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 subunition 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_2HPO_4, 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

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CHAPTER 1

1.1 INTRODUCTION AND HISTORICAL REVIEW

ATP citrate lyase (E.C 4.1.3.8) catalyses the following reaction:

citrate + CoASH + ATP ----------> Oxaloacetate + AcetylCoA + ADP + Pi

(12,89,113,117,150,164,168,176,199,211)

This enzyme is of physiological importance because of its role in providing acetyl units for lipogenesis from glucose.

In animals acetylCoA is formed by the oxidative decarboxylation of pyruvate within the mitochondria, whereas lipogenesis occurs in the cytosol (19). Since acetylCoA cannot penetrate the inner mitochondrial membrane its export to the cytoplasm is accomplished by condensation with oxaloacetate to form citrate which is then transported across the mitochondrial membrane by a specific carrier (19). In the cytosol, citrate is reconverted to oxaloacetate and acetylCoA by ATP citrate lyase (ATPCL) (12,50,71,73,124,156,169,174,200). The acetylCoA formed in the cytosol can then be utilized for fatty acid biosynthesis.

Thus ATPCL can be regarded as the first committed enzyme of lipogenesis from carbohydrate in the cytosol in non-ruminant mammals (40, 43,89,94,98,99,104).

ATPCL has been shown to be widely distributed in nature. The enzyme was initially found in lipogenic tissues such as liver (2,5,12,14,15,19, 40,63,87,89,92,98,99,111,140,147,164,168,172,173,174,178, 189, 194, 205, 209,211,213) adipose tissue (2,13,14,15,18,19,29,95, 135, 136, 140, 143, 164,166,180,184,185) and lactating mammary gland (70,71,172).

ATPCL has also been demonstrated in non lipogenic tissues such as brain (12,74,157,172,186,187,188,189,190,191), adrenal (29,172), and testicular tissue (10,11,29,57). The enzyme was also shown to be present in microbiological sources. ATPCL activity is present when these bacteria (9,91,165,179) and fungal (24,25,26,28,69,119,165,179) sources are grown under optimal conditions when lipogenesis is occurring.

Two plant sources that contain ATPCL are mango fruit (121, 172) and

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 @-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-

nuing 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.

3S-citrylCoA ----------> AcetylCoA + Oxaloacetate

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 32P injection of orthophosphate in vivo (2,111,113).

In rat liver the structural phosphate, resulting from labelling with 32P 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).

citrate + ATP + CoASH ----------> (3S)-citrylCoA

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 similiar 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 similiar 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

-thr-ala-ser[p]-phe-ser-glu-ser-arg- (4,12,132)

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).

Phosphorlyation 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-Li preadipocytes by partial proteolysis,analyses of complete tryptic digests of [32P]-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 lipogensis.

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 refed 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 10uM 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.