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THE PURIFICATION AND IMMUNOLOGICAL  
ISOLATION OF ATP CITRATE LYASE  
FROM RAT LIVER

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ABSTRACT

ATP CITRATE LYASE (E.C 4.1.3.8) has been purified from rat hepatocyte cytoplasm by a combination of existing published procedures. The final purification method produced homogeneous ATPCL with specific activity of 10-16 units/mg.

Antibodies were raised in rabbits against purified ATPCL eluted from reactive Blue Sepharose CL-6B or DEAE anion exchange column.

The purified antibodies were tested for their specificity for ATPCL. This was accomplished by Ouchterlony double diffusion analysis and also by disruption of antibody-antigen complexes and visualizing the generated protein bands on detergent gels.

The equivalence point of the purified antibody was determined by immunotitration with both purified enzyme and crude extract. The equivalence point was later confirmed by immunotitration of radiolabelled proteins.

Antibodies were then used to immunochemically isolate and quantitate the amount of (35-S) methionine or (14-C) radiolabelled ATPCL in the cytosolic fraction of rat liver.

Pulse labelling of rat liver proteins in vivo and then precipitation of radiolabelled proteins demonstrated that the purified antibodies precipitated proteins other than just the ATPCL subunit. The amount of ATPCL present in the cytosolic fraction could be calculated after immunoprecipitation and excision of radiolabelled ATPCL subunit on SDS-PAGE. The proportion of ATPCL protein to the total TCA precipitable protein could then be calculated since the immunoprecipitation was carried out under conditions of antibody excess.

Radiolabelled ATPCL was then immunoprecipitated from the cytosolic fractions of rats that had been subjected to different nutritional regimes.

The results of this set of experiments showed that induction of ATPCL activity resulted from an increase in immunologically reactive

protein. Increasing amounts of radiolabelled immunoreactive ATPCL protein could be precipitated by antibodies as the enzyme was induced. Induction of ATPCL activity resulted from increased rate of synthesis or decreased rate of degradation of immunoreactive protein and not from the activation of pre-existing enzyme protein.

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## LIST OF ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'- triphosphate
BPB	Bromophenol blue
cDNA	Circular DNA
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
HIgG	Immunoglobulin heavy chain
LIgG	Immunoglobulin light chain
	mRNA Messenger RNA
PBS	Phosphate buffered saline (10 mM Na <sub>2</sub> HPO <sub>4</sub> , 150 mM NaCl pH 7.4)
PEG	Polyethylene glycol
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N",N" Tetramethylethylenediamine
Tris	Tris(Hydroxymethyl)aminomethane

## LIST OF FIGURES

FIGURE		PAGE
1	SDS Polyacrylamide gel separation of individual purification fractions obtained using preparative method 1(b).	42
2	Elution Pattern of Purified ATPCL eluted from a column of reactive Blue Sepharose.	46
3(a)	SDS polyacrylamide gel electrophoresis of ATPCL prepared by Purification Method 1(a).	49
3(b)	Microdensitometer scan of 7.5% SDS PAGE gel of ATPCL eluted from a reactive Blue Sepharose column.	49
4	Elution pattern of purified ATPCL eluted from the DEAE 52 anion exchange column.	57
5	Photograph of 7.5% SDS PAGE slab gel of ATPCL eluted from DEAE 52 anion exchange column using Preparative Method 2.	60
6	Microdensitometer scan of 7.5% SDS PAGE gel track containing ATPCL eluted from a DEAE 5 anion exchange column.	62
7	Electrophoretic separation of undialyzed ATPCL prepared by Preparative Method 3.	66
8	Electrophoretic separation of dialyzed sample of ATPCL prepared by Preparative Method 3.	66
9	SDS PAGE gel comparing ATP citrate lyase eluted from Blue Sepharose column and DEAE 52 column to SDS PAGE molecular weight standards.	72
10	Estimation of the ATPCL Subunit molecular weight by SDS gel electrophoresis.	74

		vii
11	Ouchterlony Plate of Purified anti-rat Albumin challenging rat serum	84
12	Immunotitration of ATPCL activity from the cytosol fraction. Percentage activity remaining in supernatant vs amount of antibody added.	96
13	Electrophoretic separation (SDS PAGE) of antibody - antigen complexes immunoprecipitated from rat liver cytosol.	100
14(a) and 14(b)	Microdensitometer scan of SDS PAGE gel track of disrupted antibody-antigen complex cytosol vs purified IgG.	102
15	SDS-PAGE separation of the immunoprecipitate formed between the IgG fraction of Rabbit 2 and rat liver cytosol.	106
16	Microdensitometer scan of the SDS-PAGE separation of the proteins from an immunoprecipitate formed from rat liver cytosol with purified IgG fraction from Rabbit 3.	108
17	Microdensitometer scan of purified ATPCL immunoprecipitated by purified IgG fraction obtained by mixing Rabbit 1 and 2 antiserum.	111
	Photography of Ouchterlony plates.	
18	Partially purified ATPCL vs purified IgG fraction from Rabbit 1 antiserum.	113
19	Partially purified ATPCL vs purified IgG fraction from Rabbit 2 antiserum.	113
20	Cytosolic fraction vs purified IgG fraction obtained from Rabbit 1 antiserum.	113



## LIST OF TABLES

	PAGE
1 Alternative strategies for the recovery of ATPCL activity from the 50.Ti supernatant	39
2 Alternative strategies for the recovery of ATPCL activity from the Blue Sepharose column.	40
3 Purification of rat liver ATPCL. Preparative Method 1(a).	44
4 Purification of rat liver ATPCL. Preparative Method 1(b).	45
5 Recovery of ATPCL activity from the Sephacryl S200 using a DEAE-52 column.	54
6 Purification of rat liver ATPCL . Preparative Method 2.	56
7 Purification of rat liver ATPCL. Preparative Method 3.	65
8 Comparison of all purification methods.	70
9 Effect of dietary regime on the induction of ATP citrate lyase in rat liver.	79
10 Determination of the equivalence point of the purified anti-albumin antibodies by immunotitration. Immunotitration of radiolabelled rat sera using rabbit anti-rat albumin IgG.	86
11 Immunoprecipitation of (35-S) rat albumin from rat sera using anti-rat albumin IgG.	87
12 Equivalence point and total amount of purified IgG solutions from individual rabbits immunized against ATPCL.	98

13	Characterisation of recentrifuged cytosolic fraction from a rat refed a high carbohydrate diet for 2 days.	120
14	Radioactive protein bands present in SDS-PAGE gel slices. Analysis of radiolabelled proteins immunoprecipitated by purified IgG solutions.	122
15	Radioimmunoassay. Counts present in the ATPCL subunit vs constant amount of antibody. Rabbit 3 IgG (0.6 mg) vs cytosolic supernatant.	126
16	Characterisation of recentrifuged cytosolic fraction of rat refed a high carbohydrate diet for 3 days.	128
17	Radioactive labelled (35-S) methionine proteins immunoprecipitated by Rabbit 3 purified IgG.	129
18	Proportion of ATPCL in rat liver starved 2 days and refed a high carbohydrate diet for 2 days as determined by different methods.	132
19	Proportion of ATPCL in a rat refed a high carbohydrate diet for 3 days as determined with Rabbit 3 IgG.	134
20	Purification table of 30 minute pulse chase experiment. Characterisation of the ultracentrifuge fraction used for immunoprecipitating ATPCL.	138
21	Examination of radiolabelled (35-S) methionine proteins precipitated by purified antibodies or TCA. Percent of total precipitable protein.	140
22	Immunoreactive radiolabelled (35-S) methionine ATPCL in relation to enzyme activity.	144
23	Immunoreactive radiolabelled (35-S) methionine ATPCL subunit in relation to enzyme specific activity.	145
24	Statistical analysis of the data within Table 23. Correlation of radiolabelled present with immunoreactive ATPCL subunit	147

compared with the induction of enzyme activity.

- 25 Comparison of the proportion of ATP citrate lyase to the TCA precipitable protein in rats with different nutritional status as determined by the assay and by immunoprecipitation using purified IgG. 149

TABLE OF CONTENTS.

	Page
ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
LIST OF ABBREVIATIONS	v
LIST OF FIGURES	vi
LIST OF TABLES	viii
 CHAPTER 1	
1.1 INTRODUCTION AND HISTORICAL REVIEW	1
1.2 OBJECTIVES OF THIS STUDY	11
 CHAPTER 2 GENERAL METHODS	 12
2.0 INTRODUCTION	12
2.1 BUFFERS	12
2.2 EXPERIMENTAL ANIMALS	12
2.3 CENTRIFUGATION	12
2.4 SPECTROPHOTOMETRY	12
2.5 PROTEIN DETERMINATION	13
2.6 POLYACRYLAMIDE GEL ELECTROPHORESIS	
2.6.1 Discontinuous non-denaturing gels	13
2.6.2 Resolving gel	14
2.6.3 Stacking gel	14
2.6.4 Polymerization	14
2.6.5 Electrode resevoir buffer	14
2.6.6 Sample preparation	14
2.6.7 Sodium dodecyl sulphate continuous denaturing gels	 15
2.6.8 Sample preparation	15
2.6.9 Electrophoresis	15

2.6.10	Gel staining	15
2.6.11	Gel scanning	16
2.7	ESTIMATION OF PROTEIN SUBUNIT MOLECULAR WEIGHTS	16
2.8	DETERMINATION OF RADIOACTIVITY	16
2.9	DETERMINATION OF THE RADIOACTIVITY WITHIN SDS-PAGE GEL SLICES	17
2.10	DETERMINATION OF THE RADIOLABELLED TRICHLOROACETIC ACID-PRECIPITABLE PROTEIN	17
2.11	CALIBRATION AND MAINTENANCE OF CHROMTOGRAPHY COLUMNS	17
2.11.1	Ion exchange chromatography columns	18
2.11.2	Calibration of the gel filtration column	18
2.11.3	Regeneration of the Sephacryl S-200 column	19
2.11.4	Regeneration of Blue Sepharose CL-6B affinity chromatography column	18
CHAPTER 3 PURIFICATION OF ATP-CITRATE LYASE FROM RAT LIVER		
3.0	INTRODUCTION	20
3.1	ELUTION OF ATPCL FROM REACTIVE BLUE SEPHAROSE CL-6B	21
3.2	MATERIALS	22
3.3	METHODS	22
3.3.1	Assay of ATP-citrate lyase	22
3.3.2	Purification of ATPCL: METHOD 1.	25
3.3.3	: METHOD 2	31
3.3.4	: METHOD 3	33
3.4	RESULTS AND DISCUSSION	
3.4.1	Discussion of the assay	35
3.5	RESULTS AND DISCUSSION OF PURIFICATION PROCEDURES	
3.5.1	Addition of magnesium ions to the homogenisation buffer	35
3.5.2	DEAE-32 cellulose chromatography	35
3.5.3	Ammonium sulphate and PEG fractionation	38
3.5.4	Gel filtration	39
3.6	ELUTION OF ATP CITRATE LYASE FROM BLUE SEPHAROSE USING PREPARATIVE METHODS 1(a) AND 1(b)	39
3.7	RESULTS AND DISCUSSION OF THE ELUTION OF	

ATPCL FROM DEAE-52 ANION EXCHANGE CELLULOSE USING PURIFICATION METHOD 2	53
3.8 RESULTS AND DISCUSSION OF PREPARATIVE METHOD 3	64
3.9 CONCLUSION OF PURIFICATION PROCEDURES	69
CHAPTER 4 THE EFFECT OF DIET ON THE INDUCTION OF ATP CITRATE LYASE	
4.1 INTRODUCTION	77
4.2 ANIMALS FEEDING REGIMES	77
4.3 METHODS	77
4.4 RESULTS AND DISCUSSION OF THE EFFECT OF DIET ON ATPCL INDUCTION	78
CHAPTER 5 PURIFICATION OF RAT ALBUMIN AND PRODUCTION OF RABBIT ANTI-RAT ALBUMIN ANTIBODY.	
5.1 INTRODUCTION	81
5.2 MATERIALS	81
5.3 METHODS	82
5.4 RESULTS AND DISCUSSION OF ANTI-ALBUMIN IgG PRODUCTION AND IMMUNOPRECIPITATION OF ALBUMIN FROM RAT SERA	83
CHAPTER 6 PREPARATION OF ANTI-ATP CITRATE LYASE ANTIBODIES	
6.1 INTRODUCTION	89
6.2 MATERIALS	89
6.3 IMMUNOLOGICAL PROTOCOLS	
6.3.1 Preparation of anti-ATPCL sera	89
6.3.2 Preparation of serum	90
6.3.3 Preparation of Gamma globulin fraction.	91
6.4 CHARACTERISATION OF THE ANTI-ATP CITRATE LYASE ANTIBODY INTERACTION	
6.4.1 Introduction	92
6.4.2 Determination of the equivalence point: antibody titer. Preparation of antibody-antibody complexes for SDS-PAGE.	
6.4.3 Immunodiffusion	93

6.5	RESULTS AND DISCUSSION OF THE IMMUNIZATION PROTOCOLS	94
6.6	IMMUNOTITRATION RESULTS AND DISCUSSION	95
6.7	ANALYSIS OF DISRUPTED ANTIGEN - ANTIBODY COMPLEXES.	
6.7.1	Discussion of proteins immunoprecipitated by purified IgG fraction from Rabbit 1.	99
6.7.2	Discussion of proteins precipitated by purified IgG fraction from Rabbit 2	104
6.7.3	Discussion of proteins precipitated by purified IgG fraction from Rabbit 3	105
6.7.4	Immunoprecipitation of purified ATPCL	107
6.8	RESULTS AND DISCUSSION OF OUCHTERLONY DOUBLE IMMUNODIFFUSION	117
6.9	CONCLUSION OF THE IMMUNOLOGICAL PROTOCOLS AND QUANTITATION OF THE ANTIBODY-ANTIGEN INTERACTION	112
CHAPTER 7 INTRAPORTAL LABELLING OF LIVER PROTEINS IN VIVO		
7.1	INTRODUCTION	117
7.2	MATERIALS	117
7.3	METHODS	
7.3.1	Intraperitoneal (24 hour) labelling	118
7.3.2	Immunoprecipitation of ATPCL	119
7.4	IMMUNOPRECIPITATION OF ATPCL FROM RAT LIVER CYTOSOL	
7.4.1	Results and discussion of 24 hour intraperitoneal labelling of a rat refed a carbohydrate diet for 2 days	120
7.4.2	Discussion of the (14-C) radiolabelled protein that are precipitated by TCA from the recentrifuged cytosolic fraction.	123
7.4.3	Discussion of the immunoprecipitated (14-C) radiolabelled proteins.	123
7.5	CALCULATION OF THE PROPORTION OF IMMUNOREACTIV ATPCL IN THE ULTRACENTRIFUGE SUPERNATANT	125
7.6	DISCUSSION OF A RADIOIMMUNOTITRATION	125
7.7	RADIOACTIVE IMMUNOPRECIPITATION OF (35-S) METHIONINE LABELLED ATPCL FROM A RAT REFED A CARBOHYDRATE DIET FOR THREE DAYS BY RABBIT 3 PURIFIED IgG.	127
7.8	CALCULATION OF THE PROPORTION OF ATPCL TO THE TOTAL TCA PRECIPITABLE PROTEIN.	130
7.9	CONCLUSION OF IMMUNOPRECIPITATION OF (14-C) LABELLED ATPCL	

FROM A REFED RAT.	131
7.10 CONCLUSION OF THE IMMUNOPRECIPITATION OF (35-S) METHIONINE LABELLED ATPCL FROM A RAT ON A REFED REGIME	134
CHAPTER 8	
INTRAPORTAL (30 MINUTE) LABELLING OF A RAT ON THE STARVATION / REFEEDING REGIME	
8.1 INTRODUCTION	136
8.2 METHODS	136
8.3 ANALYSIS OF THE LABELLED PRODUCTS	137
8.4 RESULTS AND DISCUSSION OF 30 MIN PULSE CHASE EXPERIMENT	137
8.4.2 Immunoprecipitation of albumin from rat sera	139
8.4.3 Proportion of ATPCL to the TCA precipitable protein	139
CHAPTER 9	
IMMUNOPRECIPITATION OF ATP CITRATE LYASE FROM RATS WITH DIFFERENT NUTRITIONAL STATUS	
9.1 INTRODUCTION	142
9.2 METHODS	142
9.3 RESULTS AND DISCUSSION	143
9.4 THE PROPORTION OF ATP CITRATE LYASE TO THE TCA PRECIPITABLE PROTEIN	148
CHAPTER 10	
CONCLUSION	151
10.2 EXTENSIONS OF THIS WORK	154
APPENDICES	
1 TABLE OF BUFFERS	156
2 ACTUAL RADIOLABELLED IMMUNOPRECIPITATION DATA	159



BIBLIOGRAPHY

160-178



germinating castor bean endosperm (58).

ATPCL has been purified to homogeneity from several tissues including rat liver (89,113,150,189), adipose tissue (140, 148), mammary gland (71) and brain (189).

Mammalian ATPCL is a 440-480,000 molecular weight phosphoprotein (2,5,14,18,113,140,148,151). The enzyme is a tetramer of four identical subunits (12,71,111,113,140,148). It has been suggested that the enzyme has multiple chromatographic forms (40). The tetrameric form mentioned above is stable at basic pH and is the predominant form of the enzyme. About 10% of the enzyme in rat liver exists as a polymer with an apparent molecular weight of ten million which is stable at acid pH (40). ATPCL has also been reported to exist in a high molecular weight complex with fatty acid synthetase and acetylCoA carboxylase (61,175,177).

The activity and concentration of ATPCL has been shown to change with variation in diet (1,8,12,56,59,71,88,98,104,105,124,151,156,167, 168,170,193,211,212,213). Induction of the enzyme is also sensitive to the administration of hormones (8,11,28,29,59,64,88,90,95,103,105, 124,167,168,170,) and the expression of cellular programmes for differentiation (16,68,98,105,106,164,174,207,208).

The change in the activity of ATPCL has been shown to parallel the change in the rate of lipid synthesis in live (59,92,104,151,167,212), adipocytes (208), and lactating mammary gland (71) when previously starved rats are refed a high carbohydrate diet.

The amount of ATPCL found in a liver homogenate declines when rats are starved for two or more days (2,8,56,59,98,102,104,124,167,170,211, 212) as shown by enzyme assay, rocket immunoelectrophoresis or radial immunodiffusion. Induction of alloxan diabetes in rats also results in a pronounced decrease in the ATPCL activity when compared to rats fed a standard chow diet. When alloxan induced diabetic or starved rats are refed a high carbohydrate diet (2,8,59,102,104,105,113, 140,167,170,193, 204,211,212) or administered insulin (40,59,78,174, 185,211), ATPCL is restored to the same level as that found in chow fed rats.

The levels of ATP citrate lyase, Fatty acid synthetase, Malate

dehydrogenase, AcetylCoA carboxylase, and Glucose-6-phosphate dehydrogenase have been shown in rat liver to rise and fall co-ordinately in response to dietary variation (59,171,201,204). Thus ATPCL and the other lipogenic enzymes are coordinately induced.

In a number of cases it has been shown that the induction of ATP Citrate lyase is paralleled by proportional increases in translatable messenger RNA's (35,59,71,138,155,181,208).

Recent dot translation hybridization analysis show that the relative content of rat liver ATPCL mRNA (4.9kb) increased when previously starved mice are refed a high carbohydrate diet. There was a 25 fold increase in ATPCL mRNA after a 15 hour refeeding in rat liver and a 30 fold increase in murine ATPCL. The increased abundance of ATPCL immunoreactive protein paralleled this induction of the mRNA (53,155,206,208).

The blockage of the induction of immunoreactive protein by puromycin (59,71), actinomycin D (90), cordycepin (60) and  $\alpha$ -amanitin (an inhibitor of RNA polymerase II (52)), is indirect evidence that de novo synthesis of ATPCL messenger and protein are necessary for induction (60,99,211).

Previous work, (Gibson 1973 (71)) has shown that the change in the total ATPCL activity in rat liver is a result of a change in the rate of synthesis of the enzyme with very little change in the rate of degradation of the protein.

These reports indicate that the synthesis of ATP citrate lyase is controlled at a pretranslational level (155).

The plasma signal which mediates the observed effect of diet on the level of the "lipogenic" enzymes is generally considered to be insulin, which is elevated in the blood of well fed animals. However glucagon, which is elevated in the blood of starved animals inhibits the synthesis of the lipogenic enzymes (reviewed 124). In cultured hepatocytes, insulin and thyroxine stimulate the accumulation of ATPCL and this stimulation is counteracted by glucagon (99,185).

The mechanism by which these individual hormones regulate the synthesis and/or degradation of ATPCL mRNA in vivo is the subject of conti-

ning investigation using hormone sensitive cultures of rat hepatocytes (206,208).

These data provide evidence that the regulation of ATPCL depends on the long term adaptive changes based on hormone dependent (123,168) and diet dependent (59,156,193,212) induction and degradation (59,104,169,212).

The overall rate of fatty acid biosynthesis is subject to short term regulatory control by regulation of acetylCoA carboxylase. The activity of this enzyme is controlled by allosteric effectors and by reversible phosphorylation (30,35,72,73,79,100). ATPCL has also been shown to be subject to reversible phosphorylation in response to the actions of hormones (2,5,8,12,15,43,70,95,140,143,145).

Several workers have suggested that phosphorylation of ATPCL may well be significant in the regulation of the enzyme, which represents a crucial link between carbohydrate and lipid metabolism. Inactivation and activation of ATPCL at the crossroads of the fate of dietary carbon is therefore vitally important in considering the control of lipogenesis. Phosphorylation requires an expenditure of energy in the cellular economy and are usually strictly controlled and have a defined function.

However, to date, no regulatory significance has been conclusively assigned to the phosphorylations within ATPCL.

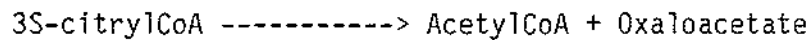
The phosphorylation sites within ATPCL are separate and can be distinguished by their chemical properties, and by their different susceptibilities to phosphoprotein phosphatases (4,71,137).

The phosphorylation sites of ATP citrate lyase are defined as:

### 1. Catalytic phosphate.

A phosphorylated histidine (0.5molP/mol of subunit)(42,140,151,164, 165) is found at the catalytic site. The phosphate is derived directly from ATP as a result of the reaction mechanism (42,89,90,134, 147,205). This phosphate is acid labile (5) as well as being sensitive to hydrolysis by hydroxylamine and substrates (4,42,46,89,90,113,140,143,151,165).

The catalytic phosphate is located on a 68,000 molecular weight tryptic peptide from the enzyme that catalyses acylCoA lyase activity.



### 2. Structural phosphate.

The reversible phosphorylation of ATPCL in the structural site is the subject of active investigation, and as a consequence, the published literature is often confusing and controversial. There is also a great deal of confusion and uncertainty as to the specific role and mechanism of the structural site phosphorylations. Different preparative methods and analytical techniques for the preparation of ATPCL from different tissues may also contribute to the inconsistency of the published data.

Structural phosphates are at sites on the enzyme distant from the catalytic site. Structural site phosphorylations are catalysed by kinases and fall into two classes:

- (a) Those catalysed by glucagon-cAMP dependent protein kinase(s).
- (b) Those catalysed by insulin sensitive-cAMP independent protein kinase, ATPCL kinase, calcium - independent and calmodulin - insensitive kinase(s).

In rat liver both structural phosphates reside on an acid stable phospho-serine residue (113,143,164). In rat adipose tissue, structural phosphates reside in both serine and threonine residues (148).

In rat liver and fat pad there is 0.5 mol P/mol of subunit (113,149)

but only 0.2 mol P/ mol of subunit in rat mammary gland ATPCL (71).

Labelling of structural serine phosphate can be detected after  $^{32}\text{P}$  injection of orthophosphate *in vivo* (2,111,113).

In rat liver the structural phosphate, resulting from labelling with  $^{32}\text{P}$  orthophosphate *in vivo*, resides in a 57,000 molecular weight peptide. This peptide is formed by the action of trypsin on native ATPCL (2,113) and contains citrate thiokinase activity (111).

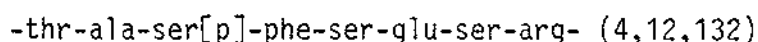


ATPCL is phosphorylated at sites distinct from the catalytic site in response to the actions of glucagon, insulin, and B-agonists but by apparently different mechanisms in different tissues.

Glucagon and B-adrenergic agonists (2,3,4,5,13,14,15,18,21,31, 54,71,85,86,95,113,140,168) mediate phosphorylation through a cAMP dependent proteinkinase (4,5,12,70) which increases the amount of serine structural phosphate in the enzyme from hepatocytes (148), but not in the enzyme from mammary gland (71). This difference may be a tissue specific phenomenon and not a difference in enzyme structure, since the physiochemical properties (subunit molecular weight, subunit structure, absorbance index and specific activity) and the amino-acid composition of rat liver is very similar to rat mammary ATPCL (71).

cAMP-dependent protein kinase phosphorylates ATPCL to the extent of 0.5 mol P/ mol of ATPCL subunit from hepatocytes (12). The rate of phosphorylation of ATPCL by the cAMP-dependent protein kinase is similar to the observed rate of phosphorylation of the enzyme caused by glucagon in hepatocytes (5).

The site of phosphorylation by purified cAMP-dependent protein kinase has been shown to be located in a unique tryptic peptide of 1,000 molecular weight (132,133,146,185) with the following sequence



In rat hepatocytes (2,95,113) and adipocytes (14,21,54,85,140) this phosphate can be removed by a specific phosphatase which has been par-

tially purified from rat liver (113,164).

Experimental evidence suggests that insulin stimulates the phosphorylation of a specific serine residue of ATPCL (2,4,14,18,19,20,21, 49,54,95,133,140,142,185) via an insulin-sensitive protein kinase (cAMP-independent protein kinase, ATPCL kinase) (4,12,15,19,20,21,71,143,144).

Ramakrishna and Benjamin 1981 (143,144) have reported the partial purification of a cAMP-independent protein kinase from rat adipose tissue. This kinase phosphorylates rat adipose tissue ATPCL at a site distinct from that phosphorylated by the cAMP-dependent protein kinase.

The site phosphorylated by the insulin-sensitive cAMP-independent protein kinase occurs on a tryptic peptide of 8,500 molecular weight. This has been determined by phospho-aminoacid analysis of radio-labelled tryptic peptides separated by HPLC (135,145,154). Both a serine and a threonine residue in this peptide are phosphorylated in the enzyme from rat adipo-cytes, (135,136,145,146,148).

The cAMP-dependent protein kinase phosphorylates only a single serine residue in a peptide of 1,000 molecular weight (135,136).

Phosphorylation of the larger peptide was shown to be absolutely dependent upon the prior phosphorylation of a residue within the smaller peptide by the cAMP-dependent protein kinase. The degree of phosphorylation of ATPCL by both kinases together is more than their sum when incubated separately (146). Whether the smaller peptide is contained within the larger peptide is still the subject of investigation.

A number of reports suggest that within rat liver ATP citrate lyase, the same site is phosphorylated in response to either insulin or hormones that increase cAMP (12,133). These workers have used a number of different techniques including analysis of phosphopeptides released from ATPCL isolated from liver cells or 3T3-L1 preadipocytes by partial proteolysis, analyses of complete tryptic digests of  $[^{32}\text{P}]$ -ATPCL by reverse phase HPLC and a combination of electrophoresis and chromatography.

These observations are consistent with the possibility that phosphorylation of one serine (or threonine) within ATPCL phospho-peptide



excludes the subsequent phosphorylation of a second residue (185). These data are inconsistent if the phosphorylations are of regulatory significance because these hormones have entirely different overall effects on lipogenesis.

Avruch (2,14) and Benjamin (19) raise the possibility that one pathway of response of ATPCL to insulin might involve one or more of the following :

- (a) inhibition of a phosphatase,
- (b) the activation of a cAMP - independent protein kinase,
- (c) an alteration in the ability of ATPCL to serve as a substrate for the protein phosphatase or
- (d) an indirect effect on cAMP - dependent protein kinase.

The dephosphorylation of the insulin sensitive phosphorylation site in rat liver ATP citrate lyase can be catalysed by protein phosphatases 1, 2a and 2c in vivo (214). Isolated rabbit skeletal muscle phosphorylases can dephosphorylate this site in lactating mammary gland ATPCL (71). In both cases the specific dephosphorylations of ATPCL has no observable effect on the activity of the enzyme (214).

The following roles have been proposed for the phosphorylated and dephosphorylated forms of ATP citrate lyase :

- (a) Dephosphorylation could be a signal for the association of ATPCL with the outer mitochondrial membrane.

Only 4-10% of the total ATPCL has been shown to bind to mitochondria (39,92,94,147).

Janski and Cornell (92,94) reported that citrate would release 80% of the ATPCL bound to the mitochondria in the absence of magnesium in vitro.

When added simultaneously, ATP, citrate and CoA released 98% of the ATPCL bound to the mitochondria.

In the presence of magnesium ions, CoA released 100% whereas ATP, CoA, and citrate released 91% of ATPCL bound to the mitochondria.

They also showed (94) that in starved rats, 52% (0.6 of 1.22 units) of ATPCL was bound to the mitochondria and this is a higher proportion than rats re-fed a fat free diet (7.8 of 32 units). The increase was attributed to elevation of CoA levels in the fed state.

This result was confirmed by the use of the fatty acid analogue 5-(tetradecyloxy) 2 - furoic acid, which lowers CoA levels in liver cells and results in an increase in binding of ATPCL to mitochondria.

Inactivation of the enzyme by binding to the membrane may represent a mechanism for controlling the rate of citrate cleavage either in the cytosol or as it leaves the mitochondria (39,92,93,113,143,147).

- (b) An association with microsomes (112,114) or endoplasmic reticulum (114).

In vitro, ATPCL binds to isolated rat liver microsomes (114). This binding phenomenon appears to be both temperature and time and dependent and is a saturable process that requires the presence of a specific membrane protein. However individual preparations of microsomes bind ATPCL to variable extents. When purified rough and smooth endoplasmic reticulum were prepared both fractions bound ATPCL to the same extent. There is sufficient microsomal protein in the hepatocyte to allow most, if not all, the ATPCL to be bound to the endoplasmic reticulum even when the enzyme has been induced over ten fold by dietary manipulations (114).

In vitro relatively high salt concentrations inhibit binding of ATPCL to microsomes. At 50 mM potassium phosphate and 60 mM KCl, 40% of the enzyme is bound, whereas 10 $\mu$ M CoASH completely eliminates binding (114).

There is no compelling evidence for the association of ATPCL with microsomes in vivo.

(c) A signal for protein degradation or that phosphorylation affects turnover or subcellular localization (2,71,113,147,185,208).

Dunaway and Segal (51) have isolated a 3500 Mr stabilizing factor that protects phosphofructokinase (PFK) from lysosomal and thermal inactivation.

Phosphorylation of PFK does not alter the allosteric properties of this enzyme but decreases the affinity for the stabilization factor which in turn controls the activity of PFK.

Osterland and Bridger (126,127) isolated the same stabilizing factor and showed that this peptide also stabilizes ATPCL against thermal and lysosomal degradation. No evidence was presented to suggest that phosphorylation of ATPCL affects either the affinity of ATPCL for the stabilizing factor, or any turnover phenomenon.

To date, no alteration in ATPCL function has been demonstrated to result from hormone stimulated phosphorylation of the enzyme (12,95). In vitro, there has been no observed alteration in the kinetic properties of the various phosphorylated and dephosphorylated forms of ATPCL (12,70,71,95,98,113,117,142,144,146,147,185,208).

Ranganathan et al (1980) (147) could demonstrate no difference in the manner in which they are degraded by lysosomal proteases (147). Even complete removal of the phosphopeptide from ATPCL by proteolytic nicking does not alter any kinetic property of the enzyme (164).

The phosphorylation of ATPCL is slow and hence any effect on regulation would have to be long term (149).

The reversible phosphorylations of ATPCL in both sites is of relevance in this thesis if these phosphorylations affect the affinity of the enzyme for the DEAE anion exchange chromatography column or if it alters its behaviour in the various preparative procedures used.

Hoffman et al (76) located only one peak of activity after anion exchange chromatography. Corrigan et al (40) located 3 individually resolvable peaks. Two of these peaks were retained by anion exchange

columns while the first was eluted in the void volume peak. There was no data presented comparing the degree of phosphorylation between these protein peaks.

## 1.2

## OBJECTIVES OF THIS INVESTIGATION

The purpose of this thesis is to investigate the molecular basis of the regulation of ATP citrate lyase by dietary manipulation. To achieve this aim, it was found necessary to isolate milligram quantities of homogeneous ATPCL and prepare high-titre ATP citrate lyase antiserum.

The antibodies can then be used to confirm that the increase in inducible enzyme activity paralleled the increase in ATPCL immunoreactive protein.