AN INVESTIGATION INTO THE BINDING OF SHEEP HEART PHOSPHOFRUCTOKINASE TO AN INTRACELLULAR COMPONENT.

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ABSTRACT

Phosphofructokinase (E.C.2.7.1.11) is an ambiquitous enzyme; ie it can be located either in a cytosolic or particulate form, depending on the metabolic status of the cell. Two indepedent methods were used to attempt to determine the particulate location of sheep heart PFK.

The first method involved an adaptation of the method of subcellular fractionation of liver, for use with heart tissue. A crude fractionation procedure was first used, in which the PFK was found to be located in a fraction which precipitated at 800g. Specific enzyme assays of acid phosphatase, succinic dehydrogenase and 5'-nucleotidase, were performed to determine the presence of lysosomes, mitochondria and plasma membrane respectively, in the fraction containing PFK. These assays revealed that most of the enzyme activity corresponding to the above organelles, had also precipitated in this fraction. Both mitochondria and myofibrils were purified to determine if PFK copurified with these organelles, but the results obtained were inconclusive.

The second method involved immunochemical localisation of PFK. Antibodies against purified sheep heart PFK were raised in rabbits, and an attempt was made to separate the anti-sheep heart PFK antibodies from the other antibodies by affinity chromatography. Both CDI-activated and cyanogen bromide activated sepharose columns were made and used. Successful separation was not achieved. Western blotting followed by staining with a second antibody labelled with alkaline phosphatase, revealed that the antibody preparation was sufficiently specific to proceed to immunochemical localisation. Sections of fresh sheep heart were embedded at low temperature in Lowicryl K4M resin, then incubated with the anti-sheep heart antibodies followed by secondary antibodies labelled with 15nm gold particles. Electron micrographs of the sections revealed the electron dense gold particles, and hence the location of the PFK.

By comparing the density of gold particles on the myofibrils with that of the background it was shown that the density on myofibrils was 4 to

4.5 times higher than on the background. Thus it was tentatively shown that the myofibrils was the site of localisation of PFK in sheep heart cells.

Investigations into the effect of metabolites on the solubilisation of PFK from the membrane, by including various salts in the extraction buffer, revealed that divalent anions, specifically sulphite, sulphate and thiosulphate ions, were effective in releasing PFK into the cytosol. The effect of fructose-2,6-bisphosphate on the solubilisation and activity of sheep heart PFK was uncertain, but it appeared to inhibit the activity of cytosolic PFK and have no effect on the solubilisation.

The possible cross reactivity of the anti-sheep heart antibodies with other sheep tissues and species was studied via polyacrylamide gel electrophoresis of immunoprecipitates, Micro-Ouchterlony plates and alkaline phosphatase staining of Western Blots. Cross reactivity was observed with sheep muscle and to a less extent, rat heart. Only minor cross reactivity was observed against liver PFK (rat or sheep), though this may be due to low levels of PFK in that tissue.

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LIST OF ABBREVIATIONS

ADP	Adenosine 5′-diphosphate
AMP	Adenosine 5′-monophosphate
ATP	Adenosine 5′-triphosphate
Bisacrylamide	N′N′-methylenebisacrylamide
BSA	Bovine serum albumin
CDI	1′1-carbonyldiimidazole
CMC	1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-ρ-toluene-sulphonate
CoQ	Coenzyme Q
DCPIP	2,4-dichlorophenolindophenol
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
F-2,6-P_2	Fructose-2,6-bisphosphate
α-GDP	α-glycerophosphate dehydrogenase
KCNS	Potassium thiocyanate
PBS	Phosphate buffered saline
PFK	Phosphofructokinase
Pι	Inorganic Phosphate
SDS	Sodium dodecyl sulphate
TEMED	N′N′N′N′-tetramethylethylenediamine
TPI	Triosephosphate isomerase
Tween 20	Polyoxyethylenesorbitan Monolaurate

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CHAPTER 1 GENERAL INTRODUCTION

Glycolysis has two main roles. The first is the degradation of glucose in order to generate ATP, and the second is to provide carbon skeletons for synthetic reactions. The process of glycolysis needs to be regulated to suit the needs of the cell for these components. Those enzymes which catalyse reactions which are virtually irreversible, tend to play a regulatory as well as catalytic function in biochemical pathways, and in glycolysis these enzymes are hexokinase (E.C.2.7.1.1), phosphofructokinase (E.C.2.7.1.11) and pyruvate kinase (E.C.2.7.1.40).

The most important regulatory enzyme in the glycolytic pathway, is phosphofructokinase (PFK), which catalyses the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate with the concomitant dephosphorylation of ATP to ADP. PFK, rather than hexokinase, is the main glycolytic control enzyme because it regulates the committing step of glycolysis. Glucose-6-phosphate can be used in glycogen synthesis, or oxidised by the pentose phosphate pathway as well as be converted to pyruvate. The conversion of fructose-6-phosphate to fructose-2,6-bisphosphate however, commits the 6 carbon skeleton to glycolytic catabolism.

The Discovery and Structure of PFK

PFK was first identified in 1935 by Dische (Dische, 1935), after it had been shown that the phosphorylation of fructose-6-phosphate to fructose-2,6-bisphosphate was an essential reaction of glycolysis in yeast (Harden and Young, 1908; Young, 1909). PFK was later identified in animal tissues (Ostern *et al*, 1936) and in plant tissue (Axelrod *et al*, 1952).

Catalytically active mammalian PFK is a tetramer of molecular weight 320,000 to 360,000, (Aaronson and Frieden, 1972; Mansour,1972), although polymers of up to $2x10^6$ have been reported (Mansour, 1970). Brennan *et al* (1974) determined a range of molecular weights of sheep heart PFK protomers of 80,000 to 85,000 by SDS gel electrophoresis. Peptide mapping of tryptic peptides was consistent with a 85,000 molecular weight form composed of identical subunits. Preliminary sequencing by Walker *et al* (1976) lead to the conclusion that rabbit muscle PFK was a tetrameric enzyme consisting of four similar (probably identical) subunits with a molecular weight of 85,000, and to the proposal that gene duplication could have occurred in the evolution of eukaryotic PFKs.

Rabbit skeletal muscle PFK and sheep heart PFK undergo association-dissociation reactions to catalytically active and inactive forms (Mansour and Ahlfors, 1968; Aaronson and Frieden, 1972); the active form being the polymer and the inactive form the dimer. Strong activators such as fructose-2,6-bisphosphate and fructose-1,6-bisphosphate stabilise the tetramer, whereas inhibitors such as citrate, depolymerise the active tetramer to inactive dimers. Fructose-6-phosphate modulates the effect of citrate by stabilising a mixture of aggregates of intermediate size and activity (Lad *et al*, 1973).

PFK is more active in those tissues with a high rate of glycolysis such as muscle and brain, rather than gluconeogenic tissues such as liver (Brock, 1969). However, regardless of its source PFK is controlled by the same allosteric effectors (Lowry and Passonneau, 1964). When cellular energy is low (indicated by a low ATP/AMP ratio), or the availability of cellular building blocks is limited (indicated by low citrate levels), then PFK is activated. If the cell is rich in energy and carbon skeletons (high ATP/AMP ratio and high citrate levels), then PFK is inactivated.

ATP is a potent inhibitor of PFK activity below pH7.5 and at low fructose-6-phosphate concentrations. The ATP-inactivated enzyme can be reactivated by cyclic 3',5'-AMP, 5'-AMP, ADP and inorganic phosphate, since these compounds antagonize this inhibition. ATP inhibition and cyclic AMP reactivation are not observed at high fructose-6-phosphate concentrations, or at an alkaline pH. (Mansour, 1963; Mansour, 1965). A review of the strucure and function of PFK is presented by Goldhammer and Dies (1979).

Regulation by pH Induced Dimerisation

Both Trevedi and Danforth (1966) and Ui (1966) independently demonstrated *in vitro*, the suppression of PFK activity as the assay pH was lowered. They suggested that this PFK inhibition was the cause of pH-induced glycolytic inhibition.

Bock and Frieden (1974) demonstrated the reduction of PFK activity in rabbit skeletal muscle as the pH was lowered. They later (Frieden *et al*, 1976a,b, and c) studied the kinetics of inactivation and reactivation as a function of pH and enzyme concentration at constant temperature. From the enzyme concentration dependence they concluded that the mechanism involved at least a protonation step followed by isomerisation to an inactive form, and then dissociation into a species one half the molecular weight. Ionisable groups control the inactivation process and the apparent pK for these groups is temperature dependent so that the enzyme is cold labile below pH 7.0. Reactivation of the inactive enzyme occurs by a kinetically different pathway. The inactive, dissociated form is protonated to a form which may either isomerise to another inactive form, or dimerise to the active enzyme. Frieden *et al*, (1976a,b and c), postulated a mechanism containing four species (two with four subunits and two with two subunits), each of which can be protonated or unprotonated. Inactivation or reactivation is induced by changes in pH or

temperature. They also concluded that substrates and allosteric effectors exert their effect on pH-dependent inactivation by binding preferentially to either the protonated or unprotonated forms, and thus shifting the apparent pK for the process.

Hand and Somero (1983) showed that the acidification which occurs when squirrels enter hibernation, acting synergistically with a fall in body temperature, promotes PFK inhibition. They showed that the loss of activity was due to a freely reversible conversion of the active, tetrameric form of the enzyme to the catalytically inactive, but structurally active, dimeric form of PFK, as proposed by Bock and Frieden (1976a,b and c). When the squirrels return to their euthermic state the tetrameric form is restored. Hand and Somero (1984) in a similar study to the above on thornbacked rays, showed that the particulate form of PFK had a marked reduction in pH induced sensitivity compared with the soluble form. They believed this reduced sensitivity may have been due to an aggregation of monomers. This particulate PFK non-sensitivity was demonstrated only *in vitro* however.

Hand and Carpenter (1986a and b), showed reversible dissociation and inactivation of PFK in ischemic rat hearts, and decided that the enzyme dissociation represented dimerisation of PFK tetramers, rather than dissociation into four protomers. This suggestion was based on several pieces of evidence. The sedimentation value for the inactive form was consistent with that of a two protomer species (80,000-90,000 Mwt each), and the dimer-dimer contact site appeared to be hydrophobic as compared with the protomer-protomer contact which is ionically bonded. Thirdly, both the inactive and active forms were immunoprecipitable, whereas PFK protomers are not. Hand and Carpenter (1986a and b) also showed that certain metabolites, such as fructose-2,6-bisphosphate, stabilized the enzyme against pH-induced dimerisation. Citric acid, on the other hand, enhances the inactivation process.

Regulation of PFK by Phosphorylation-Dephosphorylation

Incorporation studies by Brand and Soling (1975), showed that rat liver PFK was activated by a kinase catalysed phosphorylation, and inactivated by a phosphatase catalysed dephosphorylation. Brand and Soling (1976), also showed that *in vivo*, both active and inactive forms of PFK exist, and that the proportion of the two forms depends on the nutritional state of the animal. The sum of the two forms, however, remains constant. Hofer and Furst (1976), isolated the phosphorylated form of PFK from rabbit skeletal muscle and suggested that a possible role of the phosphate residue may be as an interconversion mechanism.

Tryptic digestion of the phosphorylated PFK, removed a peptide of less than 30 amino acids from the carboxyl terminus. This peptide contained a phosphoserine. It was found that the susceptibility of the terminal region to proteolysis was dependent on the conformational state of the enzyme (Riquelme and Kemp, 1980). Kemp *et al* (1981), found that rabbit skeletal muscle PFK was phosphorylated by the catalytic subunit of a cyclic AMP dependent protein kinase, and that the rate of phosphorylation was increased in the presence of allosteric activators eg AMP, and decreased in the presence of inhibitors eg ATP. The activators and inhibitors alter the V_{max} of phosphorylation but not the K_m. Kemp *et al* (1981) also determined that the exact site of phosphorylation was a serine residue, six amino acid residues from the carboxyl terminus.

The question of whether or not phosphorylation-dephosphorylation of PFK does actually play a regulatory role is debatable. Furuya and Uyeda (1980) suggest that the degree of phosphorylation determines the binding of activators and hence the activity of PFK would be directly controlled by intracellular metabolite levels. Since phosphorylated PFK is more susceptible to proteolysis (Riquelme and Kemp, 1980),

phosphorylation-dephosphorylation may act to limit the levels of activated PFK. Soling and Brand (1981), in their review of the covalent modification of PFK by phosphorylation-dephosphorylation suggest the possibility that PFK phosphorylation is involved in the spatial arrangement of the enzyme within the cell. Luther and Lee (1986) believe that phosphorylated PFK has a higher affinity for F-actin than the dephosphorylated form. They found that upon muscle contraction the enzyme become more phosphorylated and binding to the muscle matrix increased. However, the experimental evidence of Riquelme *et al* (1978), and Kemp and Foe (1983), suggests that phosphorylation has little effect on enzyme activity.

Intracellular Distribution as a Regulatory Mechanism

Until recently, research into enzyme-structure interactions has been limited to those enzymes which are strongly bound or integral components of membranes, and those which are compartmentalised in subcellular organelles. However, over recent years there has been study and discussion on the possibility of cytosolic enzymes interacting with the cell matrix, or being organised into multi-enzyme complexes.

Historically, the study of interactions between cytosolic components and cellular structure can be traced back to the investigations of Green *et al* (1965). Green and his co-workers described membrane fractions derived from erythrocytes and yeast cells, which were capable of catalysing glycolysis and put forward the postulate that all metabolic sequences may be membrane bound.

John E. Wilson coined the term 'ambiquitous' to describe those enzymes 'whose distribution between soluble and particulate forms may vary with the metabolic status of the cell as reflected in the levels of certain metabolites capable of influencing that distribution' (Wilson, 1978).

Brain hexokinase is the prototype ambiquitous enzyme (Wilson, 1980). Association of hexokinase with brain mitochondria was first reported many years ago by Crane and Sols (1953) and Johnson (1960). They found that about 80% of the total hexokinase activity in brain homogenates was particulate. Rose and Warms (1967) found that the mitochondria-bound enzyme could be solubilised by the addition of glucose-6-phosphate or ATP. Wilson (1968) reported that a change in soluble-particulate distribution was a factor in the regulation of hexokinase activity *in vivo*. Brain hexokinase has been shown to definitely exhibit ambiquitous behaviour *in vivo*, by Knull *et al* (1973, 1974) in their study of chick and mouse brains. Their data indicated that an increased glycolytic rate due to ischaemia or insulin treatment resulted in a rapid and reversible shift to increased proportions of the particulate enzyme. Felgner *et al* (1979) have isolated a protein from the outer mitochondrial membrane, which they believe is the binding site for hexokinase.

Aldolase is another ambiquitous enzyme which has been well studied. Arnold and Pette (1968) first noted that aldolase bound to the particulate fraction in rabbit muscle. In rat brain it was found that one isoenzyme of aldolase preferentially bound to the microsomes, whereas another isoenzyme was located primarily in the cytosol (Clarke *et al*, 1970). Since then aldolase has been shown to bind to F-actin and troponin-tropomyosin complexes in bovine and rabbit muscle (Arnold and Pette, 1970; Arnold *et al*, 1971; Clarke *et al*, 1974; Morton *et al*, 1977). Walsh *et al* (1977) showed that the binding of aldolase to F-actin-tropomyosin-troponin filaments produced major alterations in the kinetic parameters of aldolase. Aldolase has also been shown to bind to other cell components depending on the tissue being studied. Using immunohistochemical techniques, Foemmel *et al* (1975) demonstrated the association of aldolase with endoplasmic reticulum, and Strapazon and Steck (1976, 1977) have shown that rabbit muscle aldolase binds to Band 3, the predominant polypeptide of the human erythrocyte membrane.

Aldolase can be specifically released from the membrane by relatively low levels of its substrate, fructose-1,6-bisphosphate, but not by any other common metabolite (Arnold and Pette, 1970; Strapazon and Steck, 1977).

In 1974, Craven and Basford found that PFK bound to purified rat brain mitochondria in the presence of ADP, and to a certain extent in the presence of ATP. Inorganic phosphate had a slight effect on the solubilisation of PFK, whereas fructose-6-phosphate had no effect.

Later Karadsheh and Uyeda (1977) noted a specific interaction between PFK and the inner surface of erythrocyte membrane, and that this binding to the membrane was associated with a change in the allosteric properties of the enzyme. The membrane bound enzyme was apparently not inhibited by ATP or 2,3-diphosphoglycerate, and its fructose-6-phosphate curve reverted to a non-sigmoidal shape. It seemed that the interaction had reduced the conformational flexibility of the enzyme. Their results implied that the regulatory role of PFK may be influenced by adsorption to cellular structure. Jenkins *et al* (1985), showed that the polyacidic amino-terminal sequence of Band 3 of erythrocyte membranes, bound electrostatically to the polybasic adenine nucleotide-activation site of PFK. ADP and NADH displaced PFK from the membrane by binding to this adenine nucleotide activation site. From their results, it was concluded that Band 3 protein binds PFK dimers with a higher affinity than the tetramers, thus shifting the cytosolic tetramer-dimer equilibrium towards the inactive dimer, and so resulting in a decrease in PFK activity.

By applying subcellular fractionation techniques to sheep heart homogenates, Choate *et al* (1985), showed that PFK appeared to be associated with myofibrils. Their results also indicated that PFK binding to the particulate fraction was enhanced when the pH was lowered, and solubilised by increased pH or ATP concentrations. Choate *et al* suggested that the shifting of the equilibrium of PFK

from soluble to particulate may be a mechanism to regulate PFK activity. Binding to the particulate fraction during acidosis may prevent irreversible inactivation of the enzyme, and ensure continued production of fructose-1,6-bisphosphate.

The main aim of this thesis is to determine the particulate location of PFK by two independent means. The first method involved adapting the subcellular fractionation techniques used for liver tissue (Fleischer and Kervina, 1974), for use with sheep heart. Once the range of possible locations for PFK have been narrowed by crude cell fractionation, the location could be pinpointed by more refined fractionation methods. Procedures for heart fractionation are not as well developed as those for liver, but sheep heart was chosen for study, since large supplies of highly purified sheep heart PFK could be readily obtained. Having a supply of pure PFK was essential for the second part of the study, in which antibodies specific against sheep heart PFK are raised, for use in determining the location of PFK by Immunogold Localisation and Electron Microscopy (Robertson *et al*, 1985).