition of F26BP to the homogenate supernatant and subsequent heating to 62-63°C resulted in a significant increase in specific activity, and this step was therefore introduced into the sheep liver PFK purification procedure.

3.1.3.2 DOUBLE HEAT STEP TRIAL

A double heat step trial was performed to investigate if further heating of the supernatant from the initial heat step, after the addition of a further 2mg of F26BP, could result in an additional increase in specific activity. Using 1kg of frozen sheep liver, PFK was prepared as described in Section 3.1.3.1, and heated with constant stirring in an 80°C water bath to 62-63°C, following the addition of 2mg of F26BP. The heat treated suspension was rapidly cooled to 5°C before being centrifuged at 9 000rpm for 25min. The supernatant was then used for the second heat treatment trial. The supernatant was heated with a further 2mg of F26BP to 76°C over a period of 8min in a 90°C water bath with constant stirring, and temperature readings taken at 30sec intervals. Samples were removed at 1min intervals, and immediately placed on ice. Following (at 0-5°C) the completion of heating, the samples were centrifuged at 9 000rpm for 25min, and the supernatant retained for protein and PFK activity determinations.

The results from the protein and PFK enzyme assays show that the protein concentration remained constant up to 4min (60°C), after which it decreased at a steady rate up to 8min (76°C)(Fig. 4). This decline was paralleled by a drop in PFK activity, with all of the PFK activity being lost after 8min. The loss of PFK activity occurred at temperatures below 60°C, suggesting that the heat stabilizing effect brought about by the addition of F26BP had been lost, rather than the stabilizing effect being counteracted by high temperatures. Since all of the PFK activity was rapidly lost this double heat step procedure was not included in the purification procedure.

3.1.4 AMMONIUM SULPHATE PRECIPITATION TRIAL

1kg of frozen sheep liver was minced, homogenized, centrifuged and heated to 60°C upon addition of 2mg of F26BP as described in Section 3.1.3.2. Sufficient ammonium sulphate was added to the supernatant over 20min to give a 20% saturated solution.

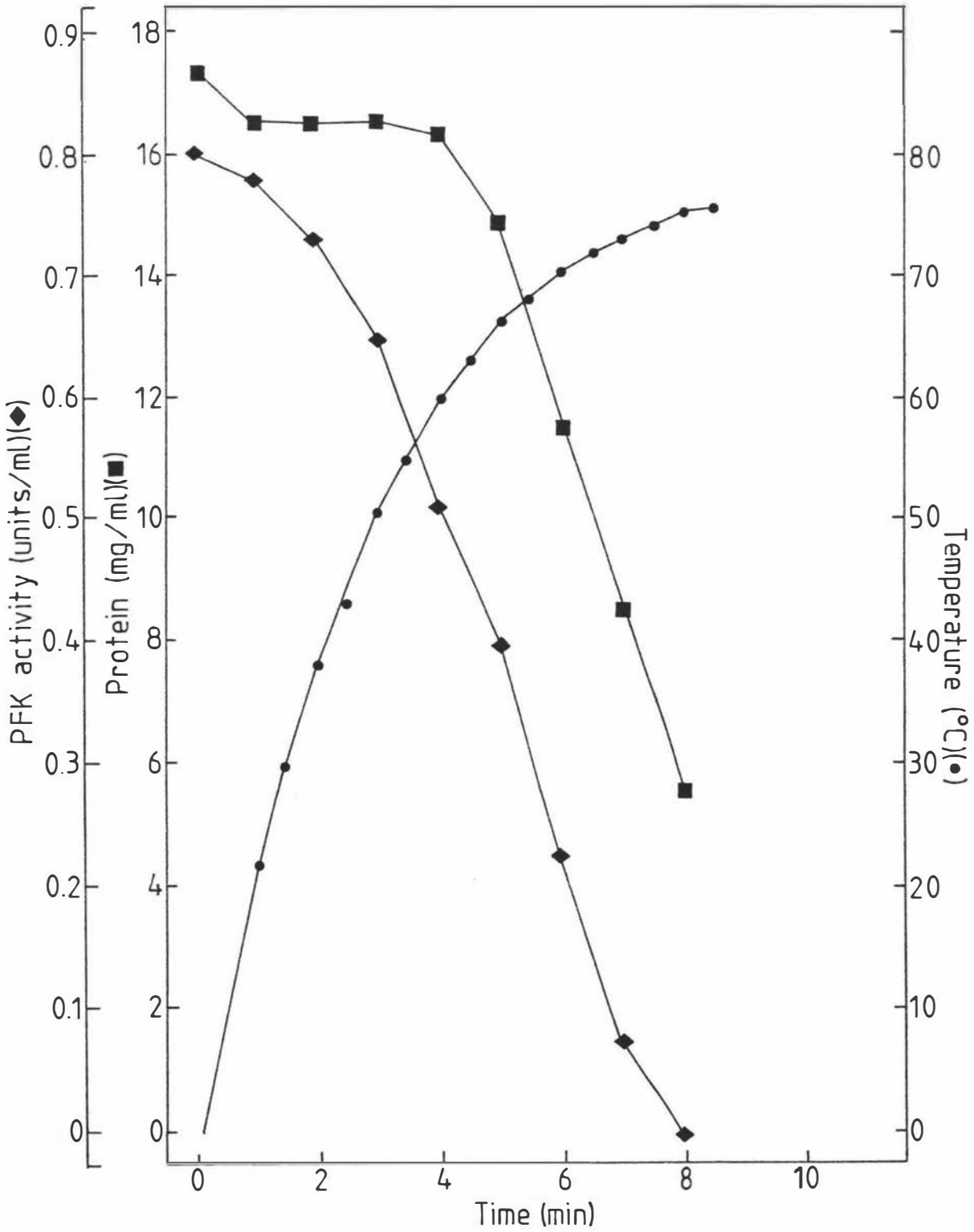


Figure 4: Effect of a Second Heat Step on the Purification of Sheep Liver PFK.

1kg of frozen sheep liver was prepared as described in Section 3.1.3.2 up to the conclusion of the heat treatment step. Following centrifugation of the heat treated material, the supernatant was heated to 76°C in a 90°C water bath after the addition of 2mg of F26BP. Temperature readings were taken at 30sec intervals. Samples were removed at 1min intervals and centrifuged as described in Section 3.1.3.2 and PFK enzyme assays and the protein determinations carried out as described in Sections 3.3 and 3.4.

The material was then equilibrated for 30min in a 0-5°C methanol bath, before being centrifuged at 9 000rpm for 20min. The precipitate was then dissolved in homogenization buffer and dialysed against 1litre of the same buffer, to remove the ammonium sulphate, since ammonium sulphate is an activator of PFK (Section 1.4.4). Ammonium sulphate was then added to the supernatant to give a 30% saturated solution. Following a 30min equilibration period, the material was centrifuged and the precipitate redissolved and dialysed against the same homogenization buffer as above. The ammonium sulphate concentration of the supernatant was then raised in turn to 40%, 50% and 60% saturation, providing fractions of 0-20%, 20-30%, 30-40%, 40-50% and 50-60% saturated ammonium sulphate. Each fraction was then assayed for protein content and PFK enzyme activity.

The majority of the PFK activity present was precipitated between 20-50% saturated ammonium sulphate (Fig. 5), with a sharp peak of activity being precipitated between 30-40% (cf 38-55% for sheep heart PFK). This broad precipitation pattern is similar to that described for rat liver PFK (Dunaway et al., 1974), in which the majority of the PFK precipitated between 30-40%, while some of the enzyme was still being precipitated by 60% saturated ammonium sulphate. However in other reported purifications of liver PFK (Table XVI) high yields of activity were achieved using narrower ammonium sulphate concentration ranges. This discrepancy may result from either differences in the properties of the liver PFKs from different species and strains, or the influence of different protein concentrations at the precipitation stage.

The precipitation of PFK activity in this study followed the precipitation of total protein over the ammonium sulphate concentration range examined. Although there was a 5-fold increase in specific activity between 30-40% ammonium sulphate, 46% of the enzyme activity was not precipitated in this concentration range. Since a high yield, as well as a high specific activity was desirable, ammonium sulphate fractionation was considered unsuitable for the purification of sheep liver PFK, and an alternative procedure was investigated.

3.1.5 POLYETHYLENE GLYCOL PRECIPITATION TRIAL

Polyethylene glycol-6000 (PEG) is a commonly used precipitation agent due to its non-toxic and non-denaturing properties. It has been found to prevent the spontaneous inactivation of rat liver PFK which occurs at low protein concentrations, and at pH 7.0 in the absence of substrates. PFK may even be activated by high levels of PEG to a

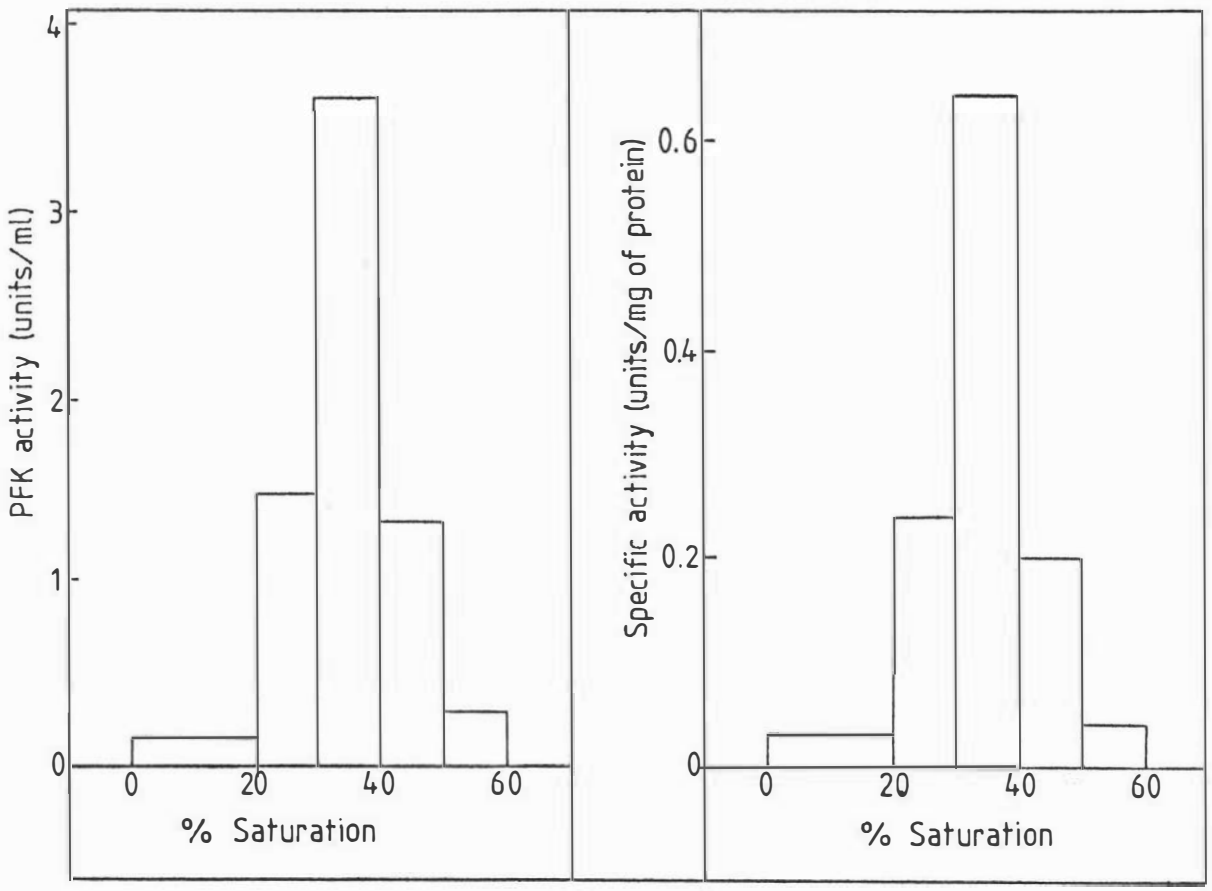


Figure 5: Precipitation of Sheep Liver PFK by Ammonium Sulphate.

Sheep liver PFK was prepared as described in Section 3.1.3.2 up to the end of the centrifugation following the heat step. To the supernatant 0-60% ammonium sulphate was added in steps of 0-20%, 20-30%, 30-40%, 40-50% and 50-60% over 20min, and allowed to equilibrate for 30min before centrifugation at 9 000rpm for 20min. Precipitates were resuspended in homogenization buffer, and dialysed in preparation for assaying. Each dialysed fraction was assayed for PFK enzyme activity and protein concentration determined as described in Section 3.3 and 3.4.

TABLE XVI

AMMONIUM SULPHATE FRACTIONATIONS USED IN THE PURIFICATION OF LIVER PFK FROM DIFFERENT SPECIES, AND THE RESULTING PFK YIELDS.

Species	% $(NH_4)_2SO_4$	% yield compared to previous step	Ref
Rat	33	124	(i)
Rat	40	96	(ii)
Rat	26-55	83	(iii)
Rat	32.5	71	(iv)
Rat	32.5	95	(v)
Rabbit	50-26	96	(vi)
Sheep	50	89	(vii)

Reported ammonium sulphate fractionations used for the purification of liver PFK from different species. The yield is the PFK activity following the precipitation step, as compared to the previous step.

(i) Furuya and Uyeda, 1980
(ii) Kagimoto and Uyeda, 1979
(iii) Brand and Söling, 1974
(iv) Dunaway and Weber, 1974a
(v) Kasten et al., 1983
(vi) Kemp, 1975a
(vii) Brock, 1969

small extent (Reinhart, 1980), therefore if would seem to be an ideal precipitant to use in the purification of liver PFK.

Sheep liver PFK was prepared as described in Section 3.1.3.2. Sufficient PEG was added to the supernatant over 20min to achieve a 2% (w/v) solution which was then equilibrated for 30min in a 0-5°C methanol bath before further PEG was added in 0.5% steps up to 8.5% (w/v). Samples were removed at each concentration interval, centrifuged at 9 000rpm for 20min at 0-5°C and the supernatants assayed for protein and PFK enzyme activity.

65% of the original PFK activity, and only 5% of the original protein was precipitated between 4-5% PEG (Fig. 6), resulting in a 13-fold increase in specific activity in this PEG fraction. Although the 4-5% PEG fraction appeared to be optimal for the precipitation of sheep liver PFK, when this fractionation was scaled up, it was necessary to increase the fractionation range to 3-7.5% PEG in order to maximize the yield of PFK activity. This PEG concentration range was similar to the 3.5-8.0% PEG fractionation used in the purification of rat liver PFK (Sakakibara and Uyeda, 1980).

3.2 PURIFICATION OF SHEEP LIVER PHOSPHOFRUCTOKINASE

The method of PFK purification used in this study from sheep liver was a combination of the methods devised from trials as described in Sections 3.1.1-3.1.5, and modifications to the chromatography steps used by Tsai and Kemp (1974) and Kasten et al. (1983). Fresh sheep livers were collected from the freezing works packed on ice. The livers were cut into small pieces before being frozen and stored at -15°C. Unless otherwise indicated all operations were carried out at 0-4°C, and all % are (w/v).

2kg of frozen sheep livers was minced into 2litre of cold homogenization buffer containing 50mM Tris-PO_4 at pH 8.5, 50mM NaF, 1mM DTT, 1mM ATP, 20mM βME, and 174mg PMSF. NaF, DTT, βME and ATP were present since they are known to stabilize the enzyme (Section 1.3.7), while PMSF was added to reduce proteolytic cleavage from the large number of proteases that are known to be present in the liver.

The minced liver was homogenized in a stainless steel Waring Blender for 30sec at low speed, and centrifuged at 9 000rpm at 0°C for 25min in a Sorvall RC2-B centrifuge. The supernatant was filtered through glass wool to remove floating fatty material. An early study revealed that up to one quarter of the PFK activity present in the

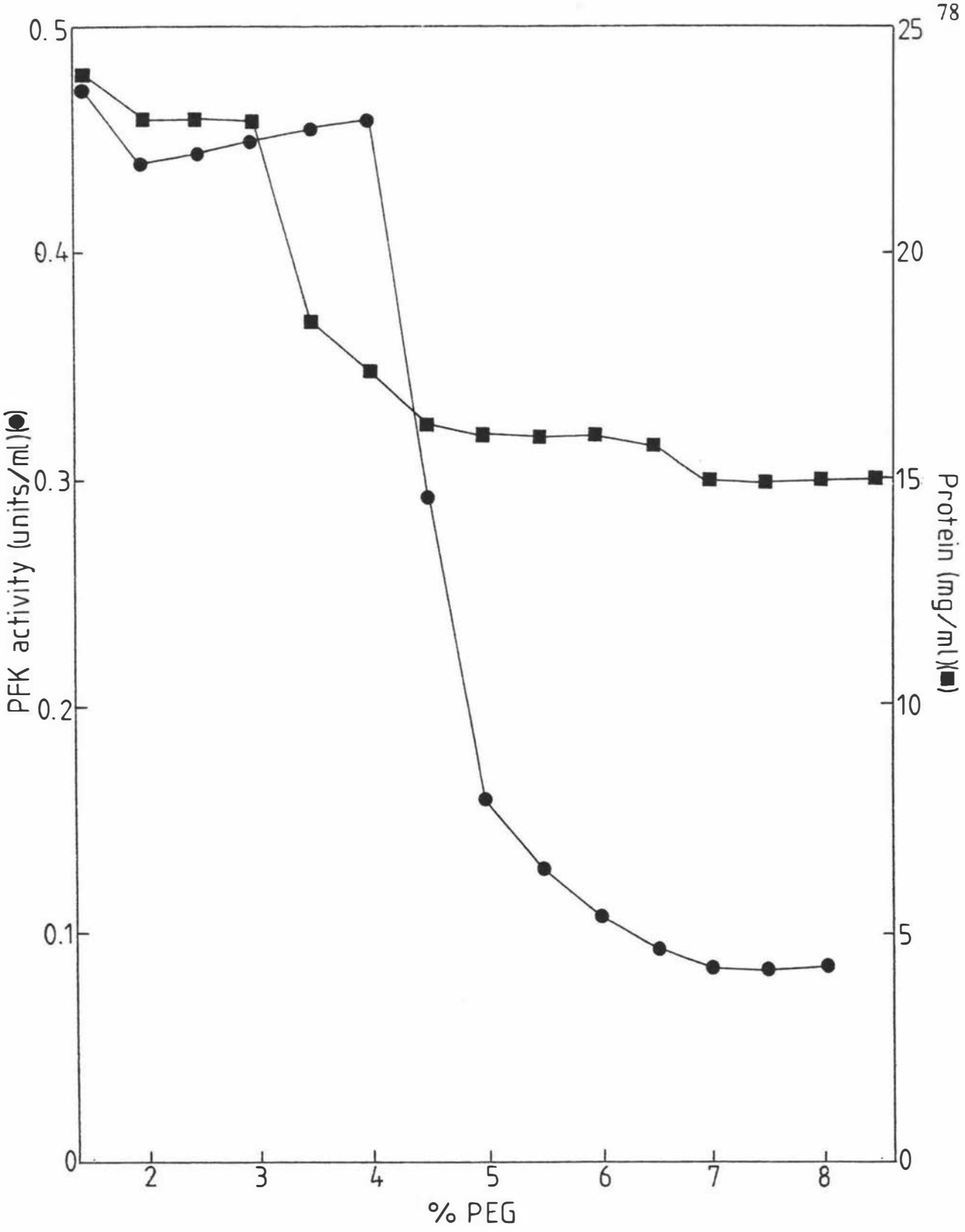


Figure 6: Precipitation of PFK Activity and Protein by PEG.

Frozen sheep liver was prepared as described in Section 3.1.3.2 up to the end of the centrifugation step, following a single heat treatment. 0-8.5% (w/v) PEG was initially added from 0-2%, and thereafter in steps of 0.5% to the supernatant, over a 20min period and allowed to equilibrate for 30min. Samples were removed at each interval, and centrifuged as described in Section 3.1.5. PFK enzyme assays and protein determinations on the samples were carried out as described in Sections 3.3 and 3.4.

homogenate remained associated with the precipitate after the first centrifugation. Therefore the precipitate was resuspended in a further 1litre of homogenization buffer and recentrifuged at 9 000rpm for 25min. The original supernatant together with the supernatant from the re-extracted precipitate were pooled (Fraction I).

1mg/litre of fructose-2,6-bisphosphate was added to the combined supernatants and 1litre batches heated to 62°C in an 80°C water bath, with constant stirring. The optimum time for this step was found to be 9-10min. The heat treated suspension was then immediately tranferred to a stainless steel beaker held in a methanol bath at -5°C and gently stirred to facilitate rapid cooling to 4°C. Heat precipitated material was removed by centrifugation at 9 000rpm for 20min, and re-extracted by suspension in a further 1litre of extraction buffer, and the centrifugation repeated as described above, since up to a quarter of the PFK activity remained associated with the precipitate following the initial centrifugation. The precipitate was discarded, and the dark red supernatant (Fraction II) made up to 3% (w/v) by the addition of solid PEG. After a 30min equilibration period at 0°C, the precipitated material was removed by centrifugation at 9 000rpm for 15min. The resultant supernatant (Fraction III) was brought to 7.5% (w/v) PEG, and equilibrated for 30min. The precipitated PFK was collected by centrifugation at 9 000rpm for 15min, the supernatant (Fraction IV) was discarded.

The precipitated material was resuspended in approximately 100ml of "DEAE-cellulose column buffer" (20mM Tris-PO_4 at pH 8.0, 1mM $(NH_4)_2SO_4$, 0.2mM EDTA, 0.1mM F16BP, 0.1mM ATP, 1mM DTT, 6mM citrate), and centrifuged at 12 000rpm for 10min before the supernatant (Fraction V), was loaded onto a DEAE-cellulose column (29cm x 5.5cm) equilibrated with DEAE-cellulose column buffer, at a rate of 70-80ml/hr. The column was washed with 1litre of column buffer, which resulted in the elution of white iridescent material. No activity was present in this breakthrough volume.

The above steps were repeated on a further 2kg of sheep liver. After washing the DEAE-cellulose column with column buffer, a concave gradient made using 2litre of column buffer in the mixing chamber of the gradient maker, and 1litre of column buffer containing 0.4M ammonium sulphate in the other chamber, was started (flow rate was 70-80ml/hr). Ammonium sulphate was used for the gradient based on the observation that this salt helped to stabilize the enzyme (Kemp, 1971). Following completion of the gradient, the conductivity of the fractions was determined. The majority of the PFK activity was found to be eluted in a large volume between 2-8mmho. Fractions were

assayed for PFK activity and those containing activity were pooled (Fraction VI), and dialysed against 5litre of Cibacron Blue column buffer (50mM Tris-PO_4 at pH 8.0, 0.1mM EDTA, 0.05mM F16BP, 1mM DTT) until the conductivity of the dialysate dropped to below 3mmho. The dialysed material (700-800ml) was loaded onto a column (27.5cm x 2.0cm), which had been packed with Cibacron Blue gel (Section 3.5) and equilibrated with Cibacron Blue column buffer at a flow rate of 60ml/hr. After loading, the column was washed with Cibacron Blue column buffer until the absorbance of the eluant at 280nm returned to baseline. Following this, the column was washed with the same buffer containing 0.15mM ADP (300ml) (Kagimoto and Uyeda, 1979). PFK was eluted from this column using Cibacron Blue column buffer containing 2mM ATP and 2mM F6P. Fractions were assayed for PFK activity and those with the greatest activity were pooled (Fraction VII), and the PFK precipitated with 60% saturated ammonium sulphate. The precipitated material was then dialysed against Cibacron Blue column buffer until the conductivity was below 3mmho, and then loaded and eluted from the Cibacron Blue column again. The eluted PFK was stored as a 60% ammonium sulphate suspension. A flow diagram for the purification procedure is shown in Fig. 7.

3.3 PHOSPHOFRUCTOKINASE ENZYME ASSAYS

PFK activity was measured by following the decrease in NADH concentration in a coupled reaction requiring aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase (Fig. 8). The reaction was followed at 340nm in an Unicam SP800 spectrophotometer, using 0.5ml quartz cuvettes containing 0.45ml of assay mixture consisting of 50mM Tris-HCl at pH 8.0, 1mM $MgCl_2$, 0.01% BSA, 5mM βME and the substrates, 2mM F6P, 1mM ATP, 0.25mM NADH and the coupling enzymes, 1Unit/ml α-GPD/TPI, and 0.275Units/ml aldolase. The reaction was started by the addition of 5µl of the PFK fraction, diluted if necessary with 10mM Tris-HCl at pH 8.0 containing 0.01% BSA and 100mM βME (Massey and Deal, 1973).

Since one mole of F16BP yields 2moles of oxidised NAD^+, the definition of one unit of enzyme is the amount of enzyme which catalyses the reaction of 1µmole of F6P/min, and this equals the oxidation of 2µmoles of NADH/min. The activity is calculated using the molar extinction coefficient of NADH of 6.22×10^3.

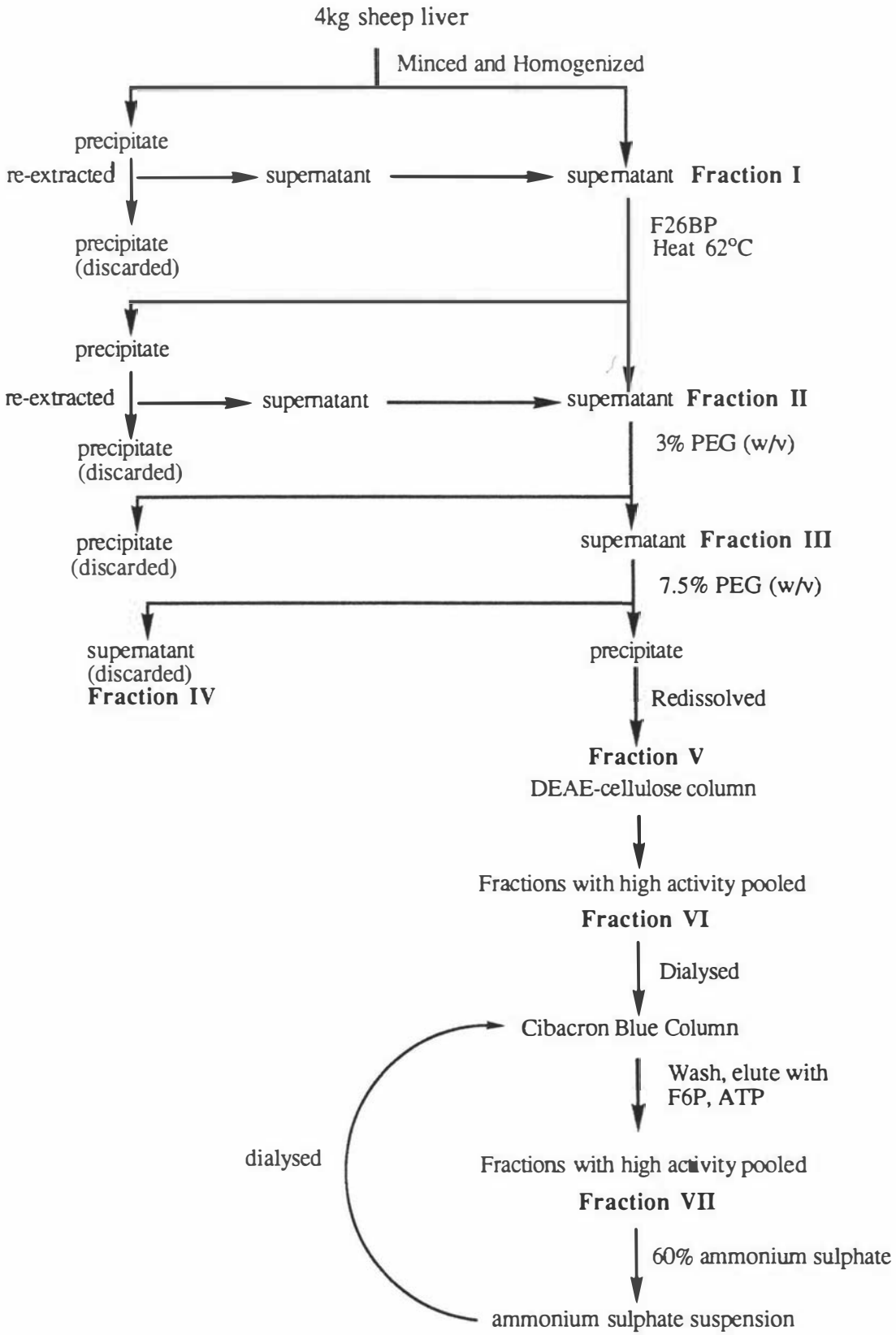


Figure 7: Flow Diagram of the Purification Procedure for Sheep Liver PFK.

The procedure is as described in Section 3.2.

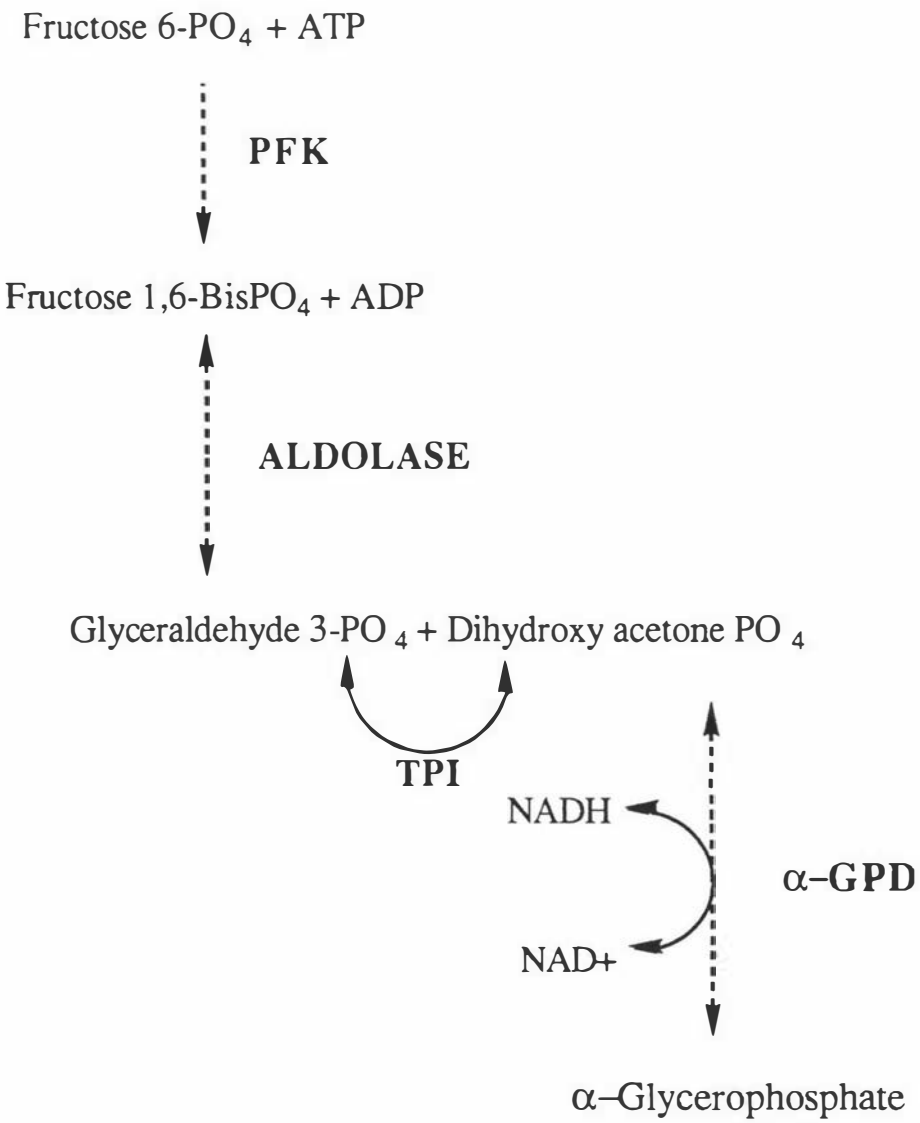


Figure.8: The Phosphofructokinase Enzyme Assay.

Flow diagram of the coupled reaction used to assay PFK activity as described in Section 3.3.

TPI =Triose Phosphate Isomerase

α-–GPD= α-Glycerophosphate Dehydrogenase

PFK=Phosphofructokinase

3.4 DETERMINATION OF PROTEIN LEVELS

Protein concentrations were determined by the Coomassie Blue dye binding method. Coomassie Blue was prepared by dissolving 100mg Coomassie Brilliant Blue G-250 in 50ml of 95% (v/v) ethanol, then adding 100ml of 85% (w/v) phosphoric acid, and diluting with distilled water to 1litre (Read and Northcote, 1981). 0.5ml aliquots of sample were added to 5ml of Coomassie Blue reagent, and the absorbance read at 595nm. Bovine serum albumin was used to standardize the Coomassie Blue.

3.5 PREPARATION OF THE CIBACRON BLUE COLUMN

Cibacron Blue gel was prepared according to Atkinson et al. (1981), using Sepharose 4B-CL. 6g of Cibacron Blue dye dissolved in 200ml of 4M NaCl was added to 400g of Sepharose 4B-CL, which had been suspended in 1.4litre of distilled deionised water. 20ml of 10M NaOH was added and the suspension heated to 55°C with gentle stirring for 16hr. Following completion of the incubation, the gel was sequentially washed with excess 1) water, 2) 1M NaCl in 25% ethanol, 3) water, 4) 1M NaCl in 0.2M phosphate pH 7.0, 5) water, before being equilibrated with Cibacron Blue column buffer.

3.6 RESULTS AND DISCUSSION OF THE PURIFICATION OF SHEEP LIVER PHOSPHOFRUCTOKINASE

3.6.1 DEAE-CELLULOSE CHROMATOGRAPHY OF SHEEP LIVER PHOSPHOFRUCTOKINASE

A typical elution profile of sheep liver PFK from DEAE-cellulose is shown in Fig. 9. This shows the majority of PFK activity eluting in a large volume between 2-8mmho. Of the enzyme activity loaded onto the column approximately 76% was eluted in the pooled fraction. A large amount of protein eluted prior to the PFK, which appeared to elute in dual peaks. Since PFK is less susceptible to proteolysis in its inhibited form, citrate was included in the DEAE-cellulose column buffer to protect against proteolysis at this step (Riquelme and Kemp, 1980).

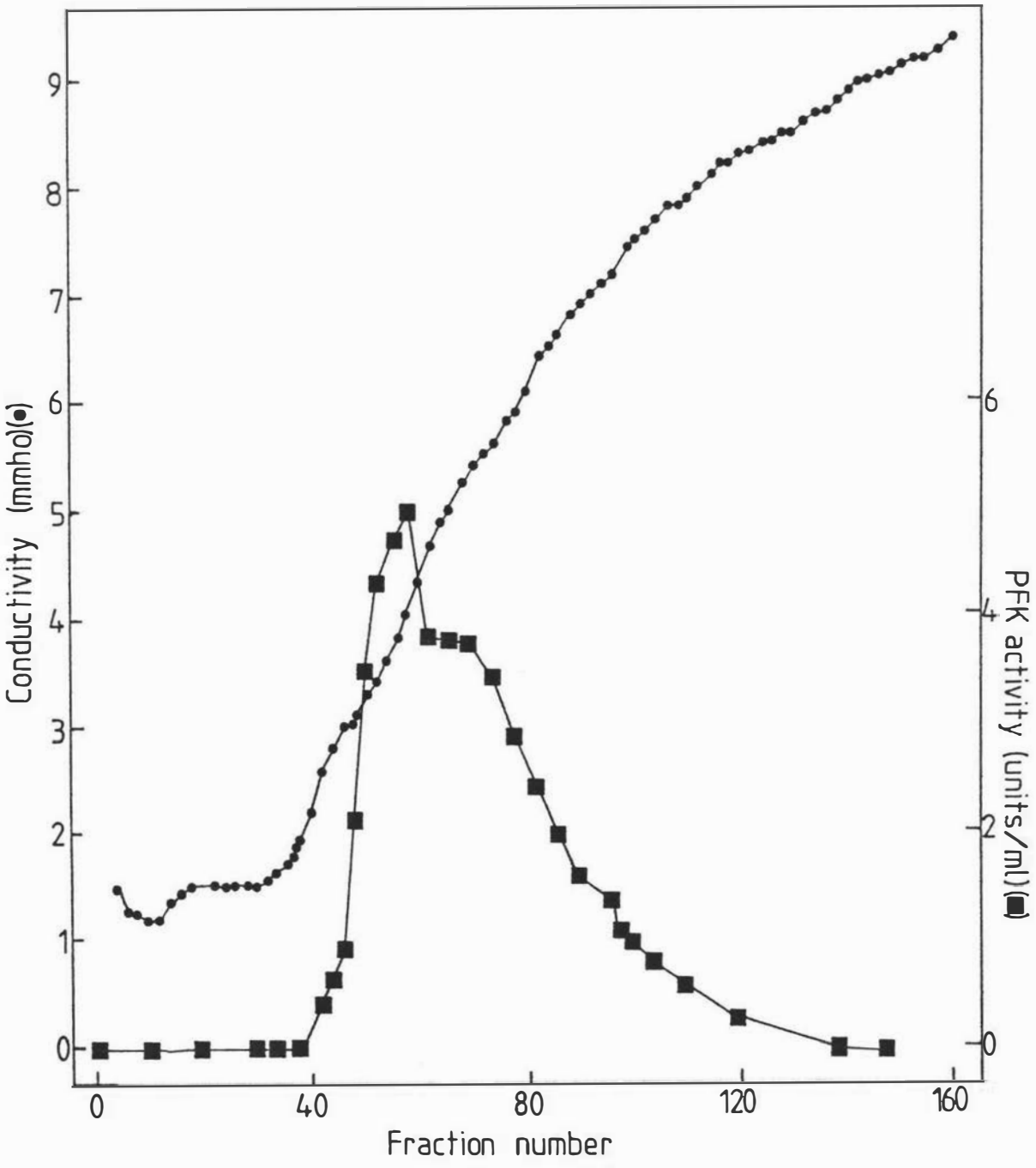


Figure 9: Elution Profile of Sheep Liver PFK From DEAE-Cellulose

The redissolved 3-7.5% PEG precipitate was loaded onto a the DEAE-cellulose column which was washed with DEAE-cellulose column buffer, and the PFK eluted with a gradient from 1mM-0.4M ammonium sulphate as described in Section 3.2. Fraction size was 15ml.

3.6.2 CIBACRON BLUE CHROMATOGRAPHY OF SHEEP LIVER PHOSPHOFRUCTOKINASE

A typical elution profile of sheep liver PFK from the Cibacron Blue column is shown in Fig. 10. The majority of the PFK activity is eluted in a fairly sharp peak starting approximately 50-60ml after the introduction of the elution buffer. As can be seen from Table XVII approximately 93% of the activity loaded onto the column, and 48% of the original activity was recovered at this point. SDS-PAGE of this preparation indicated the presence of 5-6 minor bands and a major band which corresponded to a molecular weight of approximately 80-84 000, and which was presumably the liver PFK subunit. PFK in the pooled fractions from this step was precipitated by the addition of ammonium sulphate to 60% saturation, and the precipitated enzyme then collected by centrifugation, and the precipitate redissolved in Cibacron Blue column buffer and rechromatographed. Following rechromatography of this fraction, SDS-PAGE showed that a single band was prevalent with only one minor band of a slightly higher molecular weight being present. Investigation into the nature of this band is discussed in Section 5.2-5.2.2.

3.6.3 SUMMARY OF THE PURIFICATION OF SHEEP LIVER PHOSPHOFRUCTOKINASE.

Data from a typical PFK purification from 4kg of frozen sheep liver is shown in Table XVII. Compared to the sheep heart muscle preparation which yields 120-130mg PFK/1.8kg of sheep heart, the yield from sheep liver is very low at 25-30mg/4kg (Table XVII). This is due to the low level of PFK found in the liver, since the liver is primarily a gluconeogenic tissue. González et al. (1975) have studied the tissue distribution of PFK from several different animal sources including mouse, rat, guinea pig and rabbit. In each case the liver contained the lowest level of PFK while skeletal muscle contained the highest. Studies of the distribution of PFK in rabbit tissues showed that the kidney, adipose tissue and liver contained the lowest amounts of PFK of all the tissues investigated (Table XVIII). Whereas, as expected, the activities were highest in those tissues considered to have high rates of glycolysis such as skeletal muscle, heart, and brain.

Comparison of the purification achieved for sheep liver PFK in this study compares favourably with those from other liver sources by other workers (Table XIX), and is significantly better in a number of cases. The purification of rat liver PFK by Kasten et

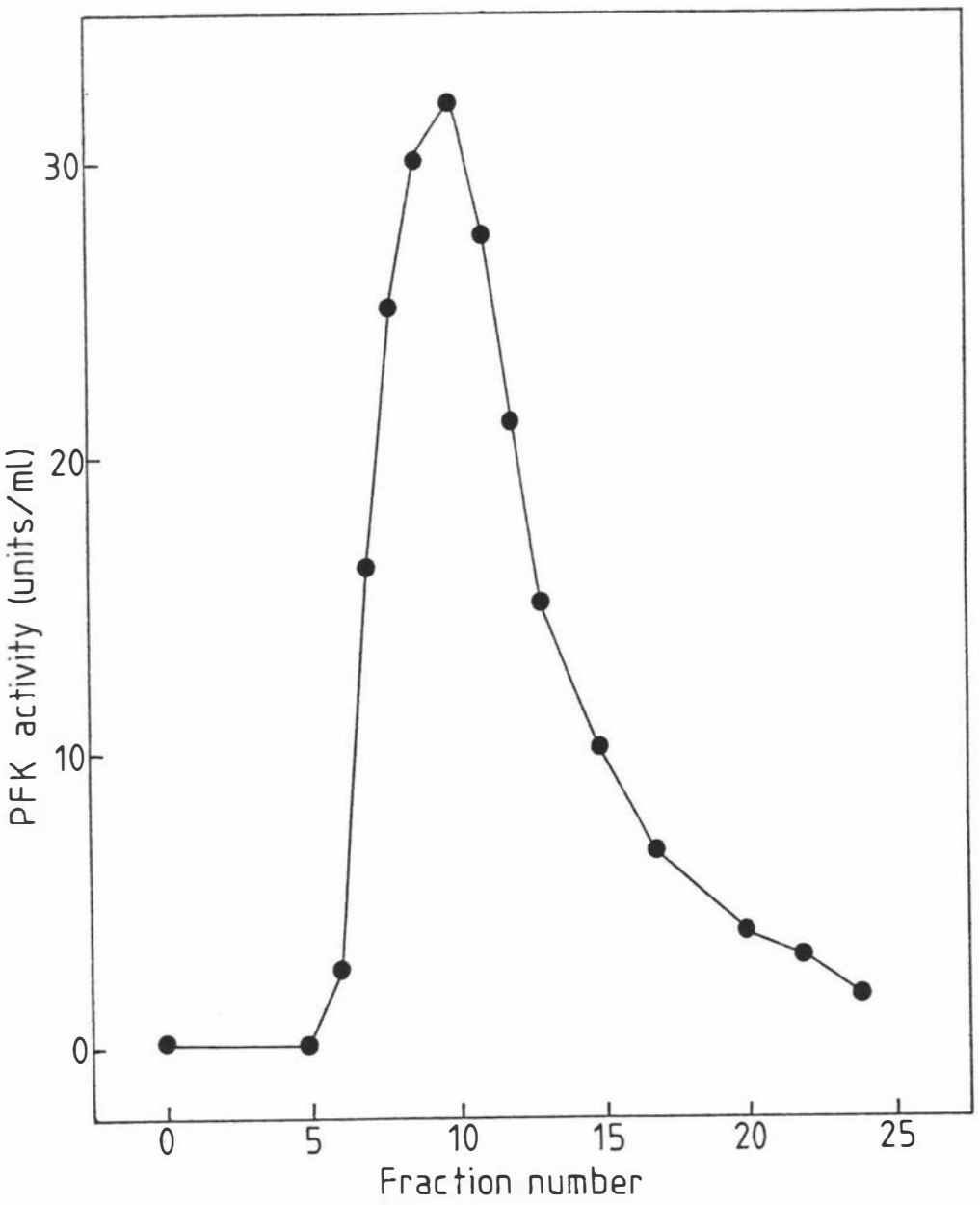


Figure 10: Elution Profile of Sheep Liver PFK from Cibacron Blue.

The pooled, dialysed fraction from the DEAE-cellulose chromatography step was appled to the Cibacron Blue column at a flow rate of 60ml/hr. Following washing of the column with Cibacron Blue column buffer containing 0.15mM ADP, the PFK was eluted with Cibacron Blue column buffer containing 2mM ATP and 2mM F6P as described in Section 3.2. Fraction size was 10ml.

TABLE XVII

PURIFICATION OF SHEEP LIVER PFK.

Fraction	Total Protein mg	Total Activity Units	Specific Activity Units/mg	Yield %
I	355520	5464	0.015	100
II	110720	4936	0.045	90.3
III	100980	4368	0.043	79.9
IV	91430	552	0.006	10.1
V	2904	3686	1.27	67.5
VI	476	2813	5.91	51.5
VII	26.3	2625	99.8	48

4kg of sheep liver tissue used

Fraction
- I Combined supernatants after homogenization
- II Combined supernatants after heat step
- III Supernatant after 3% PEG precipitation step
- IV Supernatant after 7.5% PEG precipitation step
- V Redissolved precipitate, loaded onto the DEAE-cellulose column
- VI Pooled material eluted from the DEAE-cellulose column
- VII Pooled material eluted from the Cibacron Blue column

TABLE XVIII

DISTRIBUTION OF PFK ACTIVITY IN RABBIT TISSUES.

Tissue	PFK Activity Units/Wet weight of tissue (g)	Specific Activity Units/mg
Skeletal muscle	156	2.70
Heart	22.4	0.35
Cerebrum	10.5	0.26
Testes	5.4	0.093
Lung	4.4	0.070
Spleen	3.1	0.064
Kidney cortex	2.8	0.027
Liver	2.4	0.023
Stomach	2.0	0.038
Uterus	1.4	0.039
Adipose tissue	0.5	0.031

The PFK activities were measured in 18 000xg supernatant fractions of crude homogenates prepared in 30mM KF, 4mM EDTA, 5mM DTT, at pH 7.5 (Tsai and Kemp, 1973).

TABLE XIX

PURIFICATION OF LIVER PFK FROM DIFFERENT SOURCES.

Source	Protein mg/kg tissue	Specific Activity Units/mg	Yield %	Ref
Sheep	5.70	18.5	22	(i)
Rabbit	2.42	48.0	11	(ii)
Rat	2.60	86.9	15	(iii)
Rat	7.71	206.0	30	(iv)
Rat	1.70	90.0	8.5	(v)
Rat	3.30	85.0	28	(vi)
Rat	2.00	95.0	28	(vii)
Pig	1.53	100.0	18	(viii)

Final yields and specific activities achieved for the purification of liver PFK from different species as reported from various laboratories.

(i) Brock, 1969
(ii) Kemp, 1971
(iii) Brand and Söling, 1974
(iv) Kasten et al., 1983
(v) Kagimoto and Uyeda, 1979
(vi) Reinhart and Lardy, 1980a
(vii) Dunaway and Weber, 1974a
(viii) Massey and Deal, 1973

al. (1983) resulted in a specific activity of 90.1Units/mg after the Cibacron Blue chromatography step, which is comparable to the 99.8Units/mg achieved for sheep liver PFK in this study. The subsequent gel filtration step used by Kasten et al. (1983), more than doubled the specific activity of their preparation, but lost half of the activity present after the previous step. This step was therefore not implemented for sheep liver PFK.

3.7 PURIFICATION OF SHEEP HEART PHOSPHOFRUCTOKINASE

The method used to purify sheep heart PFK was a combination of the methods used by Kemp (1975b), Hussey et al. (1977) and Kasten et al. (1983). Hearts from freshly killed sheep were collected from the freezing works packed in ice. Fat and connective tissue were removed before the hearts were sliced into small pieces, frozen and stored at -15°C. Unless otherwise stated all operations were carried out at 0-4°C, and all % are (w/v).

1.8kg of sheep heart tissue was minced into 4litre of cold homogenisation buffer (10mM Tris-HCl at pH 8.0, 2mM EDTA). The material was homogenized at low speed for 30sec, followed by 30sec at high speed in a 4litre stainless steel Waring blender. At this stage sheep heart PFK was located in the sedimentable cellular fraction in an inactive state (Mansour et al., 1966). The homogenate was therefore centrifuged at 8 500rpm for 10min in a Sorval RC2-B centrifuge, and the supernatant discarded. The precipitated PFK was solubilized by homogenizing in a Waring blender for 15sec at low speed in 1.8litre of cold extraction buffer containing ATP and $MgSO_4$ (10mM Tris-HCl pH 8.6, 50mM $MgSO_4$, 5mM βME, 0.5mM ATP, 0.1mM EDTA), this also resulted in its reactivation.

The pH of the re-extracted precipitate was adjusted to 8.0 using saturated Tris base. One litre batches were heated with constant stirring to 57°C in a 78°C water bath, the optimum heating time being 7 to 8 min. Each batch was then maintained at 57°C for 3min by transferring to a 57°C water bath. The heat-treated suspension was rapidly cooled to 4°C by transferring to a -5°C methanol bath, and stirring. Heat precipitated material was removed by centrifugation at 8 500rpm for 15min. The red supernatant was filtered through Miracloth (Fraction I) and made up to 38% saturation with ammonium sulphate. After a 30min equilibration period the precipitated material was removed by centrifugation at 8 500rpm for 10min (Fraction II). The resultant supernatant was adjusted to 55% saturated ammonium sulphate, and equilibrated for a further 30min before centrifuging at 8 500rpm for 10min to remove the precipitated

material containing most of the PFK activity. The supernatant (Fraction III), was discarded. The precipitate was resuspended in 100ml of cold phosphate buffer (10mM PO_4 at pH 8.0, 5mM βME), and dialysed against 5litre of the same buffer overnight. The dialysate was centrifuged for 10min at 12 000rpm to remove any precipitated material, and the PFK then precipitated (Fraction IV) by reducing the pH of the dialysate to 6.1 with saturated KH_2PO_4, and collected by centrifugation at 8 500rpm for 10min. The supernatant (Fraction VI) was discarded.

The pH 6.1 acid precipitate (Fraction V), containing PFK activity was dissolved in a minimum volume of Cibacron Blue column buffer (50mM Tris PO_4 at pH 8.0, 0.1mM EDTA, 0.05mM F16BP, 1mM DTT), and dialysed against 5litre of this buffer until the conductivity of the dialysate was below 3mmho. The dialysed material (Fraction VII) was then loaded onto a Cibacron Blue column (27.5cm x 2.0cm) which had previously been equilibrated with Cibacron Blue column buffer. Following loading, the column was washed with Cibacron Blue column buffer until the absorbance of the eluant at 280nm returned to baseline. The column was then washed with 300ml of Cibacron Blue column buffer containing 0.15mM ADP. Elution of the PFK was then achieved by washing with Cibacron Blue column buffer containing 2mM ATP and 2mM F6P. PFK enzyme assays were carried out, and fractions containing high levels of activity were pooled (Fraction VIII), and stored as a 60% saturated ammonium sulphate suspension. A flow diagram of the purification procedure is shown in Fig. 11.

3.8 RESULTS AND DISCUSSION OF THE SHEEP HEART PHOSPHOFRUCTOKINASE PURIFICATION

Data from a typical purification of 1.8kg of frozen sheep heart are shown in Table XX. The apparent increase in activity in Fraction II was typical of all preparations after the addition of 38% saturated ammonium sulphate. This increase has been suggested to be due to enzyme activation by either the NH_4^+ or the SO_4^{2-} ions (Mansour et al., 1966), by a change in subunit association due to protein concentration changes, or the precipitation of some inhibitory factor. The inclusion of the Cibacron Blue chromatography step instead of the previously used high and low salt gel filtration columns (Hussey et al., 1977), resulted in an increased specific activity from approximately 120 to 185-200Units/mg, while maintaining a similar yield.

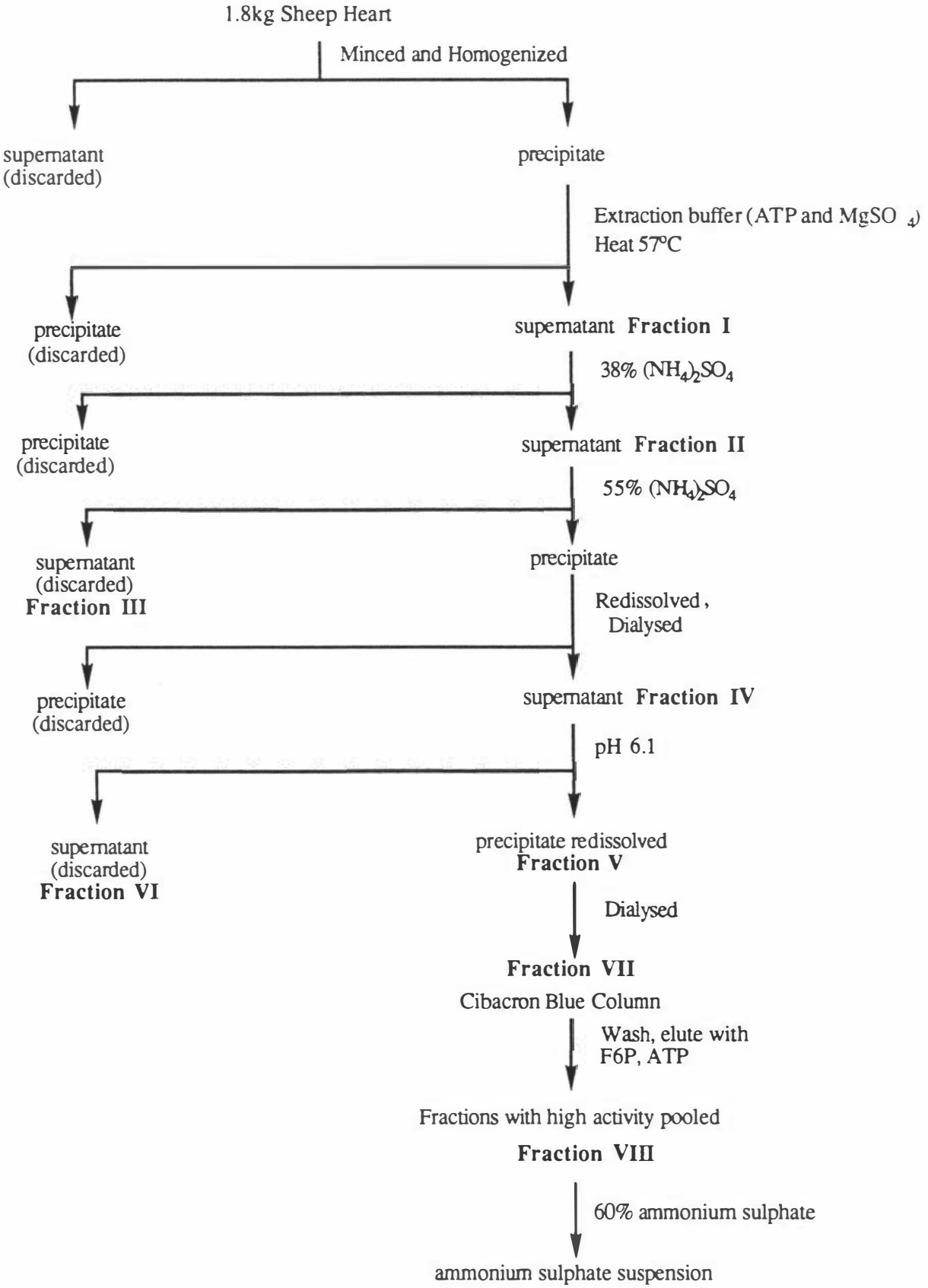


Figure.11: Flow Diagram of the Purification Procedure for Sheep Heart Muscle PFK.

The procedure is as described in Section 3.7.

TABLE XX

PURIFICATION OF SHEEP HEART PFK.

Fraction	Total Protein mg	Total Activity Units	Specific Activity Units/mg	Yield %
I	14120	62428	4.42	100
II	13536	63473	4.69	102
III	3840	6174	1.61	9.9
IV	2459	33159	13.48	53
V	390	24971	64	40
VI	1436	2865	2.0	4.6
VII	390	25424	65.2	41
VIII	121.5	22478	185	36

1.8kg of sheep heart tissue used

Fraction
- I Heat step supernatant
- II Supernatant, 38% ammonium sulphate precipitation step
- III Supernatant, 55% ammonium sulphate precipitation step
- IV Dialysed 38-55% ammonium sulphate precipitate
- V Redissolved pH 6.1 precipitate
- VI Supernatant from pH 6.1 precipitate
- VII Redissolved pH 6.1 precipitate after dialysis
- VIII Pooled fraction from the Cibacron Blue column

CHAPTER FOUR

METHODS

4.1 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Electrophoresis of SDS-7.5% polyacrylamide slab gels (16cm x 12cm) was carried out using a modified version of that described by Laemmli (1970). The 7.5% running gel (7.5% acrylamide, 0.25% bis-acrylamide, 0.375M Tris-HCl pH 8.9, 1% SDS, 0.1% TEMED, 0.1% ammonium persulphate), which was degassed prior to the addition of SDS and the polymerizing agents, was poured to within 3cm of the top of the gel plates. To produce an even surface, water was overlaid over the running gel. Following polymerization the water layer was poured off and the stacking gel solution (5% acrylamide, 0.5% bis-acrylamide, 0.0625M Tris-HCl pH 6.7, 1% SDS, 0.1% TEMED, 0.1% ammonium persulphate) added. A sample slot comb was inserted immediately into the stacking gel, and the gel allowed to polymerize. Following polymerization, the sample comb was removed and the slots washed with electrode buffer (0.01M Tris-glycine pH 8.3, 0.1% SDS). The gel was then clipped into a slab gel electrophoresis tank and the upper and lower reservoirs filled with electrode buffer. Following the application of samples, the gel was electrophoresed at 18mA until the samples had entered the running gel, and then at 20mA until the dye indicator marker had migrated to the bottom of the gel. Electrophoresis was then stopped, and the gel removed and placed in Coomassie Blue staining solution (0.125% Coomassie Brilliant Blue R-250, 5% Acetic acid, 45% methanol), and allowed to stain overnight. The gel was then destained by diffusion in 47.5% methanol and 7% acetic acid. The gel could then be photographed and dried.

4.1.1 PREPARATION OF SAMPLES FOR SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Samples stored as $(NH_4)_2SO_4$ precipitates were dialysed overnight against a $^1/_{12}$ dilution of the stacking gel buffer. Samples were then diluted 2:1 (v/v) with sample buffer (0.0625M Tris pH 6.7, 10% glycerol, 5% βME, 3% SDS and 1 drop of bromophenol blue) and heated to 100°C for 3min. Between 5-50µl of the samples

were loaded onto the gels, which were then electrophoresed as described in Section 4.1.

4.2 CARBOXYMETHYLATION

PFK was dissolved in, and dialysed against 0.1M Tris-HCl to remove ammonium sulphate and low molecular weight compounds. Following dialysis, solid guanidine hydrochloride was added to the dialysate to give a concentration of 7.0M. This was carried out in a round bottom flask under nitrogen. After 10min DTT (0.05µmol/mg enzyme) was added, and if necessary the pH adjusted to 8.0. Reduction was allowed to proceed under nitrogen for 2-3hr at room temperature. 1µmol [2^{14}C]-iodoacetic acid containing approximately 10.8µCi was added per 1µmol of total thiol groups, and carboxymethylation allowed to proceed in the dark, under nitrogen for 45min. A further 3µmol of cold iodoacetic acid per 1µmol of total free thiol groups was then added, and allowed to react for a further 45min. The reaction was terminated by the addition of excess βME. The solution was extensively dialysed against 4 x 5litre of distilled H_2O, to remove excess reagents, during which time protein precipitation occurred. The material was then freeze-dried in preparation for digestion.

4.3 AMINO ACID ANALYSIS

Analyses were performed on a Beckman Model 119BL amino acid analyser with an automatic sample injector.

Analyses on the whole protein were carried out by dissolving a known amount (0.88mg) of protein in 1.4ml of 70% formic acid. 200µl aliquots were then transferred into pyrex hydrolysis tubes (12mm x 100mm) and dried. Duplicate 24, 48 and 72hr hydrolyses were performed under vacuum in 250µl of 5.9M glass distilled HCl containing 1% phenol at 110°C. The hydrolysates were then dried under vacuum over NaOH pellets, and analysed.

4.4 TRYPTIC DIGESTION OF PFK

Carboxymethylated PFK was digested with TPCK treated trypsin at an enzyme to substrate ratio of 1:100, in 0.5% ammonium bicarbonate. Digestion was allowed to proceed for 4-5hr at 37°C, during which time the pH was maintained at 8.0. As digestion proceeded the turbid solution cleared, indicating that digestion was occurring.

The reaction was terminated by lyophilization, in preparation for HPLC mapping and sequencing.

4.5 CNBr DIGESTION OF PFK

The carboxymethylated substrate was dissolved in a small volume of 70% formic acid, and gassed with oxygen free nitrogen. CNBr was added in 200-fold molar excess over the total methionine content, and the reaction allowed to proceed overnight in a stoppered vessel at 20°C. The sample was then transferred to a conical centrifuge tube and centrifuged at 2 000 rpm for 5min, and the supernatant was removed. The precipitate was further digested by resuspending in 70% formic acid, and adding a further 100-fold molar excess of CNBr. The reaction was allowed to continue for 24hr before undergoing centrifugation to remove the precipitate. The supernatants containing the digested PFK were pooled in preparation for FPLC (Sections 4.6.3 and 4.8.1), HPLC (Section 4.6.2), or sequencing (Section 4.8.1).

4.6 PEPTIDE MAPPING OF SHEEP HEART AND LIVER PFK

A Waters Assoc. (Milford, MA,USA) high performance liquid chromatography (HPLC) system was used for mapping the trypsin and CNBr digested muscle and liver PFK peptides. This consisted of two M600A solvent delivery units, an M660 solvent programmer and a U6K universal liquid chromatography injector, coupled to an M450 variable wavelength UV spectrophotometer and an Omniscribe two channel recorder (Houston Instruments, Austen, TX, USA). Sample injections were made using a 250µl syringe.

The columns used for the separation were a Resolve Radial compression C-18 reverse phase (spherical 10µ, 60-175Å pore size, with a C load of 12%, which was not endcapped), or a Vydac C-4 reverse phase column, which was especially packed into a rad-pak cartridge (spherical 20µ, 300Å pore size, endcapped).

4.6.1 MAPPING OF TRYPTIC PEPTIDES USING HPLC

Samples of [^{14}C]-carboxymethylated muscle and liver PFK were digested in parallel with TPCK treated trypsin as described in Section 4.4. Following lyophilization the digested material was dissolved in a small volume of 0.5% ammonium bicarbonate (Am Bic), and centrifuged at 2 000rpm for 10min. The supernatant was removed and the precipitate washed 3 times with ammonium bicarbonate. The supernatants

containing the ammonium bicarbonate-soluble tryptic peptides were combined and freeze-dried. The precipitate, containing the ammonium bicarbonate-insoluble tryptic peptides was freeze-dried separately.

The muscle and liver PFK ammonium bicarbonate-soluble peptides were dissolved in 0.1M ammonium bicarbonate in preparation for HPLC mapping. Duplicate 200µl aliquots of each sample were injected onto a Resolve RC C-18 column. The sample was washed onto the column using buffer A (0.1M Am Bic) at a rate of 1ml/min. Elution of peptides was achieved using a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1) run over 2hr at a rate of 1ml/min. The eluant was monitored at 230nm, and fractions were collected manually in scintillation vials in preparation for the determination of radioactivity (Section 4.7).

The ammonium bicarbonate-insoluble tryptic peptides were dissolved in 250µl of 70% formic acid, and any insoluble material was removed by centrifugation at 2 000rpm for 10min. The supernatant was then used for HPLC mapping using a Vydac C-4 column. Following injection (200µl), the sample was washed onto the column using buffer A (5% formic acid) at a rate of 1ml/min. Elution of peptides was achieved using a linear gradient from 0-100% B (5% formic acid/isopropanol/acetonitrile 1:1:1) run over 2hr at a rate of 1ml/min. The eluant was monitored at 280nm, and fractions collected manually in scintillation vials in preparation for the determination of radioactivity (Section 4.7).

4.6.2 MAPPING OF CNBr PEPTIDES USING HPLC

Samples of [^{14}C]-carboxymethylated muscle and liver PFK were digested with CNBr in parallel as described in Section 4.5. 200µl of the supernatants from the CNBr digests were injected onto a Vydac C-4 reverse phase column, and washed on with buffer A (5% formic acid), at a rate of 1ml/min. A linear gradient from 0-100% B (5% formic acid/isopropanol/acetonitrile 1:1:1) was run over 2hr at a rate of 1ml/min. Monitoring of the eluant was carried out at 280nm, and fractions collected manually in scintillation vials in preparation for the determination of radioactivity (Section 4.7).

4.6.3 MAPPING OF CNBr PEPTIDES USING FPLC

Samples of [^{14}C]-carboxymethylated muscle and liver PFK were digested with CNBr in parallel as described in Section 4.5. The supernatants from the CNBr digests were then used for mapping on a Fast protein liquid chromatography system (FPLC),

Pharmacia (Sweden). This system was resistant to the high levels of formic acid necessary for the efficient separation of the CNBr peptides of PFK.

200µl of the supernatants were loaded onto a Pharmacia Superose 12 gel filtration column previously equilibrated with 50% formic acid, at a rate of 0.4 ml/min. The eluant was monitored at 280nm, and fractions collected manually in scintillation vials in preparation for the determination of radioactivity (Section 4.7).

4.7 DETERMINATION OF RADIOACTIVITY

^{14}C-radioactivity was determined using a Beckman LS8000 scintillation counter. Samples were dried under vacuum in the scintillation vials and counted in 5ml Scintillation solvent (Triton X-100/toluene 1:2 v/v, containing 0.4% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis [2-(5-phenyloxazolyl)]benzene (POPOP)).

4.8 PREPARATION OF PFK PEPTIDES FOR SEQUENCING

4.8.1 CNBr PEPTIDES

20mg of purified liver PFK was carboxymethylated with [^{14}C]-iodoacetic acid as described in section 4.2. The freeze-dried material was then subjected to CNBr digestion as described in Section 4.5. FPLC as described in Section 4.6.3 was used for the initial separation of the CNBr digested liver PFK. Approximately 2ml of the supernatant from the CNBr digest was loaded in 200µl aliquots onto a Pharmacia Superose 12 gel filtration column, previously equilibrated with 50% formic acid, at a rate of 0.4ml/min. The eluant was monitored at 280nm and identical fractions collected manually and pooled from each run. Fractions were then freeze-dried in preparation for further purification by HPLC.

The low molecular weight fractions (Fig. 30, fractions H-N), were further purified by HPLC on a Resolve RC C-18 column, and the higher molecular weight fractions (Fig. 30, fractions A-G), were separated by HPLC on a Vydac C-4 column as described for the ammonium bicarbonate-soluble and insoluble tryptic peptides respectively in Section 4.6.1. Peaks were collected manually by observing the absorbance at 230nm or 280nm respectively, and were freeze-dried in preparation for sequencing. A flow diagram of the preparation of the CNBr peptides for sequencing is shown in Fig. 12.

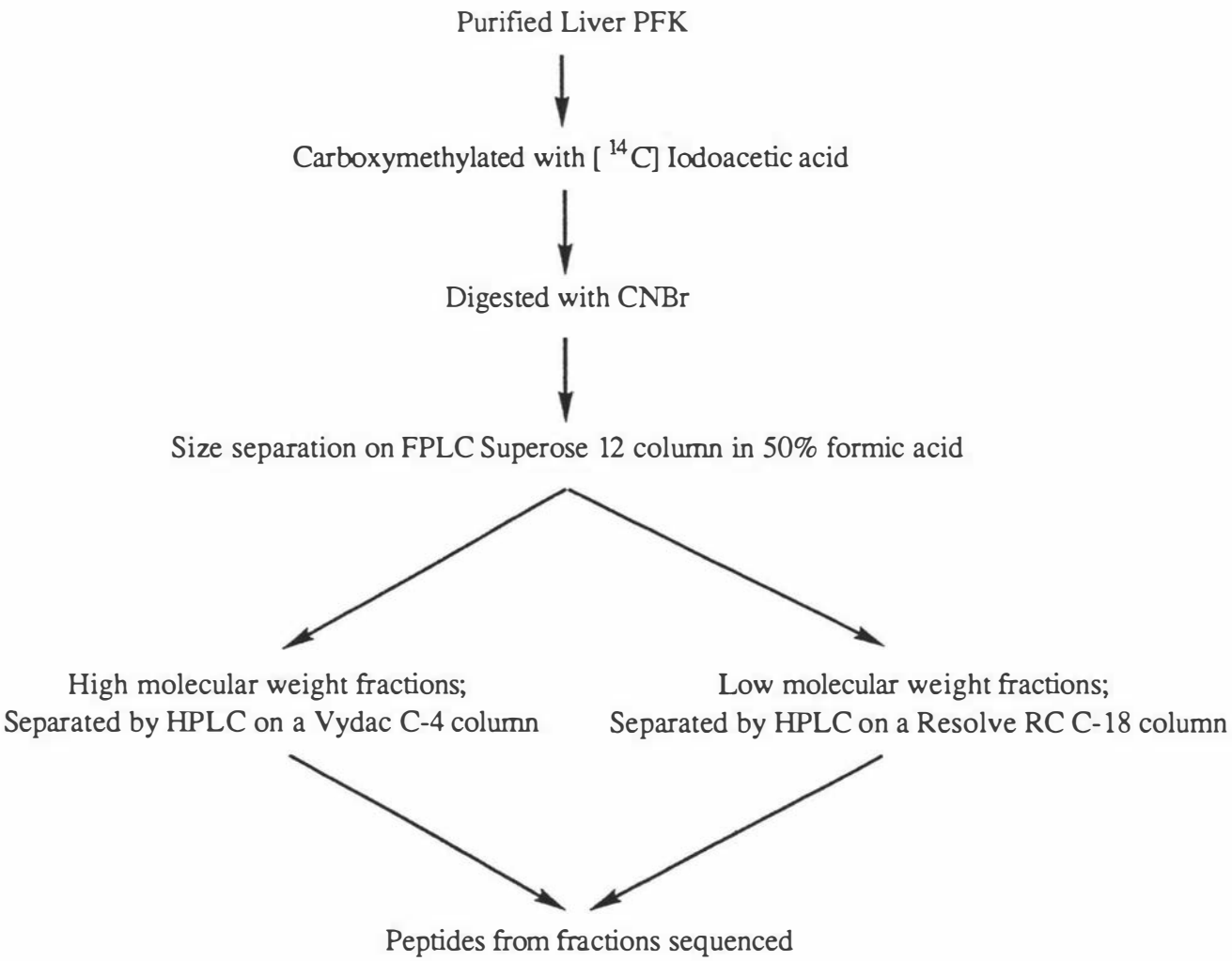


Figure 12: Flow Diagram of the Preparation of the CNBr Peptides for Sequencing.

The procedure is as described in Section 4.8.1.

4.8.2 TRYPTIC PEPTIDES

5mg of [^{14}C]-carboxymethylated liver PFK was digested with trypsin as described in Section 4.4. Only the ammonium bicarbonate-soluble peptides were purified for sequencing. This was carried out as described in Section 4.6.1, and peptides for sequencing were collected manually by observing the absorbance at 230nm. Fractions were freeze-dried in preparation for sequencing. A flow diagram of the preparation of the tryptic peptides for sequencing is shown in Fig. 13.

4.8.3 MALEYL-TRYPTIC PEPTIDES OF LIVER PFK

4.8.3.1 MALEYLATION OF LIVER PFK

40mg of [^{14}C]-carboxymethylated liver PFK was dissolved in 8ml of 6M guanidine hydrochloride. The pH was adjusted to 9.0 before the addition of small quantities of maleic anhydride. The solution was stirred constantly and the pH maintained between 8.5 and 9.0 using dilute NaOH. Sufficient maleic anhydride was added over the period of 1hr to provide a 500-fold molar excess over the total lysine content. Following the completion of the reaction, the protein was dialysed extensively against 4 x 5litre of distilled water with ammonia added to maintain a pH of between 8.5 and 9.0. The material was then freeze-dried in preparation for digestion with trypsin.

4.8.3.2 TRYPTIC DIGEST OF MALEYLATED LIVER PFK

The tryptic digest of maleylated liver PFK (Section 4.8.3.1) was carried out as described in Section 4.4, except that following the completion of the digestion, the material was centrifuged at 8 000rpm for 10min, and the supernatant decanted and heated in a boiling water bath for 6min, to inactivate the trypsin. The material was then cooled, and freeze-dried in preparation for the removal of the maleyl groups.

4.8.3.3 REMOVAL OF MALEYL GROUPS

The digested material from Section 4.8.3.2 was dissolved in a small volume of distilled deionised water, and the pH adjusted to 3.5 using dilute acetic acid. Demaleylation was achieved by heating at 60°C overnight at pH 3.5. During demaleylation some precipitation occurred. This precipitated material was removed by centrifugation at 8 000rpm for 10min. The precipitate was washed twice with dilute acetic acid and recentrifuged, and the combined supernatants containing the acid-soluble peptides were

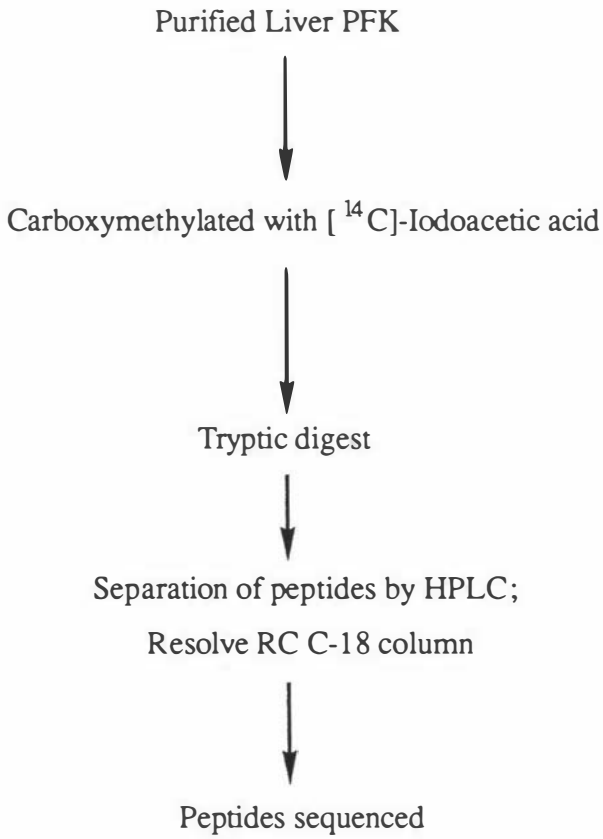


Figure 13: Flow Diagram of the Preparation of the Tryptic Peptides for Sequencing.

The procedure is as described in Section 4.8.2.

freeze-dried. The precipitate containing the acid-insoluble peptides was freeze-dried separately in preparation for purification by ion-exchange chromatography and HPLC.

4.8.3.4 SEPARATION OF ACID-SOLUBLE MALEYL-TRYPTIC PEPTIDES

A Spectra Physics SP8800, high pressure liquid chromatography system, coupled to a SP8490 dual wavelength UV spectrophotometer, was used to separate the maleyl-tryptic peptides.

Columns used for separation were a Pharmacia Mono-Q ion-exchange, and a Vydac C-18 reverse phase column (spherical 10µ, 300Å pore size, endcapped). The acid-soluble material was dissolved in 0.5ml of 20mM Tris-HCl pH 8.0, and undissolved material removed by centrifuging at 2 000rpm for 10min. A 25µl aliquot was injected onto the Mono-Q column, and washed on with buffer A (20mM Tris-HCl pH 8.0), at a rate of 0.5ml/min. Elution was achieved by a linear gradient from 100% A to 100% B (20mM Tris-HCl pH 8.0, 1M NaCl), over 2hr at a rate of 0.5ml/min. The eluant was monitored at 280nm, and fractions collected manually in scintillation vials in preparation for the determination of radioactivity (Section 4.7). The remainder of the acid-soluble material was separated under the same conditions and 12 fractions (Fig. 42) were collected manually, these were then freeze-dried, awaiting further purification.

Samples 1-12 collected from the Mono-Q ion-exchange column were each dissolved in 0.1M ammonium bicarbonate, and undissolved material removed by centrifuging at 2 000rpm for 10min. Each individual sample was then loaded onto a Vydac C-18 column by a series of multiple injections. The sample was washed onto the column with buffer A (0.1M Am Bic, 5% acetonitrile) at a rate of 1ml/min. Elution was achieved by a linear gradient from 0-100% B (0.1M Am Bic /isopropanol/acetonitrile 1:1:1) in 2hr. The eluant was monitored at 230nm and 280nm, and fractions collected manually whenever a significant peak was detected on the recorder. The fractions were lyophilized and stored at -4°C awaiting sequencing.

4.8.3.5 SEPARATION OF ACID-INSOLUBLE MALEYL-TRYPTIC PEPTIDES

The acid-insoluble maleyl-tryptic peptides were dissolved in 20mM Tris-HCl pH 8.0, and undissolved material removed by centrifuging at 2 000rpm for 10min.

Acid-insoluble maleyl-tryptic peptides were then separated on a Mono-Q ion-exchange column as described in Section 4.8.3.4. Nine separate fractions were collected (Fig. 48), and each of these individual fractions (1-9), underwent further purification on a Vydac C-18 column as described in the previous section. A flow diagram of the preparation of the maleyl-tryptic peptides for sequencing is shown in Fig. 14.

4.9. SEQUENCING OF PEPTIDES

Peptides were sequenced on an ABI 470A gas phase sequencer with an on-line ABI 120A PTH analyzer (Hewick et al., 1981), using the standard PTH program version 3.0. With the exception of 5% PITC in n-heptane and HiPerSolv grade acetonitrile from BDH Chemicals, solvents and reagents were purified as described by Esch (1984).

1-2nmoles of sample, were dissolved in either distilled water or 50% formic acid as in the case of the CNBr peptides, and loaded onto a conditioned ABI TFA-treated glass cartridge filter loaded with ABI BioBrenePlus. Amino acid residues were determined by comparison with the PTH amino acid standard. Cysteine residues were determined by the presence of the derivative carboxymethyl-cysteine, and the presence of radioactivity in the appropriate fraction following sequencing.

4.10 FAB MASS SPECTROMETRY

Fast atom bombardment (FAB MS) of liver PFK peptides was carried out on a Kratos MS9 mass spectrometer with an Ion Tech fast atom gun operating at an accelerating voltage of 6-8kV to generate a stream of xenon atoms. Solid samples were dissolved either in glycerol with oxalic acid and lithium oxalate, or HCl, and applied to gold plated probe. The probe was introduced into the ion source via a vacuum lock. All the spectra obtained were of positive ions.

4.11 SEPARATION OF PHOSPHOFRUCTOKINASE ISOZYMES USING NON-DISSOCIATING CONDITIONS

Separation of PFK isozymes was carried out according to the method of Karadsheh et al. (1977). A DEAE-cellulose column (DE 52)(18cm x 0.9cm), was equilibrated with equilibration buffer (25mM Tris-HCl pH 8.0, 5mM $(NH_4)_2SO_4$, 5mM βME, 1mM F6P, 0.5mM F16BP, 1mM EDTA). Samples were prepared by dissolving the PFK from an $(NH_4)_2SO_4$ suspension in equilibration buffer and then dialysing against the

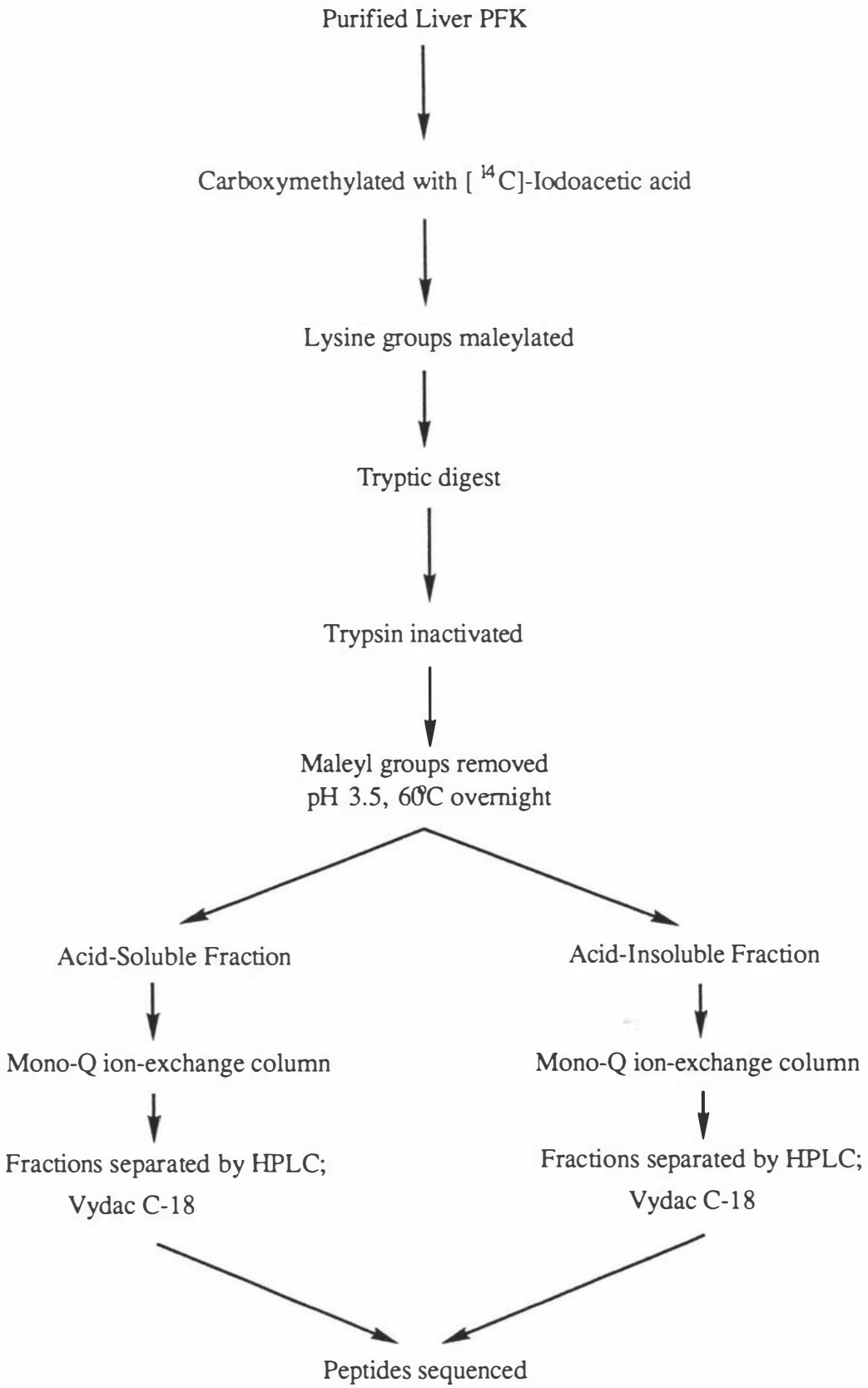


Figure 14: Flow Diagram of the Preparation of the Maleyl-Tryptic Peptides for Sequencing.

The procedure is as described in Sections 4.8.3.2-4.8.3.5.

same buffer overnight. The sample was then loaded, and washed onto the column with equilibration buffer containing 50mM Tris-HCl pH 8.0 instead of 25mM Tris-HCl. The column was washed further with equilibration buffer containing 100mM Tris-HCl , before the linear gradient of 100mM-300mM Tris-HCl in equilibration buffer was started. 2.5-3ml fractions were collected and assayed for PFK activity, fractions containing PFK activity were pooled and made to 60% saturated $(NH_4)_2SO_4$, before undergoing dialysis in preparation for electrophoresis on SDS-polyacrylamide gels (Section 4.1).

4.12 SEPARATION OF PHOSPHOFRUCTOKINASE ISOZYMES USING DISSOCIATING CONDITIONS

Sheep heart and liver PFK were dissociated in 6M guanidine-HCl containing 100mM βME and 10mM Tris-HCl pH 8.0, then dialysed against 2 changes of 8M urea containing 10mM βME and 10mM Tris-HCl pH 8.0. The sample was loaded onto a DEAE-cellulose column (18cm x 1.5cm), which had been previously equilibrated with 8M urea containing 20mM βME and 10mM Tris-HCl pH 8.0. The sample was eluted using a 400ml linear gradient of 0-0.15M NaCl in equilibration buffer. Since PFK is inactive in the dissociated form, the enzyme was located by absorbance at 280nm. Peaks were pooled, dialysed against distilled water to remove the urea, before being freeze-dried and electrophoresed on SDS-polyacrylamide gels (Section 4.1).

CHAPTER FIVE

RESULTS

CHARACTERIZATION OF SHEEP LIVER PHOSPHOFRUCTOKINASE

5.1 SDS-PAGE OF PURIFIED SHEEP LIVER PHOSPHOFRUCTOKINASE

The purity of the sheep liver PFK preparations were examined on SDS-7.5% polyacrylamide gels. In each case the sheep liver PFK preparations showed two bands; a major band of slightly lower molecular weight than sheep heart PFK, and a minor band of slightly higher molecular weight (Fig. 15). The major band was assumed to be the liver PFK isozyme. The nature of the minor band was uncertain, with two possibilities; a) a contaminant which is co-purified with PFK, i.e. binds to Cibacron blue, and is eluted under the same conditions as PFK. This would seem to be rather unlikely in view of the extensive purification procedure used, and the high specific activity of the purified PFK, b) that the band is also a PFK. This possibility would seem to be the more likely of the two. The hypothesis that the upper band is a PFK, and its possible origins are discussed and investigated in Section 5.2.

Tracks in which both heart muscle and liver PFK were loaded failed to resolve the heart and liver isozymes, and a broad band resulted.

5.1.1 MOLECULAR WEIGHT DETERMINATIONS OF SHEEP HEART AND LIVER PFK

The plot of molecular weight versus mobility (Fig. 16) indicated that the molecular weight of the major liver PFK band was approximately 81 000 ± 500, and the minor band approximately 87 000 ± 500, while the sheep heart PFK had a molecular weight of 84 000 ± 500. The molecular weight for sheep heart PFK reported in this study compares favourably with the published molecular weight values (82-85 000) of other muscle PFKs determined by SDS-PAGE (Table I), and is very similar to the value of

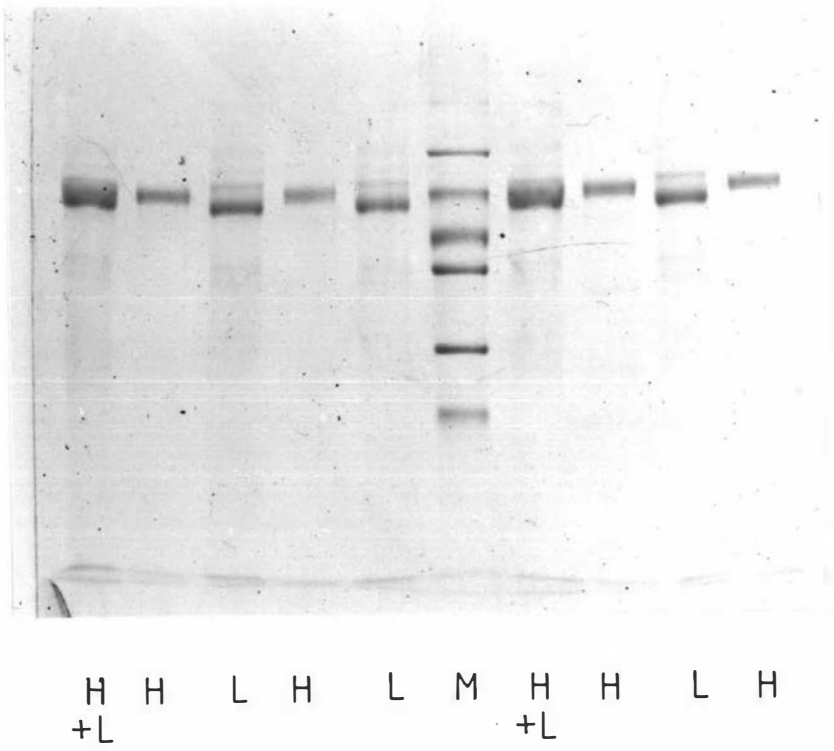


Figure 15: SDS -7.5% Polyacrylamide Gel Showing Purified Sheep Liver PFK.

Samples of purified sheep liver PFK and sheep heart PFK along with molecular weight markers were prepared for electrophoresis as described in Section 4.1.1. Molecular weight markers used were; phosphorylase b (97 400), transferrin (80 000), bovine serum albumin (66 000), catalase (57 000) citrate synthase (43 000) and malate dehydrogenase (37 000). Electrophoresis and staining with Coomassie Blue was as described in Section 4.1.

H=Sheep heart muscle PFK.
L=Sheep liver PFK.
M=Molecular weight markers.

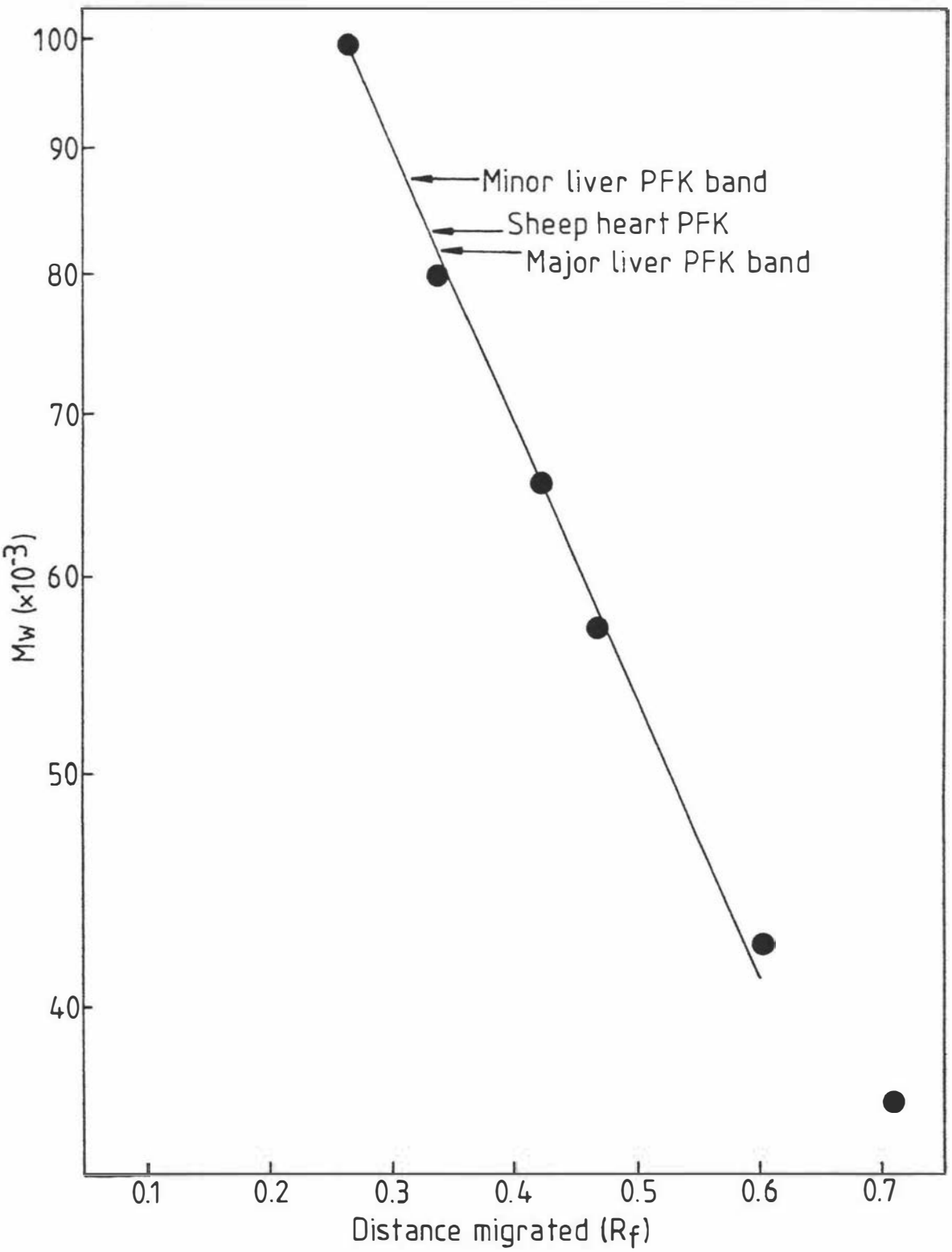


Figure 16: Electrophoretic Mobilities of Standard Proteins Calculated for the SDS-7.5% Polyacrylamide Gel Shown in Fig. 15.

Standard protein molecular weight markers were those listed in Fig. 15.

84 975 calculated from the amino acid sequence, derived from the DNA sequence of rabbit muscle PFK (Lee et al., 1987). The molecular weight of sheep liver PFK reported in this study was also similar to published molecular weight values, with the exception of the value of 65 000 determined by Dunaway and Weber (1974a). This low value was possibly obtained from a liver PFK preparation which had undergone proteolytic cleavage during the purification procedure, as no proteolytic inhibitors were present during any stage of their purification. Indeed early attempts in this laboratory to purify sheep liver PFK in the absence of PMSF resulted in the purification of an enzyme of molecular weight 60 000 ± 2 000 (Figs. 17 and 18), with no protein of molecular weight 80 000 present at all. The gel containing this material shown in Fig. 17 indicated that all the sheep liver PFK had undergone proteolytic cleavage during purification to yield the major band of molecular weight 60 000, as well as several minor bands of molecular weight 52 000, 18 000 and 14 000 (result not shown). Following this result, PMSF was added to the homogenization buffer, and subsequent preparations of sheep liver PFK yielded the higher molecular weight proteins of 81 000 and 87 000.

Despite the fact that the liver PFK had undergone proteolytic cleavage, loss of PFK activity did not occur. This suggests that the cleaved fragment(s) did not dissociate from the tetramer under the purification and assay conditions used. This is in contrast to limited proteolysis experiments performed on muscle PFK with subtilisin, where an enzyme of molecular weight 74 000, which retained its tetrameric form, but lost its activity, was observed (Riquelme and Kemp, 1980). This loss in activity was due to the dissociation of fragment(s) containing the active site.

The difference in apparent molecular weight between sheep heart and sheep liver PFK (2 000-4 000), could be manifest in a number of ways. Firstly, differences in the amino acid composition between the two isozymes (i.e. larger numbers of lower molecular weight residues, and fewer high molecular weight residues in the liver enzyme). Secondly, differences in the number of amino acid residues making up the proteins, in which 25-35 amino acid residues could be lost from sheep liver PFK to account for the 2 000-4 000 difference in molecular weight. Thirdly, a combination of both changes in the amino acid composition, and differences in the number of residues making up the two proteins could explain the difference in molecular weight. Differences in amino acid composition between the two isozymes should be made apparent by a comparison of amino acid analyses. However, a difference in the number of residues making up the proteins can only be ascertained by obtaining the complete amino acid sequence of both proteins.

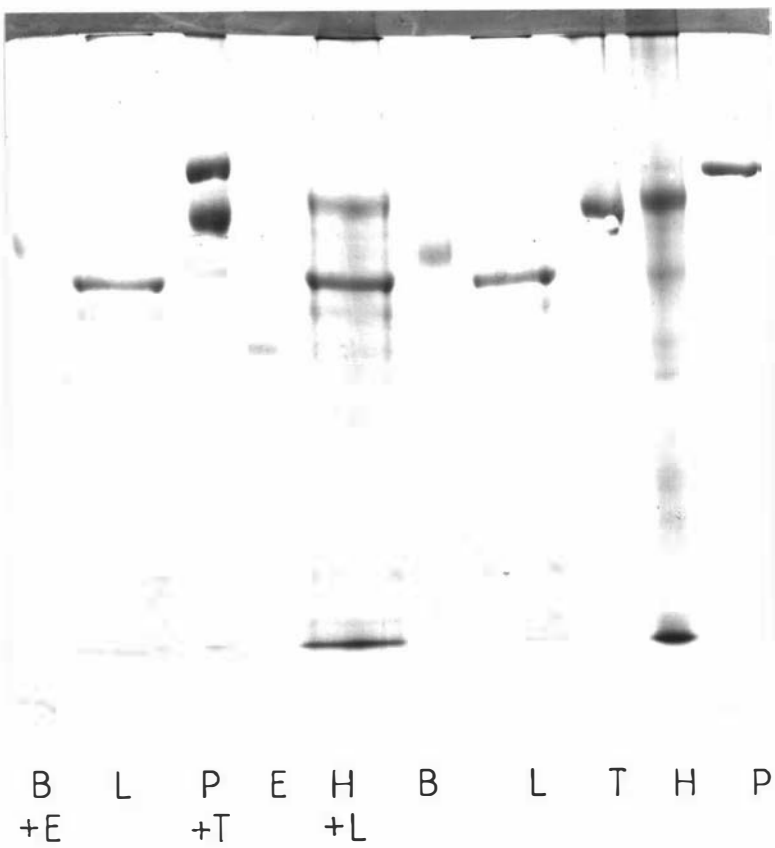


Figure 17: SDS-7.5% Polyacrylamide Gel of Purified Sheep Liver PFK which had Undergone Proteolytic Cleavage During Purification.

Samples of purified sheep liver and sheep heart muscle PFK along with molecular weight markers were prepared for electrophoresis as described in Section 4.1.1. Molecular weight markers used were; phosphorylase b (97 400), transferrin (80 000), bovine serum albumin (66 000) and egg albumin (45 000). Electrophoresis and staining with Coomassie Blue was as described in Section 4.1.

H=Sheep heart muscle PFK.
L=Sheep liver PFK.
P=Phosphorylase B.
T=Transferrin.
B=Bovine serum albumin.
E=Egg albumin.

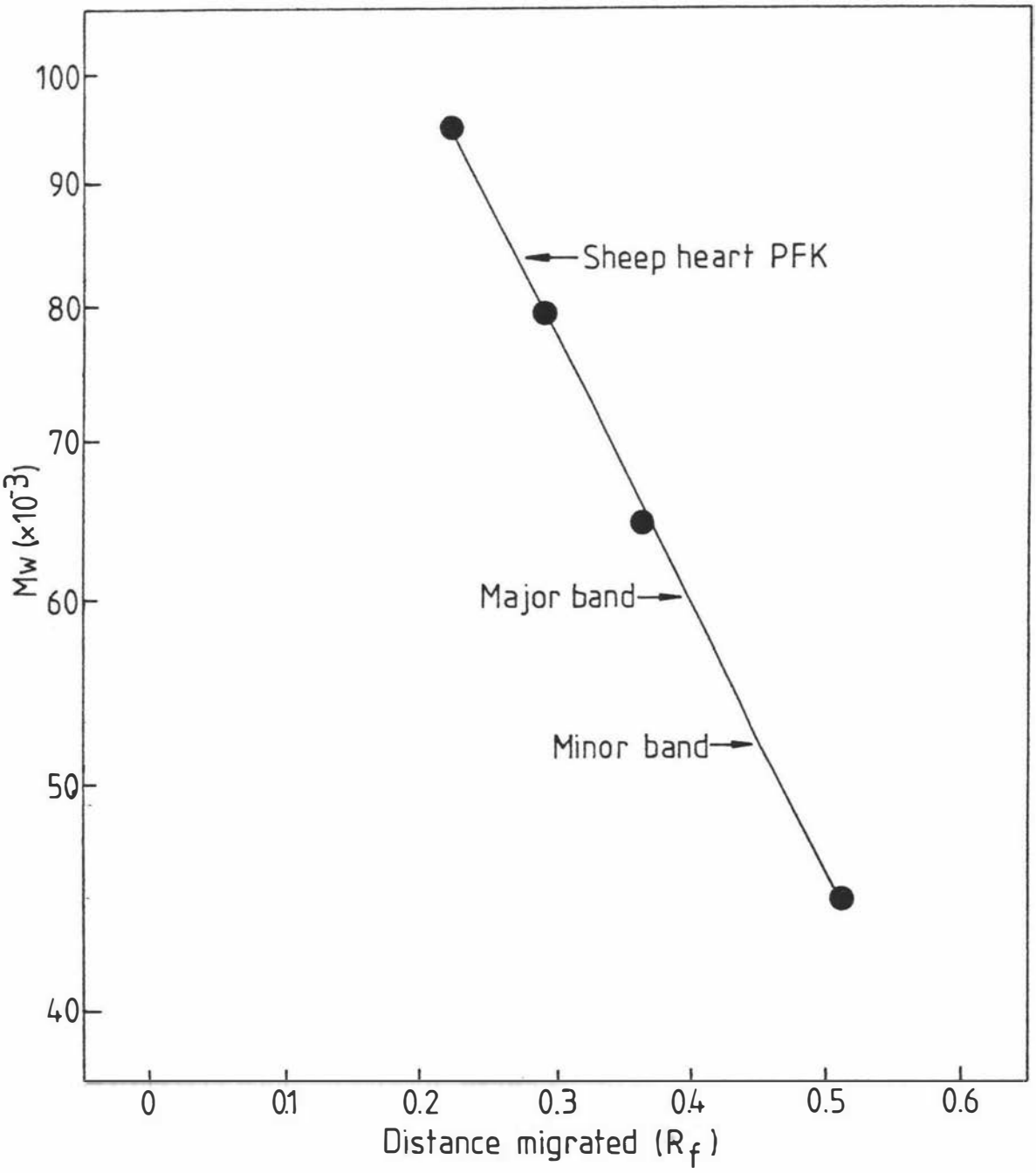


Figure 18: Electrophoretic Mobilities of Standard Proteins Calculated for the SDS-7.5% Polyacrylamide Gel Shown in Fig. 17.

Standard protein molecular weight markers were those listed in Fig. 17.

Analysis of the amino acid composition of sheep liver PFK indicated that it is unlikely that the apparent difference in molecular weight between sheep heart and sheep liver PFK is due to the change in amino acid composition (Section 5.3).

5.2 SEPARATION OF PHOSPHOFRUCTOKINASE ISOZYMES

Purified sheep liver PFK electrophoresed on SDS-7.5% polyacrylamide gels always resulted in the presence of two bands; a major band of molecular weight 81 000 and a minor band of molecular weight 87 000 The major band was considered to be the liver PFK isozyme, but the nature of the minor band was uncertain. As mentioned in Section 5.1 there were two possible sources of the band; a) that it was a contaminant which co-purified with liver PFK, b) that it was an isozyme of PFK.

If the minor band was a PFK, there are several ways in which it could have eventuated:

1) The band could result from the presence of platelet or erythrocyte PFK due to blood remaining in the liver. Platelet PFK consists of hybrids of P and L subunits (Section 1.3.1), while erythrocyte PFK consists of either the L subunit, in which case only one band would have been observed on the gel containing liver PFK, or M plus L hybrids (Section 1.3.1). If these PFKs were electrophoresed with sheep liver PFK on SDS-polyacrylamide gels then this would result in a liver subunit band and a platelet subunit band for platelet PFK. If erythrocyte PFK was present then a broad band consisting of both the muscle and liver subunits would result, since as described in Section 5.1 the sheep heart muscle and sheep liver PFK bands could not be resolved completely. Since two distinct bands were obtained when liver PFK was electrophoresed on SDS-polyacrylamide gels, it is unlikely that the minor band was the result of contamination from erythrocyte PFK.

2) The two bands could arise due to variation in the phosphorylation content of sheep liver PFK. Reports in the literature (Sakakibara and Uyeda, 1983) suggest that phosphorylation content does not cause any change in the apparent electrophoretic mobilities of the PFK subunits.

3) The enzyme from sheep liver itself could consist of a hybrid of P and L subunits, in a ratio such that the liver subunit predominates. This would seem to be the logical choice since the existence of more than one type of PFK subunit has been reported in the livers of several different animals (Section 1.3.1).

Experiments were designed to investigate these options. By chromatographing undissociated enzyme on a DEAE-cellulose (DE52) column it is possible to separate PFK into its constituent heterotetramers. If either erythrocyte or platelet PFK were present, then at least some of the M_4 or P_4 species should be found, along with all the M and L or P and L hybrids. If the PFK from sheep liver consists of hybrids, then the predominant form should be L_4, with hybrids present in lesser amounts. Studies of hybrid liver PFKs from other species (Section 1.3.1) have not found a tetramer composed solely of the minor subunit to exist, therefore it is unlikely that such a species should be present in sheep liver PFK. If contaminating PFK from the blood is present, then hybrids, plus a homotetramer of either M or P subunits will be present. If the liver enzyme itself consists of hybrids, then no such homotetramer composed from the minor subunit should be found.

5.2.1 SEPARATION OF PFK ISOZYMES USING NON-DISSOCIATING CONDITIONS

The elution profile of sheep heart PFK from chromatography on a DEAE-cellulose (DE52) column chromatographed using non-dissociating conditions (Section 4.11), is shown in Fig. 19. The peak containing sheep heart PFK, detected by enzyme activity, eluted early in the gradient and was characterized by a shoulder on the right hand side of the peak. No enzyme activity was eluted after this peak. Of the activity loaded onto the column, 92% was recovered in the eluted peak. The peak was pooled in two fractions (i and ii) as shown in Fig. 19. When electrophoresed on an SDS-7.5% polyacrylamide gel no difference in molecular weight could be detected between the PFK subunits collected in fractions i and ii (result not shown). The peak shape and elution position were similar to those described for PFK from human muscle (Karadsheh et al., 1977; Vora et al., 1980), and rat muscle, both of which were identified as M_4 (Taylor and Bew, 1970; Oskam et al., 1985; Vora et al., 1985), therefore this peak was also identified as a homotetramer of M_4. It is possible that the presence of the shoulder on the muscle PFK peak could have resulted from different phosphorylation states of sheep heart PFK.

The elution profile of sheep liver PFK after chromatography on a DEAE-cellulose (DE52) column, chromatographed using non-dissociating conditions, is shown in Fig. 20. Two overlapping peaks containing PFK activity, which eluted much later in the gradient compared to sheep heart PFK, were observed. These peaks were collected in three fractions (A, B, and C) as shown in Fig. 20. No PFK activity was recovered in the region where heart PFK was found to elute. Since no muscle PFK tetramers

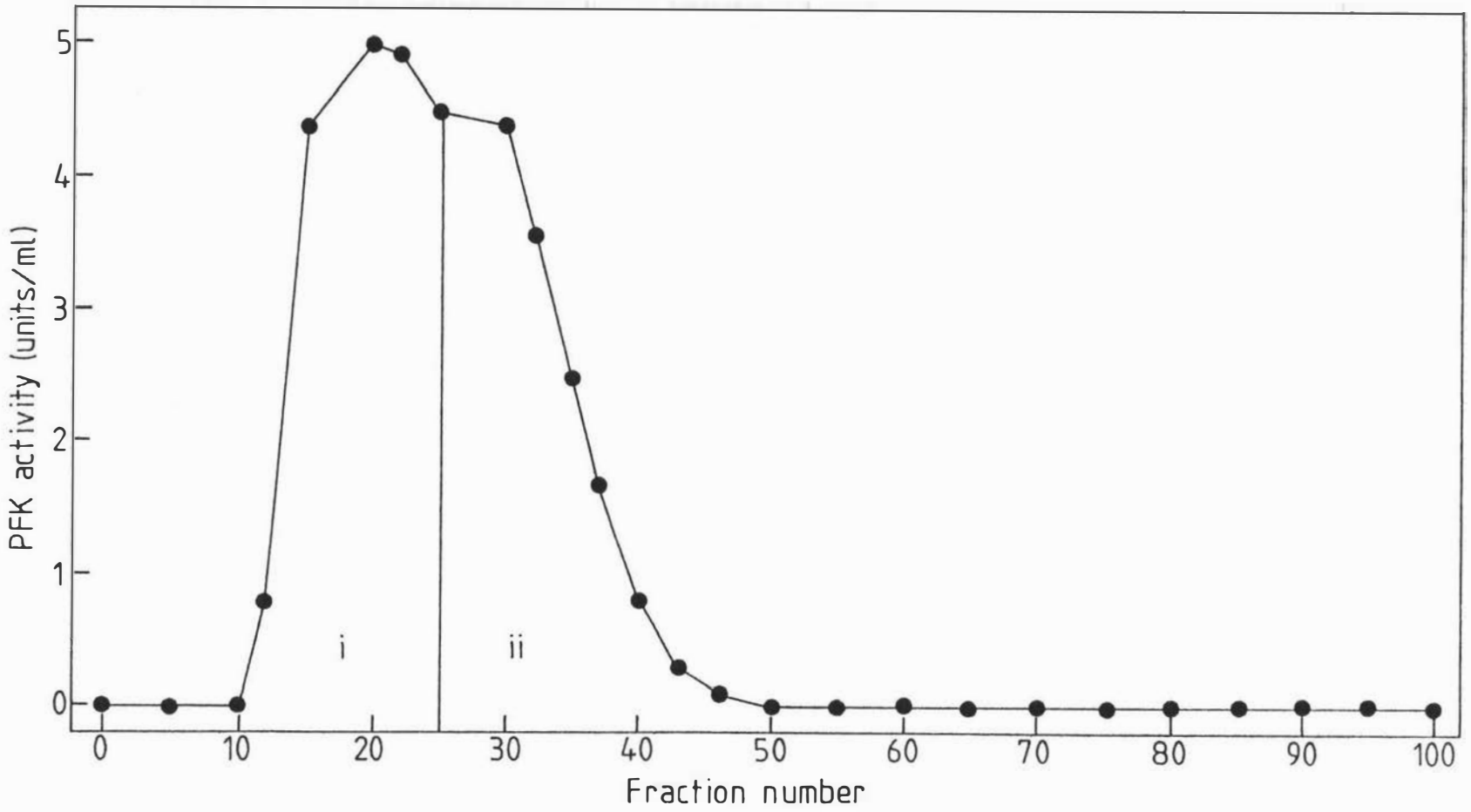


Figure 19: Separation of Sheep Heart PFK on DEAE-Cellulose Using Non-Dissociating Conditions.

Purified sheep heart PFK was eluted from a DEAE-cellulose column with a linear gradient from 100mM to 300mM Tris-HCl buffer as described in Section 4.11. PFK enzyme activity was detected as described in Section 3.3. Fraction size was 2.5-3.0ml.

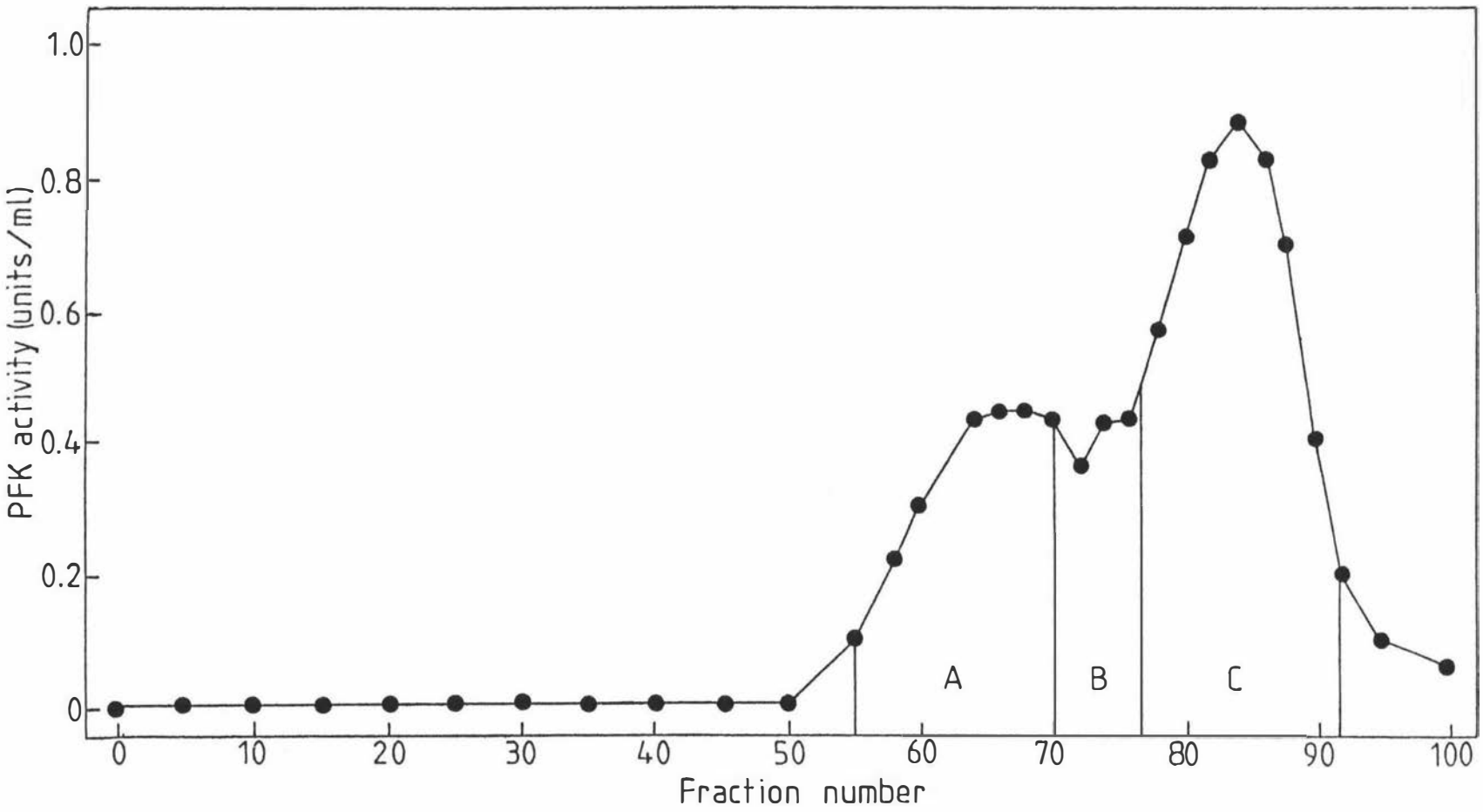


Figure 20: Separation of Sheep Liver PFK on DEAE-Cellulose Using Non-Dissociating Conditions.

Purified sheep liver PFK was eluted from a DEAE-cellulose column with a linear gradient from 100mM to 300mM Tris-HCl buffer as described in Section 4.11. PFK enzyme activity was detected as described in Section 3.3. Fraction size was 2.5-3.0ml.

were eluted it is highly unlikely that the source of the minor band is a contaminating PFK from the blood.

Comparison of the elution profile of sheep liver PFK with profiles published in the literature show many features in common. Human liver PFK (Vora et al., 1980) also eluted in a similar position in the gradient, and comprised a main peak with a shoulder on the left hand side, this entire fraction was described by the authors as being L_4. Rat liver PFK (Vora et al., 1985; Oskam et al., 1985), also elutes in a similar position late in the gradient and consisted of a main peak with 2 small peaks adjoined on the left hand side. The authors identified the main peak as L_4, and the two minor peaks as P_2L_2 and PL_3 (Vora et al., 1985) or a similar combination of hybrids with the M and L subunits, or a combination of all three L, P and M subunits (Oskam et al., 1985). Taylor and Bew (1970), found that a relatively sharp peak was eluted when separating rat liver PFK subunits, as well as a small amount of activity which eluted in the M_4 position.

An SDS-7.5% polyacrylamide gel of the three pooled fractions from the sheep liver PFK separation is shown in Fig. 21. Fraction A contained two bands, which correspond to the major and minor bands identified from the SDS-polyacrylamide gel containing liver PFK shown in Fig. 15. In this case, however, fraction A contained significantly higher levels of the upper band. This occurred to such an extent that the lower band was present only in minor amounts. Fraction C shows the lower band only, confirming this peak contained the L_4 tetramer, while Fraction B also contained both bands, but in more equal amounts. The profile of sheep liver PFK from DEAE-cellulose suggests that liver PFK is composed of both L_4 and PL_3 tetramers. P rather than M due to the molecular weight difference observed between the upper minor band and sheep heart PFK (M_4)(Fig. 15). The main problem with concluding that peak A contains the hybrid PFK PL_3, is that such a hybrid should produce P:L bands in a ratio of 1:3, and the band intensities on the gel do not support this. The presence of some P_2L_2 as well would still produce more of the lower band compared to the upper. On a DEAE-cellulose column, P_4 elutes very close to M_4 (Vora et al., 1985), therefore any P_3L present should have been eluted between 100 and 125ml. Because of these contradictions, the gel pattern remains to be explained. It is possible that the P subunit binds Coomassie Blue stain to a greater degree than does the L subunit, thus creating a misleading ratio between the two subunits. This has been reported to occur with other proteins (Domenech et al., 1978).

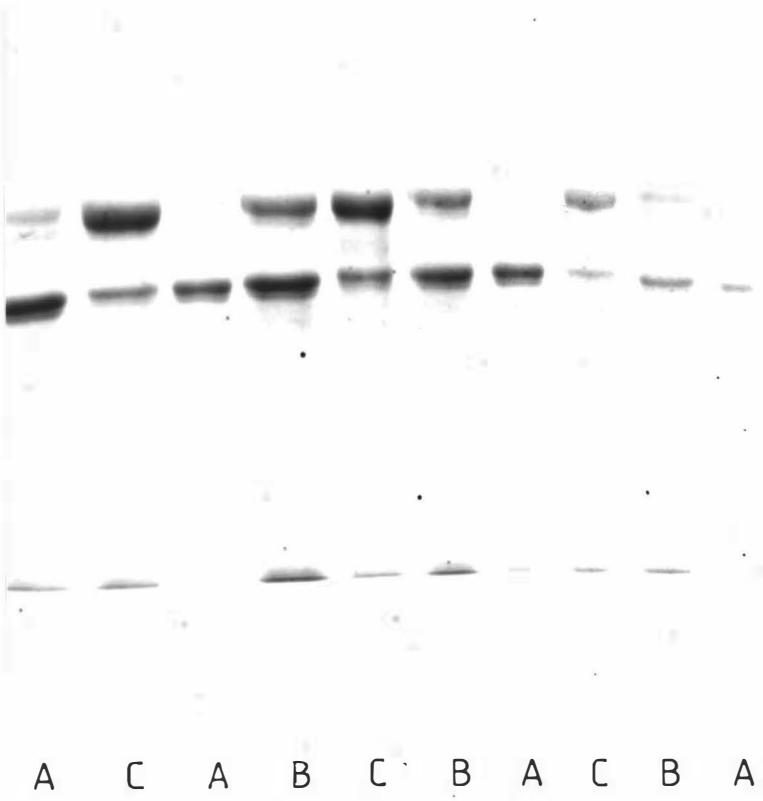


Figure 21: SDS-7.5% Polyacrylamide Gel of Sheep Liver PFK Fractions Separated on a DEAE-Cellulose Column Using Non-Dissociating Conditions

Fractions A, B, and C, as shown in Fig. 20 were prepared, electrophoresed and stained with Coomassie Blue as described in Sections 4.1 and 4.1.1.

NB: The lower band was due to proteolysis of the sheep liver PFK.

When both muscle and liver PFK were chromatographed together on a DEAE-cellulose column, the elution pattern of each PFK isozyme remained the same, such that the profile was similar to that achieved by overlaying Figs. 19 and 20.

5.2.2 SEPARATION OF PFK ISOZYMES USING DISSOCIATING CONDITIONS

In order to obtain further information about the nature of the liver PFK composition, a number of dissociation experiments were carried out. Since under dissociating conditions using DEAE-cellulose (Section 4.12), 2 peaks should be observed, one of the L subunits and one proposed to be the P subunit. However the results from this experiment were inconclusive due to the difficulty in detecting the protein. Alternative methods of separating the subunits using dissociating conditions on a Superose 12 gel filtration column and Mono-Q and Mono-S ion-exchange columns failed to separate the subunits. In part this may have been due to the use of unsuitable dissociating conditions, since experiments (not shown) showed that 8M urea failed to fully dissociate the liver enzyme, while the muscle enzyme was fully dissociated by 8M urea. The use of 6M guanidine-HCl to dissociate the enzyme resulted in the precipitation of the liver PFK, while the muscle PFK, under the same conditions remained in solution. In order to successfully carry out such dissociation experiments a better method of dissociation would first need to be determined.

5.3 AMINO ACID COMPOSITIONS OF SHEEP HEART AND LIVER PHOSPHOFRUCTOKINASE

Amino acid analyses were carried out on duplicate samples hydrolyzed for 24, 48 and 72hr as described in Section 4.3. Since destruction of serine and threonine occurs during hydrolysis, values for these residues were obtained by extrapolating back to zero time. Values for valine, isoleucine and leucine were obtained from the maximum readings after 72hr hydrolysis. Cysteine and tryptophan values were not determined.

Calculations of the amino acid compositions were carried out based on molecular weights of 84 000 and 81 000 for the sheep heart muscle and sheep liver PFK respectively. The amino acid composition values of liver PFK were then converted to mol/84 000g to allow a direct comparison with that of the muscle PFK (Table XXI).

TABLE XXI

AMINO ACID COMPOSITION OF SHEEP HEART MUSCLE AND SHEEP LIVER PFK.

Amino acid	Heart (mol/84 000g)	Liver (mol/84 000g)
Lysine	41.30 ± 0.35	37.33 ± 0.24
Histidine	18.45 ± 0.31	17.33 ± 0.18
Arginine	60.55 ± 0.78	54.89 ± 0.46
Aspartic acid	74.19 ± 1.30	69.54 ± 1.10
Threonine	52.37 ± 1.34	44.49 ± 0.44
Serine	42.35 ± 0.66	48.99 ± 0.70
Glutamic acid	81.18 ± 0.92	84.06 ± 0.79
Proline	30.32 ± 0.89	29.37 ± 0.68
Glycine	82.26 ± 0.82	80.90 ± 1.35
Alanine	59.51 ± 0.27	64.03 ± 0.72
Valine	61.31 ± 0.99	64.47 ± 1.44
Methionine	22.21 ± 0.22	21.96 ± 0.38
Isoleucine	44.37 ± 0.36	41.51 ± 0.54
Leucine	66.50 ± 0.73	67.54 ± 0.84
Tyrosine	18.92 ± 0.44	22.64 ± 0.64
Phenylalanine	27.40 ± 0.76	27.22 ± 0.63

Amino acid composition of sheep liver and sheep heart PFK calculated from analyses of duplicate 24, 48 and 72hr hydrolysates. The amino acid composition of liver PFK was converted to mol/84 000g in order to allow direct comparison with heart muscle PFK. Values are the mean ± standard error.

Using the method of Cornish-Bowden (1983), for comparing amino acid compositions for assessing the relatedness of proteins, a coefficient was obtained which almost certainly indicates that sheep heart and sheep liver PFK are related proteins.

A Student t-test, which was designed to allow for small sample sizes, was carried out to ascertain statistical variability in the levels of each amino acid between muscle and liver PFK. The t-test results showed that the levels of proline, glycine, leucine, phenylalanine, valine and methionine were not statistically different. The t-test values for histidine, aspartic acid, and glutamic acid were at the limit of the test, and upon consideration of the standard deviation values were also classified as "not statistically different".

Amino acids which were found to be present in statistically different amounts in sheep muscle and liver PFK were; lysine, arginine, threonine, serine, isoleucine and tyrosine. Liver PFK contains lesser amounts of both lysine and arginine compared to heart muscle PFK. The positions of these losses within the molecule are very important since the loss of a positive charge around an active site could conceivably alter the binding capacity of the molecule, hence leading to altered kinetic properties. The net charge of the molecule could also be altered. With fewer arginine and lysine residues, a tryptic digest of sheep liver PFK should result in fewer peptides compared to muscle PFK.

The number of threonine residues is significantly lower in liver PFK compared to muscle PFK, however muscle PFK has fewer serine residues than liver PFK. Overall the number of these hydroxyl residues present in the two isozymes remains approximately the same. A threonine/serine substitution would tend to be a conservative change, and therefore should not cause disruption of the secondary structure, or if occurring at the active or effector site would probably not cause significant alteration of the binding capacity.

There are more alanine residues in liver PFK than muscle PFK, with its small uncharged side chain, an alanine substitution for a more bulky residue could cause considerable changes in the packing of the molecule. A charged residue replaced by an alanine could also result in significant changes depending on its position within the molecule.

There are fewer isoleucine residues present in liver PFK compared to muscle PFK, with its bulky side chain, changes in packing could occur if it is substituted by a residue

with a smaller side chain. The t-test also indicates that there are more tyrosine residues in liver PFK than muscle PFK. With its bulky side chain, substitution of any residue other than an aromatic is likely to cause significant disruption of the molecule.

The amino acid composition of sheep heart muscle PFK were similar to those obtained from PFK from rabbit muscle (Tarui et al., 1972; Walker et al., 1976) and human muscle (Karadsheh et al., 1977), with the exception of the number of tyrosine residues in human muscle which was very low.

Comparison of the amino acid composition between sheep liver and rat liver PFK (Pilkis et al., 1982) shows some vast differences. The values presented by Pilkis et al. (1982), were reported as equivalents/83 000g, and they reported over 1 000 amino acids constituting this molecular weight. This would equate to an average amino acid molecular weight of approximately 83. Therefore comparison of the sheep liver PFK amino acid composition with this set of data from rat liver PFK is not valid.

Comparison of the amino acid composition of sheep liver PFK with a more recent set of values for rat liver PFK (Domenech et al., 1988), shows some marked differences. The reported value for glycine in rat liver PFK is 115mol/84 000g, much higher than that of 81mol/84 000g found for sheep liver PFK. This high glycine value is probably due to the fact that the rat liver PFK used for the amino acid analyses was purified by SDS-PAGE, using buffer systems which included glycine. This erroneous glycine result would then lead to all the other amino acid composition values being low. Indeed, with the exception of glycine and serine, all the reported amino acid composition values for rat liver PFK were lower than those found for sheep liver PFK.

5.4 PEPTIDE MAPPING OF CNBr PEPTIDES OF MUSCLE AND LIVER PFK ON FPLC

CNBr digests of [^{14}C]-muscle PFK and [^{14}C]-liver PFK were mapped on a Superose 12 gel filtration column as described in Section 4.6.3. The peptide maps are shown in Fig. 22 and Fig. 23 for sheep heart muscle PFK and sheep liver PFK respectively.

Comparison of the two profiles show very few features in common. There is a small amount of protein of high molecular weight (eluting at 7ml and 9ml for muscle and liver PFK respectively), which is probably undigested enzyme in each case. The muscle profile shows a trend for higher molecular weight fragments (9-16ml), than the liver

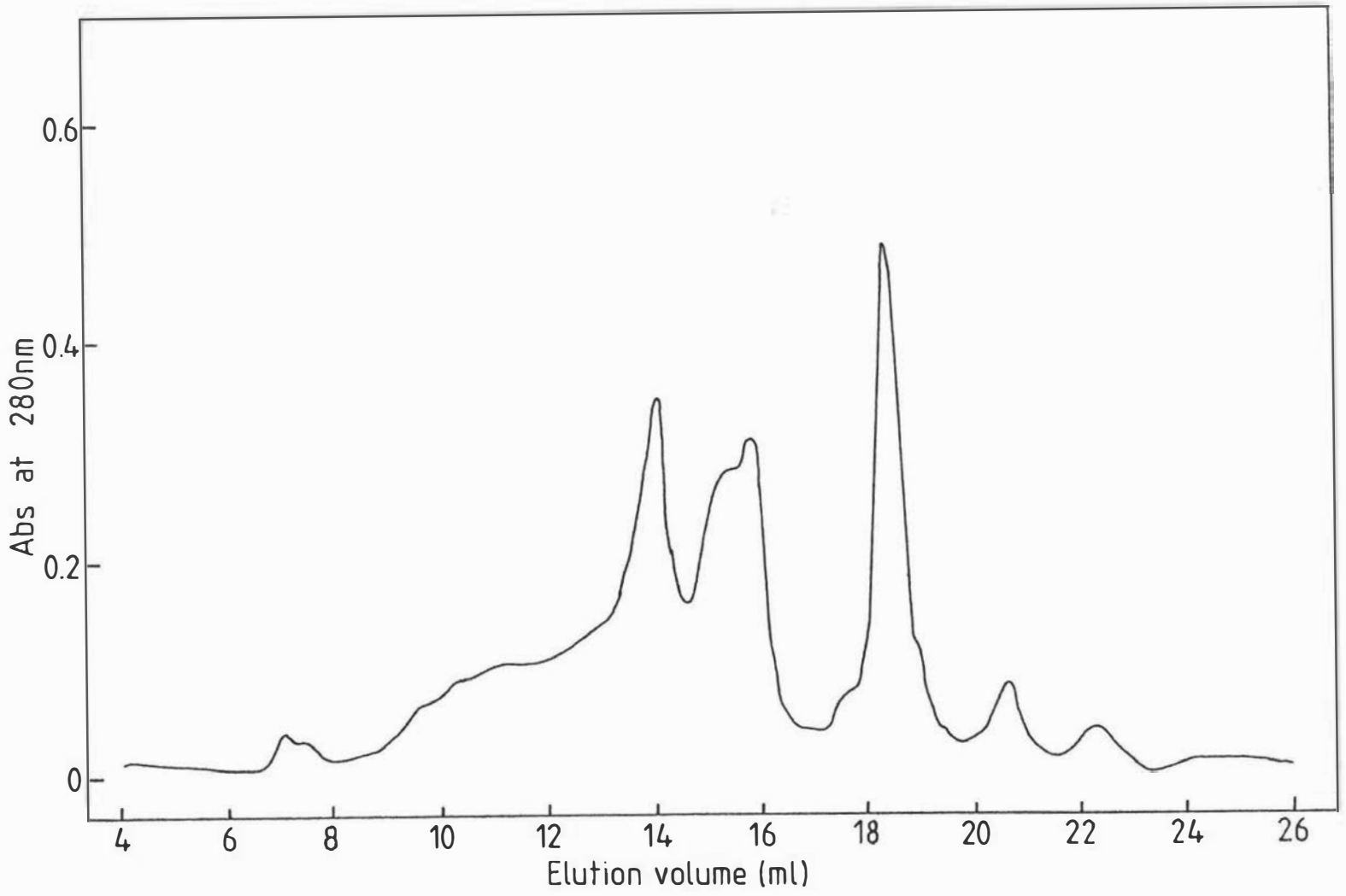


Figure 22: FPLC Peptide Map of a CNBr Digest of Sheep Heart PFK.

The CNBr digested sample (Section 4.5), was washed onto a Superose 12 gel filtration column and eluted with 50% formic acid at a rate of 0.4ml/min as described in Section 4.6.3.

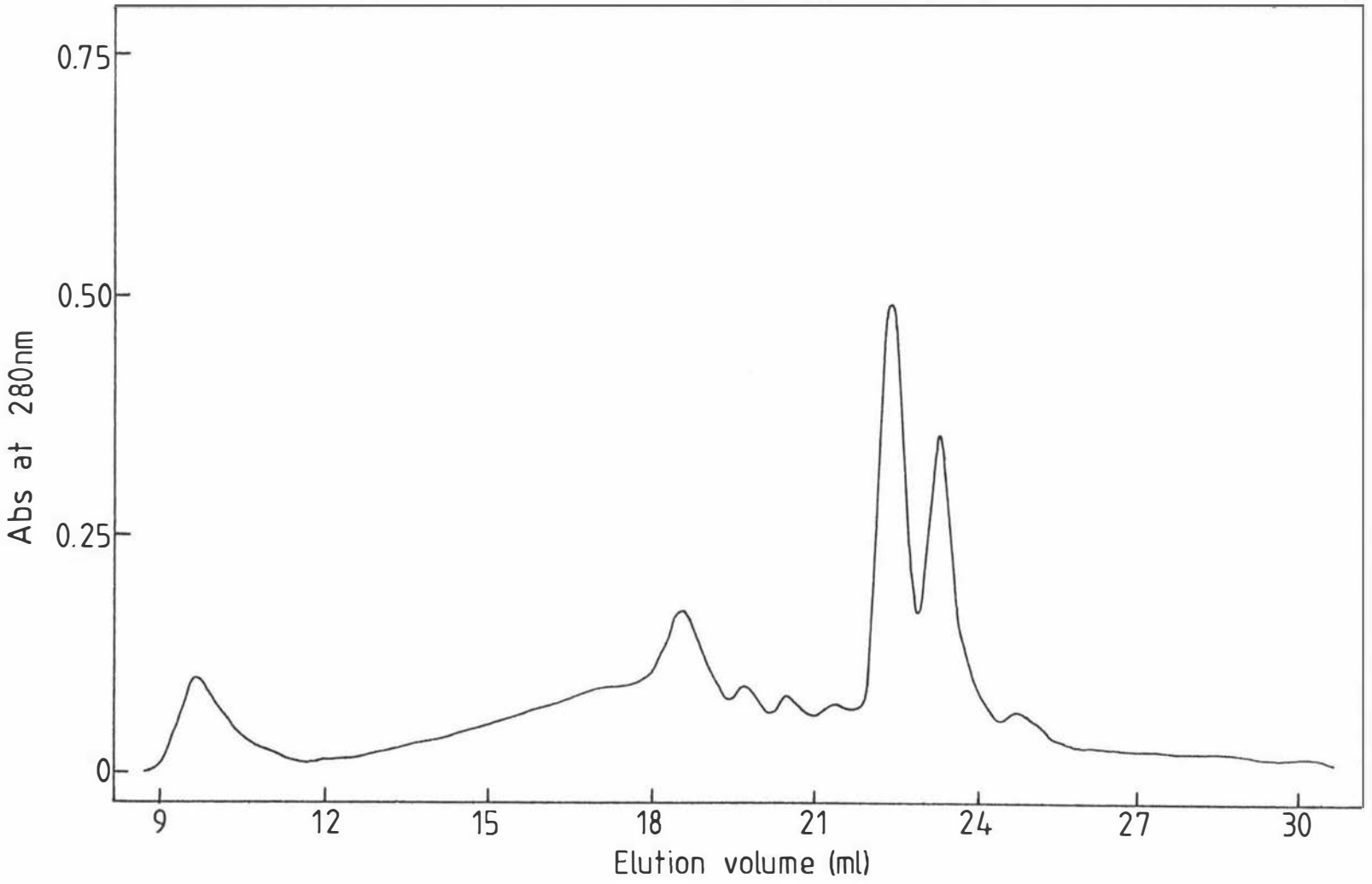


Figure 23: FPLC Peptide Map of a CNBr Digest of Sheep Liver PFK.

The CNBr digested sample (Section 4.5), was washed onto a Superose 12 gel filtration column and eluted with 50% formic acid at a rate of 0.4ml/min as described in Section 4.6.3.

enzyme. Since the number of methionine residues in muscle and liver PFK are similar this would indicate that the large molecular weight peptides of muscle PFK: 108, 98, 90, and 65 residues in length have been cleaved into smaller fragments in the liver enzyme due to a redistribution of methionine residues. The liver enzyme exhibits a peak at 23-24ml which represents a much smaller size fragment than is present in the muscle enzyme, adding further support to the idea of liver PFK being cleaved into smaller peptides by CNBr, than muscle PFK. By further separating each FPLC fraction of the CNBr digest by HPLC and sequencing the resultant peptides, evidence was obtained which proved the above hypothesis to be correct (Sections 6.1.1, 6.1.2).

5.5 PEPTIDE MAPPING OF TRYPTIC DIGESTS OF MUSCLE AND LIVER PFK ON HPLC

Carboxymethylation of PFK with [^{14}C]-iodoacetic acid, leads to [^{14}C]-labelling of the cysteine residues. This enables the comparison of the elution patterns of peptides containing the labelled residues from muscle and liver PFK, and therefore gives some indication of whether any similarity exists between the labelled species of the two enzymes.

Peptide mapping of tryptic digests of [^{14}C]-cysteine labelled muscle and liver PFK was carried out in two fractions ammonium bicarbonate-soluble, and insoluble peptides. Peptide maps of ammonium bicarbonate-soluble peptides of muscle and liver PFK (prepared as described in Section 4.6.1), which were separated by HPLC on a Resolve RC C-18 column, are shown in Fig. 24 and Fig. 25 respectively. Comparison of the HPLC profiles show few features in common. Both exhibit seven radioactively labelled fractions (shaded) which elute with the gradient. Of these, possibly three have eluted in similar positions, one with approximately 18-19%, one with 34-35%, and one with 43% of the gradient. Generally, the labelled muscle peptides have eluted later in the gradient than the liver peptides, indicating that the labelled muscle peptides are more hydrophobic than the labelled liver peptides.

Sequencing of the radioactively labelled tryptic peptides should give insight into the nature of the changes which have occurred to produce the different peptide maps.

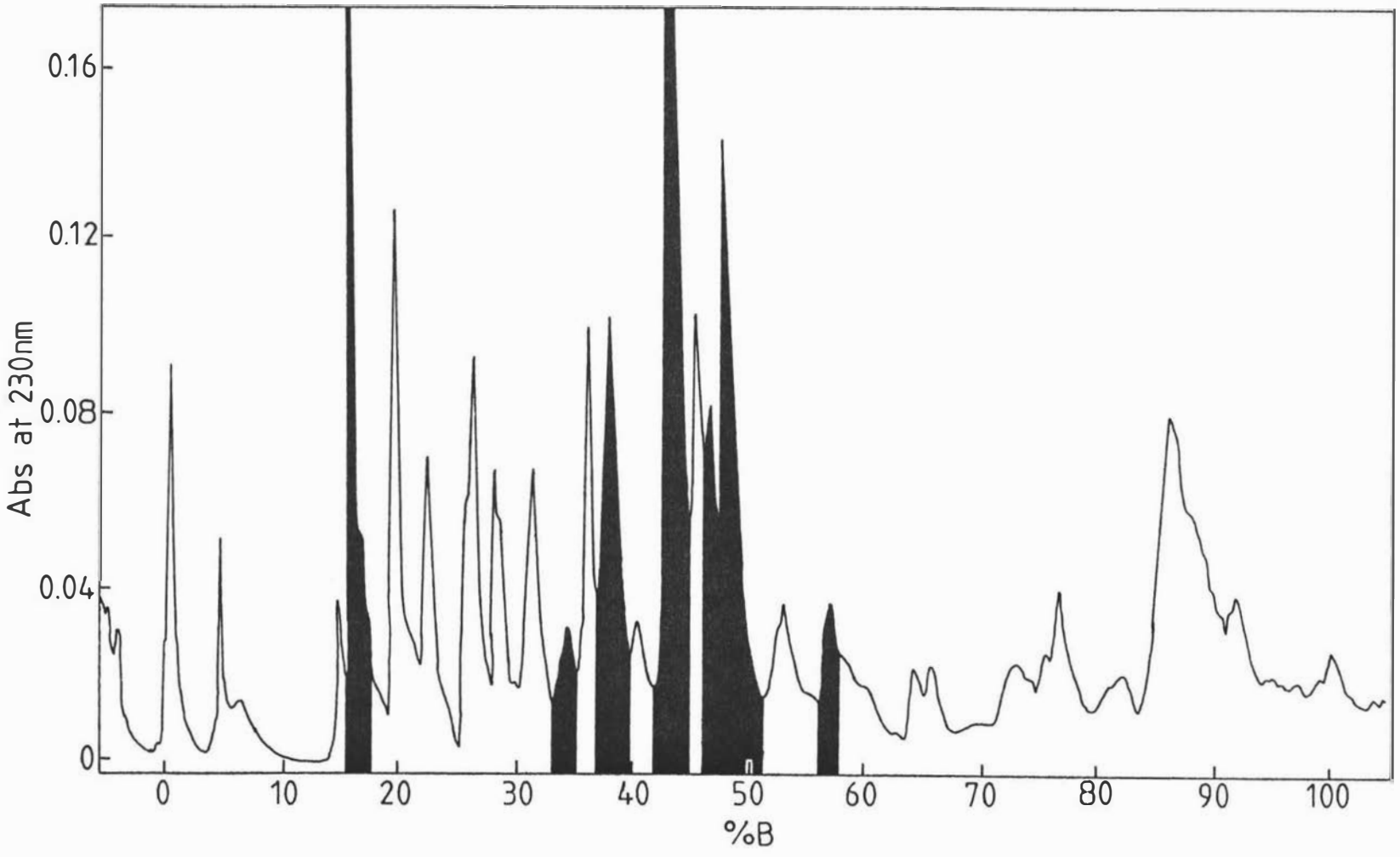


Figure 24: HPLC Peptide Map of the Ammonium Bicarbonate-Soluble Peptides from a Tryptic Digest of Sheep Heart PFK.

The ammonium bicarbonate-soluble tryptic peptides were prepared as described in Section 4.6.1. The sample was washed onto a Resolve RC C-18 column with buffer A (0.1M Am Bic), and eluted with a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1) run over 2hr at a rate of 1ml/min as described in Section 4.6.1. Shaded peaks are those containing radioactivity.

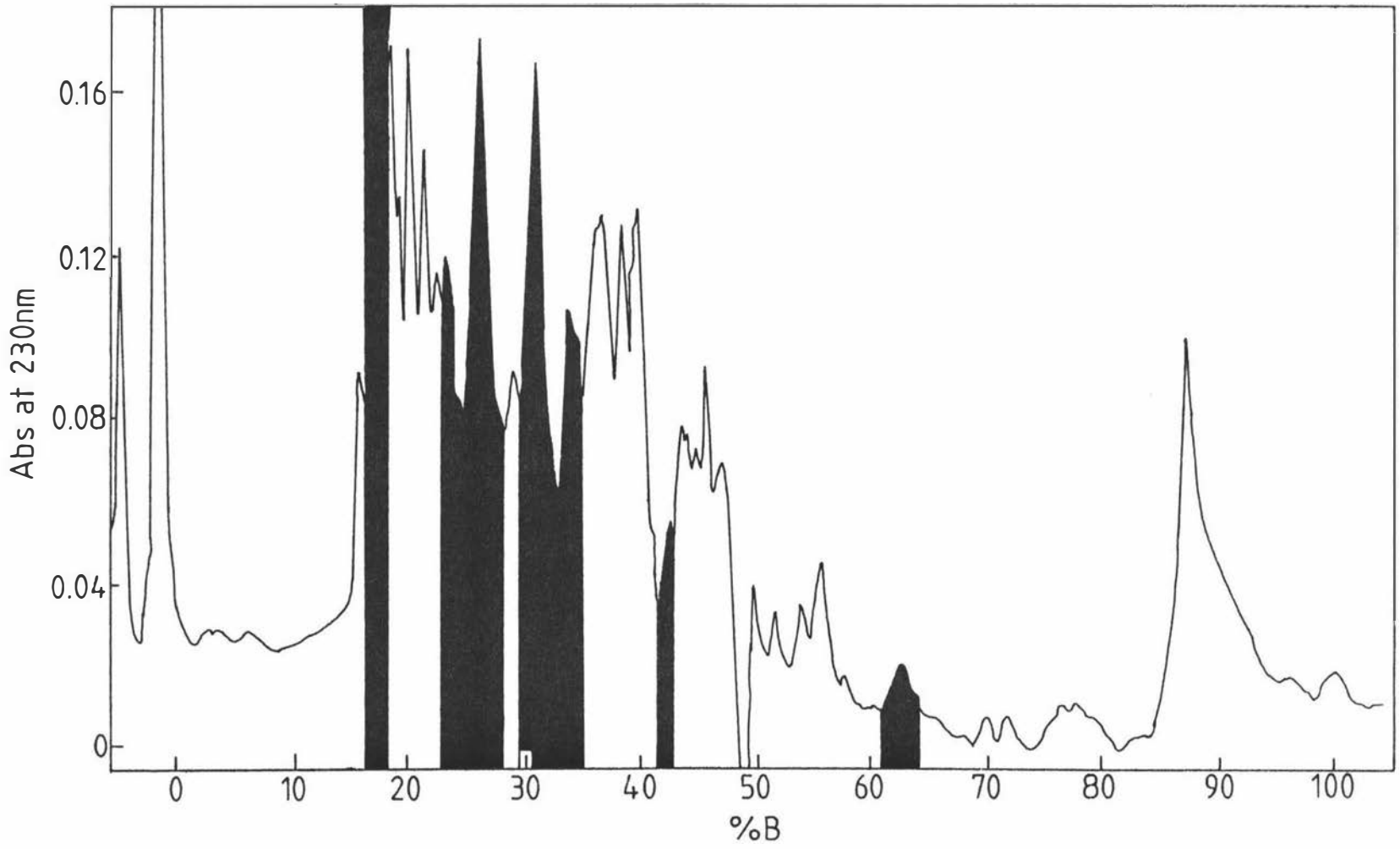


Figure 25: HPLC Peptide Map of the Ammonium Bicarbonate-Soluble Peptides from a Tryptic Digest of Sheep Liver PFK.

The ammonium bicarbonate-soluble tryptic peptides were prepared as described in Section 4.6.1. The sample was washed onto a Resolve RC C-18 column with buffer A (0.1M Am Bic), and eluted with a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1) run over 2hr at a rate of 1ml/min as described in Section 4.6.1. Shaded peaks are those containing radioactivity.

5.6 PEPTIDE MAPPING OF AMMONIUM BICARBONATE-INSOLUBLE TRYPTIC PEPTIDES ON HPLC

The ammonium bicarbonate-insoluble tryptic peptides of [^{14}C]-cysteine labelled muscle and liver PFK were separated by HPLC, on a Vydac C-4 column, in a formic acid gradient system as described in Section 4.6.3. The profiles (Figs. 26, 27) contain some fairly broad peaks, but exhibit several similarities. Four peaks containing radioactivity (shaded) were eluted in similar positions in both the muscle and liver PFK profiles, although the muscle profile has a peak containing radioactivity which was eluted at 60-62% of the gradient, which was not present on the liver profile. Overall the ammonium bicarbonate-insoluble tryptic peptides appear to be relatively similar.

5.7 PEPTIDE MAPPING OF CNBr PEPTIDES OF MUSCLE AND LIVER PFK ON HPLC

Peptide maps of the CNBr digested muscle and liver PFK were obtained by chromatography on a Vydac C-4 column using a formic acid gradient system (Section 4.6.2). The profiles contain relatively broad peaks and exhibit several similarities, a peak containing radioactivity (shaded) eluted late in the gradient (65-69%)(Fig. 28 and Fig. 29), and three other peaks containing radioactivity eluted early in the gradient at 15, 20 and 25%. In both the muscle and liver PFK profiles, there are five peaks containing radioactively labelled material which were eluted between 37% and 55% of the gradient. Four of these appear to be of both similar shape and elution position; those eluting at 38, 41, 45 and 52% of the gradient. The other peak containing radioactivity in each profile has eluted in a totally different position, with the muscle PFK profile having a sharp peak which is eluted at 47% of the gradient which does not occur in the liver PFK profile. The liver PFK profile had a peak containing radioactivity which was eluted with 41% of the gradient, which appeared as a shoulder in the muscle enzyme profile which did not contain radioactivity. The liver profile also had a peak containing radioactivity that eluted early on in the gradient at 10%, which was not radioactively labelled in the muscle enzyme.

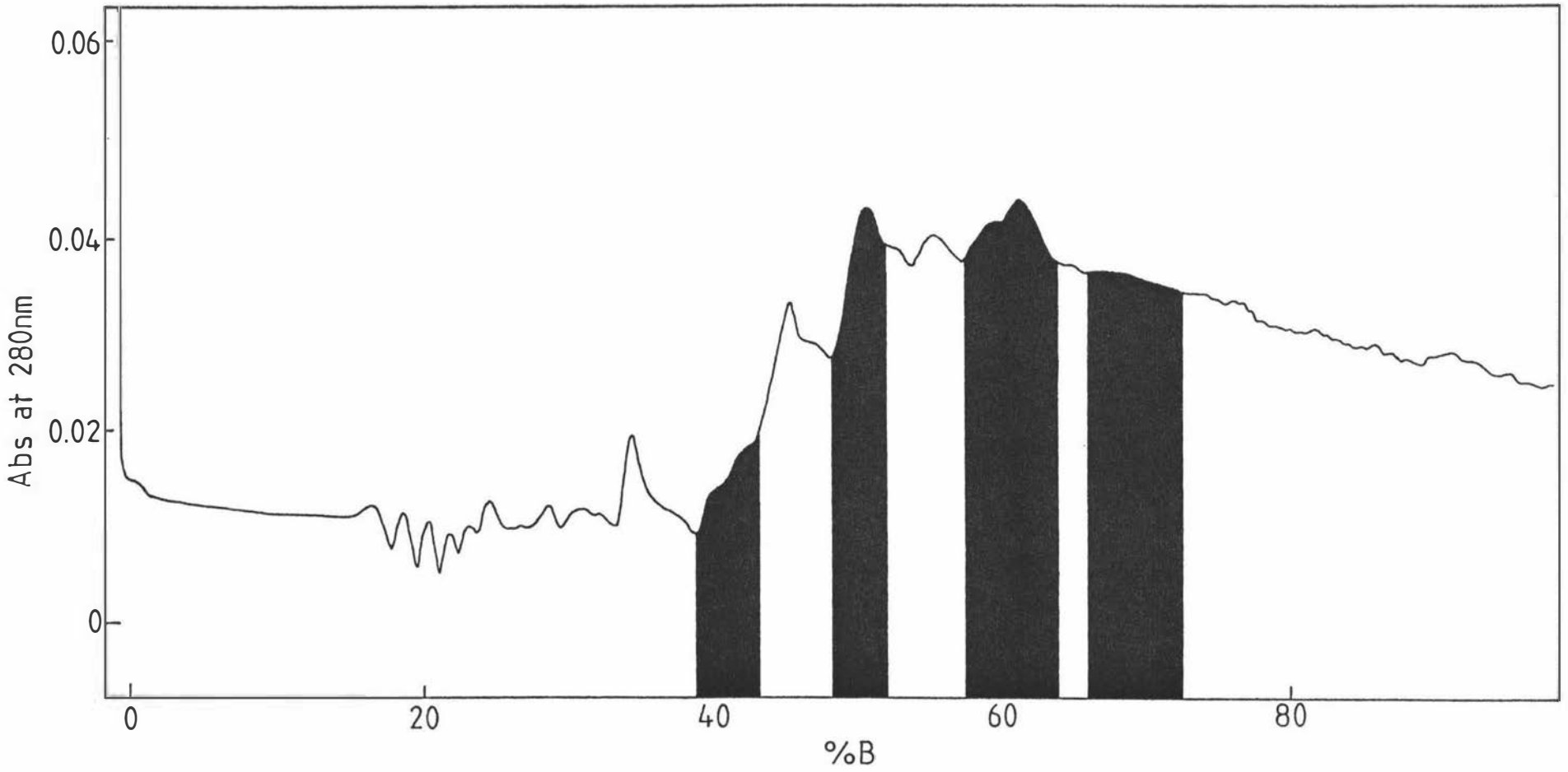


Figure 26: HPLC Peptide Map of the Ammonium Bicarbonate-Insoluble Peptides from a Tryptic Digest of Sheep Heart PFK.

The ammonium bicarbonate-insoluble tryptic peptides were prepared as described in Section 4.6.1. The sample was washed onto a Vydac C-4 column with buffer A (5% formic acid), and eluted with a linear gradient from 0-100% B (5% formic acid/isopropanol/acetonitrile 1:1:1) run over 2hr at a rate of 1ml/min as described in Section 4.6.1. Shaded peaks are those containing radioactivity.

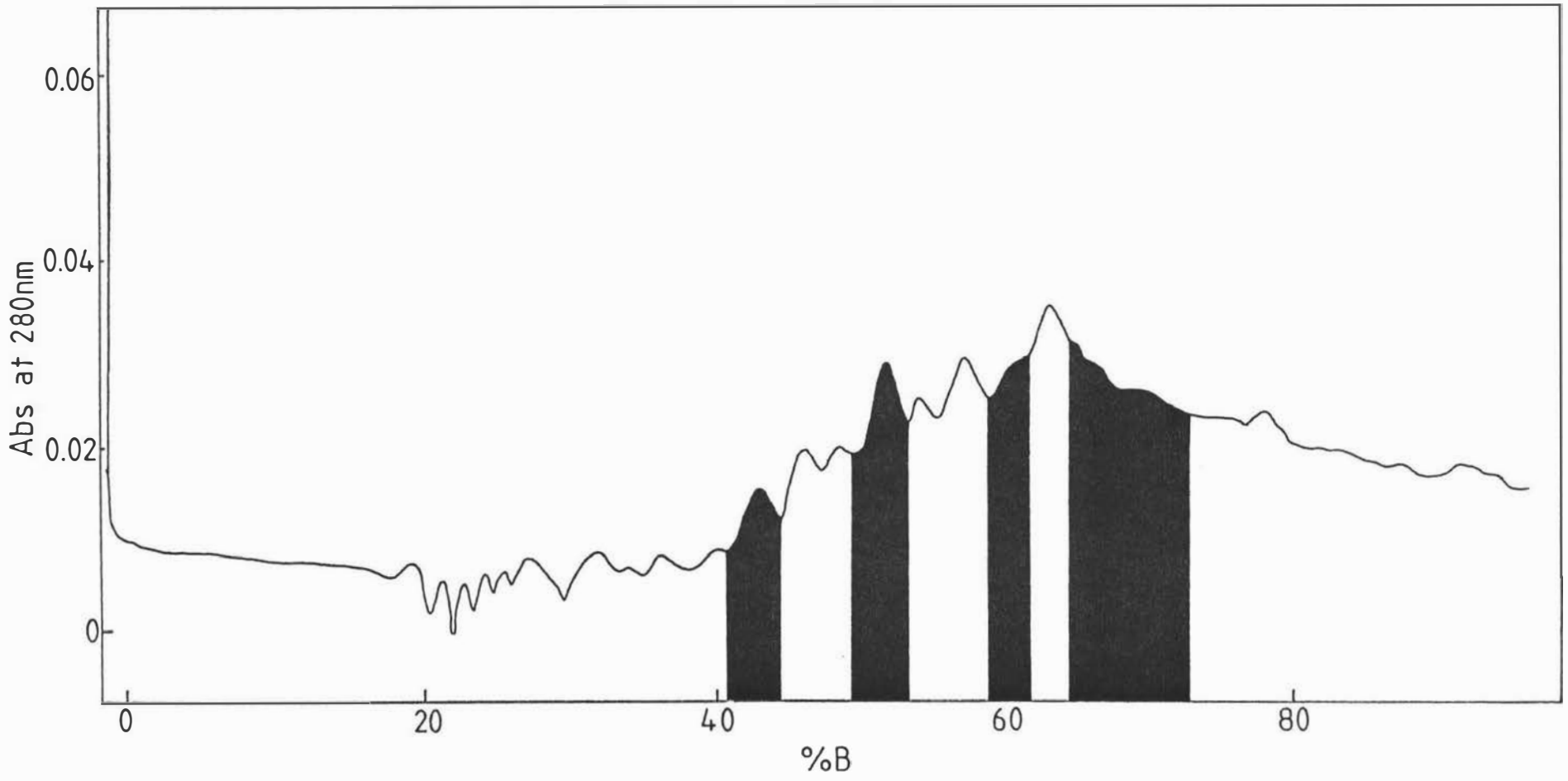


Figure 27: HPLC Peptide Map of the Ammonium Bicarbonate-Insoluble Peptides from a Tryptic Digest of Sheep Liver PFK.

The ammonium bicarbonate-insoluble tryptic peptides were prepared as described in Section 4.6.1. The sample was washed onto a Vydac C-4 column with buffer A (5% formic acid), and eluted with a linear gradient from 0-100% B (5% formic acid/isopropanol/acetonitrile 1:1:1) run over 2hr at a rate of 1ml/min as described in Section 4.6.1. Shaded peaks are those containing radioactivity.

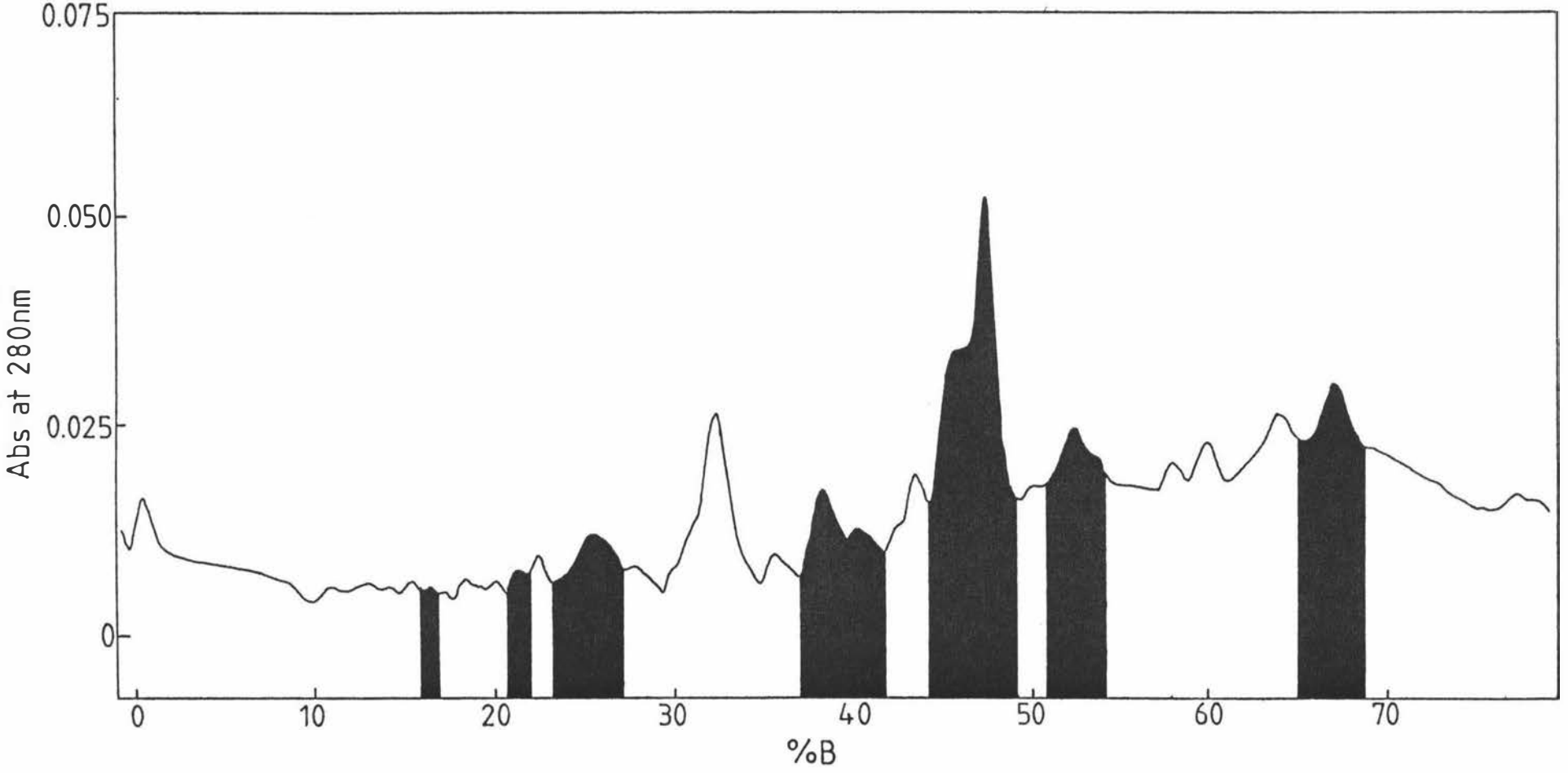


Figure 28: HPLC Peptide Map of a CNBr Digest of Sheep Heart PFK.

The CNBr digested sample (Section 4.5) was washed onto a Vydac C-4 column with buffer A (5% formic acid). Elution consisted of a linear gradient from 0-100% B (5% formic acid/isopropanol/acetonitrile 1:1:1) run over 2hr at a rate of 1ml/min as described in Section 4.6.2. Shaded peaks are those containing radioactivity.

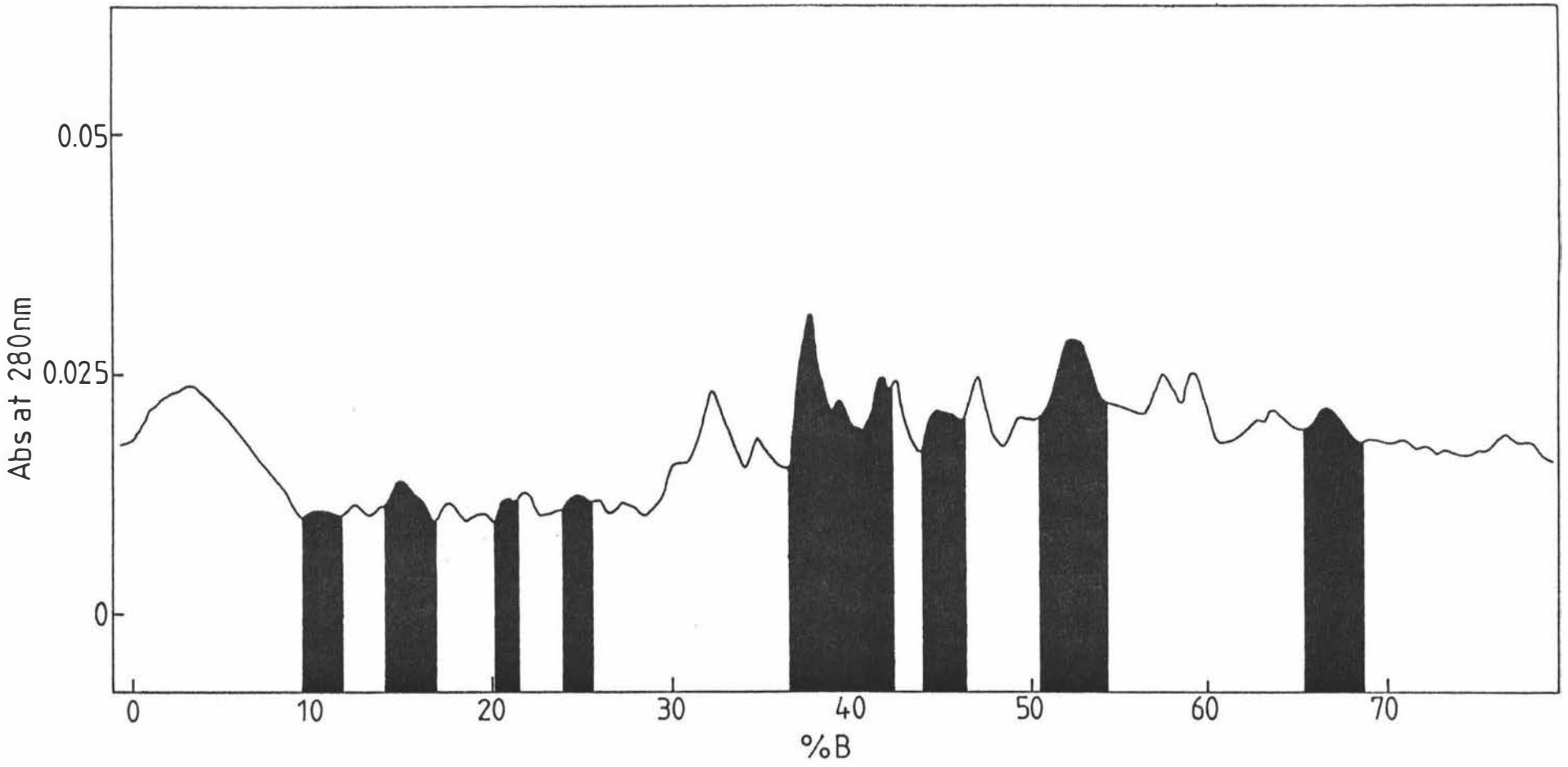


Figure 29: HPLC Peptide Map of a CNBr Digest of Sheep Liver PFK.

The CNBr digested sample (Section 4.5) was washed onto a Vydac C-4 column with buffer A (5% formic acid). Elution consisted of a linear gradient from 0-100% B (5% formic acid/isopropanol/acetonitrile 1:1:1) run over 2hr at a rate of 1ml/min as described in Section 4.6.2. Shaded peaks are those containing radioactivity.

CHAPTER SIX

RESULTS

AMINO ACID SEQUENCE OF SHEEP LIVER PHOSPHOFRUCTOKINASE

Due to the large amount of sample required to carry out amino acid analyses in this department, and the relatively small amount of liver PFK which was used for the generation of peptides in this study, it was not possible to confirm the amino acid composition of the peptides by amino acid analysis. The sequencing process therefore relied upon the repetitive sequencing of the peptides, and the generation of overlapping peptides. Toward the end of this study, a mass spectrometer with a fast atom bombardment source (FAB MS), became available, and the confirmation of the molecular weights of a number of peptides were obtained by this means.

In a number of cases, particularly the high molecular weight CNBr peptides, it was not possible to sequence to the end of the peptide due to a lack of material.

The N-terminal of sheep liver PFK was found to be blocked (results not shown), as has been found to be the case for both the rabbit muscle and sheep heart muscle enzymes.

6.1 CNBr PEPTIDES

The usefulness of CNBr digestion as a sequencing tool is well known. Due to its high specificity for cleavage on the C-terminal side of methionine residues, coupled with the relatively infrequent occurrence of methionine residues in proteins, digestion generally leads to a smaller number of larger molecular weight peptides than other digestion methods.

Peptides from a CNBr digest of [^{14}C]-liver PFK were initially separated by gel filtration (Section 4.8.1), on a Superose 12 gel filtration column (Fig. 30), followed by HPLC, as described in Section 4.8.1. The nomenclature used for these peptides is as

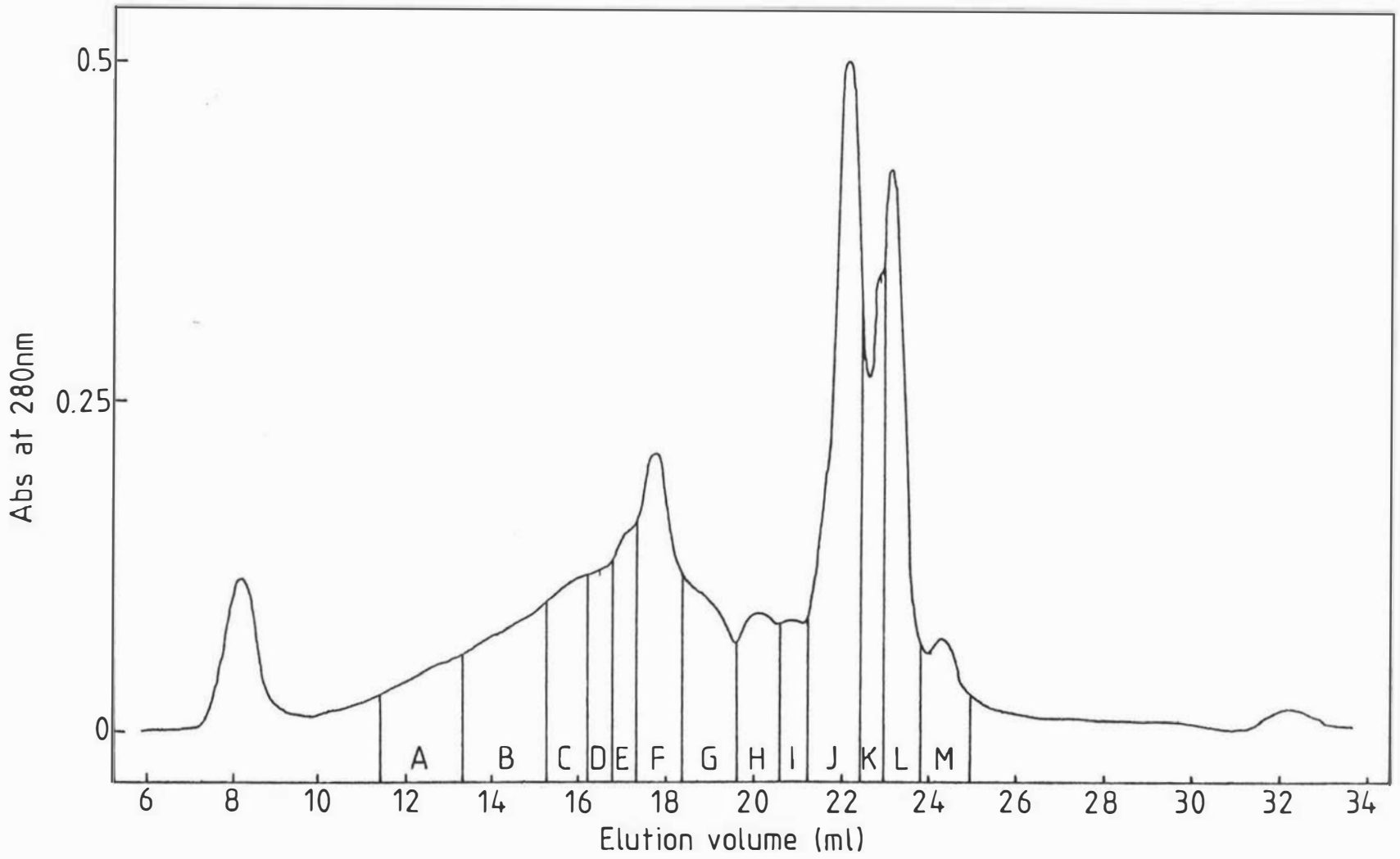


Figure 30: FPLC Elution Profile of a CNBr Digest of Sheep Liver PFK.

The CNBr digested sample (Section 4.5) was washed onto a Superose 12 gel filtration column and eluted with 50% formic acid at a rate of 0.4ml/min as described in Section 4.6.3. The Fractions which underwent further purification by HPLC are labelled A-M.

follows: CNBr followed by the appropriate fraction letter from the gel filtration elution profile (Fig. 30), followed by the appropriate peak number from the HPLC elution profile eg: CNBr F-7, meaning a CNBr peptide isolated from fraction F (gel filtration profile), peak 7 (HPLC profile of CNBr F).

6.1.1 SEPARATION AND AMINO ACID SEQUENCE OF THE LOW MOLECULAR WEIGHT CNBr PEPTIDES

The low molecular weight fractions from FPLC (G-M) were further separated by HPLC on a Resolve RC C-18 column as described in Section 4.8.1. Peaks from these, were collected, and freeze-dried ready for sequencing as described in Section 4.9. The HPLC elution profiles, along with the amino acid sequences of the isolated peptides are shown in Fig. 31, 32 and 33 and Tables XXIIa, XXIIIa, XXIVa respectively. Tables showing the alignment of the liver peptides with the corresponding sequence in the rabbit muscle and sheep heart muscle PFK are shown in Tables XXIIb and XXIIIb. The position of the peptide within the sequence is shown, along with the amino acid residues which are different in the muscle PFKs compared to the liver sequence. Blank spaces represent identical amino acids in the three sequences while a hyphen indicates residues which are yet to be determined. Since the majority of the peptides were sequenced more than once, the alignment with the muscle sequence is shown only for the first occurrence of the peptide. Despite the high absorbances of the peaks in fractions J-M, when chromatographed on HPLC, these fractions did not contain any peptides.

The peptides sequenced from fraction CNBr I, were each found to be identical to those previously sequenced in either CNBr G or CNBr H, the sequence alignment of these peptides can therefore be found in Tables XXIIb and XXIIIb.

Most of the peptides isolated and sequenced from the fractions CNBr G-I were found to end with a derivative of methionine (homoserine or homoserinelactone), as would be expected from a CNBr digest. A noteable exception to this, was the peptide CNBr G-2 (ADYVSGELEHVTRR), which was isolated and sequenced a number of times, and in no case was found to be terminated by a methionine residue. The lack of a methionine at the C-terminus implies that this peptide could be the C-terminal of the protein. Using FAB-MS as described in Section 4.10, the amino acid composition of this peptide, as well as the double arginine sequence at the C-terminus of this peptide was confirmed, adding further support to this peptide being the C-terminal of sheep liver PFK.

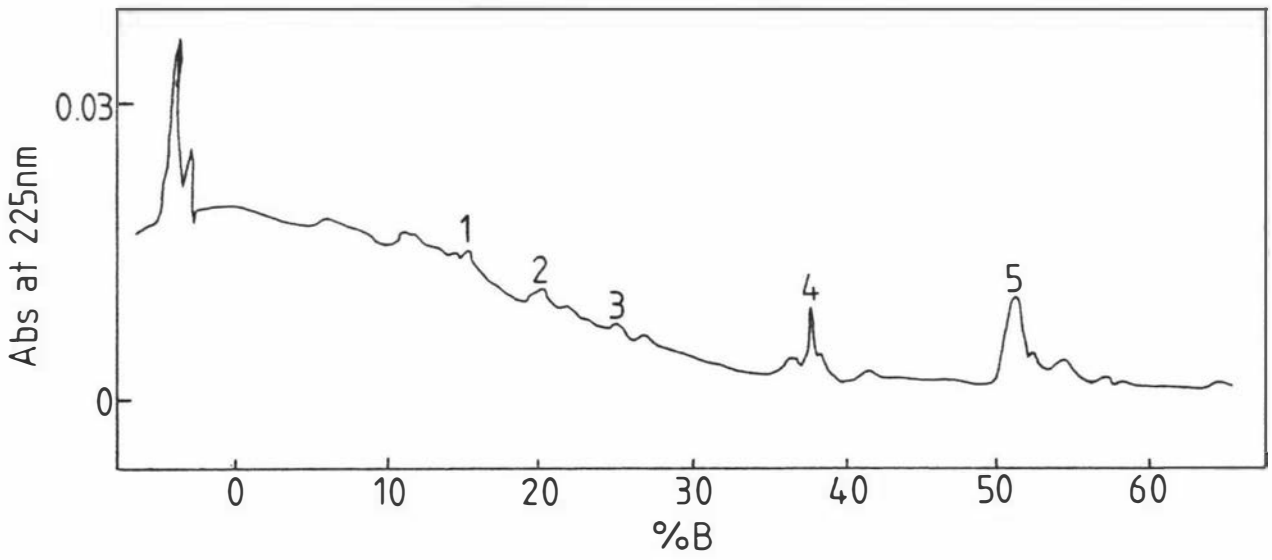


Figure 31: HPLC Elution Profile of Fraction CNBr G Chromatographed on a Resolve RC C-18 Column.

Labelled peaks are those for which sequence information is shown in Fig. XXIIa. Peptides in Fraction CNBr G were dissolved in .1M Am Bic. Elution consisted of a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXIIa

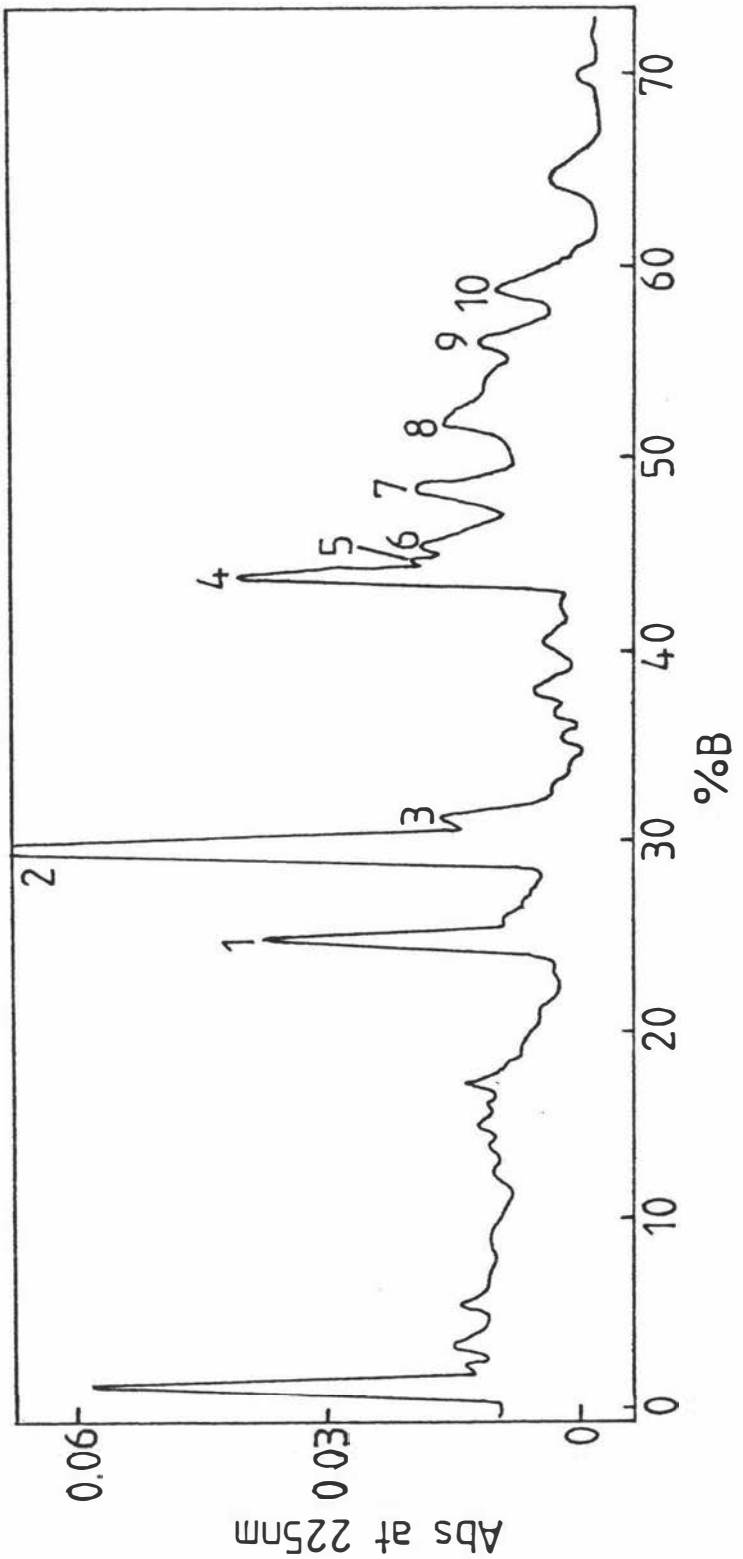
AMINO ACID SEQUENCES OF LIVER PFK PEPTIDES ISOLATED FROM FRACTION CNBr G FOLLOWING HPLC

Fraction	Sequence
CNBr G-1	NVGAPAAGM
CNBr G-2	ADYVSGELEHVTRR
CNBr G-3	ADYVSGELEHVTRR
CNBr G-4	LAHYRISM
CNBr G-5	LAHYRISM

TABLE XXIIb

ALIGNMENT OF SHEEP LIVER PFK PEPTIDES FROM FRACTION CNBr G WITH RABBIT MUSCLE AND SHEEP HEART PFK

Peptide		Sequence Alignment
CNBr G-1	SL	406 NVGAPAAGM 414
	RM	
	SH	
CNBr G-2	SL	759 ADYVSGELEHVTRR 772
	RM	DTSEHAH I S K
	SH	DTSEHAH I K
CNBr G-3	SL	751 LAHYRISM 758
	RM	K E DL
	SH	K E DL


Abs at 225nm
0.06
0.03
0
0
10
20
30
40
50
60
70
%B
1
2
3
4
5
6
7
8
9
10

Opposing Page.

Figure 32: HPLC Elution Profile of Fraction CNBr H Chromatographed on a Resolve RC C-18 Column

Labelled peaks are those for which sequence information is shown in Fig. XXIIIa. Peptides in Fraction CNBr H were dissolved in .1M Am Bic. Elution consisted of a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXIIIa

AMINO ACID SEQUENCES OF LIVER PFK PEPTIDES ISOLATED FROM FRACTION CNBr H FOLLOWING HPLC

Fraction	Sequence
CNBr H-1	TKEVQKAM
CNBr H-2	ADYVSGELEHVTRR
CNBr H-3	ADYVSGELEHVTRR
CNBr H-4	TIGTDSALHRIM
CNBr H-5	ALLEATPDTPACVVSLSGNQAVRL
CNBr H-6	NAAVRAVTRM
CNBr H-7	NAAVRAVTRM
CNBr H-8a	LGTKRTLPKGYM
CNBr H-8b	NAAVRAVTRM
CNBr H-8c	ALLEATPDTPACVVSLSGNQAVRL
CNBr H-8d	EVIDAITTTAQ
CNBr H-9a	LGTKRTLPKGYM
CNBr H-9b	LAHYRISM
CNBr H-9c	ALLEATPDTPACVVSLSGNQAVRL
CNBr H-10a	ALLEATPDTPACVVSLSGNQAVRL
CNBr H-10b	LGTKRTLPKGYM

TABLE XXIIIb

ALIGNMENT OF SHEEP LIVER PFK PEPTIDES FROM FRACTION CNBr H WITH RABBIT MUSCLE AND SHEEP HEART PFK

Peptide			Sequence Alignment	
CNBr H-1	SL	354	TKEVQKAM	361
	RM		D T	
	SH		D TR	
CNBr H-4	SL	174	TIGTDSALHRIM	185
	RM		T	
	SH		----	
CNBr H-5	SL	322	ALLEATPDTPACVVSLSGNQAVRL	345
	RM		G	
	SH		G Q	
CNBr H-6	SL	30	NAAVRAVTRM	39
	RM		V V	
	SH		V V	
CNBr H-8a	SL	466	LGTKRTLPKGYM	477
	RM		S KSF	
	SH		----- -SF	
CNBr H-8d	SL	186	EVIDAITTTAQ	196
	RM		IV	
	SH		----------	

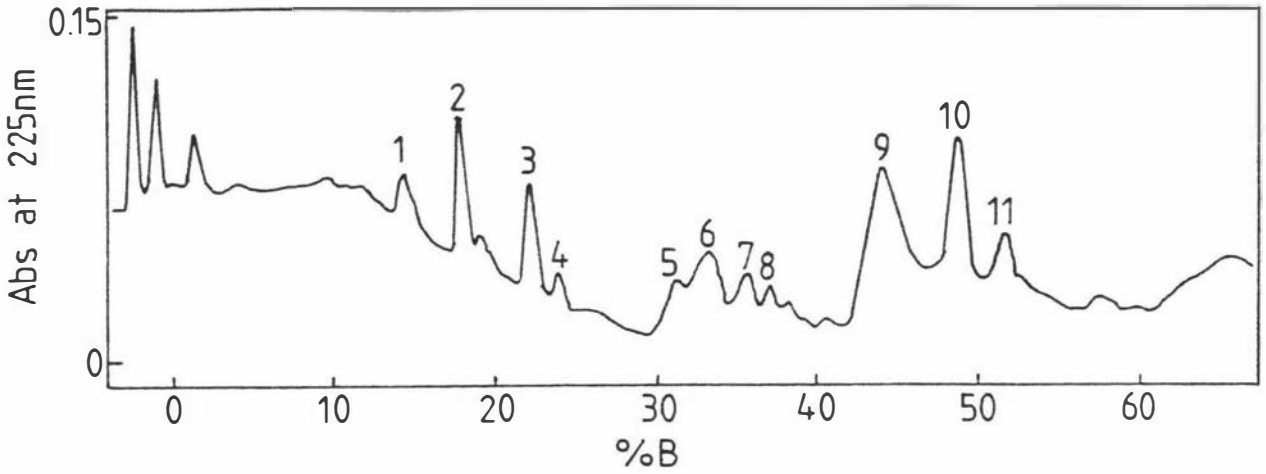


Figure 33: HPLC Elution Profile of Fraction CNBr I Chromatographed on a Resolve RC C-18 Column

Labelled peaks are those for which sequence information is shown in Fig. XXIVa. Peptides in Fraction CNBr I were dissolved in .1M Am Bic. Elution consisted of a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXIVa

AMINO ACID SEQUENCES OF LIVER PFK PEPTIDES ISOLATED FROM FRACTION CNBr I FOLLOWING HPLC

Fraction	Sequence
CNBr I-1	NVGAPAAGM
CNBr I-2	ADYVSGELEHVTRR
CNBr I-3	ADYVSGELEHVTRR
CNBr I-4	ADYVSGELEHVTRR
CNBr I-5	LAHYRISM
CNBr I-6	LAHYRISM
CNBr I-7a	LAHYRISM
CNBr I-7b	ADYVSGELEHVTRR
CNBr I-7c	TIGTDSALHRIM
CNBr I-8a	LAHYRISM
CNBr I-8b	ADYVSGELEHVTRR
CNBr I-8c	TIGTDSALHRIM
CNBr I-9a	LAHYRISM
CNBr I-9b	NAAVRAVTRM
CNBr I-10	LAHYRISM
CNBr I-11	LAHYRISM

6.1.2 SEPARATION AND AMINO ACID SEQUENCE OF THE HIGH MOLECULAR WEIGHT CNBr PEPTIDES

The high molecular weight fractions (A-F), were further separated by HPLC on a Vydac C-4 column as described in Section 4.8.1. Peaks from these were collected, freeze-dried and sequenced as described in Section 4.9. The HPLC elution profiles, along with the amino acid sequences of the isolated peptides are shown in Fig. 34, 35, 36, 37, 38 and Tables XXVa, XXVIa, XXVIIa, XXVIIIa, XXIXa respectively. Tables showing the alignment of the liver PFK peptides with the corresponding sequence of the rabbit muscle and sheep heart muscle sequence along with the amino acid differences between these species are shown in Tables XXVb, XXVIIb, XXVIIIb and XXIXb. No useful sequence information was obtained from peaks in fraction CNBr A.

Alignment of all the CNBr peptides from sheep liver PFK with the amino acid sequence of rabbit muscle and sheep heart muscle PFK is shown in Fig. 39.

CNBr F-6 corresponded exactly to the peptide at 713-738 of muscle PFK. This peptide also covers part of the sequence already obtained from peptide CNBr B-1b, and it is likely that it has resulted from the purification and concentration of the peptide from the contaminating subunit from the hybrid liver PFK.

Following alignment of the amino acid sequences of the CNBr peptides from sheep liver PFK with the sequences of rabbit and sheep muscle PFKs, it is clear that as suggested by the results from CNBr peptide mapping using FPLC (Section 5.4), a redistribution of methionine residues in sheep liver PFK has indeed occurred.

The sheep muscle PFK CNBr peptides can be grouped into families according to their size (Table XXX). Of the four high molecular weight peptides (67, 94, 98, 108 residues in length), only the peptide 98 residues in length does not have information indicating a substitution of a methionine into the sequence, thereby reducing the apparent size of these CNBr peptides in sheep liver PFK.

Comparison of the peptide 66 residues in length (713-779), in muscle PFK with the corresponding sequence in liver PFK, shows that the methionine at position 712 has been substituted by a leucine, thereby joining the 67 residue long peptide with the adjoining 26 residue long peptide (687-712). However two "new" methionines have also been introduced into the liver sequence, one at position 750, and the other at

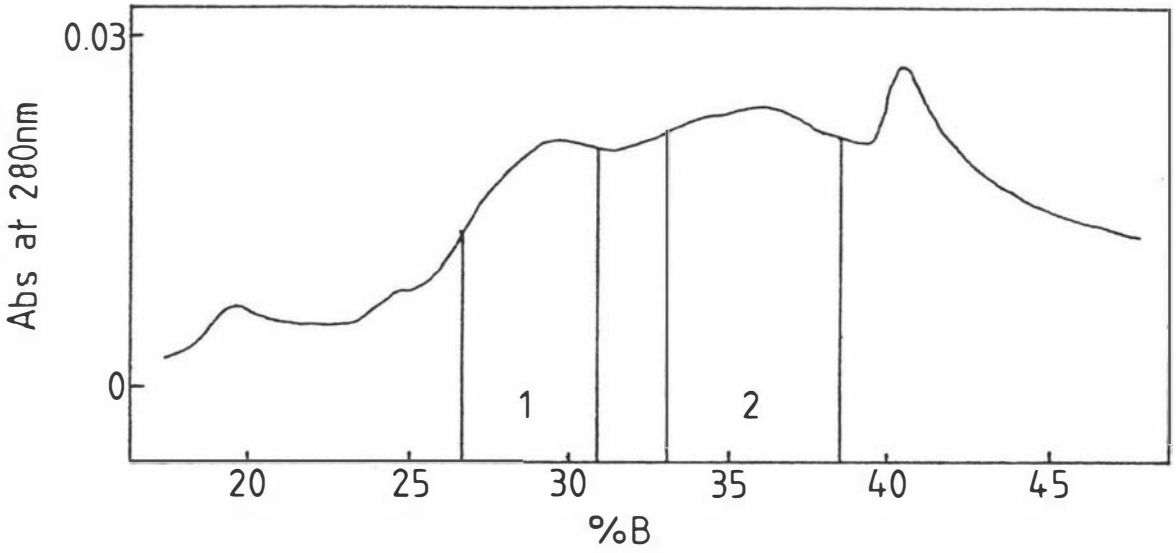


Figure 34: HPLC Elution Profile of Fraction CNBr B Chromatographed on a Vydac C-4 Column

Labelled peaks are those for which sequence information is shown in Fig. XXVa. Peptides from Fraction CNBr B were dissolved in 5% formic acid. Elution consisted of a linear gradient from 0-100% B (5% formic acid/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXVa

AMINO ACID SEQUENCES OF LIVER PFK PEPTIDES ISOLATED FROM FRACTION CNBr B FOLLOWING HPLC

Fraction	Sequence
CNBr B-1a	KTE I QKGLVLRNEKCNENYTTEF I YNLYSEEGKGVFDSR LNVLGHLQ
CNBr B-1b	SEKLRAVYRNGRVFANAPDSACV I GLQKKVVAF SPVTE
CNBr B-2	GRHCGYLALVTSLACGADWVF I PECPPN I QW

TABLE XXVb

ALIGNMENT OF SHEEP LIVER PFK PEPTIDES FROM FRACTION CNBr B WITH RABBIT MUSCLE AND SHEEP HEART PFK

Peptide	Sequence Alignment
CNBr B-1a	
	616 ... 662
SL	KTE I QKGLVLRNEKCNENYTTEF I YNLYSEEGKGVFDSRLNVLGHLQ
RM	TVKR D F I K M
SH	TVKR D D F I K M
CNBr B-1d	
SL	687 SEKLRAVYRNGRVFANAPDSACV I GLQKKVVAFSPVTE 724
RM	AG IKES I T G L MR RALL Q
SH	G IKES I T G L MR RALL Q
CNBr B-2	
SL	208 GRHCGYLALVTSLACGADWV I PECPPN I QW 238
RM	S DDN
SH	S Q DDN

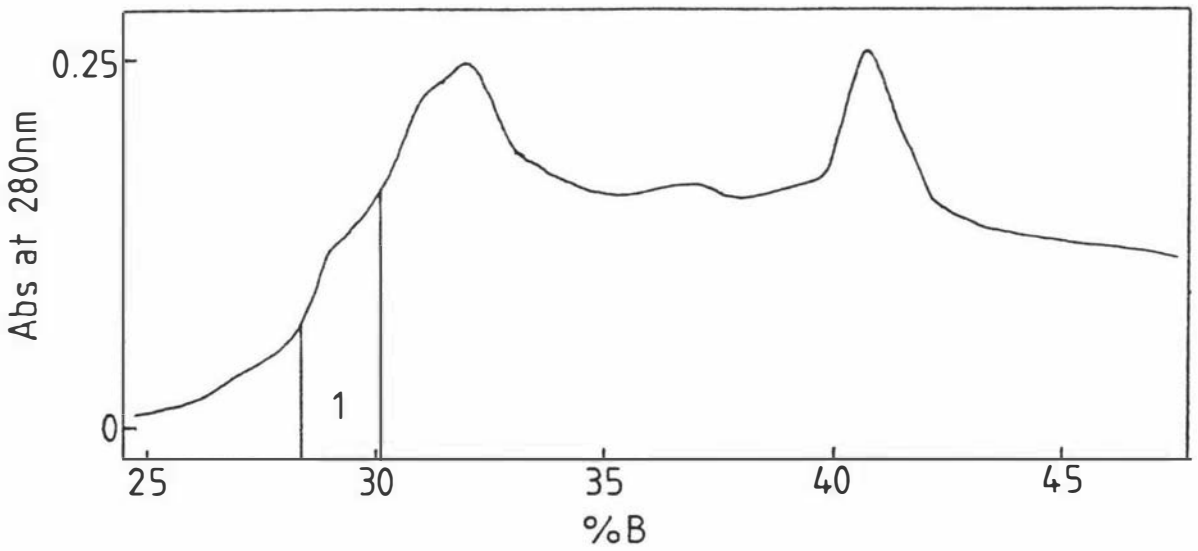


Figure 35: HPLC Elution Profile of Fraction CNBr C Chromatographed on a Vydac C-4 Column

Labelled peaks are those for which sequence information is shown in Fig. XXVIa. Peptides from Fraction CNBr C were dissolved in 5% formic acid. Elution consisted of a linear gradient from 0-100% B (5% formic acid/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXVIa

AMINO ACID SEQUENCES OF LIVER PFK PEPTIDES ISOLATED FROM FRACTION CNBr C FOLLOWING HPLC

Fraction	Sequence
CNBr C-1a	KTE I QKGLVLRNEKCNENYTTEF I YNLYSEEGKGVFDSR LNVLGHLQ
CNBr C-1B	SEKLRAVYRNGRVFANAPDSACV I GLQKKVVAFSPVTE

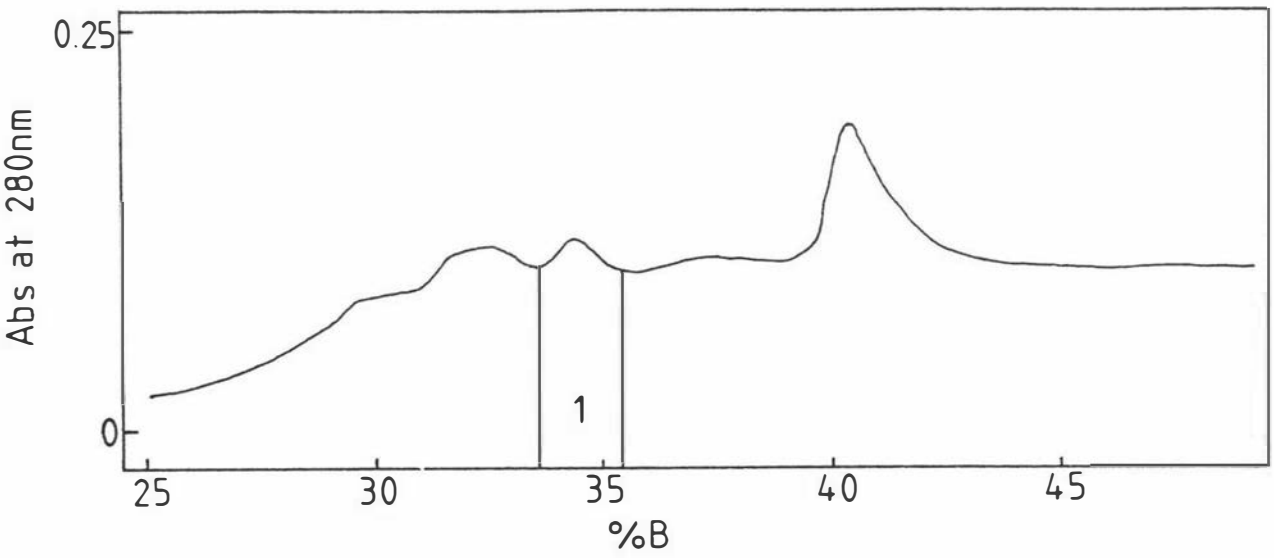


Figure 36: HPLC Elution Profile of Fraction CNBr D Chromatographed on a Vydac C-4 Column

Labelled peaks are those for which sequence information is shown in Fig. XXVIIa. Peptides from Fraction CNBr D were dissolved in 5% formic acid. Elution consisted of a linear gradient from 0-100% B (5% formic acid/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXVIIa

AMINO ACID SEQUENCES OF LIVER PFK PEPTIDES ISOLATED FROM FRACTION CNBr D FOLLOWING HPLC

Fraction	Sequence
CNBr D-1	CERLGETRSRGSRLN I I I AEGA I DRNGKS I TSRY

TABLE XXVIIb

ALIGNMENT OF SHEEP LIVER PFK PEPTIDES FROM FRACTION CNBr D WITH RABBIT MUSCLE AND SHEEP HEART PFK

Peptide		Sequence Alignment
CNBr D-1	SL	243 CERLGETRSRGSRLN||||AEGA|DRNGKS|TSRY 287
	RM	R S T V P EG
	SH	R- S IL ----------P EG

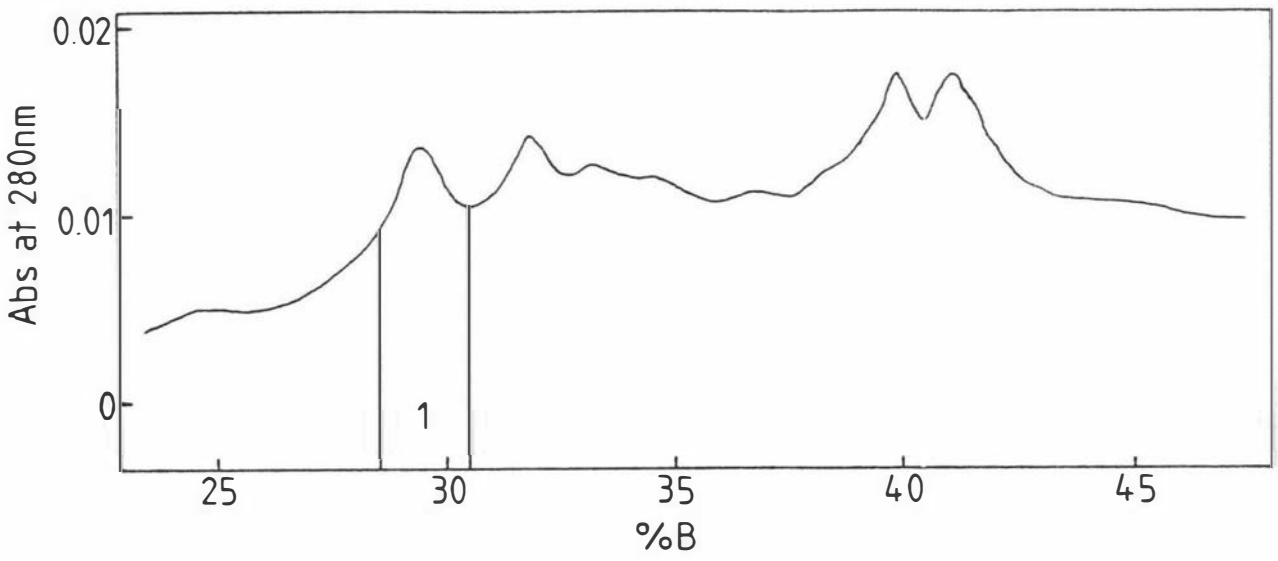


Figure 37: HPLC Elution Profile of Fraction CNBr E Chromatographed on a Vydac C-4 Column

Labelled peaks are those for which sequence information is shown in Fig. XXVIIIa. Peptides from Fraction CNBr E were dissolved in 5% formic acid. Elution consisted of a linear gradient from 0-100% B (5% formic acid/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXVIIIa

AMINO ACID SEQUENCES OF LIVER PFK PEPTIDES ISOLATED FROM FRACTION CNBr E FOLLOWING HPLC

Fraction	Sequence
CNBr E-1a	NAAVRSAVRSG I SQGHTVYVVHDGFEGLAKNQVQEVSWHD VAGWLGRGGS
CNBr E-1b	SEKLRAVYRNGRVFANAPDSACV I GLQKKVVAFSPVTE

TABLE XXVIIIb

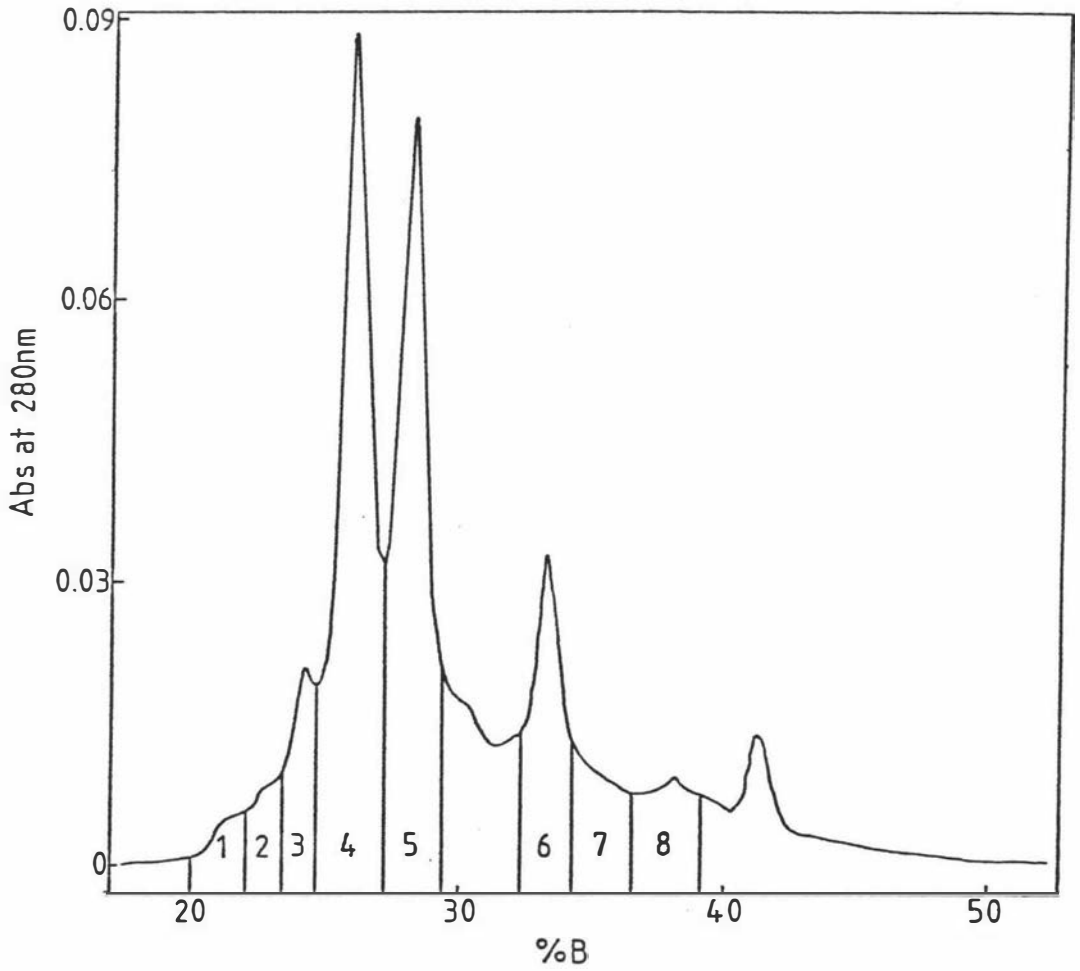
ALIGNMENT OF SHEEP LIVER PFK PEPTIDES FROM FRACTION CNBr E WITH RABBIT MUSCLE AND SHEEP HEART PFK

Peptide	Sequence Alignment
CNBr E-1a	
415	464
NAAVRSAVRSG I SQGHTVYVVHDGFEGLAKNQVQEVSWHDVAGWLGRGGS	
T I LI NR L G IE AG SY G T Q	
T I LI NR L ----------	

Line 1=Sheep liver PFK

Line 2=Rabbit muscle PFK.

Line 3=Sheep heart PFK.


0.09
0.06
0.03
0
Abs at 280nm
1
2
3
4
5
6
7
8
20
30
40
50
%B

Opposing Page.

Figure 38: HPLC Elution Profile of Fraction CNBr F Chromatographed on a Vydac C-4 Column

Labelled peaks are those for which sequence information is shown in Fig. XXIXa. Peptides from Fraction CNBr F were dissolved in 5% formic acid. Elution consisted of a linear gradient from 0-100% B (5% formic acid/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXIXa

AMINO ACID SEQUENCES OF LIVER PFK PEPTIDES ISOLATED FROM FRACTION CNBr F FOLLOWING HPLC

Fraction	Sequence
CNBr F-1	NAAVRSAVRSG I SQGHTVYVVHDG
CNBr F-2a	NAAVRSAVRSG I SQGHTVYVVHDG
CNBr F-2b	SEKLRAVYRNGRVFANAPDSAC
CNBr F-2c	ALLEATPDTPACVVSLSGNQAVRL
CNBr F-3a	NAAVRSAVRSG I SQGHTVYVVHDG
CNBr F-3b	SEKLRAVYRNGRVFANAPDSAC
CNBr F-3c	ALLEATPDTPACVVSLSGNQAVRL
CNBr F-4a	NAAVRSAVRSG I SQGHTVYVVHDGFEGLAKNQVQEVSWHD VAGWLGRGGS
CNBr F-4b	SEKLRAVYRNGRVFANAPDSACV I GLQKKVVAFSPVTE
CNBr F-5	DEKRFDEA I QLRGRSFENNWN I YKLLAHQK I SKENTWN GGAVVM
CNBr F-6	RKRALLFQPVTELQEQTDFEHR I PKE
CNBr F-7	GRHCGYLALVTSLACGADWVF I PECPPN I QW
CNBr F-8a	EQ I VES I RLHN I HALLV I GGFEAYEGVLQLVEARGRY
CNBr F-8b	LV I PATLSNNVPGTDFSVASDTALNT

TABLE XXIXb

ALIGNMENT OF SHEEP LIVER PFK PEPTIDES FROM FRACTION CNBr F WITH RABBIT MUSCLE AND SHEEP HEART PFK

Peptide	Sequence Alignment
CNBr F-5	
SL	362 DEKRFDEA|QLRGRSFENNWN|YKLLAHQK|SKENTWNGGAVV 404
RM	MK M EV |RPPAPKSGSYTVAV
SH	R MK M EV VRPPKSKSGLHTVAV
CNBr F-8a	
SL	478 EQ|VES|RLHN|HALLV|GGFEAYEGVLQLVEARGRY 514
RM	SAN TKF QG VI T G E M G KQF
SH	E TAD ---------- G KQ
CNBr F-8b	SL 521 LV|PATLSNNVPGTDFSVASDTALNT 547
	RM V V S GA
	SH V V S GA

```
    1                                                                50
RM  THEEHHAARTLGVGKAIAVLTSGGDAQGMNAAVRAVVRVGIFTGARVFFV
SH  THEEHHEAKTLGIGKAIAVLTSGGDAQGMNAAVRAVVRVGIYTGARVFFV
SL                                        NAAVRAVTRM

    51                                                           100
    HEGYQGLVDGGDHIREATWESVSMMLQLGGTVIGSARCKDFREREGRLRA
    HEGYQGLVDGGDNIREATWES---------------CKDFREREGRLRA

    101                                                          150
    AHNLVKRGITNLCVIGGDGSLTGADTFRSEWSDLLSDLQKAGKITAEEAT
    AHNLVK-GIGNLCVIGADGSLTGGDTFRSEWGDLLSDLQKSGKITAEEAT

    151                                                          200
    RSSYLNIVGLVGSIDNDFCGTDMTIGTDSALHRITEIVDAITTTAQSHQR
    KSSYLNIV-------------MTIGTDSAL------------------
                           TIGTDSALHRIMEVIDAITTTAQ

    201                                                          250
    TFVLEVMGRHCGYLALVTSLSCGADWVFIPECPPDDNWEDHLCRRLSETR
    ---------HCGYLALVTSLSCGADWVFIPQCPPDDNWEEHLCR-LSETR
            GRHCGYLALVTSLACGADWVFIPECPPNIQW    CERLGETR

    251                                                          300
    TRGSRLNIIIVAEGAIDRNGKPITSEGVKDLVVRRLGYDTRVTVLGHVQR
    ILGSR----------------PITSEGVKDLVVKRLGYDTRVTVLGVEHR
    SRGSRLNIIIIAEGAIDRNGKSI-SRY

    301                                                          350
    GGTPSAFDRILGSRMGVEAVMALLEGTPDTPACVVSLSGNQAVRLPLMEC
    GGTPSAFDRILGSRMGVEAVMALLEGTPQTPACVVSLSGNQAVRLPLMEC
                           ALLEATPDTPACVVSLSGNQAVRL

    351                                                          400
    VQVTKDVTKAMDEKRFDEAMKLRGRSFMNNWEVYKLLAHIRPPAPKSGSY
    VQVTKDVTRAMDERRFDEAMKLRGRSFMNNWEVYKLLAHVRPPKSKSGLH
       TKEVQKAMDEKRFDEAIQLRGRSFENNWNIYKLLAHQKISKENTWNG
```

```
401                                                          450
TVAVMNVGAPAAGMNAAVRSTVRIGLIQGNRVLVVHDGFEGLAKGQIEEA
TVAVMNVGAPAAGMNAAVRSTVRIGLIQGNRVLVVHDGFEGLAK------
GAVVMNVGAPAAGMNAAVRSAVRSGISQGHTVYVVHDGFEGLAKNQVQEV

451                                                          500
GWSYVGGWTGQGGSKLGSKRTLPKKSFEQISANITKFNIQGLVIIGGFEA
--------------------TLPK-SFEEITADI---------------
SWHDVAGWLGRGGS LGTKRTLPKGYMEQIVESIRLHNIHALLVIGGFEA

501                                                          550
YTGGLELMEGRKQFDELCIPFVVIPATVSNNVPGSDFSVGADTALNTICT
--------EGRKQYDELCIPFVVIPATVSNNVPGSDFSVGADTALNTICM
YEGVLQLVEARGRY        LVIPATLSNNVPGTDFSVASDTALNT

551                                                          600
TCDRIKQSAAGTKRRVFIIETMGGYCGYLATMAGLAAGADAAYIFEEPFT
TCDRIK------------IETMGGYCGYLATMAGLAAGADAAYIFEEPFT


601                                                          650
IRDLQANVEHLVQKMKTTVKRGLVLRNEKCNENYTTDFIFNLYSEEGKGI
VRDLQANVEHLVQ-MKTTVKRGLVLRDEKCNENYTTDFIFNLYSEEGKGI
               KTEIQKGLVLRNEKCNENYTTEFIYNLYSEEGKGV

651                                                          700
FDSRKNVLGHMQQGGSPTPFDRNFATKMGAKAMNWMAGKIKESYRNGRIF
FDSRKNVLGHMQQGGSPTPFDRNFATKMGAKAMNWMSGKIKESYRNGRIF
FDSRLNVLGHLQ                                SEKLRAVYRNGRVF

701                                                          750
ANTPDSGCVLGMRKRALVFQPVTELQNQTDFEHRIPKEQWWLKLRPILKI
ANTPDSGCVLGM-KRALLFQPVTELQEQTDFEHRIPKEQWWLK-RPILK-
ANAPDSACVIGLQKKVVAFSPVTE

751                          779
LAKYEIDLDTSEHAHLEHISRKRSGEATV
LAKYEIDLDTSEHAHLEHITRKRSGEADI
LAHYRISMADYVSGELEHVTRR
```

Figure 39: Sequence Obtained From CNBr Peptides

Alignment of the CNBr peptides from sheep liver PFK with the amino acid sequence of rabbit muscle and sheep heart muscle PFK.

TABLE XXX

SHEEP HEART MUSCLE PFK CNBr PEPTIDES

Peptide Family	Peptide Length No of Residues	Position of Peptide in Sequence	Number of Cysteines
1-10	1	75	-
	3	684-686	-
	5	679-683	-
	6	316-321	-
	8	371-378	-
	9	362-370	-
	9	406-414	-
	10	573-582	1
11-20	13	349-361	1
	17	662-678	-
21-30	22	551-572	1
	26	379-405	1
	26	678-712	-
	27	322-348	1
	29	1-29	-
31-40	33	583-651	-
	34	174-207	-
41-50	42	509-550	2
	45	30-74	-
	46	616-661	1
60-70	67	713-779	-
90-100	94	415-508	-
	98	76-173	3
101-110	108	208-315	4

position 758, as well, there may have been 7 residues lost from the C-terminus of liver PFK. Overall, the redistribution of methionines in the region from 687 to the C-terminus in liver PFK has resulted in the generation of three peptides of 8, 14, and 64 residue in length, as opposed to two in muscle PFK which are 26 and 67 residues long.

The sheep liver PFK sequence corresponding to the peptide which is 94 residues in length (415-508), from sheep heart PFK shows the introduction of two "new" methionine residues, one at position 465, and the other at position 477. The methionine at position 508 in the sheep heart PFK has been substituted for a valine, but a further methionine residue has been introduced into the liver sequence at position 521. Hence the two muscle peptides, 94 residues (415-508) and 42 residues (509-550) in length, correspond to four peptides in sheep liver PFK which are 51 residues (414-465), 12 residues (466-477), 44 residues (478-521), and 29 residues (522-550) long. So again, the resultant liver PFK peptides are generally smaller than the corresponding sheep heart peptides.

The 108 residue peptide (208-315), in sheep heart PFK corresponds to two peptides in the sheep liver PFK, with a methioine introduced at position 242 of the liver PFK sequence. Hence peptides which are 35 residues (208-242), and 73 residues (243-315) in length occur in sheep liver PFK in this region.

Other CNBr peptides from sheep muscle PFK which correspond to smaller peptides in the liver sequence are shown in Table XXXI. The substitution of methionines in the heart muscle enzyme, by other residues in the liver enzyme has also occurred, resulting in longer peptides (Table XXXI). The substitution of methionine 370 in the sheep heart enzyme by an isoleucine, and methionine 378 by a glutamic acid residue in the liver enzyme has resulted in the joining of three small peptides in the muscle sequence, to yield one longer peptide in the liver sequence (Table XXXI). The liver PFK sequence corresponding to the peptide 46 residues in length (616-661) in the muscle PFK sequence has also "lost" a methionine at position 661, resulting in a longer peptide of 63-71 residues in length (615-678 or 686). Overall, the sequencing of the CNBr peptides has shown that the liver PFK peptides are generally smaller than those of muscle PFK. This explains the different FPLC peptide maps obtained from the CNBr digests of sheep heart muscle and sheep liver PFK.

TABLE XXXI

CHANGES IN THE POSITION OF METHIONINE RESIDUES IN SHEEP LIVER PFK COMPARED TO SHEEP MUSCLE PFK

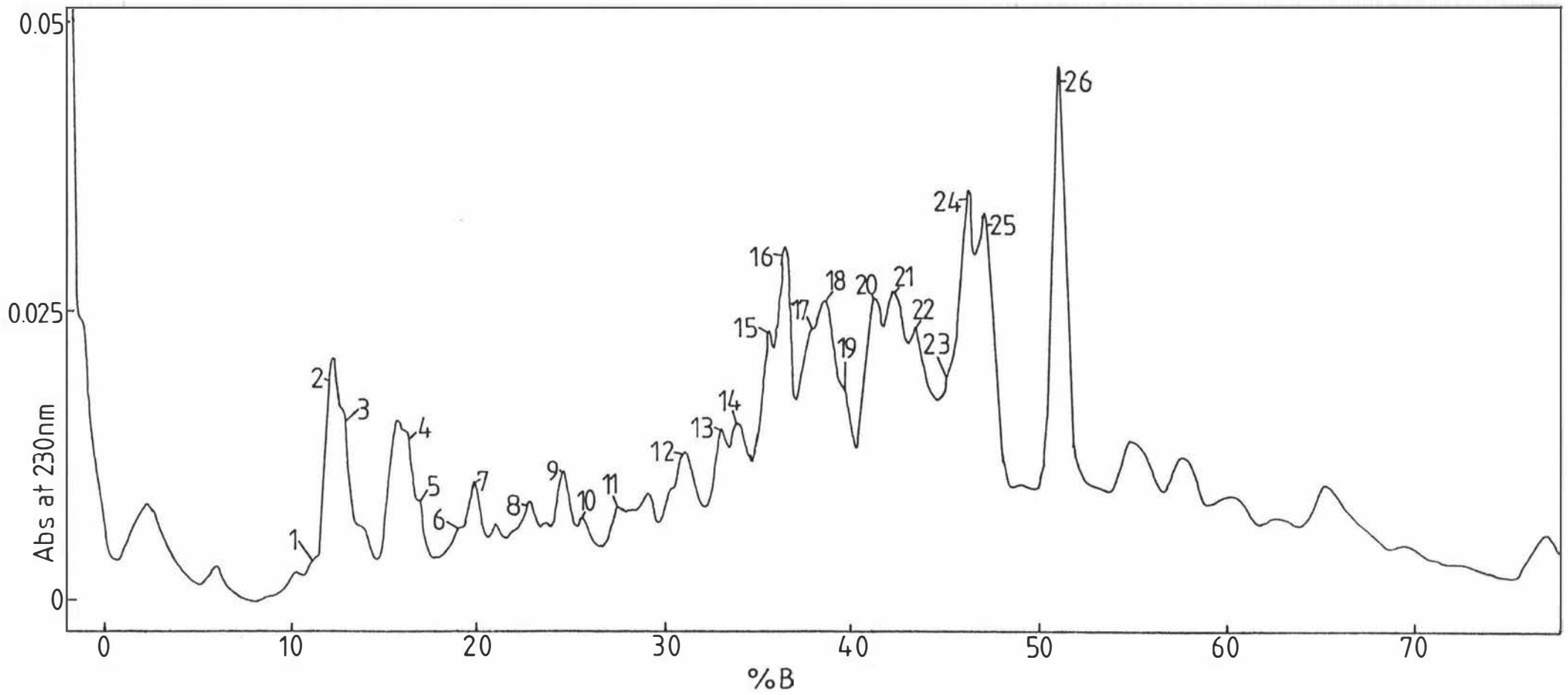
Muscle		Liver	
Peptide length No of Residues	Position in Sequence	Peptide length No of Residues	Position in Sequence
34	174-207	12	174-185
		22	186-207
45	30-74	10	30-39
		35	40-74
9	362-370	44	362-405
8	371-378		
27	379-405		
46	616-661	63-71	615-678 or 686

6.2 TRYPTIC PEPTIDES

Peptides of a tryptic digest of liver PFK were separated by HPLC as described in Section 4.8.2. These peptides are designated T (representing a tryptic digest), followed by the peak number 1-26, eg T 22. The HPLC elution profile, along with the amino acid sequences of the isolated peptides are shown in Fig. 40 and Table XXXIIa respectively. Table XXXIIb shows the alignment of the liver PFK tryptic peptides with the corresponding sequence in rabbit muscle and sheep heart muscle PFK. The position of the peptide within the whole sequence is shown, along with the amino acid residues which are different in the muscle PFKs. Blank spaces represent identical amino acids in the sequence, while hyphens indicate residues which have as yet not been determined.

Comparison of the tryptic peptide maps of sheep heart and liver PFK showed that the radioactively labelled muscle peptides tended to be more hydrophobic, and elute later in the gradient during HPLC than the radioactively labelled liver peptides (Section 5.5). From the amino acid sequence of sheep heart PFK, it is possible to predict the number of [^{14}C]-peptides which should be obtained from a tryptic digest (Table XXXIII).From the sequence information obtained for sheep liver PFK, it is possible to compare the sequences of the [^{14}C]-labelled tryptic peptides of liver PFK with those of sheep heart PFK.

Of the ten radioactively labelled peptides of sheep heart PFK, only one remained totally unchanged in the liver enzyme. This is the peptide 2 residues in length at positions 88-89, which would elute either very early in the gradient, or not be retained on the column at all. Of the other nine peptides, changes in the amino acid sequence of liver PFK resulted in four of these peptides being eluted earlier than the corresponding peptides from the muscle sequence (as predicted using the retention coefficients published by Meek and Rossetti, 1981), two of these the 11 residue peptide (345-355) and the 42 residue peptide (513-554) are much shorter peptides in the liver enzyme due to the repositioning of lysine and arginine residues. In fact, the 42 residue peptide which in muscle PFK contains 3 cysteine residues, in the liver has been divided into at least two cysteine containing peptides, due to the substitution of an arginine for a proline at position 520. Although the confirmation of the two cysteines at positions 549 and 552 has not been obtained, it is highly likely that these two cysteines will also be conserved. Since all the 12 cysteine residues sequenced from sheep liver PFK are in identical positions to those in the sheep heart muscle enzyme.


0.05
0.025
0
Abs at 230nm
0
10
20
30
40
50
60
70
%B
1
2
3
4
5
6
7
8
9
10
11
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Opposing Page.

Figure 40: HPLC Elution Profile of a Tryptic Digest of Sheep Liver PFK Chromatographed on a Resolve RC C-18 Column.

Labelled peaks are those for which sequence information is shown in Fig. XXXIIa. The ammonium bicarbonate-soluble tryptic peptides were dissolved in 0.1M Am Bic Elution consisted of a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXXIIa

AMINO ACID SEQUENCES OF TRYPTIC PEPTIDES FROM SHEEP LIVER PFK FOLLOWING HPLC

Fraction	Sequence
T 1	AVYR
T 2	ANVEHMTEK
T 3	ANVEHMTEK
T 4	GGTPSAFDR
T 5a	GGTPSAFDR
T 5b	DLVVQR
T 6a	GGSMLGTK
T 6b	DLVVQR
T 6c	MKTEIQK
T 7a	SGISQGHTVY
T 7b	AFTTR
T 8a	LGFDTR
T 8b	TLPK
T 9	LGFDTR
T 10a	GLVLR

Table continued overleaf...

Table cont...

T 10b	LLAHQK
T 11	GLVLR
T 12	MG I YVGAK
T 13a	MG I YVGAK
T 13b	RFDEA I QLR
T 13c	SFENNWN I YK
T 14a	MG I YVGAK
T 14b	RFDEA I QLR
T 15a	MLAHYR
T 15b	VTVLGHVQR
T 15c	VFANAPSACV I GLQK
T 16a	SFENNWN I YK
T 16b	VTVLGHVQR
T 17a	A I GVLTSGGDAQGMNAAVR
T 17b	SG I SQGHTVYVVHDGFEGLAK
T 18a	A I GVLTSGGDAQGMNAAVR
T 18b	VVAFSPVTELQEQTDFEHR
T 18c	I SMADYVSGELEHVTR
T 19a	A I GVLTSGGDAQGMNAAVR
T 19b	VVAFSPVTELQEQTDFEHR
T 20	VFL I YEGYEGLVEGGEN I R
T 21	VFL I YEGYEGLVEGGEN I R
T 22	GYMEQ I VES I R
T 23a	TFVLEVMGR
T 23b	LNVLGHLQQGGAPTPFDR
T 24a	TFVLEVMGR
T 24b	LNVLGHLQQGGAPTPFDR
T 24c	EQWWLNLR
T 25	SEWGSLLEELVSEGK
T 26	NQVQEVSWHDVAGWLGR

TABLE XXXIIb

ALIGNMENT OF THE TRYPTIC PEPTIDES FROM SHEEP LIVER PFK WITH RABBIT MUSCLE AND SHEEP HEART MUSCLE PFK SEQUENCE

Peptide	Sequence Alignment		
T 1	SL	692 AVYR	695
	RM	ES	
	SH	ES	
T 2	SL	606 ANVEHMTEK	614
	RM	LVQ	
	SH	LVQ-	
T 4	SL	301 GGTPSAFDR	309
	RM		
	SH		
T 5b	SL	280 DLVVQR	285
	RM	R	
	SH	K	
T 6a	SL	462 GGSMLGTK	474
	RM	K S	
	SH	--------	
T 6c	SL	615 MKTEIQK	621
	RM	TVKR	
	SH	TVKR	
T 7b	SL	90 AFTTR	94
	RM	D RE	
	SH	D RE	
T 8a	SL	286 LGFDTR	291
	RM	Y	
	SH	Y	

Table continued overleaf...

Table cont...

T 8b	SL	471	TLPK	474
	RM			
	SH			
T 10a	SL	622	GLVLR	626
	RM			
	SH			
T 12	SL	39	MGIYVGAK	46
	RM		V FT R	
	SH		V T R	
T 13b	SL	365	RFDEAIQLR	373
	RM		MK	
	SH		MK	
T 13c	SL	376	SFENNWNIYK	385
	RM		M EV	
	SH		M EV	
T 15a	SL	750	MLAHYR	755
	RM		I K E	
	SH		- K E	
T 15b	SL	292	VTVLGHVQR	300
	RM			
	SH		VEH	
T 15c	SL	699	VFANAPDSACVIGLQK	714
	RM		I T G L MR	
	SH		I T G L MR	
T 17a	SL	16	AIGVLTSGGDAQGMNAAVR	34
	RM		A	
	SH		A	
T 17b	SL	424	SGISQGHTVYVVHDGFEGLAK	444
	RM		I LI NR L	
	SH		I LI NR L	

Table continued overleaf...

Table cont...

T 18b	SL	716	VVAFSPVTELQEQTDFEHR 734
	RM		ALV Q N
	SH		ALL Q
T 18c	SL	756	ISMADYVSGELEHVTR 771
	RM		DLDTSEHAH IS
	SH		DLDTSEHAH I
T 20	SL	47	VFLIYEGYEGLVEGGENIR 65
	RM		FVH Q D DH
	SH		FVH Q D D
T 22	SL	475	GYMEQIVESIR 485
	RM		KSF SAN T
	SH		-SF E TAD-
T 23a	SL	201	TFVLEVMGR 209
	RM		
	SH		---------
T 23b	SL	655	LNVLGHLQQGGAPTPFDR 672
	RM		K M S
	SH		K M S
T 24c	SL	738	EQWWLNLR 745
	RM		K
	SH		K-
T 25	SL	129	SEWGSLLEELVSEGK 143
	RM		SD SD QKA
	SH		D SD QKS
T 26	SL	445	NQVQEVSWHDVAGWLGR 461
	RM		G IE AG SY G T Q
	SH		----------

TABLE XXXIII

PREDICTED [^{14}C]-CYSTEINE CONTAINING PEPTIDES FROM A TRYPTIC DIGEST OF SHEEP HEART PFK

Peptide Length No of residues	Position in Sequence Residues numbers	Number of Cysteine Residues
2	88-89	1
11	345-355	1
15	699-713	1
19	630-648	1
21	108-128	1
30	315-344	1
32	152-183	1
35	210-244	4
37	566-602	1
42	513-554	3

The 15 residue long peptide (699-713) in liver PFK exhibited several differences in the amino acid sequence from that in the muscle peptide, but both should still elute at similar positions in the gradient. The 19 residue long peptide (630-648) in liver PFK displayed only one amino acid difference to that of the corresponding muscle sequence. Retention coefficients suggest that the muscle peptide should elute slightly earlier in the gradient than the liver peptide.

Comparison of the elution positions of three peptides, the 35 residue (210-244), the 37 residue (566-602) and the 30 residue (315-344) long peptides cannot be made due to an incomplete sequence of the liver PFK in these regions.

Overall, comparison of the amino acid sequences around the [^{14}C]-cysteines of sheep muscle and liver PFK has revealed that the positions of the cysteine residues within the sequence have remained unchanged, but other amino acid substitutions have occurred which could explain the different tryptic peptide maps obtained for the two isozymes. The rearrangement of lysine and arginine residues in liver PFK, may also result in a greater number of [^{14}C]-labelled cysteine-containing tryptic peptides in sheep liver PFK than in the muscle enzyme.

The amino acid sequence of two tryptic peptides from human liver PFK have recently been determined (Levanon et al., 1987). In both cases these peptides were found to be identical to the corresponding sheep liver PFK sequence. One peptide corresponded to T 4, which has exactly the same sequence in the muscle PFKs also. The other corresponded to T 8a, which has a phenylalanine at position 288 in the liver enzyme whereas the muscle PFKs have a tyrosine residue. It is interesting to note that a peptide corresponding exactly to T 4 has also been isolated and sequenced from chicken muscle PFK (Green and Midwinter, Personal communication). Since this peptide is identical in all the mammalian PFKs sequenced, and is very similar to that in Bs PFK (shown below), it is tempting to speculate that this region may be of vital importance to the functional integrity of the enzyme.

Mammalian	GGTPSAFDR
Bs	GGSPTAFDR

Peptides corresponding to T 13c, T 18c and T 24c have also been isolated and sequenced from chicken muscle PFK. In a number of cases the chicken muscle peptides appear to be intermediate between the rabbit muscle and sheep liver peptides,

with some amino acids the same as those in rabbit muscle and others as those in sheep liver PFK.

Liver	SFENNWNI
Chicken	SFQNNWNV
Rabbit	SFMNNWEV

This may merely be coincidence or may reflect the importance of the conservation of the amino acids concerned.

The N-terminal residue of the peptide T 13b and T 14b is an arginine, the failure of trypsin to cleave after this arginine at position 365, was probably due to the presence of the lysine residue at position 364, since the presence of a double basic sequence such as this often results in incomplete cleavage by trypsin.

Alignment of all the sheep liver PFK tryptic peptides with the amino acid sequence of rabbit muscle and sheep heart PFK is shown in Fig. 41.

6.3 MALEYL-TRYPTIC PEPTIDES

Maleic anhydride readily acylates α-and ε-amino groups in proteins at mildly alkaline pH. Maleyl-proteins have a large excess of negative charge at or above neutral pH, and the resulting electrostatic repulsion has a dissociating and solubilizing effect. Maleylation of the lysine residues allows specific cleavage by trypsin at the arginine bonds. The maleyl-amino group is very stable at alkaline pH and is therefore unlikely to be hydrolyzed during prolonged enzymic digestions at alkaline pH. Cleavage at some arginyl residues in maleylated proteins seems to occur much more slowly than at others, probably because of inhibition by adjacent negative charges (Butler and Hartley, 1972), and this kinetic specificity can be an advantage in obtaining large peptides for sequence studies.

The maleyl group is readily removed by hydrolysis of the amide bond at mildly acid pH, as a result of an intramolecular catalysis by the adjacent, locked carboxylic acid group. The unblocking conditions although generally specific for the maleyl-amino bond, may cause deamidation of particularly labile amide groups (eg in -Asn-Gly-).

```
   1                                                                  50
RM THEEHHAARTLGVGKAIAVLTSGGDAQGMNAAVRAVVRVGIFTGARVFFV
SH THEEHHEAKTLGIGKAIAVLTSGGDAQGMNAAVRAVVRVGIYTGARVFFV
                  AIGVLTSGGDAQGMNAAVR     MGIYVGAKVFLI

   51                                                                 100
HEGYQGLVDGGDHIREATWESVSMMLQLGGTVIGSARCKDFREREGRLRA
HEGYQGLVDGGDNIREATWES----------------CKDFREREGRLRA
YEGYEGLVEGGENIR                          AFTTR

  101                                                                 150
AHNLVKRGITNLCVIGGDGSLTGADTFRSEWSDLLSDLQKAGKITAEEAT
AHNLVK-GIGNLCVIGADGSLTGGDTFRSEWGDLLSDLQKSGKITAEEAT
                                  SEWGSLLEELVSEGK

  151                                                                 200
RSSYLNIVGLVGSIDNDFCGTDMTIGTDSALHRITEIVDAITTTAQSHQR
KSSYLNIV--------------MTIGTDSAL------------------


  201                                                                 250
TFVLEVMGRHCGYLALVTSLSCGADWVFIPECPPDDNWEDHLCRRLSETR
---------HCGYLALVTSLSCGADWVFIPQCPPDDNWEEHLCR-LSETR
TFVLEVMGR

  251                                                                 300
TRGSRLNIIIVAEGAIDRNGKPITSEGVKDLVVRRLGYDTRVTVLGHVQR
ILGSR----------------PITSEGVKDLVVKRLGYDTRVTVLGVEHR
                                  DLVVQRLGFDTRVTVLGHVQR

  301                                                                 350
GGTPSAFDRILGSRMGVEAVMALLEGTPDTPACVVSLSGNQAVRLPLMEC
GGTPSAFDRILGSRMGVEAVMALLEGTPQTPACVVSLSGNQAVRLPLMEC
GGTPSAFDR

  351                                                                 400
VQVTKDVTKAMDEKRFDEAMKLRGRSFMNNWEVYKLLAHIRPPAPKSGSY
VQVTKDVTRAMDERRFDEAMKLRGRSFMNNWEVYKLLAHVRPPKSKSGLH
               RFDEAIQLR  SFENNWNIYK
```

```
401                                                                450
TVAVMNVGAPAAGMNAAVRSTVRIGLIQGNRVLVVHDGFEGLAKGQIEEA
TVAVMNVGAPAAGMNAAVRSTVRIGLIQGNRVLVVHDGFEGLAK------
                          SGISQGHTVYVVHDGFEGLAKNQVQEV

451                                                                500
GWSYVGGWTGQGGSKLGSKRTLPKKSFEQISANITKFNIQGLVIIGGFEA
--------------------TLPK-SFEEITADI----------------
SWHDVAGWLGRGGSMLGTK TLPKGYMEQIVESIR

501                                                                550
YTGGLELMEGRKQFDELCIPFVVIPATVSNNVPGSDFSVGADTALNTICT
--------EGRKQYDELCIPFVVIPATVSNNVPGSDFSVGADTALNTICM


551                                                                600
TCDRIKQSAAGTKRRVFIIETMGGYCGYLATMAGLAAGADAAYIFEEPFT
TCDRIK------------IETMGGYCGYLATMAGLAAGADAAYIFEEPFT


601                                                                650
IRDLQANVEHLVQKMKTTVKRGLVLRNEKCNENYTTDFIFNLYSEEGKGI
VRDLQANVEHLVQ-MKTTVKRGLVLRDEKCNENYTTDFIFNLYSEEGKGI
      ANVEHMTEKMKTEIQKGLVLR

651                                                                700
FDSRKNVLGHMQQGGSPTPFDRNFATKMGAKAMNWMAGKIKESYRNGRIF
FDSRKNVLGHMQQGGSPTPFDRNFATKMGAKAMNWMSGKIKESYRNGRIF
    LNVLGHLQQGGAPTPFDR                          AVYR   VF

701                                                                750
ANTPDSGCVLGMRKRALVFQPVTELQNQTDFEHRIPKEQWWLKLRPILKI
ANTPDSGCVLGM-KRALLFQPVTELQEQTDFEHRIPKEQWWLK-RPILK-
ANAPDSACVIGLQK VVAFSPVTELQEQTDFEHR   EQWWLNLR    M

751                          779
LAKYEIDLDTSEHAHLEHISRKRSGEATV
LAKYEIDLDTSEHAHLEHITRKRSGEADI
LAHYRISMADYVSGELEHVTR
```

Figure 41: Sequence Obtained From Tryptic Peptides

Alignment of the tryptic peptides from sheep liver PFK with the amino acid sequence of rabbit muscle and sheep heart muscle PFK.

Maleylation and tryptic digestion followed by demaleylation of liver PFK was carried out as described in Sections 4.8.3.1.-4.8.3.3. The separation of these peptides was carried out in three stages: Stage 1) separation into acid-soluble and acid-insoluble peptides, 2) separation of the above samples by ion-exchange chromatography, and 3) separation of the fractions obtained from stage 2, by HPLC, as described in Sections 4.8.3.4. and 4.8.3.5.

The acid-soluble maleyl-tryptic peptides are named by the following system; M designates a maleyl-tryptic peptide, while S designates an acid-soluble fraction, 1-12 depending on the fraction from the Mono-Q column, followed by the peak number from HPLC, eg MS 2-12, meaning a acid-soluble maleyl-tryptic peptide from fraction 2 off the ion-exchange column and peak 12 from HPLC.

The acid-insoluble maleyl-tryptic peptides are named M (maleyl-tryptic), I for acid-insoluble fraction, 1-9 depending on the fraction from the Mono-Q column, followed by the peak number from HPLC elution profile eg MI 3-4.

The elution profile of the acid-soluble maleyl-tryptic fraction from the Mono-Q ion-exchange column, and the fractions which were collected for further purification by HPLC is shown in Fig. 42. The HPLC elution profiles, and the amino acid sequences of the peptides isolated from fractions MS 1-MS 5 are shown in Fig. 43, 44, 45, 46 and 47, and Tables XXXIVa, XXXVa, XXXVIa, XXXVIIa and XXXVIIIa respectively. Alignment of these peptides with the corresponding sequence in rabbit muscle and sheep heart muscle PFK is shown in Tables XXXIVb, XXXVb, XXXVIIb and XXXVIIIb.The position in the sequence is shown, along with the residues which are different in the muscle sequence.

The elution profile of the acid-insoluble maleyl-tryptic fraction from the Mono-Q ion-exchange column, and the fractions which were collected for further purification by HPLC is shown in Fig. 48. The HPLC elution profiles, and the amino acid sequences of the peptides isolated from fractions MI 1-MI 3 are shown in Fig. 49, 50, and 51, and Tables XXXIXa, XLa, and XLIa, respectively. Alignment of these peptides with the corresponding sequence in rabbit muscle and sheep heart muscle PFK is shown in Table XLIb.The position in the sequence is shown, along with the residues which are different in the muscle sequence.

Alignment of all the sheep liver PFK maleyl-tryptic peptides with the amino acid sequence of rabbit muscle and sheep heart PFK is shown in Fig. 52.

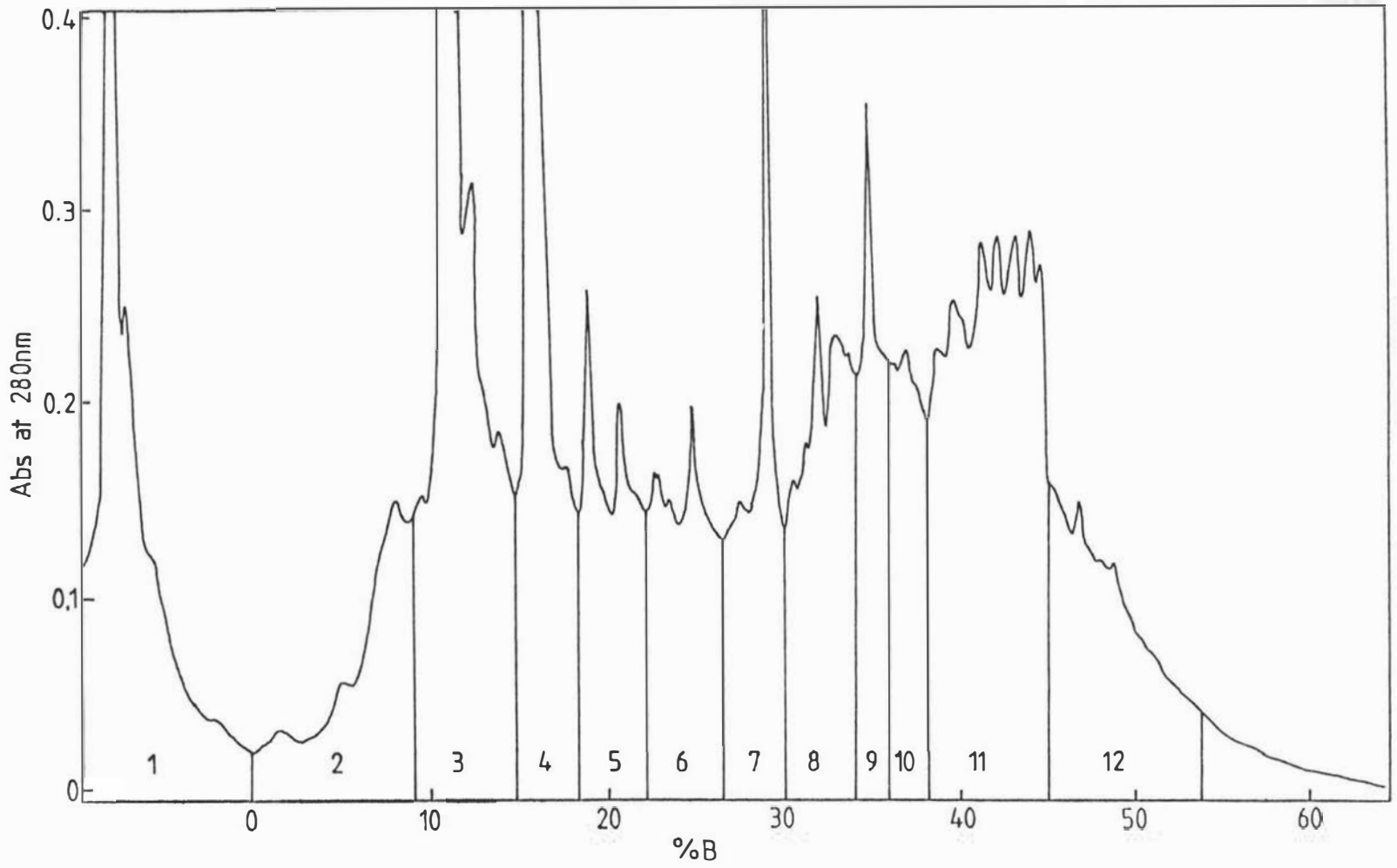
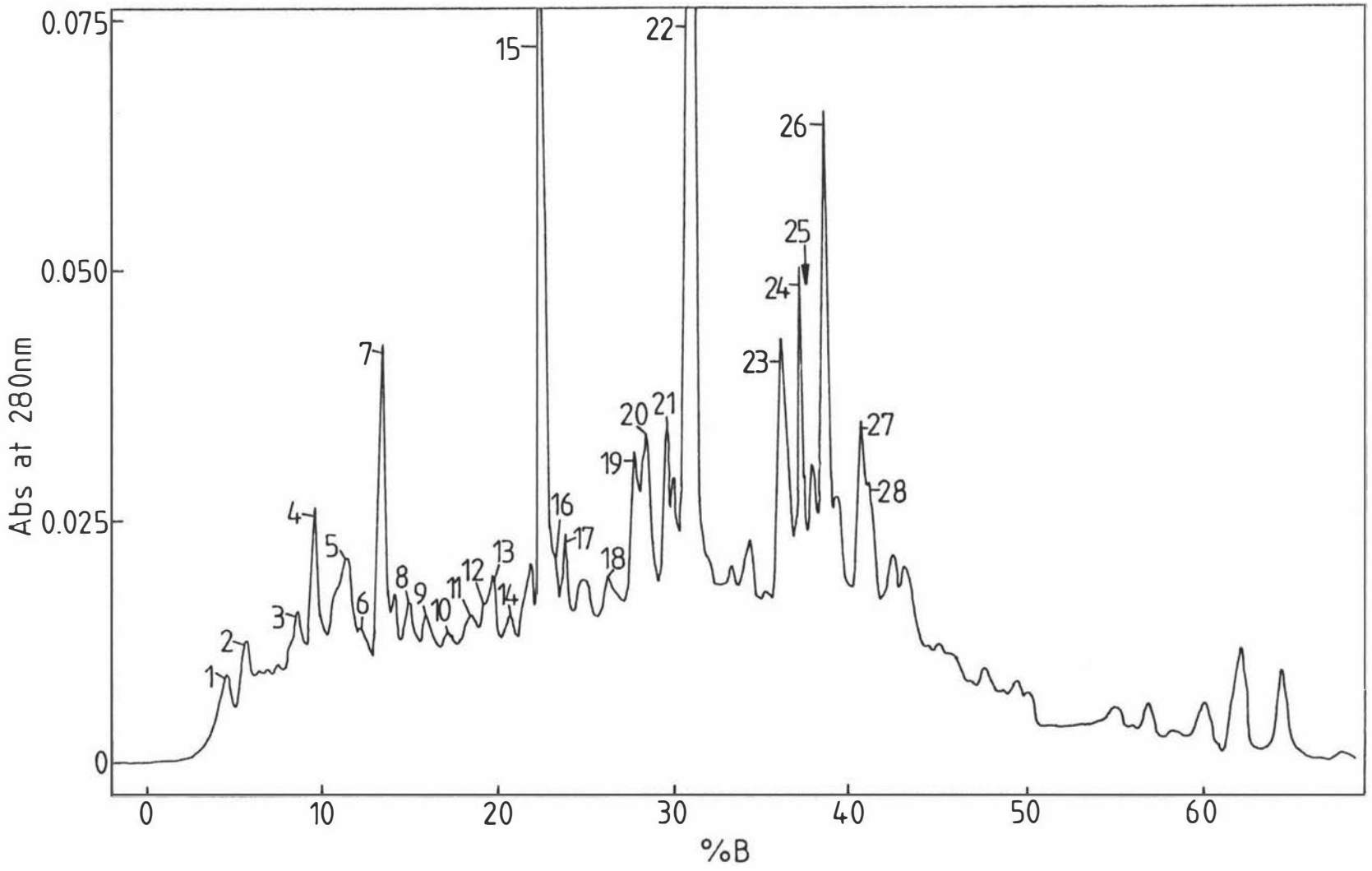


Figure 42: HPLC Elution Profile of the Acid-Soluble Maleyl-Tryptic Peptides Chromatographed on a Mono-Q Ion-exchange Column.

The Acid-soluble maleyl-tryptic peptides were dissolved in Buffer A (20mM Tris-HCl pH 8.0). Elution consisted of a linear gradient from 0-100% B (20mM Tris-HCl pH 8.0, 1M NaCl) run over 2hr at a rate of 0.5ml/min. The Fractions which underwent further purification by HPLC are labelled 1-12.


0.075
0.050
0.025
0
Abs at 280nm
0
10
20
30
40
50
60
%B
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
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28

Opposing Page.

Figure 43: HPLC Elution Profile of Fraction MS 1 Chromatographed on a Vydac C-18 Column.

Labelled peaks are those for which sequence information is shown in Fig. XXXIVa. Peptides in Fraction MS 1 were dissolved in 0.1M Am Bic, 5% acetonitrile. Elution consisted of a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXXIVa

AMINO ACID SEQUENCES OF ACID-SOLUBLE MALEYL-TRYPTIC PEPTIDES FROM SHEEP LIVER PFK ISOLATED FROM FRACTION 1

Peptide	Sequence
MS 1-1	LGYSTR
MS 1-2	GGTPSAFDR
MS 1-3a	CKAFTTR
MS 1-3b	LGETR
MS 1-4a	IKQSAAGTVQR
MS 1-4b	GGSMLGTKR
MS 1-5	LGFDTR
MS 1-6	GLVLR
MS 1-7	GLVLR
MS 1-8	ILGSR
MS 1-9	AAHNLVKR
MS 1-10	FDEAIQLR
MS 1-11	VTVLGHVQR
MS 1-12	VTVLGHVQR
MS 1-13	VTVLGHVQR
MS 1-14	TFVLEVMGR
MS 1-15a	TSGAGKAIGVLTSGGDAQGMNAAVR
MS 1-15b	TLPKGYMEQIVESIR

Table continued overleaf...

Table cont...

MS 1-15c	YVKDLVVQR
MS 1-16	TLPKGYMEQIVESIR
MS 1-17	TSGAGKAIGVLTSGGDAQGMNAAVR
MS 1-18	TSGAGKAIGVLTSGGDAQGMNAAVR
MS 1-19	LNVLGHLQQGGAPTPFDR
MS 1-20	LNVLGHLQQGGAPTPFDR
MS 1-21	TFVLEVMGR
MS 1-22	EQWWLNLR
MS 1-23	TLPKGYMEQIVESIR
MS 1-24	TLPKGYMEQIVESIR
MS 1-25	VFANAPDSACVIGLQKKVVAFSPVTE
MS 1-26a	GITNLCVIGGDASLTGANTFR
MS 1-26b	VFANAPDSACVIGLQKKVVAFSPVTE
MS 1-27a	VFANAPDSACVIGLQKKVVAFSPVTE
MS 1-27b	GITNLCVIGGDASLTGANTFR
MS 1-27c	IMEVIDAITTTAQSHQR
MS 1-28a	IMEVIDAITTTAQSHQR
MS 1-28b	GITNLCVIGGDASLTGANTFR
MS 1-28c	VFANAPDSACVIGLQKKVVAFSPVTE

TABLE XXXIVb

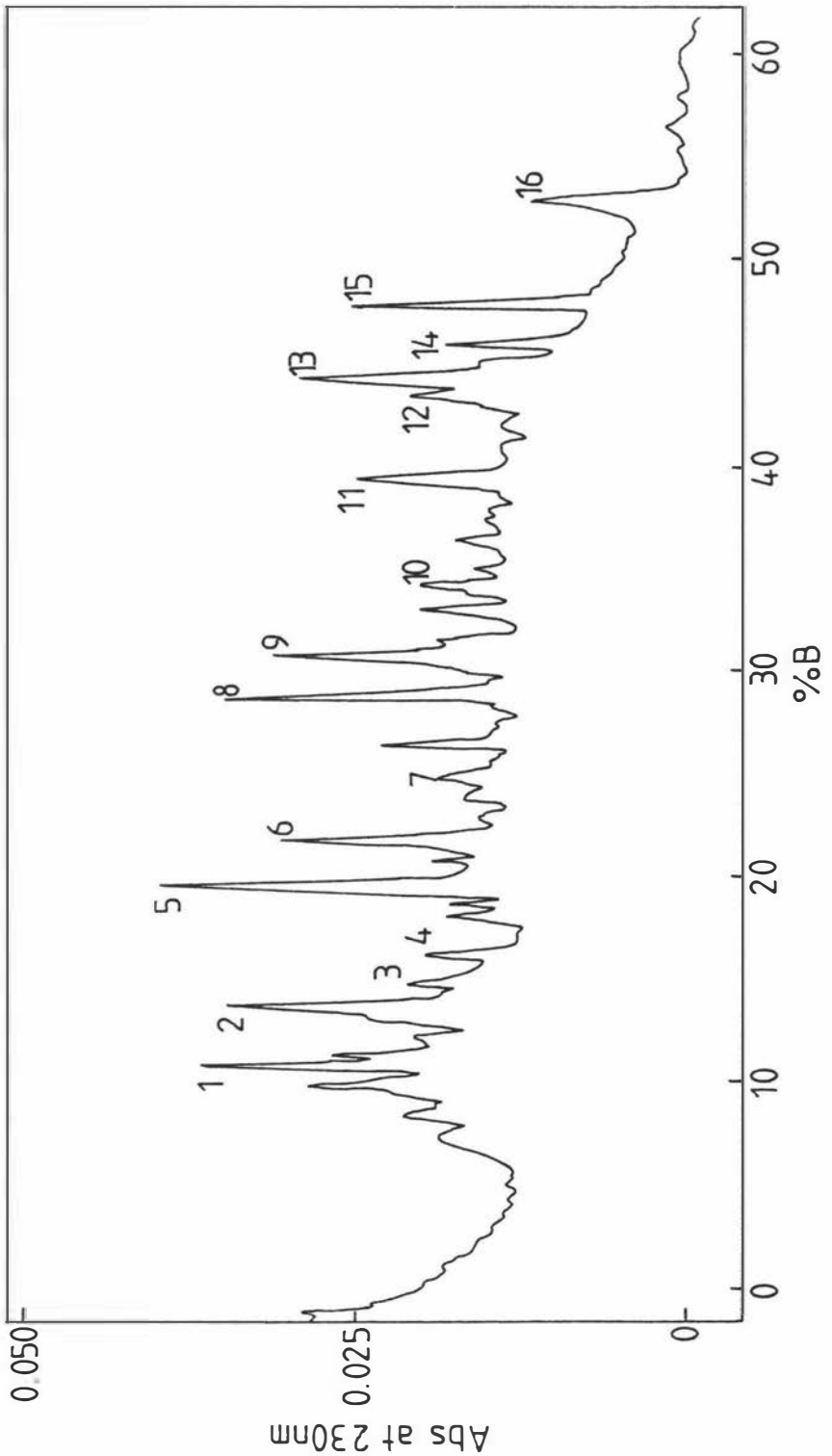
ALIGNMENT OF THE SHEEP LIVER PFK MS 1 MALEYL-TRYPTIC PEPTIDES WITH RABBIT MUSCLE AND SHEEP HEART MUSCLE PFK

Peptide name		Sequence Alignment
MS 1-2	SL	301 GGTPSAFDR 309
	RM	
	SH	
MS 1-3a	SL	88 CKAFTTR 94
	RM	D RE
	SH	D RE
MS 1-4a	SL	555 IKQSAAGTVQR 565
	RM	KR
	SH	----------
MS 1-4b	SL	462 GGSMLGTKR 470
	RM	K S
	SH	---------
MS 1-5	SL	286 LGFDTR 291
	RM	Y
	SH	Y
MS 1-6	SL	622 GLVLR 626
	RM	
	SH	
MS 1-8	SL	310 ILGSR 314
	RM	
	SH	
MS 1-9	SL	100 AAHNLVKR 107
	RM	
	SH	-
MS 1-10	SL	363 FDEAIQLR 373
	RM	MK
	SH	MK

Table continued overleaf...

Table cont...

MS 1-11	SL	292	VTVLGHVQR	300
	RM			
	SH		VEH	
MS 1-14	SL	201	TFVLEVMGR	209
	RM			
	SH		---------	
MS 1-15a	SL	10	TSGAGKAIGVLTSGGDAQGMNAAVR	34
	RM		L V A	
	SH		L I A	
MS 1-15b	SL	471	TLPKGYMEQIVESIR	485
	RM		KSF SAN T	
	SH		-SF E TAD -	
MS 1-15c	SL	277	YVKDLVVQR	285
	RM		G R	
	SH		G K	
MS 1-19	SL	655	LNVLGHLQQGGAPTPFDR	672
	RM		K M S	
	SH		K M S	
MS 1-22	SL	738	EQWWLNLR	745
	RM		K	
	SH		K-	
MS 1-25	SL	699	VFANAPDSACVIGLQKKVVAFSPVTE	724
	RM		I T G L MR RALV Q	
	SH		I T G L MR RALL Q	
MS 1-26a	SL	108	GITNLCVIGGDASLTGANTFR	128
	RM		G D	
	SH		G A G GD	
MS 1-27c	SL	184	IMEVIDAITTTAQSHQR	200
	RM		T IV	
	SH		----------	


Abs at 230nm
0.050
0.025
0
0
10
20
30
40
50
60
%B
1
2
3
4
5
6
7
8
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10
11
12
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14
15
16

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Figure 44: HPLC Elution Profile of Fraction MS 2 Chromatographed on a Vydac C-18 Column.

Labelled peaks are those for which sequence information is shown in Fig. XXXVa. Peptides in Fraction MS 2 were dissolved in 0.1M Am Bic, 5% acetonitrile. Elution consisted of a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXXVa

AMINO ACID SEQUENCES OF ACID-SOLUBLE MALEYL-TRYPTIC PEPTIDES FROM SHEEP LIVER PFK ISOLATED FROM FRACTION 2

Peptide	Sequence
MS 2-1	GGTPSAFDR
MS 2-2	LGFDTR
MS 2-3	LGFDTR
MS 2-4	LGFDTR
MS 2-5	ISMADYVSGELEHVTR
MS 2-6	TLSIETGFR
MS 2-7	GRYEETCIR
MS 2-8	ISMADYVSGELEHVTR
MS 2-9a	TLPKGYMEQIVESIR
MS 2-9b	LNVLGHLQQGGAPTPFDR
MS 2-10	LPKMECVQMTKEVQK
MS 2-11	TLPKGYMEQIVESIR
MS 2-12	IMEVIDAITTTAQSHQR
MS 2-13	IMEVIDAITTTAQSHQR
MS 2-14	IMEVIDAITTTAQSHQR
MS 2-15a	IMEVIDAITTTAQSHQR
MS 2-15b	VVHDGFEGLAKNQ
MS 2-16	IMEVIDAITTTAQSHQR

TABLE XXXVb

ALIGNMENT OF THE SHEEP LIVER PFK MS 2 MALEYL-TRYPTIC PEPTIDES WITH RABBIT MUSCLE AND SHEEP HEART MUSCLE PFK

Peptide name		Sequence Alignment
MS 2-5	SL	756 ISMADYVSGELEHVTR 771
	RM	DLDTSEHAH IS
	SH	DLDTSEHAH I
MS 2-6	SL	566 TLSIETGFR 574
	RM	VFI MGG
	SH	--- MGG
MS 2-7	SL	512 GRYEETCIR 520
	RM	KQFD L P
	SH	KQ D L P
MS 2-10	SL	345 LPKMECVQMTKEVQK 359
	RM	L V D T
	SH	L V D T
MS 2-15b	SL	434 VVHDGFEGLAKNQ 446
	RM	G
	SH	--

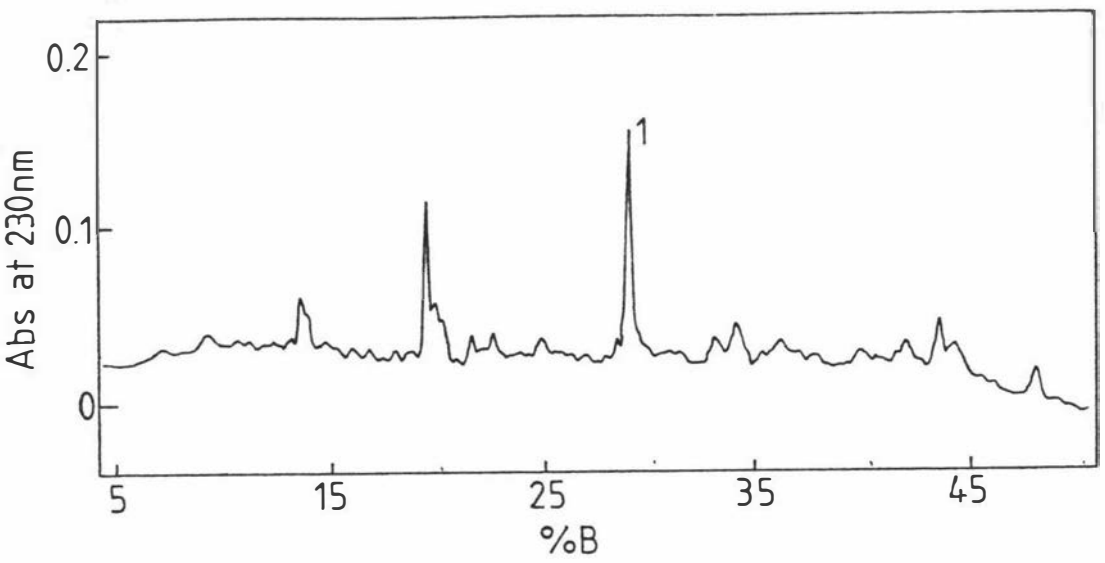


Figure 45: HPLC Elution Profile of Fraction MS 3 Chromatographed on a Vydac C-18 Column.

Labelled peaks are those for which sequence information is shown in Fig. XXXVIa. Peptides in Fraction MS 3 were dissolved in 0.1M Am Bic, 5% acetonitrile. Elution consisted of a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXXVIa

AMINO ACID SEQUENCES OF ACID-SOLUBLE MALEYL-TRYPTIC PEPTIDES FROM SHEEP LIVER PFK ISOLATED FROM FRACTION 3

Peptide	Sequence
MS 3-1	I SMADYVSGELEHVTR

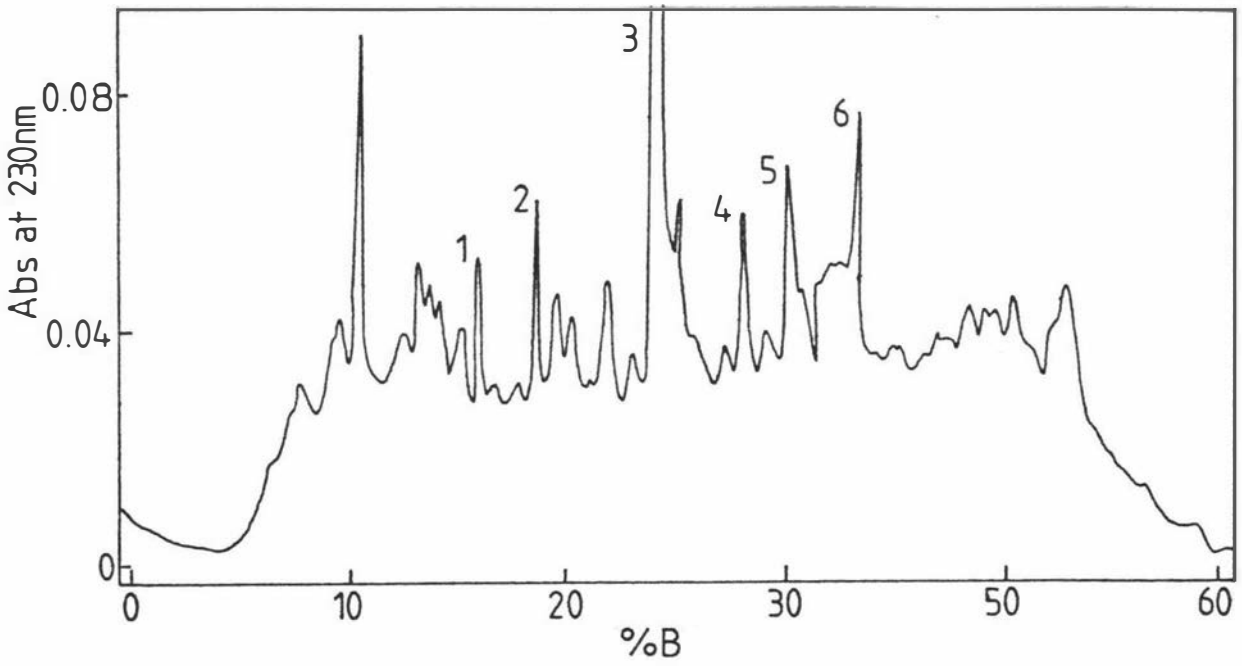


Figure 46: HPLC Elution Profile of Fraction MS 4 Chromatographed on a Vydac C-18 Column.

Labelled peaks are those for which sequence information is shown in Fig. XXXVIIa. Peptides in Fraction MS 4 were dissolved in 0.1M Am Bic, 5% acetonitrile. Elution consisted of a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXXVIIa

AMINO ACID SEQUENCES OF ACID-SOLUBLE MALEYL-TRYPTIC PEPTIDES FROM SHEEP LIVER PFK ISOLATED FROM FRACTION 4

Peptide	Sequence
MS 4-1	I SMADYVSGELEHVTR
MS 4-2	LGEDN I NVVEGNEQF I SASK
MS 4-3	SFENNWN I YK
MS 4-4	I SMADYVSGELEHVTR
MS 4-5	ALLFQPVTELQEQTDFEHR
MS 4-6	EQWWLNLR

TABLE XXXVIIb

ALIGNMENT OF THE SHEEP LIVER PFK MS 4 MALEYL-TRYPTIC PEPTIDES WITH RABBIT MUSCLE AND SHEEP HEART MUSCLE PFK

Peptide name		Sequence Alignment
MS 4-3	SL	376 SFENNWNIYK 385
	RM	M EV
	SH	M EV

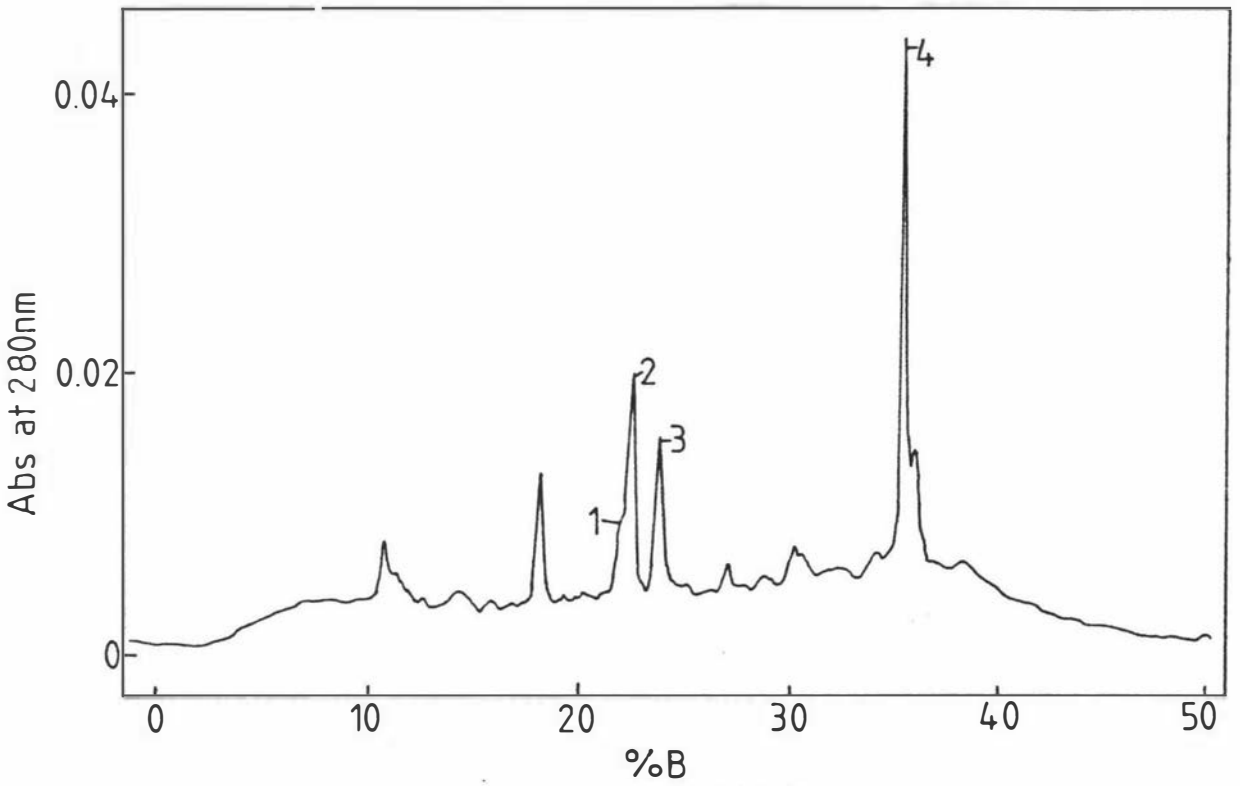


Figure 47: HPLC Elution Profile of Fraction MS 5 Chromatographed on a Vydac C-18 Column.

Labelled peaks are those for which sequence information is shown in Fig. XXXVIIIa. Peptides in Fraction MS 5 were dissolved in 0.1M Am Bic, 5% acetonitrile. Elution consisted of a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXXVIIIa

AMINO ACID SEQUENCES OF ACID-SOLUBLE MALEYL-TRYPTIC PEPTIDES FROM SHEEP LIVER PFK ISOLATED FROM FRACTION 5

Peptide	Sequence
MS 5-1a	LIYEGYEGLVEGGENIR
MS 5-1b	SFENNWNIYKLLAHQK
MS 5-2	LIYEGYEGLVEGGENIR
MS 5-3a	SEWGSLLEELVSEGKISEGTAQYR
MS 5-3b	SHLNIAGLVGSIDNDFCGTDMTIG
MS 5-4a	SHLNIAGLVGSIDNDFCGTDMTIG
MS 5-4b	SEWGSLLEELVSEGKISEGTAQYR

TABLE XXXVIIIb

ALIGNMENT OF THE SHEEP LIVER PFK MS 5 MALEYL-TRYPTIC PEPTIDES WITH RABBIT MUSCLE AND SHEEP HEART MUSCLE PFK

Peptide name		Sequence Alignment
MS 5-1a	SL	49 LIYEGYEGLVEGGENIR 65
	RM	FVH Q D DH
	SH	FVH Q D D
MS 5-1b	SL	376 SFENNWNIYKLLAHQK 391
	RM	M EV IR
	SH	M EV VK
MS 5-3a	SL	129 SEWGSLLEELVSEGKISEGTAQYR 152
	RM	SD SD QKA TAEEATRS
	SH	D SD QKS TAEEATKS
MS 5-3b	SL	153 SHLNIAGLVGSIDNDFCGTDMTIG 176
	RM	Y V
	SH	Y V----------

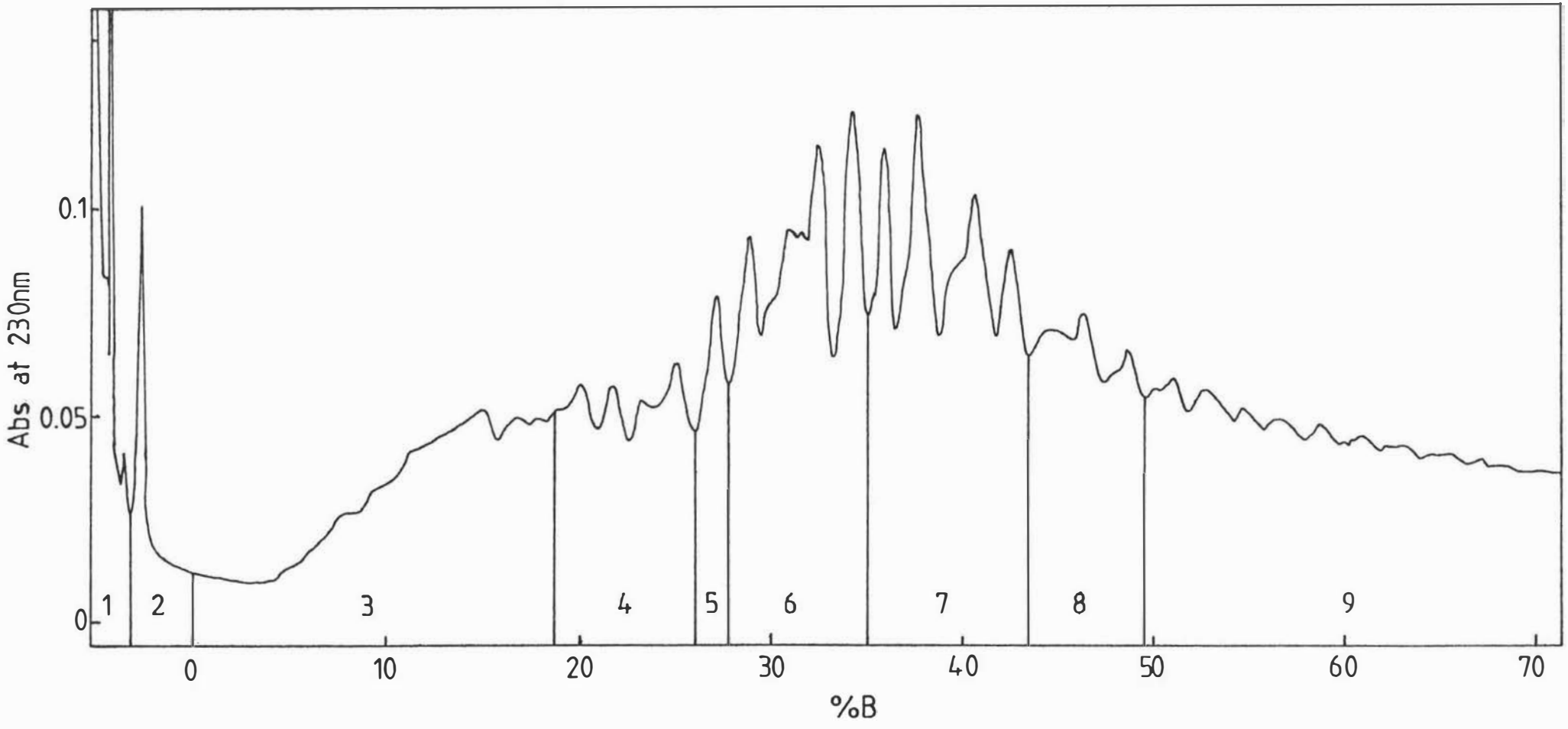
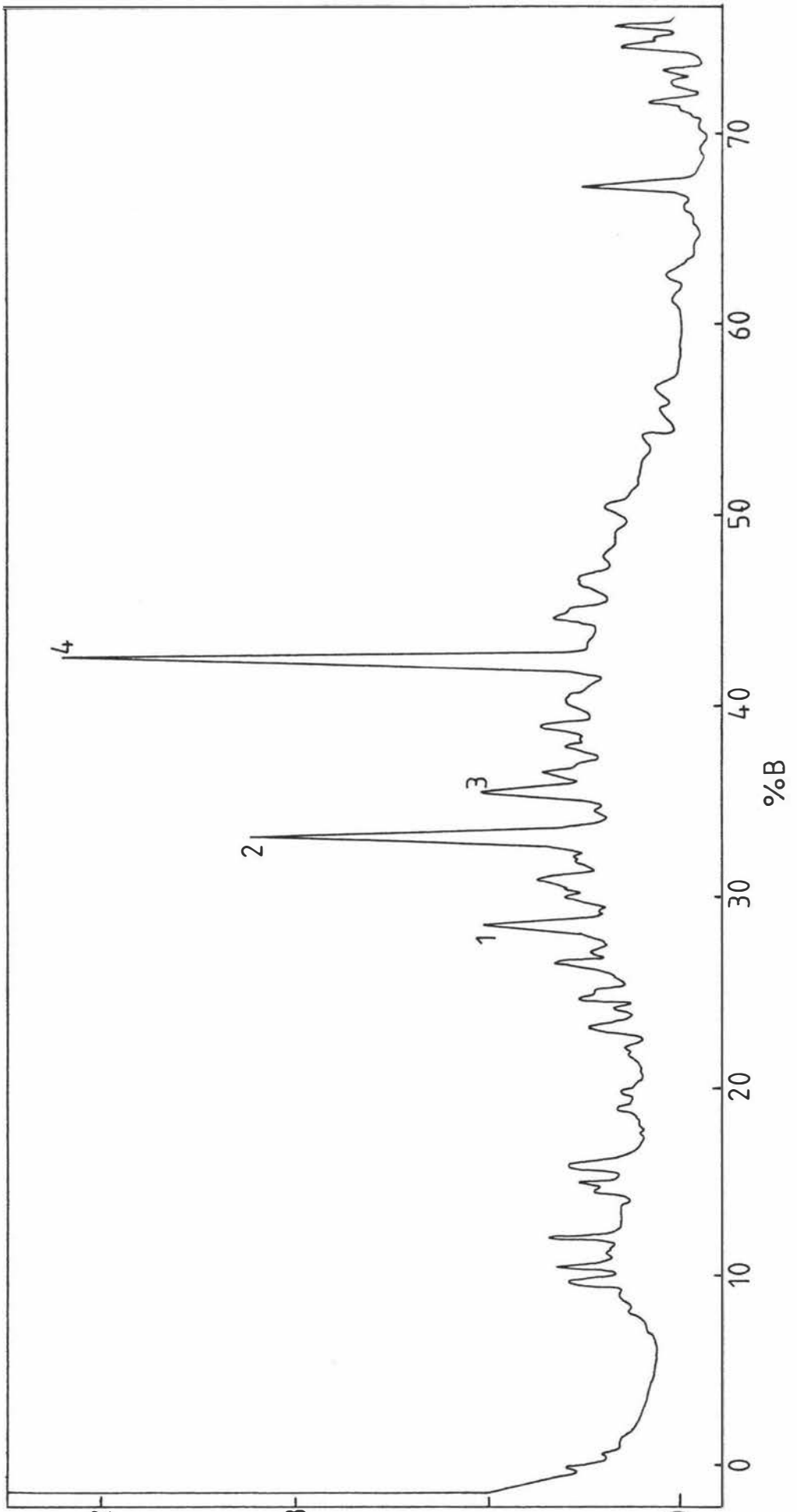


Figure 48: HPLC Elution Profile of the Acid-Insoluble Maleyl-Tryptic Peptides Chromatographed on a Mono-Q Ion-exchange Column.

The Acid-insoluble maleyl-tryptic peptides were dissolved in Buffer A (20mM Tris-HCl pH 8.0). Elution consisted of a linear gradient from 0-100% B (20mM Tris-HCl pH 8.0, 1M NaCl) run over 2hr at a rate of 0.5ml/min. The Fractions which underwent further purification by HPLC are labelled 1-9.


Abs at 230nm
0.12
0.08
0.04
0
0
10
20
30
40
50
60
70
%B
1
2
3
4

Opposing Page.

Figure 49: HPLC Elution Profile of Fraction MI 1 Chromatographed on a Vydac C-18 Column.

Labelled peaks are those for which sequence information is shown in Fig. XXXIXa. Peptides in Fraction MI 1 were dissolved in 0.1M Am Bic, 5% acetonitrile. Elution consisted of a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XXXIXa

AMINO ACID SEQUENCES OF ACID-INSOLUBLE MALEYL-TRYPTIC PEPTIDES FROM SHEEP LIVER PFK ISOLATED FROM FRACTION 1

Peptide	Sequence
MI 1-1	SFENNWN I YK
MI 1-2	A I LWMSEKLR
MI 1-3	TFVLEVMGR
MI 1-4	NQVQEVSWHDVAGWLGR

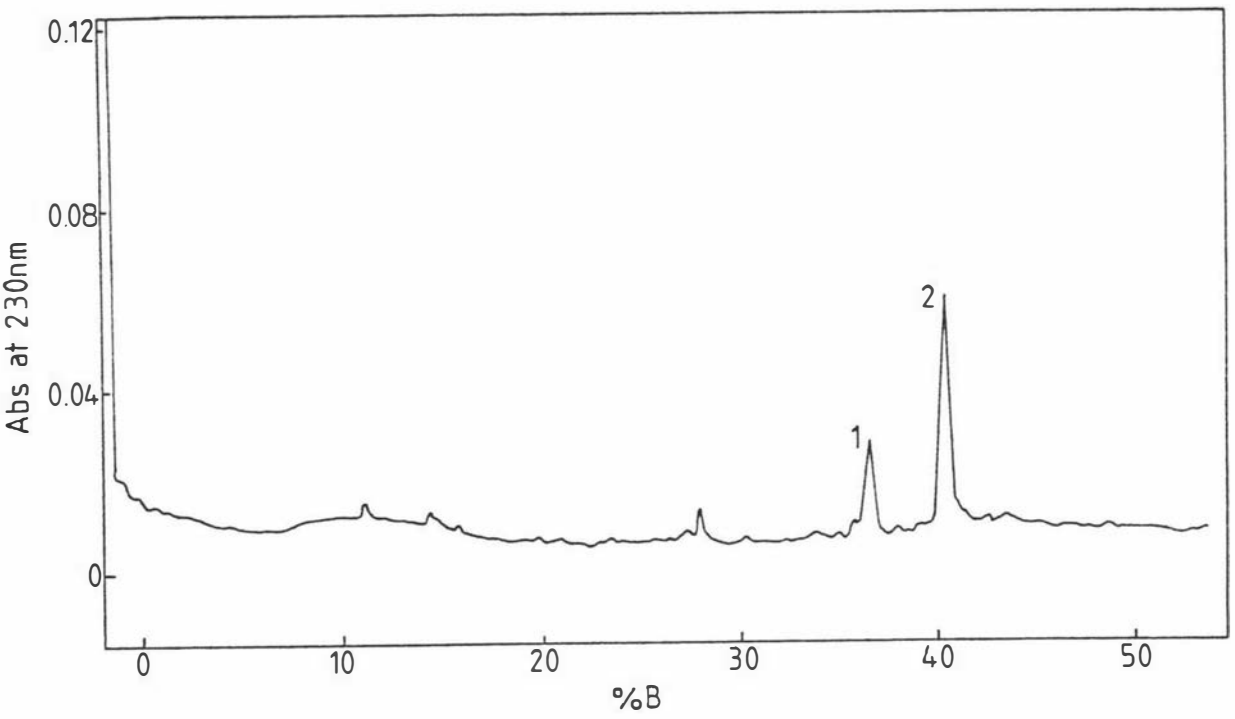


Figure 50: HPLC Elution Profile of Fraction MI 2 Chromatographed on a Vydac C-18 Column.

Labelled peaks are those for which sequence information is shown in Fig. XLa. Peptides in Fraction MI 2 were dissolved in 0.1M Am Bic, 5% acetonitrile. Elution consisted of a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XLa

AMINO ACID SEQUENCES OF ACID-INSOLUBLE MALEYL-TRYPTIC PEPTIDES FROM SHEEP LIVER PFK ISOLATED FROM FRACTION 2

Peptide	Sequence
MI 2-1	VFLIYEGYEGLVEGGENIR
MI 2-2	SEWGSLLEELVSEGKISEGTAQYR

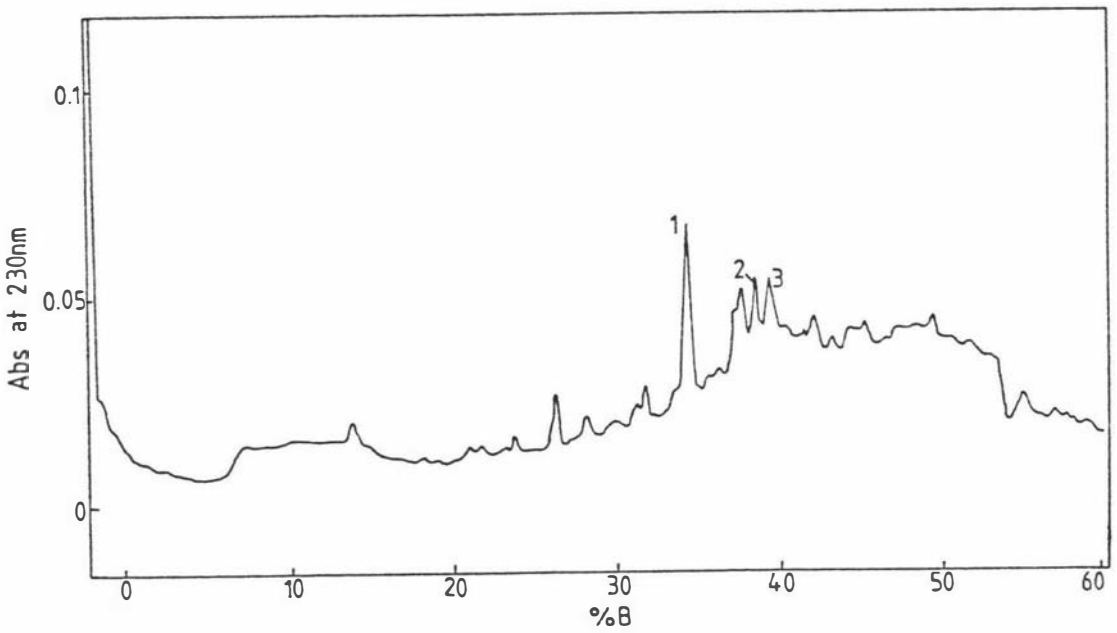


Figure 51: HPLC Elution Profile of Fraction MI 3 Chromatographed on a Vydac C-18 Column.

Labelled peaks are those for which sequence information is shown in Fig. XLIa. Peptides in Fraction MI 3 were dissolved in 0.1M Am Bic, 5% acetonitrile. Elution consisted of a linear gradient from 0-100% B (0.1M Am Bic/isopropanol/acetonitrile 1:1:1), run over 2hr at a rate of 1ml/min.

TABLE XLIa

AMINO ACID SEQUENCES OF ACID-INSOLUBLE MALEYL-TRYPTIC PEPTIDES FROM SHEEP LIVER PFK ISOLATED FROM FRACTION 3

Peptide	Sequence
MI 3-1	VFL I YEGYEGLVEGGEN I R
MI 3-2	SEWGSLLEELVSEGK I SEGTAQYR
MI 3-3a	SEWGSLLEELVSEGK I SEGTAQYR
MI 3-3b	MG I YVGAKVFL I YEGYEGLVEGGEN I R

TABLE XLIb

ALIGNMENT OF THE SHEEP LIVER PFK MI 3 MALEYL-TRYPTIC PEPTIDES WITH RABBIT MUSCLE AND SHEEP HEART MUSCLE PFK

Peptide name		Sequence alignment
MI 1-2	SL	682 AILWMSEKLR 690
	RM	MN AG IK
	SH	MN G IK
MI 1-4	SL	445 NQVQEVSWHDVAGWLGR 461
	RM	G IE AG SY G T Q
	SH	----------
MI 2-1	SL	47 VFLIYEGYEGLVEGGENIR 65
	RM	FVH Q D DH
	SH	FVH Q D DH
MI 3-3b	SL	39 MGIYVGAKVFLIYEGYEGLVEGGENIR 65
	RM	V FT R FVH Q D DH
	SH	V T R FVH Q D D

```
    1                                                            50
RM  THEEHHAARTLGVGKAIAVLTSGGDAQGMNAAVRAVVRVGIFTGARVFFV
SH  THEEHHEAKTLGIGKAIAVLTSGGDAQGMNAAVRAVVRVGIYTGARVFFV
              TSGAGKAIGVLTSGGDAQGMNAAVR     MGIYVGAKVFLI

    51                                                           100
    HEGYQGLVDGGDHIREATWESVSMMLQLGGTVIGSARCKDFREREGRLRA
    HEGYQGLVDGGDNIREATWES---------------CKDFREREGRLRA
    YEGYEGLVEGGENIR                          CKAFTTR     A

    101                                                          150
    AHNLVKRGITNLCVIGGDGSLTGADTFRSEWSDLLSDLQKAGKITAEEAT
    AHNLVK-GIGNLCVIGADGSLTGGDTFRSEWGDLLSDLQKSGKITAEEAT
    AHNLVKRGITNLCVIGGDASLTGANTFRSEWGSLLEELVSEGKISEGTAQ

    151                                                          200
    RSSYLNIVGLVGSIDNDFCGTDMTIGTDSALHRITEIVDAITTTAQSHQR
    KSSYLNIV--------------MTIGTDSAL------------------
    YRSHLNIAGLVGSIDNDFCGTDMTIG         IMEVIDAITTTAQSHQR

    201                                                          250
    TFVLEVMGRHCGYLALVTSLSCGADWVFIPECPPDDNWEDHLCRRLSETR
    ---------HCGYLALVTSLSCGADWVFIPQCPPDDNWEEHLCR-LSETR
    TFVLEVMGR

    251                                                          300
    TRGSRLNIIIVAEGAIDRNGKPITSEGVKDLVVRRLGYDTRVTVLGHVQR
    ILGSR----------------PITSEGVKDLVVKRLGYDTRVTVLGVEHR
                                 YVKDLVVQRLGFDTRVTVLGHVQR

    301                                                          350
    GGTPSAFDRILGSRMGVEAVMALLEGTPDTPACVVSLSGNQAVRLPLMEC
    GGTPSAFDRILGSRMGVEAVMALLEGTPQTPACVVSLSGNQAVRLPLMEC
    GGTPSAFDRILGSR                                   LPKMEC

    351                                                          400
    VQVTKDVTKAMDEKRFDEAMKLRGRSFMNNWEVYKLLAHIRPPAPKSGSY
    VQVTKDVTRAMDERRFDEAMKLRGRSFMNNWEVYKLLAHVRPPKSKSGLH
    VQMTKEVQK       FDEAIQLR  SFENNWNIYKLLAHQK
```

```
401                                                          450
TVAVMNVGAPAAGMNAAVRSTVRIGLIQGNRVLVVHDGFEGLAKGQIEEA
TVAVMNVGAPAAGMNAAVRSTVRIGLIQGNRVLVVHDGFEGLAK------
                                          VVHDGFEGLAKNQVQEV

451                                                          500
GWSYVGGWTGQGGSKLGSKRTLPKKSFEQISANITKFNIQGLVIIGGFEA
--------------------TLPK-SFEEITADI---------------
SWHDVAGWLGRGGSMLGTKRTLPKGYMEQIVESIR

501                                                          550
YTGGLELMEGRKQFDELCIPFVVIPATVSNNVPGSDFSVGADTALNTICT
--------EGRKQYDELCIPFVVIPATVSNNVPGSDFSVGADTALNTICM
            GRYEETCIR

551                                                          600
TCDRIKQSAAGTKRRVFIIETMGGYCGYLATMAGLAAGADAAYIFEEPFT
TCDRIK------------IETMGGYCGYLATMAGLAAGADAAYIFEEPFT
    IKQSAAGTVQRTLSIETGFR

601                                                          650
IRDLQANVEHLVQKMKTTVKRGLVLRNEKCNENYTTDFIFNLYSEEGKGI
VRDLQANVEHLVQ-MKTTVKRGLVLRDEKCNENYTTDFIFNLYSEEGKGI
                      GLVLR

651                                                          700
FDSRKNVLGHMQQGGSPTPFDRNFATKMGAKAMNWMAGKIKESYRNGRIF
FDSRKNVLGHMQQGGSPTPFDRNFATKMGAKAMNWMSGKIKESYRNGRIF
    LNVLGHLQQGGAPTPFDR          AILWMSEKLR        VF

701                                                          750
ANTPDSGCVLGMRKRALVFQPVTELQNQTDFEHRIPKEQWWLKLRPILKI
ANTPDSGCVLGM-KRALLFQPVTELQEQTDFEHRIPKEQWWLK-RPILK-
ANAPDSACVIGLQKKVVAFSPVTE               EQWWLNLR

751                          779
LAKYEIDLDTSEHAHLEHISRKRSGEATV
LAKYEIDLDTSEHAHLEHITRKRSGEADI
      ISMADYVSGELEHVTR
```

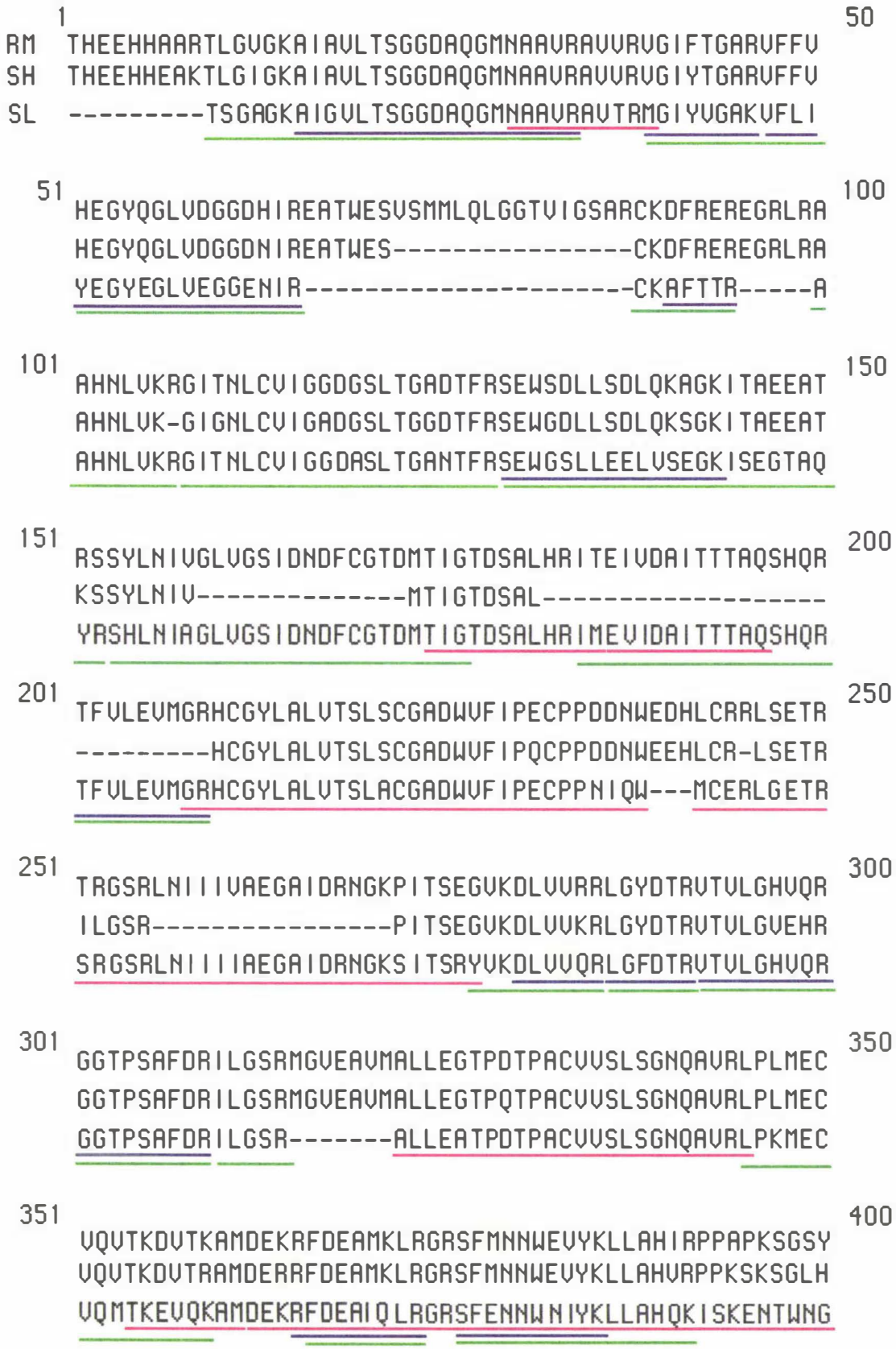
Figure 52: Sequence Obtained From Maleyl-Tryptic Peptides

Alignment of the maleyl-tryptic peptides from sheep liver PFK with the amino acid sequence of rabbit muscle and sheep heart muscle PFK.

Two maleyl-tryptic peptides (MS 1-1 and MS 1-3b), could not be aligned with the muscle sequence, however this does not rule out their existence in sheep liver PFK. Peptide MS 4-5 corresponded to a peptide from the contaminating PFK subunit. Generally such peptides were obtained in low yields and the corresponding peptides from liver PFK were obtained in greater amounts and also isolated from more than one digest. Peptide MS 4-2 was found to be analogous to a region of trypsin, so it is likely that the trypsin was undergoing auto-proteolytic activity. Despite the fact that the trypsin used had been TPCK treated to inactivate chymotryptic activity, it appears that some remained as evidenced by peptides MS 5-1a and MS 2-15b, where cleavage after phenylalanine and tyrosine residues respectively had occurred.

The extent of the maleylation of the lysine residues did not appear to be 100% since peptide MS 4-3 contained a C-terminal lysine residue, and peptide MI 1-4 has resulted from cleavage after a lysine residue. Both these peptides were obtained in low yields however.

The amino acid sequence of sheep liver PFK derived from the CNBr, tryptic and maleyl-tryptic digests and the sequence overlaps formed from these peptides are shown in Fig. 53.


1
50
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SH THEEHHEAKTLGIGKAIAVLTSGGDAQGMNAAVRAVVRVGIYTGARVFFV
SL ---------TSGAGKAIGVLTSGGDAQGMNAAVRAVTRMGIYVGAKVFLI
51
100
HEGYQGLVDGGDHIREATWESVSMMLQLGGTVIGSARCKDFREREGRLRA
HEGYQGLVDGGDNIREATWES----------CKDFREREGRLRA
YEGYEGLVEGGENIR----------CKAFTTR-----A
101
150
AHNLVKRGITNLCVIGGDGSLTGADTFRSEWSDLLSDLQKAGKITAEEAT
AHNLVK-GIGNLCVIGADGSLTGGDTFRSEWGDLLSDLQKSGKITAEEAT
AHNLVKRGITNLCVIGGDASLTGANTFRSEWGSLLEELVSEGKISEGTAQ
151
200
RSSYLNIVGLVGSIDNDFCGTDMTIGTDSALHRITEIVDAITTTAQSHQR
KSSYLNIV----------MTIGTDSAL----------
YRSHLNIAGLVGSIDNDFCGTDMTIGTDSALHRIMEVIDAITTTAQSHQR
201
250
TFVLEVMGRHCGYLALVTSLSCGADWVFIPECPPDDNWEDHLCRRLSETR
---------HCGYLALVTSLSCGADWVFIPQCPPDDNWEEHLCR-LSETR
TFVLEVMGRHCGYLALVTSLACGADWVFIPECPPNIQW---MCERLGETR
251
300
TRGSRLNIIIVAEGAIDRNGKPITSEGVKDLVVRRLGYDTRVTVLGHVQR
ILGSR----------PITSEGVKDLVVKRLGYDTRVTVLGVEHR
SRGSRLNIIIIAEGAIDRNGKSITSRYVKDLVVQRLGFDTRVTVLGHVQR
301
350
GGTPSAFDRILGSRMGVEAVMALLEGTPDTPACVVSLSGNQAVRLPLMEC
GGTPSAFDRILGSRMGVEAVMALLEGTPQTPACVVSLSGNQAVRLPLMEC
GGTPSAFDRILGSR-------ALLEATPDTPACVVSLSGNQAVRLPKMEC
351
400
VQVTKDVTKAMDEKRFDEAMKLRGRSFMNNWEVYKLLAHIRPPAPKSGSY
VQVTKDVTRAMDERRFDEAMKLRGRSFMNNWEVYKLLAHVRPPKSKSGLH
VQMTKEVQKAMDEKRFDEAIQLRGRSFENNWNIYKLLAHQKISKENTWNG

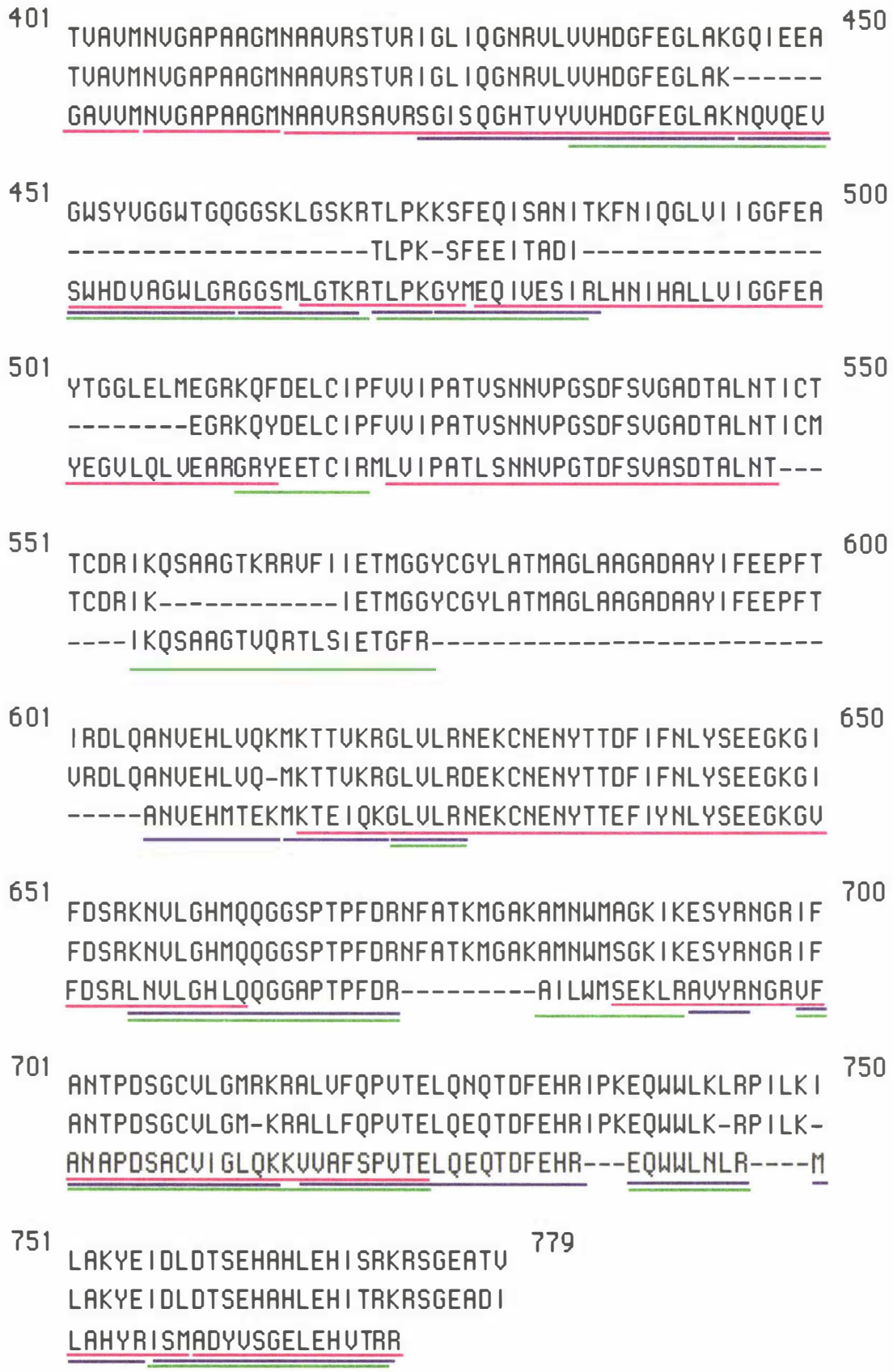


Figure 53: Amino Acid Sequence of Sheep Liver PFK Obtained from the Three Digestion Methods Used.

The determined amino acid sequence of sheep liver PFK, aligned with the rabbit muscle and sheep heart sequences. CNBr peptides are underlined in red, while tryptic peptides are underlined in blue and maleyl-tryptic peptides in green, indicating the occurrence of overlaps.

CHAPTER SEVEN

DISCUSSION

7.1 GENERAL DISCUSSION

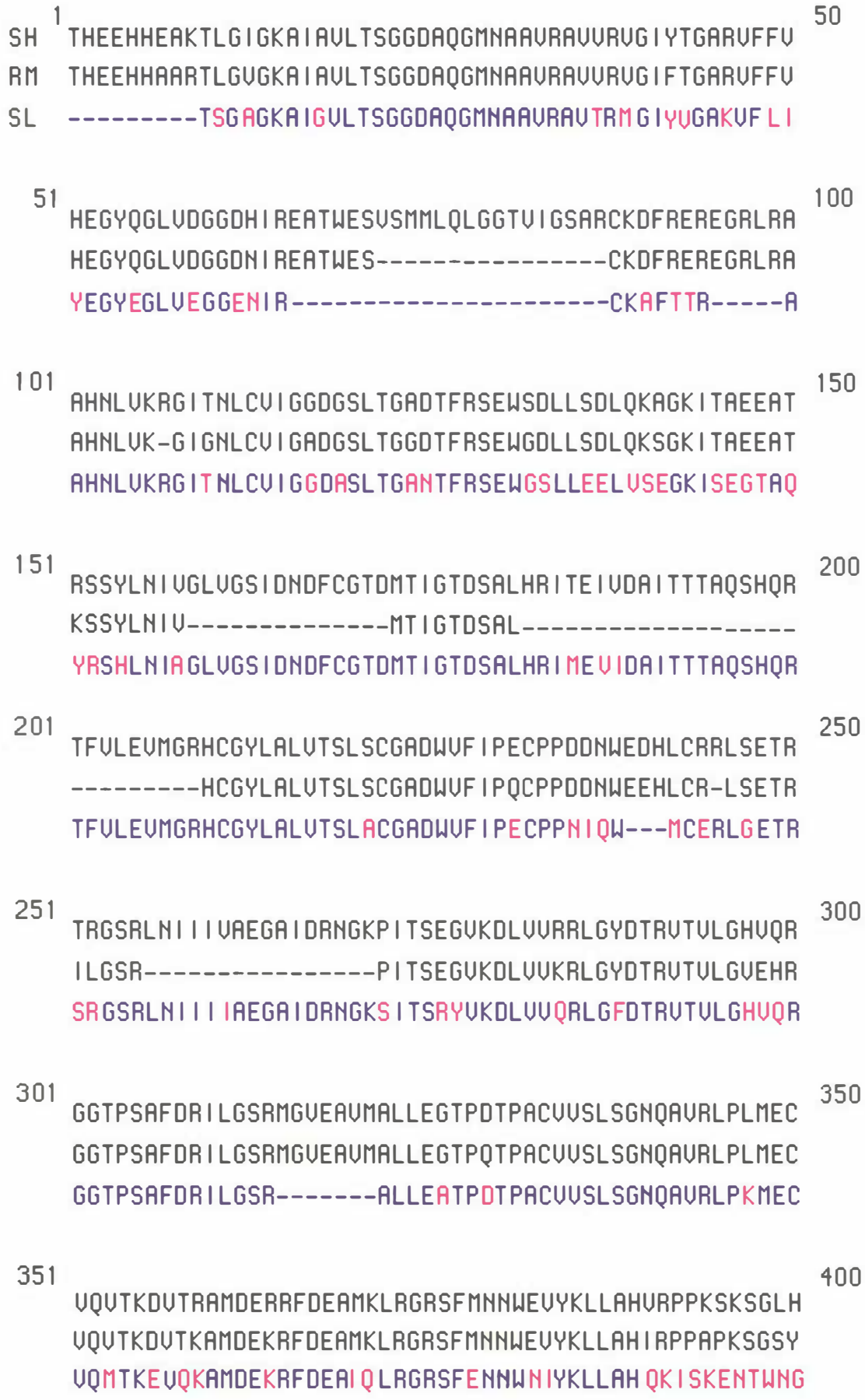
Molecular weight determinations showed that sheep liver PFK was of a slightly lower molecular weight than sheep heart muscle PFK. Comparison of amino acid compositions, and peptide maps all suggested that some amino acid differences existed between the isozymes.

A study of the isozyme composition of sheep liver PFK indicated that although the L_4 species was probably the predominant form, at least some hybrid species, most likely composed of liver and platelet subunits exist.

87% of the amino acid sequence of sheep liver PFK has been determined in this study. Fig. 54 shows the amino acid sequence of sheep liver PFK aligned with the sequences of rabbit muscle and sheep heart PFK. Residues which are different in the sheep liver sequence as compared to muscle PFKs are shown in red, while those which are identical are shown in blue.

A table showing the percentage of each amino acid which has been sequenced compared to that obtained from the amino acid composition is shown in Table XLII. Most of the values are of a similar proportion to the amount of sequence obtained. Exceptions to this are threonine, isoleucine and histidine, for which all the expected residues have been determined, and proline for which only 57% of the expected residues have sequenced. This may reflect the distribution of these residues in sheep liver PFK.

Rabbit muscle and sheep heart muscle PFK both contain three histidine residues in the first nine residues of the N-terminal segment. This region has not been sequenced from sheep liver PFK, however since it appears that all the histidine residues present in the sheep liver PFK molecule have been sequenced, it may indicate that these three histidine residues are absent from the N-terminal segment of sheep liver PFK.


SH 1 THEEHHEAKTLGIGKAIAVLTSGGDAQGMNAAVRAVVRVGIYTGARVFFV 50
RM THEEHHAARTLGVGKAIAVLTSGGDAQGMNAAVRAVVRVGIFTGARVFFV
SL ---------TSGAGKAIGVLTSGGDAQGMNAAVRAVTRMGIYVGAKVFLI
51 HEGYQGLVDGGDHIREATWESVSMMLQLGGTVIGSARCKDFREREGRLRA 100
HEGYQGLVDGGDNIREATWES----------CKDFREREGRLRA
YEGYEGLVEGGENIR----------CKAFTTR-----A
101 AHNLVKRGITNLCVIGGDGSLTGADTFRSEWSDLLSDLQKAGKITAEEAT 150
AHNLVK-GIGNLCVIGADGSLTGGDTFRSEWGDLLSDLQKSGKITAEEAT
AHNLVKRGITNLCVIGGDASLTGANTFRSEWGSLLEELVSEGKISEGTAQ
151 RSSYLNIVGLVGSIDNDFCGTDMTIGTDSALHRITEIVDAITTTAQSHQR 200
KSSYLNIV----------MTIGTDSAL----------
YRSHLNIAGLVGSIDNDFCGTDMTIGTDSALHRIMEVIDAITTTAQSHQR
201 TFVLEVMGRHCGYLALVTSLSCGADWVFIPECPPDDNWEDHLCRRLSETR 250
---------HCGYLALVTSLSCGADWVFIPQCPPDDNWEEHLCR-LSETR
TFVLEVMGRHCGYLALVTSLACGADWVFIPECPPNIQW---MCERLGETR
251 TRGSRLNIIIVAEGAIDRNGKPITSEGVKDLVVRRLGYDTRVTVLGHVQR 300
ILGSR----------PITSEGVKDLVVKRLGYDTRVTVLGVEHR
SRGSRLNIIIIAEGAIDRNGKSITSRYVKDLVVQRLGFDTRVTVLGHVQR
301 GGTPSAFDRILGSRMGVEAVMALLEGTPDTPACVVSLSGNQAVRLPLMEC 350
GGTPSAFDRILGSRMGVEAVMALLEGTPQTPACVVSLSGNQAVRLPLMEC
GGTPSAFDRILGSR-------ALLEATPDTPACVVSLSGNQAVRLPKMEC
351 VQVTKDVTRAMDERRFDEAMKLRGRSFMNNWEVYKLLAHVRPPKSKSGLH 400
VQVTKDVTKAMDEKRFDEAMKLRGRSFMNNWEVYKLLAHIRPPAPKSGSY
VQMTKEVQKAMDEKRFDEAIQLRGRSFENNWNIYKLLAHQKISKENTWNG

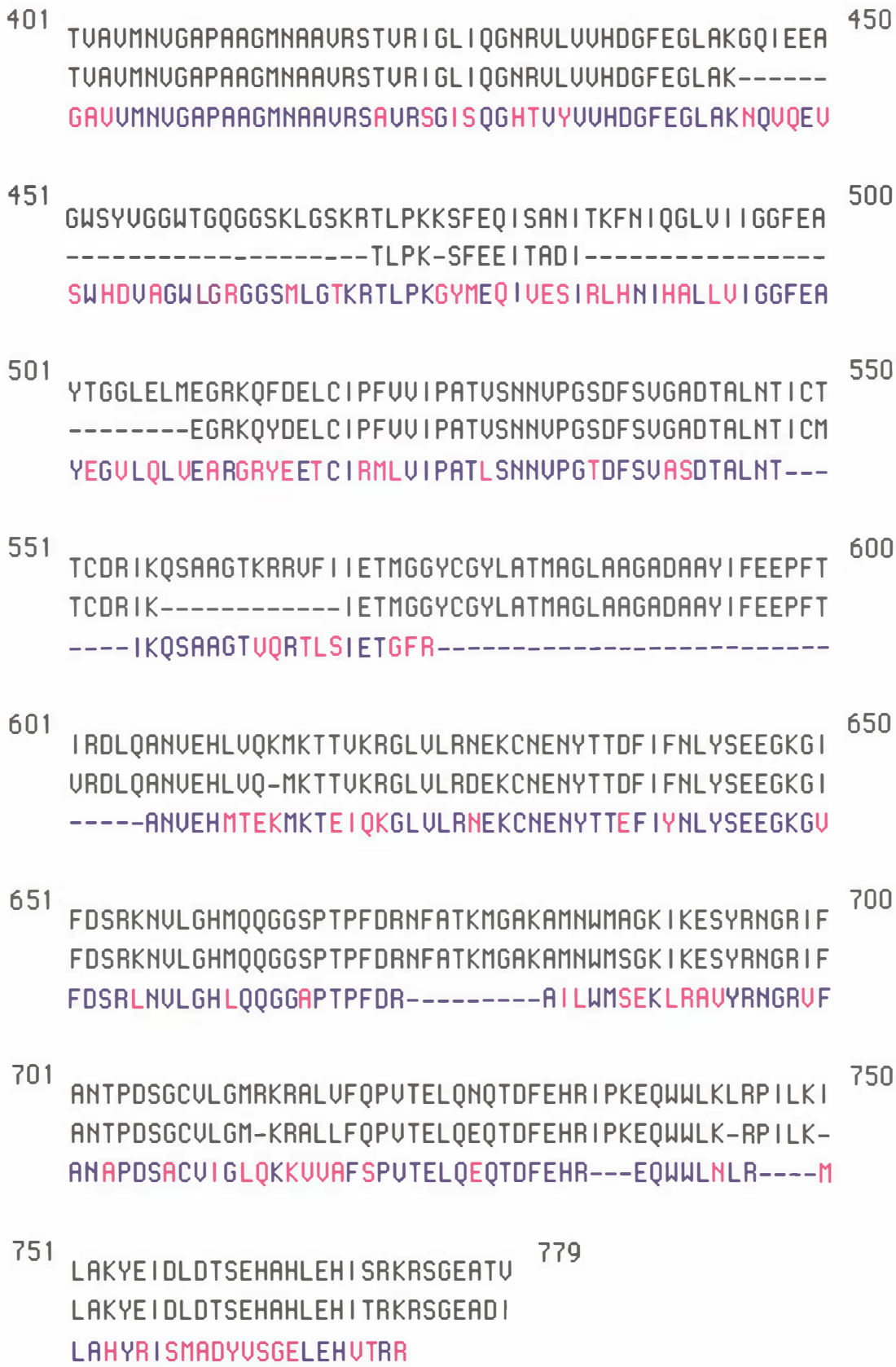


Figure 54: Amino Acid Sequence of Sheep Liver PFK

Residues which are different in the sheep liver sequence compared to the muscle PFKs are shown in red, while those which are identical are shown in blue. Hyphens represent residues yet to be determined.

TABLE XLII

NUMBER OF EACH AMINO ACID RESIDUE SEQUENCED COMPARED TO THE AMINO ACID COMPOSITION

Residue	Number sequenced	% of amino acid composition
Lysine	27	75
Histidine	17	100
Arginine	46	87
Aspartic acid	56	84
Threonine	43	100
Serine	40	85
Glutamic acid	73	90
Proline	16	57
Glycine	67	86
Alanine	57	92
Valine	57	92
Methionine	20	95
Isoleucine	40	100
Leucine	56	86
Tyrosine	17	77
Phenylalanine	21	81

To date, PFK sequences from E. coli (Hellinga and Evans, 1985), B. stearothermophilus (Kolb et al., 1980), rabbit muscle (Poorman et al., 1984; Lee et al., 1987) and human muscle (Nakajima et al., 1987) have been published. The sequences of two peptides from human liver PFK have also been reported in the literature (Levanon et al., 1987). In this laboratory, approximately 80% of the sheep heart muscle PFK sequence has been determined, along with a small number of peptides from chicken muscle PFK (Green and Midwinter, personal communication), while this project reports 87% of the amino acid sequence of sheep liver PFK.

The sequence of peptide CNBr G-2 suggests that the sheep liver enzyme isolated in this study may be 7 residues shorter than the muscle enzymes at the C-terminal end. An alternative to this is that this region of the enzyme is very susceptible to proteolysis, and 100% cleavage of the C-terminal fragment has occurred, due to the presence of a protease which is not inactivated by PMSF.

7.2 SEQUENCE AND STRUCTURAL HOMOLOGY WITH BACTERIAL PHOSPHOFRUCTOKINASE

Early speculation that mammalian PFKs have evolved by duplication of a prokaryotic gene (Paetkau et al., 1968; Coffee et al., 1973; Emerk and Frieden, 1974), has been supported by the high degree of sequence homology which has been found to exist between the amino acid sequence of Bacillus stearothermophilus and rabbit muscle PFK (Poorman et al., 1984; Lee et al., 1987). Comparison of the sequence of the N-terminal half of rabbit muscle PFK with Bs PFK shows 44% sequence homology (Poorman et al., 1984), while the C-terminal half displays only 34% homology. Comparison between the two halves of rabbit muscle PFK shows 32% sequence homology (Poorman et al., 1984), and this is similar to the values of 35-40% homology reported between the two halves of the transferrin molecule for this species, for which gene duplication of an ancestral gene has been proposed. Therefore, it is feasible that the mammalian PFK subunit has arisen by gene duplication of an ancestral gene related to that of Bs PFK, followed by fusion and mutation. With the N-terminal of the muscle PFK being constrained to retain the catalytic properties of PFK.while the C-terminal half, left freer to mutate, has developed the additional ATP and citrate inhibitory sites and the fructose bisphosphate activator site (Poorman et al., 1984).

Proteins which serve the same function in all organisms frequently exhibit strong homology in tertiary structure, but similarities at the level of the primary structure are not always apparent, for example, NADH-dependent dehydrogenases (Rossman et al.,

1975). Distantly related proteins such as papain and actinidin which exhibit approximately 48% sequence homology also exhibit a similar tertiary structure (Kamphuis et al., 1985), with sites of insertions or deletions in these proteins corresponding to surface loops, with the secondary structure elements showing strong conservation.

Since the degree of homology between Bs PFK and rabbit muscle PFK is similar to that observed between related proteins which have the same tertiary structure, it seems plausible that provided insertions can be accommodated in surface loops, and no disruption of the secondary structural elements due to sequence changes occurs (Poorman et al., 1984), the N and C halves of rabbit muscle PFK and the Bs PFK subunit will share a similar conformation (Fig.55 and 56).

Alignment of the sheep liver PFK sequence with that of Bs PFK results in an alignment which in the majority is the same as that proposed for rabbit muscle PFK (Poorman et al., 1984). The exceptions to this are :

1) A proposed 7 residue insert between residues 104 and 105 (based on the Bs numbering system) in the C-terminal half of rabbit muscle PFK has been suggested to be situated in the helix α-5 (Poorman et al., 1984). However in sheep liver PFK an insert of the same length but moved 6 residues towards the C-terminal is more likely, since it could be better accommodated in the loop between α-5 and β-E.

2) The proposed deletion at position 271 (Bs) which is postulated to be in α-11 in the C-terminal half of rabbit muscle PFK (Poorman et al., 1984), would result in greater sequence homology in the sheep liver enzyme if the deletion were at 273 (Bs).

3) The proposed 1 residue insert between residues 287 and 288 (Bs), in both the N and C-terminal halves of rabbit muscle PFK, which is postulated to be located in the loop between β-J and β-K (Poorman et al., 1984), would result in greater sequence homology if the insert was between residues 284 and 285 (Bs) which is located within β-J. This alignment was confirmed using the Genbank data base and applying the Gap computer program (Devereux et al., 1984).

(4) The proposed 3 residue insert between residues 215 and 216 (Bs) in both the N and C-terminal halves of the rabbit muscle enzyme, which is postulated to be within the loop between α-8 and β-H (Poorman et al., 1984), would result in greater sequence

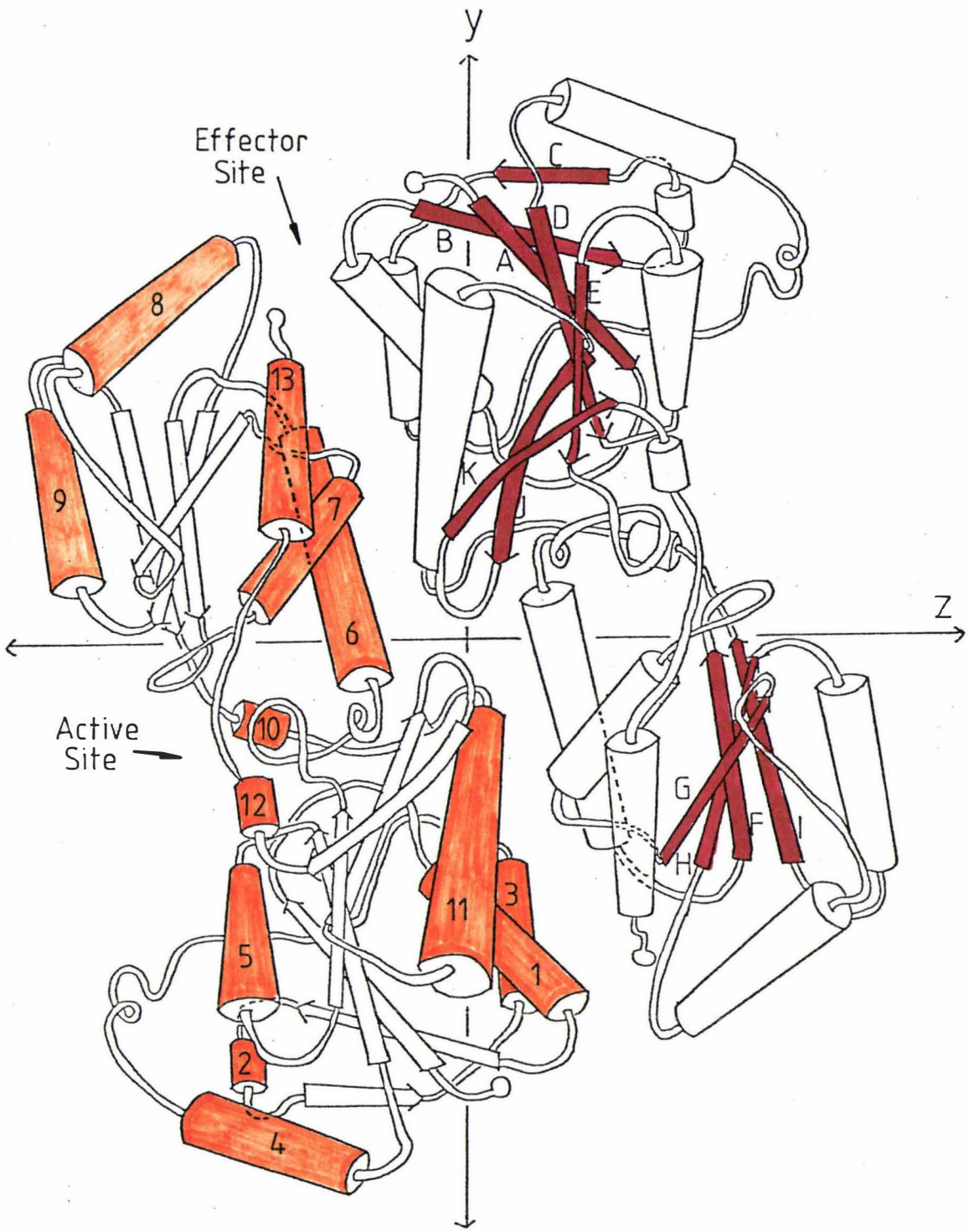
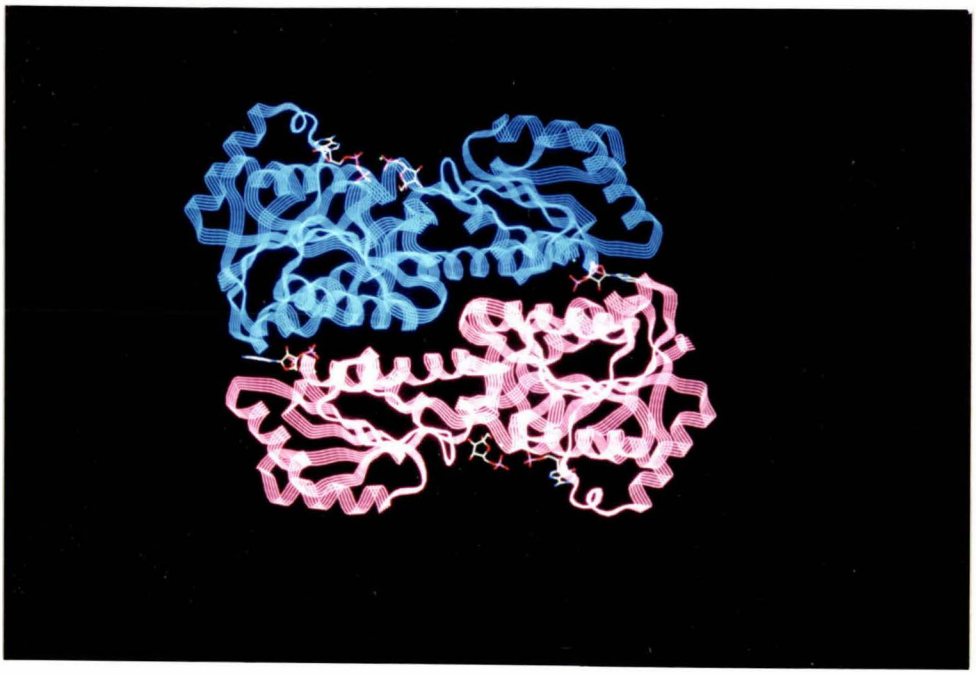


Figure 55: Schematic View of Two Subunits in the Bs-PFK Tetramer, Viewed Along the x-axis.

Numbers 1-13 represent α-helices, while letters A-K represent β-sheet.
From Evans and Hudson, (1979).

A



B

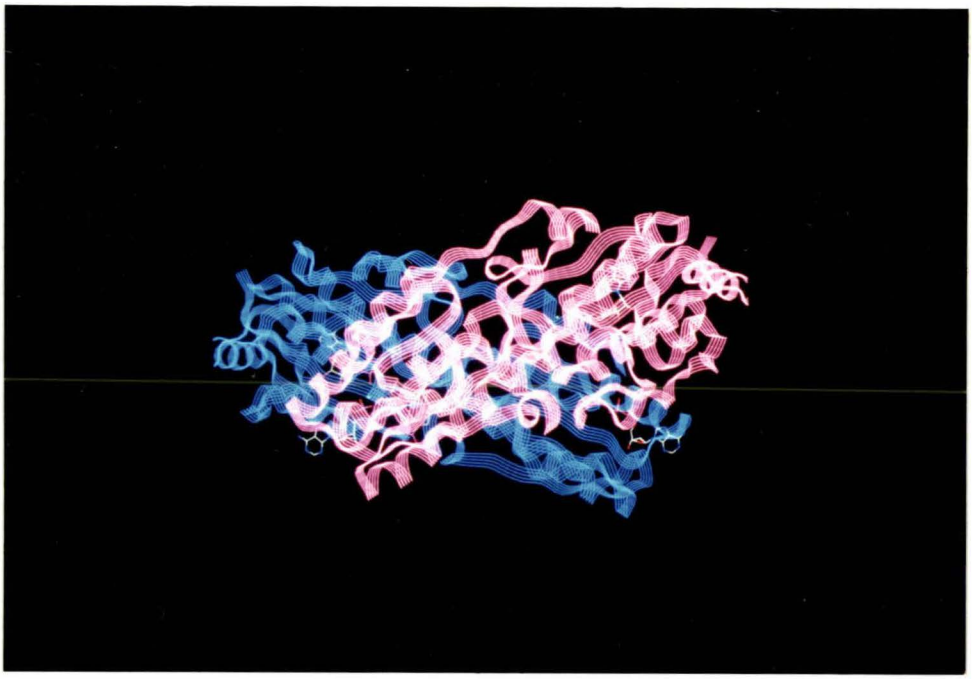


Figure 56: Computer Graphic View of Two Subunits of E. coli PFK Viewed along the x-axis (A), and y-axis (B), from the Computer Program FRODO.

The substrates and activators are shown, and can be identified by reference to Fig. 55.

homology if the insert were between residues 213 and 214 (Bs) which would still lie within the same loop.

The positions of all of the inserts in liver PFK and their proposed positions in the elements of secondary structure of the Bs PFK are shown in Table XLIII. Most of the inserts (Table XLIII) are external with the two exceptions being the 1 residue inserted in β-F and β-J. Most of the inserts are in regions of the sequence which are distant from all the binding sites. There are however 4 exceptions; 1) The extensions of the sequence on the end of the C-terminus, which are near the proposed citrate and ATP inhibitory sites. 2) The loop between α-9 and β-I in both the N-terminal and the C-terminal halves of the molecule, which may affect the active and the fructose bisphosphate binding sites respectively. 3) The extension which joins the two halves of the molecule ("hinge"), and 4) the insertion between α-8 and β-H which may affect binding at the ADP effector site. These combined insertions may in fact lead to the different allosteric properties between prokaryotic and eukaryotic PFKs.

Following alignment of the muscle and liver PFK sequences with the Bs enzyme, it was possible to superimpose the regions of secondary structure onto the mammalian enzyme. Using the Chou and Fasman (1974) model for the prediction of secondary structure, it was possible to check if any amino acid residue changes in the mammalian enzymes compared to the Bs enzyme, were likely to cause disruption of any of the secondary structural elements.

Consideration of the areas proposed to form β-sheet, showed that residue changes in these areas were unlikely to cause disruption of the structure. Likewise for the majority of the α-helices, disruption will probably not occur. Exceptions to this are α-3 in the C-terminal half of the rabbit muscle enzyme, in which two glycine residues occur at the C-terminal end of the proposed helix, and may result in a slightly shorter helix. However, in the liver enzyme, only one glycine is present, so disruption is less likely to occur. There are 2 glycine residues present in the region of the sequence which is proposed to contain the helix α-5 in the C-terminal half, however, due to the strong helix forming residues around these 2 glycine residues, the helix will most likely remain intact.

Secondary structure predictions were also carried out on the regions of the mammalian PFK deemed to be inserts, which were of at least 5 residues in length. Of these inserts, only the N-terminal, the loop between α-5 and β-E of the N-terminal half, the "hinge", and the C-terminal regions exhibited a likelihood of forming secondary structural

TABLE XLIII

LOCATION OF THE INSERTED AMINO ACID SEQUENCES IN SHEEP LIVER PFK COMPARED TO Bs PFK

Position of Insert Relative to 2° structure	Size (No of residues)	Effects/Conformation
N-Terminal Half of Enzyme		
N-terminal segment	12	Exterior secondary structure
Between α-2 and β-C	2	Exterior loop
Between α-5 and β-E	23	Exterior secondary structure
Between α-8 and β-H	3	Exterior loop
Between β-H and α-9	7	Exterior loop
Within β-J	1	Interior β-sheet
Hinge segment	32	Exterior secondary structure
C-Terminal Half of the Enzyme		
Between α-5 and β-E	7	Exterior loop
Within β-F	1	Interior
Between α-8 and β-H	3	Exterior loop
Between β-H and α-9	6	Exterior loop
Between α-9 and β-I	1	Exterior loop
Between α-11 and β-J	16	Exterior loop
Within β-J	1	Interior
C-terminal segment	28	Exterior secondary structure

elements. The N-terminal "insert" segment of muscle PFK, which forms an extension on the N-terminal of the Bs PFK, consisted of 12 residues, included 7 helix forming residues in a row, which suggests that an α–helical structure may be adopted by this segment. The insert in the loop between the α-5 and β-E also exhibits a large number of helix forming residues. In this case, either two small, or one longer helix is likely to form. The "hinge" region also contains a segment likely to form an α-helical stucture,as 6-7 helix forming residues were present, while the C-terminal extension may form either, two α-helices of 7-9 residues joined by a short segment of β-sheet or, one long helix.

It is likely that a mammalian PFK monomer is similar to two Bs monomers that are joined by an α-helical hinge region, having α-helical extensions on both the N and C-terminal ends of the protein (Fig. 57), since a) the rabbit muscle and sheep liver PFKs each exhibit approximately 30-40% sequence homology with the Bs PFK. b) The majority of the inserts in the mammalian enzyme are external. c) It is unlikely that the amino acid differences between the mammalian and Bs PFKs will disrupt any secondary structure elements.

Based on the known D2 symmetry of the mammalian PFK tetramer (Foe and Trujillo, 1980), and results from limited proteolysis studies in which the N-terminal of each subunit was susceptible to proteolysis, while the intersubunit forces maintaining the tetramer remained intact (Gottschalk et al., 1983), Poorman et al. (1984), proposed that the rabbit muscle PFK dimers must be brought together along the y-axis with all the connecting peptides on the inner faces (Fig. 58).

Based on this model of mammalian PFK it should be possible to compare changes in the primary structures of the mammalian PFKs, and relate them to positions in the tertiary structure, based on the Bs structure. Therefore it should be possible to provide some insight into the roles of amino acid residue changes, in leading to the different kinetic properties of muscle and liver PFK.

Crystallographic studies of Bs PFK have revealed the regions for the binding of the substrates F6P and ATP, and the allosteric effector site for ADP, as well as the residues which are important for ligand binding (Evans and Hudson, 1979). Hellinga and Evans (1985), have prepared a table showing the proposed function of the ligand binding residues in the Bs enzyme, and the analogous residues in both the rabbit muscle and the E. coli PFK, thus indicating how they are conserved or changed in the three PFK molecules.

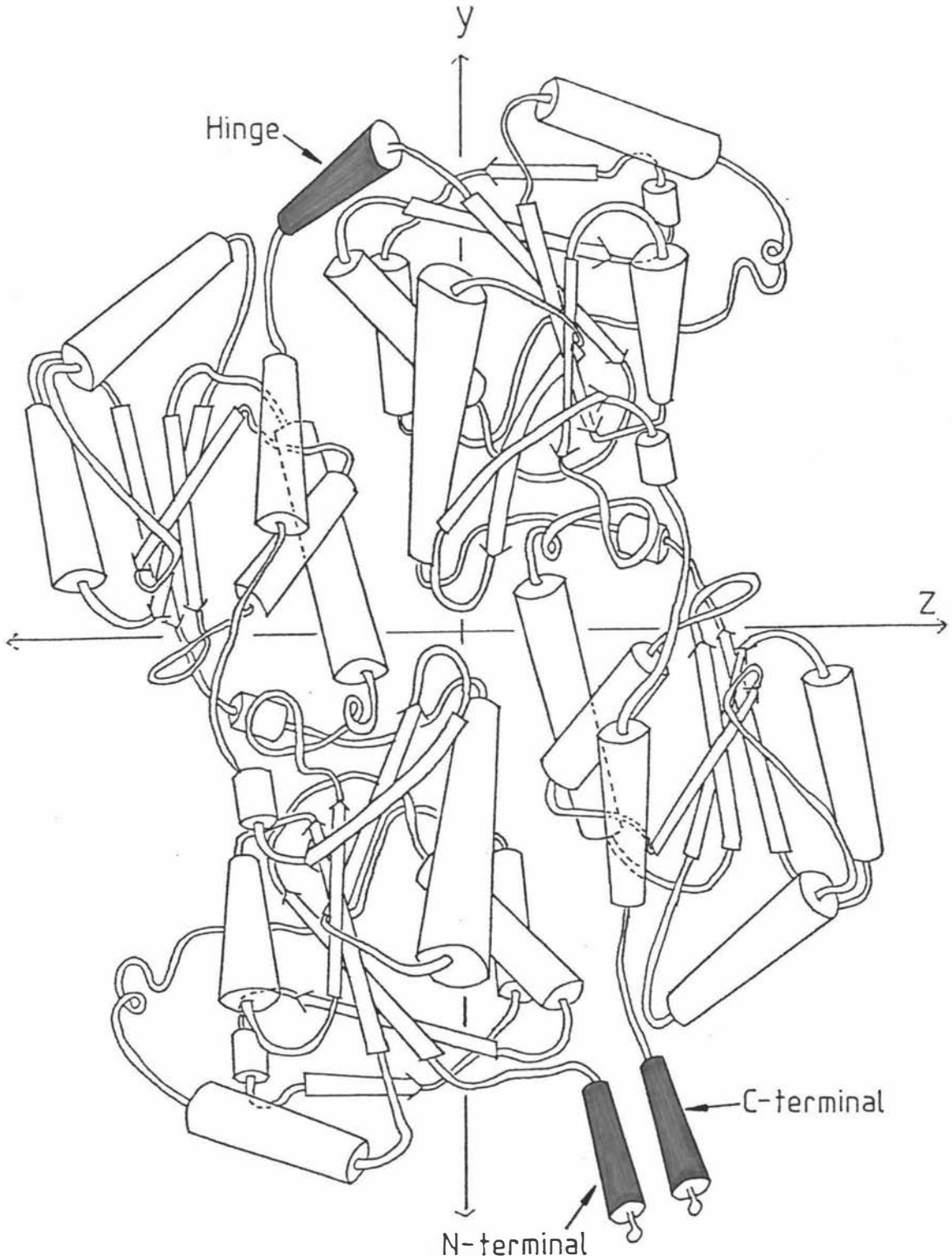


Figure 57: Schematic Diagram of the Proposed Tertiary Structure of a Mammalian PFK Monomer.

It is likely that a mammalian PFK monomer is similar to two Bs monomers joined by an α-helical region, with an α-helical extension on both the N and C-terminal ends of the protein.

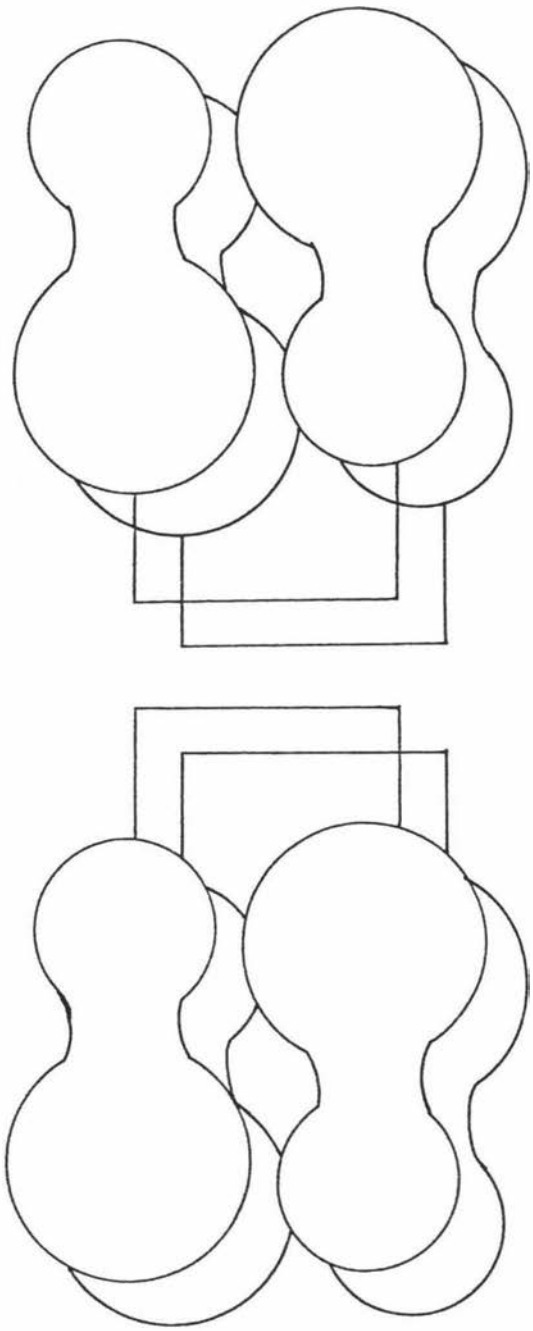


Figure 58: Schematic Diagram of the Proposed Mammalian PFK Tetramer

It has been proposed that the mammalian PFK dimers are brought together along the y-axis with the hinge regions on the inner faces (Poorman et al., 1984).

The ligand-binding residues in the N-terminal half of rabbit muscle PFK are more conserved than those in the C-terminal half. This is consistent with the N-terminal half being more homologous to the Bs enzyme and retaining the important catalytic site. The C-terminal half has lost the catalytic residues from its active site, but has retained most of the F6P binding residues, suggesting this site has lost its catalytic function, and has adapted to become a fructose bisphosphate binding site (Poorman et al., 1984; Hellinga and Evans, 1985).

The ADP effector site residues are less well conserved between the Bs and the rabbit muscle enzymes. This probably reflects the different allosteric effects that ADP has on the prokaryote and eukaryote enzyme.

Table XLIV shows the percentage sequence homology between the N and C-terminal halves of the four mammalian PFKs compared with the Bs enzyme. In all cases the N-terminal half exhibits greater sequence homology with the Bs PFK than does the C-terminal half. This is also consistent with the C-terminal half mutating at a faster rate than the N-terminal half.

7.3 SEQUENCE HOMOLOGY BETWEEN MAMMALIAN PHOSPHOFRUCTOKINASES

The sequence homology between the three muscle PFKs (Table XLV) is very high at 94-96%. This suggests a slow rate of evolution for PFK as has been found for the other enzymes of the glycolytic pathway (Fothergill-Gilmore, 1987). The sequence homology between sheep liver PFK and each of the three muscle PFKs is somewhat lower at 72-73%. These values are similar to those reported for another glycolytic enzyme pyruvate kinase (PK), where a comparison of cat and chicken muscle PK showed approximately 90% sequence homology, while the muscle and rat liver isozymes exhibited 70% homology (Muirhead et al., 1986). Comparison of the amino acid sequences of rabbit muscle and human liver phosphorylase, an enzyme slightly larger than PFK, showed 80% sequence homology (Rath et al., 1987).

The amino acid sequence of rat liver PFK-2/F26BPase displays no sequence homology with either the bacterial or mammalian PFKs. (Lively et al., 1988).

As with PK (Muirhead et al., 1986), the amino acid changes between the muscle and liver isozymes of PFK are not randomly distributed throughout the molecule. By dividing the PFK molecule into two halves, and excluding the "hinge" region, it can be

TABLE XLIV

SEQUENCE HOMOLOGY BETWEEN THE N AND C-TERMINAL HALVES OF MAMMALIAN PFKs COMPARED TO *Bs* PFK

	N-terminal half	C-terminal half
Rabbit muscle	44%	34%
Human muscle	42%	35%
Sheep heart muscle	39%	35%
Sheep liver	46%	33%

TABLE XLV

SEQUENCE HOMOLOGY BETWEEN MAMMALIAN PFKs

	Rabbit muscle	Human muscle	Sheep heart
Human muscle	96%	-	-
Sheep heart	94%	94%	-
Sheep liver	73%	72%	73%

seen that the degree of homology of the N-terminal half (76-82%) is at least 5% higher than for the C-terminal half (66-71%). This is in contrast to the comparison of the three muscle PFKs which exhibit a slightly higher degree of homology (2%) in the C-terminal half compared to the N-terminal half. This points towards the liver enzyme, particularly the C-terminal half, evolving at a faster rate than the muscle enzyme. It appears to be a general phenomenon that liver isozymes evolve at a faster rate than their muscle counterparts (Fothergill-Gilmore, 1987).

Many of the amino acid changes are probably functionally neutral, or result in effects which are too subtle to interpret. However there are three areas in sheep liver PFK which show a large number of sequence differences compared to the muscle enzymes (Fig. 54). These lie in regions regarded as inserts compared to the bacterial structure, and as such lie on the exterior of the protein. These areas are; the loop between α-5 and β-E, the "hinge" and the C-terminal. It is interesting to note that despite the lack of sequence homology between muscle and liver PFK in these regions each were still predicted to form secondary structure elements.

The amino acid differences between sheep liver PFK and the three muscle PFKs were categorized as conservative or strictly conservative changes based on the groupings of amino acids by Rath et al. (1987) (conservative amino acid residue changes (G, A, V, L, I, F, P, M, C), (S, T, Y, W, N, Q), (K, R, H), (D, E), and strictly conservative changes based on the volume of the side chains (V, L, I), (G, A), (T, N, Q), (C, S) and (D, E)). These along with the number of substitutions involving charge changes are shown in Table XLVI.

Of the amino acid differences between liver and muscle PFK, approximately 44% are conservative. Taking into consideration changes in the volume or branching of side chains then approximately 21% are strictly conservative. Amino acid substitutions resulting in charge changes range from 27-35% of the residue differences.

The most frequently occurring amino acid differences between sheep liver and the muscle PFKs are shown in Table XLVII. All of these changes, with the exception of isoleucine->serine are common amino acid interchanges in proteins, as evidenced by their appearance in the list of the most frequently observed amino acid replacements table prepared by Doolittle (1979). Most of these are like for like amino acid changes for example acidic for acidic, and as such should result in minimum disruption of the structure and function of the enzyme.

TABLE XLVI

TYPES OF AMINO ACID CHANGES

Type of change	SH->L	RM->L	HM->L
Conservative	44%	43%	45%
Strictly conservative	21%	20%	21%
Charge changes	35%	27%	23%

TABLE XLVII

MOST FREQUENTLY OBSERVED AMINO ACID REPLACEMENTS BETWEEN MUSCLE PFKs AND SHEEP LIVER PFK

Amino acid Replacement	Sheep heart	Rabbit muscle	Human muscle	No. of base changes
D->E	6	6	8	1
I->V	6	6	7	1
G->A	5	6	6	1
V->I	5	6	6	1
R->K	6	4	4	1
I->S	4	4	4	1
A->V	3	4	4	1

There are three major charge changes which occur between muscle and liver PFK (Table XLVIII), which result in an opposite charge being present in the liver enzyme as opposed to the muscle enzyme. Despite these rather dramatic charge changes, the effects are likely to be relatively minor, due to the positions of these substitutions in the tertiary structure. Residue 244 is proposed to lie in α–8, but lies on the outside of the helix, as does residue 276, which lies in α-9. The glutamic acid to arginine amino acid change which occurs in the C-terminal region may affect the ATP-inhibitory site depending on its position in the tertiary structure. Arginine 755 in liver PFK may be an additional stabilizing force in ATP binding if it lies close enough to the γ–phosphate to bind to it, whereas the glutamic acid residue in muscle PFK would have a destabilizing effect. So this could add to the liver enzyme having a greater affinity for ATP than the muscle enzyme.

The majority of other charge changes (i.e. either gains or losses of charged groups) occur on the exterior of the protein where the effect is not likely to be significant. It seems to be a general trend that changes which are internal are those classified as conservative or semi-conservative, while those occurring on the exterior of the protein are of a more dramatic nature, for example R->E. This is what would be expected in light of the enzyme maintaining the same structure and catalytic function.

The lack of cross-reactivity between muscle PFK antibodies and liver PFK, and vice versa led to a belief that some structural differences existed between them. Work with the influenza virus antigen neuraminidase has led to the discovery that one amino acid residue change on the exterior of a protein is sufficient to completely abolish the antibody-protein reaction (Coleman, 1988). The charge changes such as R->E and E->R in α-helices 8 and 9 in liver PFK could therefore result in sufficient change to prevent a muscle antibody from binding to liver PFK. Therefore the lack of reaction between the antibodies does not necessarily indicate large structural differences between proteins.

As can be seen in Table XLIX, approximately 70% of the amino acid substitutions can be accounted for by single base changes. The remainder are double base changes, with no changes occurring which require three base changes.

TABLE XLVIII

RESIDUE CHANGES INVOLVING MAJOR CHARGE CHANGES BETWEEN SHEEP LIVER AND MUSCLE PFKs

Residue number	Muscle	Liver	Location
276	E	R	α–9
244	R	E	α–8
755	E	R	C-terminal insert

TABLE XLIX

PERCENTAGE OF AMINO ACID CHANGES RESULTING FROM SINGLE AND DOUBLE BASE CHANGES

	Sheep heart	Rabbit muscle	Human muscle
Single base changes	69%	71%	72%
Double base changes	31%	29%	28%

7.4 COMPARISON OF THE SUBUNIT INTERACTION AND BINDING SITES

7.4.1 COMPARISON OF THE RESIDUES INVOLVED IN SUBUNIT INTERACTIONS

It is interesting to note that a number of residues thought to be at the subunit interface, and contacting other subunits have been conserved in the mammalian PFKs compared to the bacterial enzymes (Table L). This is consistent with the findings of Klotz (1970) (Section 1.10), since mutations preventing subunit association would abolish enzyme activity.

With the insertions that occur in the mammalian enzymes compared to the Bs enzyme, it is possible that additional interactions between the N and C-terminal halves occur. One possibility is that the loops between β-J and β-K, which are both extended by one residue in the mammalian sequence may be close enough to interact and result in additional stability between the two halves of the enzyme. Inserts in external loops may enable further interactions to occur between subunits, when associating to the dimeric or tetrameric forms, depending on the conformations which they adopt. It is possible that the peptide T 4 (Table XXXIIa), which is highly conserved between both mammalian and Bs PFK (Section 6.2), may be important for contacts between the N and C-terminal halves of the molecule, since this region is proposed to correspond to the loop between α-10 and α-11 as well as part of α-11, which lies at the subunit interface.

7.4.2 THE CALMODULIN BINDING SITES

Two calmodulin binding sites from rabbit muscle PFK have been isolated and sequenced (Buschmeier et al., 1987). The high affinity site corresponds to residues 371-405 of the muscle sequence, while the low affinity site represents residues 731-779. The high affinity site is an insert compared to the Bs enzyme, while the first 14-15 residues of the low affinity site correspond to the C-terminal of the Bs PFK, the remainder also being an additional segment.

TABLE L

CONSERVATION OF RESIDUES INVOLVED IN SUBUNIT INTERACTIONS COMPARED TO THE Bs ENZYME

Bs	Asp 12	Thr 156	His 160	Arg 63
E. coli	Asp	Thr	His	Arg
Rabbit muscle				
N-terminal Half	Asp	Thr	His	Leu
C-terminal Half	Pro	Ser	Thr	Gln
Sheep heart				
N-terminal Half	Asp	-	-	-
C-terminal Half	Pro	-	-	-
Sheep liver				
N-terminal Half	Asp	Thr	His	-
C-terminal Half	Pro	Ser	Thr	Arg

From Hellinga and Evans (1985).

Comparison of the calmodulin binding sites of muscle PFK with the corresponding regions in sheep liver PFK, shows that the tryptophan residues which have been found in segment "A" of all the calmodulin binding peptides, are conserved in the liver PFK.

High affinity calmodulin binding site:

```
                    "A"                "B"
RM   371 KLRGRSFMNNWEVYKLLAHIRPPAPKSGSYTVAVM 405
SL       QLRGRSFENNWNIYKLLAHQKISKENTWNGGAVVM
```

Low affinity calmodulin binding site:

```
                    "A"                          "B"
RM   731 FEHRIPKEQWWLKLRP...DLDTSEHAHLEHISRKRSGEATV779
SL       FEHR---EQWWLNLR-...SMADYVSGELEHVTRR
```

In segment "B" the three residue cluster neighboured on the N-terminal by basic residues is found in both the high and low affinity sites of muscle PFK. In the high affinity site of sheep liver PFK a large number of amino acid substitutions have occurred, resulting in a region which no longer resembles that of muscle PFK. Therefore the "signal" residues in this site of liver PFK have been lost. Comparison of the low affinity site from muscle PFK with the corresponding region in sheep liver PFK indicates that the serine present in segment "B" in the muscle enzyme is absent in the liver enzyme. There is a serine residue in the liver enzyme six residues upstream from this position, however the occurrence of the N-terminal basic residues which normally characterize this site are not present. It would therefore appear that the "signal" for calmodulin binding has been lost from the low affinity site of sheep liver PFK also. Since Ca^{2+}-CaM-dependent proteins do occur in the liver (Smith et al., 1983),it is possible that regulation of liver PFK by Ca^{2+}-CaM is unnecessary in sheep liver and the "signal" sequences have therefore been lost from the sheep liver PFK.

7.4.3 THE PHOSPHORYLATION SITE

The phosphorylation site of rabbit skeletal muscle PFK which has been isolated and sequenced (Kemp et al., 1981), has been found to contain the usual recognition sequence of cAMP-dependent protein kinase; a serine residue bounded on the N-terminal side by 2-3 basic residues. With the exception of the serine and basic residues

no sequence homology exists between the muscle PFK-1 and PFK-2/F26BPase phosphorylation sites (Murray et al., 1984).

Comparison of the C-terminal regions of muscle and liver PFK shows that if the peptide CNBr G-2 is the true C-terminal of sheep liver PFK, then the phosphorylation site corresponding to that in muscle PFK has been lost. However an SGE sequence as found at the muscle PFK phosphorylation site is located at residues 763-765 of the liver sequence.

```
                          *
RM          .........HISRKRSGEATV 779
SL    759 ADYVSGELEHVTRR
```

* Phosphorylated serine

So the question arises, has the phosphorylation site been moved upstream slightly in the liver enzyme? As can be seen from the sequence in this region, there are no basic residues on the N-terminal side of this serine, so the recognition sequence for cAMP-dependent protein kinase would seem to be lacking in this region. An experiment designed to phosphorylate sheep liver PFK with [^{32}P]-ATP, and isolate the phosphorylated peptide from a limited tryptic digest as described for muscle PFK (Kemp et al., 1981), failed to yield any phosphorylated peptide. So the question as to whether this is a site capable of being phosphorylated remains unanswered. To determine this, a phosphorylation experiment performed with both muscle and liver PFK in parallel would need to be carried out. If the liver enzyme was not phosphorylated under conditions where the muscle enzyme was, then it would be likely that sheep liver PFK has either lost, or not developed a phosphorylation site. It is interesting to note that as yet no liver PFK phosphorylation has been isolated and sequenced, despite the numerous reports of the phosphorylation of liver PFK.

Since a number of papers have reported that liver PFK has been isolated in a phosphorylated form, such a species was looked for on the FAB MS data. However no molecular ions corresponding to the CNBr G-2 peptide containing a phosphate group were found, again indicating the questionable nature of this being a phosphorylation site.

7.4.4 THE ACTIVE SITE

7.4.4.1 THE ATP BINDING SITE

The conservation of residues at the ATP binding site between both the bacterial, and the N-terminal half of the mammalian PFKs is quite remarkable (Table LI).

Comparison of the bacterial ATP binding residues with those in the C-terminal half of the mammalian PFK show very little homology, and it was this lack of homology that prompted Poorman et al. (1984), to suggest that this was no longer an ATP binding site in the mammalian enzyme. Homology between the C-terminal halves of the mammalian enzymes is apparent however.

Between the muscle and liver PFKs two amino acid differences exist in the N-terminal half. An arginine in the muscle enzyme (77 Bs), has been substituted for a threonine in the liver enzyme. In E. coli this residue is also an arginine, which forms a H-bond with a water molecule, which in turn forms contacts to the adenine, thus providing a stabilizing effect. In liver PFK the loss of this basic residue and substitution for a threonine may represent a major change.

The other amino acid difference between muscle and liver PFK occurs at position 104 (Bs), which is a glycine in both the bacterial and muscle enzymes, but is an alanine in the liver sequence. The role of the glycine is thought to be in forming a main chain amide H-bond to the β-phosphate (Hellinga and Evans, 1985)(Fig. 59). But it has been stated that there is no room for a side chain in this position. With an alanine introduced in this position, unless other residue changes in this vicinity occur to compensate for this extra side chain, some disruption at this site will occur.

By replacing residues 77 and 104 (Bs) with a threonine and an alanine respectively, with the aid of the computer program FRODO (Jones, 1982), which superimposes common atoms of the substituted side chain with those of the native structure, it was possible to examine the likely effects of these amino acid changes. These changes resulted in the alanine residue being very close to the α-phosphate of ATP. There was sufficient room in this area for the phosphate to move away from the alanine residue. This may affect the orientation of the adenine, and it is possible that the Arg->Thr residue change at position 77 may provide sufficient flexibility in this region to compensate for the Gly->Ala substitution (Baker, personnal communication).

TABLE LI

RESIDUES INVOLVED WITH THE BINDING OF ATP

Bs	His*	Ser	Gly	Tyr	Cys	Lys	Asp	Gly	Gln	Arg
	160	9	11	41	73	77	103	104	107	171
Ec	His	Ser	Gly	Tyr	Cys	Arg	Asp	Gly	Met	Arg
RM										
Nt	His	Ser	Gly	Tyr	Cys	Arg	Asp	Gly	Thr	Arg
Ct	Thr	Val	Ala	Phe	Thr	Lys	Phe	Glu	Thr	Gly
SH										
Nt	-	Ser	Gly	Tyr	Cys	Arg	Asp	Gly	Thr	-
Ct	-	Val	Ala	Phe	Thr	-	-	-	-	Gly
SL										
Nt	His	Ser	Gly	Tyr	Cys	Thr	Asp	Ala	Thr	Arg
Ct	Thr	Val	Ala	Phe	Thr	Gly	Phe	Glu	Glu	Phe

* residues not in the subunit containing the main part of the site.

From Hellinga and Evans (1985).

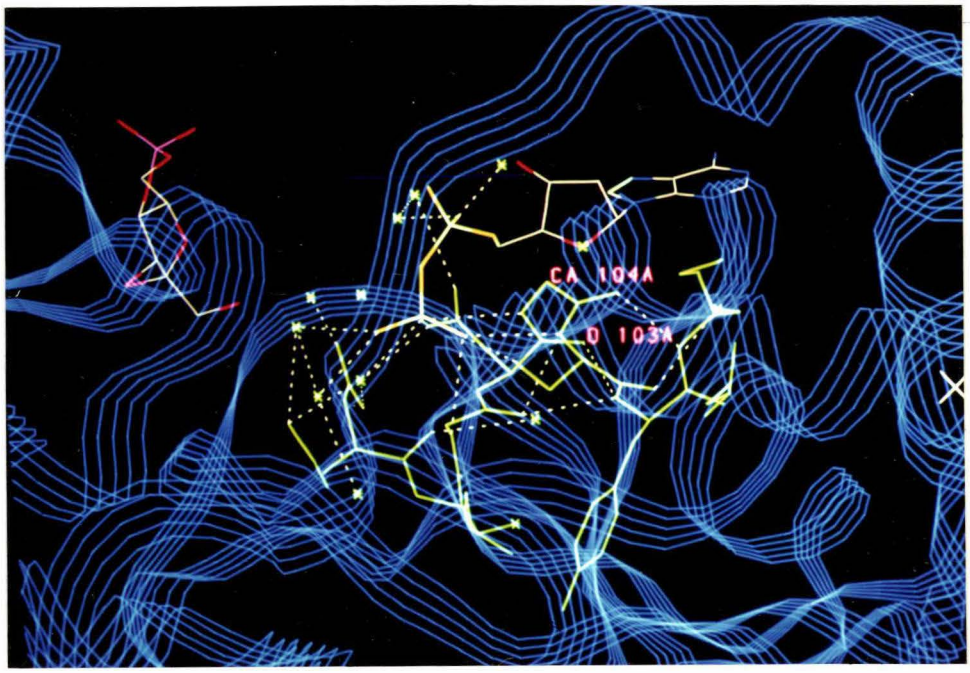


Figure 59: Computer Graphic View of the ATP Binding Site of E. coli PFK from the Computer Program FRODO.

This shows the proximity of residue 104, which is a glycine in the bacterial and muscle PFKs but is an alanine in sheep liver PFK, to the ATP binding site. This residue is labelled 104A.

Glutamine 107 in Bs PFK has been suggested as forming a possible H-bond to adenine N6 (Hellinga and Evans, 1985). The lack of complementarity between this residue in the Bs PFK and that in the E. coli and mammalian enzymes suggest that perhaps this is not an important residue for ATP binding.

7.4.4.2 FRUCTOSE 6-PHOSPHATE BINDING SITE

As can be seen by the comparison of the F6P binding residues (Table LII), the conservation of the N-terminal residues between the bacterial and the mammalian PFKs is almost absolute. The exception being the Val->His substitution in sheep heart PFK at position 249 (Bs). This high degree of homology suggests that the F6P binding site in the N-terminal half is conserved as part of the catalytic site of mammalian PFKs.

Studies of site directed mutations at the active site of E. coli PFK, involving changing the catalytic aspartic acid residue to a serine, as in the C-terminal half of the mammalian enzyme, resulted in a reduction of the turnover number by a factor of 18 000 (Hellinga and Evans, 1987), indicating that the aspartic acid is a key residue in the rate enhancement of the enzyme. The alteration of the Michaelis constant for F16BP in the reverse direction, by a factor of 45, also indicated that the aspartic acid residue destabilizes the product complex. The mutation of arginine 171 to a serine resulted in only a small decrease in the turnover number, indicating that this residue has only a minor effect on catalysis.

The evolution of regulation in proteins is likely to proceed not by changes in residues contacting the substrates, but more through subtle alterations, in perhaps distant regions of the enzyme. This is demonstrated in the conservation of the residues at the active site of PFK where the majority of the residues contacting the ATP and F6P have been conserved between the bacterial and mammalian enzymes (Fig. 60 and 61a,b). The residues which are different in the mammalian enzyme still tend to be of a similar nature to those in the bacterial enzyme, for example threonine 125 (Bs) which corresponds to a serine in the mammalian enzyme. Therefore the substituted residue can probably still maintain the role of H-bonding to the oxygen of the phosphate.

7.4.5 THE FRUCTOSE BISPHOSPHATE SITE

Comparison of the residues which bind F6P in the Bs enzyme with the corresponding residues in the C-terminal half of the mammalian PFKs (Table LII), shows that there is less sequence homology in this half than with the N-terminal half. Differences between

TABLE LII

RESIDUES INVOLVED WITH THE BINDING OF FRUCTOSE 6-PHOSPHATE

Bs	Asp	Arg*	Met	Glu	Arg*	His	Arg
	127	162	169	222	243	249	252
Ec	Asp	Arg	Met	Glu	Arg	His	Arg
RM							
Nt	Asp	Arg	Met	Glu	Arg	His	Arg
Ct	Ser	Arg	Met	Glu	Arg	His	Gln
SH							
Nt	-	-	-	-	Arg	Val	Arg
Ct	Ser	-	Met	Glu	Arg	His	Gln
SL							
Nt	Asp	Arg	Met	Glu	Arg	His	Arg
Ct	Ser	Gln	Gly	Glu	Arg	His	Gln

* residues not in the subunit containing the main part of the site.

From Hellinga and Evans (1985).

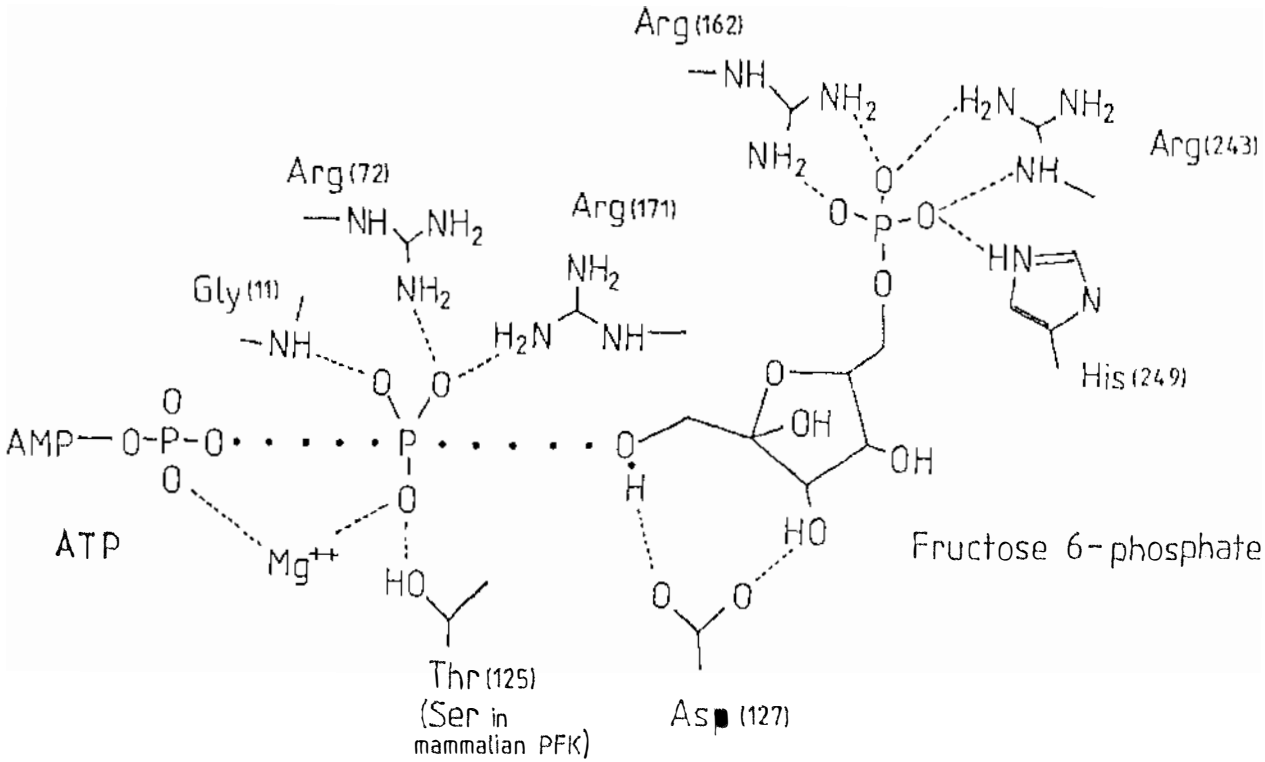


Figure 60: Conservation of the Residues at the Active Site of Mammalian PFK as Compared to the Bs Enzyme

All residues depicted are conserved between Bs PFK and mammalian PFKs, with the exception of threonine 125 which is a serine in mammalian PFK. Modified from Hellinga and Evans (1987).

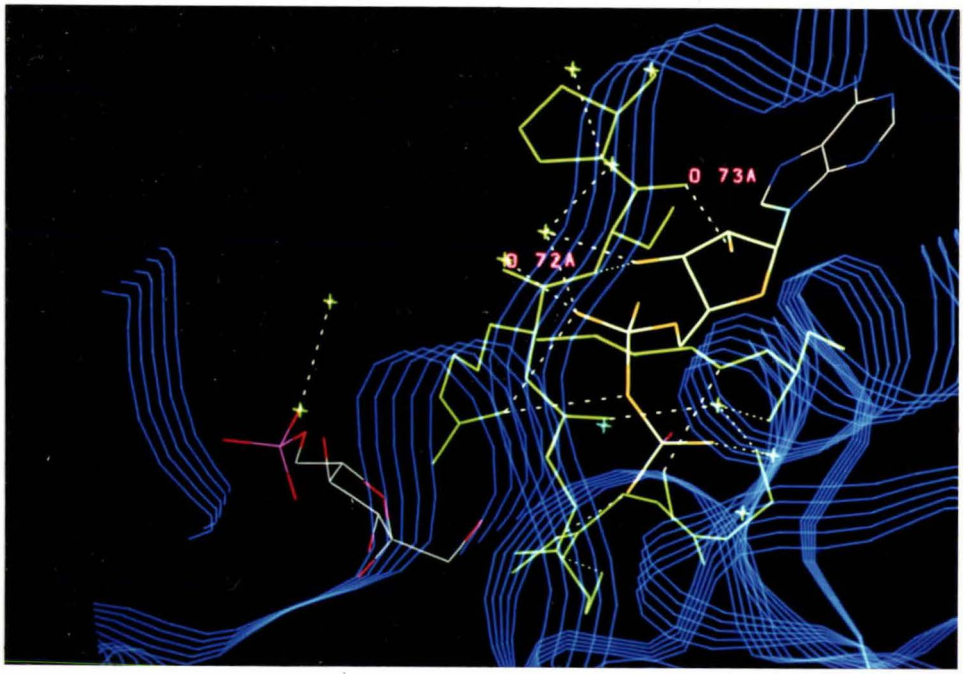


Figure 61a: Computer Graphic View of the ATP Binding Site of E. coli PFK from the Computer Program FRODO.

Both residues arginine 72 and cysteine 73, which form H-bonds with the ATP are conserved in mammalian PFKs. These residues are labelled 72A and 73A respectively.

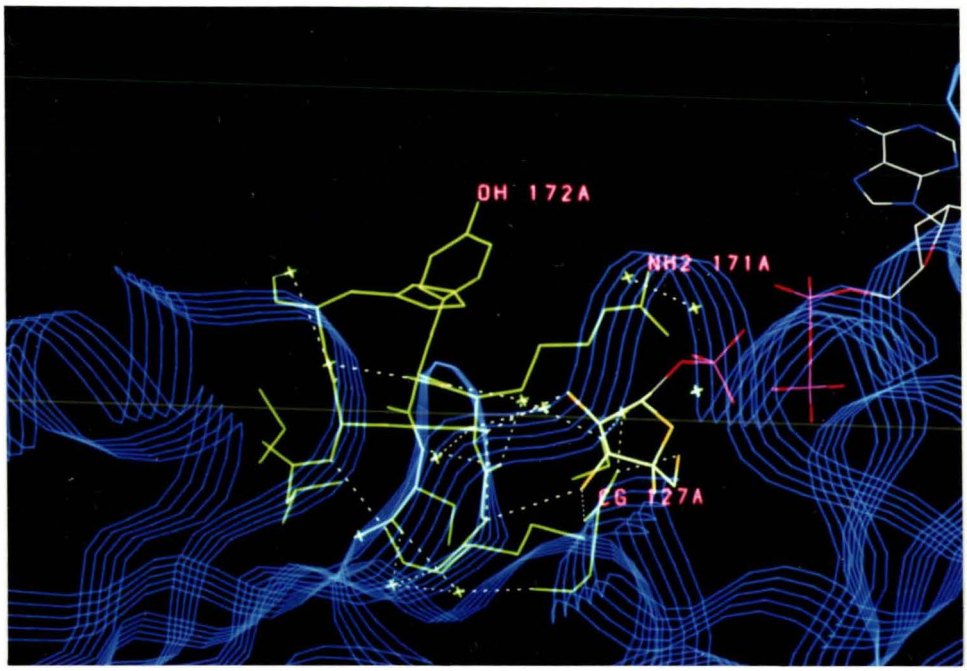


Figure 61b: Computer Graphic View of the F6P Binding Site of E. coli PFK from the Computer Program FRODO.

The catalytic residue aspartic acid 127, and arginine 171 which forms an H-bond with the ATP, are both conserved in the mammalian PFKs. These residues are labelled 127A and 171A respectively.

the Bs and the mammalian enzyme at this site are the catalytic aspartic acid (Bs 127) which corresponds to a serine in the C-terminal half of the mammalian enzymes, and the arginine at 252 (Bs) in the bacterial enzyme which corresponds to a glutamine in the mammalian enzymes. There are two amino acid residue differences between the muscle and sheep liver enzymes. The arginine at 564 (Bs 162) in the rabbit muscle enzyme has been substituted for a glutamine in the liver PFK which will result in the loss of a H-bond to the 6-phosphate, but other amino acid residue changes in this area may provide additional stabilizing forces. The methionine at 572 (Bs 169), in muscle has been substituted for a glycine in the liver enzyme.

Poorman et al. (1984), have suggested that the C-terminal half F6P binding site has mutated to an allosteric activator site for fructose bisphosphate. The loss of the catalytic aspartic acid residue supports this, since its role is thought to be in increasing the nucleophilicity of the O-1 hydroxyl of F6P for attack on the γ-phosphate of ATP, and then an electrostatic repulsion of F16BP. With the replacement of the aspartic acid by a serine in the C-terminal half, the negative charge and therefore the repelling force is lost, as well as providing more room for the binding of the fructose bisphosphate. A diagrammatic representation of this site, which is now thought to bind fructose bisphosphates is shown in Fig.62. As can be seen from this diagram the two arginines and the histidine which bind to the 6-phosphate have been conserved.

7.4.6 THE ADP BINDING SITE

Comparison of the ADP binding residues of the bacterial and the mammalian PFKs shows that a number of these residues have been conserved (Table LIII), in particular the arginines at positions 21 and 25 (Bs). The nature of some of the amino acid residues have also been conserved, such as glutamic acid 187 (Bs), which corresponds to an aspartic acid in the mammalian enzymes. The ADP activation site is found at the interface between the N and C-terminal halves, and is composed of polypeptide chains from both monomers (Fig. 55).

Site directed mutagenesis of residues at the ADP binding site of E. coli PFK have helped to define the relative importance of certain residues at this binding site. Tyrosine 55 was situated in such a position that it was postulated that it may be involved in a hydrophobic interaction with the adenine moiety of ADP, and that the hydroxyl group of the tyrosine may possibly form an H-bond with the adenine. The mutation of this residue to a phenylalanine and a glycine in turn, showed that this tyrosine has a minimal involvement in the binding of ADP (Lau et al., 1987). This is also reflected in the lack

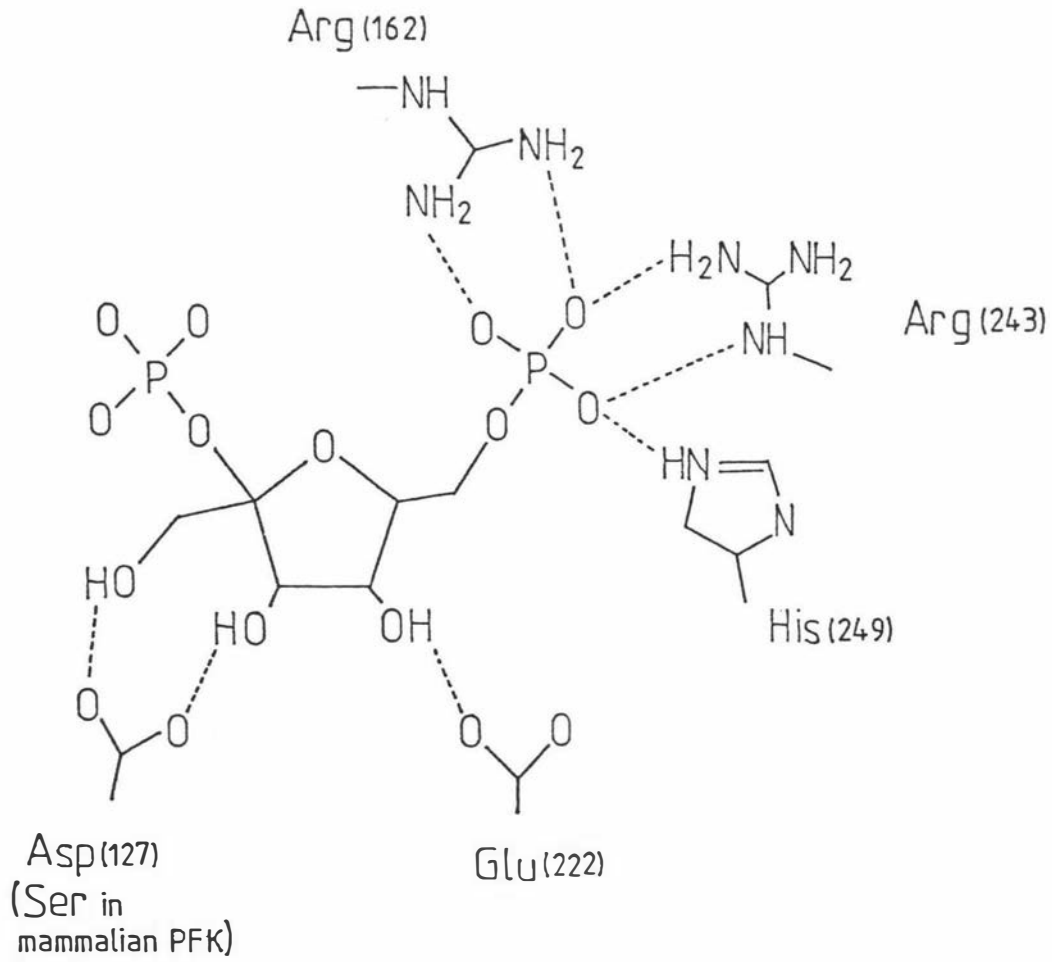


Figure 62: Residues at the Proposed F26BP Binding Site

All residues depicted are conserved between Bs PFK and mammalian PFKs, with the exception of the catalytic residue aspartic acid 125 which is a serine in mammalian PFK.

TABLE LIII

RESIDUES INVOLVED WITH THE BINDING OF ADP

Bs	Lys	Arg*	Arg*	Asp*	Arg*	Arg	Gly	Glu	Arg	Lys	Lys
	111	21	25	59	63	154	185	187	211	213	214
Ec	Arg	Arg	Arg	Asp	Arg	Arg	Gly	Glu	Lys	Lys	Lys
RM											
Nt	Thr	Arg	Arg	Met	Leu	Thr	Gly	Asp	Thr	Thr	Arg
Ct	Gln	Arg	Arg	Gly	Gln	Lys	Gly	Asp	Lys	Lys	Thr
SH											
Nt	Thr	Arg	Arg	-	-	-	Gly	Asp	Thr	Ile	Leu
Ct	Gln	Arg	Arg	-	-	Lys	Gly	Asp	-	Lys	Thr
Sl											
Nt	Thr	Arg	Arg	-	-	Thr	Gly	Asp	Thr	Ser	Arg
Ct	Arg	Arg	Arg	Gly	Arg	Lys	-	-	Lys	Lys	Thr

* residues not in the subunit containing the main part of the site.
From Hellinga and Evans (1985).

of conservation of this residue in both the bacterial and mammalian PFKs, since this residue is different in each of these PFKs.

Other mutations at the ADP binding site of E. coli involve Glu 187->Ala (in mammalian PFK this residue is an aspartic acid)(Lau and Fersht, 1987). This mutation resulted in the reduction of GDP binding, but resulted in PEP being an activator of E. coli PFK, indicating that this residue is important in the structure of the ADP effector site. Mutation of Lys 213->Ala led to a loss in both GDP and PEP binding, such that no binding was detected at concentrations below 3mM GDP and PEP. In the mammalian enzymes, sequence alignment results in this lysine corresponding to a threonine in rabbit muscle, isoleucine in sheep heart and a serine in sheep liver PFK. This lack of homology, and the results of the above mutation suggest that perhaps this residue does not occupy the same position in the mammalian enzymes as it does in the bacterial enzymes, since the mammalian enzymes do still bind ADP. There are a number of arginine residues in this area so perhaps one of these carries out the same function as this lysine, thus maintaining the nature of the binding group.

Mutation of arginine 25 to an alanine resulted in a weakening of both GDP and PEP binding, while Arg 21->Ala abolished their binding altogether, showing the importance of both these residues in ADP binding (Lau and Fersht, 1987). It is interesting to note that both of these residues have been conserved in the mammalian PFKs.

Modification of the lysine at position 677 (Bs 366) of sheep heart PFK with a [^{14}C]-carboxybenzenesulfonyl group caused a loss of the allosteric properties , leading the authors to suggest that modification had occurred at the ADP binding site. Although this residue does not correspond to any of the bacterial ADP binding site residues, the fact that its modification blocks ADP binding suggests that the site is localized in this area, and probably remains in much the same position as that in the bacterial enzymes.

A diagrammatic representation of the ADP site (Fig. 63) shows that the nature of the three basic residues, and the acidic residue at 187 (Bs) have remained conserved, and probably form the basis of the ADP site in mammalian PFK. Other amino acid changes localized in this area probably form additional H-bonds and hydrophobic interactions with the ADP and therefore stabilize it.

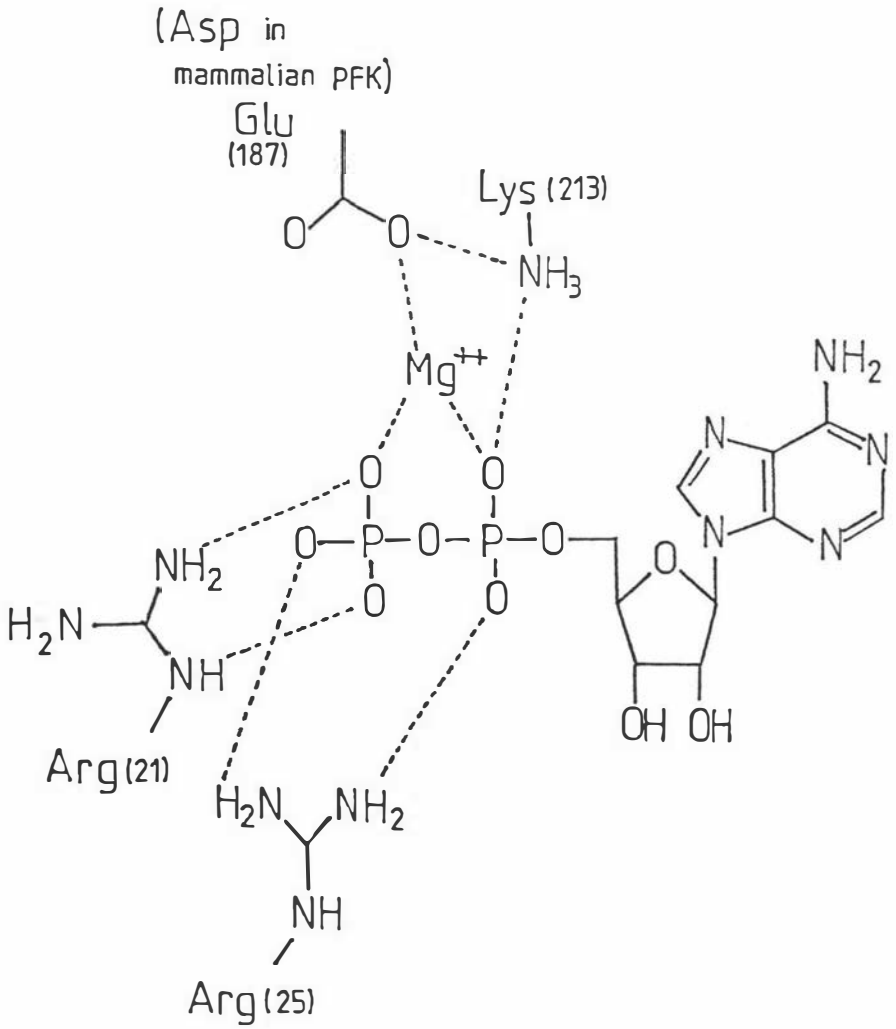


Figure 63: Conservation of the Residues at the ADP Effector Site of Mammalian PFK as Compared to the Bs Enzyme

All residues depicted are conserved between Bs PFK and mammalian PFKs, with the exception of glutamic acid 187 which is an aspartic acid in mammalian PFK. Modified from Lau and Fersht (1987).

7.4.7 THE CITRATE BINDING SITE

Modification of the citrate binding site with pyridoxal phosphate resulted in the labelling of a lysine at position 556 of rabbit muscle PFK (Kemp et al., 1987), localizing this site within the cleft between the two halves of the molecule (Fig. 55). It is possible that the second ADP site in the bacterial enzyme has mutated to form the citrate inhibitor site. In support of this, the lysine residue which was modified, corresponds to arginine 154 in Bs PFK which H-bonds to the β-phosphate of ADP. With the large number of charged residues in this interface region it is possible that these could bind to, and stabilize a citrate molecule. Citrate inhibition results in the decreased affinity of the enzyme for F6P at the active site (Pettigrew and Frieden, 1979a). The crystal structure of Bs PFK in its inhibited conformation (Evans et al, 1986), shows that the tetramer has twisted about its long axis such that one pair of subunits in the tetramer rotates relative to the other pair by about 8° around one of the molecular dyad axes, so that the F6P site is reduced in size. Within the subunit, one domain rotates relative to the other by 4.5°, which further closes the F6P site, without closing the cleft between the domains of the same subunit. It is possible therefore that citrate by inserting into the cleft between the two halves of the mammalian PFK results in a twisting effect due to the additional interactions of the molecule with citrate. Hence citrate binding, forces the PFK into the inhibited conformation.

The adenine nucleotides which can relieve citrate inhibition, may act by binding in the opposite cleft, and reversing the twisting effect by producing interactions that counteract this effect. This is highly speculative however.

7.4.8 THE ATP INHIBITORY SITE

The ATP inhibitory site has been localized to the C-terminal of rabbit muscle PFK, in a region which is in the 35 residue extension beyond that of the homologous Bs enzyme. Valaitis et al. (1987), showed that the cleavage of 17 residues from the C-terminal resulted in the loss of ATP inhibition in muscle PFK. They further localized the inhibitory site to a region 9 residues in length, containing three histidine residues, which they have suggested may contribute to MgATP binding at the inhibitory site.

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Histidines were previously implicated in the binding of ATP at the inhibitory site of sheep heart PFK on the basis of modification by diethylpyrocarbonate (Setlow and

Mansour, 1970). It is also interesting to note that the ionization of histidine residues has been reported in the effect of pH on activation and inactivation of PFK, and also the effect of activators and inhibitors in altering the pK of the ionizable groups.

Predictions of secondary structure in this C-terminal region, resulted in a high likelihood of this region forming an α-helix, particularly the 9 residue segment isolated by Valatais et al. (1987). By drawing a helical wheel, it can be seen that 2 of the histidine residues lie on one side of the helix, while the other histidine is on the opposite side. The homologous region in liver PFK contains only one histidine residue, but despite the low degree of sequence homology between these regions in the liver and muscle enzymes, the liver region is still likely to form an α-helical structure.

The histidine residue could bond to the oxygens in the phosphate groups of ATP, while the C-terminal arginine of liver PFK and corresponding arginine in the muscle enzyme could interact with the hydroxyl groups in the sugar ring of the adenine. An additional stabilizing force could be the glutamic acid residue in liver PFK, which has been substituted for the histidine at position 765. This glutamic acid could interact with the Mg^{2+}, whereas with the histidine no such interaction could occur. This may help to explain the greater affinity of the liver enzyme for MgATP.

The extension on the N-terminal of the mammalian PFKs compared to the bacterial enzymes could also provide residues from the N-terminal half of the molecule to help stabilize the binding of ATP at the inhibitory site. Secondary structure predictions of the muscle PFK N-terminal sequence indicate the formation of an α-helix in this region. In the first 6 residues of muscle PFK, three histidine residues are found. In a helix all three would lie on one side, and therefore these may be involved in interactions with the 3 phosphate groups on ATP. A basic residue found at residue 9 in the muscle enzyme may also interact with the γ-phosphate of ATP. Depending on the nature of the N-terminal of sheep liver PFK similar interactions may or may not occur. If more basic residues occur in the sheep liver enzyme in this region, then this could result in a stronger interaction occurring than with the muscle enzyme, and could also explain the greater affinity of liver PFK for ATP at the inhibitory site.

The blocked N-terminus which is a feature of mammalian PFKs may also have a role to play in the ATP inhibitory site. The acetyl group may H-bond to the adenine, thus providing an additional stabilizing force. If the N-terminal was not blocked, the α-amino group of the threonine residue may have had an opposing, repulsive effect,

thus destabilizing the binding of ATP. A diagrammatic representation of the postulated ATP inhibitory site for mammalian PFK is shown in Fig. 64.

The inhibited form of PFK has been found to be less susceptible to limited proteolysis than the active form of the enzyme. This suggests that the N and C-termini are more tightly bound in the inhibited form, as would be expected if the postulated ATP inhibitory site was correct.

The inhibitory action of ATP may occur by a similar mechanism to that proposed for citrate inhibition. ATP binds to the ATP inhibitory site in the C-terminal segment of liver PFK with greater affinity than the muscle enzyme due to the presence of a glutamic acid residue, the ATP phosphates then attract the histidine residues in the N-terminal segment, and result in the closing of the F6P binding site due to the twisting caused by the additional interactions between the ATP and the two halves of the molecule. ADP binding at the effector site may relieve ATP inhibition by binding to the opposite cleft and reversing the twisting effect by producing interactions that counteract this effect. Due to the greater affinity of the liver enzyme for ATP, a greater concentration of the ADP is required to destabilize the ATP.

Citrate and ATP inhibition is known to be synergistic (Underwood and Newsholme, 1965). This could be explained by there being a greater degree of interaction between the N and C-terminal halves of the molecule when ATP and citrate bind. Therefore the PFK is pulled and held in the inhibited conformation with a greater intensity, and subsequently, to relieve the inhibition a greater opposing force (greater concentration of activator) is necessary.

7.4.9 THE HINGE

The "hinge" is the insertion segment which joins the two halves of the PFK formed by gene duplication. This segment of approximately 30 residues, displays low sequence homology between the muscle and liver PFKs (53%). This is perhaps unusual for a region which is thought to be internal in the tetramer, and forming contact surfaces when two dimers associate to the tetramer (Fig. 58). Secondary structure predictions show that in a segment which is reasonably conserved between muscle and liver PFK, a region of α-helix is likely.

Differences in the sequences between muscle and liver PFK at this interface could affect the interactions between the subunits, and alter the equilibrium between the active and

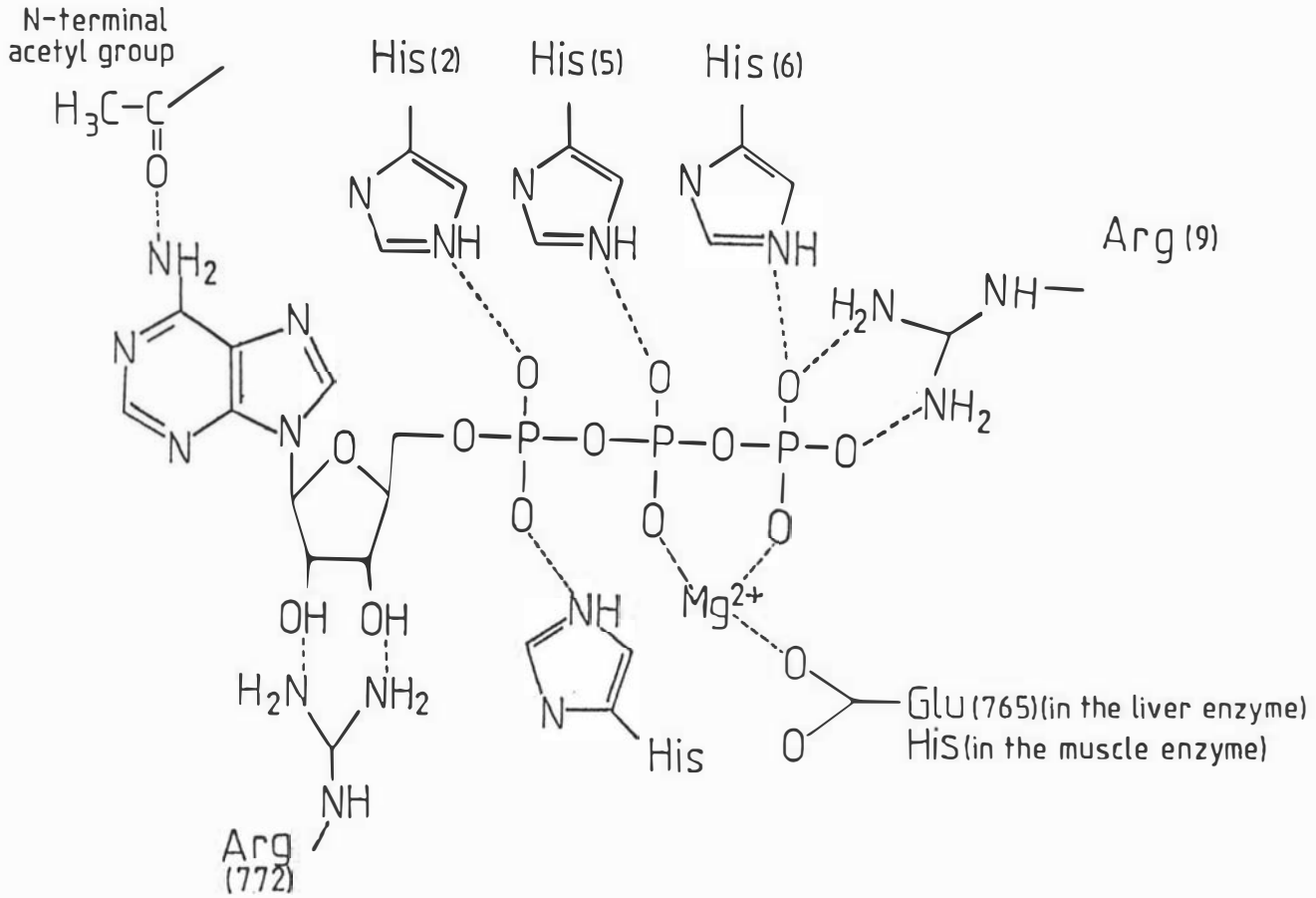

Figure 64: Schematic Diagram of the Postulated ATP Inhibitory Site of Mammalian PFK

inhibited forms of the enzyme. With the liver enzyme being forced into a conformation which is generally more inhibited than the muscle enzyme (hence its lower affinity for F6P), and also more susceptible to ATP inhibition. It has been suggested that differences in sequence between muscle and liver pyruvate kinase, in a region which is a major intersubunit contact may mediate the different allosteric properties of the two isozymes (Muirhead et al., 1986).

The lack of inhibition caused by PEP, phosphocreatine and citrate on liver PFK compared to the muscle enzyme could also be explained by the effect of the "hinge". Since the liver PFK is already partially inhibited, a greater degree of binding of these metabolites is therefore required to achieve any further inhibitory action on the enzyme.

7.5 GENERAL SUMMARY

75% of the residues which are different in sheep liver PFK as compared to the muscle PFKs are found on the surface of the structure. This is similar to the residue distribution found for liver phosphorylase (Rath et al., 1987). As expected, residues involved in binding the substrates at the active site have tended to be conserved between the two isozymes. The conservation of residues at the ADP site is also apparent, but to a lesser extent than for the active site.

This project suggests that the observed differences in allosteric properties between muscle and liver PFK may be due to amino acid residue changes in the "hinge" region, leading to altered intersubunit contacts which result in the liver enzyme being in a more inhibited conformation than the muscle enzyme. Changes at the ATP inhibitory site may also lead to the liver enzyme having a greater affinity for ATP at this site than the muscle enzyme. Alternatively, differences in the allosteric properties may be the cumulative result of amino acid replacements throughout the molecule. Conformational changes which result from the binding of allosteric ligands are distributed throughout the structure, therefore all amino acid substitutions, even conservative ones, may play some role in their propagation. Substitutions which are chemically similar, but involve even small differences in volume or branching of side chains may result in the repacking of α-helices. Adjustments such as these could therefore alter the communication routes between allosteric effector sites.

In order to determine more precisely the cause of the liver and muscle PFKs having different kinetic properties, it would be necessary to determine the crystallographic structure of both isozymes, and compare them.. But until a satisfactory means of

producing suitable PFK crystals for study is found, the speculation on the amino acid changes resulting in the altered kinetic properties of muscle and liver PFK must be confined to comparisons based on the crystal structure of Bacillus stearothermophilus PFK.

APPENDIX

Following the completion of this dissertation, the amino acid sequence of mouse liver PFK, established from the cDNA clone was published (Gehnrich et al., 1988).

The mouse liver sequence was composed of 780 residues, which is one greater than rabbit muscle PFK. This involved an extra residue on both the N and C-terminal of the protein, and a deletion at residue 398. This gave a molecular weight of 85 000, which is similar to that of muscle PFK. The authors suggested that the apparent difference in molecular weight of muscle and liver PFK was probably due to their anomalous behaviour on SDS-PAGE.

The fact that the mouse liver sequence does not terminate with the peptide CNBr G-2 suggests that the C-terminal 7-8 residues may have been cleaved from the sheep liver PFK by a trypsin-like protease during the purification procedure. Gehnrich et al. (1988) have postulated that the C-terminal sequence contains the phoshphorylation site (RRTLS).

Comparison of the sheep liver sequence with that of mouse liver shows 86% sequence homology.

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