THE REGULATION OF MAMMALIAN LIPOGENESIS VIA REVERSIBLE PHOSPHORYLATION BY THE cAMP-DEPENDENT AND AMP-ACTIVATED PROTEIN KINASES

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ABSTRACT

Lipid biosynthesis in the mammal is stringently regulated according to nutritional status. The rate limiting step in fatty acid synthesis is believed to be that catalysed by acetyl-CoA carboxylase (ACC). The activity of ACC is regulated both allosterically and by reversible phosphorylation. The work described in this thesis was conducted to ascertain which protein kinases are responsible for the in vivo phosphorylation and inactivation of ACC in the major lipogenic tissues i.e. lactating mammary gland and liver.

Glucagon-mediated inhibition of ACC in hepatocytes, and phosphorylation and inactivation of purified ACC in vitro by purified cAMP-dependent protein kinase (cAMP-PK) from bovine heart suggested cAMP-PK as a good candidate for the physiological ACC kinase. However, intensive study of the catalytic subunit of cAMP-PK from mammary gland showed that it is probably an isozymic form of cAMP-PK characterised by poor stability and different substrate specificity to its cardiac counterpart, in particular a low affinity for ACC. Furthermore it appears that there is present in lactating mammary gland a factor that specifically inactivates the free catalytic subunit of cAMP-PK which may also be a reason for its lack of effect on ACC in vivo.

Under physiological conditions eg over time courses of starvation, the inactivation of ACC in lactating rat mammary gland or rat liver more closely correlated with activation of AMP-activated protein kinase (AMP-PK) than cAMP-PK. This provided further evidence that AMP-PK and not cAMP-PK is the physiological ACC kinase. This work also suggested that the primary control point in the lactating mammary gland is pyruvate dehydrogenase (PDH) and its associated kinase, but in the liver ACC is the primary control point.

AMP-PK itself is regulated by reversible phosphorylation, being phosphorylated and activated by a separate kinase kinase activity. A partial purification and characterisation of the AMP-PK kinase from mammary gland is described, together with its stimulation by fatty acyl-CoA.

The final products of mammalian lipid biosynthesis are triglycerides and cholesteryl ester, these are assembled together to form very low density lipoprotein (VLDL) in the liver and are then secreted from the liver into the blood stream. The major structural protein of VLDL is apolipoprotein B-100 (apo B). Apo B is known to be a phosphoprotein in vivo. This work describes the in vitro phosphorylation of apo B by cAMP-PK and AMP-PK and identifies the sites of phosphorylation. It also reports the partial characterisation of a possibly novel apo B kinase.

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LIST OF ABBREVIATIONS

APO	-	Apolipoprotein
AMP	-	Adenosine Monophosphate
ATP	-	Adenosine Triphosphate
AMP-PK	-	AMP-Activated Protein Kinase
ATP-CL	-	ATP-Citrate Lyase
A-V	-	Arterio-Venous
ACK-2	-	Acetyl-CoA Carboxylase Kinase-2
ACON	-	Acetonitrile
A_{280}	-	Absorbance at 280nm
ACC	-	Acetyl-CoA Carboxylase
BSA	-	Bovine Serum Albumin
BZ	-	Benzamidine
cAMP	-	Cyclic 3'5' Adenosine Monophosphate
cAMP-PK	-	cAMP-Dependent Protein Kinase
C-subunit	-	Catalytic Subunit of cAMP-PK
DEAE-cellulose	-	Diethylaminoethyl cellulose
DTT	-	Dithiothreitol
DNA	-	Deoxyribonucleic Acid
cDNA	-	Complementary Deoxyribonucleic Acid
FPLC	-	Fast Protein Liquid Chromatography
FSBA	-	Fluorosulphonyl Benzoyl Adenosine
GPAT	-	Glycerol-3-Phosphate Acyltransferase

GTP	-	Guanosine Triphosphate
HPLC	-	High Pressure Liquid Chromatography
HEPES	-	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HMG-CoA	-	Hydroxymethyl Glutaryl Co-enzyme A
HMG-CoAR	-	Hydroxymethyl Glutaryl Co-enzyme A Reductase
HDL	-	High Density Lipoprotein
HSL	-	Hormone Sensitive Lipase
IBMX	-	3-isobutyl-1-methyl xanthine
IDL	-	Intermediate Density Lipoprotein
LDL	-	Low Density Lipoprotein
PAGE	-	Polyacrylamide Gel Electrophoresis
PCA	-	Perchloric Acid
PMSF	-	Phenylmethylsulphonyl fluoride
PDH	-	Pyruvate Dehydrogenase
PEG	-	Polyethyleneglycol
PKC	-	Protein Kinase C
R-subunit	-	Regulatory Subunit of cAMP-PK
RNA	-	Ribonucleic Acid
mRNA	-	Messenger Ribonucleic Acid
r.ER	-	Rough Endoplasmic Reticulum
s.ER	-	Smooth Endoplasmic Reticulum
SBTI	-	Soya Bean Trypsin Inhibitor
SDS	-	Sodium Dodecyl Sulphate

TCA	-	Trichloroacetic Acid
TFA	-	Trifluoroacetic Acid
TLCK	-	Nα-p-tosyl-L-lysine chloromethyl ketone
TPCK	-	N-tosyl-L-phenylalanine chloromethyl ketone
VLDL	-	Very Low Density Lipoprotein
NADPH	-	Nicotinamide Adenine Dinucleotide Phosphate (reduced)

CHAPTER ONE

Introduction

1.1. Biosynthesis of Lipids in Mammals

There are three important lipogenic tissues, the liver is the most important site in most mammals and birds, but in some mammals white adipose tissue also contributes a large proportion of whole body fatty acid synthesis (Hardie, 1989). For lactating mammals the mammary gland is also an extremely active site of lipogenesis, especially in those mammals that produce a lipid rich milk (Williamson, 1980). The liver has mainly a secretory role, white adipose tissue has mainly a storage role and the major function of the mammary gland is to synthesize milk lipids.

The common fatty acids that occur in mammals are monocarboxylic acids with a long unbranched hydrocarbon chain with a terminal carboxyl group. The chain usually has an even number of carbon atoms and may be unsaturated by the introduction of one or more double bonds. The most commonly occuring fatty acids are palmitate (16.0) and oleate (18.1).

Fatty Acids

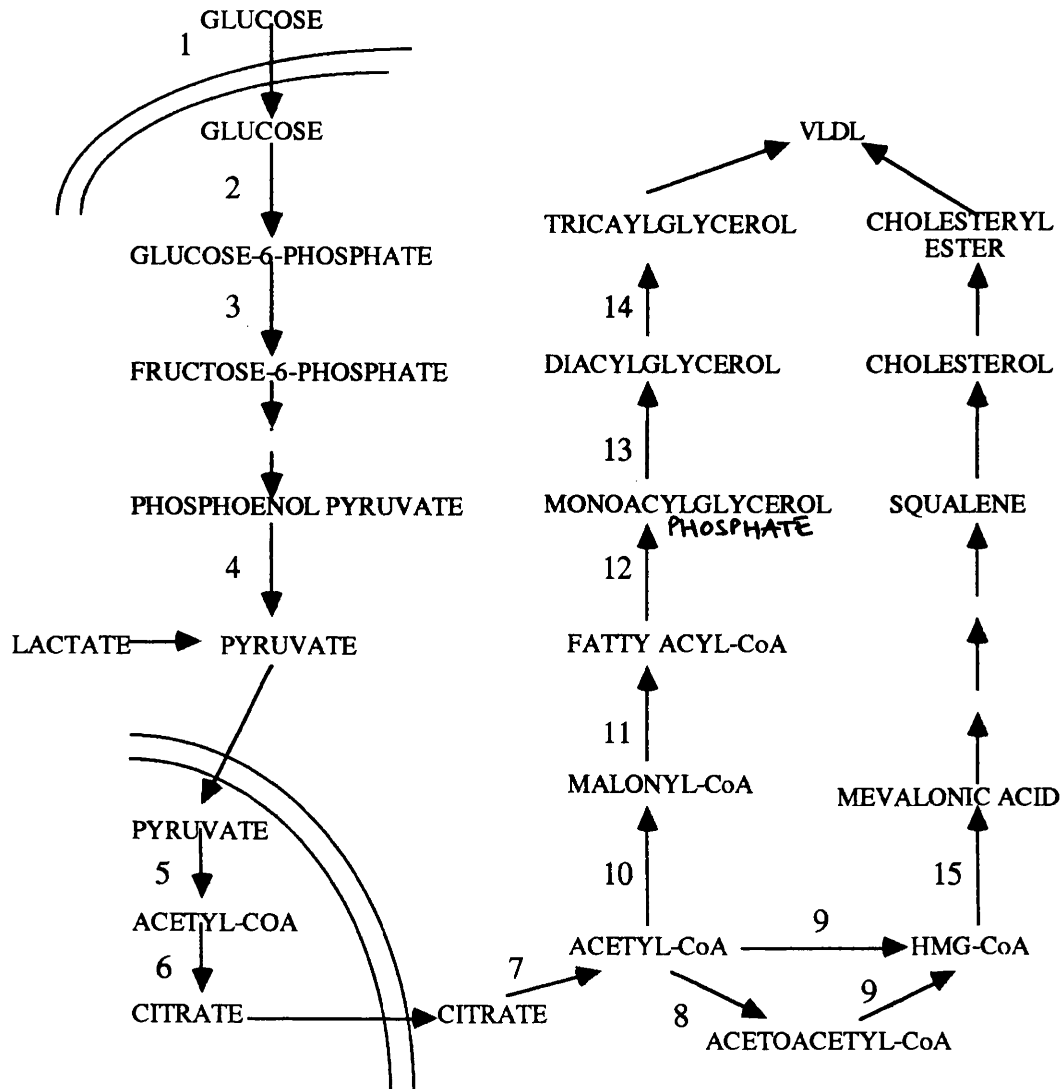
It was originally believed that fatty acid biosynthesis was a simple reversal of the degradative pathway. However the discovery of malonyl-CoA (Wakil, 1958) was the

first step in the elucidation of the fatty acid biosynthetic pathway. ß-oxidation of fatty acids occurs in the mitochondria and peroxisomes whereas biosynthesis occurs almost exclusively in the cytosol, elongation beyond sixteen carbon atoms and also desaturation are accomplished by microsomal enzyme systems. The major pathway of fatty acid synthesis in mammals is illustrated in Fig. 1.1. The first step that is committed to fatty acid synthesis, the carboxylation of acetyl-CoA to malonyl-CoA is catalysed by the biotin containing enzyme acetyl-CoA carboxylase (ACC) which is the rate limiting enzyme for fatty acid synthesis. The process that successively condenses C2 units from malonyl-CoA and reduces them with NADPH is catalysed by the multifunctional enzyme, fatty acid synthase. The mammary gland is unique in that it contains medium chain acylthioesterase (Smith and Abraham, 1975, Libertine and Smith, 1978) which causes early chain termination and thus changes the fatty acid synthase product from C-16 fatty acids to a mixture of C-8 to C-12 fatty acids. Therefore a substantial proportion of the fatty acids synthesized by the mammary gland are of medium chain length. The reasons for the high concentration of medium chain fatty acids in milk are unclear although there is evidence that neonates utilize medium chain triglycerides better than long chain triglycerides (Odle et al, 1989).

Cholesterol

Cholesterol is an important structural component of all cellular membranes and is also a precursor for steroid hormones and biliary steroids. The biosynthesis of cholesterol is a complex pathway of some 25 reaction steps, some of which are summarised briefly in Figure. 1.1. The reactions occur in the cytosol where the condensation of acetyl-CoA with acetoacetyl-CoA forms hydroxymethylglutaryl-CoA (HMG-CoA).

Fig. 1.1. Pathways of Lipogenesis in Mammals



1. Glucose Transporter
2. Hexokinase
3. Phosphoglucoisomerase
4. Pyruvate kinase
5. Pyruvate Dehydrogenase
6. Citrate Synthase
7. ATP-Citrate Lyase
8. Acetoacetyl-CoA Thiolase
9. HMG-CoA Synthase
10. Acetyl-CoA Carboxylase
11. Fatty Acid Synthase
12. Glycerol-3-phosphate Acyltransferase
13. Lysophosphatidate Acyltransferase
14. Diacylglycerol Acyltransferase
15. HMG-CoA Reductase

This is reduced to mevalonic acid which gives rise to 3,3 dimethylallyl pyrophosphoric acid. Successive condensation and reduction of this 5 carbon compound gives rise to the open chain intermediate squalene, which by internalisation of bonds closes 4 rings and ultimately forms cholesterol. Most of the cholesterol formed in higher organisms is esterified at the hydroxyl group with fatty acyl-CoA. The pathway of cholesterol synthesis, in common with the lipogenic pathway (see Fig. 1.1), has cytosolic acetyl-CoA as its precursor and uses NADPH as its reducing power. The rate limiting enzyme of cholesterol synthesis in most tissues is HMG-CoA reductase which catalyses the conversion of HMG-CoA to mevalonic acid.

Approximately 16 mg of cholesterol are secreted in rat milk per day (Clarenburg and Chaikoff, 1966). Cholesterol synthesis is low in the rat mammary gland even though HMG-CoA reductase, the rate limiting enzyme for cholesterol synthesis, has high activity in the tissue (Gibbons et al, 1983). A maximum of 30-40% of the cholesterol secreted into rat milk is synthesized within the mammary gland, and when rats are maintained on a normal, low fat, high carbohydrate chow diet, about 11% is derived from the diet, the rest is synthesized in the liver (Gibbons et al, 1983).

Triacylglycerol

The sequential esterification of sn-glycerol-3-phosphate (supplied by the glycolytic pathway) with three molecules of fatty acyl-CoA is the predominant pathway by which triacylglycerol is formed in tissues (Figure. 1.1).

Tri-acylglycerol is the major form of milk lipid (Davies et al, 1983) and its biosynthesis is an important process. The stereospecific distribution of fatty acids in the tri-

acylglycerol is one of the processes unique to the mammary gland and although this varies according to species, the pattern of distribution in milk usually differs from that in tissue lipids. GPAT appears to have a preference for long chain acyl groups in rat mammary gland (Tanoika et al, 1974), and the respective specificities of GPAT and lysophosphatidate acyl transferase for the sn-1 and sn-2 positions of glycerol appear to direct this stereospecific distribution of fatty acids. The triacylglycerol formed is deposited in a liquid droplet which is ultimately secreted from the apical surface of the cell into the lumen.

1.2. Regulation of Fatty Acid Synthesis

1.2.1 Hepatic Fatty Acid Synthesis

Precursors:

In non-lactating rats, the conversion of glucose to fatty acids in the post-absorptive state is high in adipose tissue. In contrast, in rat liver it appears that neither glucose, nor glycogen, are utilized significantly as precursors for fatty acid synthesis (Geelen and Hendriks, 1984). This may seem a little surprising, but it must be remembered that the liver is normally metabolizing in the set direction of gluconeogensis rather than glycolysis although there is evidence for a zonation of intact liver into periportal "gluconeogenetic" and perivenous "glycolytic" areas (Thurman et al, 1986). The reason for this is probably because the liver places a higher priority on glycogen repletion than on fatty acid synthesis, therefore not only glucose is used for glycogen synthesis but also lactate is diverted firstly for glycogen synthesis and only secondly for fatty acid synthesis (Hardie, 1989).

The major precursor for fatty acid synthesis in the liver is almost certainly lactate. Although a portion of the lactate required for fatty acid synthesis may be derived from erythrocytes and anaerobic muscle metabolism, the major portion comes from the metabolism of glucose in the intestinal mucosa. During starvation, the lactate concentration in the portal vein is 1-2 mM, and this increases to 3-4 mM after a carbohydrate-rich meal (Hopkirk and Bloxham, 1977). If isolated hepatocytes are presented with lactate in this concentration range, they will use it preferentially as a carbon source for fatty acid synthesis and there is little or no flow of carbon from glucose into fatty acids (Clark et al, 1974).

Amino acids are also utilised by isolated hepatocytes as precursors for fatty acid synthesis (Clark et al, 1974), and this is probably significant for animals on high protein diets. This may be especially important for the ketogenic amino acids which are broken down to acetyl-CoA and cannot be used as gluconeogenic precursors.

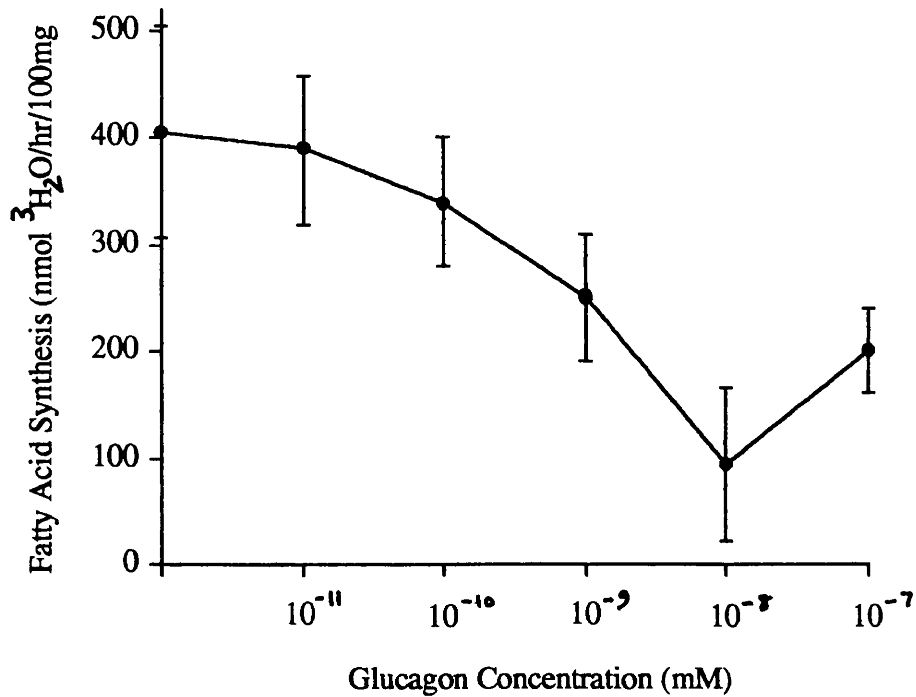
Hormonal Regulation of Hepatic Lipogenesis:

Short term regulation of fatty acid synthesis was first shown in experiments conducted on liver slices (Bloch and Kramer, 1948), with experiments showing that insulin would increase the incorporation of [^{14}C] acetate into fatty acids under certain conditions. This effect of insulin i.e. rapid stimulation of fatty acid synthesis has now been shown in isolated rat hepatocyte preparations where insulin stimulates the rate of fatty acid synthesis by 40% (Holland and Hardie, 1985) and more markedly in isolated rat adipocytes where insulin stimulates the rate of incorporation of radioactivity from glucose into fatty acids by at least 25-fold (Haystead and Hardie, 1988). This

stimulatory effect of insulin is inhibited by glucagon in hepatocytes, and glucagon and adrenaline in adipocytes.

Glucagon has been shown to inhibit fatty acid synthesis in liver in vivo, in the perfused organ and in isolated hepatocytes (for review see Hardie et al, 1984). In isolated rat hepatocytes glucagon inhibits the rate of fatty acid synthesis (measured as the incorporation of radioactivity from tritiated water into saponifiable lipid) by 50% (Holland et al, 1984). The dose-dependent inhibition of fatty acid synthesis by glucagon in rat hepatocytes is shown in Figure. 1.2. The inhibition occurs at physiological glucagon concentrations (with half-maximal effects at approximately 10^{-9}M glucagon). In isolated rat hepatocytes supplied with glucose the action of glucagon on fatty acid synthesis has been explained by its inhibitory effect on glycolysis, which causes a decreased availability of pyruvate and lactate for fatty acid synthesis (Beynen et al, 1979). Under these experimental conditions this is reflected in turn, by a decreased concentration of citrate, a precursor of fatty acid synthesis further down stream in the pathway (Beynen et al, 1979). The addition of lactate and/or pyruvate only partially relieves the glucagon-induced inhibition of fatty acid synthesis whereas the glucagon mediated decrease of cellular citrate levels is totally reversed (Watkins et al, 1977, McGarry et al, 1978). This would suggest that part of the inhibitory effect of glucagon on lipogenesis is exerted at a point beyond the formation of citrate. This idea is supported by the fact that, with some hepatocyte preparations, glucagon inhibits both glycolysis and lipogenesis but also causes an increase in citrate levels (Harris, 1975). Citrate levels probably increase under these conditions because its use for fatty acid synthesis is more greatly inhibited by

Fig. 1.2. Inhibition of Fatty Acid Synthesis by Glucagon



The dose dependent inhibition of fatty acid synthesis in hepatocytes by glucagon is shown above. Fatty acid synthesis was measured as the incorporation of of ^3H from ^3H_2O into saponifiable lipid.
Data taken from Hardie et al (1984).

glucagon than its formation from substrates obtained from glycolysis. The explanation for the fact that lactate relieves glucagon inhibition of lipogenesis is unclear. Lactate, does however, seem to have a hormone-like activity with respect to fatty acid synthesis that is distinct from its role as a precursor of acetyl-CoA i.e. it not only increases the rate of fatty acid synthesis but also enhances the activity of ACC (Table 1.1), this is in agreement with the previously explained role for lactate. Lactate does increase cytosolic citrate levels which may act to modulate the states of polymerisation and/or phosphorylation of ACC.

Table 1.1 Effect of Lactate on Fatty Acid Synthesis and the Activity of ACC in Isolated Hepatocytes

Addition (mM)	Fatty Acid Synthesis (%)	ACC Activity (%)
None	100	100
Lactate (10)	162	183

ACC activity and fatty acid synthesis are expressed in arbitrary units, fatty acid synthesis was measured as $^{3}H_2O$ incorporation into fatty acids. Data taken from Geelen et al., (1980)

However it is also argued that the inhibition of glycolysis is unlikely to be a significant factor in the effects of glucagon on hepatic fatty acid synthesis in vivo, because the major precursor for the pathway is probably extrahepatic lactate (Hardie et al, 1984). It is also thought that a major proportion of the glucose absorbed by the

intestinal mucosa is converted to lactate in that tissue (Nicholls et al, 1983) and therefore, as already stated, lactate concentrations can be as high as 4mM in the portal vein and even in starved animals can be 1-2mM (Hopkirk and Bloxham, 1977). Hardie et al (1984) propose that since glucagon and adrenaline (the latter acting through beta-adrenergic receptors) are examples of hormones that exert their actions through elevating intracellular cAMP concentrations, that cAMP-dependent protein kinase (cAMP-PK) must be involved since it is a major intracellular receptor for cAMP. They therefore suggest that a reversible phosphorylation of ACC is the means by which glucagon and adrenaline exert their inhibitory effects on fatty acid synthesis.

1.2.2 Mammary Gland Fatty Acid Synthesis

Precursors:

In rat adipose tissue and lactating rat mammary gland, glucose is the most important precursor for fatty acid synthesis. At peak lactation (10-15 days post parturition) the mammary gland of the rat utilises of the order of 30 mmol of glucose per day which is roughly equivalent to the whole body glucose consumption of an adult rat of the same weight (Williamson, 1980). Of this 30 mmol, 7 mmol are used for lactose synthesis (Carrick and Kuhn, 1978), another 10% is oxidised completely (Katz and Walk, 1972), the rest is used for lipogenesis, primarily for fatty acid and triglyceride synthesis (Williamson, 1980). The rate of lipogenesis in the lactating rat mammary gland is five times higher, expressed per gram wet weight, than in liver (Robinson et al, 1978b). Glucose metabolism in the mammary gland is therefore stringently regulated to ensure that milk is only synthesised when substrates are available and to make maximum use of available substrates e.g. to use dietary fat instead of de novo

synthesis, for example a high fat diet fed for 7 days depresses mammary lipogenesis by up to 80% and the rat milk composition reflects that of the diet (Grigor and Warren, 1980). These changes are due to inhibition of specific lipogenic enzymes such as ACC (Munday and Hardie, 1986b) and glucose-6-phosphate dehydrogenase (Young et al, 1990) as well as reduced expression of tissue lipogenic enzymes as has been shown for fatty acid synthase, in adipose tissue and liver (Schillabeer et al, 1990).

Hormonal Regulation of Mammary Gland Lipogenesis

The lactating rat mammary gland posseses a large number of insulin receptors (Flint, 1982), and is a highly insulin sensitive and insulin responsive tissue (Burnol et al, 1983, Jones et al, 1984a, 1984b). Lipogenesis in the mammary gland, is sensitive to insulin (Bussmann et al, 1984). Short-term insulin deficiency inhibits the rate of lipogenesis (Robinson and Williamson, 1977a, Freed et al, 1988) and insulin administration with glucose can reactivate lipogenesis in insulin deficient or starved lactating rats (Munday and Williamson, 1981). The number of insulin receptors on lactating mammary gland epithelial cells rises during lactation (O'Keefe and Cuatrecasas, 1974, Flint, 1982) while the numbers of receptors on peripheral adipocytes is unchanged (Flint et al, 1979). Glucose is therefore diverted to the gland. Furthermore, despite its hyperphagia, the lactating rat has a 50% lower plasma insulin concentration than virgin or non-lactating rats (Robinson et al, 1978). However the lactating mammary gland can still maintain its responsiveness to insulin and this relative hypoinsulinaemia may prevent other tissues, such as white adipose tissue, from responding so rapidly to insulin and which may help to divert glucose to

the lactating mammary gland. Signals that are antagonistic to insulin in the lactating rat mammary gland are not well defined. In liver and white adipose tissue glucagon, and glucagon and adrenaline, respectively, are antagonistic to the insulin stimulation of lipogenesis. These hormones elevate cAMP levels and activate cAMP-PK (Holland et al, 1984). The role of cAMP in mediating hormonal effects in lactating mammary gland is less clearly defined. Hormones that act via raised cAMP concentrations have no effect on the rate of fatty acid synthesis in the lactating mammary gland in vivo (Bussman et al, 1984) or in isolated mammary acini in vitro (Williamson et al, 1983). Mammary gland has no glucagon receptors (Robson et al, 1984) but does possess a complete and competent cAMP signalling system. In the presence of ß-adrenergic agonists and phosphodiesterase inhibitors it is possible to raise the basal cAMP level of mammary acini by 20-fold (Clegg and Mullaney, 1985). However these elevated cAMP levels have no effect on the rate of lipogenesis in these acini (Clegg et al, 1986) even though cAMP-PK is activated and phosphorylase is inactivated (Clegg and Ottey, 1990). The role of cAMP and cAMP-PK is discussed in Chapter 3.

Prolactin appears to have no role in the short term regulation of mammary lipogenesis, changes in its plasma concentration do not always parallel changes in lipogenesis (Robson et al, 1978b). It appears to be involved in the longer-term control of lipogenic enzyme concentrations (McNeillie and Zammit, 1982), and there is evidence that it mediates the insulin resistance of adipose tissue which occurs during lactation (Ros et al, 1990).

Other hormonal signals e.g. thyroxine and corticosteroids play no role in the acute

regulation of lipogenesis, they are involved in the initiation and maintenance of lactogenesis and also mammary gland development during pregnancy and early lactation (Topper and Freeman, 1980).

1.3 Regulatory Steps in the Pathway of Fatty Acid Synthesis

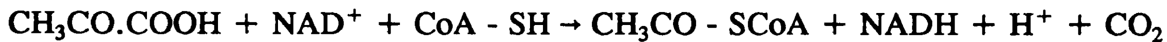
From Fig. 1.1 it is clear that there a number of steps in the lipogenic pathway that could be subject to short-term hormonal regulation. In the lactating mammary gland where glucose is converted to fatty acid these could be the rate of glucose transport across the plasma membrane, and/or the relative activities of hexokinase, 6-phosphofructo-1-kinase (6-PF-1-K), pyruvate dehydrogenase (PDH), and acetyl-CoA carboxylase (ACC). In the liver since lactate is the major precursor of fatty acid synthesis the steps likely to be under short-term hormonal control are those catalysed by PDH and ACC. Whilst any of these steps may be important in regulating fatty acid synthesis ACC catalyses the step that irreversibly commits substrate to fatty acid synthesis and the discussion herein focuses mainly on ACC and its regulation together with a brief discussion of PDH. The importance of these enzymes in lipogenic regulation will be discussed below.

1.3.1 Pyruvate Dehydrogenase Complex (PDH)

The PDH complex is located in the inner mitochondrial matrix space. It is a multienzyme complex which catalyses the oxidative decarboxylation of pyruvate to

acetyl-CoA via a co-ordinated series of reactions. The overall reaction is essentially irreversible and regulates the supply of acetyl-CoA which is available for oxidation for fatty acid synthesis or for lipid biosynthesis when transported via citrate to the cytosol.

The PDH reaction is catalysed sequentially by three components of the complex. Pyruvate decarboxylase (E1); dihydrolipoyl transacetylase (E_2) and dihydrolipoyl dehydrogenase (E_3). The overall reaction catalysed is described below:



$$CH_3CO.COOH + NAD^+ + CoA - SH \rightarrow CH_3CO - SCoA + NADH + H^+ + CO_2$$

The structure of the PDH complex from mammary gland has not been studied but so far PDH structure has shown only small variations in different tissues. Three to five molecules of PDH kinase and phosphatase are associated with the complex. The kinase is tightly bound to E_2 and copurifies with the PDH complex. The phosphatase only binds to E_2 in the presence of Ca^{+2} (Pettit et al, 1972). The PDH complex is inhibited when phosphorylated by its intrinsic kinase (Sugden and Randle,1978).

The PDH complex is also inhibited by its reaction products acetyl-CoA and NADH (Pettit et al, 1975, Kerbey et al, 1976, 1977). End product inhibition may play a role in the acute regulation of PDH in lactating mammary gland but is believed to be quantitatively less important than regulation by reversible phosphorylation (Munday and Hardie, 1987). Moreover the relative importance of direct end product inhibition is difficult to quantify because elevated $NADH/NAD^+$ and acetyl-CoA/CoA ratios

stimulate PDH kinase (Pettit et al, 1975).

For many years it was believed that there existed a PDH kinase activator protein, KAP (Kerbey and Randle, 1982). KAP had been defined as a distinct protein product which was slow in its onset of activity and required new protein synthesis (Kerbey and Randle, 1982). Furthermore, in mammary gland, synthesis of KAP was reported to increase in response to starvation and diabetes (Kerbey and Randle, 1981, 1982, Kerbey et al, 1984), this was logical given that KAP was thought to render PDH-kinase insensitive to pyruvate inhibition (Hutson and Randle, 1978). However recent reports have identified KAP as free PDH kinase (Mistry et al, 1991, Jones and Yeaman, 1991), although the physiological role of such a form remains unexplained.

Hepatic PDH, in the active form, increased from 12% in the fed state by 77% upon short-term exposure to insulin (0.5hr) and PDH activity decreased significantly from 12% by 75% upon 6-hr starvation and this decrease was accompanied by a decrease in lipogenesis. The hepatic PDH activity after short-term exposure to insulin (0.5hr) was less in 6-hr starved than in fed rats but the absolute increases in activity were similar over the time course of insulin exposure. Increases in PDH activity were matched by increases in lipogenesis. Reactivation of PDH in 48-hr starved rats was complete upon 4-hr chow refeeding or insulin treatment and was accompanied by increased lipogenesis (Holness et al, 1988 and Holness and Sugden, 1990).

The link between lipogenesis and the covalent modification of PDH is illustrated by the three fold increase in the proportion of active complex which occurs from

parturition to peak lactation (Coore and Field, 1974). This change is superimposed on a 7-fold increase in the total amount of PDH over the same period. Thus the mammary gland has the potential for a 21-fold increase in the rate of acetyl-CoA synthesis. Lipogenesis and PDH activity in the mammary gland falls when lactating rats are made insulin deficient (Field and Coore, 1976) or starved (Kankel and Reinauer, 1976, Baxter and Coore, 1978) and is rapidly restored by insulin treatment (Field and Coore, 1976) or by refeeding (Munday and Williamson, 1981). These changes are mediated by reversible phosphorylation of PDH. The proportion of mammary PDH in its active (dephosphorylated) form falls by more than 70% after 24 hours starvation (Kankel and Reinauer, 1976, Baxter and Coore, 1978), furthermore starvation and insulin deprivation both correlate with an increase in PDH kinase activity (Baxter and Coore, 1978) and a reduction in PDH phosphatase activity (Baxter and Coore, 1979a). It has been shown that PDH kinase from the mammary tissue of starved rats has lost its sensitivity to pyruvate inhibition (Baxter et al, 1979, Baxter and Coore, 1979a).

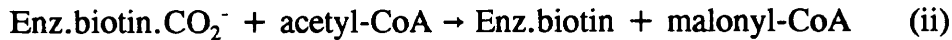
1.3.2. Acetyl-CoA Carboxylase

Structure and Function

Acetyl-CoA carboxylase (ACC) is a cytosolic biotinyl enzyme whose activity was first described by Wakil in 1958. ACC catalyses the committed step in the synthesis of fatty acids from acetyl-CoA, namely the carboxylation of acetyl-CoA to malonyl-CoA. Malonyl-CoA is not used in any pathway other than fatty acid synthesis. The overall

reaction catalysed by ACC is the sum of two partial reactions:

$$ATP + HCO_3^- + Enz.biotin \rightarrow Enz.biotin.\ CO_2^- + ADP + Pi \quad (i)$$



$$Enz.biotin.CO_2^- + acetyl\text{-}CoA \rightarrow Enz.biotin + malonyl\text{-}CoA \quad (ii)$$

It has been shown that mammalian ACC, purified from liver, adipose and mammary gland tissue is composed of a single type of subunit (of molecular mass 250 KDa), which forms a homodimer of molecular mass 500 KDa (Tipper and Witters, 1982). However, Bianchi et al (1990) have recently identified a biotin containing cytosolic protein with a M_r of 280 KDa which they suggest is an isozymic form of ACC. The ACC 280 KDa form is uniquely expressed in rat cardiac and rat skeletal muscle but is co-expressed with the ACC 250 KDa form in rat liver, mammary gland and brown adipose tissue. In the fed rat white adipose tissue contains solely the 250 KDa form. The two isozymes differ in that the 280 KDa form has a higher K_a for citrate (concentration of citrate required for half-maximal activation) and a higher K_m for acetyl-CoA than the 250 KDa form. The explanation for these observations is not clear but it may be due to varying degrees of phosphorylation. Bianchi et al (1990) speculate that since the 280 KDa form is found in cardiac and skeletal tissue, where rates of fatty acid synthesis are low, it may have an alternative role in these tissues such as the regulation of fatty acid oxidation or microsomal fatty acid elongation.

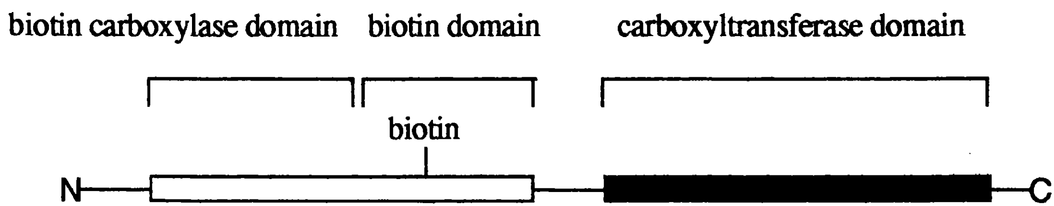
The partial reactions, shown above, can still be measured independently using radio isotope exchange reactions, and it seems very likely that ACC from eukaryotic sources is a multifunctional polypeptide that contains two active sites which have been combined by gene-fusion events. Data obtained from the recent cloning and

sequencing of cDNAs encoding rat and chicken ACC have supported this view (Takai et al, 1988). A weak homology exists between the amino terminal region of carboxylases and carbamoyl phosphate synthetase (Figure 1.3). Carbamoyl phosphate synthetase catalyses an ATP-dependent carboxylation reaction analogous to reaction (i) above, therefore it is possible the amino terminal regions may represent the biotin carboxylase domain. The biotin binding region is the centre of the molecule and this probably corresponds to a carboxyl carrier domain. A weak homology exists in the carboxyl-terminal region with the ß-subunit of propionyl-CoA carboxylase (Fig. 1.3). Propionyl-CoA carboxylase contains distinct α and ß subunits, with the α-subunit containing the biotin and being able to catalyze the biotin carboxylase reaction but not the overall reaction (Haase et al, 1982). It is therefore possible that the ß-subunit of propionyl-CoA carboxylase is the carboxyl transferase subunit and that the carboxyl-terminal domain of ACC may be the carboxyl transferase domain of ACC. ACC can be cleaved by proteinases to yield two fragments of an approximate molecular weight of 120 KDa (Wada and Tanabe, 1985). The sites of cleavage have not been clearly defined but it would appear likely that they are between the putative carboxyl carrier and carboxyl transferase domains and that this cleavage may occur because there is an exposed 'hinge-region' between the domains.

Long Term Regulation

The activity of ACC is regulated in the long term by varying the quantity of enzyme in the cell. Hicks et al, (1965) showed that the rise in ACC concentration that occurs in liver upon refeeding after starvation can be prevented by treatment with puromycin,

Fig. 1.3. Model of the Domain Structure of Acetyl-CoA Carboxylase



A model for the domain structure of ACC is shown above and is based on the location of the covalently-bound biotin, and homologies with carbamoyl phosphate synthetase and propionyl-CoA carboxylase.
Modified from Hardie (1989).

a protein synthesis inhibitor or actinomycin D, a transcription inhibitor. Nakamishi and Numa (1970) showed that the amount of immunotitratable ACC in rat liver is more than halved by a 48 hour fast and increased nearly 3-fold above control values when the 48 hour fasted animals were refed with a fat-free diet. This was due to simultaneous changes in the rate of degradation and synthesis of ACC (Table 1.2). Similarly, diet induced changes in the concentration of ACC in liver or epididymal fat pads correlate positively with changes in the amount of ACC mRNA (Pape et al, 1988).

After parturition rat mammary ACC content and activity increases as a result of increased synthesis of the enzyme (MacKall and Lane, 1976). This is accompanied by a 7-fold elevation in translatable ACC mRNA (Lopez-Cassillas, 1987).
The mechanism controlling the gene expression of ACC and also the turnover of the enzyme have not been fully elucidated though anabolic hormones have a role to play. The absence of either glucocorticoids, insulin or thyroxine significantly reduces the increase in lipogenic capacity that occurs in liver upon refeeding after a fast of more than 36 hours (Wurdeman et al, 1978, Bouillon and Berdanier, 1980). Furthermore transcription and translation of the enzyme glucose-6-phosphate dehydrogenase is increased by these hormones (Fritz et al, 1986, Kletzien et al, 1985, Fritz and Kletzien, 1987). It is highly probable that other lipogenic enzymes including ACC are influenced similarly.

Table 1.2 Degradation Rates of ACC

	Total ACC (arbitrary units)	t½ for degradation (hours)	Relative rate of synthesis (hours)
Control	1.0	59	1.0
48 hour fast	0.5	31	0.5
48 hour fast/ 72 hour refed fat free diet	2.7	55	4.0

Total ACC was measured by immunotitration and synthesis was estimated by ^{3}H-leucine incorporation,(data from Nakimishi and Numa, 1970).

Short Term Regulation

Allosteric Modification

ACC can be allosterically activated in vitro by citrate (Martin and Vagelos, 1962, Moss and Lane, 1971) and inhibited by fatty acyl-CoA thioesters (Bortz and Lynen, 1963, Lunzer et al, 1977, Ogiwara et al, 1978). This evidence would suggest that feed forward activation by citrate (the immediate precursor for acetyl-CoA) and end product inhibition by fatty acyl-CoA thioesters is a plausible model for ACC regulation.

There is no conclusive evidence showing a close correlation between fatty acid synthesis and citrate levels. Nishikori et al, (1973) reported a rapid rise in citrate, ACC and fatty acid synthesis in the livers of starved rats in response to refeeding. In contrast, in rat epididymal fat pads, stimulation of fatty acid synthesis by insulin was associated with an unchanged or decreased tissue concentration of citrate whereas adrenaline caused an increase in citrate concentrations along with a decrease in fatty acid synthesis (Denton and Halperin, 1968, Saggerson and Greenbaum, 1970, Halestrap and Denton, 1974). Fluoroacetate is metabolised to fluorocitrate, a potent inhibitor of aconitase, and increases citrate levels ten-fold in epididymal fat pads without increasing ACC activity (Brownsey et al, 1977). Prolonged starvation of lactating rats increases tissue citrate concentrations over a time course during which ACC is markedly inhibited (Hagopian et al, 1991).

An inverse relationship between the rate of fatty acid synthesis and intracellular levels of fatty acyl-CoA esters has been demonstrated by some authors e.g. Denton and Halperin (1968) and Saggerson and Greenbaum (1970) using rat epididymal fat pads treated with insulin, and Nishikori et al, (1973) in the livers of starved rats refed a fat-free diet. Others find no such relationship (Jacobs and Majerus, 1973). The role of fatty acyl-CoA esters in vivo will be extremely difficult to resolve largely because the true free intracellular levels are undetermined. They are modulated by fatty acid binding proteins (Lunzer et al, 1977) and compartmentalization within the cell.
Several other potential regulators of ACC have been described. These include GTP and other guanine nucleotides (Witters et al, 1981, Beuchler and Gibson, 1984), polyphosphoinositides especially phosphatidyl inositol 4,5-bisphosphate (Heger and

Peter, 1977, Blyth and Kim, 1982), an autocrine factor tentatively identified as an oligosaccharide (Witters et al, 1988), small molecular weight substances released from liver plasma membranes by insulin binding (Saltiel et al, 1983), and ADP-ribosylation (Witters and McDermott, 1986). The physiological relevance of these potential effectors to the regulation of ACC and fatty acid synthesis is, as yet, unclear.

In the presence of the allosteric activator, citrate, ACC polymerises to form linear helices of up to 32 dimers in length in an extended helical array (Ahmad et al, 1978). These polymers have been observed by electron microscopy, viscometry and ultracentrifugation of the purified enzyme and can also be detected in crude extracts by gel filtration or sucrose gradient velocity sedimentation (Ahmad and Ahmad, 1978). It was widely accepted that the polymeric species was the only active species of ACC but recent experiments have shown that the activation of the ACC subunits by citrate precedes their polymerisation (Beaty and Lane, 1983). There is evidence that the polymerisation of ACC occurs when it is activated irrespective of the presence of citrate. Thus the ACC activation by Coenzyme A (Yeh et al, 1981) or limited trypsinolysis (Iritani et al, 1969) both induce polymerisation. Borthwick et al, (1987) have reported that ACC from insulin-treated adipose tissue which has an elevated ACC activity is more highly polymerised, estimated by gel filtration on FPLC, in the absence of citrate than that from control tissue or tissue treated with the ß-agonist isoprenaline which inhibits ACC. Ashcraft et al, (1980) have reported that, in avian liver, fat feeding or starvation, both of which decrease lipogenesis, cause a decrease in the amount of polymeric ACC in vitro. Thus indicating that the polymerisation of ACC may be of significance in vivo. Indeed Tanake et al, (1977) have reported that ACC is less susceptible to proteolysis in the presence of citrate which may mean that

it is protected by polymerisation.

Allred and coworkers have published several reports describing the regulation of ACC by the translocation of an inactive form of the enzyme from the mitochondrial outer membrane to the cytosol whereupon the enzyme becomes active (Allred and Roman-Lopez, 1988). The proportion of active to inactive ACC is decreased by fasting and increased by refeeding (Allred et al, 1985, Roman-Lopez et al, 1989). These workers also show short-term insulin deficiency increases the amount of inactive ACC associated with the mitochondria (Roman-Lopez and Allred, 1987) thus providing a mechanism for the decreased lipogenesis seen in diabetic animals. However Moir and Zammit (1991) claim to observe no translocation from the mitochondria to the cytosol in response to refeeding in rat liver. It has also been shown that the increased activity characteristic of genetically obese Zucker rats is not due to a greater amount of enzyme but is the result of a greater proportion of ACC being in the active cytosolic-form. There are no reports addressing possible interactions between enzyme translocation, and the better understood allosteric and covalent modification mechanisms of controlling ACC activity.

Covalent Modification

The first indications that ACC is phosphorylated in vivo came from reports that ACC purified from rat liver contained covalently bound phosphate (Inoue and Lowenstein, 1972) and from the observations that crude preparations of rat liver ACC became inactivated in a time-dependent fashion when incubated with MgATP (Carlson and Kim, 1973). Using antiserum against ACC, they later showed that the inactivation was

associated with the incorporation of radioactivity from [$\gamma^{32}P$] ATP into immunoprecipitable protein. They further showed that both the inactivation and the incorporation of radioactive phosphate was reversed by treatment with a protein phosphatase preparation.

Phosphorylation was clearly apparent in control hepatocyte cells incubated without hormones but was also stimulated by glucagon. In experiments on enzyme purified to homogeneity glucagon treatment was shown to increase the phosphate content of ACC from 4.5 to 5.2 mol per subunit and this increase in phosphate was associated with a decrease in the Vmax and an increase in the Ka for citrate. At physiological citrate concentration (0.5mM) the degree of inhibition is about 50% which correlates well with the decrease in the rate of fatty acid synthesis observed under the same conditions (Hardie et al, 1984). Phosphorylation of ACC in isolated rat adipocytes was first reported by Brownsey et al (1979), both glucagon and adrenaline cause phosphorylation of ACC increasing the phosphate content from 3.3 mol per subunit to 3.7 and 4.7 mol per subunit respectively. This resulted in a decreased ACC Vmax, increased Ka for citrate and inhibition of fatty acid synthesis (Holland et al, 1984, 1985).

Insulin is the counter-regulatory signal to glucagon and adrenaline in hepatocytes and adipocytes and therefore stimulates the rate of lipogenesis (Hardie et al, 1984). The well characterised effect of phosphorylation on ACC is to decrease activity therefore it would be expected that insulin would cause a dephosphorylation and activation of ACC resulting in the stimulation of lipogenesis. However it was reported that insulin

increased the phosphorylation of ACC at a site termed the 'I' site (Brownsey and Denton, 1982). This was confirmed in adipocytes (Haystead et al, 1988) and hepatocytes (Holland and Hardie, 1985).

It was also reported (Brownsey et al, 1981) that incubation of an adipocyte membrane fraction with partially purified adipocyte ACC produced a time and MgATP-dependent activation of the ACC. They also reported a phosphorylation of the ACC but this preceded the activation. This group reported increased ACC activity in cell free extracts of cells treated with insulin and, although there was no evidence that this was related to the membrane bound kinase, proposed a model in which insulin caused the translocation from the membrane to the cytosol of a kinase which phosphorylated and activated ACC. It is also accepted that under conditions where insulin does bring about activation of ACC in adipocytes it does not bring about dephosphorylation. It has recently been reported (Witters et al, 1988) that in the insulin-sensitive Fao Reuber hepatoma cell line, insulin does cause an activation of ACC which is stable during purification and involves dephosphorylation of the peptide containing the site phosphorylated in response to glucagon in vivo. Similar observations have been made in mammary cells (Munday and Hardie, 1986b). It has been suggested that the rapid effects of insulin on ACC activity in adipocytes must be due to changes in the concentration of a small molecular weight effector, which at present remains unidentified. Saltiel et al (1983) have reported that a low molecular weight activator of ACC is released when rat liver membranes are incubated with insulin, however this remains unproven and the true mechanism of insulin action remains to be discovered. Another possible explanation is that insulin may bring about phosphorylation followed

by polymerisation and thus activation of ACC leading to stimulation of lipogenesis.

Starvation is a physiological situation which results in the inhibition of lipogenesis in rat liver and this inhibition is reversed upon chow refeeding for 2-4 hours (Holness et al, 1988, Holness and Sugden, 1990). It has been shown in the livers of starved rats that the concentration of ACC is decreased and this is probably due to both decreased synthesis and increased degradation (Nakanishi and Numa, 1970) but it has also been shown that the phosphorylation state of hepatic ACC varies with nutritional condition. Hepatic ACC activity decreased upon starvation and there was a concomitant increase in phosphate content with a value of 7.6 molP/ mol subunit obtained for 48-hour starved rats. Refeeding of the starved rats resulted in increased hepatic ACC activity and a decreased phosphate content of 5.1 molP/ mol subunit after 24-hour refeeding. ACC purified from the livers of 48-hour starved rats could be reactivated to fed control levels upon dephosphorylation with protein phosphatases therefore suggesting that the low activity of hepatic ACC from starved rats was due to an increased phosphate content and that ACC is regulated by phosphorylation under physiological situations such as starvation (Thampy and Wakil, 1988).

Starvation for 24 hours and feeding a high fat diet are two situations which result in inhibition of fatty acid synthesis in lactating rat mammary gland. The inhibition upon starvation is totally reversed by 2 hour refeeding, an effect which is totally abolished in streptozotocin-diabetic animals (Robinson et al, 1978) which suggests that the inhibition is due to low plasma insulin concentrations. It has been shown (Munday and Hardie, 1986, Munday and Hardie, 1987) that the decrease in ACC activity due to both starvation and high fat feeding results from increased phosphorylation, although

in the case of 24 hour starvation there is also some decrease in total enzyme concentration. The increased phosphorylation could be reversed by protein phosphatase-2A. The total phosphate content of enzyme purified from chow-fed rats increased from 3.3 to 4.5 mol per subunit after 24 hour starvation and 4.3 mol per subunit after 14 days on a high fat diet. The low specific activity and the high phosphate content from 24 hour starved rats were both reversed by refeeding for 2 hours but this did not occur in rats made diabetic with streptozotocin (Munday and Hardie, 1987). This shows that the effects of starvation or fat feeding on ACC activity are due to increased phosphorylation and at least in the case of the starved animals may be due to their low plasma insulin concentrations. The effects of fat feeding in vivo may be due to changes in the plasma concentration of triglycerides rather than in the plasma insulin concentration but treatment of isolated acinar cell preparations with insulin can reverse the effects of high fat feeding (Munday and Hardie, 1986b). Insulin causes both an increase in the specific activity and a decrease in the phosphate content of ACC purified from the cells.

The regulation of ACC by phosphorylation-dephosphorylation is now firmly established as a method of controlling ACC activity and is probably the major mechanism of ACC regulation in vivo. Purified ACC has been shown to be phosphorylated in vitro by at least seven distinct protein kinases. These will now be discussed followed by the protein phosphatases which dephosphorylate and activate ACC.

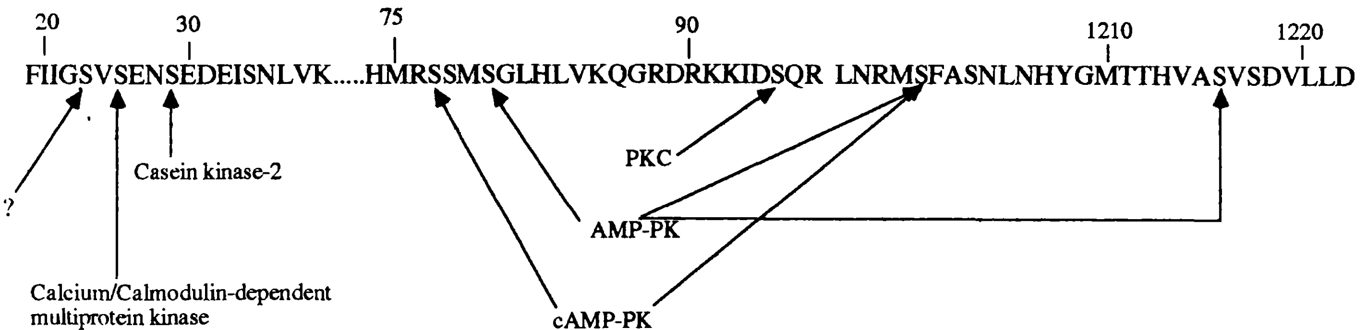
Protein Kinases that Phosphorylate ACC

Casein Kinases-1 and 2

Casein Kinases-1 and 2 have now been purified and characterised from a number of tissues and appear to have a widespread distribution (Hathaway and Traugh, 1982). These protein kinases are assayed against commercial casein as a substrate which has led to their naming. However, there is a casein kinase in mammary gland whose physiological role is the phosphorylation of milk casein and it does not utilize commercial casein as a substrate unless it is first partially dephosphorylated and it is likely to be a distinct protein kinase from casein kinases-1 and 2 (Mackinley et al, 1977). Casein kinases-1 and 2 are present in rat mammary gland (Munday and Hardie, 1984).

There is no effect on the kinetic properties of ACC as a result of phosphorylation by either casein kinase-1 or 2, (Munday and Hardie, 1984) therefore the physiological significance of these reactions is unclear. Casein kinase-2 has been shown to phosphorylate at a single site on ACC which is termed Serine-29 (Haystead et al, 1988) which is adjacent to the site phosphorylated by calmodulin-dependent protein kinase (Fig. 1.4). Serine-29 is also phosphorylated in intact, isolated adipocytes and phosphorylation at this site increases in response to insulin (Haystead et al, 1988). Phosphorylation at serine-29 fits well with the consensus sequence that has been established for casein kinase-2 i.e. a serine residue immediately followed, C-terminally, by a number of acidic residues (Marin et al, 1986). At present the casein-kinase-1 phosphorylation site on ACC has not been fully characterised.

Fig. 1.4. Map of the Phosphorylation Sites on Acetyl-CoA Carboxylase



The location of phosphorylation sites on ACC is shown above. The regions of sequence containing the phosphorylation sites have been illustrated in detail utilizing the single letter amino acid code. The question mark indicates the serine residue found phosphorylated in intact adipocytes (Haystead et al, 1988). The protein kinase that is responsible for phosphorylating this site has yet to be identified. PKC = Protein kinase C, cAMP-PK = cyclic AMP-dependent protein kinase and AMP-PK = AMP-activated protein kinase. Modified from Davies et al (1990)

The Calcium Calmodulin-dependent Multiprotein Kinase

This protein kinase (sometimes named the calcium calmodulin-dependent protein kinase II) was purified and characterised independently in several different laboratories and from several different tissues (Cohen, 1988). The enzyme possesses at least three different isozymic forms (Shenolikar et al, 1986) but all three isozymes possess apparently identical substrate specificities. Substrates which are phosphorylated in vivo include enzymes (e.g. glycogen synthase and tyrosine hydroxylase), cytoskeletal proteins (e.g. tubulin and microtubule associated protein-2) and proteins involved in neurotransmitter release (Synapsin-1). This broad specificity contrasts with the very restricted substrate specificity of myosin light chain kinase and phosphorylase kinase, which are two other calcium and calmodulin dependent protein kinases (Cohen, 1988).

The calcium calmodulin-dependent multiprotein kinase phosphorylates ACC purified from rat mammary gland in a calcium and calmodulin dependent manner (Hardie et al, 1986). The phosphorylation is stoichiometric and, at concentrations of protein substrate of 2 μM, the initial rate is approximately 20% of the initial rate of phosphorylation of glycogen synthase by this kinase. Hardie and coworkers (1986) were unable to show any alteration in the Ka for citrate, or in the Vmax, of ACC as a result of phosphorylation by this protein kinase. The major site of phosphorylation was found to be at serine-25 of rat ACC (Lopez-Casillas et al, 1988). This is a surprise, since it does not fit with the sequence Arg-X-Ser(P) which is thought to be the minimal recognition sequence for calcium calmodulin-dependent multiprotein kinase (Pearson et al, 1985). So it would appear that phosphorylation at serine-25 has no effect on the activity of ACC.

Protein Kinase C

Protein kinase C was originally found in rat brain extracts and described as being active only after treatment with proteases. It was later reported that the enzyme could be activated reversibly (i.e. without proteolysis) by calcium and a crude sonicated suspension of membrane phospholipids (Takai et al, 1979). It was later found that full activity could be achieved at physiological calcium concentrations using suspensions of pure phosphatidyl-serine containing trace amounts of diacylglycerol (Kishimoto et al, 1980). Protein kinase C is activated by phorbol esters (Castagna et al, 1982) and may be the only high affinity "receptor" for phorbol esters in cells (Parker et al, 1984).

Protein kinase C phosphorylates ACC stoichiometrically which gives a modest depression of Vmax (Hardie et al, 1986). The inactivation is completely dependent on the presence of calcium and phospholipid and is reversed by protein phosphatase-2A, therefore the inactivation must be through a phosphorylation reaction. Protein kinase C phosphorylates two sites on ACC, identified as serine-77 and serine-95 (Haystead and Hardie, 1988, Lopez-Casillas et al, 1988). Since serine-77 is also phosphorylated by cAMP-PK (Munday et al, 1988) it is possible that phosphorylation at this serine residue is responsible for the lowered Vmax produced by protein kinase C.

Although protein kinase C does cause a small inhibition of ACC activity it is not likely to be the physiological kinase that regulates ACC activity in the intact cell. There is no phosphorylation of ACC in intact cells at serine-77 or serine-95 even after

phorbol ester stimulation (Haystead and Hardie, 1988). Significantly serine-95 is the only one of the nine phosphorylation sites on the rat ACC which is not conserved in the predicted sequence of the chicken enzyme where it is replaced by a valine (Takai et al, 1988).

Cyclic AMP-dependent Protein Kinase (cAMP-PK)

The free catalytic subunit of cAMP-PK purified from rabbit skeletal muscle or bovine heart was the first enzyme identified to phosphorylate highly purified ACC (Hardie and Cohen, 1978, Munday et al, 1988). There was stoichiometric phosphorylation which was completely blocked by the heat stable protein inhibitor of cAMP-PK (Munday and Hardie, 1984). It was originally believed that there was a single major phosphorylation site (Holland et al, 1984), however recent amino acid sequencing studies in the Hardie laboratory have shown that there are in fact two sites of phosphorylation (Munday et al, 1988). By comparison with the complete sequence of the rat enzyme, derived from cDNA cloning, these phosphorylation sites are identified as serine-77 and serine-1200 (Fig. 1.4). Furthermore these sites are conserved in the sequence of chicken ACC, with the corresponding residues being at serine-80 and serine-1193.

cAMP-PK phosphorylates and inactivates ACC. This inactivation is a modest decrease in Vmax coupled with an increase in the Ka for citrate (Munday et al, 1988). The maximum effects of phosphorylation are, therefore observed at low, subsaturating concentrations of citrate (0.1 - 1 mM) which corresponds well with the range of estimated physiological concentrations of citrate in hepatocyte cytosol (Holland et al,

1984). The inactivation is definitely a result of the phosphorylation since it can be reversed by dephosphorylation using purified protein phosphatases (1, 2A or 2C) (Ingebritsen et al, 1983) and is prevented by the protein inhibitor of cAMP-PK (Brownsey and Hardie, 1980).

It has been shown that ACC purified from rat or rabbit mammary gland, rat liver or rat adipose tissue is phosphorylated and inactivated in vitro by cAMP-PK purified from skeletal or heart tissue (Hardie, 1989). Some workers however, have claimed that they observe no direct phosphorylation and inactivation of ACC by cAMP-PK (Lent and Kim, 1983) and have suggested that ACC is in fact phosphorylated by a specific ACC kinase which is itself phosphorylated and activated by cAMP-PK. It has also been shown (Clegg and Ottey, 1990) that activation of cAMP-PK in vivo has no effect on ACC phosphorylation. The available evidence now suggests very strongly that direct phosphorylation of ACC by cAMP-PK does not occur (See Chapter 3).

Acetyl-CoA Carboxylase Kinase-2 (ACK-2)

ACK-2 was originally identified as a contaminant of ACC preparations purified from rabbit mammary gland and has been partially purified (Munday and Hardie, 1984). This enzyme has not yet been fully characterised but it can be distinguished from the catalytic subunit of cAMP-PK, since it has an apparent molecular weight on gel-filtration of 76,000 Da compared to 40,000Da for the catalytic subunit of cAMP-PK, is sensitive to inhibition by heparin and is completely insensitive to the specific inhibitor of cAMP-PK (Munday and Hardie, 1984). It was initially reported that the phosphorylation sites for cAMP-PK and ACK-2 were similar, judged by HPLC

analysis of chymotryptically derived phosphopeptides (Munday and Hardie, 1984). Recent experiments utilizing a double digestion protocol (trypsin + chymotrypsin) have confirmed that ACK-2 does phosphorylate sites within the TC1 and TC2 peptides although TC2 is phosphorylated much more rapidly than TC1. This is different to cAMP-PK which phosphorylated both peptides at similar rates. Amino acid sequencing has confirmed that serine-1200 is the major site of phosphorylation by ACK-2 (Munday et al, 1988). Both cAMP-PK and ACK-2 have similar effects on ACC activity (mainly an increase in Ka for citrate) suggesting this effect may be through a phosphorylation at serine-1200. The exact physiological role of ACK-2 remains unclear (see also Chapter 4).

The AMP-activated Protein Kinase

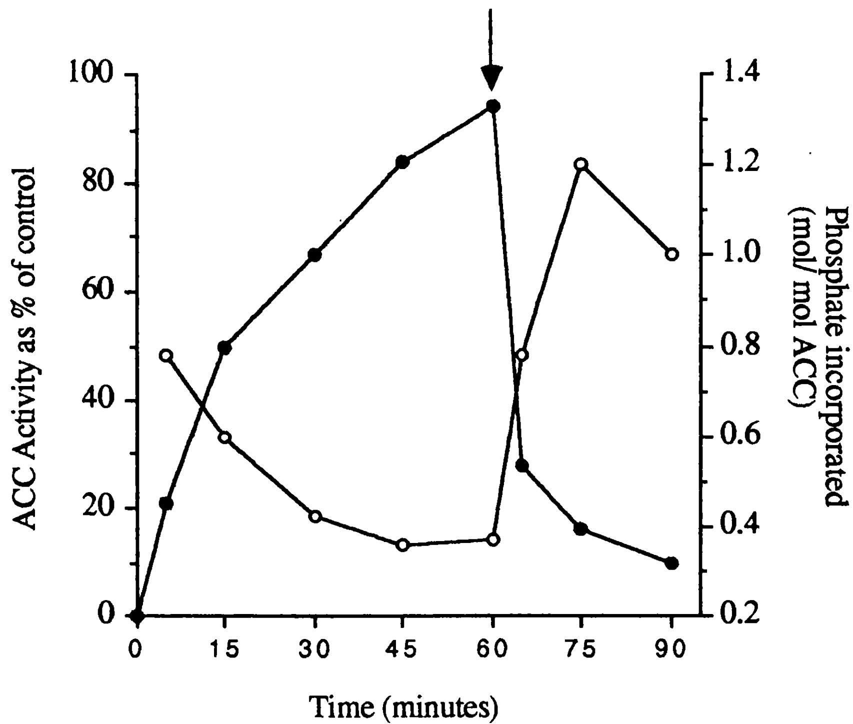
From studies conducted on rat hepatocytes it was clear that ACC was present in a low specific activity form that had a high phosphate content (4-5 molecules phosphate per subunit), even in cells under basal conditions (Holland et al, 1984). A proportion of the phosphate content was clearly of regulatory importance because treatment of the purified enzyme with protein phosphatases produced a significant activation as shown by an increase in Vmax and a decrease in Ka for citrate (Holland et al, 1984, Sim and Hardie, 1988). This basal endogenous phosphorylation could not have been produced by cAMP-PK or by ACK-2 since neither produced such a large inhibition of Vmax in vitro. This therefore suggested the presence of another ACC kinase in rat liver.

Although there are a number of reports of rat liver kinase activities that produce large decreases in the V_{max} of ACC (Allred et al, 1983, Jamil and Madsen, 1987, Lent and

Kim, 1982, Shiao et al, 1981) only the kinase activity described by Lent and Kim was characterised in any detail. These workers reported that kinase activity copurified with ACC on polyethylene glycol precipitation and DEAE-cellulose chromatography but could be separated on Sepharose-2B. As judged by SDS-PAGE the kinase was reported to be pure at this stage and to have a subunit molecular weight of 170,000 Da. There was however, no evidence that the 170 KDa polypeptide correlated with the kinase activity and the very low specific activity makes the argument for homogeneity dubious. It was later reported by Lent and Kim that phosphorylation was stimulated by the binding of coenzyme-A to ACC and that the kinase was phosphorylated and activated by cAMP-PK (Lent and Kim, 1983a,b).

A protein kinase has recently been purified from rat liver which accounts for greater than 90% of the cAMP-independent ACC kinase activity recovered after PEG fractionation of a crude extract of the tissue (Carling and Hardie, 1986, Carling et al, 1987). The kinase has not yet been purified to homogeneity although the reported specific activity is nearly 2 orders of magnitude greater than that reported by Lent and Kim for their preparation. There is a very good correlation between inactivation of ACC and its phosphorylation by this protein kinase as shown in Fig. 1.5. Phosphorylation results in both an increase for Ka citrate and a large decrease in Vmax (Munday et al, 1988) Phosphorylation of ACC by this kinase reduces its activity, measured at citrate concentrations that are close to physiological levels (0.5 mM), by more than 95%. This effect is totally reversed by dephosphorylation with protein phosphatase 2A(Carling et al, 1987). This protein kinase is stimulated by micromolar concentrations of AMP and is called the AMP-activated protein kinase (AMP-PK).

Fig. 1.5. Phosphorylation and Inactivation of ACC by AMP-PK



The above figure shows the phosphate content (closed circles) and the activity of ACC (open circles) during phosphorylation by AMP-PK and dephosphorylation by protein phosphatase-2A. The arrow marks the addition of purified protein phosphatase.
Modified from Carling et al (1987).

Rat ACC is phosphorylated by AMP-PK at three sites, which have been defined by direct amino acid sequencing (Munday et al, 1988) as serines-79, 1200 and 1215 (Davies et al, 1990). Data obtained from peptide mapping studies using the trypsin/chymotrypsin double digestion protocol suggested the AMP-PK phosphorylated sites within the TC1 and TC2 peptides identical to those phosphorylated by cAMP-PK. This was odd, because for the same level of phosphorylation inactivation by the AMP-PK was much greater than by cAMP-PK. This apparent anomaly was resolved by sequencing studies which showed that AMP-PK phosphorylates exclusively the fourth residue within the TC1 peptide (corresponding to serine-79 in the rat enzyme) whereas cAMP-PK phosphorylates exclusively the second residue, serine-77. cAMP-PK and AMP-PK both phosphorylate the TC2 peptide which contains one phosphorylatable serine residue, serine-1200. The increased phosphorylation of serine-79 has been shown in isolated hepatocytes and adipocytes in response to glucagon and adrenaline respectively (Davies et al, 1990, Sim and Hardie, 1988, Haystead et al, 1990) and furthermore, serine-79 has been reported to contain phosphate in vivo in the liver (Davies et al, 1991). Further evidence for the importance of serine-79 comes from the fact that limited trypsin treatment of the 240 kDa subunit completely reactivated the enzyme by removing the N-terminal segment containing serine-79 (Davies et al, 1990). The 220 kDa core which contains serine-1200 and -1215 is unaffected by this proteolytic treatment therefore suggesting that phosphorylation at these serine residues is not important in determining the kinetic parameters of ACC. Serine-77 is not phosphorylated in intact cells even when cAMP levels are increased (Sim and Hardie, 1988, Haystead et al, 1990) so it would seem that, in liver and adipose tissue, AMP-PK and not cAMP-PK, is the physiological ACC kinase.

Similar evidence showing increased serine-79 phosphorylation in the lactating mammary gland has not, as yet, been produced.

Protein Phosphatases Acting on ACC

Studies published by Cohen and others have suggested that four classes of protein phosphatase, i.e. phosphatases-1, -2A, -2B and -2C account for all of the cytosolic protein phosphatase activity which is active against a range of phosphoserine/phosphothreonine-containing enzymes involved in lipid metabolism, glycogen metabolism, glycolysis/gluconeogenesis, amino acid breakdown and protein synthesis (Cohen, 1988, Ingebritsen and Cohen, 1983). Protein phosphatase-2A is now thought to be the major phosphatase acting on ACC *in vivo* (Clarke *et al*, 1991).

1.4 Regulation of Triacylglycerol Biosynthesis

GPAT esterifies fatty acyl-CoA to glycerol-3-phosphate to produce monoacylglycerolphosphate and is thought to be the rate limiting enzyme in triacylglycerol synthesis. The incubation of rat adipocytes with adrenaline decreases GPAT activity by up to 50% after 60 minutes. The effect is dose-dependent and is antagonised by insulin and blocked by propranolol (Saggerson *et al* 1979). Although there is a build up of non-esterified fatty acids in these incubations as a result of stimulation of lipolysis by adrenaline the decrease in GPAT activity does not correlate with elevated fatty acid levels and is not mimicked by the addition of fatty acids. Since nearly 90% of rat adipocyte GPAT is located in the microsomes (Schlossman and Bell, 1976) the significant change seen with adrenaline is probably microsomal. There is a major

inactivation of GPAT upon incubation of adipose tissue microsomes from starved rats with cAMP, cAMP-PK and MgATP (Nimmo and Houston, 1978). This inactivation can be reversed by the addition of alkaline phosphatase. However, Rider and Saggerson (1983) have subsequently reported that the noradrenaline induced inactivation of GPAT in adipocytes is not reversed by either alkaline phosphatase or protein phosphatase-1. They also report that although incubation with cAMP-PK and MgATP increases the incorporation of ^{32}P into microsomal protein, there is no inactivation of GPAT, and therefore suggest that the inactivation of GPAT in response to noradrenaline is not through phosphorylation. Nimmo and Nimmo (1984) have claimed that the lack of inactivation observed by Rider and Saggerson (1983) is due to the latter authors' not using a high enough concentration of cAMP-PK.

1.5 Regulation of Cholesterol Biosynthesis

1.5.1 Hydroxymethylglutaryl-Coenzyme A Reductase

Hydroxymethylglutaryl-coenzyme A (HMG-CoA) is synthesized from acetyl-CoA by the enzyme HMG-CoA synthase. HMG-CoA is then converted to mevalonate by the enzyme HMG-CoA reductase (HMG-CoAR). HMG-CoAR is the rate limiting enzyme of cholesterol biosynthesis and catalyses what is thought to be the principal controlled step in the synthesis of cholesterol, namely the conversion of HMG-CoA to mevalonate. Mevalonate also serves as the precursor for three other metabolic pathways including the formation of ubiquinones, dolichols and isopentenyl-tRNAs (Beg and Brewer, 1981). HMG-CoAR is, in mammalian cells, a transmembrane glycoprotein with its active site orientated towards the cytosol and a carbohydrate

containing site facing the luminal surface of the endoplasmic reticulum (Brown and Simoni, 1984). The molecular weight of native HMG-CoAR in CHO cells deduced from the amino acid sequence is 97,092 Da and proteolysis of the native protein generates a 53,000 Da fragment that contains the active site of the enzyme (Chin et al, 1982).

1.5.2 Regulation of HMG-CoA Reductase

Control of HMG-CoAR activity is acheived through regulation of both the amount and the expressed activity of HMG-CoAR. The basic control mechanisms for regulation of HMG-CoAR activity are:

(1) Diurnal variation involving diurnal increases in HMG-CoAR activity during feeding (Rodwell et al, 1976) which are attributed mainly to an elevated rate of synthesis of HMG-CoAR (increased HMG-CoAR mRNA concentration) and a decreased rate of degradation of the enzyme (Gibson and Parker, 1987).

(2) Control of HMG-CoAR activity or degradation through changes in the fluidity of the membrane structure in the immediate microsomal environment of the enzyme (Richert et al, 1984) and through changes in the membrane composition.

(3) Short term regulation involving phosphorylation and dephosphorylation of HMG-CoAR (Beg and Brewer, 1981).

Regulation of HMG-CoA Reductase by Reversible Phosphorylation

Protein Kinases Acting on HMG-CoA Reductase

Reductase Kinase

It was reported in 1973 by Beg et al, that rat liver microsomal reductase activity was diminished following incubation with MgATP and a protein fraction from rat liver

cytosol. Repeated centrifugation of the microsomes did not affect the loss in activity. When the pretreated microsomes were incubated with a second cytosolic fraction in the absence of MgATP, HMG-CoA reductase activity was restored. From these observations it was suggested that the inactivation and reactivation of HMG-CoAR was due to reversible phosphorylation. The reversible inactivation of HMG-CoAR has now been confirmed in a variety of tissues and animal species (Beg et al, 1987). Nordstrom et al, (1977) found that the activator enzyme was severely inhibited by fluoride and that isolation of liver microsomes in the absence of fluoride would give an activation of reductase. It was confirmed that reversible phosphorylation was involved by the fact that the activation system could be replaced with fluoride sensitive phosphorylase a phosphatase from liver (Ingebritsen et al, 1978). The inactivating enzyme was termed reductase kinase.

It was observed by Ingebritsen et al (1978) that microsomal reductase kinase existed in active and inactive forms. The phosphorylation (activation) and dephosphorylation (inactivation) of cytosolic reductase kinase was established by Beg et al (1979). The enzyme catalysing the phosphorylation of reductase kinase was termed reductase kinase kinase. Dephosphorylation of reductase kinase was catalysed by a cytosolic

reductase kinase phosphatase. It has now also been demonstrated that reductase kinase from human liver cytosol exists in active and inactive forms with the phosphorylated form being the active form (Beg et al, 1984). Based on these observations it has been proposed that HMG-CoA reductase is regulated via a bicyclic cascade system consisting of reductase kinase and reductase kinase kinase, reductase phosphatase and a reductase kinase phosphatase (Beg et al, 1987).

It was reported by Brown et al (1975) that a factor in the extracts of cultured human fibroblasts or rat liver cytosol caused the inactivation in vitro of HMG-CoAR and that this inactivation was dependent on the presence of ATP or ADP and Mg^{+2} or Mn^{+2}. The inactivation was prevented by the addition of either an ATP or an ADP regenerating system which suggested that both nucleotides were required for the inactivation. It was postulated that ATP functioned as the phosphoryl donor for the reductase kinase while ADP fuctioned as an allosteric activator of the reductase kinase (Brown et al, 1975). It was subsequently shown that AMP was a much more potent activator of reductase kinase than ADP (Ferrer et al, 1985) with the affinity of reductase kinase for AMP being 20-fold greater than for ADP (Harwood et al, 1984). It has now been shown that the reductase kinase has the same sensitivity to AMP and to protein phosphatase treatment as the AMP-activated protein kinase (AMP-PK) (Hardie, 1989). It has been shown that highly purified AMP-PK will phosphorylate and inactivate HMG-CoAR and also that the AMP-PK and HMG-CoAR kinase activities exactly copurify during a six-step, 4800-fold purification from rat liver and are inactivated with similar kinetics by treatment with the reactive ATP analogue fluorosulphonylbenzoyladenosine (Carling et al, 1989). The reductase kinase is now

believed to be the AMP-PK. Rat HMG-CoAR is phosphorylated by AMP-PK at serine-871 and phosphorylation at this site inactivates the enzyme in vitro. It has been shown that this site can be phosphorylated in intact liver (Clarke and Hardie, 1990). AMP-PK is thought to be the kinase responsible for the phosphorylation and inactivation of HMG-CoAR.

Protein Kinase C

In addition to the AMP-PK system for the modulation of HMG-CoAR activity two other protein kinases have been reported to modulate HMG-CoAR activity via phosphorylation. The first of these is the calcium and phospholipid-dependent protein kinase, protein kinase C (PKC). PKC purified from rat brain cytosol has been shown to phosphorylate and inactivate both microsomal native (M_r 97,092) and protease cleaved, soluble, purified (Mr 53,000) HMG-CoAR. Beg et al (1975) have reported that the optimal phosphorylation of purified HMG-CoAR by PKC was associated with the incorporation of 0.56 mol of phosphate/ mol 53,000 purified fragment of HMG-CoAR.

Calcium Calmodulin-Dependent Protein Kinase

Another protein kinase that modulates HMG-CoAR by reversible phosphorylation is the calcium and calmodulin-dependent protein kinase. The calmodulin-dependent protein kinase catalysed inactivation of purified HMG-CoAR correlated directly with the incorporation of radiolabelled phosphate; 0.35 mol of phosphate was incorporated per mol of the 53,000 Da fragment of HMG-CoAR and this produced a 72% inactivation of enzyme activity (Beg et al, 1987). Maximal phosphorylation by the

calcium calmodulin-dependent protein kinase resulted in the incorporation of nearly 0.5 mol of phosphate per mol of the Mr 53,000 fragment of HMG-CoAR and nearly total inactivation of the enzyme (Beg et al, 1987).

Protein Phosphatases Acting on HMG-CoA Reductase

It has been reported that protein phosphatases-2A (30%) and -2C (66%) account for virtually all of the HMG-CoAR phosphatase activity in fractions of rat liver and experiments with okadaic acid, a phosphatase inhibitor, in intact cells suggest that, similar to ACC another AMP-PK substrate, protein phosphatase-2A is the important HMG-CoAR phosphatase (Clarke et al, 1991).

1.6 Regulation of VLDL Assembly and Secretion

In the liver triacylglycerol and cholesteryl ester are end products of cholesterol and fatty acid biosynthesis (Fig. 1.1). Triacylglycerol and cholesteryl ester are assembled together to form very low density lipoproteins (VLDL) which are then secreted from the liver into the blood stream to supply triacylglycerol and cholesterol to the peripheral tissues. In the periphery they are used as biosynthetic precursors, energy sources or stored (Gibbons, 1990). Triacylglycerol and cholesterol are not soluble in aqueous media they are therefore transported as VLDL- a macromolecular complex of a hydrophobic triacylglycerol and cholesterol ester core surrounded by a hydrophilic shell of phospholipids and cholesterol bound to a number of polypeptides (the apoproteins) (Knott et al, 1986). VLDL triacylglycerol is hydrolysed by

lipoprotein lipase on the vascular endothelium of the peripheral tissues eg. adipose tissue, muscle. The products of the hydrolysis, fatty acids, are taken up by the tissue leaving cholesterol-enriched intermediate density lipoproteins (IDL). Approximately half of the IDL produced is rapidly cleared through a receptor-mediated endocytosis by the liver involving the apo E receptor. The remaining 50% loses its apoprotein E component and becomes low density lipoprotein (LDL). LDL is the major cholesterol-transporting lipoprotein that performs the important physiological function of supplying cholesterol to tissues. LDL is taken up by the LDL-receptor, apo B is the sole apoprotein present in LDL and is thought to form the recognition site for the LDL-receptor (Brown and Goldstein, 1986).

During feeding, part of the triacylglycerol found in adipose tissue originates in the liver from which it is secreted as VLDL. During starvation VLDL triacylglycerol is an important fuel source for muscle tissue, in the rat 50% of the total lipid energy requirement of muscle is met by VLDL triacylglycerol (Wolfe and Durkot, 1985). The mechanisms by which signals arising out of changes in nutritional status alter the rate of assembly and secretion of VLDL is an important issue, and is focused on apolipoprotein B (apo B), a polypeptide which provides the structural skeleton for the VLDL particle and without which hepatic triacylglycerol secretion is not possible. However the physiological purpose of VLDL production is the need to secrete hepatically synthesised triacylglycerol therefore it is very likely that, normally, the availability of functional apo B is secondary or linked with changes in triacylglycerol available for secretion (Gibbons, 1990).

Secretory proteins are targeted to the endoplasmic reticulum (ER) by translation of a signal sequence from the appropriate mRNA on cytosolic ribosomes (Gibbons, 1990). The signal sequence binds to a signal recognition particle, this recognises a "docking protein" on the ER which then facilitates the targeting of the whole complex to the organelle's cytosolic face. After cleavage of the signal peptide, translocation occurs via a transient channel in the ER membrane. It appears that apo B is not completely translocated during translation and that it remains bound to the membrane for a significant time whilst passing through the secretory apparatus (Gibbons, 1990). This may play a role in maintaining the conformation of apo B required for triacylglycerol binding and for the assembly of "signal patches" that interact with receptors in the secretory apparatus (Lodish et al, 1983). These interactions may determine the intracellular transport rate and the final destination (e.g. membrane, lysosomes, secretory vesicle) of a particular protein. This is also a period during which the protein will be exposed to the action of protein kinases.

1.6.1 Synthesis and structure of apo B

The complete amino acid sequence of apo B, deduced by sequence analysis of cDNA clones has now been published (Yang et al, 1986, Chen et al, 1986 and Knott et al, 1986). The polypeptide is encoded for by a 14.1 kilobase mRNA, which produces a 4563-residue amino acid sequence (including a 27 amino acid signal peptide) of M_r 514,000.

Two major molecular mass forms of apo B are synthesized in mammals, the M_r

514,000 molecular form is named apo B-100 and a smaller form is named apo B-48. Apo B-48 is so called because it is the N-terminal 48% of the apo B-100 polypeptide (Gibbons, 1990). In humans apo B-100 is synthesized exclusively in the liver and apo-B48 exclusively in the small intestine, but in the rat both forms are synthesized and secreted hepatically (Gibbons, 1990). It has recently been shown that apo B-48 in rabbit and human intestine is produced via a unique post-transcriptional modification of the apo B-100 message and this modification is ~~a truncation of~~ an insertion of a stop codon into the mRNA (Powell et al, 1987).

Post-translational modification of apo B

Glycosylation

At least 20 potential glycosylation sites exist on apo B (Yang et al, 1986, Knott et al, 1986). The oligosaccharide chains are N-linked and are either asparagine linked chains containing a high proportion of mannose units or "complex oligosaccharide" chains (Vauhkonen et al, 1985). Inhibition of N-linked glycosylation by tunicamycin does not prevent VLDL secretion (Suita-Mangano et al, 1982), but a possible function may be linked to apo B-LDL receptor binding (Yang et al, 1986).

Fatty acylation

Apo B-100, isolated from Hep G2 cells is acylated with stearic and palmitic acids (Hoeg et al, 1988) possibly via a cysteine linked thioester bond. The functional importance of apo B acylation, if any, is unclear, although by analogy with other acylated proteins, a role in promoting interaction with intracellular membranes or in

lipid binding during VLDL maturation has been proposed (Hoeg et al, 1988).

Phosphorylation

Apo B-48 is secreted from rat hepatocytes as a phosphoprotein (phosphorylated on serine), but no phosphorylated apo B-100 could be detected (Davis et al, 1984). Recently it has been shown that both apo B-48 and apo B-100 are phosphorylated and secreted by rat hepatocytes (Sparks et al, 1988), phosphoserine and phosphotyrosine residues were detected. Glucagon and, in the short-term (less than 24 hours), insulin both cause inhibition of apo B secretion (Gibbons, 1990). This is a very interesting observation with respect to phosphorylation of apo B since glucagon is thought to exert its effects through elevation of intracellular cAMP levels leading to activation of cAMP-PK. VLDL and apo B secretion is hormonally regulated and there is a possibility that this may be mediated by apo B phosphorylation. Phosphorylation may play a functional role in apo B secretion and there are examples of proteins where phosphorylation is a prerequisite for secretion, eg. the egg yolk protein, vitellogenin (Wang and Williams, 1982), and the milk protein casein (Bingham and Farrell, 1977). Therefore phosphorylation might act to change the conformation of apo B and consequently affect:

(1) the rate of apo B mRNA translation,

(2) the rate of translocation of apo B into the ER,

(3) the rate of lipid binding to apo B and maturation of the VLDL particle, and

(4) the rate of secretion of apo B and VLDL.

However, once VLDL is secreted into the bloodstream it is rapidly dephosphorylated by serum phosphatases (Davis et al, 1984), therefore it is likely that phosphorylation

plays little or no role in the metabolism of apo B.

Topography of VLDL assembly

A major problem in understanding VLDL assembly is whether or not the site of apo B synthesis is removed from the site(s) at which it becomes associated with the bulk of the lipid. The work of Alexander et al. (1976) provided immunocytochemical data that apo B synthesized in the rough endoplasmic reticulum (rER), came into contact with triacylglycerol synthesized in the smooth endoplasmic reticulum, (sER) only at the junction of the two compartments and from here the lipoprotein was transferred to the Golgi in specialised tubules. This may be a specialized or transition region of the ER. A comprehensive explanation of VLDL assembly has been given by Oloffson et al, (1987), this is summarized below.

There is a hydrophobic interaction between the newly translated apo B and the membranal lipids of the ER, which leads to a conformation which is conducive for binding triacylglycerol. The eventual exit of apo B from the ER may depend upon at least some binding of triacylglycerol to apo B at this stage. The binding of apo B to the membrane of the intracellular transport system may reflect the requirement for a specific ~~confirmation~~ conformation, until enough triacylglycerol has been bound to allow the conformation to persist, unsupported, in the aqueous lumen.

There are two different views as to the major site of apo B transition from the membrane associated form to the lipoprotein associated form. One view suggests the Golgi as the point of lipid association especially the point of transfer from the medial

to the trans-Golgi and a second view suggests an important role for the ER during tri-acylglycerol transfer (evidence for both these theories is reviewed by Gibbons, 1990). Whether VLDL transport from the trans-Golgi to the cell surface is constitutive or regulated is not very clear. It is possible that secretory vesicles containing VLDL "bud off" from the Golgi, suggesting a regulated mechanism (Alexander et al, 1976). Regulated transport of VLDL to the cell membrane will not occur unless there is a hormone mediated signal.

There are a number of unresolved questions about VLDL assembly and its regulation. For example, what determines the stage at which apo B is released from the membrane of the secretory apparatus and what are the signals that guide apo B through the secretory pathway and target it for secretory vesicles. It is possible that post-translation modifications of apo B may play a part in the explanation of these questions hence studies on apo B phosphorylation (and glycosylation and acylation) may prove to be very important.

1.6.2 VLDL Secretion and Nutritional Status

The rate of VLDL triacylglycerol output is dependent on the nutritional status of the subject. Generally conditions which favour a high rate of de novo triacylglycerol synthesis, from small carbohydrate derived precursors are associated with high rates of VLDL generation. There is evidence (Duerden and Gibbons, 1988) that an increase in newly synthesized fatty acid, is itself not the direct cause of increased VLDL triacylglycerol secretion but that the two events are linked and simultaneously co-

ordinated. Davis et al, (1984) have shown that an increase in newly synthesized fatty acids, but not of extracellular pre-formed fatty acids (Davis and Boogaerts, 1982), is linked with an increase in the secretion of newly synthesized apo B. Also, starvation, a condition characterised by a decreased rate of fatty acid synthesis, results in a decreased synthesis and secretion of apo B (Davis et al, 1985). The stimulation in vitro of fatty acid synthesis and VLDL triacylglycerol output, however, did not result in a change in the rate of secretion of newly synthesized apo B (Boogaerts et al, 1984). Therefore it is possible that some in vivo factor produces the co-ordinated increase in the rates of fatty acid and apo B synthesis. The identity of this factor is unknown.

1.6.3 Effects of Hormones on VLDL Assembly and Secretion

Insulin

Insulin stimulates lipogenesis in rat liver and there have been reports of insulin, under some conditions, enhancing VLDL triacylglycerol secretion from perfused livers in situ (Topping and Mayes, ~~1989;~~ 1982 Topping et al, 1988). However insulin administration to human subjects decreased hepatic VLDL output (Pietri et al, 1983) and this was not due to a decreased rate of uptake of plasma free fatty acids by the liver. Studies on rat hepatocytes have also suggested an inhibitory effect of insulin on VLDL output during periods of less than 24 hours insulin treatment (Pullinger et al, 1989).

Therefore it appears that insulin does stimulate triacylglycerol synthesis but, certainly in the short term, inhibits triacylglycerol secretion from the hepatocyte. This therefore

leads to intracellular triacylglycerol accumulation and its mobilization gives an enhanced VLDL secretion, upon the subsequent removal of insulin (Duerden et al, 1989) (Table 1.3).

Table 1.3 The Effects of Isulin on VLDL Triacylglycerol(Gibbons, 1990)

Culture conditions		Relative VLDL triacylglycerol
Previous 48 hours	Final 24 hours	secretion during final 24 hours
+	-	1 (highest)
+	+	2
-	-	3
-	+	4 (lowest)

Key: + insulin present - insulin absent

The mechanism by which insulin uncouples the synthesis and secretion of triacylglycerol is not clear. It may be due to the fact that the release of fatty acids by hepatic lysosomal lipolysis of cytosolic triacylglycerol contributes to VLDL triacylglycerol and inhibition of this lipase by insulin may suppress the secretion of triacylglycerol. There may also be some impairment of apo B association with triacylglycerol. The de novo synthesis or post-translational modification of apo B may also be impaired, however insulin does not affect apo B gene expression in Hep G2 cells (Pullinger et al, 1989).

The short term inhibitory effect of insulin contrasts with the long term effect. A 24-48

hour exposure to insulin results in an enhanced VLDL secretion by a culture of primary rat hepatocytes (Duerden et al, 1989), this may be due to a down regulation of insulin receptor levels (Patsch et al, 1986). The exact response to insulin may depend on the extent of stimulation of triacylglycerol synthesis by the hormone, the different effects of insulin on VLDL triacylglycerol secretion rates are summarized in table 1.3, insulin therefore has a biphasic effect of short-term inhibition and long-term activation (Gibbons, 1990).

Glucagon

Glucagon has an inhibitory effect on VLDL triacylglycerol secretion and esterified and non-esterified cholesterol in isolated preparations of rat liver (Pullinger and Gibbons, 1985). This matches with the rapid inhibition of de novo fatty acid synthesis by glucagon (Geelen et al, 1980, Gibbons et al, 1984). However there is no evidence that long term exposure to glucagon or cAMP suppresses total cellular triacylglycerol synthesis. When circulating glucagon levels are raised in vivo, extracellular fatty acids would be an important source of hepatic triacylglycerol and it is likely that these are directed into a storage pool rather than a secretory pool (Gibbons, 1990). Studies on glucagon effects on apo B secretion in Hep G2 cells have been hampered by the insensitivity of this cell line to glucagon and cAMP (Pullinger et al, 1989). The physiological role of glucagon, if any, in regulating hepatic VLDL secretion is, at present, unclear. However it is interesting that hyperglucagonaemia is characteristic of insulin-dependent diabetes mellitus, a condition in which hepatic VLDL secretion is decreased, and in obese Zucker rats, an elevated VLDL output is associated with

a depressed plasma glucagon concentration (Gibbons, 1990).

It is clear that apo B is a phosphoprotein, that the rate of apo B synthesis does not always match VLDL secretion rates and that there is hormonal regulation of VLDL output and apo B secretion. On the basis of these observations it is possible that VLDL secretion and assembly is dependent on apo B phosphorylation.

1.7 Protein Kinases and Lipid Metabolism

1.7.1 General Protein Kinase Structure

The eukaryotic cell is a highly regulated entity that responds to its immediate environment as well as to external stimuli. Regulation is mainly mediated, directly or indirectly, by conformational changes in proteins. The equilibrium between active and non-active conformations can be altered by both allosteric and covalent mechanisms. One of the most common covalent mechanisms is phosphorylation.

The enzymes that catalyse phosphorylation reactions are known as protein kinases and they represent a structurally diverse group of proteins. They differ widely in size, subunit structure, subcellular localisation, mechanism of activation and substrate specificity. There are two general classes of protein kinases, those that transfer phosphate to serine or threonine residues and those that transfer phosphate to tyrosine residues. Tyrosine protein kinases not only play a central role in many types of cellular transformation but also probably participate in normal growth regulation, even

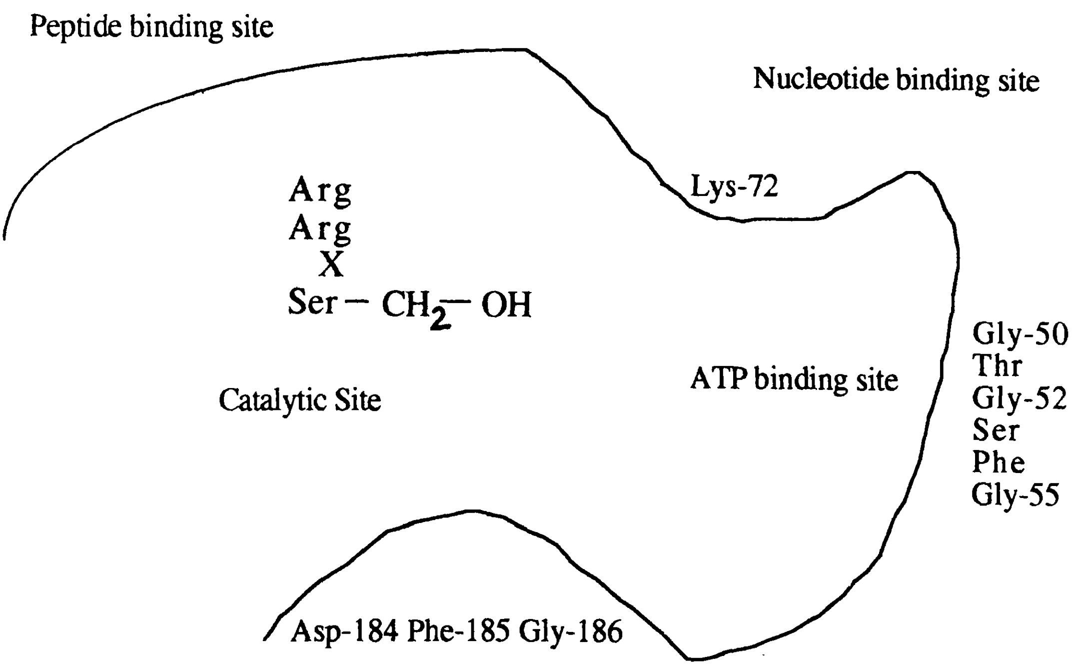
though tyrosine phosphorylation accounts for less than 0.1% of total eukaryotic cell phosphorylation. The subsequent events that lead to neoplastic transformation are still unclear. Many physiological substrates of the oncogene kinases have been identified such as the 36kDa protein that recently has been shown to be homologous to lipocortin II (Garis et al, 1986) but the functional consequences of these phosphorylations and their relationship to transformation are not yet established.

Despite their diversity, all eukaryotic protein kinases are evolutionarily related through a conserved catalytic core (Hanks et al, 1988, Taylor et al, 1988) which indicates that the protein kinases will share at least some common features of secondary and tertiary structure. Probably the best understood protein kinase is cAMP-dependent protein kinase (cAMP-PK). The catalytic subunit of this kinase is thought to represent one of the simplest protein kinases and provides a framework for studying the entire kinase family (Fig. 1.6). A close look at this conserved catalytic core shows that there are certain invariant regions which can be correlated with defined functions. The work described below was conducted on cAMP-PK but is applicable to all protein kinases.

Nucleotide Binding Site

The binding of MgATP is a common function shared by all protein kinases. There are two structural features that serve tentatively to define and localise the ATP binding site. The first was elucidated from affinity labelling studies using FSBA, which led to inactivation of the kinase by the stoichiometric covalent modification of lysine-72 (Zoller et al, 1981). The importance of this residue is confirmed by the fact that it is invariant in every protein kinase.

Fig. 1.6 Model for the Active Site of cAMP-dependent Protein kinase



A model for the active site of cAMP-PK is shown above. Gly-50, 52, and 55 are conserved in all protein kinases as are Asp-184, Phe-185 and Gly-186.
Modified from Taylor (1987).

Another striking feature of kinase structure is the conservation of a triplet of glycines, which in the catalytic subunit of cAMP-PK, correspond to glycines-50, -52 and -55. These glycines are a conserved feature of most adenine nucleotide-binding sites (Hanks et al, 1988). Rossmann et al (1974) suggested that all adenine nucleotide binding proteins would posess a common nucleotide fold consisting, in general, of 6 parallel beta-sheets with 2 alpha-helices above and below the plane formed by the beta sheets. In most cases the nucleotide binding fold contains approximately 150 amino acid residues and constitutes the amino or carboxy- terminal portion of the molecule.

Catalytic Site

There are extensive sequence homologies that exist throughout the catalytic core which strongly suggest that a common catalytic mechanism has been conserved. Kinetic studies have suggested that this is an ordered mechanism where the initial event is the binding of MgATP. It is only after the binding of MgATP that there is a high affinity binding for peptide which leads to the catalytic event. Treatment of the catalytic subunit with dicyclohexylcarbodiimide (DCCD), a hydrophobic reagent which partitions into hydrophobic regions such as those frequently associated with ATP binding sites, leads to inhibition of the catalytic subunit and, similar to the inactivation with FSBA MgATP provides protection. The amino acid residue reacting with DCCD is Asp-184 a residue which is invariant in all protein kinases (Buechler and Taylor, 1988) so this appears to be an important residue. Glu-91 also reacted with DCCD but to a lesser extent, was protected by MgATP and is conserved throughout the kinase family (Buechler and Taylor, 1988) so this may also be an important residue.

Peptide Recognition Site

The peptide recognition site is the one area where differences between the protein kinases are anticipated since they recognize different peptide substrates. The catalytic subunit of cAMP-PK, for example, requires basic amino acids to precede the site of phosphorylation with the consensus sequence for recognition being Arg-Arg-X-Ser (Bramson et al, 1984). Glu-170 is conserved in those Ser/Thr-specific kinases having a requirement for basic residues preceding the phosphorylation site, such as Protein kinase C, myosin light chain kinase and cAMP-PK, but is replaced with a basic residue in casein kinase II which requires acidic groups flanking the phosphorylation site (Hanks et al, 1988). Tyrosine kinases which normally require acidic groups to precede the site of phosphorylation (Hunter, 1982) also typically have a positively charged amino acid at that position.

Regulatory Regions

The regions that are not included within the catalytic domain do not show homologies conserved throughout the entire family of protein kinases. The non-catalytic domains are thought to be important for two reasons. Firstly they play an important role in the regulation of protein kinase activity through receiving and mediating the activating signal whether this is hormonal or through second messengers e.g. diacylglycerol, cAMP, cGMP and calcium. Secondly, the non-catalytic domains in many cases play a major role in determining the correct subcellular localisation of the protein kinase.

Kinase Regulation by Phosphorylation

Protein kinases themselves are also substrates for phosphorylation. In some cases these

phosphorylations are autocatalytic events and sometimes they involve other protein kinases. There is increasing evidence that these phosphorylations may be an important feature in regulating protein kinase activity. An example of this is the calcium calmodulin multiprotein kinase type II where autophosphorylation results in activation of the kinase which reverts to calcium calmodulin dependency after dephosphorylation (Edelman et al, 1987). Phosphorylase kinase is an example of a kinase that is phosphorylated and activated by the action of another protein kinase, in this case cAMP-PK (Edelman et al, 1987). In the case of tyrosine kinases an example is provided by the proto-oncogene $p60^{c\text{-}src}$ which differs from $p60^{v\text{-}src}$ by only 19 amino acid residues at the carboxy-terminal end. This region contains the phosphorylated amino acid Tyr-527. Phosphorylation of Tyr-527 in $p60^{c\text{-}src}$ decreases the kinase activity and this tyrosine is missing in $p60^{v\text{-}src}$. The loss of this regulatory site in $p60^{v\text{-}src}$ renders the molecule permanently active.

1.7.2 cAMP-dependent Protein Kinase

The Holoenzyme

The cyclic nucleotide cAMP is a major intracellular second messenger of hormone action (Robinson et al, 1971, Sutherland 1972). The cAMP-dependent protein kinase, cAMP-PK, (Krebs 1972) exists as an inactive tetramer consisting of two regulatory subunits and two catalytic subunits (R_2C_2) (Carlson et al, 1979). The activation of cAMP-PK is illustrated by the following equation (Corbin et al, 1978),

$$R_2C_2 + 4\ cAMP \rightleftharpoons R_2\ (cAMP)_4 + 2C$$

inactive active

The cyclic nucleotide binds to the regulatory subunits, inducing a conformational change that decreases their affinity for the catalytic subunit (by approximately four orders of magnitude) thus promoting dissociation of the holoenzyme complex. The free catalytic subunits are now active and can phosphorylate substrate proteins at serine and threonine residues (Edelman et al, 1987). Hydrolysis of cAMP by cAMP phosphodiesterases shifts the above equilibrium to the left and terminates the protein kinase activity. Protein phosphorylation by cAMP-PK is an important regulatory mechanism in the hormonal initiation and control of multiple intracellular events including the flux through many metabolic pathways. The phosphorylation and activation of hormone sensitive lipase (HSL) to stimulate lipolysis in adipose tissue and phosphorylase kinase to stimulate glycogenolysis are prime examples of such control (Cohen, 1976).

In most tissues cAMP-PK holoenzyme occurs in two major isoforms. These are termed type I and type II (Corbin et al, 1975, Flockhart et al, 1982) and are defined by their order of salt gradient elution from DEAE-cellulose. Type I typically elutes at NaCl concentrations less than 100mM (Corbin et al, 1985) whereas type II elutes at concentrations greater than 100mM. The differences are due to their different isoelectric points and differences in amino acid composition. The type I isozyme has a pI of 5.45 - 5.57 and the type II isozyme has a pI of 5.34 - 5.4 (Uno et al, 1977), the type II isozyme also has a higher acidic amino acid content than the type I isozyme (Corbin et al, 1978).

The Regulatory Subunits

The type I and II holoenzymes differ in the structure of their regulatory subunits. The R subunits, RI and RII each occur in two isoforms (RIα and RIß; RIIα and RIIß) (Clegg et al, 1988). The type I regulatory subunit from bovine skeletal muscle has been determined to have a molecular weight of 42,804 Da and the type II regulatory subunit from bovine heart a molecular weight of 45,004 Da (Beebe and Corbin, 1986). These values have been determined from amino acid sequence and are considerably lower than the apparent molecular weights determined from denaturing gel electrophoresis. The values from SDS-PAGE give a value of 49,000 Da for the molecular weight of the type I regulatory subunit from porcine, bovine and rabbit skeletal muscle, and a molecular weight of 56,000-58,000 Da for the bovine heart type II subunit. It has been shown (Robinson-Steiner et al, 1984) that the apparent molecular weight of the type II regulatory subunits determined by SDS-PAGE differs significantly among different species and tissues. Also the extent of migration differs for some forms of the type II regulatory subunit depending on the presence of phosphate in the autophosphorylation site.

The type I regulatory subunit does not show autophosphorylation and shows slightly less pronounced differences in molecular weight when ascertained by different methods (Nimmo et al, 1977). The discrepancy in SDS-PAGE measurement of the molecular weight of the type II regulatory subunits is not easily explained, it is possible that there is inadequate denaturation by SDS and therefore migration of the protein is not strictly according to molecular weight. This may suggest that the

presence or absence of phosphate at the autophosphorylation site affects the binding of SDS more to some than to other forms of the type II regulatory subunit (Robinson-Steiner et al, 1984).

The type I isozyme is more readily dissociated in the presence of high salt concentrations than the type II (Corbin et al, 1975). It has been shown (Ringheim and Taylor 1990) that the binding of MgATP prevents dissociation of the type I isozyme at physiological salt concentrations (see also Corbin et al, 1975). Without MgATP significant activation could occur in the absence of cAMP due to salt induced, dissociation of the complex. Ringheim and Taylor (1990) also suggest that MgATP accelerates the reassociation of the holoenzyme thus facilitating termination of the activation process when cAMP is removed. The regulatory subunit of the type II holoenzyme readily undergoes autophosphorylation in the holoenzyme form. The presence of phosphate in the autophosphorylation site of the type II regulatory subunit (serine-95 of the primary sequence (Takio et al, 1982)) results in a slower rate of reassociation of the regulatory subunit with the catalytic subunit (Rosen et al, 1975, Rangel-Aldao et al, 1976, 1977). The physiological significance of autophosphorylation has not, as yet, been delineated. There are also other phosphorylation sites on the type II regulatory subunit. It is phosphorylated in vitro at serine 44 and 47 by glycogen synthase kinase 3 (Hemmings et al, 1982) and at serine 74 and 76 by casein kinase II (Hemmings et al, 1982, Carmichael et al, 1982). Serine 74 and 76 are also phosphorylated in vivo (Carmichael et al, 1982). The physiological significance, if any, of these phosphorylations is, as yet, undefined.

The RI subunit is also phosphorylated, it contains one site that is phosphorylated in

vivo and one that is phosphorylated in vitro by cGMP-dependent protein kinase (Edelman et al, 1987). The latter site is serine-99 and phosphorylation at this site results in loss of inhibitory activity towards the catalytic subunit and the loss of one cAMP-binding site, but phosphorylation of this site has not been shown physiologically. Phosphorylation of the in vivo site has no apparent effect on RI function, and its position within the sequence is not known (Edelman et al, 1987).

While it is useful to classify the cAMP-PK holoenzymes into major type I and type II classes it is likely, certainly for the type II isozyme, that there are a number of microheterogenous forms. It has been shown (Robinson-Steiner et al, 1984) that a second fractionation on DEAE-cellulose is sometimes necessary to resolve both isozymes completely. There is more than one class of type II isozyme in the same animal species. It was shown that rat adipose tissue and rat heart type II isozyme elute at different NaCl concentrations from DEAE-cellulose and that mixtures of these two isozymes could be separated by chromatography on DEAE-cellulose (Beebe and Corbin, 1986). However in spite of these physical differences the kinetic properties of the catalytic subunit derived from each were similar. One classification separates type II regulatory subunits according to whether or not they change their electrophoretic mobility on SDS gels after autophosphorylation. Those that do are classified as type IIA and those that do not as type IIB.

The Catalytic Subunit

It has been generally assumed that the catalytic subunit from different tissues and different holoenzymes are identical (Bechtel et al, 1977, Krebs and Beavo 1979,

Flockhart and Corbin 1982, Nairn et al, 1985) largely because the catalytic subunits from all sources studied have had similar molecular weights of approximately 40,000 Da (Carlson et al, 1979) and similar tryptic peptide maps (Zoller et al, 1979). The catalytic subunits are also indistinguishable by their similar behaviour on many chromatography columns and similar chemical, physical and immunological properties. They can also reassociate with both type I and type II regulatory subunits (Nimmo and Cohen 1977, Corbin et al, 1975, Sugden et al, 1976). The catalytic subunit, similar to the regulatory subunits, is phosphorylated at multiple sites. Purified catalytic subunit has been shown to contain endogenous phosphate at threonine-197 and serine-338 (Beebe and Corbin, 1986), but the significance of the these phosphorylations is unknown. The catalytic subunit can be distinguished by its sensitivity to a heat and acid stable protein termed the Walsh inhibitor. The Walsh inhibitor is present in skeletal muscle and it specifically inhibits the catalytic subunit of cAMP-PK (Walsh and Ashby, 1973). The Walsh inhibitor contains segments of sequence that are very similar to the sequences of preferred substrates and is probably acting as a 'pseudo-substrate' inhibitor (Cohen, 1976). A synthetic twenty amino acid peptide, based on the active portion of the Walsh inhibitor, has been synthesised and is now widely used in cAMP-PK activity assays instead of the native Walsh inhibitor (this peptide will now be referred to as the specific peptide inhibitor of cAMP-PK). The physiological role of the Walsh inhibitor is unclear, although it may function to suppress cAMP-PK activity stimulated by basal levels of cAMP.

Two forms of the catalytic subunit, termed C_A and C_B have been separated chromatographically using a cation exchange column (Kinzel et al, 1987). These two

forms are identical with regard to their electrophoretic mobility in SDS gels, kinetic behaviour, pH dependence, inhibition by the type I and II regulatory subunits and inhibition by the specific peptide inhibitor of cAMP-PK. The catalytic subunits do differ in their susceptibility to degradation by the catalytic subunit specific protease (Alhanaty et al, 1981) with C_B being degraded faster than C_A under all conditions. Several investigators have found two or three different forms of the catalytic subunit with different isoelectric points. At least three forms of the bovine liver catalytic subunit with pI values of 6.72, 7.04 and 7.35 have been reported (Sugden et al, 1976). The charge differences of the catalytic subunits of cAMP-PK have also been utilised for the isolation of the catalytic subunits. The regulatory subunits have pI values below pH 4 and the catalytic subunits have pI values between pH 6.7 - 9.1 (Kubler et al, 1979). These different isoelectric forms of the catalytic subunit of cAMP-PK may be evidence of different isozymic forms.

There are at least two genes coding for the catalytic subunit of cAMP-PK. One gene codes for a protein containing 351 amino acids that is 98% homologous with the bovine heart catalytic subunit (Uhler et al, 1986a, b). The other gene encodes a protein closely related to the bovine heart catalytic subunit. These forms have been designated Cα and Cß. Both are found in all tissues but Cß is particularly abundant in brain tissue. These two isoforms have recently been purified using affinity chromatography (Olsen and Uhler, 1989). The affinity column consisted of the synthetic peptide that corresponds to the inhibitory domain of the heat stable Walsh inhibitor. The column was eluted using arginine which allowed a single step isolation of the purified Cα and Cß isoforms. No significant differences in the specific activity of Cα and Cß have been reported. They have 90% sequence homology and bind to

either type I or II regulatory subunits. Both isoforms had similar apparent K_m values for ATP (4 μm) and for Kemptide (5.6 μm). However there was no attempt made at characterisation of the isoforms with regard to physiological substrates (Olsen and Uhler 1989). Recently a further isoform of the catalytic subunit has been described (Weimann et al, 1991). The end parts of the cDNA 5' coding and non coding region of this isoform are unique, the rest of the coding region and the 3' noncoding region are identical to those of isoform Cß and the clone has been termed $Cß_2$. This clone contains 397 amino acids and a molecular mass of 46 kDa which is approximately 6 kDa higher than that of any known catalytic subunit. This isoform CB_2 lacks the myristoylation site usually observed in most catalytic subunits (Carr et al, 1982). The function of the myristic acid is not yet defined. Myristoylation does however play a role in the oncogene product $pp60^{v\text{-}src}$ where it determines subcellular localisation and cell transforming ability (Schmidt 1989). Van Patten and coworkers (1986, 1988) have also identified different isoforms of the catalytic subunit on the basis of its formation of one minor and two major complexes with the heat stable specific inhibitor of the cAMP-PK. These complexes have been attributed to multiple forms of the catalytic subunit. The latter workers have correlated the two major complexes with the two forms of the catalytic subunit described by Kinzel et al (1987) namely C_A and C_B. The two types of catalytic subunit-inhibitor complex may be differentiated by their dependence on Mg^{2+} and ATP for complex formation and therefore it is unlikely that Cα and Cß (which have similar Km values for ATP) correlate with the two forms of the catalytic subunit proposed by Van Patten et al, (1986). Another form of catalytic subunit, found specifically in testicular tissue has been isolated and has been termed Cγ (Beebe et al, 1990). Cγ has 83% amino acid homology with Cα and

79% with Cß. This together with its tissue specific expression suggests that Cγ may be functionally distinct from the other catalytic subunits characterised thus far.

Substrate Specificity of cAMP-PK

cAMP-PK has a very broad substrate specificty in vitro and in vivo, this discussion is limited to some of the known metabolic intracellular targets for cAMP-PK and the effect their phosphorylation has on metabolism.

Phosphorylase is the rate limiting enzyme in glycogenolysis and is converted from phosphorylase b to phosphorylase a through phosphorylation by phosphorylase kinase. cAMP-PK has a dual role, it activates the enzyme through an initial phosphorylation of one subunit of phosphorylase kinase (the beta subunit) and secondly determines the time at which inactivation can start through the secondary phosphorylation of another subunit (the alpha-subunit) (Cohen, 1976).

Glycogen synthetase is the rate-limiting enzyme in glycogen synthesis and was first shown to be regulated by reversible phosphorylation by Larner et al (1968). Glycogen synthetase is known to be phosphorylated at multiple sites by multiple kinases (Cohen, 1985) but phosphorylation of glycogen synthetase by cAMP-PK results in its inactivation (Soderling et al, 1970).

Hormone sensitive lipase (HSL) plays a major role in fatty acid mobilization from adipose tissue by catalysing the rate-limiting step in the lipolysis of stored triacylglycerols. The rate of lipolysis is under strict hormonal and neuronal control, largely exerted through regulation of HSL activity. Lipolytic agents such as noradrenaline released from sympathetic nerve endings, adrenaline, corticotropin and glucagon

stimulate lipolysis by raising the intracellular cAMP concentration which results in activation of cAMP-PK. This leads to the phosphorylation and activation of HSL and stimulation of lipolysis (Nilsson et al, 1980). Insulin is the major antilipolytic hormone and is thought to act, at least in part, by reducing intracellular cAMP levels (Londos et al, 1985). cAMP-PK phosphorylates HSL at a single residue now identified as serine-563 (Holm et al, 1988). The specific inhibitor protein of cAMP-PK which was shown to block the cAMP-PK catalysed activation of HSL immediately (Corbin et al, 1970) also interrupts the phosphorylation of HSL by cAMP-PK (Fredrikson et al, 1981) thereby confirming that cAMP-PK acts directly on HSL without any intervening lipase kinase.

The export of acetyl-CoA from the mitochondria to the cytoplasm takes place across the inner mitochondrial membrane which is impermeable to acetyl-CoA, therefore the mechanism involves conversion of acetyl-CoA to citrate using citrate synthase, transport across the mitochondrial membrane by a specific carrier and reconversion to acetyl-CoA by ATP-citrate lyase (ATP-CL) (Srere, 1972). ATP-CL becomes phosphorylated in response to glucagon in hepatocytes, adrenaline in adipocytes and insulin in both cell types (Ramakrishna and Benjamin, 1979, Alexander et al, 1979, Janski et al, 1979). ATP-CL purified from rabbit lactating mammary gland has been shown to be phosphorylated by cAMP-PK (Guy et al, 1980). The maximum extent of phosphorylation of ATP-CL by cAMP-PK reported is 2 mol of phosphate/ mol of ATP-CL (Guy et al, 1980, Alexander et al, 1981, Pierce et al 1981, Ranganathan et al, 1982). Roehrig (1980) claimed that phosphorylation caused a change in the activity of ATP-CL that is only detectable at low concentrations of CoA, but this has not been confirmed by other workers. The physiological relevance of ATP-CL

phosphorylation is at the moment rather doubtful. This is because it now appears that the sites on ATP-CL that are phosphorylated in response to insulin and glucagon in vivo and by cAMP-PK in vitro are all identical (Pucci et al, 1983). Since insulin and glucagon have opposite effects on the rates of fatty acid synthesis, this phosphorylation is unlikely to have a direct role in metabolic control. It may have other roles e.g. in controlling the degradation of the enzyme or in the binding of ATP-CL to mitochondria (Janski and Cornell, 1980) but this has yet to be shown.

1.7.3 AMP-activated Protein Kinase

Structure

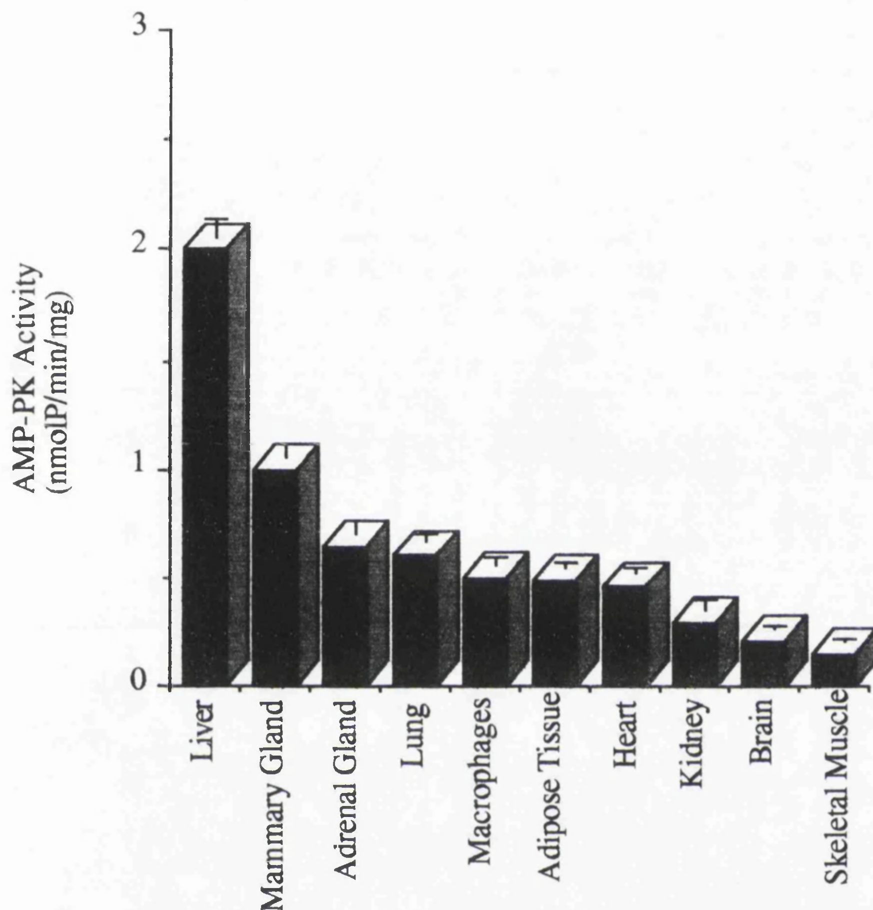
To date even the most highly purified preparations of AMP-PK from rat liver contain several polypeptides detectable by SDS-PAGE, but only one of these, with an apparent molecular mass of 63 kDa, is labelled using [^{14}C] FSBA (Carling et al, 1989). This is also the only polypeptide in the preparation that becomes significantly labelled during incubation with [γ^{32}P] ATP (Carling et al, 1989). Since virtually all protein kinases autophosphorylate (Edelman et al, 1987), this may suggest that the 63kDa polypeptide is the protein kinase catalytic subunit. FSBA appears to react at both the AMP-binding site and the catalytic (ATP) site (Ferrer et al, 1987) therefore both are probably present on the 63 kDa polypeptide. Comparison of the subunit molecular mass to the native molecular mass (100 $\pm$ 30 kDa) suggests that the AMP-PK may exist as a homodimer, but it may equally be a heterodimer of the 63 kDa subunit and one of the lower molecular mass polypeptides that are also visible when

the preparation is analysed (Carling et al, 1989b). The high specificity of the allosteric site on AMP-PK for AMP was shown by Carling et al (1989). A wide range of AMP analogues and other nucleotides were tested for their effect on AMP-PK activity. However none of the compounds tested, including cAMP, caused any stimulation of AMP-PK activity.

Studies on the AMP-PK have been greatly aided and advanced due to the development of a specific and sensitive peptide assay. From amino acid sequencing studies it is known that AMP-PK phosphorylates at serine-79 on ACC whereas cAMP-PK phosphorylates at serine-77 (Munday et al, 1988, Lopez-Casillas et al, 1988). Based on this knowledge a peptide substrate with serine-77 modified to eliminate a site for cAMP-PK was synthesised. The peptide is known as the 'SAMS' peptide and has the following sequence, His-Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg. An unmodified peptide substrate was also synthesised, the 'SSMS' peptide and this had the sequence, His-Met-Arg-Ser-Ser-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg. The two C-terminal arginine residues were added to increase the binding of the peptide to the phosphocellulose paper in the assay for AMP-PK.

Both the SAMS and the SSMS peptides are phosphorylated by AMP-PK, but the SAMS peptide is not phosphorylated by any of five other purified protein kinases so far tested (Davies et al, 1989). The sensitivity of the assay extends to measurement of AMP-PK activity in crude tissue extracts and has therefore allowed tissue distribution studies on this enzyme to be carried out. Utilizing this peptide assay, Davies et al (1989) assayed for AMP-PK activity in nine different rat tissues plus a mouse macrophage cell line and their results are summarised in Fig. 1.7. The two

Fig. 1.7 Tissue Distribution of AMP-activated Protein Kinase



The activity of AMP-PK in extracts prepared from various tissues is shown above. The results shown are the means of experiments on four independent extracts.
The vertical bars represent the standard errors of the means.
Modified from Davies et al (1990).

tissues with the highest activity were found to be liver and lactating mammary gland which are both very active in fatty acid synthesis and the liver also in cholesterol synthesis. The next group consisting of adipocytes, adrenal cortex, lung, WEHI macrophages and heart showed intermediate levels of AMP-PK activity. The observation that macrophages express AMP-PK activity is very interesting because arterial wall macrophages are believed to be the precursors of the 'foam cells' which accumulate large quantities of cholesterol ester in atherosclerosis (Brown and Goldstein, 1983). The last group of tissues consisted of brain and skeletal tissue and these showed very low levels of AMP-PK activity.

The overall conclusion to be drawn from tissue distribution studies is that there appears to be a good correlation between those tissues active in lipid metabolism and those showing high levels of AMP-PK activity.

Regulation in vitro

Two modes of regulation of the AMP-activated protein kinase itself have been reported in vitro (Carling et al, 1987). The first mechanism involves 5' AMP which stimulates AMP-PK activity by 4- 6-fold with a half maximal effect reported at 14 μM, which is within the physiological range in rat liver. A number of other nucleotides and AMP analogues including ADP and cAMP have been used but have been found to be ineffective, but the kinase is inhibited by adenosine with a half maximal effect at 200 μM (Hardie, 1989).

The second mode of regulation is by phosphorylation-dephosphorylation (Carling et al, 1987). It was initially found that the kinase was very unstable unless it was prepared in the presence of fluoride and pyrophosphate (phosphatase inhibitors) and

subsequently that purified AMP-PK was inactivated by the purified catalytic subunits of protein phosphatases. Clarke et al (1991) have reported that the protein phosphatase acting on AMP-PK is protein phosphatase-2C. Also if the kinase was partially purified in its inactive form, by omission of the phosphatase inhibitors, it can be reactivated by the addition of MgATP. This reactivation appears to be due to a distinct 'kinase-kinase' rather than to an autophosphorylation by the kinase itself, because the reactivation does not occur after subsequent purification steps. It is very interesting that the reactivation is markedly stimulated by nanomolar concentrations of palmitoyl-CoA (Carling et al, 1987) particularly in view of the effects of dietary saturated and polyunsaturated fatty acids to decrease plasma cholesterol levels and the inhibit the rates of fatty acid and cholesterol synthesis in rat liver (Gibbons and Pullinger, 1986).

Regulation of AMP-PK in vivo.

Regulation by Hormones

Measurements of HMG-CoAR activity both in isolated hepatocytes (Gibson and Parker, 1987) and in vivo (Easom and Zammit, 1987) suggest that phosphorylation of HMG-CoAR is decreased by insulin and increased by glucagon. Phosphorylation of the AMP-PK site in ACC in response to glucagon treatment of hepatocytes has been shown by direct sequencing (Sim and Hardie, 1988). The mechanism by which cAMP-elevating hormones effect phosphorylation at AMP-PK sites is unclear since cAMP-PK does not appear to cause reactivation of dephosphorylated AMP-PK

(Hardie, 1989).

Regulation by AMP

Anoxia results in a dramatic increase in AMP concentrations due to the action of adenylate kinase which responds to any small drop in ATP levels by a much larger increase in AMP concentration (Vincent et al, 1982). AMP-PK may therefore be a means of inhibiting fatty acid and cholesterol biosynthesis when ATP is limiting. However anoxia is not a physiological condition and there have been no reports of any changes in AMP concentration under physiological conditions.

Regulation by Fatty Acyl-CoA Esters

The reactivation of AMP-PK by the kinase kinase is stimulated by palmitoyl-CoA (Carling et al, 1987) and suggests that ACC is subject to direct allosteric inhibition by fatty acyl CoA esters. The drawback of the allosteric mechanism is that while the total concentration of fatty acyl-CoA in the rat hepatocyte is in the range of 1-10 μM, the free concentration is probably orders of magnitude lower due to binding to fatty acid/acyl-CoA binding proteins (Mikkelsen and Knudsen, 1987). ACC concentration varies with diet, but in the rat liver can reach concentrations as high as 1-2 μM and since this is probably much higher than the concentration of free fatty acyl-CoA esters, a kinase cascade may be required to amplify the feedback signal.

The fact that such a feedback system, activated by fatty acyl-CoAs should also inhibit HMG-CoAR may be explained by it being possible that when a high concentration of

excess fatty acyl-CoA shuts down fatty acid synthesis a simultaneous inhibition of HMG-CoAR is required to prevent the flux of acetyl units merely being diverted into cholesterol biosynthesis. The co-ordinate regulation of ACC and HMG-CoAR would also balance the rate of synthesis of the two major components of VLDL and implies a more widespread involvement of AMP-PK in regulating lipid metabolism.

Regulation by Cholesterol Metabolites

Cholesterol feeding of rats in vivo, or treatment of isolated hepatocytes with LDL, oxygenated cholesterol or cholesterol precursor mevalonolactone all lead to decreases in the activity of HMG-CoA reductase as measured by the expressed/total activity ratio (Gibson and Parker, 1987) and this may be due to changes in enzyme concentration since cholesterol metabolites are known to work via changes in mRNA levels. There may also be an allosteric effect on HMG-CoAR and this may be exerted via changes in membrane fluidity. There is no evidence for such regulation of AMP-PK but it is possible that cholesterol or a derived metabolite can also exert feedback control of HMG-CoA reductase via the AMP-PK system. Whether these effects are mediated by the binding of cholesterol metabolites to HMG-CoA reductase itself or to components of the phosphorylation cascade is unclear.

Substrate Specificity

The most important substrates for the AMP-PK are ACC and HMG-CoAR and these have been discussed previously. Another important substrate for the AMP-PK is

hormone sensitive lipase (HSL). HSL is activated by phosphorylation at serine-563 (Holm et al, 1988) by cAMP-PK and this results in activation of lipolysis or cholesterol ester breakdown in response to hormones which elevate cAMP. AMP-PK phosphorylates HSL at serine-565 (Garton et al, 1989), in a sequence that bears close similarity to that around serine-77/79 in ACC. When the peptide containing the serines-563/565 from bovine HSL was sequenced, unlabelled phosphate was found at serine-565 showing that it is at least partially phosphorylated in vivo. The effect of phosphorylation at serine-565 by AMP-PK is to inhibit phosphorylation and activation of HSL by cAMP-PK at serine-563. AMP-PK also phosphorylates in vitro a number of other substrates including casein, glycogen synthase and phosphorylase kinase. Other proteins which are good substrates for cAMP-PK (e.g. L-pyruvate kinase, 6-phosphofructo 2-kinase/fructose-2,6-bisphosphatase) are not significantly phosphorylated by AMP-PK. None of the sites phosphorylated on phosphorylase kinase are phosphorylated stoichiometrically so a physiological role for this phosphorylation is less likely.

1.8 Importance of this Research

The end products of the lipogenic pathway in mammalian liver are triacylglycerol and cholesterol ester (Fig. 1.1). These are assembled together with apo B to form VLDL particles, which are then secreted into the blood stream to supply the peripheral tissues with an energy source or a source of metabolic fuel for storage. VLDL is subsequently metabolised to LDL which is the major form in which cholesterol is supplied to peripheral tissues but it is also the major perpetrator of atherosclerosis.

Atherosclerotic lesions occur in the innermost layers of arteries and consist of plaques of proliferated smooth muscle cells infiltrated with macrophages and embedded in a matrix of basement membrane, proteoglycan and connective tissue. The formation of these plaques leads to an increased risk of thrombosis because they cause restricted blood flow. Many of the cells in the plaque are rich in cholesterol ester which gives their cytoplasm a foamy appearance. This cholesterol is derived from circulating LDL which infiltrates the site of the arterial lesion and is taken up by these cells (Brown and Goldstein, 1987). Elevated levels of LDL cholesterol in the bloodstream are therefore important risk factors in the development of atherosclerosis and coronary heart disease.

Application of the British Hyperlipidaemic Association guidelines concerning acceptable levels of plasma cholesterol and triglyceride would bring 25% of all British adults into the hyperlipidaemic category. For most individuals, this problem arises due to an increased dietary intake. Over-consumption of carbohydrate, cholesterol and saturated fat can have the effect of both overproduction of VLDL by the liver and down-regulation of hepatic LDL-receptor concentration so that LDL particles are less rapidly removed from the circulation (Brown and Goldstein, 1987). Hyperlipidaemia is a common feature of obesity and type II diabetes mellitus where hepatic over-production of VLDL is accompanied by decreased VLDL metabolism in the periphery. For these individuals dietary intervention is always the first line of therapeutic action. Some individuals exhibit a genetic predisposition to hyper-lipidaemia and show a variable response to dietary measures. Pure genetic hyper-lipidaemias include familial hypercholesterolaemia which results from an LDL-

receptor deficiency failing to adequately clear plasma LDL. Familial hyper-triglyceridaemia and familial combined hyperlipoproteinaemia are both in part the result of VLDL oversynthesis or decreased VLDL metabolism (Tait and Shepherd, 1989). The overriding consequence of hyperlipidaemias whether they are of dietary, hormonal or genetic origin is the increased disposition towards atherosclerosis and cardiovascular disease.

It would appear that lipid biosynthesis and VLDL production is regulated by reversible phosphorylation and the two protein kinases heavily implicated in this regulatory mechanism are cAMP-PK and AMP-PK. Therefore, the aim of the present investigation is to study, in some detail, the roles of cAMP-PK and AMP-PK in the regulation of mammalian lipid metabolism. Only a full understanding of the mechanism of regulation of fatty acid, triacylglycerol and cholesterol biosynthesis and VLDL assembly and secretion will allow the design of effective chemotherapeutic agents to combat the development of hyperlipidaemia and atherosclerosis.

CHAPTER TWO

Materials and Methods

2.1.1 Animals

Wistar rats were fed a standard chow diet (Special Diets Services Ltd, Kent, UK) and water ad libitum unless indicated otherwise. The animals were kept in windowless rooms, which were lit from 08.00 - 20.00 hours and dark from 20.00 - 08.00 hours. The female rats were mated at approximately 200g body weight and used at peak lactation (10-15 days post parturition).

Experimental operations were started between 09.30 and 10.00 hours and, unless otherwise stated, rats were anaesthetized with a non-recovery dose of pentobarbital (60mg/kg body weight) administered by intraperitoneal injection. Dissection was carried out under deep anaesthesia such that motor activity had ceased. Mammary tissue for freeze clamping was quickly but carefully freed from the skin and abdominal wall without cutting the blood vessels. The gland was thus sampled with the circulation intact. Liver tissue was freeze-clamped in situ. The freeze-clamped tissues were powdered under, and stored in, liquid nitrogen. When tissue was taken for enzyme purification it was often from stunned and cervically dislocated animals.

2.1.2 Chemicals

Biochemicals

Histone (type II-AS), casein, cAMP, avidin, acetyl-CoA, palmitoyl-CoA, bovine serum albumin (BSA), ATP, n-octyl-ß-D-glucopyranoside, all protease inhibitors (see section 2.1.4), gentamycin and sodium pentobarbital were all obtained from Sigma Chemical Company Ltd., Poole, Dorset, U.K. Acrylamide for gel electrophoresis and Coomassie Blue was obtained from BDH Chemicals, Poole, Dorset, U.K. Other reagents were obtained from Boehringer Mannheim (BCL), Lewes, Sussex, BDH Chemicals, Sigma Chemical Company Ltd, Poole, Dorset, UK., or Aldrich Fine Chemicals, Gillingham, UK.

Radiochemicals

[γ-^{32}P] ATP, $^{3}H_2O$, and sodium [^{14}C]-bicarbonate were obtained from ICN Radiochemicals, California, USA.

2.1.3 Peptide Substrates and Inhibitors

Kemptide, the peptide substrate of cAMP-PK, the specific peptide inhibitor of cAMP-PK and the Walsh inhibitor were obtained from the Sigma Chemical Company, Poole, Dorset, UK. The sequence of Kemptide is, Leu-Arg-Arg-Ala-Ser-Leu-Gly.

Peptide substrates based on the phosphorylation sites identified in ACC and used for assaying AMP-PK, ACK-2 and cAMP-PK activities were synthesized and purified by Dr. Gabor Toth and Dr. Paolo Mascagni, using an ABI peptide synthesizer in the

School of Pharmacy, and were also a generous gift from Dr David Carling. Two peptides, 'SSMS' and 'SAMS', were synthesised and are described below,

'SSMS': His-Met-Arg-Ser-Ser-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg

'SAMS': His-Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg

2.1.4 Protease Inhibitors

The following protease inhibitors were routinely used for all protein purifications:

For trypsin and trypsin like proteases: Benzamidine (BZ), Soya-bean trypsin inhibitor (SBTI), Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK).

For chymotrypsin: N-tosyl-L-phenylalanine chloromethyl ketone (TPCK).

For serine proteases (eg elastase,trypsin, chymotrypsin): Phenylmethylsulphonyl fluoride (PMSF).

For Metalloproteinases: EDTA and/or EGTA.

The protease inhibitors were added to buffers as indicated.

2.2 Purification and Preparation

2.2.1 Preparation of Affinity Chromatography Media

Buffers required for cyanogen bromide activation of Sepharose and coupling of ligand to gel.

Coupling Buffer

10mM sodium phosphate buffer - prepared by mixing appropriate quantities of the

mono and dihydrogen salt to give pH 7.0 at 25°C.

Blocking Buffer

1mM ethanolamine adjusted to pH 7.0 with HCl.

Monomerisation Buffer

3M guanidine in 0.2M KCl adjusted to pH 1.5 with 0.2M-HCl.

Column Buffer

20mM Tris HCl pH 7.4 at 4°C, 0.5M sodium chloride, 1mM EDTA, 5mM sodium pyrophosphate, 50mM sodium fluoride, 10% (w/v) glycerol 0.02% (w/v) and sodium azide.

Glycine Buffer

0.1M glycine adjusted at 25°C to pH 2.0 with HCl.

Method

Approximately 50ml of Sepharose CL-4B-200 gel (Sigma Chemicals) was washed with one litre of water under suction. The gel was resuspended in 550ml of 5mM Phosphate buffer (Coupling buffer diluted 1:1 with water), and the pH adjusted to pH 11.0 with 2M sodium hydroxide.

The gel suspension was then mixed with 15g of finely ground cyanogen bromide (Sigma Chemicals) in a large beaker. The temperature was maintained at 2°C by

adding chipped ice, and the pH maintained at 11.0 by the dropwise addition of 2M sodium hydroxide. After twenty minutes the mixture was filtered over ice and then washed with coupling buffer (≈700ml) using suction on a Buchner funnel. The gel was then resuspended in 135ml of coupling buffer containing 0.2mg/ml avidin or 5 mg/ml histone (type II-AS). The gel suspension was transferred to a screw top jar and mixed overnight (15 hours at 4°C) using an 'end over end' mixer to maximise coupling of ligand to the activated Sepharose. The coupled gel was then filtered under suction and resuspended in 200ml of the Blocking buffer.

For Avidin-Sepharose only

Blocking buffer was removed on a Buchner filter funnel and the gel was rinsed with Monomerisation buffer and the gel resuspended in 200ml of the Monomerisation buffer and left at room temperature overnight. The gel was poured into a column and washed through with monomerisation buffer. The column was washed with five column volumes of Column buffer until the A_{280} against Column buffer was ≤0.05. The column was then washed with five column volumes of 0.8mM biotin in Column buffer and prepared for use by washing with three volumes of Column buffer followed by three volumes of Glycine buffer. This was repeated twice more and the column finally equilibrated with column buffer. This procedure ensured that high affinity avidin sites were blocked by the 0.8mM biotin. The Glycine buffer with its low pH removes biotin from avidin, but not from the high affinity avidin sites.

Histone-Sepharose

The coupled gel was suctioned free of Coupling buffer and then resuspended in 200ml

of AMP-PK buffer (see Section 2.2.5) with 0.2% (w/v) sodium azide and stored at 4°C. Before use the gel was packed into a column and washed with five column volumes of 1M sodium chloride in 100mM Tris/HCl pH 7.0 at 4°C, followed by equilibration in AMP-PK buffer.

2.2.2 Purification of Acetyl-CoA Carboxylase (ACC) (from Tipper and Witters 1982)

ACC Homogenisation Buffer

0.25M sucrose, 2mM EDTA, 50mM NaF, 72mM Tris HCl pH 8.5 at 4°C, 5mM NaPPi, 2mM DTT, 2mM Benzamidine, 8μg/ml SBTI, 1mM PMSF, 0.1mM TPCK and 0.2mM TLCK.

Avidin-Sepharose Buffer

20mM Tris HCl pH 7.4 at 4°C, 0.5M NaCl, 5mM NaPPi, 50mM NaF, 0.2% (w/v) NaN_3, 10% (w/v) glycerol, 2mM-DTT, 2mM Benzamidine, 8μg/ml SBTI and 0.2mM TLCK.

Method

Dissected mammary gland or liver was freeze-clamped and the tissue pulverised under liquid nitrogen. 1.0-1.2g of the tissue powder was homogenised in 10 volumes of ACC homogenisation buffer using 30 strokes of a hand held pyrex/teflon homogeniser. The homogenate was centrifuged at 50,000g for 20 minutes at 4°C.

The supernatant was filtered through glass wool and then taken to 40% saturated ammonium sulphate by the gradual addition of the solid salt, (0.243g/ml). The suspension was kept on ice for 5 minutes then centrifuged at 4°C for 10 minutes at 50,000g, the supernatant was discarded and the pellet flash frozen in liquid nitrogen before storage at -70°C if it was not to be used immediately.

The ammonium sulphate pellet was resuspended in 5ml of avidin-Sepharose buffer. Undissolved material was removed by centrifugation at 25,000g for 15 minutes. The resuspension was applied directly to a 2ml avidin-Sepharose column. The column was washed with 40mls of the same buffer, and the protein was then eluted in avidin-Sepharose buffer containing 3mM biotin and no glycerol. Fractions of 0.5ml were collected, and the fraction having the highest protein concentration (typically 60-100μg/ml) as determined by the Coomassie blue protein assay (section 2.4.1) was taken for immediate assay of ACC activity (section 2.3.2). The fraction was diluted to 15μg/ml of ACC using 100mM Tris HCl pH 7.4 at 37°C containing 1% BSA. At this concentration of ACC the assay is linear with respect to time.

2.2.3 Preparation of Enzymes for use as Protein Kinase Substrates

ACC was prepared from lactating rat mammary gland using a scaled up version of avidin-Sepharose chromatography as described above, ATP-Citrate lyase was prepared from lactating rat mammary gland as described by Guy et al (1981), glycogen synthase was prepared as described by Stewart et al (1981), phoshorylase kinase was

prepared as described by Cohen (1973), glycogen synthase and phosphorylase kinase were also generous gifts from Dr. David Carling.

2.2.4 Purification of cAMP-Dependent Protein Kinase

The catalytic subunit of cAMP-PK was purified from bovine and rat heart as described by Reimann and Beham (1983). The holoenzyme forms of cAMP-PK were ~~purified~~ isolated from lactating rat mammary gland as described by Clegg and Ottey (1990).

2.2.5 Purification of AMP-Activated Protein Kinase (AMP-PK) from Lactating Rat Mammary Gland

The purification and characterisation of AMP-PK from rat liver is described in considerable detail by Carling et al (1989). The purification of AMP-PK from lactating rat mammary gland is based on the published methodology for rat liver.

Homogenisation Buffer

50mM Tris HCl pH 8.4 at 4°C, 0.25M mannitol, 50mM NaF, 5mM NaPPi, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.1mM PMSF, 4μg/ml SBTI, 1mM Benzamidine.

AMP-PK Buffer

20mM Tris HCl pH 7.2 at 4°C, 0.5mM EDTA, 0.5mM EGTA, 5mM NaPPi, 10% (w/v) glycerol, 5mM DTT, 0.02% (w/v) Brij 35, 1mM Benzamidine,

4μg/ml SBTI, 0.1mM TLCK.

Method

Dissected liver or mammary gland tissue was rapidly rinsed in ice-cold homogenisation buffer where it was finely chopped using a pair of scissors. The tissue was homogenised in a domestic kitchen blender using three thirty second bursts at top speed, with a minute on ice in between each burst. All steps subsequent to homogenisation were performed at 4°C in the buffer described above. The homogenate was centrifuged at 3,000g for 10 minutes, and polyethylene glycol 6000 was added to a final concentration of 2.5% (w/v). After centrifugation at 30,000g for 15 minutes, the supernatant was retained and the pellet discarded. Polyethylene glycol was added to the supernatant to give a final concentration of 6% (w/v). The homogenate was centrifuged at 30,000g for 15 minutes and the 6% PEG pellet retained and the supernatant discarded. The 6% pellet was resuspended in AMP-PK buffer and the suspension clarified by centrifuging at 25,000g for 15 minutes. The supernatant was applied to a DEAE-Sepharose fast flow column, equilibrated in AMP-PK buffer. The column was washed with AMP-PK buffer until the A_{280} was <0.05, and eluted with AMP-PK buffer containing 200mM sodium chloride. Fractions showing the greatest AMP-PK activity were pooled and dialysed to remove sodium chloride and then applied to a Blue-Sepharose column equilibrated in AMP-PK buffer. The protein was eluted with a linear gradient from 0-1M sodium chloride in AMP-PK buffer. Once again the fractions showing the highest AMP-PK activity were pooled and concentrated using an Amicon concentrator and dialysed into AMP-PK buffer to remove sodium chloride. This was then applied to a Mono-Q column (HR 5/5)

previously equilibrated in AMP-PK buffer at 1ml/min, the column was washed with AMP-PK buffer until the A_{280} was <0.05 and AMP-PK eluted with a gradient from 0 - 0.4M sodium chloride in AMP-PK buffer. Fractions showing the greatest AMP-PK activity were pooled and concentrated using an Amicon concentrator.

This was then applied to a histone-Sepharose affinity gel, prepared as described in Section 2.2.1. The column of histone-Sepharose was then washed with AMP-PK buffer until the A_{280} was 0.05. Protein was eluted from the column in a batchwise fashion, using AMP-PK buffer containing 0.25M and 0.5M-sodium chloride. The first elution eliminated 50% of the protein applied but less than 10% of the kinase activity. The 0.5M eluate exhibited 90% of the kinase activity applied. The fractions showing the highest AMP-PK activity were pooled and concentrated by using an Amicon concentrator followed by a centricon unit with a molecular weight cut off at 30,000 Da, the concentrated eluate was stored at 4°C.

2.2.6 Preparation of Lipoproteins by Sequential Ultracentrifugation of Plasma

The method used in the work reported here used plasma as a source of lipoproteins. Under even the most optimal conditions the isolation of a particular class of lipoproteins will take time e.g. 20 hours are required for VLDL isolation and 40 hours for LDL isolation, during which time they will be liable to degradation in three ways.

1) by enzymes that are native to the plasma or of microbiological origin

2) by oxidation

3) since the components of the lipoprotein complex are bound together only by non-covalent forces, it is possible that some disproportion may occur during preparative manipulations.

The first two degradative effects can be counteracted by storing the plasma between 0 and 4°C, although ideally it should be used as soon as possible after its preparation, and by the inclusion of protease inhibitors e.g. Benzamidine, SBTI and PMSF, NaN_3 which is a bactericide, and DTT and EDTA which are antioxidants. EDTA greatly reduces any oxidation processes by sequestering heavy metal catalysts and also inhibits metalloproteinases. Oxidation is probably the most important hazard once isolation has begun, and EDTA must be added to all the solutions used.

Routinely 500-1000ml of blood (St. Bartholomew's Hospital, London), was collected in the presence of citrate or EDTA to prevent clotting. The blood was centrifuged at 5,000g for 20 minutes at 15°C to sediment red cells. In practice half of the blood volume is plasma. The plasma was removed and recentrifuged if necessary, EDTA (0.02% w/v), sodium azide (0.02% w/v) in sodium chloride (0.85% w/v), gentamycin sulphate (0.02% w/v), 1mM benzamidine, 4ug/ml SBTI and 2mM PMSF were added to the plasma.

The plasma was centrifuged at 150,000g for 20 hours at 15°C. A white layer of triglyceride-rich VLDL and chylomicra collected at the top of the sample. This was removed by clamping the centrifuge tube to keep it stable (any jarring of the tube causes the lipoproteins to edify into the rest of the contents) and drawing off the top

layer of lipoprotein very carefully with a very thin bore Pasteur pipette.

To isolate LDL the density of the infranatant plasma was adjusted to 1.063g/ml with potassium bromide and the plasma centrifuged at 150,000g for 20 hours at 15°C. The yellow oily cholesterol-rich LDL layer collected at the top of the sample and was removed by aspiration as decribed above. After collection each lipoprotein fraction was dialysed into 50mM Tris HCl pH 7.4 at 4°C containing EDTA (0.5mM), sodium azide (0.02% w/v), sodium chloride (0.85% w/v) for 24 hours. After dialysis, gentamycin sulphate (0.02% w/v), 1mM benzamidine, 4μg/ml SBTI and 2mM PMSF were added to the lipoproteins. The lipoproteins were stored at 4°C, after their protein concentration had been determined. 100mls of blood will yield approximately 10mg of LDL.

The quantity of potassium bromide to be added for density adjustments is calculated using the following formula:

$$(V2 \times D2) - (V1 \times D1) / A = Y$$

$(Y - 1)(1.45)(A)$ = grams of KBr required, where

D1 = density of sample D2 = density required

V1 = volume of sample V2 = volume after density adjustment

(V2 - V1) = the difference in volume, A.

The KBr is added to the sample, mixed and the sample brought up to volume with water. The difference in volume must be estimated in advance because addition of

the solid KBr will increase the volume of the sample and this must be considered in the calculation.

2.3 Assays

2.3.1 Assay of Pyruvate Dehydrogenase

The active form of the PDH complex (PDHa) and the activity of citrate synthase and total activity after incubation with PDH phosphatase were measured in freeze clamped tissue extracts as described by Holness et al (1988). One unit of PDH activity converts 1μmol of substrate into product per minute at 30°C and is expressed relative to citrate synthase activity in order to correct for possible differences in the efficiency of extraction of mitochondria.

2.3.2 Assay of Acetyl-CoA Carboxylase (ACC)

Principle of Assay

ACC catalyses the synthesis of malonyl-CoA from acetyl-CoA and bicarbonate. The overall reaction can be summarised by the following half reactions:

1) $ATP{:}Mg^{2+} + HCO_3^- + \text{E-biotin} \rightarrow \text{E-biotin } CO_2 + ADP + Pi$

2) $\text{E-biotin} - CO_2^- + \text{acetyl-CoA} \rightarrow \text{malonyl-CoA} + \text{E-biotin}$

The activity of ACC is quantified by measuring the incorporation of ^{14}C-bicarbonate into malonyl-CoA.

2.3.2a Assay of ACC in Crude Homogenates (Initial/ Total)

Initial Activity

Powdered frozen liver or mammary gland was homogenised in 10 volumes of buffer containing 0.3M mannitol, 100mM Tris HCl buffer pH 7.4 at 4°C, 2mM EDTA, 50mM NaF, 2mM NaPPi, 1mM DTT, 1mM benzamidine, 4μg/ml SBTI and 1mM PMSF. Homogenisation was achieved using 30 strokes of a pyrex-teflon hand held homogeniser. The homogenate was spun in a bench centrifuge at 12,000g for one minute and the supernatant was removed. Poly(ethylene glycol) was added to the liver supernatant to give a final concentration of 6% (w/v). The mixture was whirlimixed and left on ice for 2 minutes and then spun in a bench centrifuge at 12,000g for 3 minutes. The supernatant was discarded and the poly(ethylene glycol) pellets were frozen in liquid nitrogen.

Supernatants from lactating mammary gland prepared in the presence of 50mM NaF and 2mM NaPPi (phosphatase inhibitors) were assayed for initial ACC activity immediately at 0.5mM and 10mM citrate by the ^{14}C fixation assay described below. For the liver, measurements of initial activity required the resuspension of the 6% poly(ethylene glycol) pellet. The 6% poly(ethylene glycol) pellet was resuspended in 150μl of buffer containing 0.15M mannitol, 100mM Tris HCl pH 7.4 at 37°C, 1mM EDTA, 1mM DTT, 100mM NaF, 2mM NaPPi, 1mM benzamidine, 2μg/ml-SBTI. An aliquot of this resuspension was taken for a protein concentration assay. A 10μl aliquot of the resuspended PEG pellet, or of the mammary gland supernatant, was then assayed for ACC activity in buffer containing 20mM Tris HCl pH 7.4 at 37°C,

0.3mM acetyl-CoA, 4mM-ATP/2mM $MgCl_2$, 1% BSA, 2mM $NaH^{14}CO_3$, $MgCl_2$ and citrate were added to give the desired final concentrations of citrate, i.e. 0.5mM and 10mM. Blanks were conducted in the absence of acetyl-CoA. The assay was stopped after 90 seconds at 37°C by the addition of 50μl of a 7% perchloric acid solution and left on ice for at least 3 minutes. The tubes were then spun in a bench centrifuge at 13,000rpm for 3 minutes. A 125μl aliquot of the supernatant was removed into a clean "Eppendorf tube" and dried down in a Gyrovap (V.A. Howe and Co. Ltd., London) at 40°C. 100μl of water was added to resolubilise the dry residues, followed by 1ml of scintillation fluid, the samples were mixed on a whirlimixer and counted on a LKB-Rackbeta Scintillation Counter.

Total Activity

Powdered frozen liver or mammary gland tissue was homogenised in 10 volumes of buffer containing 0.3M mannitol, 100mM Tris HCl pH 7.4 at 4°C, 2mM EDTA, 2mM DTT, 1mM benzamidine, 4μg/ml SBTI and 1mM PMSF. The tissue was homogenised using 30 strokes of a hand-held pyrex-teflon homogeniser. The homogenate was centrifuged at 13,000rpm for 1 minute using a bench centrifuge and the resulting supernatant was removed. The liver supernatant was used for the production of 6% (w/v) poly(ethylene glycol) pellets as described above. For measurement of total activity the mammary gland supernatant was diluted with incubation buffer and the 6% (w/v) poly(ethylene glycol) pellet was resuspended in incubation buffer to give, in both cases final concentrations of 0.3M mannitol, 100mM Tris HCl pH 7.4 at 37°C, 2mM EDTA, 1mM DTT, 1mM benzamidine, 4μg/ml SBTI, 1mM PMSF, 20mM sodium citrate, 20mM $MgCl_2$ and 20 U/ml protein

phosphatase-2A. After incubation at 37°C for 30 minutes to achieve total dephosphorylation and activation of ACC the activity of ACC was assayed at 20mM Mg.citrate concentration using the method described above for initial enzyme activity.

2.3.2b Assay of Purified ACC (Holland et al 1984)

ACC purified by avidin-Sepharose affinity chromatography was diluted to between 15 and 20μg/ml with 0.1M Tris HCl, pH 7.4 at 37°C, containing-1mM DTT and 2% BSA. The activity of ACC was then measured at 37°C in a total volume of 100μl containing 0.1mM Tris HCl, pH 7.4 at 37°C, 1mM DTT, 0.3mM acetyl-CoA, 1% BSA, 4mM ATP, 2mM $MgCl_2$, 20mM $NaH^{14}CO_3$ and citrate/$MgCl_2$ ranging in concentration from 0 to 20mM. The assay was started by the addition of 10μl of ACC solution, to give a final protein concentration of 1.5 - 2.0μg/ml. After a 4 minute incubation the reaction was stopped by the addition of 50μl of 6% (v/v) perchloric acid. The terminated assays were centrifuged in a bench centrifuge at 12,000 rpm for 3 minutes. A 125μl aliquot of each supernatant was transferred to a clean "Eppendorf tube", dried down in a rotary vacuum evaporator (Gyrovap, V.A. Howe and Co. Ltd., London) at 40°C. The residue was redissolved in 100μl of water and then 1.0ml of scintillation fluid was added before being counted on a Rackbeta (LKB) Scintillation Counter. The results were expressed as μmol of ^{14}C incorporated into acid stable product per min per mg of ACC protein.

V_{max}, K_a for citrate and the Hill co-efficient (h) were determined using computational fitting of the initial rates (v) at each citrate concentration (c) to the following equation:

$$v = \frac{Vmax\,[c]^h}{Ka + [c]^h}$$

2.3.3 Calculation of Specific Radioactivity of $NaH^{14}CO_3$

Stock $NaH^{14}CO_3$ (200mM) was diluted 1:10 with H_2O. A 10μl aliquot of this was pipetted into 1ml of scintillation fluid in an "Eppendorf tube", to which had previously been added 90μl of 1M NaOH to ensure a slightly alkaline pH. The mixture was whirlimixed and counted using a LKB-Rackbeta Scintillation Counter. The resulting dpm (typically about 400,000 dpm) represented the dpm of the 200nmol in the 10ul aliquot and consequently the specific radioactivity of the $NaH^{14}CO_3$ solution was typically about 2000 dpm/nmol.

2.3.4 Assay of Plasma Insulin Concentration

Plasma insulin concentrations were determined by Dr. Mary Sugden using published methods (Sugden et al, 1990).

2.3.5 Assay of Glycogen Concentrations and Rates of Synthesis

Hepatic glycogen concentrations and rates of synthesis were measured by Dr. Mary

Sugden as described in Holness et al (1988).

2.3.6 Assay of cAMP-PK Activity (modified from Roskoski 1983)

2.3.6a Preparation of Tissue Extracts

Powdered frozen liver or mammary gland tissue was homogenised in 10 volumes of buffer, containing 10mM potassium phosphate pH 6.8 (made from an equimixture of the mono and di-hydrogen salts), 1mM EDTA, 1mM IBMX, 1mM DTT, 1mM benzamidine, 4ug/ml SBTI and 1mM PMSF, using 30 strokes of a pyrex-teflon hand held homogeniser. The homogenate was diluted with 10mg/ml BSA such that the total final dilution, including that in the assay, would be one that gave a linear rate of phosphorylation of the peptide substrate, Kemptide. An aliquot of this diluted homogenate was immediately transferred to the assay incubation described below to determine the activity of cAMP-PK.

2.3.6b Assay for cAMP-PK Activity

Diluted tissue extracts, holoenzyme forms or purified catalytic subunits of cAMP-PK were assayed at 37°C for 4 minutes, in a 25μl incubation containing 10mM HEPES pH 7.0, 150μM Kemptide, 0.2mM [γ^{32}P] ATP (specific radioactivity 2-4 x 10^6 cpm/nmol), 4mM $MgCl_2$, 50mM NaF, 1mM NaPPi and 0.2mM EDTA and in assays of the holoenzyme 10μM cAMP (to dissociate the holoenzyme). The initial activity

of cAMP-PK was measured as the linear incorporation of ^{32}P into Kemptide over a 4 minute incubation from which incorporation in the presence of the specific peptide inhibitor of cAMP-PK (4.0 Units/μl) had been subtracted. In diluted tissue extracts the total cAMP-PK activity was measured in a similar manner but in the presence of cAMP (10μM). At the end of each incubation, reactions were stopped with excess EDTA (5mM) to chelate the magnesium and an aliquot of the reaction mixture was spotted onto a square of P81 phosphocellulose paper (Whatman). The papers were washed three times in 75mM phosphoric acid and immersed in 4ml of a toluene based scintillant in an LKB-Rackbeta Liquid Scintillation Counter. This procedure was identical for other substrates except that ACC, ATP-Citrate lyase, casein, histone, glycogen synthase, LDL and phosphorylase kinase were precipitated with TCA (25% w/v) at the end of each reaction. BSA (300μg) was added as a carrier and after centrifugation (13,000 rpm in a bench microfuge for 3 minutes), the protein pellet was washed extensively with TCA (25% w/v) and counted by Cerenkov counting. One unit of cAMP-PK activity is defined as the incorporation of 1nmol of radiolabelled phosphate into the appropriate substrate per minute. The activity ratio of cAMP-PK is defined as the ratio of initial (or basal) : total cAMP-PK activity.

2.3.7 Assay of AMP-activated Protein Kinase

2.3.7a Preparation of Tissue Extracts

Powdered frozen liver or mammary gland tissue was homogenised in 10 volumes of buffer containing 0.25M mannitol, 100mM Tris HCl pH 7.2 at 4°C, 50mM NaF,

1mM EDTA, 1mM benzamidine, 4μg/ml SBTI and 1mM PMSF, using 30 rapid strokes of a pyrex-teflon hand held homogeniser. The homogenate was diluted with BSA such that the total final dilution, including that in the assay, for either tissue homogenate was one that would give a linear rate of phosphorylation of the specific peptide substrate of AMP-PK. A 5μl aliquot was transferred to the assay incubation described below to determine the activity of AMP-PK.

2.3.7b Assay for AMP-PK Activity

Diluted tissue extracts or purified AMP-PK were assayed at 37°C for 4 minutes in a 25μl incubation containing 10mM HEPES pH 7.0, 100μM synthetic peptide substrate "SAMS", 0.2mM [γ-^{32}P] ATP (specific radioactivity 2-4 x 10^6 cpm/nmol), 4mM $MgCl_2$, 50mM NaF and 0.2mM EDTA, 200μM 5'AMP was added to some experiments to show activation of AMP-PK, but not in experiments with tissue extracts. ^{32}P icorporation into the "SAMS" synthetic peptide and into other protein substrates was analysed as described for cAMP-PK above. The activity of AMP-PK is expressed as the incorporation of 1nmol of radiolabelled phosphate into the appropriate substrate per minute.

2.3.8 Assay of Acetyl-CoA Carboxylase Kinase 2 (ACK-2) Activity

Partially purified ACK-2 was assayed at 37°C in a 25μl incubation containing 20mM Tris HCl pH 7 at 4°C, synthetic peptide substrate "SSMS", 0.2mM-[γ^{32}P] ATP (specific radio activity 1-2.5 x 10^5 cpm/nmol), 4mM $MgCl_2$, 50mM NaF, 0.2mM

EDTA and 2.1 units of the specific peptide inhibitor of cAMP-PK. Incubations with "SSMS" peptide or protein substrates were stopped and analysed as described for cAMP-PK and AMP-PK above. The activity of ACK-2 is expressed as the incorporation of 1nmol of radiolabelled phosphate into the substrate peptide "SSMS" per minute. The specific inhibitor of cAMP-PK is included in the assay because this synthetic peptide is also phosphorylated by cAMP-PK. This synthetic peptide is also phosphorylated by AMP-PK and the inability to distinguish between AMP-PK and ACK-2 phosphorylation of the peptide means that this assay for ACK-2 is not as specific as that for cAMP-PK or AMP-PK.

2.3.9 Assay of Novel Apo B Kinase Activity

The novel apo B kinase was incubated with LDL apo B-100 presented at a final concentration of 1mg/ml, 0.2mM [γ^{32}P] ATP (specific radioactivity 2.5 x 10^5 cpm/nmol) and 4mM $MgCl_2$. The assay was carried out in buffer containing 20mM Tris HCl, pH 7 at 37°C, 50mM NaF, 0.5mM EDTA, 1mM benzamidine, 4μg/ml SBTI and 1mM PMSF. Assays were incubated at 37°C and terminated by the addition of 1ml of TCA (25% w/v), 300μg of BSA were added and the assay mixture centrifuged in a bench centrifuge at 10,000g for 3 minutes. The precipitate was washed 3 times with TCA (25% w/v) and then counted by Cerenkov counting in a LKB-Rackbeta Scintillation counter. One unit of apo B kinase activity is defined as the incorporation of 1nmol of radiolabelled phosphate into LDL apo B per minute. All assays conducted for the determination of protein kinase activity were performed with quantities of kinase which produced linear rates of phosphorylation with respect

to both time and protein added.

2.3.10a Determination of the Specific Radioactivity of ATP

The concentration of ATP was determined spectrophotometrically from its absorbance at 260nm. Using the Beer-Lambert law where A is the absorbance at 260nm, c is the concentration of the solution, l is the path length of the cell (usually 1cm) and ϵ is the molar extinction coefficient, the concentration of ATP was determined using the equation below,

$$A = \epsilon . c. l.$$

A 5μl aliquot of the ATP solution was counted in a LKB-Rackbeta scintillation counter by Cerenkov counting. The specific radioactivity was therefore calculated as cpm/nmol ATP. If the concentration of substrate and its level of phosphorylation by a protein kinase, i.e. the net incorporation of cpm taking account of blanks, is known, it is possible to calculate the stoichiometry of phosphorylation. For example to calculate the stoichiometry of phosphorylation of LDL apo B.

2.3.10b Example of a Calculation of Stoichiometry of Phosphorylation of LDL Apo B

Absorbance at 260nm of ATP = 0.145

Concentration of ATP solution = 0.145/(0.15 x 1) = 0.97 nmol/μl

cpm per 5μl ATP solution = 1.25 x 10^6

= 250,000 cpm/μl

Specific radioactivity = 250,000/ 0.97 = 257,732 cpm/nmol

LDL solution = 6.17 mg per ml

In an assay using 5μl of LDL = 6.17 x 5 = 30.85μg of LDL

Molecular weight of Apo B protein = 512,000g = 1mol

512μg = 1nmol

therefore nmols Apo B in assay = 30.85/512 = 0.06 nmols

cpm for nmolP/nmol Apo B = 0.06 x 257,732 = 15,464 cpm

Net cpm incorporation into LDL = 9529.3 cpm

nmolP/nmol apo B = 9529.3/ 15,464 = 0.6 nmolP/nmol

or molP/mol apo B

Therefore the stoichiometry of Apo B phosphorylation is 0.6 molP/mol apo B.

2.3.11 Phosphorylation of Low Density Lipoprotein (LDL)

LDL apoB was phosphorylated using highly purified preparations of the catalytic subunit of cAMP-PK and of AMP-PK. The phosphorylations were usually allowed to proceed for 1 hour at 37°C, and the incubation mixtures typically contained 20mM-Tris HCl pH 7.0 at 37°C, 0.2mM [γ^{32}P] ATP (specific radioactivity 1-2.5 x 10^5 cpm/nmol), 4mM $MgCl_2$, 50mM NaF, 1mM NaPPi, 0.2mM EDTA, 0.2mM EGTA, (for assays involving the AMP-PK 200μm AMP was also included) and LDL at a final concentration of approximately 1 mg/ml since this was found to be a very good concentration (and was certainly above the Km) for phosphorylation of LDL. Reactions were stopped by the addition of TCA (25% w/v) and LDL apoB protein was precipitated by adding BSA (300μg) and centrifuging in a bench centrifuge at

12,000g for 3 minutes. The precipitated apo B was washed 3 times using TCA (25% w/v), the ^{32}P incorporation into apoB was determined by Cerenkov counting in a LKB-Rackbeta Scintillation counter and subsequently the stoichiometry of phosphorylation of apoB determined.

2.3.12 Digestion of LDL ApoB-100 by Thrombin

LDL-apoB-100 was phosphorylated using purified cAMP-PK catalytic subunit or AMP-PK as described above. After a 60 minute incubation, an aliquot of the reaction mixture was removed to determine the stoichiometry of phosphorylation of the LDL-apoB-100 as described above. Another equal aliquot was removed from the reaction mixture and boiled with SDS-sample buffer as described in Section 2.4.2a, for five minutes and then stored at -20°C until required for subsequent electrophoresis.

To the remaining volume of the reaction mixture, EDTA was added to a final concentration of 10mM to chelate Mg^{+2} ions and thereby terminate the phosphorylation reaction. To this terminated reaction mixture an aliquot of thrombin solution was added (Sigma Chemical Co. Ltd., UK) in the ratio 1:50 (w/w) thrombin: apo B. This mixture was incubated at 37°C overnight and then SDS-sample buffer was added to the thrombin digest mixture, the sample boiled for five minutes and then stored at -20°C until required for subsequent electrophoresis. Polyacrylamide gel electrophoresis was carried out on 10% gels, as described in Section 2.4.2a. The gels were fixed, stained and destained as described in Section 2.4.2b.

2.3.13 Release of ^{32}P from Radiolabelled LDL ApoB-100 using Trypsin

LDL-apoB-100 was phosphorylated using purified cAMP-PK catalytic subunit or AMP-PK as described in Section 2.3.11, and the reaction was terminated with excess EDTA (5mM). A solution of trypsin (10mg/ml, stored in hydrochloric acid, pH 3.0) was added to the [^{32}P] labelled LDL-apoB-100 to give a ratio of 1:50 trypsin:apo B. This meant a large dilution of the protease into Tris HCl buffer at pH 7 was required, which in turn meant the highly acidic pH of the trypsin solution was diluted away and was not a problem in the subsequent parts of the experiment.

This reaction mixture was incubated at 37°C, and at set times aliquots were removed from the mixture and pipetted into 1ml of TCA (25% w/v), 300μg BSA was added as a carrier and the mixture centrifuged at 12,000g for 3 minutes. The pellet was washed 3 times with TCA (25% w/v) and counted by Cerenkov counting as described previously. The time course of release of radiolabel was determined by comparing the degree of phosphorylation at each time point to the initial level of phosphorylation, and it was found that there was virtually total release of all [^{32}P] label from phosphorylated LDL-apoB-100 within 4 hours of incubation with trypsin.

2.4 General Methods

2.4.1 Assay of Protein Concentration

Protein concentration was assessed by two techniques:

1) The direct absorbance of protein solutions at 280nm. This method was typically used where quick, but not highly accurate measurements of protein concentration were required e.g. for reading protein concentrations of large numbers of fractions collected after chromatography. This method utilises the ability of aromatic amino acids to act as chromophores with ultra-violet radiation. There is a direct relationship between concentration and absorbance at 280nm.

2) The use of the Coomassie Blue dye binding assay (Bradford, 1976). Coomassie Blue is a dye which binds to basic amino acids and results in the formation of a chromophore which absorbs strongly at 595nm. This method allows accurate measurements of protein concentration to be made. The protein sample is prepared in a plastic cuvette by adding 90μl of water to 10μl of the sample and finally adding 1ml of Coomassie Blue. The contents of the cuvette are mixed and the absorbance at 595nm determined. Blanks are prepared in the same way except there is 100μl of water present to allow for the missing protein sample. Once the absorbance of a protein sample at 595nm is known the protein concentration can be calculated from a standard calibration curve constructed using the absorbance at 595nm of accurately measured amounts of BSA.

2.4.2a Polyacrylamide Gel Electrophoresis (PAGE) of Proteins

The sodium dodecyl sulphate (SDS) Tris-glycine discontinuous buffer system as described by Laemmli (1970) was employed. The ion detergent SDS coats all proteins with a dense negative charge so that upon application of a potential difference

all proteins migrate towards the positive electrode at rates determined only by the size of the protein. SDS also acts as a denaturing agent causing unfolding of the protein and also dissociation of any subunit based holoenzymes into their constituent components. The separating gel was prepared using the appropriate percentage (w/v) polyacrylamide solution, 0.375M Tris HCl, pH 8.8 at 37°C and 0.1% (w/v) SDS. The stacking gel solution was prepared using 3% or 5% (w/v) polyacrylamide, 0.125M Tris HCl, pH 6.8 at 37°C and 0.1% (w/v) SDS. Both solutions were thoroughly degassed before use. The separating and stacking gels were cross-linked with 5μl/10ml of TEMED (N N N' N'tetramethylethylenediamine, Sigma) and 0.05% (w/v) ammonium persulphate which were added just before use. Slab gels of 15 x 15 x 0.2cm were cast using a standard casting kit (LKB). The stacking gel serves to stack the protein samples loaded so that they enter the separating gel at the same time, the separating gel then separates out protein samples according to their molecular weights. The gels were placed in a vertical slab cell unit (LKB) and run typically, with voltage being the limiting factor, at 30mA through the stacking gel and 40mA through the separating gel, with the gel run complete in ≈5-6 hours. Gradient gels were usually run at concentrations of polyacrylamide between 5 and 15%. A gradient maker (LKB) was utilised for pouring gradient gels and the gel was typically poured from approximately 8-10 inches above the base of the gel stand.

The proteins for analysis were solubilised by boiling in SDS-sample buffer for approximately 3-5 minutes. SDS-sample buffer contained 62.5mM-Tris HCl (pH 6.8) with 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-Mercaptoethanol and 0.05mg/ml of bromophenol blue as a marker. Prior to electrophoresis samples were

heated at 70-80°C for 5-7 minutes. Electrophoresis was performed in reservoir buffer containing 50mM Tris HCl pH 8.3 at 37°C, 0.2M glycine and 0.1% (w/v) SDS. The molecular weight markers routinely used were BSA (66,000 Da), ovalbumin (45,000 Da), glyceraldehyde-3-phosphate dehydrogenase (36,000 Da), carbonic anhydrase (29,000 Da), trypsinogen (24,000 Da), trypsin inhibitor (20,000 Da) and lactalbumin (14,200 Da).

2.4.2b Protein Detection After PAGE by the Coomassie Blue Dye Stain

After completion of electrophoresis, the proteins in the gel were fixed by allowing the gel to soak for at least one hour in a 50% (v/v) methanol, 10% (v/v) acetic acid solution. They were then stained by soaking the gels for 10-20 minutes in 0.6% (w/v) Serva blue R. The bands of protein were visualised by washing the gel in several changes of 10% (v/v) acetic acid, 10% (v/v) methanol until the background had destained sufficiently to allow visualisation of the protein bands.

2.4.3 Autoradiography of Radiolabelled Proteins

Autoradiography of radiolabelled samples separated by SDS-PAGE, after labelling with [$\gamma^{32}P$] ATP was carried out using Kodak X-Omat film exposed in a Kodak X-Omatic cassette at -70°C. The film was developed for 2 minutes with LX-24 developer and then fixed using FX-40 fixer. [^{32}P] labelled samples typically required 24-72 hours exposure in order to give a sufficiently strong signal on the autoradiograph.

2.4.4 Analysis of Phosphorylation Sites on [^{32}P] Labelled Peptides Proteolytically Derived from LDL Apo B-100

LDL-apoB-100 was phosphorylated using purified cAMP-PK catalytic subunit or AMP-PK as described in Section 2.3.11. The reaction was terminated with excess EDTA (5mM) and trypsin added to the phosphorylated apoB-100, the mixture was incubated up until the time point, at which there was 90-100% release of the [^{32}P] label, i.e. 4 hours. This proteolytic digest was carried out with at least 0.5mg of [^{32}P] labelled LDL-apoB-100. At the end of the incubation the reaction mixture was made 10% (w/v) TCA by adding 100% (w/v) TCA, at a 1:10 dilution. This precipitated the majority of the protein but left the tryptic peptides in solution. The mixture was centrifuged for 2 minutes at 10,000g in a bench centrifuge and the supernatant removed as completely as possible using a needle and syringe. The entire volume of this supernatant was then loaded onto a Sep-Pak cartridge, a reverse phase C-18 column (Waters), which had been previously washed with a solution of acetonitrile (80% w/v) containing 0.1% (v/v) TFA but was then equilibrated in water containing 0.1% (v/v) TFA. After loading of the supernatant the Sep-pak cartridge was washed through with water containing 0.1% (v/v) TFA, using about 5-10 syringe volumes to remove unreacted [γ^{32}P] ATP but not the tryptic peptides since these will have bound to the column. The Sep-pak cartridge was then washed through with a solution of acetonitrile (80% w/v)containing 0.1% (v/v) TFA in order to elute the radiolabelled tryptic peptides, 1ml fractions were collected (typically 5-6 fractions were collected) and counted for [^{32}P] by Cerenkov counting in an LKB-Rackbeta Scintillation Counter. The fractions showing the highest activity were dried down,

using a rotary vacuum evaporator (Gyrovap, V.A. Howe & Co. Ltd., London) at 40°C, to a total combined volume of <50μl.

To this dried down solution of radiolabelled tryptic peptides, derived from apoB-100, an aliquot of water containing 0.1% (v/v) TFA was added such that the total volume did not exceed 100μl. The solution of peptides in water/TFA (0.1% v/v) was loaded onto a reverse phase C18 column (Vydac), previously equilibrated in water containing 0.1% (v/v) TFA on a Gilson twin pump HPLC system. The radiolabelled peptides were eluted at a flow rate of 1ml/min, using a gradient running from 0-80% (v/v) acetonitrile/water containing 0.1% (v/v) TFA. Fractions of 1ml were collected and counted for [^{32}P] label by Cerenkov counting. Absorbance at 215nm was used to monitor the eluate and gave a plotted profile of the peptide concentration via a linked chart recorder.

CHAPTER THREE

The role of cAMP-dependent Protein Kinase in Regulating Fatty Acid Synthesis in the Mammary Gland of the Lactating Rat

3.1 Introduction

From studies on adipocytes and hepatocytes it is well established that lipogenesis is inhibited by beta adrenergic agonists and the hormone glucagon (Holland et al, 1983, Robson et al, 1984). These effectors all cause an increase in intracellular cAMP, by the stimulation of adenylate cyclase, and thus stimulate cAMP-PK activity.

However, acini prepared from lactating rat mammary tissue do not respond to adrenaline, glucagon or adrenergic agonists with a decrease in lipogenesis (Robson et al, 1984, Williamson et al, 1983, Clegg and Mullaney 1985). Mammary tissue has been shown to possess no glucagon receptors (Robson et al, 1984) but it does possess β_2-adrenergic receptors which are coupled to adenylate cyclase in a functional manner (Clegg and Mullaney 1986). Despite the presence of this system, it required the treatment of mammary acini with ß-agonists simultaneously with phosphodiesterase inhibitors to produce significant twenty-fold increases in intracellular cAMP levels (Clegg and Mullaney,1985). Administration of isoprenaline to rats, 30 minutes before sampling caused a 4-fold increase in the activity ratio of cAMP-PK in the

lactating mammary tissue, the same effect is observed in isolated mammary acini (Clegg and Ottey 1990). Recent evidence (Clegg and Ottey 1990) has shown that in isolated acini cAMP-PK is able to phosphorylate and activate phosphorylase, a known cellular target protein of cAMP-PK. These results demonstrate that treatment known to increase intracellular cAMP concentrations does cause activation of cAMP-PK which does phosphorylate target proteins.

Withdrawal of food from lactating rats for 24 hours results in a significant inhibition of mammary gland ACC activity (40% inhibition when measured in crude tissue extracts and 73% inhibition when measured using purified enzyme) associated with an increase in the alkali-labile phosphate content of 1mol/ mol of ACC subunit (Munday and Hardie, 1986). This is responsible at least in part for the 98% inhibition of mammary gland lipogenesis. However there is no activation of cAMP-PK at 24-hour starvation (Clegg and Ottey, 1990). This evidence suggests that the signal to the lactating mammary gland at 24-hour starvation to shut down lipogenesis does not involve raised cAMP concentrations, or that the increase in cAMP concentration and cAMP-PK activity is transient. Even when mammary gland cAMP-PK is activated, as described above, there is no inhibition of lipogenesis nor of ACC activity and there is no increase in the phosphate content of ACC in acini (Clegg et al, 1986, 1987, Clegg and Ottey, 1990). This does not correlate with the documented phosphorylation and inactivation of ACC in vitro by cAMP-PK purified from bovine heart and rabbit skeletal muscle (Hardie and Guy, 1980, Munday and Hardie, 1984). This chapter describes experiments designed to show why cAMP-PK from mammary gland may not play a role in regulating ACC activity in the lactating rat mammary

gland.

3.2 Results and Discussion

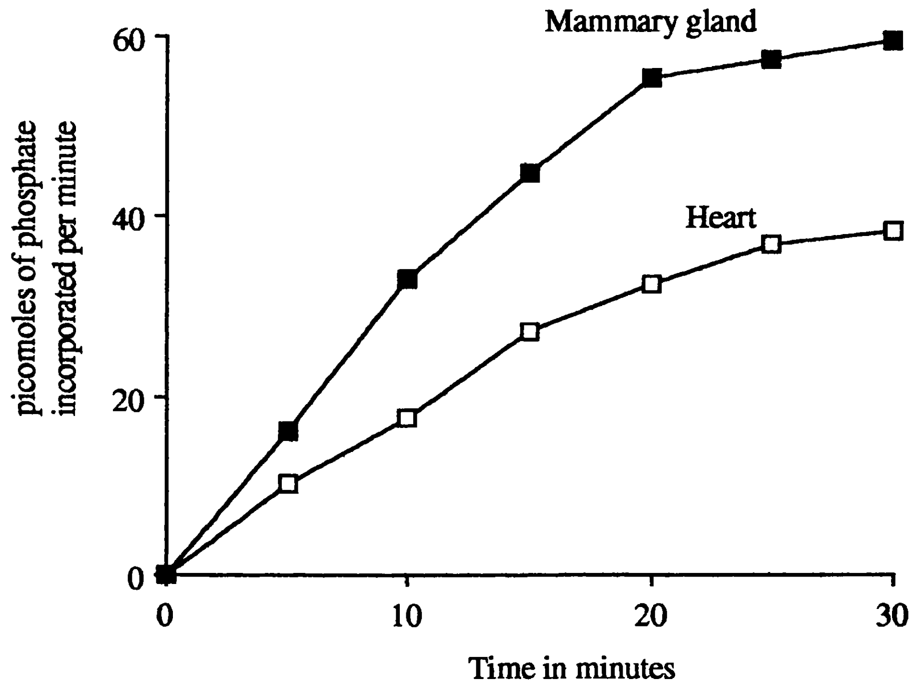
3.2.1 Time course of Kemptide phosphorylation by cAMP-PK

In order to achieve linearity in the measurement of cAMP-PK activity, a time course of phosphorylation of kemptide was performed. Crude tissue extracts of lactating rat mammary gland and rat heart at various dilutions were incubated with Kemptide and [γ^{32}P] MgATP. The phosphorylation of Kemptide at various time points was determined. The results (Fig. 3.1) show the dilutions that have the best combination of phosphate incorporation and time of incubation whilst ensuring that the rate of phosphorylation was linear. These conditions, namely a five minute assay, 10μM cAMP and a 100-fold dilution of the mammary gland extract or a 400-fold dilution of the heart extract were employed in subsequent assays of cAMP-PK activity.

3.2.2 Dependence of cAMP-PK activity on cAMP

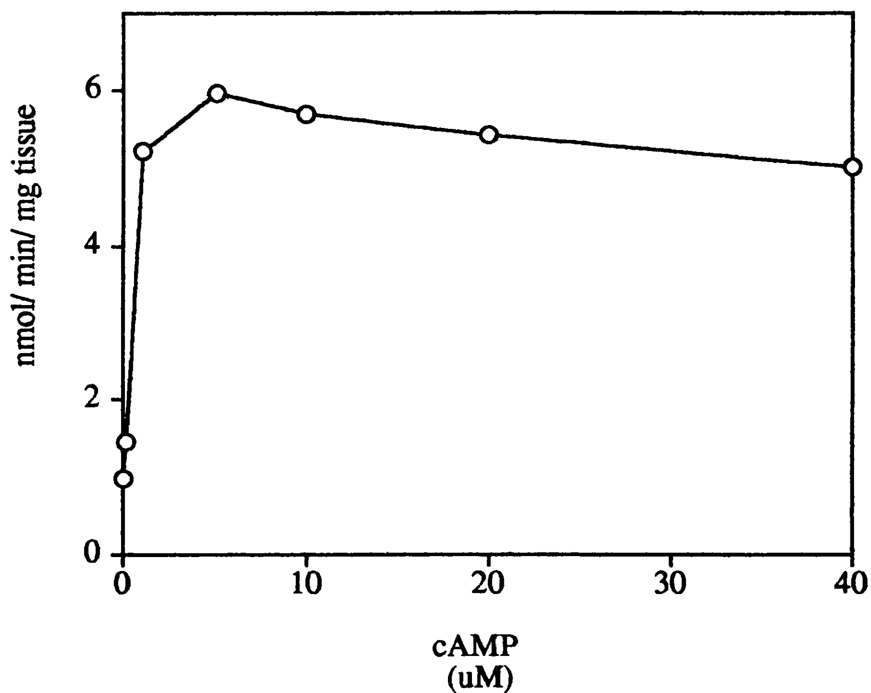
In order to find the maximally effective concentration of cAMP, crude tissue extracts of lactating rat mammary gland were incubated with varying concentrations of cAMP. cAMP-PK activity was assayed using the incorporation of radioactive phosphate into kemptide as described in the Methods section. The results (Fig. 3.2) show that the basal activity of cAMP-PK was maximally stimulated by cAMP at a final concentration of 5-10μM. Concentrations of cAMP exceeding 10μM started to show

Fig. 3.1 Time Course of Phosphorylation of Kemptide by Crude Tissue Extracts of Rat Heart and Lactating Mammary Gland



The phosphorylation of kemptide by crude tissue extracts of rat heart and lactating rat mammary gland was studied in order to obtain a linear rate of phosphorylation for future experiments. The above graph shows that a 1 in 400 dilution of rat heart homogenate and a 1 in 100 dilution of lactating rat mammary gland gave a linear rate of phosphorylation of kemptide for 10 minutes. These dilutions were then routinely used in assays measuring cAMP-PK activity. The details of how cAMP-PK activity was assayed are described in Chapter 2

Fig. 3.2 Dependence of cAMP-PK Activity on cAMP



The effect of cAMP concentration on the expressed activity of cAMP-PK in crude tisssue extracts from lactating rat mammary gland is shown above. Mammary gland tissue extracts were prepared as described in Chapter 2. cAMP-PK activity was assayed using the synthetic peptide kemptide as substrate as described in Chapter 2.

an inhibition of maximum cAMP-PK activity, with 40μM cAMP causing a 20% inhibition.

3.3 cAMP-PK Activity Ratio

The regulatory subunits of cAMP-PK have a very high affinity for cAMP, with a K_m for activation of 2 x 10^{-8} M (Corbin et al, 1975). This means that the dissociation and activity of the kinase is directly correlated with the concentration of cAMP, and is expressed as an activity ratio. Measurements of cAMP-PK activity ratios were first introduced by Corbin et al (1973). The activity ratio is the cAMP-PK activity measured in the absence of cAMP as a fraction of that measured in the presence of cAMP. A ratio of 0.0 suggests that the enzyme is totally in its holoenzyme form, a ratio of 1.0 suggests that the enzyme is fully activated and dissociated. Routinely, tissues that have not been stimulated to increase intracellular cAMP concentration exhibit an activity ratio of 0.1 showing that approximately 10% of cAMP-PK is dissociated (Corbin, 1983). However, measurement of activity ratio can only be taken as an indication of changes in intracellular cAMP concentration and protein kinase activation if steps are taken to ensure that the tissue homogenisation and assay procedure does not artificially increase or decrease the extent of holoenzyme dissociation.

Corbin et al (1975) have shown that 150mM NaCl is sufficient to cause significant dissociation of isoenzyme type I. However, dissociated type II isoenzyme will reassociate in low ionic strength buffer. Workers have attempted to overcome these

problems by extracting tissues in buffer containing phosphodiesterase inhibitors in the absence of NaCl for assays of the type I isoenzyme (Corbin, 1983). For the assay of the type II isoenzyme 0.5M NaCl is included, to overcome the problem of reassociation. Intermediate conditions; 0.15M NaCl and phosphodiesterase inhibitors were used when both isoenzymes were being assayed (Corbin, 1983).

Palmer et al (1980) have reported that under the conditions of the assay these precautions are not necessarily sufficient to prevent artefactual activity ratio measurements because there is the potential for dissociation of cAMP-PK during the extraction procedure. They suggest the inclusion of exogenous protein kinase as an internal standard to tissue extracts in order to correct for activation-inactivation artifacts. Further potential pitfalls in the determination of activity ratios are detailed by Corbin (1983) and includes ATPases, phosphodiesterases, phosphatases, artificial holoenzyme dissociation, binding of the catalytic subunit to particulate matter, salt inhibition of the kinase, isozyme distribution (tissue and species), presence of endogenous substrates and proteases.

In the initial work on measurements of cAMP-PK activity ratios, histone was used as a substrate for the kinase since it was inexpensive and readily available from commercial sources. In recent years however it has become clear that there are severe disadvantages in using histone for measuring cAMP-PK activity; the phosphorylation of histones by many different protein kinases present in a crude tissue extract, the activation of cAMP-PK holoenzyme by histone and that histone is a relatively poor substrate for cAMP-PK (Giembycz and Diamond, 1990)

It is generally accepted that the use of a specific synthetic peptide substrate e.g kemptide or malantide for measuring cAMP-PK activity gives considerable advantages over histone. Murray et al (1990) offer a study detailing the advantages of the peptide malantide and suggest that NaCl must be present in order to obtain a meaningful activity ratio.

3.3.1 Effect of Starvation on the Activity of cAMP-PK and ACC in the Lactating Rat Mammary Gland.

The activity ratio of cAMP-PK in extracts of freeze clamped mammary tissue from fed lactating rats was 0.12 and did not change significantly when the animals were starved for 24 hours (Table 3.1). Over the same time course lipogenesis is inhibited by 98% (Robinson et al, 1978) and ACC is inhibited. There is a 50% fall in the V_{max} of ACC after 24 hour starvation. This is accompanied by an increase of 1.5 moles of alkali labile phosphate from 5.9 to 7.4 moles per mole ACC (Munday and Hardie, 1987). These changes in ACC activity occur although there is no change in the activity of cAMP-PK (Table 3.1). This is direct in vivo evidence that cAMP-PK plays no role in the control of lipogenesis and ACC activity in the lactating rat mammary gland during starvation. Further doubt is cast by the observation that over a period of 24 hour starvation the proportion of the two major isozymes of cAMP-PK which are present in the lactating rat mammary gland did not change (Clegg and Ottey, 1990). Approximately 30% of the total activity occurs as the type I isozyme and 70% of the total activity as the type II isozyme. Therefore, the selective activation of cAMP-PK isozymes as seen in other tissues (Livesey et al, 1982, 1984,

Ekanger et al, 1988) does not occur in lactating rat mammary gland during starvation. Thus the constancy of activity ratio is not due to a differential inactivation of one isozyme together with activation of the other thereby producing no overall change in cAMP-PK activity (Clegg and Ottey 1990).

Table 3.1 Activity of cAMP-PK in Mammary Tissue Extracts from Fed and 24h Starved Rats.

	Basal Activity (no cAMP)	Total Activity (10μM cAMP)	Activity Ratio Basal:Total
Fed (n = 5)	0.25±0.05	1.97±1.05	0.12±0.04
24hr starved (n=5)	0.29±0.06	2.53±0.3	0.13±0.05

For experimental details see Materials and Methods.

Results are mean values ± S.E.M., n = number of observations. The assay measured the incorporation of ^{32}P from [$\gamma^{32}P$] MgATP into kemptide/min/g wet weight of tissue.

3.4 The Mammary Gland Catalytic Subunit

The refractory nature of the normal cAMP cascade in the lactating rat mammary gland especially with regard to the phosphorylation of ACC suggests that the kinase may

have unusual properties conferred upon it, either by the special environment of actively secreting mammary epithelial cells or maybe even some inherent property of the kinase itself. Workers who have shown the phosphorylation and inactivation of ACC by cAMP-PK in vitro (Hardie and Guy 1980, Munday and Hardie, 1984) have all used the catalytic subunit of cAMP-PK purified from bovine heart or rabbit skeletal muscle. This was because it was believed that the catalytic subunit was an invariant enzyme but this belief is now known to be incorrect and a number of isoenzymic forms of the catalytic subunit have been reported (see Chapter 1).

The catalytic subunit of cAMP-PK from the lactating rat mammary gland was therefore purified and partially characterised to determine its specific molecular properties.

3.4.1 Purification of the Catalytic Subunit of cAMP-PK from Lactating Rat Mammary Gland.

The initial attempts at purifying the catalytic subunit of cAMP-PK from mammary gland using the Reimann and Beham (1983) method were unsuccessful, in that active catalytic subunit could not be purified. After a number of attempts at purification it became clear that the reason for this was that the mammary gland catalytic subunit was very labile and did not survive the published procedure which took two days to complete. Therefore a modified procedure, which could be completed in 7-8 hours, was developed, this method produced active catalytic subunit from both rat heart and rat mammary gland and allowed studies to be carried out on the mammary gland

subunit. This procedure is described below.

Separation on DEAE-cellulose

The inguinal and thoracic mammary tissue from rats at peak lactation, killed by an intra peritoneal injection of sodium pentobarbitone (60mg/kg body weight), was dissected out and the tissue was homogenised in 0.25M sucrose, 50mM Tris HCl pH 7.0 at 4°C, 50mM NaF, 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM BZ, 4μg/ml SBTl, 0.1mM TLCK and 0.1mM PMSF. The tissue was homogenised using a domestic kitchen blender using three bursts at top speed with one minute on ice in between bursts. The homogenate was centrifuged at 30,000g for 30 minutes at 4°C. The supernatant was poured through a plug of glass wool, to remove floating fat and other debris, into a plastic beaker containing DEAE-cellulose which had been previously equilibrated in buffer (containing 25mM Tris HCl pH 7.0 at 4°C, 50mM NaF, 1mM DTT, 1mM EDTA, 1mM EGTA, 1mM BZ, 4ug/ml SBTI, 0.1mM TLCK, 0.1mM PMSF and glycerol 10% (w/v) and was allowed to stir for 30 minutes at 4°C. The DEAE-cellulose which is an anion exchanger will bind the holoenzyme form of cAMP-PK.

The DEAE-cellulose was then extensively washed on a Buchner funnel (Whatman, hardened ashless filter paper) with the buffer described above. The DEAE-cellulose was washed until no further protein washed off the column, as judged by an A_{280} reading <0.05.

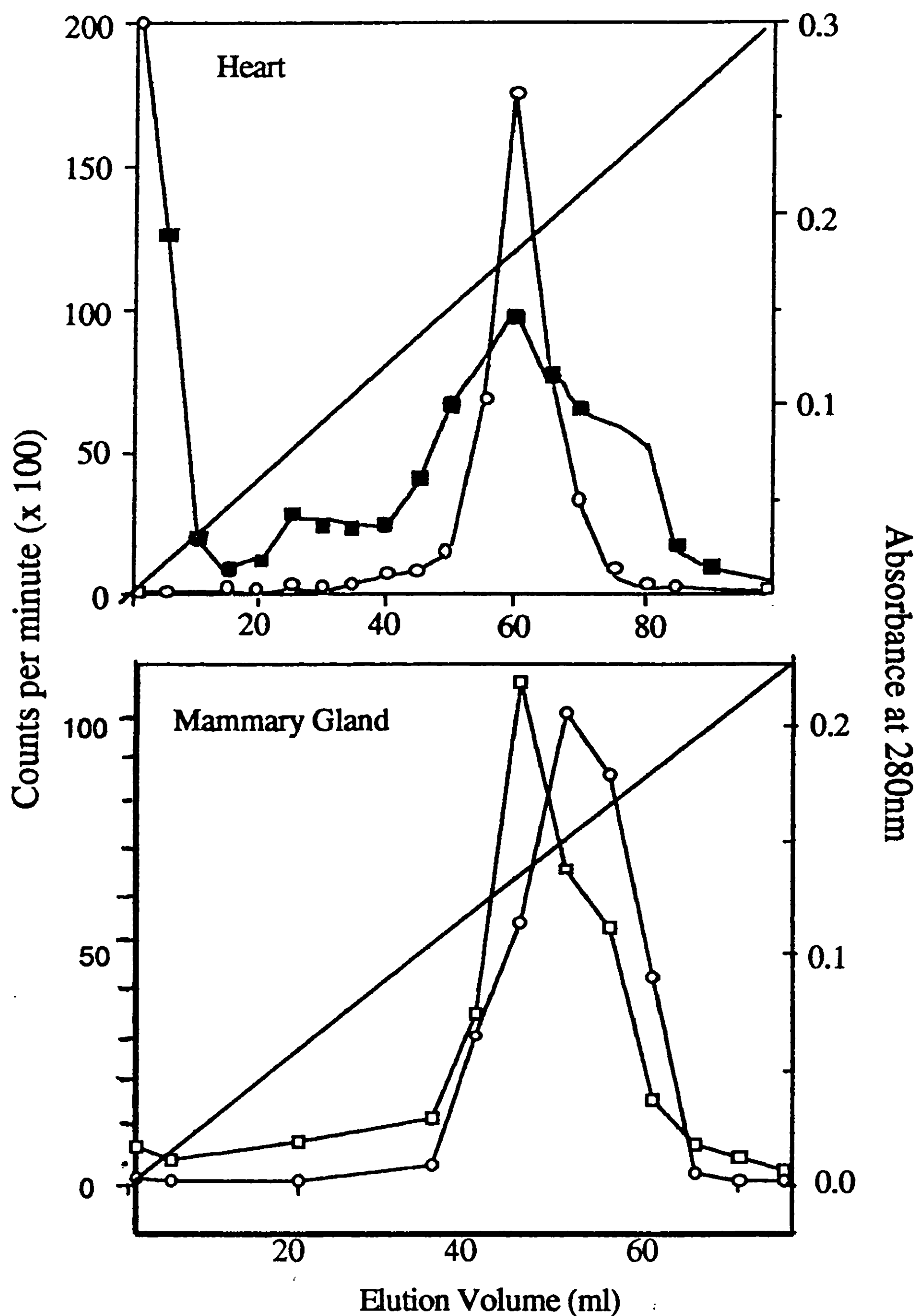
The DEAE-cellulose was then eluted whilst still on the Buchner funnel using 100-200ml of buffer, containing 20-50μM cAMP. The eluate was collected into a siliconised Buchner flask which contained phosphocellulose (Whatman, type Pll) equilibrated in the same buffer in the absence of cAMP.

Separation on Phosphocellulose

Phosphocellulose is a cation exchanger although it appears to have a specific affinity for protein kinases. The catalytic subunit being positively charged at pH 7.0 will therefore bind to the phosphocellulose (Munday and Hardie, 1984). The phosphocellulose was stirred for 5-7 minutes at 4°C and then washed extensively on a Buchner funnel, with buffer containing 150mM NaCl. The phosphocellulose was then packed into a glass chromatography column (12 x 2.0cm) and then further washed with buffer containing 150mM NaCl until the A_{280} reading was less than 0.05.

The column was developed using a linear salt gradient from 150mM NaCl to 600mM NaCl in the buffer described above in order to elute the catalytic subunit of cAMP-PK (see Fig. 3.3a, b). For the lactating rat mammary gland there was a peak of protein eluting at approximately 330mM NaCl followed closely by a peak of protein kinase activity eluting at approximately 350mM NaCl (Fig. 3.3a). For the rat heart there was a broad protein peak eluting at approximately 420mM NaCl, the peak of protein kinase activity coincided with the protein peak but was sharper (Fig. 3.3b). The fractions were assayed for cAMP-PK activity and those showing the greatest activity were pooled and concentrated using an Amicon concentrator, and then dialysed into buffer containing 25mM Tris HCl pH 7.0 at 4°C, 50mM NaF, 1mM DTT, 1mM

Fig. 3.3 Purification of cAMP-PK Catalytic Subunits from Lactating Rat Mammary Gland and Rat Heart on Phosphocellulose



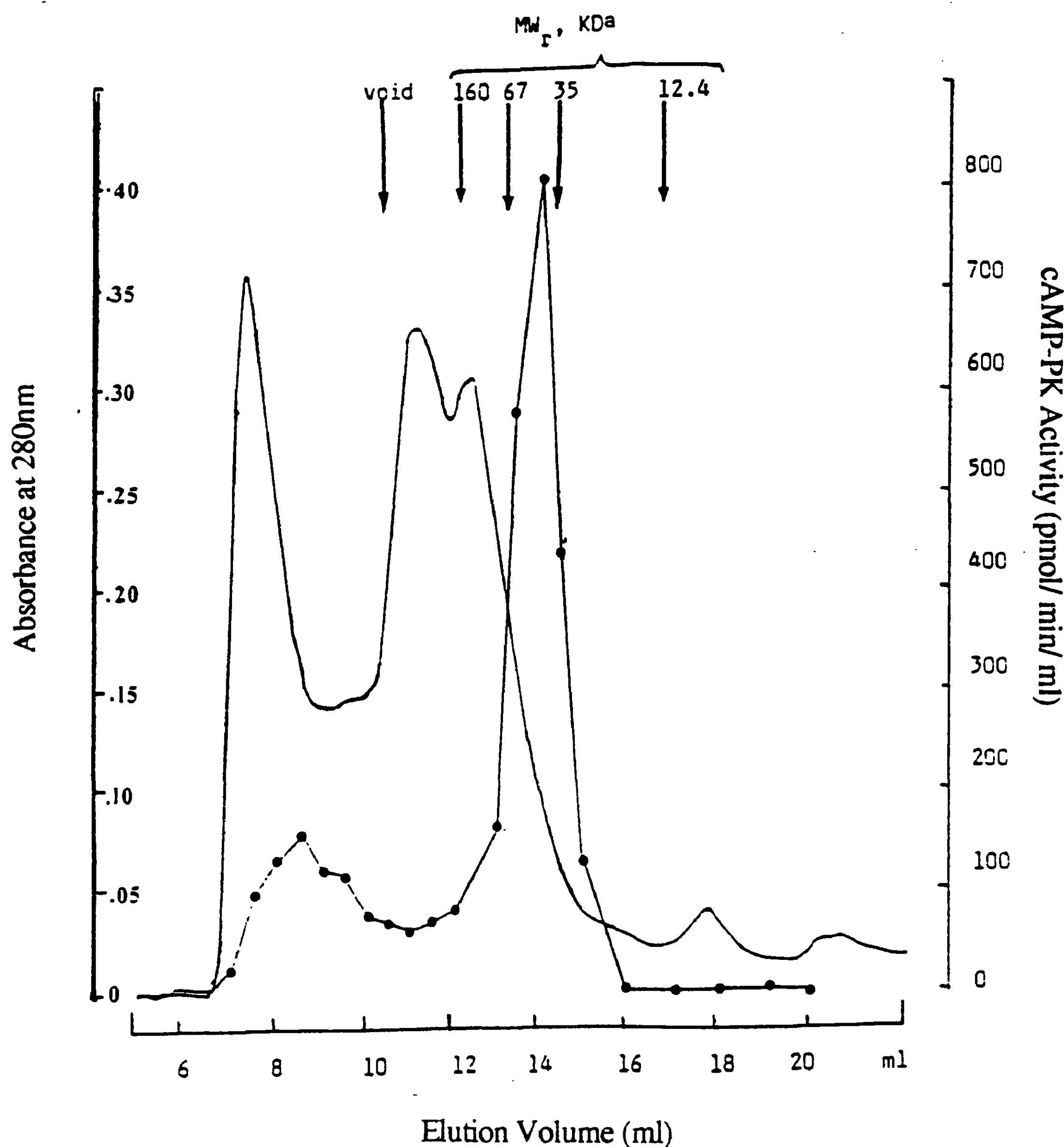
The elution profile obtained after chromatography of cAMP-PK, from lactating rat mammary gland and rat heart, on phosphocellulose is shown above. The absorbance of the collected protein solution at 280nm is indicated by the squares and cAMP-PK activity is indicated by the circles. The solid diagonal line on both panels indicates the salt gradient running from 0 - 0.6M.

EDTA, 1mM EGTA and 50% w/v glycerol. The cAMP-PK preparation was stored at -20°C. From these purification profiles it appears that the catalytic subunits from rat heart and lactating rat mammary gland are eluting at different salt concentrations from phosphocellulose with the former eluting at 420mM NaCl and the latter at 350mM. This differing behaviour on phosphocellulose may indicate that the two catalytic subunits are not identical.

Separation by Gel Filtration

A sample of the mammary gland cAMP-PK preparation was analysed using a Superose-12 gel filtration column (Pharmacia, Milton Keynes, U.K) which had been previously equilibrated in column buffer containing 25mM Tris HCl pH 7.0 at 37°C, 50mM NaF, 1mM DTT, 1mM EDTA, 1mM EGTA, 1mM BZ, 4ug/ml SBTI, 0.1mM TLCK, 0.1mM PMSF, 10% (w/v) glycerol and 0.5M NaCl. Fig. 3.4 shows a sharp peak of activity that resolved at 14ml which is equivalent to a Mr of 45,000. The molecular weight of the catalytic subunit from a variety of sources is generally quoted as approximately 41,000 Da as judged by SDS-polyacrylamide gel electrophoresis. The difference of 4,000 is within the range of accuracy possible with Superose-12 gel filtration. The elution volume of the mammary gland catalytic subunit did however exactly correlate with that of a homogenous preparation of bovine heart catalytic subunit.

Fig. 3.4 Purification of the Mammary Gland Catalytic Subunit by Superose-12 Gel - Filtration.



The concentrated peak fractions from the phosphocellulose step were dialysed into column buffer and then chromatographed on a Superose-12 gel filtration column equilibrated in column buffer as described in the text. The column was developed at 0.5ml / min. The protein was monitored continuously at 280nm and is shown by the continuous line on the above figure. Fractions of 0.5ml were collected and cAMP-PK activity (shown by the full circles) was assayed using the synthetic peptide substrate kemptide, as described in chapter 2. The elution volumes of the molecular weight standards are indicated by the arrows at the top of the figure.

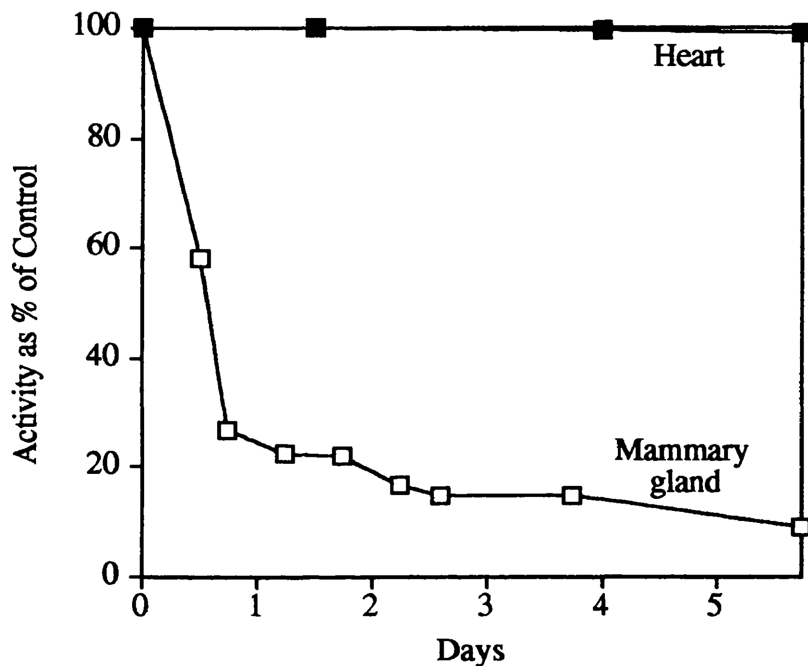
The fractions showing greatest protein kinase activity were pooled and concentrated using an Amicon concentrator and then dialysed into buffer (25mM Tris HCl pH 7.0 at 4°C, 50mM NaF, 1mM DTT, 1mM EDTA, 1mM EGTA, 1mM BZ, 4ug/ml SBTI, 0.1mM TLCK, 0.1mM PMSF and 50% (w/v) glycerol). The cAMP-PK preparation was stored at -20°C.

3.4.2 Stability of the Purified Mammary Gland Catalytic Subunit of cAMP-PK

The purified catalytic subunit of cAMP-PK from lactating rat mammary gland is very unstable compared to its cardiac counterpart (Fig. 3.5). The mammary gland catalytic subunit lost over 50% of its initial activity within about 12 hours of purification, and

only retained 9% of its initial activity after 6 days. In contrast the heart catalytic subunit shows no significant loss in activity after 6 days, and has indeed been shown to be stable for many months. It would appear that the mammary gland catalytic subunit starts to lose activity immediately upon being released from the holoenzyme form, so even the initial activity measured may be a significant underestimation of the true activity. This may account for the fact that in Fig. 3.5 100% activity for the mammary gland preparation corresponds to 2.2 $\pm$ 0.46 U/mg and for the heart preparation 53.1 $\pm$ 2.7 U/mg. However the time taken for purification was only 7-8 hours which represents 0.5 half-lives according to Fig. 3.5, so it is also very possible that there is at least some difference in the true specific activities of the catalytic subunits.

Fig. 3.5 Stability of Purified Rat Mammary Gland and Rat Heart Catalytic Subunits



The catalytic subunits of cAMP-PK from lactating rat mammary gland and rat heart were purified as described in this chapter. The stability of the purified catalytic subunits was tested at the times indicated above by assaying cAMP-PK activity and expressing it as a percentage of the initial activity. cAMP-PK activity was assayed as described in Chapter 2.

3.4.3 Substrate Specificity of cAMP-PK from Lactating Rat Mammary Gland and Rat Heart

Initial rates of phosphorylation of a range of substrates at concentrations previously described as saturating for cAMP-PK (Munday and Hardie, 1984), were measured for each catalytic subunit. The relative substrate specificity observed for the heart cAMP-PK is in agreement with that recorded for rabbit skeletal muscle catalytic subunit by other authors (Munday and Hardie 1984). Table 3.2 shows that relative to the rate of histone phosphorylation the rates of phosphorylation of glycogen synthase, phosphorylase kinase, casein and kemptide were similar for both catalytic subunits and comparable to those previously quoted for rabbit skeletal muscle cAMP-PK (Munday and Hardie, 1984). Differences were observed for ATP-citrate lyase which was

phosphorylated at a 7-fold faster rate by the mammary gland catalytic subunit, and for ACC which was at least a 17-fold poorer substrate for the mammary enzyme compared to the heart catalytic subunit (Table 3.2). To test the validity of this result the rate of phosphorylation of a synthetic peptide based on the amino acid sequence around the phosphorylation site on ACC that mediates the inhibition of enzyme activity by cAMP-PK from bovine heart *in vitro* was examined. This peptide has the sequence His-Met-Arg-Ser-Ser-Met-Ser-Gly-Leu-His-Leu-Val-Lys (the underlined serine-77 being phosphorylated by bovine heart cAMP-PK *in vitro*). Initial rates of phosphorylation of this peptide were measured in assays where catalytic subunit was liberated by 10μM cAMP from preparations of cAMP-PK holoenzyme purified from rat heart or lactating rat mammary gland. Table 3.3 shows that while the catalytic

Table 3.2 Substrate Specificity of cAMP-PK from Lactating Rat Mammary Gland and Rat Heart

Values show relative initial velocities measured at the indicated substrate concentrations and are expressed relative to the rate of phosphorylation of histone (type II-AS). The results shown are representative of those obtained with at least four different preparations of cAMP-PK purified from heart and mammary gland.

cAMP-PK relative to histone = 100%	Substrate concentration	Rate of ^{32}P incorporation by cAMP-PK relative to histone = 100%	
	mg/ml	Mammary gland	Heart
Histone	0.80	100	100
Acetyl CoA Carboxylase	0.48	<1	17
ATP-Citrate Lyase	0.24	50	7
Glycogen Synthase	0.17	130	110
Phosphorylase Kinase	0.67	310	240
Casein	2.00	53	26
Kemptide	0.13	2850	1100

subunit from heart phosphorylated this peptide at 70% of the rate achieved with kemptide, the mammary gland catalytic subunit only phosphorylated it at 12% of the rate at which kemptide was phosphorylated. Qualitatively this confirms the observations in Table 3.2 that the phosphorylation sites on ACC are worse substrates for mammary cAMP-PK than for heart cAMP-PK. However, while the peptide described above is phosphorylated at 70% and 12% of the rate of kemptide by heart and mammary catalytic subunit respectively (Table 3.3), ACC is phosphorylated at only 1.5% and 0.04% of the rate of kemptide phosphorylation by heart and mammary catalytic subunit respectively, (Table 3.2). The shortfall in phosphorylation rate by mammary catalytic subunit compared to rat heart is much greater for the sites on native ACC than it is on the synthetic ACC peptide. This confirms that secondary and tertiary structure is an important determinant in the recognition of substrate by cAMP-PK, and that the major difference in ACC substrate recognition between mammary and heart cAMP-PK lays in their ability to recognise secondary and tertiary

structural features. These results would strongly suggest that the catalytic subunit of cAMP-PK is not a functionally invariant enzyme.

The inability of cAMP-PK from mammary gland to phosphorylate ACC, yet retain activity against other substrates which is comparable to that achieved by the heart enzyme provides a clear and possibly complete explanation for the results of several authors showing that elevation of cAMP-PK activity in mammary tissue has no effect on lipogenesis or the phosphorylation state of ACC in the gland, despite the fact that the kinase is present and activatable.

Table 3.3 Phosphorylation of Synthetic Peptide Substrates by cAMP-PK from Lactating Rat Mammary Gland and Rat Heart

Table 3.3. shows the phosphorylation of synthetic peptide substrates by the catalytic subunits liberated from heart and mammary gland preparations of holoenzyme by 10μM cAMP. cAMP-PK from mammary tissue is stable in the undissociated form and the heart enzyme was also used in the holoenzyme form so that the assays could be directly comparable. The results are expressed as initial rates of phosphorylation relative to that of kemptide. Peptide 'SSMS' is the name given to the synthetic peptide corresponding to residues 73-85, inclusive on ACC; it has two extra arginine residues on the C-terminus.
[His-Met-Arg-Ser-Ser-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg] (Carling et al, 1989), underlined serine residue is phosphorylated by cAMP-PK. For heart and mammary enzyme 100% represented the same kinase activity to within ± 5%.

Substrate	Substrate concentration μM	Rat Heart cAMP-PK	Mammary Gland cAMP-PK
Kemptide	170	100	100
SSMS	100	70	12

3.4.4 Sensitivity of the cAMP-PK Catalytic Subunit from Rat Mammary Gland and Rat Heart to the Specific Peptide Inhibitor of cAMP-PK

The free catalytic subunit of cAMP-PK is inhibited by a heat stable protein known as the Walsh inhibitor (see Chapter 1). A synthetic peptide based on this protein has been synthesized and is known as the specific peptide inhibitor of cAMP-PK.

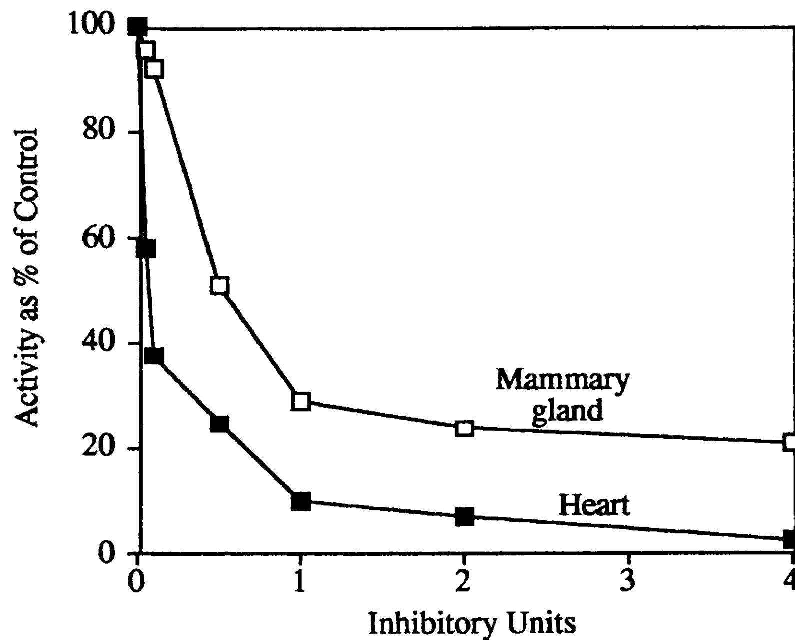
When similar amounts of cAMP-PK activity from both mammary gland and heart were assayed in the presence of the specific peptide inhibitor of the catalytic subunit it was found that the two tissues diplayed different sensitivities. The enzyme purified from the lactating rat mammary gland was found to be significantly less sensitive to inhibition than that purified from the rat heart. The ID_{50} for the mammary gland catalytic subunit was approximately ~~0.06~~ 0.55 inhibitory units versus ~~0.55~~ 0.06 inhibitory units for the heart catalytic subunit (Fig. 3.6), a 9-fold difference in sensitivity.

It was apparent from studies conducted on the purified catalytic subunit of cAMP-PK from lactating mammary gland that this enzyme was different from its heart counterpart. It showed a different sensitivity to the specific peptide inhibitor of cAMP-PK, it displayed a different substrate specificity and it also proved to be very unstable when purified.

3.5 Stability of cAMP-PK from Lactating Rat Mammary Gland

While purifying cAMP-PK from mammary gland it was noticed that enzyme activity

Fig. 3.6 Inhibition of Purified Rat Mammary Gland and Rat Heart Catalytic Subunits by the Specific Inhibitor of cAMP-PK



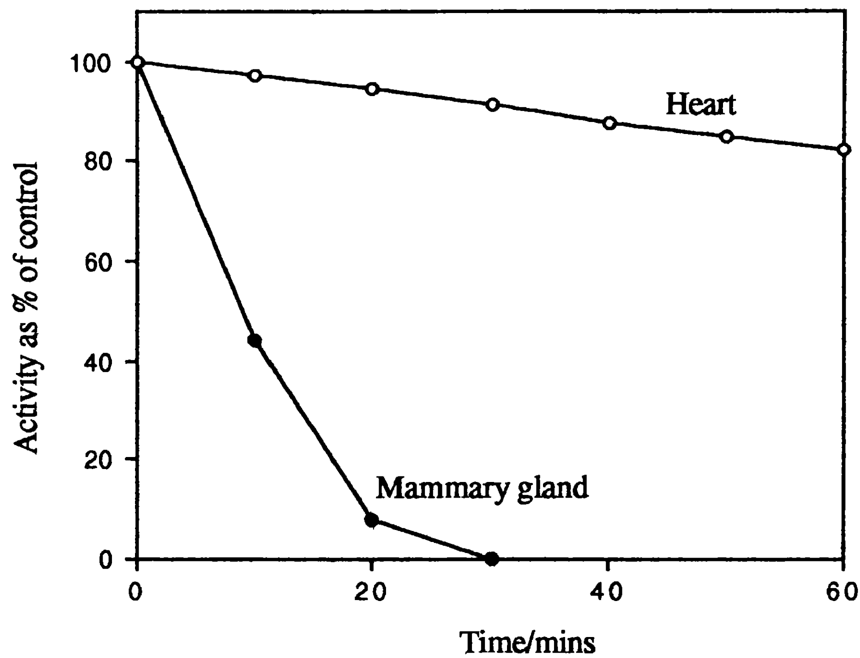
The catalytic subunits of cAMP-PK from lactating rat mammary gland and rat heart were purified as described in this chapter and incubated with varying concentrations of the specific peptide inhibitor of cAMP-PK. cAMP-PK activity at each concentration of inhibitor was determined and is expressed as a percentage of the control activity, determined in the absence of inhibitor. cAMP-PK activity was assayed as described in Chapter 2

was stable in the holoenzyme form, therefore it was decided to determine whether the enzyme was unstable only when it was dissociated to free catalytic subunit. Furthermore it was decided to determine whether the lack of stability was a consequence of the purification procedure or an intrinsic property of the catalytic subunit.

3.5.1 cAMP-PK Activity in Crude Tissue Extracts

It was found that incubation of a crude mammary gland or heart tissue extract over increasing time did not affect the rate of kemptide phosphorylation when assayed in the presence of cAMP (10μM). However when cAMP (10μM) was added to the incubation mixture to dissociate the cAMP-PK holoenzyme and release the free catalytic subunit there was a rapid loss of cAMP-PK activity in the mammary gland tissue homogenate (see Fig. 3.7). The mammary gland lost 50% of its initial activity in approximately 11 minutes, whilst the heart homogenate still retained 95% of its initial activity. The mammary gland homogenate had lost all cAMP-PK activity within 40 minutes while the heart still retained 87% of its initial activity. So although both tissue extracts showed a fall in cAMP-PK activity with respect to time, the rate of loss of activity was much more rapid in the mammary gland extract. These results show that the holoenzyme form of the cAMP-PK in mammary gland was stable and that the free catalytic subunit was very labile.

Fig. 3.7 Loss of cAMP-PK Activity in Crude Tissue Extracts of Lactating Rat Mammary Gland and Rat Heart



Crude tissue extracts were prepared from lactating rat mammary gland and rat heart as described in chapter 2. At time zero on the above graph cAMP (10uM) was added to both tissue extracts and cAMP-PK activity was determined at the time points indicated. cAMP-PK activity was assayed as described in Chapter 2.

3.6 cAMP-PK Inactivating Factor

It may be that the mammary gland catalytic subunit in its free state is intrinsically unstable. This may explain the lack of stability of the purified preparation, or there may be present in the mammary gland an activity that may force the inactivation of cAMP-PK. Such an activity has been identified in intestinal brush border membranes (Alhanaty et al, 1981, 1985) and has been termed the "kinase specific membranal protease". This is a specific protease for the catalytic subunit of cAMP-PK which rapidly degrades the native subunit (41,000 Da) to an inactive 34,000 Da fragment. The appearance of this 34,000 Da fragment quantitatively correlates with the loss of cAMP-PK activity. A similar loss of catalytic subunit, triggered by agents which activate cAMP-PK, has been reported in porcine epithelial cells (LLC-PK_1) by Hemmings (1986). He suggests that proteolysis is responsible but shows no accumulation of proteolytic products. A similar protease if present in mammary tissue might explain the loss of cAMP-PK activity observed in this tissue.

3.6.1 Preparation of a Membranal Fraction from Lactating Rat Mammary Gland

Freshly dissected inguinal and thoracic mammary tissue was used for this preparation (it was found that freeze clamped tissue was not suitable since it results in a preparation devoid of activity). Rats were killed with an intra-peritoneal injection of sodium pentobarbitone (60mg/kg body weight). The tissue was washed and minced in ice-cold homogenisation buffer consisting of 0.25M sucrose, 100mM Tris HCl pH 7.0-7.2 at 4°C, 50mM NaF, 0.1mM EDTA, 1mM BZ, 0.1mM PMSF, 0.1mM

TLCK, 4μg/ml SBTI, before being homogenised in as small a volume of buffer as possible (typically 50ml buffer per rat). The tissue was homogenised in a domestic kitchen blender using three bursts at top speed, each lasting thirty seconds, with one minute in ice in between bursts. The homogenate was centrifuged at 30,000g for 30 minutes at 4°C. The supernatant was poured through glass wool to remove any floating fat and other debris. The supernatant was then centrifuged at 100,000g for 90 minutes at 4°C. The supernatant was poured off and the 100,000g pellet was solubilised using a Pasteur pipette in as small a volume of buffer as possible (typically 4ml per rat). The buffer contained 50mM HEPES pH 7.2, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.83% (w/v) n-ocytl-ß-D-glucopyranoside, 0.1mM PMSF, 0.1mM TLCK, 4μg/ml SBTI.

The solubilised pellet was left on ice for 10-15 minutes and then centrifuged at 41,000g to bring down any material that had not dissolved. The supernatant was drawn off using a Pasteur pipette and concentrated using vacuum dialysis to a volume of 1ml. The protein concentration of this preparation was typically 4mg/ml. All steps in this preparation were carried out at 4°C and the preparation was stored at 4°C.

3.6.2 Inactivation of the Catalytic Subunit of cAMP-PK by a Membranal Fraction from Lactating Rat Mammary Gland.

As shown in Fig. 3.8 incubation of the normally stable bovine heart catalytic subunit

with the membrane preparation resulted in a time- and concentration-dependent loss of cAMP-PK activity. In the experiment shown the catalytic subunit is totally inactivated after 16 minutes whereas the control still retains nearly 90% of its activity at this time. The inactivation was not due to the presence of the detergent, n-octyl-ß-D-glucopyranoside which had no effect on the kinase activity of the catalytic subunit.

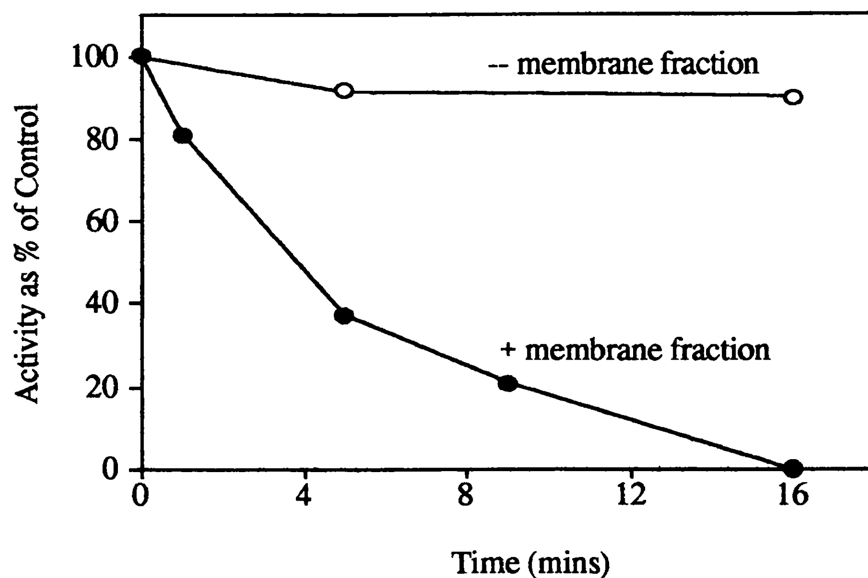
This experiment showed that there was something present in the membranal fraction prepared from the lactating rat mammary tissue that caused the inactivation of the catalytic subunit of bovine heart cAMP-PK, which is known to be very stable. This inactivating factor was further purified and partially characterised.

3.6.3 Purification of the Membrane Preparation on DEAE-cellulose

A 5ml DEAE-cellulose column was prepared and equilibrated with buffer (50mM HEPES pH 7.2, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.1% (w/v) Triton X-100, 1mM Benzamidine, 0.1mM PMSF, 0.1mM TLCK, 4μg/ml SBTI. The solubilised membrane fractions were then applied to the DEAE-cellulose resin, and the column was thoroughly washed with the buffer described above until the A280 reading was <0.05.

The column was developed using a linear salt gradient from 0 - 1M NaCl in the buffer described above and fractions were collected and then tested for their ability to inactivate the catalytic subunit of cAMP-PK purified from bovine heart tissue. There was a large and clear peak of activity eluting at a salt concentration of

Fig. 3.8 Inactivation of cAMP-PK by a Mammary Gland Membranal Fraction



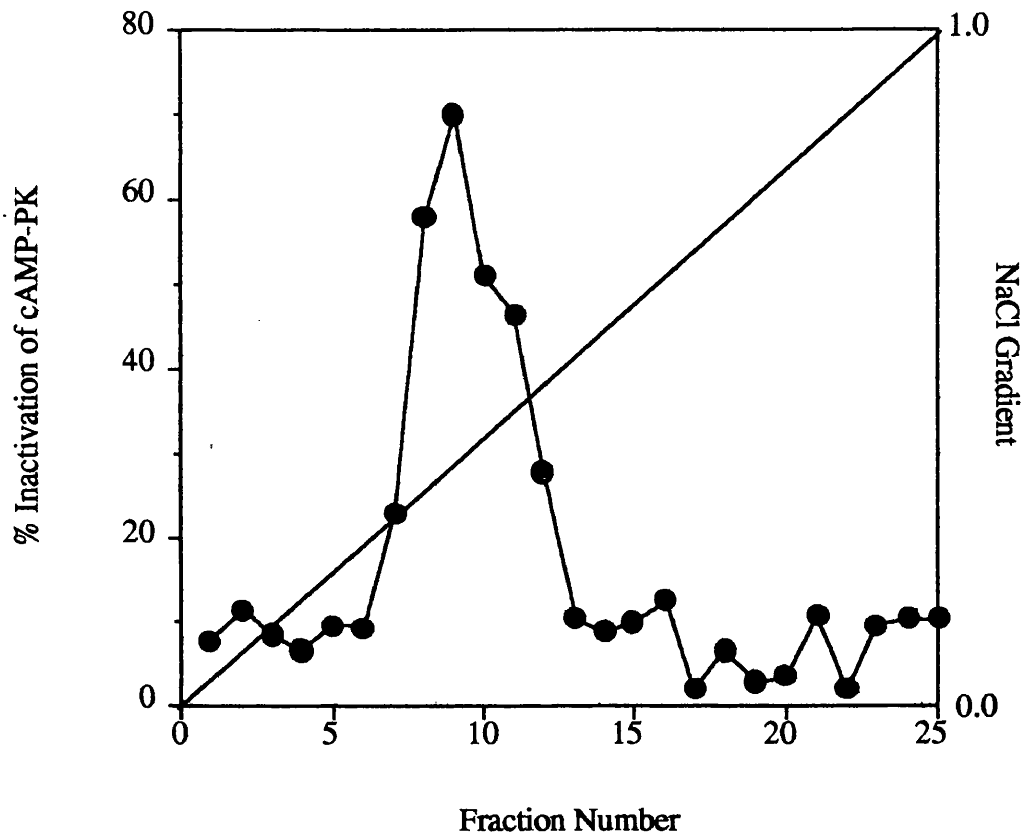
The catalytic subunit of cAMP-PK was purified from bovine heart as described in chapter 2 and incubated with the membrane fraction, prepared as described in this chapter. At the indicated time points cAMP-PK activity was determined and is expressed as a percentage of the initial activity at time zero, i.e. before the addition of the membrane fraction. cAMP-PK was assayed using the synthetic peptide kemptide as described in Chapter 2

approximately 350mM NaCl. (Fig. 3.9). The fractions showing the greatest activity were pooled and concentrated, using vacuum dialysis to a volume of 1ml. The preparation was stored at 4°C.

3.6.4 Purification of the Membrane Associated Kinase Inactivating Factor.

Table 3.4 shows the methodology and results of the purification procedure utilised to isolate this activity. There was a 6-fold purification achieved with the first centrifugation at 30,000g, with the specific activity increasing to 6.1 U/mg. In the next stage of the purification, there was an 8.5 fold increase in the specific activity and a total purification of 52-fold, to a specific activity of 52 U/mg. The most effective step in the purification was the centrifugation at 41,000g. The supernatant had a specific activity of approximately 480 U/mg, thus giving a 9-fold purification over the previous stage, and a total purification of 480-fold. This compares favourably with a 100-fold purification of the kinase inactivating activity from intestinal brush border epithelia reported by Alhanaty et al (1985). Although the post DEAE-cellulose purified membrane preparation had activity, as judged by its ability to inactivate the catalytic subunit of cAMP-PK (Fig. 3.10) the specific activity of the preparation actually decreased after this step. Hence the pre-DEAE-cellulose preparation had a specific activity of 480 U/mg compared to the post-DEAE-cellulose preparation which had a specific activity of only 220 U/mg. Also whilst the pre-DEAE-cellulose preparation retained its activity for a number of days, if stored at

Fig. 3.9 Purification of Membrane Fraction on DEAE-cellulose



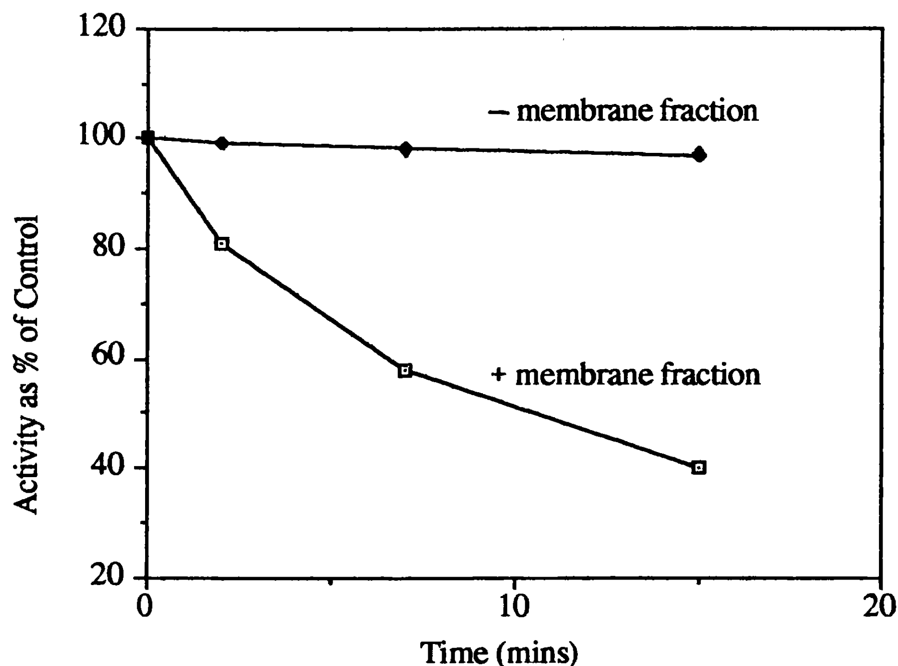
The elution profile obtained after chromatography of the membrane fraction on DEAE-cellulose is shown above. The activity of each fraction was determined by its ability to inactivate the catalytic subunit of cAMP-PK. The diagonal line represents the NaCl gradient running from 0-1M. Each fraction was incubated with cAMP-PK for 45 minutes before cAMP-PK activity was determined as described in Chapter 2

Table 3.4 Partial Purification of the Membrane Bound cAMP-PK Inactivating Activity from Lactating Rat Mammary Gland

This table shows a summary of the procedures used in the partial purification of the membrane fraction from lactating rat mammary gland. 1 Unit of activity is defined as that amount of the membrane preparation which inactivates 1 unit of cAMP-PK. 1 unit of cAMP-PK is defined as that amount of protein kinase that transfers 1nmol P/min into Kemptide..

	Total Activity (Units)	Total Protein (mg)	Specific Activity (Units/mg)	Purification -fold	Yield (%)
Homogenate	15452	15480	1.0	1.0	100
30,000g supernatant	2971	487	6.1	6.1	19
100,000g pellet resuspended in n-octyl-ß-D-glucopyranoside	1050	20	52.0	52.0	6.8
41,000g supernatant	1055	2.2	479.7	479.7	6.8
DEAE cellulose	132	0.6	220	220	1.1

Fig. 3.10 Inactivation of Catalytic Subunit of cAMP-PK by Post-DEAE Purified Membrane Fraction



The catalytic subunit of cAMP-PK was puified from bovine heart as described in Chapter 2 and incubated with the membrane fraction, purified as far as the DEAE-cellulose step, as described in this chapter. The inactivation of cAMP-PK was determined by assaying the activity of cAMP-PK at the time points indicated above. cAMP-PK activity was measured as described in Chapter 2.

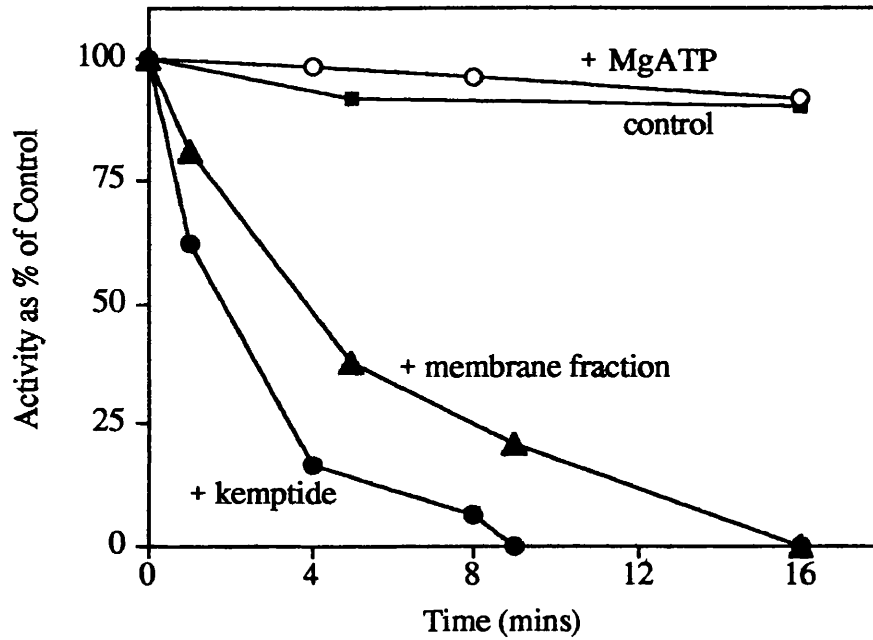
4°C, the post DEAE-cellulose preparation did not retain its activity very well. The reason for the loss of activity is unclear, it is possible that the membrane preparation is stable only in conditions of high protein concentration and therefore further purification, with its accompanying decrease in protein content, results in loss of activity. In view of this fact subsequent experiments involving the membrane preparation utilised the concentrated 41,000g supernatant.

3.6.5 Effect of MgATP and Kemptide on the Ability of the Membrane Preparation to Inactivate the Catalytic Subunit of cAMP-PK

The inactivation of the catalytic subunit by the membrane preparation was virtually totally blocked in the presence of the nucleotide substrate of the kinase, MgATP (Fig. 3.11). The nucleotide or metal ion on their own were not effective at stopping this inactivation. Thus whilst there was total inactivation of the catalytic subunit by the membrane preparation after 16 minutes, in the presence of MgATP the catalytic subunit still retained 92% of its original activity, compared to the control which still retained 90% of its initial activity. The inclusion of kemptide, a heptapeptide substrate of the cAMP-PK, actually accelerated the rate of inactivation of the catalytic subunit (Fig. 3.11). Thus while the membrane preparation caused 50% inactivation of the catalytic subunit after 4 minutes, the inclusion of kemptide resulted in a 85% inactivation of the catalytic subunit after 4 minutes.

The MgATP induced protection of cAMP-PK from inactivation by the membrane

Fig. 3.11 The Effects of MgATP and Kemptide on the Inactivation of cAMP-PK by the Membrane Fraction



The catalytic subunit of cAMP-PK was purified as described in chapter 2 and incubated with the membrane fraction (purified as far as the 41,000g supernatant) in the presence and absence of kemptide and MgATP in separate incubations. cAMP-PK activity was determined at the time points indicated above and is expressed as a percentage of the initial activity at time zero before the addition of the membrane fraction.

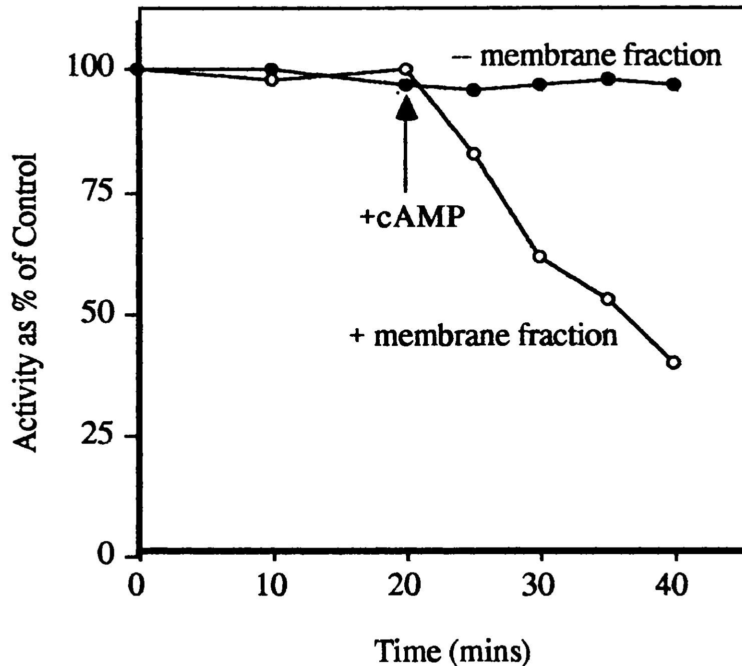
preparation, may be due to a shielding effect of the MgATP which prevents the action of the membrane preparation at a particular site in the nucleotide binding site. Alternatively or additionally, the nucleotide substrate binding may induce a conformational change in the catalytic subunit which prevents its recognition and inactivation by the membrane preparation. The binding of kemptide to the peptide binding site of the catalytic subunit of cAMP-PK, may also cause a conformational change in the enzyme that increases its susceptibility to inactivation by the membrane preparation.

3.6.6 Effect of the Membrane Preparation on the cAMP-PK Holoenzyme (Type-II)

In order to test the selectivity of action of the membrane preparation the type-II cAMP-PK holoenzyme, purified from bovine heart (using the method described by Clegg and Ottey, 1990) was incubated with the membrane preparation. At set times aliquots were removed from the incubation mixture and assayed, in the presence of 10μM cAMP, for cAMP-PK activity. After 20 minutes cAMP (10μM final) was added to the incubation mixture, aliquots were removed at set time points and assayed for cAMP-PK activity in the presence of cAMP (10μM).

It can be seen (Fig. 3.12) that there was no loss of cAMP-PK activity in the first 20 minutes corresponding to the time the catalytic subunit was associated with the regulatory subunit i.e. in the holoenzyme form. After 20 minutes the free catalytic subunit was released, upon the addition of cAMP, and the time dependent inactivation of cAMP-PK followed as was observed with the purified catalytic subunit (Fig. 3.8).

Fig. 3.12 Lack of Effect of Membrane Fraction on cAMP-PK Holoenzyme



cAMP-PK holoenzyme was purified from rat heart (as described by (Clegg and Ottey, 1990). The holoenzyme was incubated with the membrane fraction (purified as far as the 41,000g supernatant). At set times aliquots of this incubation were removed and assayed for cAMP-PK activity in the presence of cAMP (10uM). At the time point indicated above cAMP(10uM final) was added to the incubation, aliquots were removed at the times indicated, and cAMP-PK activity was determined as described in Chapter 2. The final concentration of cAMP was identical in all assays.

The control incubation, which did not have cAMP added to it showed no loss in cAMP-PK activity over a 40 minute incubation with the membrane preparation.

It therefore appears that the membrane preparation only affects the free catalytic subunit of cAMP-PK, and it does not appear to affect the holoenzyme form of the enzyme, which expressed full protein kinase activity upon the addition of cAMP. This confirms previous observations (section 3.5.1) that the holoenzyme was stable.

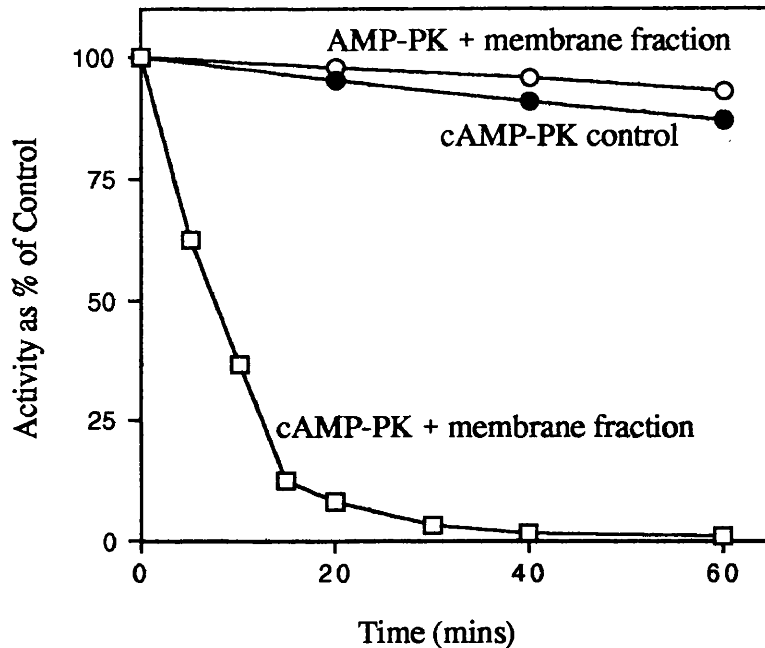
3.6.7 Effect of the Membrane Preparation on the AMP-PK

It was important to establish whether this effect was specific for the catalytic subunit of cAMP-PK or a general inactivation of protein kinases. The AMP-PK is now believed to be a very important regulatory enzyme in lipid metabolism.

AMP-PK purified from rat liver was incubated with the membrane preparation. At various times during a one hour time course, aliquots were removed from the incubation mixture and tested for AMP-PK activity, as described in Chapter 2.

As can be seen (Fig. 3.13), whilst the catalytic subunit of cAMP-PK was inactivated (100% inactivation in 30 minutes), as expected, by the membrane preparation, there was no significant loss in AMP-PK activity over 60 minutes when compared to the control incubation in the absence of membrane preparation. It would appear that the inactivating effect of the membrane preparation is specific to the catalytic subunit of cAMP-PK and that the membrane preparation does not affect other protein kinases.

Fig. 3.13 Lack of Effect of Membrane Fraction on AMP-PK



AMP-PK was purified from rat liver (Carling et al, 1989) and incubated in the presence and absence of the membrane fraction (purified as far as the 41,000g supernatant). The catalytic subunit of cAMP-PK, purified from bovine heart as decribed in chapter 2, was incubated in parallel, in the presence and absence of the membrane fraction. Both AMP-PK and cAMP-PK activity is expressed as a percentage of the control activity i.e that determined in the absence of the membrane fraction. AMP-PK activity was measured using the synthetic peptide HMRSAMSGLHLVK as substrate and cAMP-PK activity was measured using the synthetic peptide kemptide as substrate, as described in Chapter 2.

3.6.8 The Effect of DTT, ß-Mercaptoethanol, cAMP and NaCl on the Activity of the Membrane Preparation

In order to test whether the inactivation of the catalytic subunit by the membrane preparation was due to oxidative reactions, experiments were carried out in the presence of DTT and ß-Mercaptoethanol. As can be seen from Table 3.5 the inclusion of these two reagents had very little effect on the ability of the membrane preparation to inactivate the catalytic subunit. There was still a 82% inactivation in the presence of ß-Mercaptoethanol and a 78% inactivation in the presence of DTT.

In order to test whether the reaction for the observed loss in cAMP-PK activity was due to the reassociation of the catalytic subunit with regulatory subunit to form the holoenzyme, experiments were carried out in the presence of cAMP, which would prevent the reassociation of the two subunits. However, as shown in Table 3.5, there was still a 89% inactivation of the catalytic subunit of cAMP-PK.

In order to eliminate the possibility that there was targeting of the catalytic subunit into the membrane preparation and that this was responsible for the observed loss in cAMP-PK activity, experiments were conducted in the presence of NaCl to discourage such association. As can be seen from Table 3.5 there was no effect on the ability of membrane preparation to inactivate the catalytic subunit of cAMP-PK with a 84% inactivation.

Thus it would appear that the loss of cAMP-PK activity caused by the membrane

Table 3.5 The effect of 2-Mercaptoethanol, DTT, cAMP and high salt on the ability of the membrane preparation to inactivate cAMP-PK

The catalytic subunit of cAMP-PK was purified from bovine heart (as detailed in Chapter 2) and incubated with the membrane fraction (prepared from lactating rat mammary gland as described in the text) in the presence of either 2-Mercaptoethanol(5mM) or, dithiothreitol (2mM) or, cAMP (10μM) or, NaCl (150mM). After 15 minutes, aliquots were removed from each incubation and assayed for cAMP-PK activity using kemptide as a substrate as described in Chapter 2. The results are expressed as the percentage inactivation of cAMP-PK relative to the control incubation which lacked the membrane fraction.

	Concentration	% inactivation of cAMP-PK	% inactivation of cAMP-PK control
Membrane fraction	100μg/ml	87.1	1.9
2-Mercaptoethanol	5mM	82.6	2.2
Dithiothreitol	2mM	77.8	3.1
~~cAMP~~ NaCl	150mM	84.4	3.2
cAMP	10μM	89.2	2.8

preparation is not due to oxidation or free radicals. It does not appear to be reassociation with the regulatory subunits of cAMP-PK and it also does not appear to be due to selective targeting of the catalytic subunit.

3.6.9 Comparison of the Membrane Preparation and the Walsh Inhibitor

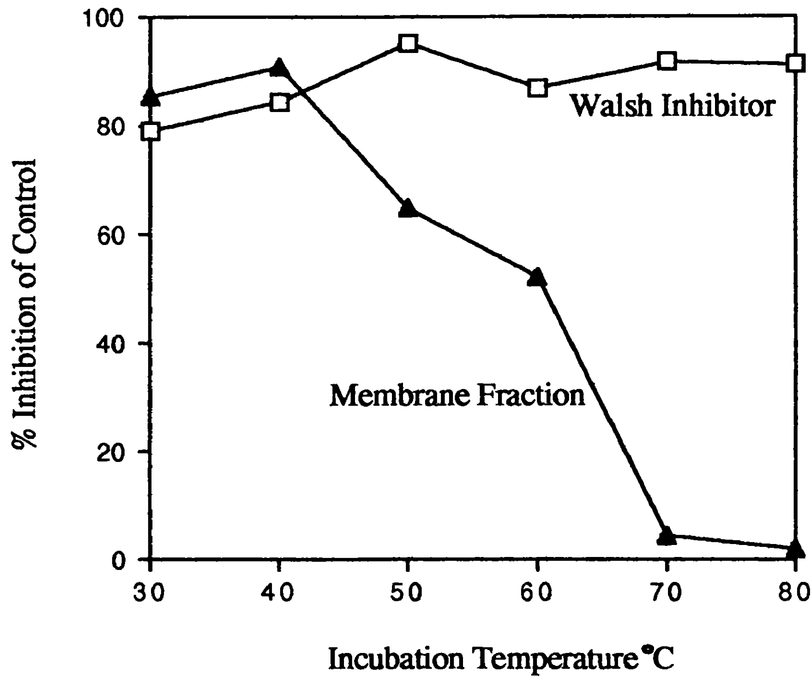
There is present in tissues an endogenous 11 kDa, heat stable, protein inhibitor of the cAMP-PK known as the Walsh Inhibitor. It was possible that the membrane fraction inactivator was the Walsh Inhibitor binding to the catalytic subunit.

A major characteristic of the Walsh Inhibitor is its heat stability. Aliquots of the membrane preparation and the Walsh Inhibitor were incubated for one hour at various temperatures ranging from 30°C to 80°C. These aliquots were then diluted accordingly and tested for their ability to inhibit the catalytic subunit of cAMP-PK.

As shown in Fig. 3.14 there is little effect of the heat treatment on the ability of the Walsh Inhibitor to inhibit the cAMP-PK, even after incubation at temperatures of 70°C and 80°C. The membrane preparation does however start to lose activity after incubation temperatures above 40°C. At 50°C there is a 64% inhibition of cAMP-PK activity, at 60°C there is a 52% inhibition, but at 70°C this is down to only a 4% inhibition of the control.

Therefore the inactivating activity present in the membrane preparation is not the Walsh Inhibitor since it is heat labile and the Walsh inhibitor is not. This heat lability

Fig. 3.14 Heat Lability of the Membrane Fraction and the Walsh Inhibitor



The membrane fraction was purified as far the 41,000g supernatant step (as described in this chapter). Aliquots of the Walsh inhibitor and the membrane fraction were incubated for one hour at the indicated temperatures and then tested for their ability to inactivate the catalytic subunit of cAMP-PK, purified from bovine heart (purification described in chapter 2). cAMP-PK activity was assayed as described in chapter 2 and the results are expressed as the percentage inhibition of cAMP-PK relative to a control incubation which lacked the membrane fraction and the Walsh inhibitor

also suggests that the activity present in the membrane is protein in nature and possibly an enzyme.

3.6.10 Proteolysis of the catalytic subunit.

It was decided to test whether the inactivation of the catalytic subunit was due to a proteolytic mechanism, as suggested by Alhanaty et al (1985, 1981). The catalytic subunit of cAMP-PK was incubated with the membrane preparation, at a dilution that would cause 100% inactivation of the catalytic subunit of cAMP-PK in 30 minutes. At various time points over this time course aliquots of the incubation mixture were taken into SDS-PAGE sample buffer and boiled for 2-3 minutes.

Electrophoresis was performed on these samples to see if there was any proteolysis of the catalytic subunit of cAMP-PK and the generation of any proteolytically derived product. There was no obvious proteolysis of the 41KDa catalytic subunit as analysed by SDS-PAGE (data not shown) however, it is possible that there is proteolysis but the proteolytic product is to small to be visualised by SDS-PAGE. These observations are in agreement with those reported by Hemmings (1986) who reported inactivation of the catalytic subunit in porcine epithelial cells but no proteolysis. However, Shaltiel and co-workers (1985) have shown that the inactivation of the catalytic subunit of cAMP-PK, by a membrane fraction prepared from brush border epithelia, is accompanied by the concomitant production of a 34 kDa band derived from the proteolysed 41 kDa catalytic subunit.

3.7 Conclusions

The activity of cAMP-PK in mammary gland does not change upon 24 hour starvation. It bears no correlation with the rate of lipogenesis or activity of ACC in vivo even when the cAMP-PK is activated. These results show by direct assay of cAMP-PK activity that this protein kinase is not directly involved in the regulation of ACC and lipogenesis in the lactating mammary gland (see chapter 4 for discussion of the role of cAMP-PK in liver lipogenesis). There is no selective activation of the type I and II isozyme in the mammary tissue during starvation unlike in the liver (Chan et al, 1979, Ekanger et al, 1988, Livesey, 1982). Different physiological roles for the type I and II holoenzyme forms of cAMP-PK have not been firmly established although selective activation of these isozymes has been reported. In the livers of rats injected with glucagon, type I cAMP-PK activation preceeded that of the type II isozyme (Schwoch, 1987). In experiments on human breast cancer cells it was shown that calcitonin selectively activated the type II isozyme (Livesey et al, 1984). Other workers report specific down regulation of the amount of rat liver type I isozyme in response to starvation or a low protein diet (Chan et al, 1979, Ekanger et al, 1988) others however find no change (McClung and Kletzien, 1981).

The cAMP-PK purified from rat mammary gland tissue has a different substrate specificity profile to that from bovine heart. Most significantly ACC does not appear to be a substrate for the mammary enzyme. This explains why in isolated mammary cells even sustained elevation of cAMP-PK activity produces no change in the activity of ACC. However cAMP-PK purified from mammary tissue does phosphorylate

kemptide and also the synthetic peptide 'SSMS' corresponding to the known cAMP-PK phosphorylation site on ACC. The results show that the secondary and perhaps the tertiary structure of ACC are important for the substrate recognition mechanism of cAMP-PK. The primary sequence of ACC also plays a part in making it a very poor substrate for mammary cAMP-PK. The heart cAMP-PK phosphorylates 'SSMS' six times more rapidly than the mammary enzyme, and nearly as well as it phosphorylates kemptide. This suggests that the heart cAMP-PK catalytic subunit is less discriminating in its requirements than the mammary cAMP-PK catalytic subunit for the recognition of phosphorylation sites. Mammary gland catalytic subunit has a lower affinity than the heart enzyme for the specific peptide inhibitor of cAMP-PK. This may be due to the subunit having a different structure or the presence of inactive catalytic subunit in the preparation which still retains the ability to bind to the inhibitor. The mammary gland catalytic subunit has a 25-fold lower specific activity than the heart enzyme. However the mammary gland enzyme is very labile and starts to lose activity immediately upon purification so it is likely that the value for specific activity is an underestimate of the true value. At the moment it is not possible to see how this problem may be overcome. The purified mammary gland catalytic subunit is also much less stable than the heart subunit. Whereas the heart enzyme is stable for many weeks and indeed months the mammary gland catalytic subunit loses 50% of its activity within 12 hours of purification. This may be due to a structural problem, with the enzyme changing conformation upon isolation from the tissue and/or an inherent lability of the catalytic subunit from mammary gland.

Crude mammary gland tissue extracts showed a greater rate of loss of cAMP-PK

activity than similarly prepared heart tissue extracts upon the addition of cAMP. This may be due to an intrinsic instability of the mammary gland catalytic subunit, which starts to lost activity immediately upon dissociation from the regulatory subunits in the holoenzyme complex, but there may also be present in mammary tissue a factor that is responsible for the inactivation of the catalytic subunit. Such a factor has been reported in brush border epithelia (Alhanaty et al, 1985, 1981). Following a modified version of the purification procedure suggested by these authors, a membrane fraction was prepared from mammary tissue. This fraction inactivated in a time dependent fashion the normally stable heart catalytic subunit. So there does appear to be present an activity in mammary tissue that inactivates cAMP-PK. This inactivation is accelerated in the presence of the substrate kemptide and inhibited in the presence of the substrate MgATP. This suggests that the binding of kemptide induces conformational changes in the catalytic subunit, that make it more susceptible to inactivation by this factor, whereas the binding of MgATP induces a conformational change that masks this site.

This mammary gland activity specifically inactivates the catalytic subunit and not the holoenzyme form of cAMP-PK, since the latter still displays full potential protein kinase activity upon the addition of cAMP. It also does not affect other protein kinases so far tested including the AMP-PK, a key regulatory enzyme in lipid metabolism. The inactivating factor in mammary gland is not simply regulatory subunit reassociating with the catalytic subunit and leading to loss of cAMP-PK activity, since it occurs even in the presence of cAMP which will prevent such reassociation. It also is not due to oxidative enzymes or free radicals since it still

occurs in the presence of high reducing conditions. It also does not appear to be simply a targeting of the catalytic subunit into the membranes since the inactivation is still seen in the presence of high salt, which should prevent any membrane reassociation. This activity is also not simply the Walsh inhibitor binding to the catalytic subunit and inactivating it. This is because the Walsh inhibitor is heat stable, but this activity begins to lose its inactivating ability, upon incubation at temperatures over 45°C, and loses all activity upon incubation at >60°C.

Thus the exact nature of this activity is still not clear, but it does appear to be protein in nature and highly specific for the catalytic subunit of cAMP-PK.

The reasons for the refractory nature of the mammary gland to adrenergic stimulation is unclear. Mammary tissue does contain highly active forms of cyclic nucleotide phosphodiesterases which have a total activity three orders of magnitude greater than that of adenylate cyclase (Clegg and Mullaney, 1984). Thus intracellular changes in cAMP concentrations may effectively be nullified, except when phosphodiesterase inhibitors are also present. It is also possible that the unusual environment of the mammary gland confers special properties upon the cAMP-PK within it. It has been reported (Gabbay and Lardy, 1987) that in rat hepatocytes, insulin can decrease the affinity of cAMP-PK for cAMP, so that the K_a is increased but the V_{max} remains unchanged. These authors suggest that insulin has this action by decreasing the binding of cAMP to the site 2 cyclic nucleotide binding site on the regulatory subunit. It may be possible that similar mechanisms operate in the mammary gland, especially since the tissue is highly insulin sensitive (Burnol et al, 1986). Compartmentalization

of cAMP-PK has been proposed as a potential regulatory mechanism for cAMP-PK (Dreyfuss et al, 1978). In human erythrocytes isoenzyme type I is mainly membrane bound while the type II isozyme is located in the cytoplasm. It is proposed that regulatory subunits bound to the membrane, can affect the substrates phosphorylated by the catalytic subunits by positioning the holoenzyme in closer proximity to certain substrates. However these authors find no difference in the catalytic properties of the cytosolic and membrane bound cAMP-PK so such compartmentalisation may not be a significant regulatory advantage in the mammary gland. It is possible that the lactating mammary gland has an unusual isoform of cAMP-PK and the data presented on stability and substrate specificity certainly support this idea and there are a number of reports of different isozymes of the catalytic subunit of cAMP-PK (see Chapter 1) but this will only be confirmed by structural, sequencing and cloning studies of mammary gland catalytic subunit forms. It may be possible that the major role of cAMP-PK during lactation is the regulation of the transcription and/or translation of genes as occurs in the liver (Quinn and Cranner 1990). The role of cAMP-PK in mammary tissue is thus not clear.

CHAPTER FOUR

Regulation of Fatty Acid Synthesis Via Phosphorylation of Acetyl-CoA Carboxylase by cAMP-Dependent and AMP-Activated Protein Kinases.

4.1 Introduction

Fatty acid synthesis in the major lipogenic tissues of the rat is stringently regulated according to the nutritional status of the animal. For example, changes in the rate of fatty acid synthesis in liver, white adipose tissue and lactating mammary gland closely parallel diurnal changes in food intake (Fukuda et al, 1985, Munday and Williamson, 1983). This regulation is accentuated in the fed to starved transition where fatty acid synthesis in the lactating rat is inhibited by 87% in the mammary gland and 65% in the liver after 6 hours and 98% in the mammary gland and 88% in the liver after 24 hours (Robinson et al, 1978). In non lactating female rats hepatic fatty acid synthesis is inhibited by 66% in response to 48 hour starvation (Holness and Sugden, 1990).

There are a number of potential regulatory steps in the pathway of glucose conversion to fatty acid in the lactating rat mammary gland and rat liver at which the inhibition in response to starvation could be effected. For the mammary gland these include: the active transport of glucose into the cell, the regulation of glycolysis by 6-

phosphofructo-kinase (6-PF-l-K), the oxidative decarboxylation of pyruvate by pyruvate dehydrogenase (PDH) and the first step committed to the synthesis of fatty acids catalysed by acetyl-CoA carboxylase, each of these steps has been implicated in the inhibition of fatty acid synthesis in the lactating rat mammary gland following a prolonged period of starvation (see Munday and Hardie, 1987 for review). Short term regulation of hepatic lipogenesis is also achieved via changes in the expressed activity of one or more regulatory enzymes in the pathway of fatty acid synthesis, two of which are the reactions catalysed by the mitochondrial pyruvate dehydrogenase complex (PDH) and ACC. Control of these enzymes may be particularly important in liver where the major precursor for fatty acid synthesis appears to be lactate (Geelen and Hindriks, 1984) whose exogenous supply is not subject to the regulatory influences of hepatic glycolysis. Each potential regulatory step, in both tissues, may be controlled by reversible phosphorylation and in the case of the mammary gland, each has been shown to be sensitive to the action of insulin, (reviewed by Munday and Hardie, 1987). Although any of the steps listed above may play a role in determining the rate of lipogenesis during starvation and refeeding, changes in PDH and ACC activity are likely to be of particular importance. Some of the reasons for this are detailed below.

It has been shown (Coore and Field, 1974) that, in parallel with increased lipogenesis and increased enzyme concentration, that the proportion of mammary gland PDH in its dephosphorylated (active) form is increased 3-fold during lactation. The short-term changes in PDH activity in the lactating mammary gland are also achieved via changes in its phosphorylation state. In response to 24hr or 48hr starvation there is

phosphorylation and inactivation of PDH. The proportion of PDH in its active form decreases by 71% and 77% respectively (Kankel and Reinauer, Baxter and Coore, 1978) and this correlates well with the decrease in mammary gland lipogenesis (Robinson et al, 1978). Induction of short-term insulin deficiency by 3hr streptozotocin treatment also causes an 85% inactivation of PDH (Baxter et al, 1979). These inactivations are very similar in extent to those previously reported for PDH in white adipose tissue of 48hr-starved alloxan-diabetic non-lactating rats (Stansbie et al, 1976). The phosphorylation and inactivation of PDH in mammary gland in response to starvation correlates with an increase in PDH kinase activity (Baxter and Coore, 1978) and a decrease in PDH phosphatase activity (Baxter and Coore, 1979a). Reactivation of lipogenesis by refeeding, or by administration of insulin with glucose, is accompanied by PDH reactivation (Munday and Hardie, 1987). The reactivation of mammary gland PDH by insulin is thought to be through a decrease in PDH kinase activity (Baxter and Coore, 1978) and an increase in PDH phosphatase activity (Baxter and Coore, 1979a).

The expressed activity of hepatic PDH decreased by 69% and 88% in response to 6 hour and 48 hour starvation respectively (Holness and Sugden, 1990) which correlates well with the rapid depletion of hepatic glycogen (Holness and Sugden, 1989) and the inhibition of lipogenesis (Holness and Sugden, 1990). Total PDH activity (i.e. that following dephosphorylation with exogenously added PDH phosphatase) does not change so that decreases in expressed activity may be attributed to increased phosphorylation and inactivation. Prolonged starvation or diabetes causes inactivation of PDH (Wieland et al, 1971) which is associated with adaptive increases in PDH

kinase activity (Kerbey and Randle, 1982). On the refeeding of chow, PDH is dephosphorylated and reactivated (Holness et al, 1988).

The major physiological mechanism that regulates ACC appears to be its phosphorylation-dephosphorylation (see Hardie, 1989). Purified ACC is phosphorylated and inactivated in vitro by the catalytic subunit of cAMP-PK from bovine heart and by AMP-PK isolated from rat liver (Munday et al, 1988b). The effect of phosphorylation by either protein kinase is to decrease the enzyme Vmax and increase the concentration of citrate required to achieve half-maximal enzyme activation (Ka citrate) (Munday et al, 1988b). However, the decrease in Vmax achieved by the AMP-PK (90%) is much greater than that achieved by cAMP-PK (20%) and is the result of phosphorylation of serine-79 (numbered from the N-terminus in the rat liver sequence, Lopez-Casillas et al (1988)) on the ACC polypeptide chain) compared with serine-77 phosphorylated by cAMP-PK (Munday et al, 1988b). The phosphorylation of these two sites is mutually exclusive (Munday et al, 1988b) and inactivation of ACC in glucagon treated hepatocytes appears to be achieved by the phosphorylation of serine-79 (Sim and Hardie , 1988). This implies that AMP-PK and not cAMP-PK is the physiologically important protein kinase responsible for hepatic ACC regulation in vivo (Sim and Hardie, 1988). AMP-PK is itself thought to be regulated by a phosphorylation-dephosphorylation mechanism achieved via a separate protein kinase activity, which is stimulated by nanomolar concentrations of palmitoyl-CoA (Carling et al, 1987) and whilst cAMP-PK and AMP-PK kinase appear to be distinct activities, it has been suggested that cAMP-PK may be involved somewhere in the cascade (Hardie, 1989, Haystead et al, 1990).

The evidence for a role for cAMP-PK comes from the observation that in isolated rat adipocytes ACC inactivation by adrenaline or the beta-agonist isoproterenol or agents which activate cAMP-PK by receptor-independent mechanisms (forskolin, an adenylate cyclase activator, cAMP analogues and isobutylmethylxanthine, a phosphodiesterase inhibitor) is mediated by phosphorylation at serine-79 (the AMP-PK site). Use of a thiophosphate analogue of cAMP, which is an antagonist of the binding of cAMP to the regulatory subunit of cAMP-PK, results in no phosphorylation and inactivation of ACC in response to adrenaline (Haystead et al, 1990). This evidence indicates that cAMP-PK is involved in the signal transduction pathway, although it clearly does not directly phosphorylate ACC. Phosphatase-2A is thought to be the major ACC phosphatase, therefore inhibition of phosphatase-1 via phosphorylation of inhibitor-1 is unlikely to be the mechanism by which cAMP-PK indirectly increases phosphorylation of ACC. Thus the precise role of cAMP-PK is, at present, unknown.

In the lactating rat mammary gland work conducted on the purified enzyme shows that ACC is phosphorylated and inactivated (73% decrease in V_{max}, 75% increase in K_a citrate and an increase of 1.2 molP/ mol enzyme subunit) in response to 24hr starvation, and dephosphorylated and reactivated by 2.5hr of chow refeeding (Munday and Hardie, 1986). Similar results have been obtained from measurements of ACC activity in crude mammary tissue extracts (McNeillie and Zammit, 1982, Munday and Williamson, 1982). In rat liver ACC is phosphorylated and inactivated (51% inhibited after 24hr starvation and 74% inhibited after 48hr starvation accompanied by an increase in the phosphate content of 1.7 and 2.6 molP/mol of subunit

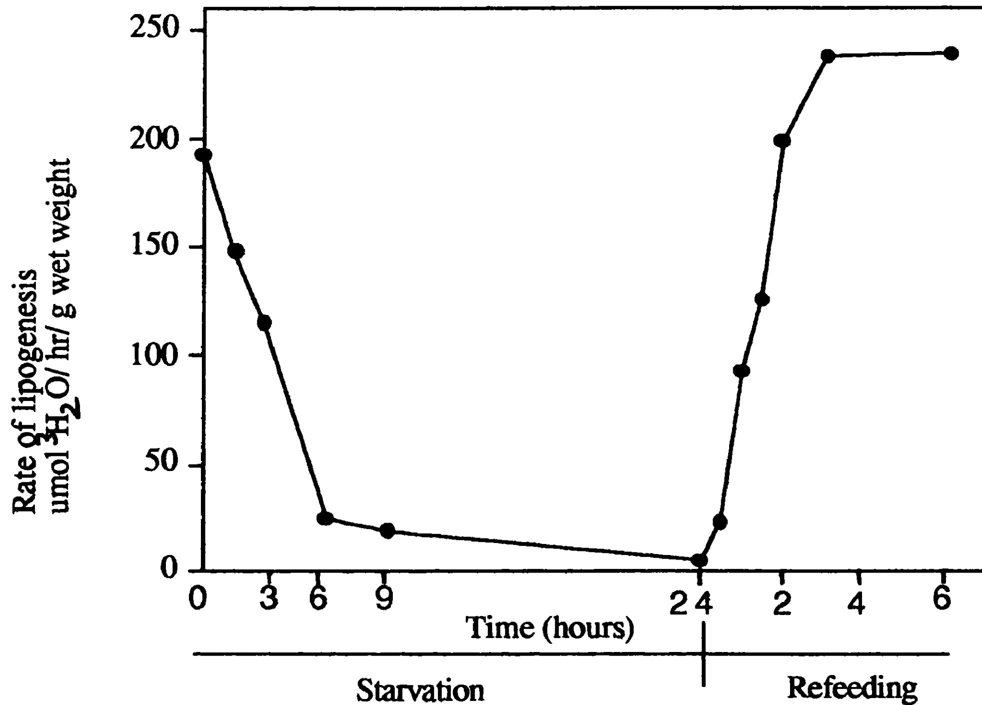
respectively) in response to starvation, and dephosphorylated and reactivated by 24hr refeeding (Thampy and Wakil, 1988).

This chapter reports the changes in both, the rates of fatty acid synthesis and in the activity of ACC, in both lactating rat mammary gland and rat liver in response to starvation and chow refeeding. The changes in ACC activity are compared to changes in PDHa activity to assess their relative importance in each tissue. The changes in purified ACC activity are further compared with changes in cAMP-PK and AMP-PK activities as these are heavily implicated in the regulation of ACC by phosphorylation. The changes in kinase activity are compared to plasma insulin concentrations. Lastly it reports the identification and partial characterisation of a protein kinase that is a potential candidate for the AMP-PK kinase.

4.2 The Regulation of Fatty Acid Synthesis in the Mammary Gland of the Lactating Rat during the Starved to Fed Transition.

The decrease in the rate of lipogenesis that occurs when food is withdrawn is shown in Fig. 4.1. The rate of fatty acid synthesis steadily declined over the first 6 hours of starvation until it was 87% inhibited relative to the fed control animals. This fall in lipogenesis was significantly greater than that due to the normal diurnal decline that also occurs over this time period (Munday and Williamson, 1983). From these authors' results it can be calculated that lipogenesis in the mammary gland of fed lactating rats falls by approximately 30% compared to the 87% inhibition caused by

Fig. 4.1. Lipogenesis in the Lactating Rat Mammary Gland During the Fed-Starved-Refed Transition



Changes in the rate of lipogenesis in the lactating rat mammary gland during starvation and refeeding are shown above. The rate of incorporation of $^3H_20$ into fatty acids (umol/ hr/ g wet weight tissue) was measured in the lactating rat mammary gland for a 30 minute time period that extended 15 minutes either side of the time point indicated. Each time point represents the mean of at least 4 observations. Modified from Hagopian et al (1991).
Food was withdrawn at 10.30 hours.

starvation over a similar time period. There was a further, slower and less dramatic decline between 6 - 24 hour starvation resulting in an eventual inhibition of lipogenesis of some 97%. These results are similar to those previously reported (Robinson et al, 1978). As can be seen from Fig. 4.1 upon refeeding of chow to the lactating rats the lipogenic inhibition was overcome and there was a stimulation of mammary gland fatty acid synthesis. 30 minute refeeding restored the rate of lipogenesis to 16% of the initial control value (and a 5-fold increase compared to the starved level) by 1-hour refeeding the rate of fatty acid synthesis was 47% of the initial control value and this increased to 70% of the control value after 1.5-hours of refeeding (a 22-fold increase compared to the starved level) and the rate of fatty acid synthesis reached fed control levels by 2-hours of refeeding. Mercer and Williamson (1986) reported that there was a 5-fold increase in lipogenesis at 0.5-hour refeeding and a 23-fold increase at 1.5-hour refeeding compared to the starved value, these results are similar to those obtained in the current study.

4.3 Activity of PDH and ACC during the Starved to Fed Transition

The fall in lipogenesis may be due to a decreased availability of substrate caused by either a fall in glucose availability and/or uptake, or inhibition of glycolysis. Lipogenesis may also be decreased by a direct inhibition of lipogenic enzymes. These factors are considered in the following sections.

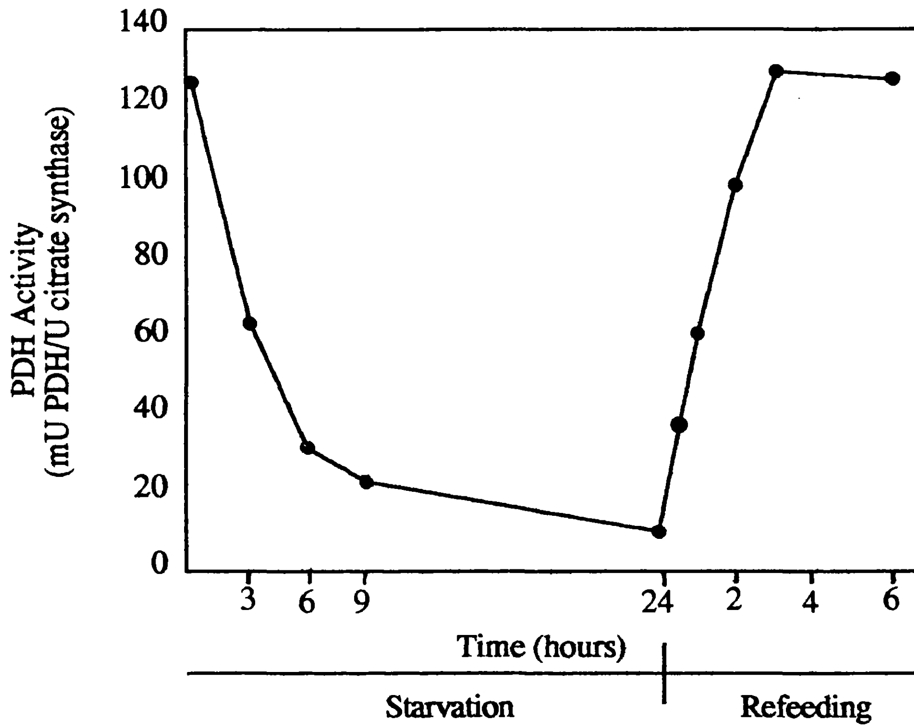
4.3.1 Pyruvate Dehydrogenase Activity

Comparison of the arterio-venous differences for glucose and pyruvate plus lactate across the mammary gland after 24 hour starvation implies that 73% of the C_3 units being taken up as glucose are subsequently released as pyruvate or lactate,assuming no pyruvate synthesis from other precursors eg amino acids, (Hagopian et al, 1991). This suggests that PDH is inactivated by 24 hour starvation. This is confirmed by current data (Fig. 4.2) and by previous investigators who reported a 71% inhibition of PDH (Baxter and Coore, 1978). PDH inactivation was considerably more rapid than this (Fig. 4.2) being 50% inhibited within 3 hour starvation, 79% by 6 hour and 92% by 24 hour starvation. This inhibition of PDH activity was reversed upon the refeeding of chow to the starved lactating rats as can be seen in Fig. 4.2. PDH activity is reactivated approximately 21% upon 30 minutes of refeeding, this figure has increased to 41% by 1-hour refeeding and 50% by 1.5-hour refeeding. By 2-hours of refeeding PDH activity is 78% of the fed control value and by 3-hours of refeeding it is restored to the original fed control levels.

4.3.2 ACC Activity in Lactating Rat Mammary Gland Homogenates

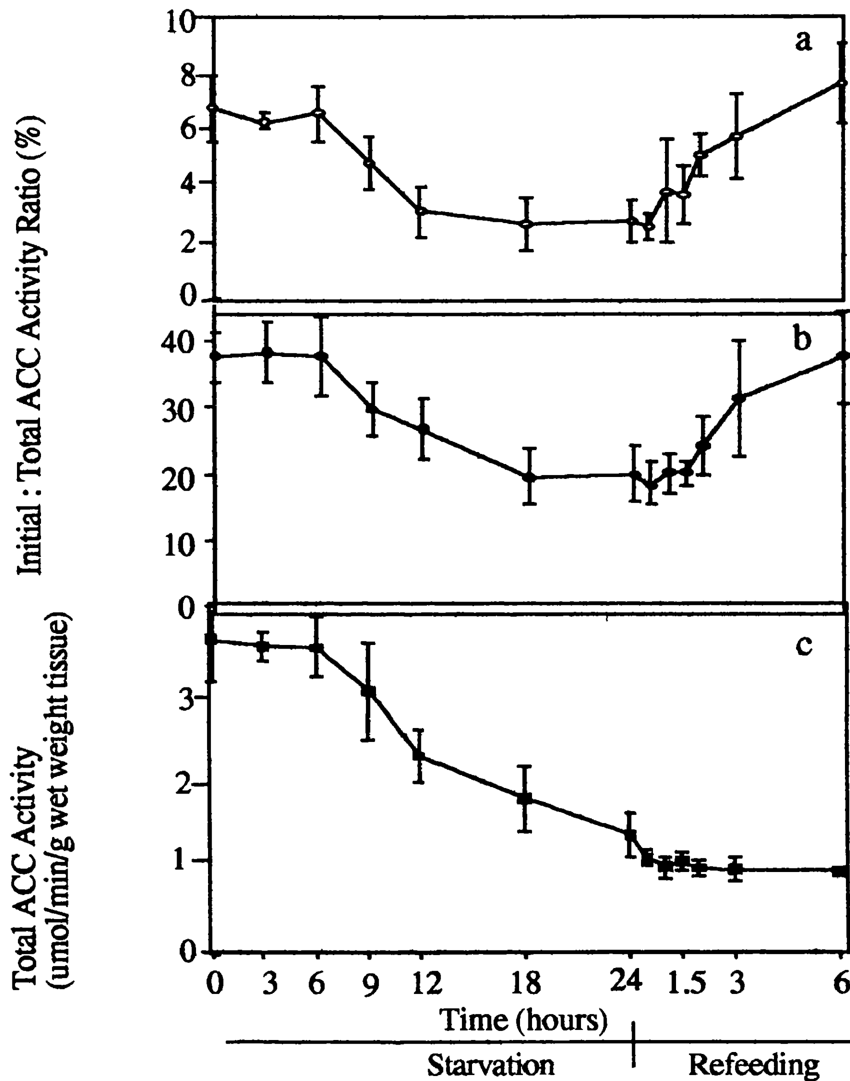
The total activity of ACC in crude homogenates decreased by approximately 63% after 24 hour starvation (Fig. 4.3c). This decrease is similar to that reported by other workers for the mammary enzyme (McNeillie and Zammit, 1982, Munday and Williamson, 1982, Munday and Hardie 1986). This decrease is likely to be due to

Fig. 4.2. Pyruvate Dehydrogenase Activity in the Lactating Rat Mammary Gland



The changes in Pyruvate Dehydrogenase (PDH) activity in the lactating rat mammary gland during starvation and refeeding are shown above. PDH activity is expressed as mUnits PDH/ Unit citrate synthase activity, where one unit of activity converts one micromole of substrate into product per minute at 30°C. Details of the assay are described in chapter 2. Each time point represents the mean of 6 observations.
Modified from Hagopian et al (1991)

Fig. 4.3 ACC Activity in Tissue Homogenates Prepared From Lactating Rat Mammary Gland



Total ACC activity was assayed in crude extracts of lactating rat mammary gland as described in chapter 2 and is expressed as umol $NaH^{14}CO_3$ incorporated/min/gram wet weight tissue (c). Initial ACC activities were assayed as described in chapter 2 at 0.5mM citrate (a) and 10mM citrate (b) and each is expressed as a percentage of the total activity. Results shown are the means of at least 4 observations at each time point. S.E.M are indicated by the vertical bars.

a decrease in the concentration of ACC (the 'total activity' of ACC is taken to be a reasonable indication of the concentration of enzyme protein since it represents the maximum available ACC activity following extensive dephosphorylation with exogenous protein phosphatase and activation and polymerisation following incubation with the allosteric activator, citrate). The decrease could be due to a decreased rate of enzyme synthesis or an increased rate of degradation. The decline in ACC concentration was not marked until 9 hours of starvation and then continued progressively over the time course of 24-hour starvation shown (Fig. 4.3c).

It can also be seen (Fig. 4.3a, b) that the expressed activity of ACC (i.e. initial:total activity ratio) showed an overall decrease over the time course of 24 hour starvation of 50-57%, this is similar to values that have been previously reported (Munday and Williamson, 1982, McNeillie and Zammit, 1982). This inhibition is indicative of inactivation of existing ACC enzyme. ACC was refractory to inhibition by starvation over the first 6-hours of starvation and the major inactivation occurred between 6 and 18 hours. Fig. 4.3a, b shows that upon 0.5-hour refeeding there was no reactivation of ACC (as judged by the expressed activity, i.e. initial:total). 1-hour refeeding caused a 18-24% reactivation of expressed ACC activity, which did not differ significantly from the reactivation at 1.5-hour refeeding. By 2-hour refeeding ACC activity was restored to 65-77% of fed control levels, this increased to 87-92% by 3-hour refeeding and expressed ACC activity was restored to fed control levels by 6-hour refeeding.

Changes in the expressed ACC activity are often indicative of changes in the

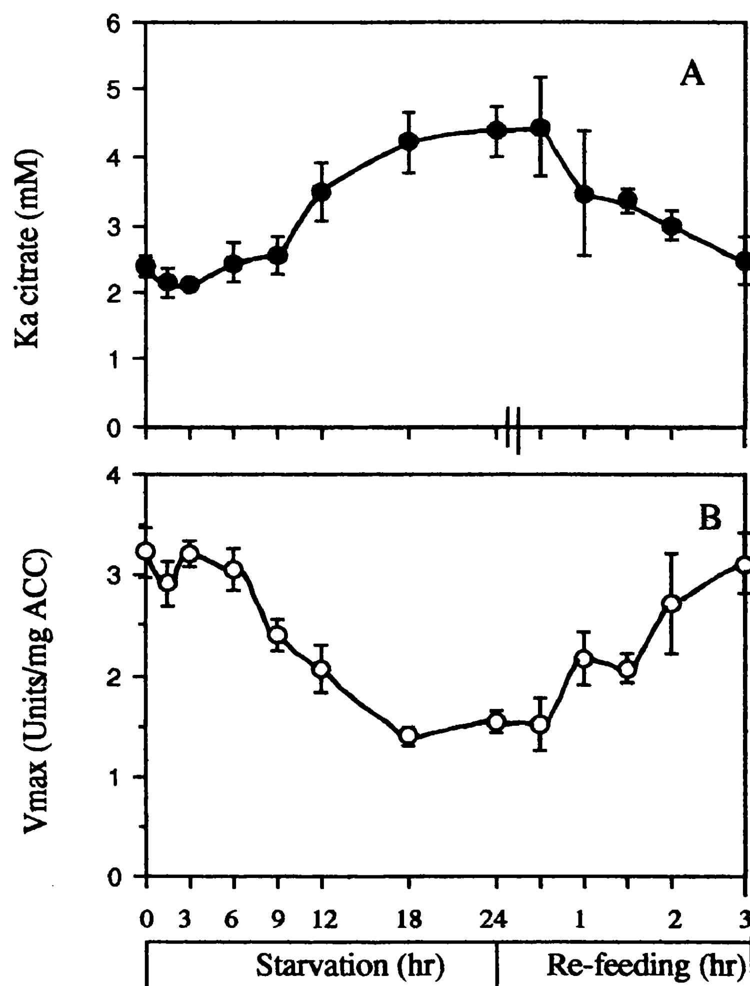
phosphorylation and activation state of ACC. Thus the results correlate quite well, with the results obtained from work conducted on the purified enzyme, discussed in section 4.4.

4.4 Activity of ACC Purified from Lactating Rat Mammary Gland

Purification of ACC by avidin-Sepharose affinity chromatography requires the monomerisation of the enzyme and also removes any possible allosteric effects of endogenous citrate or fatty acyl-CoA present in tissue extracts. As a consequence any differences in enzyme activity after the purification by avidin-Sepharose chromatography are due solely to changes in the phosphorylation status of ACC.

Over the first 6 hours of starvation there was virtually no change in the K_a citrate or V_{max} (Fig. 4.4a, b). By 12-hour starvation there was a 44% increase in the K_a citrate and a 38% decrease in the V_{max}. Maximal increases in the K_a citrate (86%) and decreases in V_{max} (57%) had occurred by 18-24-hour starvation. There was no reactivation of purified ACC activity upon 0.5-hour refeeding as reflected by no change in the V_{max} or K_a citrate of the enzyme (Fig. 4.4). At 1-hour refeeding there was a 22% decrease in the K_a citrate and the V_{max} was restored to 68% of the fed control value. Refeeding for 2-hours decreased the K_a citrate by 35% and increased the V_{max} to 81% of the fed control value. By 3-hour refeeding both parameters returned to within 3% of their respective initial values in the fed lactating rat. This data is in agreement with that suggested by measurements of initial:total activity ratio (Fig. 4.3a, b) and confirm that the observed activity changes of ACC are due to its phosphorylation.

Fig. 4.4 Purified Mammary Gland ACC Activity



The changes in the kinetic parameters of ACC purified from lactating rat mammary gland during starvation and refeeding are shown above. ACC was purified by avidin-Sepharose chromatography at the time points indicated. At each time point Vmax and the concentration of citrate required for half maximal activation, Ka citrate were measured as described in chapter 2. Each value represents the mean of at least 4 separate preparations at each time point. S.E.M is indicated by the vertical bars.

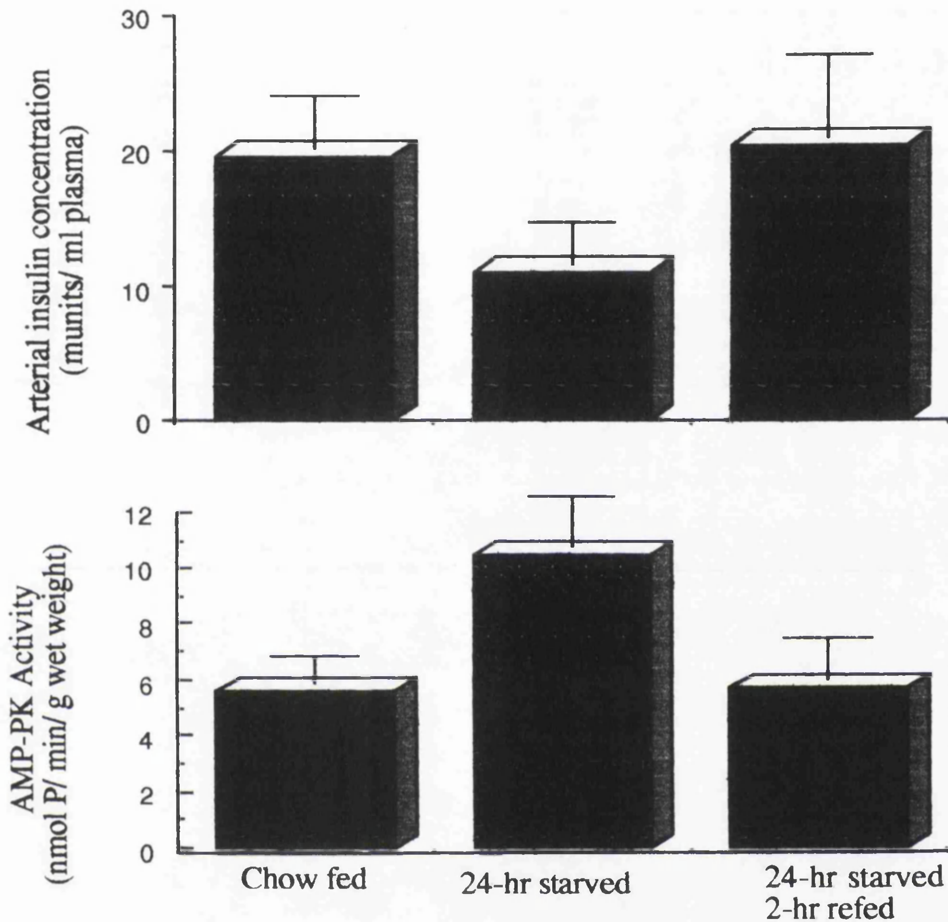
4.4.1 Mammary Gland cAMP-PK Activity

The activity ratio of cAMP-PK was measured in fed and 24-hour starved lactating rat mammary gland (as described in Chapter 2). As can be seen from Table 3.1, there is no change in the activity of cAMP-PK from the fed to the 24-hour starved state. However, after 24-hour starvation ACC was inhibited (50%) as was fatty acid synthesis (97%) (Fig. 4.4b, 4.1), therefore cAMP-PK does not appear to be responsible for the observed inhibition of ACC and fatty acid synthesis. Furthermore, the catalytic subunit of mammary gland cAMP-PK is very labile (see Chapter 3) and loses activity very quickly, the catalytic subunit from mammary gland does not phosphorylate ACC *in vitro* and the 20% decrease in Vmax of ACC as a result of phosphorylation by bovine heart catalytic subunit *in vitro* is less than the 50% decrease in Vmax observed *in vivo* after 24-hour starvation (Fig. 4.4b). Therefore it appears very unlikely that cAMP-PK has a role in the regulation of ACC and fatty acid synthesis in the lactating rat mammary gland.

4.4.2 Mammary Gland AMP-PK activity

The activity of the AMP-PK was assayed in fed and starved lactating rat mammary gland as described in Chapter 2. The AMP-PK activity increased by 97%, from 5.9 $\pm$ 0.39 to 10.6 $\pm$ 0.86 U/mg, during 24-hour starvation (Fig. 4.5). Within 3-hour refeeding the activity of AMP-PK had returned to 6.1 $\pm$ 0.45 i.e. to within ~~0.3%~~ 3.0% of the fed control value. The approximate doubling of AMP-PK activity at 24-hour starvation correlates well with the 50% inhibition of ACC. AMP-PK activity had

Fig.4.5. AMP-PK Activity and Plasma Insulin Levels in the Lactating Rat



Rat mammary gland AMP-PK activity and plasma insulin concentrations during starvation and refeeding in the lactating rat are shown above. AMP-PK activity was measured using the synthetic peptide SAMS as substrate as described in chapter 2. Each value is the mean of at least 4 observations. The data on plasma insulin concentrations is modified from Robinson et al (1978) and represents the means of at least 4 observations. S.E.M are represented by the vertical bars.

returned to fed control levels by 3-hour refeeding, at which time ACC activity was also restored (Fig. 4.4). The data shows a good inverse correlation between ACC activity and AMP-PK activity which is further proof that AMP-PK is the physiological protein kinase that phosphorylates and inactivates ACC. The liver AMP-PK is stimulated, in vitro at least, by micromolar concentrations of AMP (Carling et al, 1987). Gross changes in AMP concentration in vivo i.e millimolar changes, in response to pathological conditions such as anoxia (Hardie, 1989) and fructose feeding (Moore et al, 1991) result in activation of liver AMP-PK and phosphorylation and inactivation of ACC. While no millimolar changes in AMP concentration are observed in response to physiological situations e.g. hormonal stimulation or starvation, it is possible that there are changes in AMP concentration which can activate the kinase. Such small micromolar changes in free AMP concentration are very difficult to measure without advanced techniques e.g. nuclear magnetic resonance spectroscopy, and therefore cannot be confirmed or discounted. The liver AMP-PK is known to be phosphorylated and activated by the AMP-PK kinase (Carling et al, 1987). It is possible that in response to 24-hour starvation the AMP-PK kinase phosphorylates and activates AMP-PK in the lactating rat mammary gland. The phosphorylation and activation of AMP-PK by AMP-PK kinase in liver preparations is stimulated by nanomolar concentrations of palmitoyl CoA (Carling et al, 1987). Since the uptake of plasma free fatty acids by the mammary gland increases during starvation this is a potential mechanism for increasing AMP-PK activity. Moore et al (1991) claim that in liver, AMP not only activates AMP-PK by a direct allosteric effect but also triggers the phosphorylation of the AMP-PK by the AMP-PK kinase. They suggest that either AMP binds to the allosteric site on AMP-PK and promotes

a conformational change which exposes the site for phosphorylation by AMP-PK kinase or possibly AMP binds to and activates the AMP-PK kinase directly.

4.4.3 Insulin Concentrations and AMP-PK Activity

There is a 50% reduction in the plasma insulin concentration in the lactating rat in response to 24 hour starvation but within 2 hour refeeding the insulin concentration has returned to the fed control level (Fig. 4.5). Whilst the changes in AMP-PK activity are unaccompanied by changes in cAMP-PK activity they do match closely with the changes in insulin concentration. This may mean that there is a negative regulation of AMP-PK by insulin *in vivo*, achieved via insulin mediated changes in the phosphorylation state or concentration of AMP-PK.

4.4.4 The Effect of Starvation and Refeeding on the Activity of ACC and PDH in the Lactating Mammary Gland.

The inhibition of fatty acid synthesis in the lactating rat mammary gland is rapid and dramatic in response to starvation (Fig. 4.1). It can be seen (Figs. 4.2, 4.3) that changes in the activity of PDH and not ACC, correlate more closely with the changes in fatty acid synthesis (Fig. 4.1).

PDH activity is 79% inhibited after 6-hour starvation and 92% inhibited after 24-hour

starvation (Fig. 4.2), this corresponds with an 87% and 97% inhibition of lipogenesis respectively. This correlation between PDH activity and lipogenesis is a strong indication that the phosphorylation and inactivation of PDH is a primary event in the mediation of lipogenic inhibition during starvation. In lactating rats, increases in serum fatty acids occur after 3 hours and are significant after 6 hours (Page and Kuhn, 1986). These changes will be reflected intracellularly because there is rapid equilibration of fatty acids across the plasma membrane (Noy et al, 1986). Thus the decreased PDH activity (Fig. 4.2) is most likely to be due to both the activation of PDH kinase by the elevated NADH/NAD$^+$ and acetyl CoA/CoA ratios associated with increased intracellular fatty acid concentration (Seiss and Weiland, 1976, Baxter and Coore, 1978), and the inactivation of the PDH phosphatase reported by Baxter and Coore (1979b). Insulin is probably involved in this mechanism of PDH regulation either via direct effects on PDH kinase and phosphatase or via its modulation of serum fatty acid concentrations through its antilipolytic action in adipose tissue. Plasma insulin levels decrease by approximately 50% after 18-hour starvation (Page and Kuhn, 1986) or 24-hour starvation (Robinson et al, 1978). This decrease in plasma insulin concentration is accompanied by increases in the plasma free fatty acid concentration (Page and Kuhn, 1986). The role of insulin is supported by work reported by Baxter and Coore (1978) and Baxter et al (1979) who have shown the insulin sensitivity of PDH. Short term insulin deficiency, induced by streptozotocin, which reduces mammary gland lipogenesis also causes a large inhibition of PDH (86%) (Baxter et al, 1979). This activity is restored close to control levels by the injection of insulin. In the starved lactating rat, the administration of insulin in vivo, together with an oral glucose load completely

reactivated PDH (Munday and Hardie, 1987). Total PDH activities remained unaltered under all conditions.

The expressed activity of ACC did not fall significantly during the first 6-hours of starvation (Fig 4.3) at which time mammary gland lipogenesis was 87% inhibited (Fig. 4.1). Thus it appears that inactivation of ACC is not involved in the short term (<6 hours) metabolic responses that cope with starvation. This is in agreement with previous reports (Munday and Hardie, 1986, Williamson et al, 1983). The delayed onset of decreased ACC concentration might be predicted for an enzyme with a half-life in excess of 24 hours (Nakanishi and Numa, 1970) however the equally slow inactivation of this enzyme compared with PDH is surprising. Work conducted on the purifed enzyme confirms that there is no inactivation of ACC during the first 6-hours of starvation (Fig. 4.4). From the results presented it seems that the mechanisms responsible for ACC inactivation in mammary gland, i.e. increased phosphorylation resulting from increased protein kinase or decreased protein phosphatase activity must also initially be refractory to starvation. This delayed inhibition may represent a final "locking down" mechanism, in starvation, of the already decreased flux from glucose to fatty acids. The importance of PDH rather than ACC in the inhibition of mammary gland lipogenesis during starvation most likely represents the importance of carbohydrate precursors, especially glucose, over lipid derived sources of acetyl-CoA in this tissue.

After 0.5-hour refeeding of chow to 24-hour starved lactating rats there was a 13% reactivation of lipogenesis (Fig. 4.1). It has been reported that within 0.5-hours of

refeeding chow to 18-hour starved lactating rats there was a dramatic reactivation of the rate of glucose uptake (Page and Kuhn, 1986) and this is probably the substrate for the observed reactivation of lipogenesis (Fig. 4.1). Glucose uptake by the mammary gland is probably aided by the enhanced insulin sensitivity of the gland at this time. The increased insulin sensitivity is thought to be due to the transient hyperinsulinaemia observed upon initial refeeding (Mercer and Williamson, 1986). The 21% reactivation of PDH at 0.5-hour refeeding (Fig. 4.2) correlates well with the reactivation of lipogenesis. However, at 0.5-hour refeeding there was no reactivation of ACC as judged by expressed activity (Fig. 4.3a, b) and this is confirmed by work conducted on the purified enzyme (Fig. 4.4). This observation argues against the idea of ACC being a "locking down" step, because if this was true there should be no reactivation of lipogenesis until ACC is reactivated. It has been suggested (Mercer and Williamson, 1986, Page and Kuhn, 1986) that there is no reactivation of PDH by 0.5-hour refeeding. They also suggest that the increase in lipogenesis at 0.5-hour refeeding is not significant. The fold increase in lipogenesis reported in this study is similar to that reported by Mercer and Williamson (1986). From 0.5- 6-hour refeeding ACC and PDH are clearly reactivated (Fig. 4.2 and 4.3) and are responsible for the much larger increases in lipogenesis that occur over this period of refeeding. This result is similar to that reported by Mercer and Williamson (1986) who also reported much larger increases in lipogenesis occur from 0.5-hour refeeding onwards. Although the reactivation of PDH is faster than ACC upon refeeding (there is an approximate 0.5-hour lag in the reactivation of ACC) the pattern of reactivation of both enzymes is similar, unlike in starvation, where the time course of inhibition of PDH is clearly different to that of ACC. The mechanism for the

reactivation of PDH and ACC probably involves the raised plasma insulin concentration which is seen upon refeeding (Robinson et al, 1978). It is likely that insulin reactivates PDH and ACC through an indirect mechanism involving inhibition of protein kinases that act on these enzymes (PDH kinase and AMP-PK, respectively) and/or stimulation of protein phosphatases that act on PDH and ACC (PDH phosphatase and protein phosphatase-2A, respectively). This indirect mechanism is also likely to involve any number of other enzymes involved in the signal cascade system e.g. AMP-PK phosphatase and AMP-PK kinase.

Such co-ordinated control of similar regulatory enzymes during starvation and refeeding implies a complex interaction of hormonal and metabolite signals. If insulin is the hormonal signal of primary importance this may be achieved via multiple second messenger systems possibly involving crosstalk between different signalling systems, or different sensitivities of the target regulatory systems.

4.5 The Regulation of Fatty Acid Synthesis in the Livers of Virgin Rats during the Starved to Fed Transition.

The decrease in the rate of hepatic lipogenesis that occurs when food is withdrawn is shown in Fig. 4.6. There was a 56% inhibition of lipogenesis by 6-hour starvation and an eventual 67% inhibition of lipogenesis by 48-hour starvation. As can be seen from Fig. 4.6 upon refeeding chow to the starved rats the lipogenic inhibition was overcome and there was a stimulation of hepatic lipogenesis, 2-hour refeeding restored the rate of fatty acid synthesis to 51% of the intial control value, this increased to 84% after 4-hour refeeding and by 6-hour refeeding the rate of lipogenesis had

reached the original fed control levels.

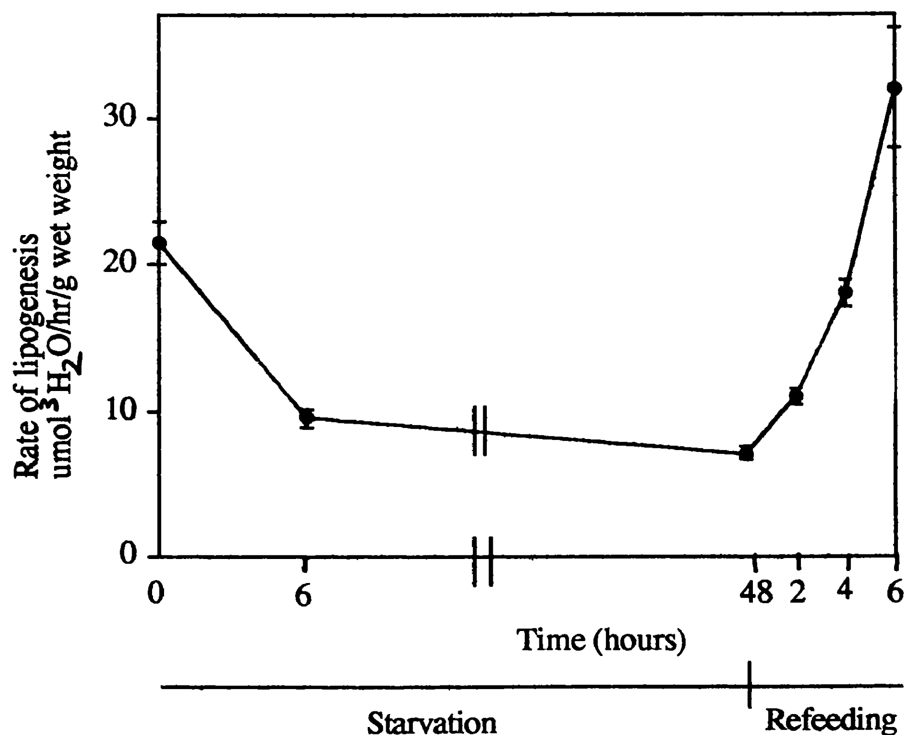
4.6 Activity of PDH and ACC in Virgin Rat Livers during the Starved to Fed Transition

The activities of the lipogenic enzymes PDH and ACC were examined over time courses of starvation and refeeding in order to see where the major point of regulation of fatty acid synthesis ocurred.

4.6.1 Pyruvate Dehydrogenase Activity

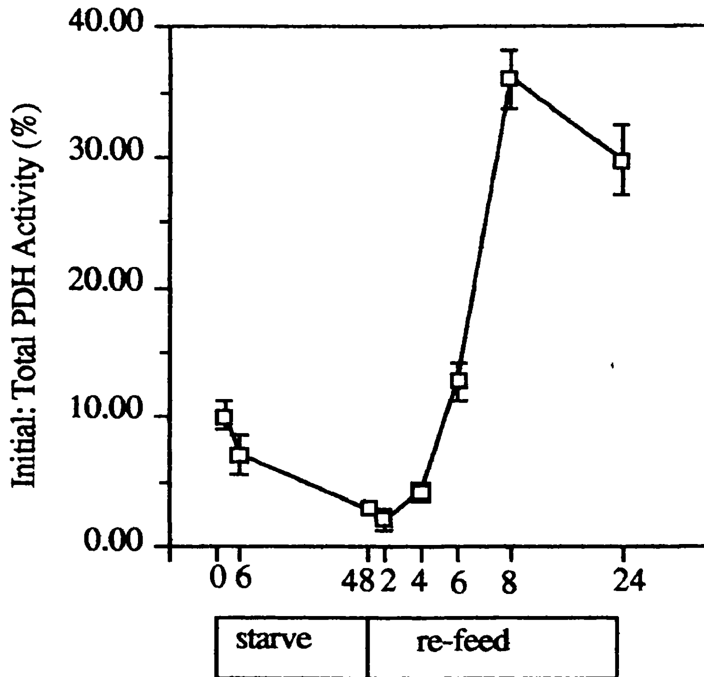
Hepatic PDH activity fell slightly in the first 6 hours of starvation (Fig. 4.7) and this was followed by a greater fall over the next 42 hours of starvation, giving a total decrease at 48-hour starvation of approximately 80%. There was no significant reactivation of PDH after 2-hour refeeding, but PDH activity returned to fed control levels within 6-hour refeeding (Fig. 4.7). However, from 6 - 8 hour refeeding there was a dramatic increase in PDH activity, such that it reached a value approximately 3.5-fold higher than the PDH activity in the fed control rats. By 24-hour refeeding PDH activity had begun to descend towards control values but was still 3-fold higher than in the fed controls. The total activity of PDH did not change across the fed to starved to refed transition and changes in the percentage of PDH in its active form represent changes in intrinsic enzyme activity i.e. changes in phosphorylation state.

Fig. 4.6 Lipogenesis in Virgin Rat Liver During the Fed-Starved-Refed Transition



The rate of incorporation of ^3H_2O into fatty acids (umol/hr/g wet weight tissue) was measured in the livers of female virgin rats at the time points indicated during time courses of starvation and refeeding. Each value represents the mean of at least 4 observations. S.E.M are indicated by the vertical bars.
Data modified from Holness and Sugden (1990) and Holness et al (1988).

Fig. 4.7. PDH Activity in Virgin Rat Livers



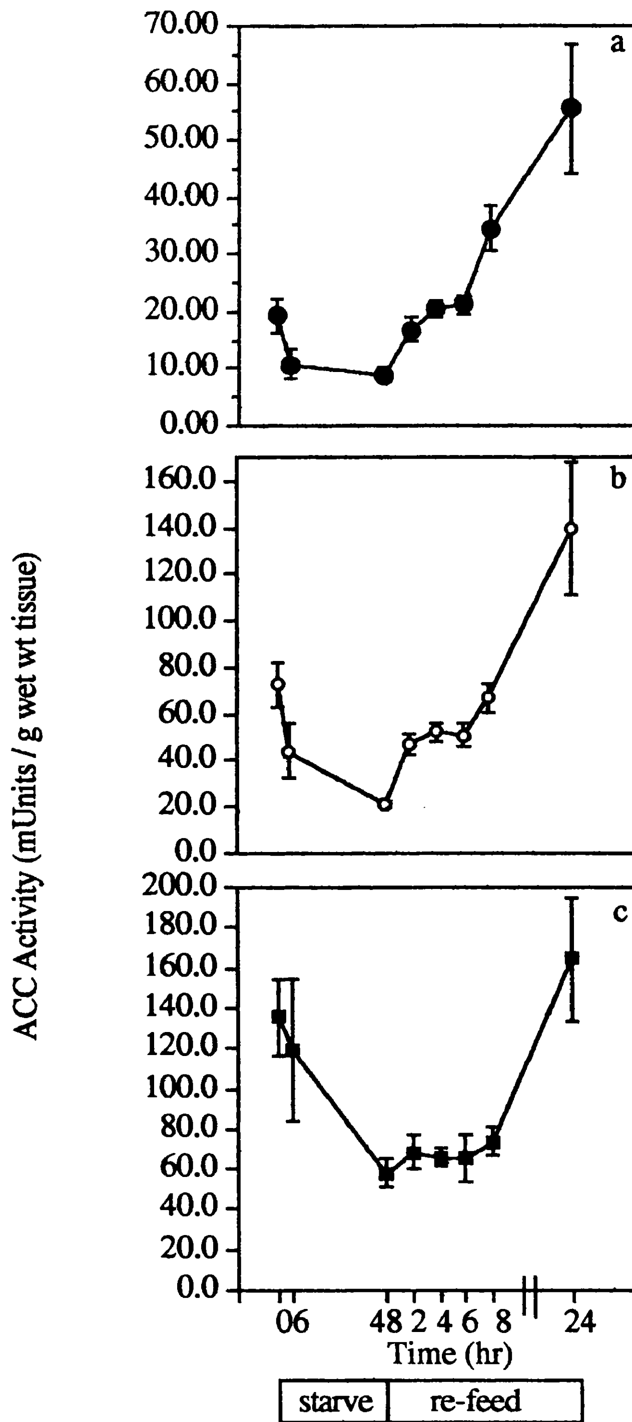
The changes in Pyruvate Dehydrogenase (PDH) activity in the livers of virgin rats during starvation and refeeding is shown above. Initial PDHa and total PDH activity was measured as described in chapter 2. PDHa activity is expressed as a percentage of the total activity at each time point. The results are the means of at least 3 observations with S.E.M represented by vertical bars.

4.6.2 ACC Activity in PEG Pellets

In response to 48 hour starvation the initial activity of ACC measured at 0.5mM (Fig. 4.8a) or at 10mM citrate (Fig. 4.8b), physiological and saturating concentrations respectively, decreased by 50 - 70%, with the major proportion of this inhibition occurring within the first 6 hours. The initial activity of ACC in the tissue rapidly increased again within 2 hours of refeeding the chow diet reaching fed control levels by 6 - 8 hours of refeeding. At 8-hour refeeding activity increased steadily such that ACC activity was 100% greater in livers of 24 hour refed rats compared with the fed control rats.

These changes in activity can be explained at least in part extent by changes in the concentration of ACC. There was no significant change in 'total activity' in the first 6 hours of starvation and this is reasonable given that ACC is an enzyme with a half-life in excess of 24 hours (Majerus and Kilburn, 1969). There was however, a drop in the 'total activity' of ACC of 60% by 48 hour starvation (Fig. 4.8c). 'Total activity' of ACC did not increase over the first 8 hours of refeeding but did increase between 8 - 24 hour refeeding to values higher than those of fed controls. These data are in agreement with previous reports that total hepatic ACC concentrations do not rise within the first 8 hours but do increase steadily over a subsequent 40 hour period of chow refeeding 48 hour fasted rats (Nishikori et al, 1973, Roman-Lopez et al, 1989). The increased concentration of cytosolic hepatic ACC during re-feeding could be due to an increased enzyme synthesis or, as reported by Allred and coworkers, due to mobilisation of mitochondrial storage forms of ACC (Roman-Lopez et al, 1989).

Fig. 4.8. ACC Activity in Polyethylene Glycol Pellets from Rat Liver

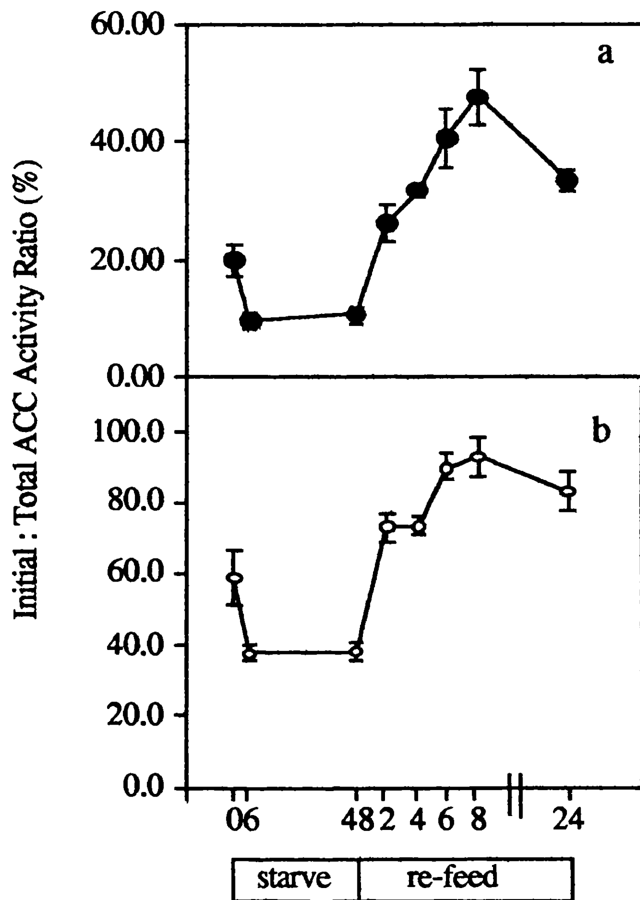


Initial ACC activity was measured in resuspended Polyethylene glycol pellets at 0.5mM citrate (a) and at 10mM citrate (b), and total ACC activity (c) was measured as described in Chapter 2. Each point represents the mean of at least six observations with S.E.M. represented by vertical bars.

The latter suggestion has been contested by Zammit and coworkers who claim to find no evidence that cytosolic ACC could be replenished from mitochondrial ACC storage pools during the refeeding of starved rats (Moir and Zammit, 1990). Whatever the mechanism responsible for changing ACC concentrations, data from this study and that reported by other workers (Nishikori et al, 1973, Roman-Lopez et al, 1989) demonstrate that the changes in initial ACC activity observed in the first 6 hours of starvation and the first 6 hours of refeeding cannot be explained by changes in the concentration of the enzyme.

When the 'initial activity' of ACC is expressed as a percentage of the 'total ACC activity' (Fig. 4.9) it is clear that within the first 6 hours of starvation there was a marked inactivation of the existing ACC protein by some 50%. This degree of inactivation did not change appreciably over the subsequent 42 hours of starvation (Fig. 4.9) suggesting therefore that the decreases in initial activity observed between 6 hour - 48 hour starvation in Fig. 4.8a and b, were due largely to decreased enzyme concentration (Fig. 4.8c). Significant inactivation of hepatic ACC has previously been reported to occur within 24 hour starvation (Moir and Zammit, 1990) but the data presented in this study suggest that inactivation actually occurs even more rapidly than this. The reactivation of ACC in response to refeeding was rapid and complete within 6 - 8 hours of refeeding. Approximately 60% of this reactivation occurred within the first 2 hours of refeeding (Fig. 4.9). This reactivation was clearly observed in a part of the time course during which ACC concentration had not changed (Fig. 4.8c). It would therefore appear to be reasonable to conclude from Fig. 4.8c that the changes in ACC activity from 0 - 6 hour starvation and from 0 -

Fig. 4.9. The Initial : Total Activity Ratio of Hepatic ACC



Initial and total ACC activity was measured as described for Fig. 4.8 and the initial:total activity ratio calculated for activities measured at 0.5mM citrate (a) and 10mM citrate (b). Each point represents the mean of at least 6 observations with S.E.M represented by vertical bars.

8 hour refeeding following 48 hour starvation are the result of changes in the intrinsic activity of existing ACC enzyme.

4.6.3 Comparison of Hepatic ACC and PDH Activity during the Starved to Fed Transition

Within the first 6-hours of starvation the expressed activity of ACC is markedly inhibited (50%) and this degree of inhibition did not change significantly over the next 42-hours of starvation (Fig. 4.9). The expressed activity of PDHa is 20% inhibited at 6-hour starvation and is 80% inactivated by 48-hour starvation (Fig. 4.7). Approximately 85% of the total decrease in lipogenesis occurs within the first 6-hours of starvation (Fig. 4.6) and this correlates with the major inactivation of ACC, therefore the inhibition of lipogenesis is probably due to inactivation of existing ACC enzyme protein.

Figures 4.7 and 4.9 show that the expressed activities of hepatic PDHa and ACC do not change in parallel in response to refeeding starved rats. Whilst the reactivation of ACC occurs within 2 hours of refeeding, the reactivation of PDH is refractory to refeeding with no increase observed in the first 2 hours, reaching control ad libitum fed levels only after 4-5 hours of refeeding (Fig. 4.7). Hence, the immediate, but limited reactivation of hepatic lipogenesis in the first 2 hours of refeeding (Fig 4.6) may be attributed to the reactivation of ACC (Fig. 4.9) in the absence of changes in PDH (Fig. 4.7). Furthermore, changes in the rate of lipogenesis are reported to

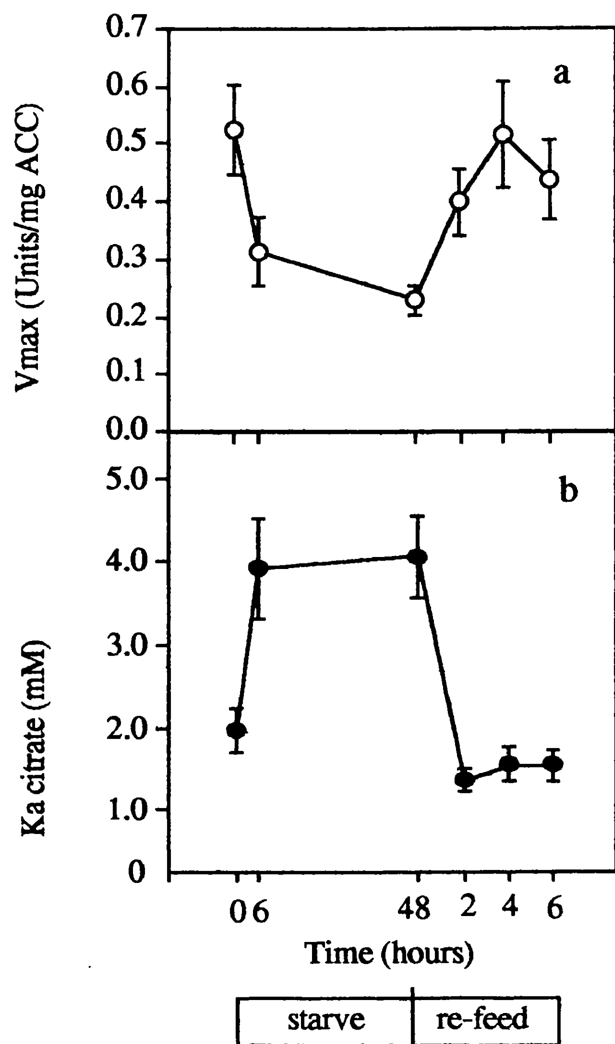
correlate with increases in the concentration of malonyl-CoA and this is probably an indication of stimulation of ACC in response to refeeding (Holness et al, 1988). The full reactivation of lipogenesis is subsequently facilitated by the reactivation of PDH which then makes acetyl-CoA derived from carbohydrate available as substrate for ACC.

It would appear reasonable to conclude from this data that ACC is the control point for carbon flux into fatty acid synthesis during the period of 48-hour starvation and the first 2-hours of refeeding. Within the first 2-hours of refeeding ACC is reactivated and this control point changes from ACC to PDH. This is in agreement with Holness et al (1988) who reported that the increase in lipogenesis from 4-hour refeeding onwards was accompanied by PDH activation and inhibited by (-)hydroxycitrate, an inhibitor of ATP-citrate lyase. This implies a change in the control point for lipogenesis from downstream to upstream ATP-citrate lyase at 4-hour refeeding and this correlates with a switch from ACC to PDH as the control point for lipogenesis.

4.7 Mechanism of ACC Inactivation in Livers during the Starved to Fed Transition

Within the first 6 hours of starvation the V_{max} of ACC decreased by 38% (Fig. 4.10a) and the K_a for citrate increased dramatically by 95% (Fig. 4.10b). There were further decreases in V_{max} and slight increases in K_a citrate in response to a further 42 hours of starvation (Fig. 4.10a, b). This supports the measurements of initial : total activity

Fig. 4.10 Purified Hepatic ACC Activity



ACC was purified and its Vmax and the concentration of citrate required for half maximal activation, Ka citrate, were measured as described in chapter 2. Each value represents the mean of at least four separate preparations at each time point with S.E.M. represented by vertical bars

ratio (Fig. 4.9) which suggest that the majority of ACC inactivation occurred within the first 6 hours of starvation, and that this must be due to its rapid phosphorylation. After 48 hour starvation the V_{max} of hepatic ACC had decreased by 53% and the K_a citrate had increased by 100%. Increased phosphorylation and inactivation of ACC has been reported in studies carried out on the livers of 2 day-fasted/2 day-refed rats in response to a subsequent period of 48 hour starvation (Thampy and Wakil, 1988). The authors report that this inactivation occurred steadily over the 48 hour period. However, the pre-treatment used to induce ACC concentration cannot be deemed a true physiological representation of fed rat liver and appeared to produce enzyme with a particularly high specific activity and considerable citrate independence compared with that measured by other groups (Sim and Hardie, 1988, Witters et al, 1988). The lack of phosphatase inhibitors in homogenisation and assay buffers possibly served to make further inaccurate estimates of true physiological phosphorylation and overestimation of activation states of ACC (Thampy and Wakil,1988).

Reactivation of hepatic ACC accompanied by a decrease in its alkali labile phosphate content has been reported in the same 2 day fasted/2 day refed rat model when rats subsequently fasted for 24 or 48 hours were refed chow for 72 hours (Thampy and Wakil, 1988). In this study, it was clear that reactivation of ACC in liver in response to chow refeeding occurred much more rapidly than this. There was a complete restoration of the K_a citrate (Fig. 4.10b) of purified hepatic ACC to fed control levels within 2 hours of refeeding and complete restoration of the V_{max} (Fig. 4.10a) within 4 hours of refeeding. Witters and coworkers have reported an equally rapid (and apparently cAMP-PK or AMP-PK site specific) dephosphorylation and activation of

ACC in Fao Reuber hepatoma cells in response to insulin (Witters et al, 1988). However, a comparison of Figures. 4.9, 4.10a and 4.10b leads to the conclusion that while dephosphorylation of the inhibitory phosphorylation sites and activation of ACC may occur within the first 2 - 4 hours of refeeding (Fig. 4.10) there is a further activation of the enzyme between this period and the increase in enzyme concentration that occurs from 8-hour refeeding (Fig. 4.9). It has been reported that in adipocytes the insulin stimulated phosphorylation of ACC, by an insulin activated serine kinase at sites different from the inhibitory sites, promotes the polymerisation and activation of ACC (Borthwick et al, 1987, 1990). It has been reported that hepatic ACC undergoes a protomer-polymer transition when 48-hour starved rats are re-fed chow for 24-hours (Thampy and Wakil, 1988), and refeeding was also observed to produce a gradual increase in the citrate-independent activity of ACC in starved rat liver (Majerus and Kilburn, 1969). Citrate-independent ACC activity was not measured in the poly(ethylene glycol) pellets of the current study but in avidin-Sepharose purified ACC it was negligible and did not vary independently of the kinetic parameters. It has been suggested that avidin-Sepharose purification may select against certain pools of ACC, particularly the citrate-independent polymeric form (Borthwick et al, 1987). These data suggests that the re-activation of hepatic ACC in chow re-feeding may consist of a number of components, including early dephosphorylation and activation, accompanied or proceded by polymerisation followed by increased ACC concentration.

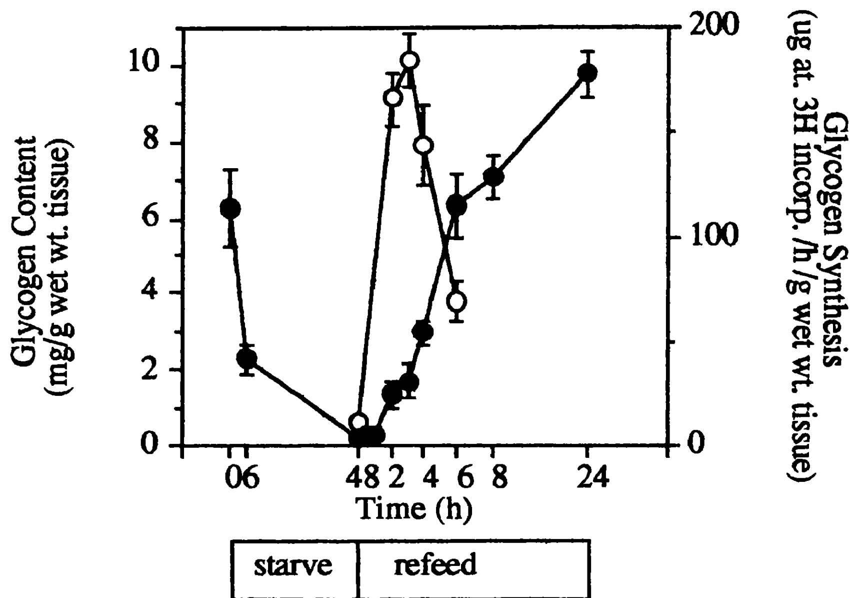
4.7.1 Hepatic Glycogen Content and Glycogen Synthesis

There was a sharp drop (67%) in liver glycogen content in the first 6 hours of starvation, followed by a further drop of 30% over the next 42 hours of starvation (Fig. 4.11). There was a steady increase in glycogen content resulting in a return to fed control levels by 6-hour refeeding. This was followed by a further increase so that after 24 hours refeeding glycogen content was some 70% higher than that of the fed controls. There was a very dramatic stimulation of the rate of glycogen synthesis in the first 2-hours of refeeding (Fig. 4.11). This rate however, started to drop within 4 hours of refeeding, and had dropped by more than half after 6 hour refeeding, compared to the maximal rate.

4.7.2 Hepatic cAMP-PK Activity

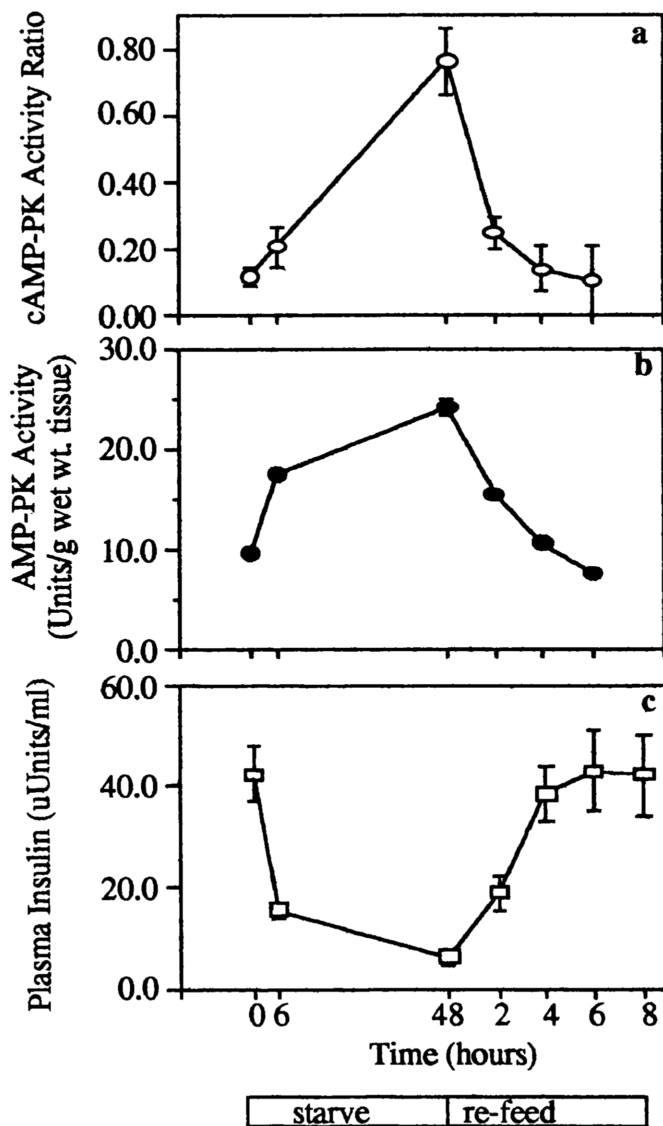
It has been suggested that cAMP-PK is somehow involved in the phosphorylation cascade that mediates the activation of AMP-PK by glucagon (Hardie 1989,). In the present study however, as the activities of cAMP-PK and AMP-PK did not increase in parallel (Fig. 4.12a, b) it is not possible to confirm this putative role. However, such a role is not necessarily excluded by the current data, since apparently insignificant increases in cAMP concentration in hepatocytes can give rise to 90% of the maximal increase in phosphorylase a activity (Blackmore and Exton, 1986). There was a small but insignificant rise in cAMP-PK activity ratio over the first 6 hours of starvation (Fig. 4.12a), but over this time period AMP-PK activity increased 75%

Fig 4.11 Hepatic Glycogen Content and Rates of Synthesis



The glycogen content (closed circles) and the rate of glycogen synthesis (open circles) were measured as described in chapter 2. Each point represents the mean of at least four observations with S.E.M shown as vertical bars.

Fig. 4.12 Hepatic cAMP-PK and AMP-PK Activity and Plasma Insulin Levels in Virgin Rats



(a) The rate of 32P incorporation into kemptide by cAMP-PK was measured in extracts of freeze clamped liver, in the presence and absence of cAMP as described in chapter 2. The ratio of activity in the absence to presence of cAMP is shown at each time point of starvation and refeeding. (b) The activity of AMP-PK was measured in extracts of freeze clamped liver, using the synthetic peptide SAMS as substrate as described in chapter 2. (c) Arterial plasma insulin concentration was measured as described in chapter 2. For each panel in the above figure every point represents the mean of at least four observations with S.E.M indicated by the vertical bars.

(Fig.4.12b) and purified ACC was inactivated (Fig. 4.10). Following 48 hour starvation, the activity ratio of cAMP-PK had significantly increased by 7-fold (Fig. 4.12a).

The delayed response of cAMP-PK to starvation is in keeping with the reported gradual increase in hepatic cAMP concentrations (14% after 6 hour starvation, 90% after 48 hour starvation) (Seitz et al, 1977). However, it is in contrast not only with the rapid activation of AMP-PK but also the rapid depletion of hepatic glycogen (Fig. 4.11). It has been postulated that a continuous cycle of glycogen synthesis and breakdown operates in the livers of fed rats (Schulman et al, 1988). Based on the data presented in this current study it would appear to suggest that the fall in hepatic glycogen content in the first 6 hours of starvation may be due to a decrease in insulin-stimulated glycogen synthesis rather than an increase of cAMP-stimulated glycogenolysis. The fall in plasma insulin concentration in response to 6 hour starvation supports this (Fig. 4.12c).

When 48 hour starved rats were refed chow, the activity of cAMP-PK decreased rapidly and had reached fed control levels within 4 hours (Fig. 4.12a). This correlated with the rapid rise in the rate of glycogen synthesis which increased 10-fold within the first 1 hour of refeeding (Fig. 4.11) and with increased plasma insulin concentration (Fig. 4.12c). It has recently been reported that an insulin stimulated protein kinase in skeletal muscle activates type-I phosphatase by phosphorylating a specific serine residue on its regulatory subunit. This results in the promotion of glycogen synthesis by dephosphorylation and activation of glycogen synthase and

dephosphorylation and inactivation of phosphorylase kinase (Dent et al, 1990). Furthermore it is reasonable to suppose that insulin deactivated cAMP-PK through its stimulation of insulin-sensitive cAMP phosphodiesterase (Wilson et al, 1983), however it is not clear whether cAMP-PK deactivation as well as stimulation of glycogen synthesis by insulin is necessary for the replenishment of hepatic glycogen stores.

4.7.3 Hepatic AMP-PK Activity

There are previous reports (Sim and Hardie, 1988) which suggest that AMP-PK is the protein kinase responsible for the glucagon-stimulated phosphorylation and inactivation of ACC in isolated hepatocytes. It can be seen (Fig. 4.12b) that the activity of AMP-PK increased by 75% in response to 6 hour starvation and by 140% following 48 hour starvation. AMP-PK activity exhibited a rapid decrease upon refeeding which was significant within 2 hours and had returned to fed control levels by 4 hours (Fig. 4.12b). These rapid changes in AMP-PK activity clearly occurred in parallel with the rapid changes in activity of purified ACC demonstrating a remarkable inverse correlation with the pattern of changes in ACC V_{max} (Fig. 4.10a). These data provide yet further support for the physiological role of AMP-PK in regulating ACC activity in vivo.

The changes in AMP-PK activity described are unlikely to be due directly to changes in cellular AMP concentration, since they survive a 500-fold dilution into the assay. The changes in AMP-PK activity are more likely to be due to changes in the

phosphorylation and activation state of AMP-PK, the concentration of AMP-PK or the concentration or activity of one or more inhibitors of AMP-PK within the cell.

4.7.4 Plasma Insulin and AMP-PK Activity

Changes in the plasma insulin concentration are shown in Fig. 4.12c. It can be seen that there was a drop of approximately 65% in the insulin concentration after 6 hour starvation. There was a further drop over the next 42 hours giving a total drop of approximately 90% in the insulin concentration after 48 hour starvation. The insulin concentration returned to 48% of its fed control level after 2 hours refeeding, and by 4 hours of refeeding had returned to virtually the fed control levels, and then remained constant up until the last time point assayed - 8 hours refeeding.

Figure. 4.12 shows that while changes in AMP-PK activity in response to starvation and refeeding do not always closely parallel changes in cAMP-PK activity, they do closely and inversely correlate with changes in plasma insulin concentration. The profiles of AMP-PK activity and plasma insulin (Fig. 4.12b, c) are almost superimposable mirror images. This would infer that insulin rather than glucagon may regulate AMP-PK activity in rat liver in vivo and this is probably achieved via insulin mediated changes in the phosphorylation state of the AMP-PK (although rapid changes in AMP-PK concentration are also a possible mechanism of regulation). However, the profile of changes in insulin concentration is also directly superimposable on the profile of changes in ACC activity, therefore it is possible that insulin may stimulate

a phosphatase that directly antagonises the phosphorylation of ACC by AMP-PK.

4.8 AMP-PK Activity and ACC Activity in Starvation

As can be seen from the above discussion the activity of ACC is an important control point in regulating fatty acid synthesis in both the lactating rat mammary gland and rat liver. The speed of changes in ACC activity differs between these tissues. In the lactating mammary gland it is not inactivated until after 6-hours of starvation whereas in the liver it is inactivated within 6-hour starvation (compare Fig. 4.4 and Fig.4.10). In the mammary gland inactivation of ACC in the latter stages of starvation may be a 'locking down' step after the inactivation of PDH. In the liver ACC appears to be the primary target enzyme in the inhibition of lipogenesis, during starvation. In starvation it would appear that an important component in the inhibition of ACC is reversible phosphorylation. The protein kinase that is thought to be responsible for the in vivo phosphorylation and inactivation of ACC is AMP-PK and the reasons for this are detailed in chapter 1. Evidence has been presented in this chapter that shows a remarkable inverse correlation between changes in AMP-PK activity and ACC V_{max} in both mammary gland and liver (Fig. 4.5, 4.4b and Fig. 4.12b 4.10a). The changes in AMP-PK activity survive through homogenisation and dilution of tissue homogenates. Therefore, changes in AMP-PK activity are likely to be due to phosphorylation of AMP-PK, unless AMP-PK has a short half-life and changes in its activity are achieved via changes in AMP-PK concentration. Hepatic AMP-PK has been shown to be phosphorylated and activated by a protein kinase, the "kinase kinase" or AMP-PK kinase (Carling et al, 1987). The existence of a similar

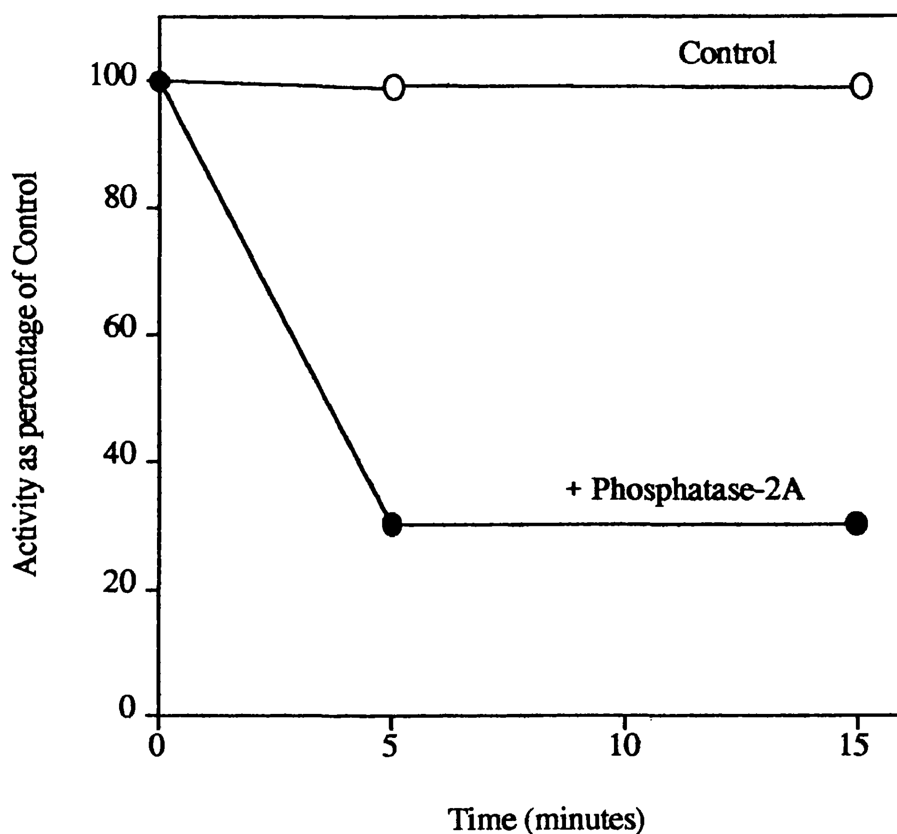
regulatory phosphorylation mechanism for the mammary gland AMP-PK was investigated as was the identity of the putative AMP-PK kinase.

4.8.1 Loss of AMP-PK activity upon Dephosphorylation

The inclusion of phosphatase inhibitors, pyrophosphate and fluoride in buffers was essential for the purification of active AMP-PK, their exclusion resulted in a preparation of AMP-PK of low specific activity. When partially purified lactating rat mammary gland AMP-PK (purified up to the Mono-Q step) was incubated with protein phosphatase 2A there was a rapid inactivation of the AMP-PK, with the activity falling to 30% of the initial activity in 5 minutes (Fig. 4.13). The control incubation lacking phosphatase showed no significant loss of AMP-PK activity (Fig. 4.13). These observations are in agreement with those reported by Carling et al, (1987), who also showed that incubation of AMP-PK from rat liver with purified protein phosphatase resulted in its inactivation and that phosphatase inhibitors, pyrophosphate and fluoride, were required in hepatic AMP-PK purification.

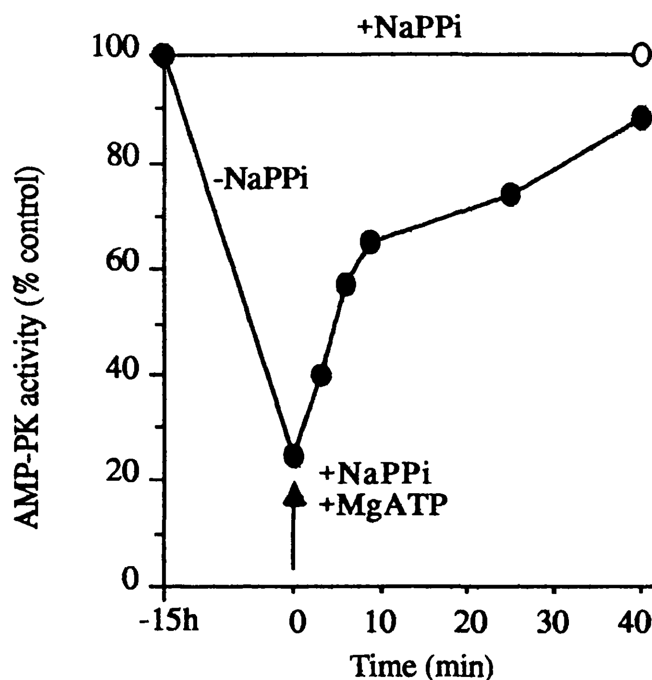
It was found that if a partially purified preparation of lactating rat mammary gland AMP-PK (purified up to the DEAE-Sepharose step, the "DEAE fraction") was dialysed into buffer that was pyrophosphate free, that there was inactivation of AMP-PK probably as a result of the activity of endogenous phosphatases (Fig. 4.14). This was further confirmed by the observation that AMP-PK that had been prepared and stored in the presence of pyrophosphate showed no loss of kinase activity (Fig. 4.14).

Fig. 4.13 Loss of AMP-PK Activity Upon Dephosphorylation



AMP-PK was purified from lactating rat mammary gland (as far as the Mono-Q step as described in chapter 2) and was incubated with protein phosphatase-2A. The control incubation lacked protein phosphatase. At the time points indicated aliquots were removed from each incubation mixture and assayed for AMP-PK activity in the presence of phosphatase inhibitors, pyrophosphate and fluoride, using the synthetic peptide SAMS as substrate as described in chapter 2.

Fig. 4. 14 Inactivation/Reactivation of Mammary Gland AMP-PK in a Post DEAE Fraction



AMP-PK was purified, in the presence of phosphatase inhibitors, from lactating rat mammary gland as far as the DEAE-Sepharose step, as described in chapter 2, but was allowed to dialyse for 15 hours into pyrophosphate free buffer. After this time the AMP-PK was incubated with MgATP in the presence of pyrophosphate. AMP-PK activity was determined during each stage of the experiment using the synthetic peptide substrate SAMS as described in chapter 2. The results are expressed relative to the control incubation i.e AMP-PK prepared and stored in the presence of pyrophosphate (5mM).

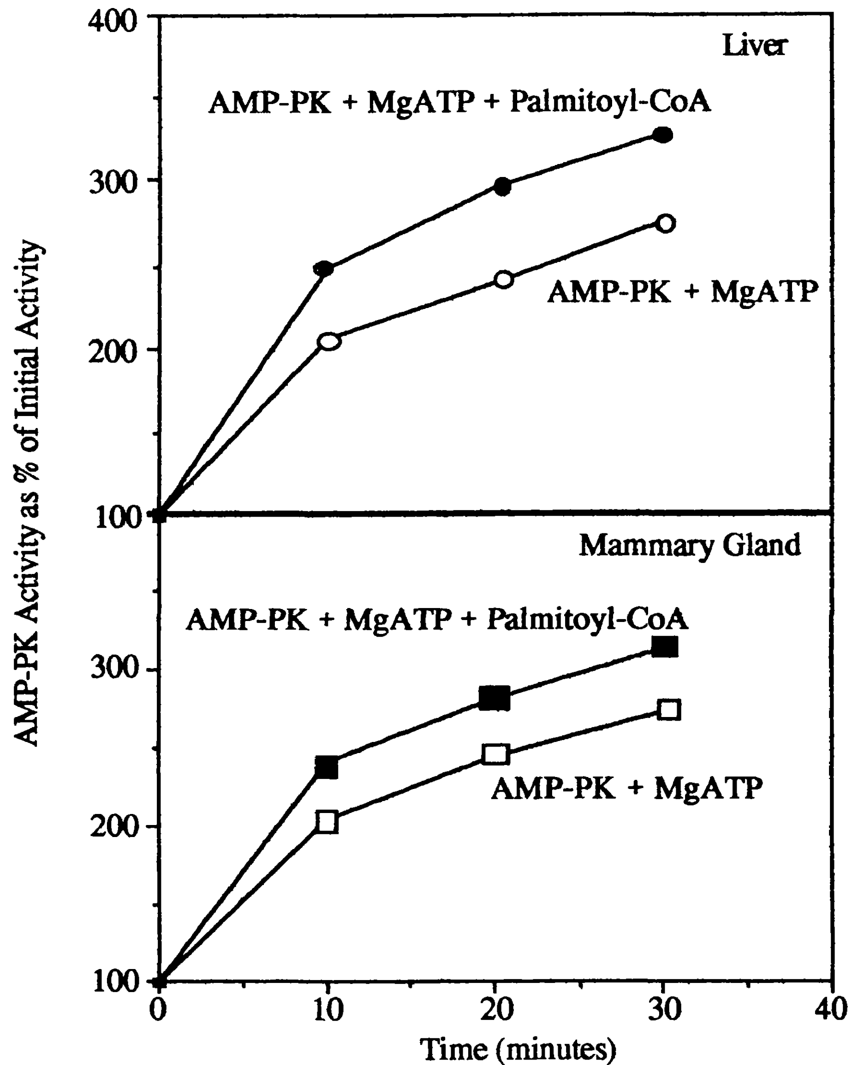
AMP-PK that had been inactivated through dephosphorylation (by dialysis into pyrophosphate free buffer) was incubated with MgATP (in the presence of pyrophosphate) and showed a time-dependent restoration of kinase activity (Fig. 4.14).

When inactive mammary gland or hepatic AMP-PK purified up to the DEAE-Sepharose step (inactivated by treatment with protein phosphatase-2A) was incubated with MgATP there was a time-dependent reactivation of AMP-PK (Fig. 4.15). These results are in agreement with those reported by Carling et al, (1987). It was also found that this MgATP dependent reactivation of AMP-PK was stimulated if 50nM palmitoyl-CoA (a C-16 fatty acyl CoA) was included in the incubation (Fig. 4.15). There was an approximate 30% stimulation of the reactivation of AMP-PK by MgATP in the DEAE fraction when palmitoyl CoA was present and this stimulation was observed with both hepatic and mammary gland AMP-PK (Fig. 4.15). Palmitoyl CoA did not have any effect on AMP-PK activity if MgATP was excluded from the preincubation.

This phosphorylation and activation may be an autophosphorylation reaction, or it may involve another protein kinase.

Incubation of a more highly purified preparation of mammary gland AMP-PK (purified beyond the DEAE-Sepharose step) with MgATP did not result in any reactivation whatsoever of AMP-PK activity (Fig. 4.16). This AMP-PK had been previously dephosphorylated and inactivated by phosphatase-2A. Therefore this result

Fig. 4.15 Reactivation of Dephosphorylated Mammary Gland and Liver AMP-PK by MgATP



The MgATP-dependent reactivation of dephosphorylated AMP-PK in the presence and absence of 50nM palmitoyl-CoA is shown above for both liver and mammary gland AMP-PK. AMP-PK was purified as far as the DEAE-Sepharose step (see Chapter 2) in the presence of phosphatase inhibitors which were then removed by dialysis. AMP-PK was dephosphorylated and inactivated by incubation with the purified catalytic subunit of protein phosphatase-2A. The inactive AMP-PK was then incubated with MgATP in the presence and absence of 50nM palmitoyl-CoA in the presence of pyrophosphate and fluoride, phosphatase inhibitors, AMP-PK activity was assayed in diluted aliquots of the incubation at time points extending over 30 minutes using the peptide SAMS as substrate as described in Chapter 2. The results are expressed as a percentage of the initial activity at time zero and are representative of 5 experiments.

would tend to suggest that the reactivation of AMP-PK is not through an autophosphorylation reaction. The reactivation seen in dephosphorylated crude DEAE preparations of AMP-PK must be due to the presence of contaminating AMP-PK kinase and this AMP-PK kinase does not copurify with AMP-PK through the latter chromatographic steps of AMP-PK purification. These results are in agreement with the those reported for rat liver by Carling et al (1987).

4.9 Is cAMP-PK the AMP-PK kinase ?

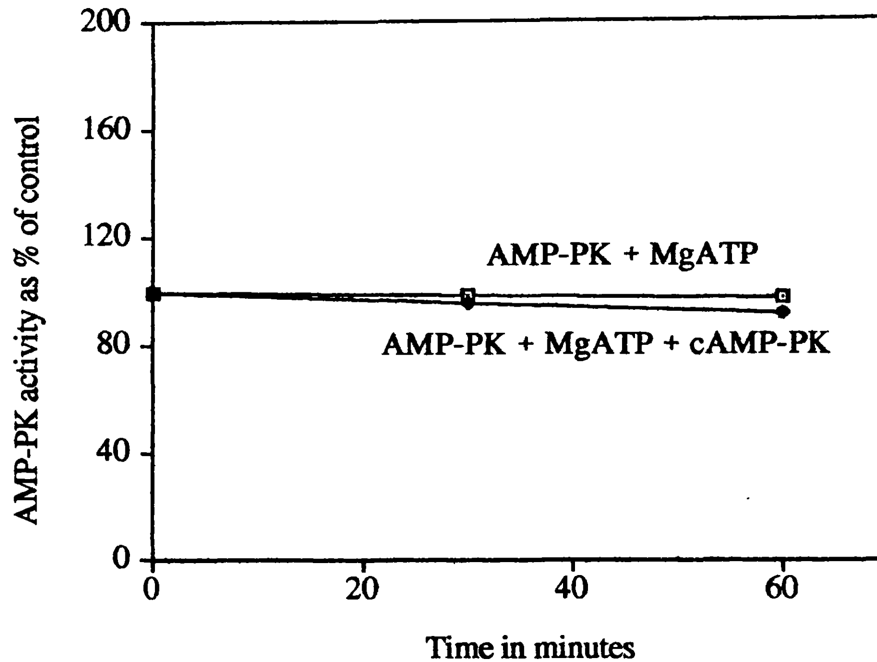
This raises the question of the identity of the AMP-PK kinase. ACC is phosphorylated and inactivated in response to glucagon in hepatocytes (Holland et al, 1984) and glucagon and adrenaline in adipocytes (Holland et al, 1985). A well known effect of glucagon is its elevation of cellular cAMP levels and this occurs in hepatocytes. Since the major intracellular receptor for cAMP is the cAMP-PK and binding of cAMP results in activation of cAMP-PK (Chapter 1) it is an attractive possibility that cAMP-PK may phosphorylate and activate AMP-PK. Also, Lent and Kim (1983) have reported the activation by cAMP-PK of a hepatic ACC kinase that may be AMP-PK. Furthermore Haystead et al (1990) have shown evidence that indicates cAMP-PK is involved in the signal transduction pathway (see section 4.1), although clearly it does not directly phosphorylate ACC. However, in the current study there was no reactivation of dephosphorylated liver or mammary gland AMP-PK (purified by DEAE-Sepharose and Mono-Q chromatography) by mammary gland cAMP-PK holoenzyme (activated by 10μM cAMP) in the presence of MgATP. There

was also no reactivation of mammary gland AMP-PK using a homogenous preparation of the catalytic subunit of cAMP-PK purified from bovine heart (Fig 4.16). Therefore it would appear very unlikely that cAMP-PK is the AMP-PK kinase. This finding is in agreement with results previously published (Sim and Hardie, 1988)), that show no reactivation of liver AMP-PK with bovine heart cAMP-PK, Protein Kinase C or the Calcium/Calmodulin-dependent multiprotein kinase.

4.10 Reactivation of AMP-PK with Acetyl CoA-Carboxylase Kinase 2 (ACK2)

ACK-2 is an ACC kinase that was originally identified and partially purified from lactating rat mammary gland (Munday and Hardie, 1984, and Chapter 1). Although it phosphorylates ACC in vitro and causes an increase in the K_a citrate, ACK-2 produces only a modest 20% inhibition of V_{max} (Munday et al, 1988). This is far below the inhibitions of V_{max} observed in vivo (see Fig. 4.4 and 4.10) thus ACK-2 is unlikely to be the physiological protein kinase that regulates ACC in vivo. The physiological role of this protein kinase is unclear and its real physiological substrates are as yet not clearly defined. It was therefore decided to test ACK-2 for any ability to reactivate AMP-PK. It has been shown (Munday et al, 1988) that ACK-2 phosphorylates within the TC1 peptide which contains the 'SSMS' sequence. On the basis that it produces only a modest decrease in the V_{max} it is probably phosphorylating serine-77. Therefore a synthetic peptide substrate containing this sequence was synthesised and ACK-2 was identified and purified on the basis of it being a 'SSMS' peptide kinase.

Fig.4. 16 Reactivation of AMP-PK is not due to Autophosphorylation or cAMP-PK



AMP-PK was purified from lactating rat mammary gland as far as the Mono-Q step (for details see chapter 2) in the presence of phosphatase inhibitors which were then removed by dialysis. The AMP-PK was then dephosphorylated and inactivated by incubation with protein phosphatase-2A. Then AMP-PK was incubated with MgATP and phosphatase inhibitors in the presence and absence of the catalytic subunit of cAMP-PK (purified from bovine heart, as described in chapter 2). Diluted aliquots of the incubation reactions were removed from each incubation and assayed for AMP-PK activity using the synthetic peptide SAMS as substrate as described in chapter 2

4.11 Purification of Acetyl CoA Carboxylase Kinase-2 (ACK-2)

The preparation described by Munday and Hardie (1984) was relatively crude and likely to be contaminated by other protein kinases therefore further purification of ACK-2 was necessary.

Lactating Wistar rats were killed by stunning and cervical dislocation. The mammary glands were rapidly dissected out and rinsed in ice-cold homogenisation buffer (0.25M sucrose, 100mM Tris HCl pH 7.5 at 4°C, 50mM NaF, 1mM EDTA, 1mM BZ, 4ug/ml SBTI). The glands were finely minced with scissors and then homogenised in cold homogenisation buffer using a domestic kitchen blender (three bursts of 30 seconds with 1 minute on ice in between each burst).

Polyethylene glycol 6000 (PEG 6000) was then added to the homogenate to give a final concentration of PEG of 2.5% (w/v). The homogenate was stirred for 10 minutes before being centrifuged at 30,000g for 20 minutes at 4°C. The supernatant was poured through a plug of glass wool to removed floating fat and other debris.

To this supernatant further PEG 6000 was added to give a final concentration of PEG of 6% (w/v). The suspension was spun at 30,000g for 20 minutes at 4°C. The supernatant was again filtered through glass wool and PEG 6000 was added to take the final concentration of PEG to 20% (w/v). This suspension was then centrifuged at 30,000g for 20 minutes at 4°C. The supernatant was removed and discarded and the 20% PEG pellet containing amongst other enzymes, cAMP-PK catalytic subunit,

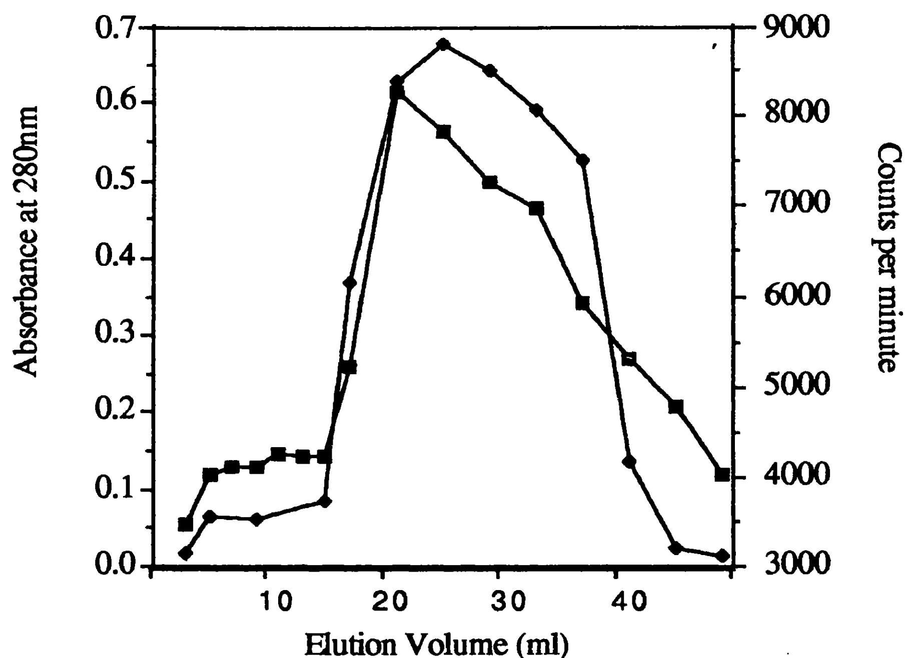
Casein Kinases I and II and ACK-2 was stored at -20°C before being used for the purification of ACK-2.

The 20% PEG pellet was resuspended in as small a volume of column buffer (50mM Tris HCl pH 7.2 at 4°C, 50mM NaF, 10% (w/v) glycerol, 1mM EDTA) as possible, this typically was 1/5th of the volume of supernatant from which the 20% pellet had been prepared. This resuspended pellet was then centrifuged at 5000 g for 10 minutes at 4°C to bring down any material that had not resuspended.

Separation on Phosphocellulose

The resuspended 20% PEG pellet was loaded onto a phosphocellulose (P11, Whatman) column which had previously been equilibrated in column buffer. The phosphocellulose column was washed with column buffer until no further protein washed off the column (as judged by the absorbance at A_{280} being <0.05). The ACK-2 was then eluted batchwise from the phosphocellulose using buffer containing 100mM NaCl. Fractions were collected and assayed for ACK-2 activity (as described in Materials and Methods) against the 'SSMS' peptide. The kinase peak eluted slightly later than the protein peak and both peaks were quite broad (Fig. 4.17). The fractions showing the greatest kinase activity were pooled and concentrated using an Amicon concentrator with a PM30 membrane and dialysed either into column buffer containing 50% (w/v) glycerol for storage at -20°C, or into column buffer for further purification.

Fig. 4.17 Purification of ACK-2 on Phosphocellulose



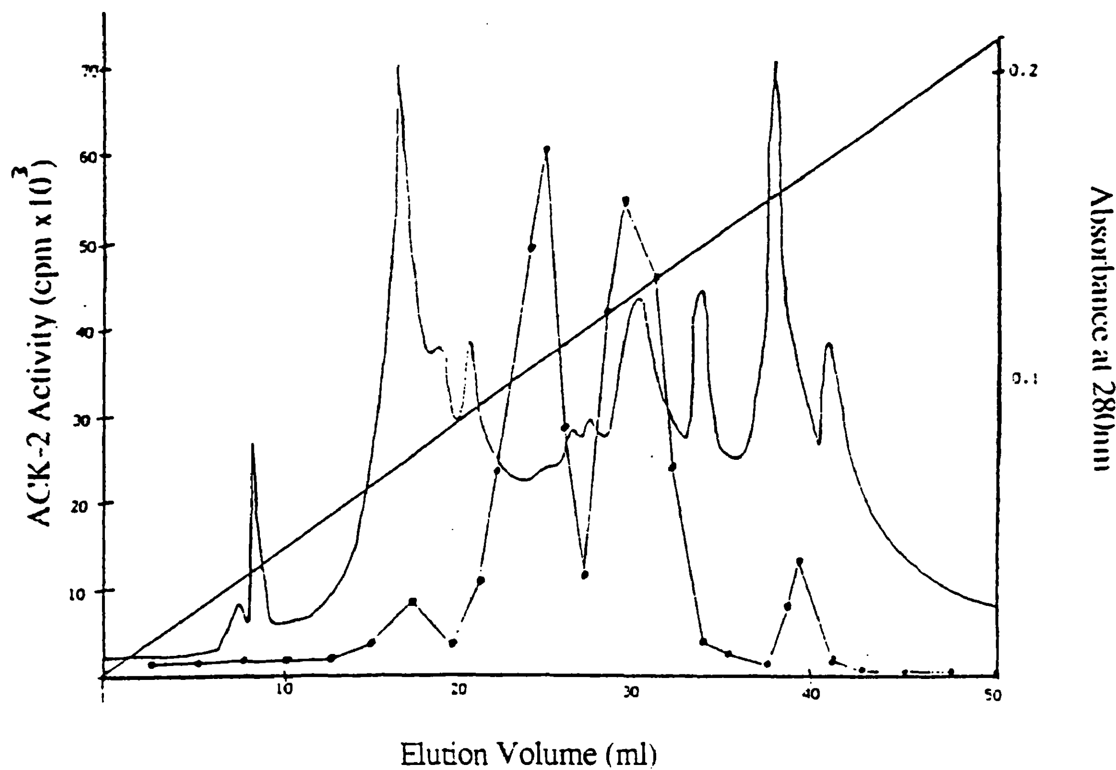
The purification of ACK-2 by phosphocellulose chromatography is shown above. ACK-2 was eluted batchwise as detailed in the text. The absorbance of the eluted protein solution at 280nm (■) and the kinase activity (◆) are shown. ACK-2 activity was measured using the synthetic peptide SSMS as substrate as described in chapter 2.

Separation on Mono-Q

The pooled peak from the phosphocellulose column was applied to a Mono-Q anion exchange column on FPLC which had previously been equilibrated in column buffer. The column was washed until no further protein eluted and ACK-2 was then eluted from the Mono-Q column using a salt gradient running from 0 to 500mM NaCl. The column was then washed up to 1M NaCl to elute all remaining protein bound to the column. Fractions of 1ml were collected and assayed for ACK-2 activity against the 'SSMS' peptide.

A large number of protein peaks, as judged by absorbance at 280nm were eluted from the Mono-Q column (Fig, 4.18). However, there was no 'SSMS' kinase activity in the breakthrough peak or in the 1M wash. There were two small peaks of 'SSMS' kinase activity eluting at 200mM NaCl and at approximately 500mM NaCl, both of these activity peaks corresponded to large protein peaks. There were two major peaks of 'SSMS' kinase activity eluting at approximately 300mM NaCl and 360mM NaCl, interestingly they did not correspond to particularly large protein peaks. The peak at 300mM NaCl is almost certainly the AMP-activated protein kinase since it was activated by AMP and phosphorylated the 'SAMS' peptide substrate that is specific for AMP-PK. The peak at 360mM NaCl did not phosphorylate the 'SAMS' peptide therefore this peak was tentatively ascribed (using the best criteria available) as ACK-2. The most active fractions around this peak were pooled and concentrated using an Amicon concentrator with a PM30 membrane to a volume of 1ml and dialysed against column buffer containing 50% (w/v) glycerol, and stored at 4°C.

Fig. 4.18 Purification of ACK-2 on Mono-Q



The concentrated peak fractions obtained after phosphocellulose chromatography of ACK-2 were further purified using a Mono-Q column. The above figure shows the purification profile obtained after chromatography of ACK-2 on Mono-Q. The absorbance at 280nm is indicated by the continuous line. The protein kinase activity was measured as the incorporation of 32P from [γ^{32}P]ATP into the synthetic peptide substrate SSMS as described in chapter 2 and is indicated by the full circles.

4.11.1 Purification Table for ACK-2

Table 4.1 shows the effectiveness of the procedures utilised for the purification of ACK-2. The ACK-2 was purified some 2100 fold. The 20% PEG pellet step, resulted in a 3-fold purification, with the specific activity of the enzyme increasing from 0.027 U/mg to 0.078 U/mg and the total protein decreasing from 10,000 mg to 1110 mg. A significant purification was achieved using the first column, phosphocellulose, which probably acts as an affinity column since it seems to bind all protein kinases. This gave almost a 700-fold purification, with the specific activity increasing from 0.078 U/mg to 18.7 U/mg, the total protein decreasing to 5.2 mg and the volume of the peak to 4mls. Thus the bulk of protein loaded did not bind to the column giving a significant purification. The Mono-Q anion exchange column also resulted in a large purification of the ACK-2. The specific activity increased from 18.7 U/mg to 57.2 U/mg giving a total purification of over 2100-fold. However there is only 0.3mg of total protein remaining at this stage, in a volume of approximately 0.5ml. From the Mono-Q profile it is clear that there are two major peaks of 'SSMS' kinase activity one of which is 'SAMS' kinase activity i.e. AMP-PK. Therefore it is very possible that the true specific activity of ACK-2 in the first three steps of the purification is an overestimation and the final purification value on Mono-Q is an underestimation, any cAMP-PK phosphorylation of the 'SSMS' peptide will be inhibited by the inclusion of the specific peptide inhibitor of cAMP-PK.

Table 4.1 Partial Purification of ACK-2 from Lactating Rat Mammary Gland

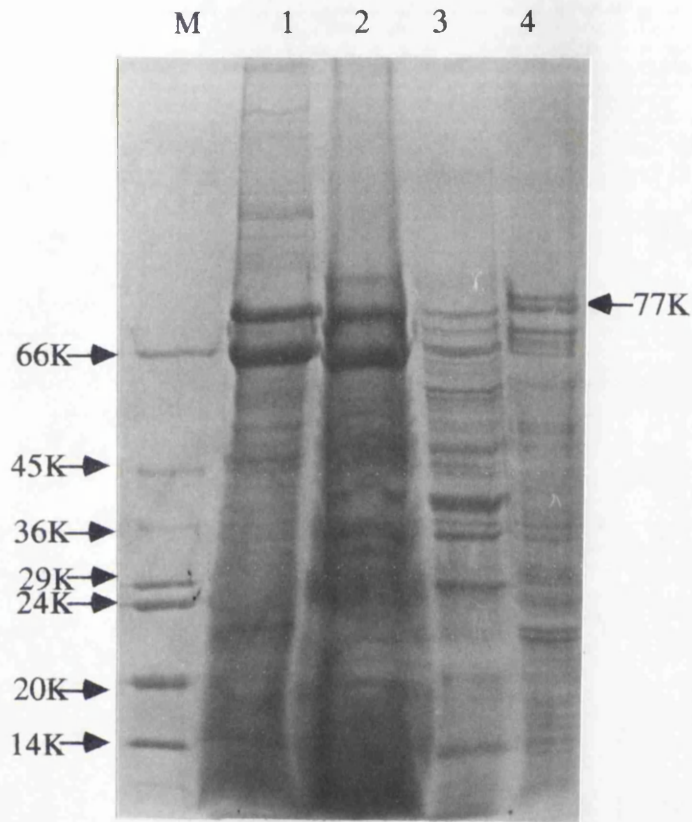
This table shows a summary of the procedures used in the purification of ACK-2. ACK-2 activity was measured by the incorporation of ^{32}P from [$\gamma^{32}P$] ATP into the synthetic peptide substrate 'SSMS' as described in Chapter 2. 1 unit of ACK-2 activity is defined as that amount of protein kinase that transfers 1 nmol P/min into the peptide substrate.

	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Purification -fold	% Yield
Homogenate	302	10068	0.03	1	100
20% PEG pellet	90	1119	0.08	3	30
Phosphocellulose	79	4.2	18.7	690	26
Mono-Q	17	0.3	57.2	2110	5

4.11.2 Analysis of ACK-2 purification by SDS-Polyacrylamide Gel Electrophoresis

Samples of the purified protein kinase from each step were dissolved in SDS-sample buffer, boiled and separated on a 7.5 - 15% polyacrylamide gel. The PEG precipitation step in the purification removes a few protein species, particularly those with a high molecular weight, as can be seen when lane 1 (crude homogenate) is compared to lane 2 (Fig. 4.19). There are two predominant bands at approximately 63 kDa and 77 kDa. Lane 3 shows the post-phosphocellulose purified ACK-2. Here it is possible to see once again the bands at 63 kDa and 77 kDa (Fig. 4.19). There are a number of protein bands present in the range running from approximately 37 kDa - 45 kDa one of which may possibly be the catalytic subunit of cAMP-PK, this generally has a M_r of 41 kDa. In lane 4 relevant to lane 3 there is a reduction in the intensity of the band at 63 kDa relative to the other bands and this therefore probably correlates with the resolution of ACK-2 from AMP-PK shown on the column profile (Fig. 4.18). Munday and Hardie, (1984) have reported that ACK-2 has a M_r of 76 kDa as determined by gel filtration.

Fig. 4. 19 SDS-Gel of ACK-2 Purification



ACK-2 was purified from lactating rat mammary gland using a combination of polyethyleneglycol precipitation and ion-exchange chromatography, for details of the purification see text. Each stage of the purification was analysed by SDS-PAGE as described in chapter 2 and the results are shown. Lane 1 is the crude homogenate prepared from mammary gland, lane 2 shows the resuspended 20% polyethyleneglycol pellet, lane 3 shows the phosphocellulose purified ACK-2 and lane 4 shows the Mono-Q purified ACK-2.

4.11.3 Substrate Specificity of ACK-2 Purified From Lactating Rat Mammary Gland

Table 4.2 Substrate Specificity of ACK-2.

The initial rates of phosphorylation of substrates by the post-phosphocellulose preparation of ACK-2 are shown and are expressed relative to the phosphorylation of ACC. The results are representative of those obtained with a number of different preparations of ACK-2 purified from lactating rat mammary gland.

	Substrate Conc. mg/ml	ACK-2
Acetly-CoA carboxylase	0.48	100
ATP-Citrate lyase	0.24	26
Glycogen Synthase	0.17	38
Phosphorylase Kinase	0.67	115
Histone	0.80	131
Casein	2.00	54
Kemptide	0.13	165

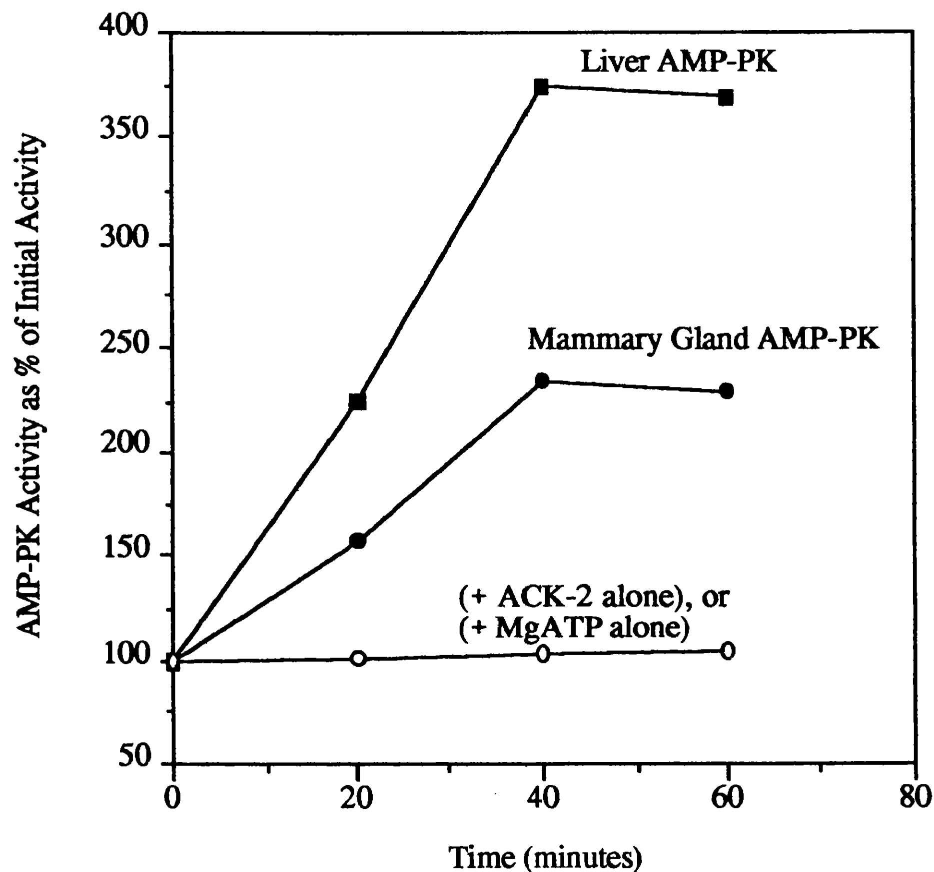
As can be seen ACK-2 does not appear to have any particularly high affinity towards any of the substrates tested, and exhibits a broad based specificity. Amongst its better substrates are acetyl-CoA carboxylase, phosphorylase kinase, histone and kemptide,

all phosphorylating at about the same rate. ATP-Citrate lyase, glycogen synthase and casein show themselves to be poorer substrates with the best of these being casein which is only phosphorylated half as well as ACC. The data demonstrates that ACK-2 shows a lack of specificity with respect to the substrates tested so far. The lack of specificity suggests that ACK-2 may have other more specific substrates especially since ACK-2 is not thought to be the protein kinase responsible for the in vivo inactivation of ACC.

4.12 Is ACK-2 the AMP-PK kinase ?

ACK-2 was purified up to the phosphocellulose step and incubated with dephosphorylated, lactating rat mammary gland AMP-PK (purified beyond the DEAE-Sepharose step and treated with protein phosphatase-2A) in the presence of MgATP. At set time points aliquots were removed from the incubation mixture and assayed for AMP-PK activity against its specific 'SAMS' peptide substrate. There was a reactivation of AMP-PK with a 90% increase in activity after 30 minutes and an eventual 200% increase after 1-hour (Fig. 4.20) this experiment was typical of a number that showed a 1.5 - 2-fold activation. There was no reactivation of AMP-PK activity in the presence of either MgATP or ACK-2 alone. This appears to be very strong evidence that this ACK-2 preparation i.e. the 0.1M NaCl eluate obtained from phosphocellulose chromatography, contains the AMP-PK kinase that can phosphorylate and activate AMP-PK. It is possible that there is contamination of this AMP-PK kinase preparation by cAMP-PK but the inclusion of the specific peptide

Fig. 4. 20. Reactivation of AMP-PK by Phosphocellulose Purified ACK-2

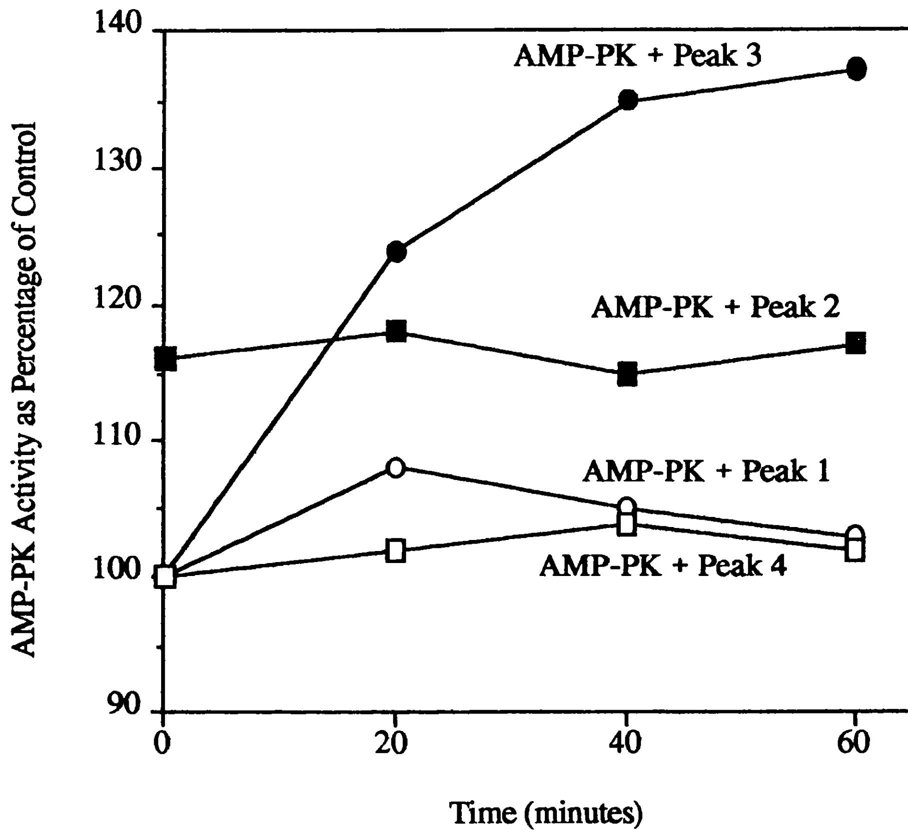


ACK-2 was purified as far as the phosphocellulose step (see text for details) and incubated with MgATP and AMP-PK (purified from either rat liver or lactating rat mammary gland as detailed in chapter 2), in the presence of the specific peptide inhibitor of cAMP-PK. Aliquots were removed from the various incubations at the indicated time points and assayed for AMP-PK activity using the synthetic peptide SAMS as substrate as described in chapter 2. The results are expressed as a percentage of the initial activity at time zero.

inhibitor of cAMP-PK inhibits this protein kinase. The presence of cAMP-PK or ACK-2 in the AMP-PK assay do not represent a problem because the activity of AMP-PK is assayed against its specific peptide substrate 'SAMS' and the other protein kinases will not phosphorylate this peptide. The problem may be that because AMP-PK contaminated the AMP-PK kinase preparation the quantity of AMP-PK being assayed would have been increased. The amount of AMP-PK added to the assay via the AMP-PK kinase preparation, taking account of dilution factors, was very small, and was also present in the control in similar amounts and therefore corrected for. Furthermore, the reactivation of AMP-PK was time-dependent and therefore could not be due merely to the addition of an increased amount of AMP-PK.

In the purification of ACK-2, four peaks of 'SSMS' peptide kinase activity were eluted from the Mono-Q column at approximately 210mM, 300mM, 350mM and 460mM NaCl (Fig. 4.18). The fractions for each peak were pooled, concentrated and tested for their ability to reactivate AMP-PK. For convenience these peaks were numbered according to their order of elution from the Mono-Q column. Lactating rat mammary gland AMP-PK (purified beyond the DEAE-Sepharose step and dephosphorylated by incubation with protein phosphatase-2A) was incubated with peaks 1 - 4 obtained after chromatography of ACK-2 on Mono-Q in the presence and absence of cold MgATP over a time course of 1-hour. At set times aliquots were removed from this incubation and assayed for 'SAMS' peptide phosphorylation with [γ32P] MgATP. Only peak 3 caused a time-dependent stimulation of AMP-PK activity (Fig. 4.21), as assayed against its specific 'SAMS' peptide substrate. Incubation with peak 2 did show higher AMP-PK activity (Fig. 4.21), but this peak

Fig. 4. 21 Reactivation of AMP-PK with Mono-Q Peaks 1-4

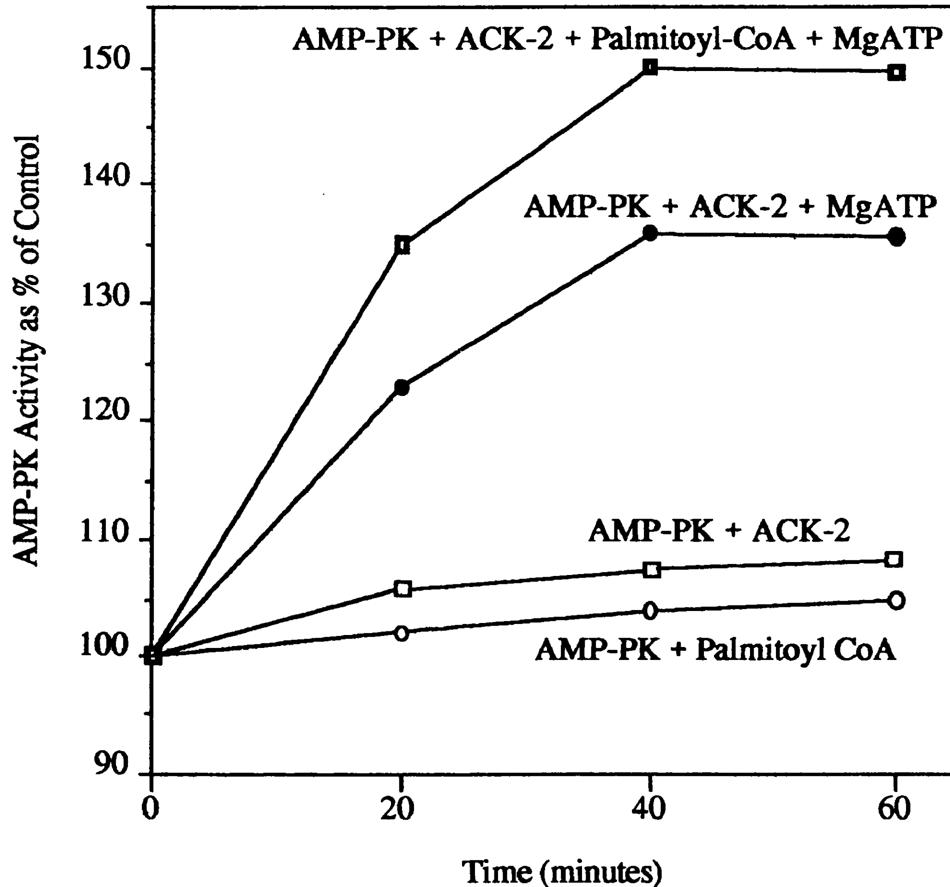


AMP-PK was purified from lactating rat mammary gland up to the Mono-Q step (for purification details see chapter 2) and was incubated with peaks 1-4 obtained after chromatography of ACK-2 on Mono-Q (for details see text) in the presence of MgATP. A control incubation consisting of AMP-PK incubated with MgATP was also performed. At the time points indicated diluted aliquots were removed from each incubation and assayed for AMP-PK activity using the synthetic peptide SAMS as substrate as described in chapter 2. The control incubation showed no reactivation over the time course and AMP-PK activities in the presence of each peak are shown as a percentage of this control activity.

has been identified as AMP-PK therefore incubation with it has simply increased AMP-PK concentration in the experiment. The reactivation of AMP-PK seen with peak 3 once again only occurred in the presence of both of the peak 3 protein and MgATP (Fig. 4.22), suggesting that this reactivation required a phosphorylation of AMP-PK. This is very strong evidence that this 'SSMS' peptide kinase i.e. peak 3, which will now be ascribed as ACK-2, is also the AMP-PK kinase.

Peak 3, obtained from the Mono-Q chromatography of ACK-2, was incubated with dephosphorylated AMP-PK (purified beyond the DEAE-Sepharose step) from both liver and lactating rat mammary gland AMP-PK and cold MgATP in the presence of 200nM palmitoyl-CoA for 1-hour. At set time points over this time course aliquots were removed from this incubation and assayed for AMP-PK activity using the 'SAMS' peptide and [γ32P] MgATP. The inclusion of palmitoyl-CoA stimulated both the rate and the final level of reactivation by ACK-2 of AMP-PK (Fig 4.22) with the final level of reactivation enhanced by approximately 50%. In the absence of ACK-2 and MgATP palmitoyl-CoA did not stimulate AMP-PK activity suggesting no direct effect on the enzyme (Fig 4.22). These observations are in agreement with those made from experiments conducted on the crude DEAE preparation of AMP-PK, where reactivation only ocurred in the presence of MgATP and was stimulated by palmitoyl-CoA in both mammary gland (Fig 4.15) and liver enzyme (Carling et al, (1987).

Fig. 4.22 Reactivation of Mammary Gland AMP-PK with Mono-Q Purified ACK-2 (Peak 3)



AMP-PK was purified from lactating rat mammary gland up to the Mono-Q step (as detailed in chapter 2), dephosphorylated by incubation with protein phosphatase-2A and then incubated with peak 3 obtained after the Mono-Q chromatography of ACK-2 and with MgATP in the presence and absence of 200nM palmitoyl-CoA. Control incubations of AMP-PK in the presence of 200nM palmitoyl-CoA and MgATP and AMP-PK with ACK-2 in the absence of MgATP were also performed. At the time points indicated diluted aliquots were removed from each incubation and assayed for AMP-PK activity using the synthetic peptide SAMS as substrate as described in chapter 2.

4.12.1 The Effect of Palmitoyl-CoA on the Activity of ACK-2

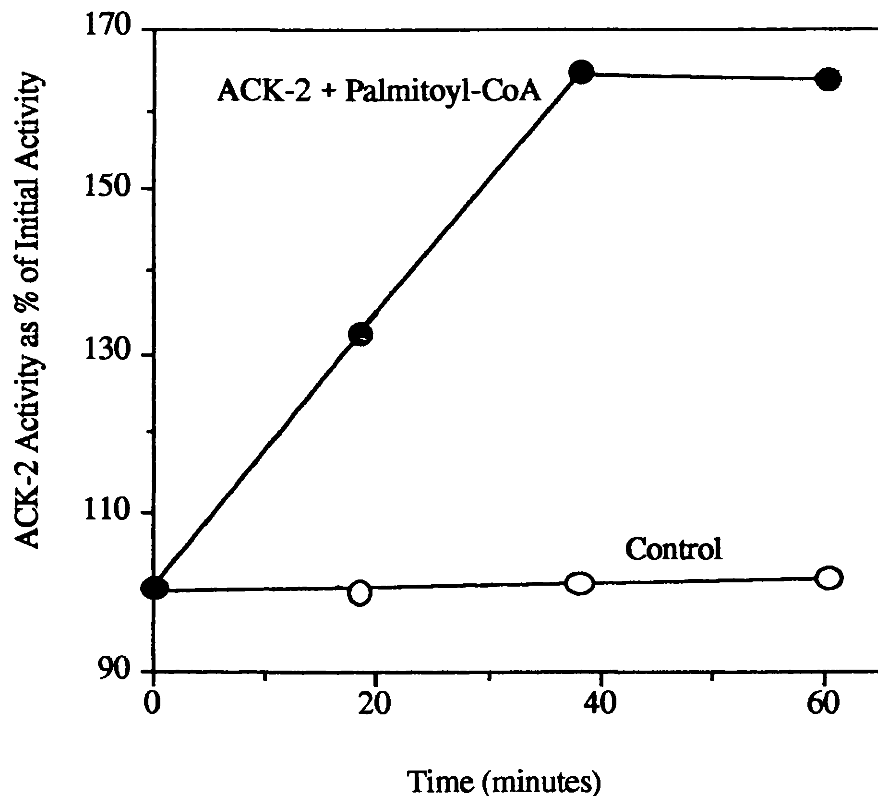
The availability of a peptide assay for ACK-2 enabled the investigation of whether the palmitoyl-CoA stimulation was a substrate (AMP-PK)-directed effect or a direct effect on ACK-2.

Peak 3, obtained from the Mono-Q chromatography of ACK-2, was incubated with cold MgATP in the presence and absence of 200nM palmitoyl-CoA. At set time points aliquots were removed from this incubation and assayed for ACK-2 activity against the 'SSMS' peptide in the presence of the specific peptide inhibitor of cAMP-PK and [γ32P] MgATP. It was found that palmitoyl-CoA caused typically a 1.5 - 2 fold stimulation of ACK-2 activity (Fig. 4.23). This is good evidence that ACK-2 is the AMP-PK kinase, since palmitoyl-CoA not only stimulates its reactivation of AMP-PK but also its phosphorylation of the synthetic 'SSMS' peptide. Furthermore, it shows that palmitoyl-CoA causes a direct stimulation of ACK-2 rather than a substrate directed effect via AMP-PK.

4.13 AMP Stimulated Reactivation of AMP-PK

The AMP-PK is stimulated in in vitro assays by micromolar concentrations of the nucleotide 5' AMP (Chapter 1). This fact is made use of in assays where it is desirable to have high AMP-PK activity since AMP can give a 4-fold stimulation of AMP-PK activity. The question is whether AMP is a physiological regulator of AMP-PK in vivo, or at least one of the potential regulatory mechanisms. In a

Fig. 4. 23 Stimulation of ACK-2 Activity by Palmitoyl-CoA



ACK-2 was purified as far as the Mono-Q step (see text for details) and incubated with 200nM palmitoyl-CoA. The control incubation lacked palmitoyl-CoA. At the time points indicated aliquots were removed from each incubation and assayed for ACK-2 activity using the synthetic peptide SSMS as substrate as described in chapter 2. The results are expressed as a percentage of the initial activity at time zero.

physiological situation such as 24-hour starvation when it is clear that AMP-PK is activated (Fig. 4.5 and Fig. 4.12b) measurements of AMP concentrations suggest that there is no change in the total intracellular concentration of this nucleotide during starvation (Hardie, 1989). However it is difficult from such parameters to estimate the changes in free intracellular AMP concentrations that would be experienced by AMP-PK due to the presence of AMP binding proteins. It is possible that only minor changes in the intracellular AMP concentration may be required to effect changes in the expressed AMP-PK activity. However, in vivo changes in AMP-PK survive homogenisation and dilution suggesting that there is covalent modification of AMP-PK. Moore et al (1991) report that raising intracellular AMP concentrations with fructose or anoxia leads to a stable activation of AMP-PK. If this was an allosteric effect it would be expected to be reversed by homogenisation and dilution into the AMP-PK assay (unless there is an unusually strong binding) therefore it is possible that AMP can stimulate the AMP-PK reactivation reaction and Moore et al (1991) claim that this may be a mechanism of regulating AMP-PK.

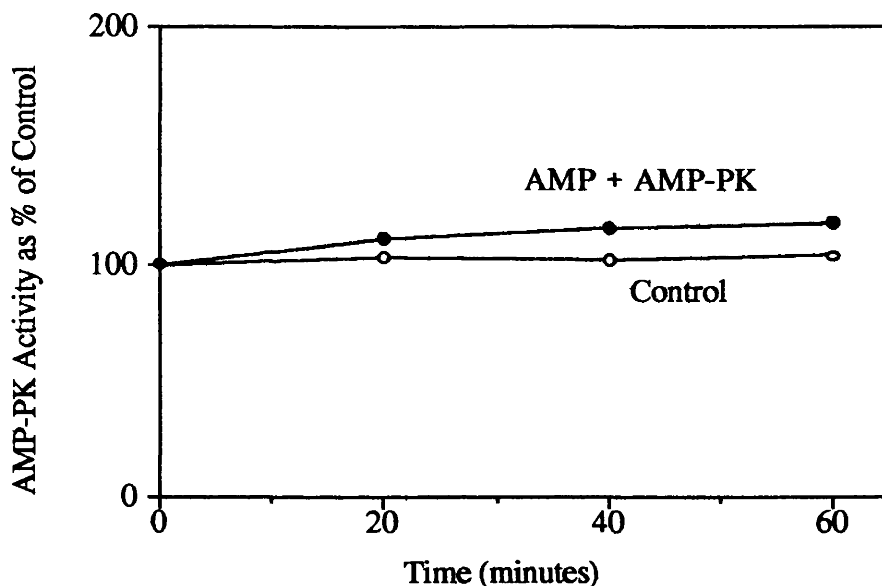
In order to see if there was any stimulation of AMP-PK reactivation by AMP, it was decided to test for this in vitro. Lactating rat mammary gland AMP-PK (purified beyond the DEAE-Sepharose step) was incubated with peak 3 (from the Mono-Q chromatography of ACK-2), cold MgATP in the presence and absence of AMP with the latter at a final concentration of 1mM. At set time points aliquots were removed from the incubation and assayed for AMP-PK activity against the specific 'SAMS' peptide using [γ32P] MgATP. There was no stimulation of the reactivation of AMP-PK by ACK-2 at this concentration of AMP. It may be that the lack of stimulation

of reactivation was due to the AMP concentration being too low, and this would have been contributed to by any AMP binding proteins that were contaminating the AMP-PK preparation, so the experiment was repeated using a final concentration of AMP of 20mM, which should be saturating even allowing for the presence of any AMP-binding proteins. Even with 20mM AMP there was still no stimulation of the reactivation of AMP-PK activity by ACK-2 (Fig. 4.24). Thus it appears that in vitro there is no stimulation of AMP-PK reactivation by AMP and changes in AMP concentration in vivo may therefore not be the normal physiological mechanism of regulating AMP-PK activity.

4.14 Conclusions

There is a rapid inhibition of fatty acid synthesis in the lactating rat mammary gland during starvation. It appears that the activity of PDH is the most important regulatory step in fatty acid synthesis in the lactating rat mammary gland since its inhibition correlates closely with the inhibition of lipogenesis during starvation. The activity of PDH is regulated by phosphorylation-dephosphorylation. The inhibition of ACC through phosphorylation plays no part in the short term regulation, < 6 hours, of mammary gland lipogenesis. The inhibition which occurs at the later stages of starvation (> 6 hours) may be promoted by free fatty acids reaching a critical level and thus activating AMP-PK via the AMP-PK kinase. The changes in the activity of AMP-PK correlate closely with the changes in ACC activity during starvation and refeeding and is further proof that AMP-PK is the physiological ACC kinase. The

Fig. 4.24 AMP Stimulated Reactivation of AMP-PK



The effect of 20mM AMP on the stimulation of the reactivation of AMP-PK is shown. AMP-PK was purified from lactating rat mammary gland (up to the Mono-Q step as described in chapter 2), dephosphorylated by incubation with protein phosphatase-2A and then incubated with peak 3 (obtained from the Mono-Q chromatography of ACK-2, for details see text) with MgATP in the presence of AMP (20mM). The control incubation was identical except that it lacked AMP. Diluted aliquots from each incubation were then assayed for AMP-PK activity using the synthetic peptide SAMS as substrate as described in chapter 2. The level of reactivation of AMP-PK by ACK-2 achieved in this experiment was actually 34%.

changes in AMP-PK activity also inversely correlate with changes in plasma insulin concentration suggesting a negative regulation of AMP-PK by insulin. cAMP-PK is not the protein kinase that phosphorylates and inactivates ACC in vivo. In the lactating mammary gland there is no change in cAMP-PK activity after 24 hours starvation, at which time there is quite clearly an inhibition of ACC activity.

It would appear reasonable to claim from the work presented in this chapter that whilst hepatic PDH and ACC may be inactivated in parallel in response to starvation, the reactivation of ACC in response to refeeding clearly precedes that of PDH. The major proportion of the ACC inactivation occurs within the first 6 hours following commencement of starvation and is the result of increased phosphorylation in response to a parallel increase in AMP-PK activity. Neither ACC phosphorylation nor AMP-PK activation can be the result of cAMP-PK activation which lags considerably behind the other responses. Furthermore AMP-PK activation more closely correlates with a decrease in plasma insulin concentration. Insulin is an antilipolytic hormone and there is a very close inverse correlation between insulin concentration and free fatty acid concentration. Therefore it is possible that decreases in plasma insulin concentration upon starvation lead to increases in free fatty acid concentration which activate AMP-PK via the AMP-PK kinase.

ACC reactivation in response to refeeding is accompanied by decreased AMP-PK activity and cAMP-PK activity and increased plasma insulin concentrations. The ACC response occurs in at least three phases; the initial rapid phase is apparent as an increased V_{max} and decreased K_a citrate of pure ACC suggestive of enzyme dephosphorylation. The second phase may involve insulin stimulated polymerisation

of the enzyme and the third phase is a longer term increase in the concentration of ACC protein.

It also appears reasonable to conclude from this work that the hepatic glycogen content during the fed to starved to refed transitions is more closely controlled by insulin than by cellular cAMP concentrations and that this is equally true of AMP-PK and ACC activity. If insulin activation of ACC and glycogen synthesis in rat liver are simultaneous on refeeding 48 hour starved rats, then the relative lag between reactivation of hepatic glycogen synthesis and lipogenesis is presumably the result of delayed reactivation of PDH.

AMP-PK activity is definitely regulated through phosphorylation. Dephosphorylation of the purified enzyme with protein phosphatases results in inactivation of the enzyme. Incubation of crude preparations of AMP-PK with MgATP results in reactivation of AMP-PK activity, this reactivation is stimulated by nanomolar concentrations of palmitoyl-CoA. The reactivation of AMP-PK is not through an autophosphorylation reaction.

cAMP-PK is not the AMP-PK kinase, since there is no reactivation of AMP-PK activity upon incubation with both the purified catalytic subunit of cAMP-PK and with catalytic subunit liberated from cAMP-PK holoenzyme by the addition of cAMP.

There was a reactivation of AMP-PK activity by ACK-2 (another ACC kinase). This was a time-dependent reactivation and only occurred in the presence of MgATP

therefore strongly suggesting a phosphorylation reaction being involved in the reactivation of AMP-PK. ACK-2 is therefore a potential candidate for the AMP-PK kinase. ACK-2 was purified on two ion exchange columns and the AMP-PK reactivating activity copurified at each stage. The purified ACK-2 was stimulated by nanomolar concentrations of palmitoyl-CoA, as assayed by its phosphorylation of the SSMS peptide. Also the reactivation of AMP-PK by ACK-2 was stimulated by palmitoyl-CoA, providing further reasons for consideration of ACK-2 as a potential AMP-PK kinase. There is no stimulation of this reactivation of AMP-PK by AMP in vitro, at saturating concentrations of AMP, even though AMP does stimulate AMP-PK activity.

CHAPTER FIVE

Phosphorylation of Apolipoprotein B, the Major Protein Component of Lipoproteins

5.1 Introduction

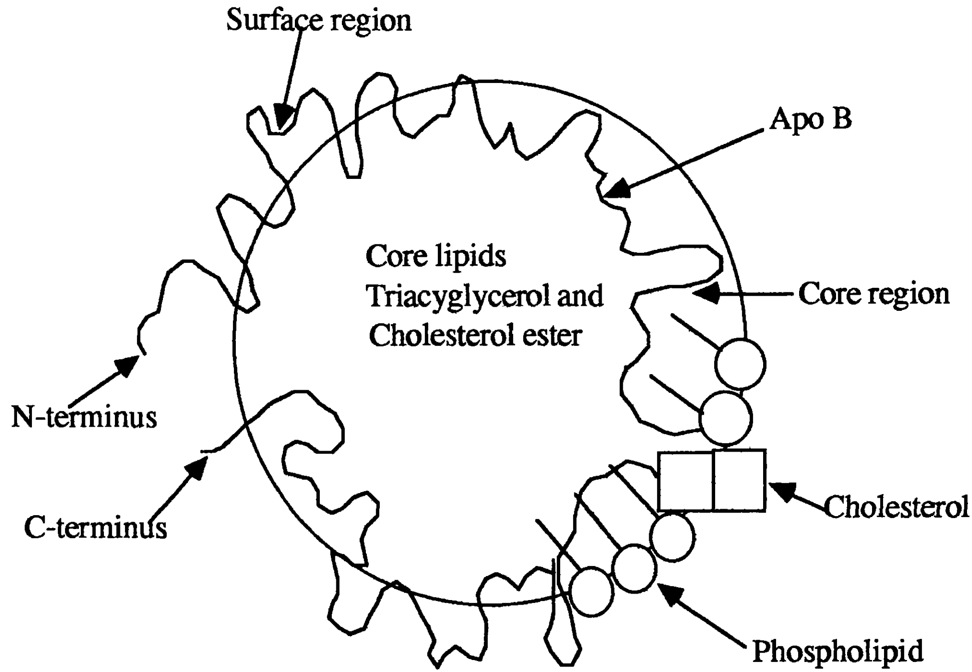
Apolipoprotein B-100 (apoB-100) is the major protein component of very low density lipoprotein (VLDL) secreted by the liver (Knott et al, 1986). Apo B-100 is synthesised in the liver and is vital for the assembly of triglyceride rich VLDL in the ER and its subsequent secretion into the plasma. Following VLDL metabolism apo B-100 is the sole protein component of low density lipoprotein (LDL) and is the ligand that binds to the LDL receptor making it responsible for the receptor mediated uptake and clearance of LDL from the circulation (Breslow, 1985). Raised plasma concentrations of LDL apoB-100 closely correlate with an elevated risk of coronary artery disease, this has been shown by epidemiological, pathological and genetic studies (LaRosa et al, 1986). The role of VLDL is to transport triglyceride to the adipose and muscle tissue, where lipoprotein lipase hydrolyses the triglyceride. The resulting particle, LDL, is therefore enriched in cholesteryl ester. The rate of production of LDL from VLDL and the rate of clearance of LDL by the liver therefore determines the net plasma concentration of LDL at any given time.

Although the clinical and biological importance of apoB-100 is well appreciated its primary structure defied elucidation mainly because of its enormous size (M_r 514,000), insolubility and tendency to aggregate. However the apo B gene has recently been cloned and the primary amino acid sequence deduced from the DNA sequence and this has revealed apo B to be a polypeptide of 4563 amino acids (Knott et al, 1986), although another report suggests there are 4536 amino acids (Yang et al, 1986). Alternating hydrophilic and hydrophobic regions on the polypeptide interact respectively with the polar surface phospholipids and the hydrophobic core of triacylglycerol and cholesteryl ester to stabilise the VLDL particle and to aid its solubilisation in the plasma (Fig. 5.1).

Dietary changes which require changes in VLDL secretion are accompanied by changes in the availability of functional apoB (Gibbons, 1990). Hormonal manipulations of isolated liver cells or whole animals have shown that changes in this functional availability are not necessarily achieved via changes in rates of apoB synthesis suggesting that some post-translational modification may be involved (Patsch et al, 1983).

Davis et al, (1984) have shown that apoB secreted by rat liver in vivo is phosphorylated on serine residues and it is possible that phosphorylation may be a general characteristic of proteins that are secreted. Other secreted proteins which associate with lipids to form lipid-protein aggregates in a similar fashion to VLDL apoB are phosphorylated e.g. casein, a major milk protein is secreted complexed with phospholipid and triacylglycerol and is phosphorylated on serine and threonine

Fig. 5.1 Model of the VLDL Particle



The above figure represents a theoretical model of the structure of the VLDL particle. Modified from Knott et al (1986).

residues (Turkington and Topper, 1966), myelin basic protein is also complexed with phospholipid and is phosphorylated on serine and threonine residues (Miyamoto and Kakiuchi, 1974). Apo B phosphorylation on serine residues has also been shown in experiments conducted on isolated rat hepatocytes (Sparks et al, 1988). The extent of apoB phosphorylation is reported, by Sparks et al (1988), to increase in apoB secreted by hepatocytes from diabetic rats, but it is also stated by Sparks et al, that this increase is due, at least in part, to phosphorylation at tyrosine residues as determined by phosphoamino acid analysis. It has been reported that, in experiments where hepatocytes were incubated with ^{32}P, insulin has decreased ^{32}P incorporation into apo B-100 (Jackson et al, 1990).

The protein kinases responsible for the phosphorylation of apo B-100 are the subjects investigated in this chapter. In general, rates of VLDL secretion correlate well with rates of de novo hepatic fatty acid and triacylglycerol synthesis (Duerden and Gibbons, 1988). Since triacylglycerol and cholesterol are the major components of VLDL the ability of the protein kinases implicated in the regulation of these biosynthetic pathways i.e. cAMP-PK and AMP-PK to phosphorylate apoB has been examined.

5.2 Results and Discussion

5.2.1 Time Course of LDL-ApoB-100 Phosphorylation

The catalytic subunit of cAMP-PK purified from bovine heart (Reimann and Beham,

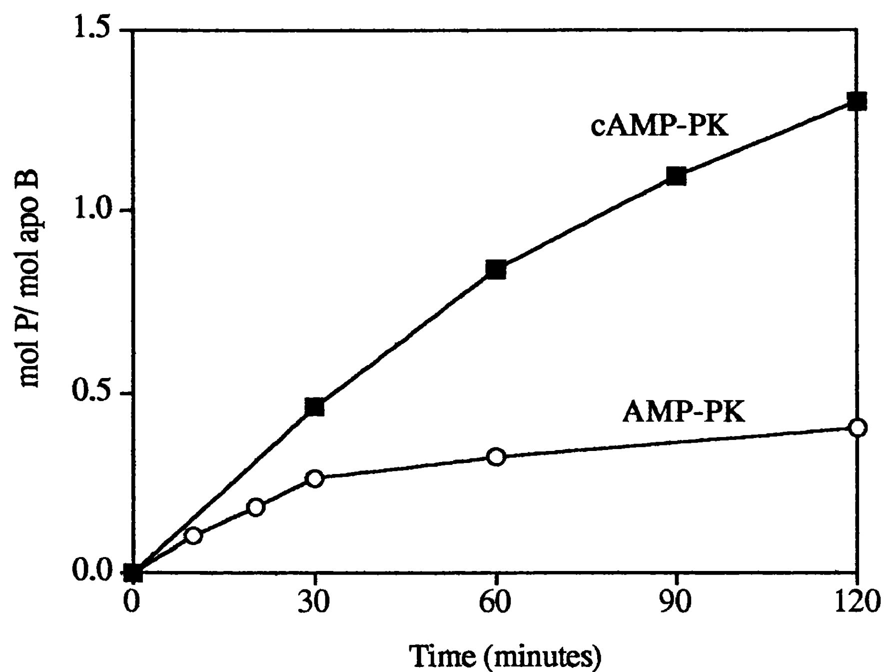
1983), and the AMP-PK purified from rat liver (see Chapter 2) both phosphorylated apo B-100 in a time dependent fashion (Fig. 5.2). The rate of incorporation of ^{32}P from [$\gamma^{32}P$] ATP into apoB by cAMP-PK and AMP-PK is shown in Fig. 5.2 the advantage of using LDL is that apoB is the sole protein present and therefore any phosphorylation detected is that of apo B. cAMP-PK incorporated approximately 1.25 molP/mol apo B and AMP-PK incorporated approximately 0.5 molP/mol apo B over a two hour time course so there is a significant level of phosphorylation of apo B in vitro by these two protein kinases. The level of phosphorylation achieved with the cAMP-PK suggests multi-site phosphorylation at, at least, two sites by the cAMP-PK.

5.2.2 Analysis of cAMP-PK and AMP-PK phosphorylation sites on Apolipoprotein B-100 by SDS PAGE of Thrombin Digests

Human plasma LDL contains one major apoprotein of M_r514,000 i.e. apo B-100. In some LDL preparations minor components termed apoB-74 ($M_r \approx 410,000$) and apoB-26 ($M_r \approx 145,000$) are seen, but the latter two have been shown to arise through proteolytic degradation of LDL-apo B-100, during collection of blood (Cardin et al, 1984).

LDL-apo B-100 was incubated with radioactive MgATP and either cAMP-PK or AMP-PK to give a stoichiometry of phosphorylation of approximately 1.0 molP/ mol and 0.4 molP/mol apo B respectively, as described in Chapter 2. The phosphorylated apo B was then incubated in a 1 : 50 ratio with thrombin (1mg thrombin : 50mg

Fig. 5.2 Time Course of Phosphorylation of Apo B
by cAMP-PK and AMP-PK

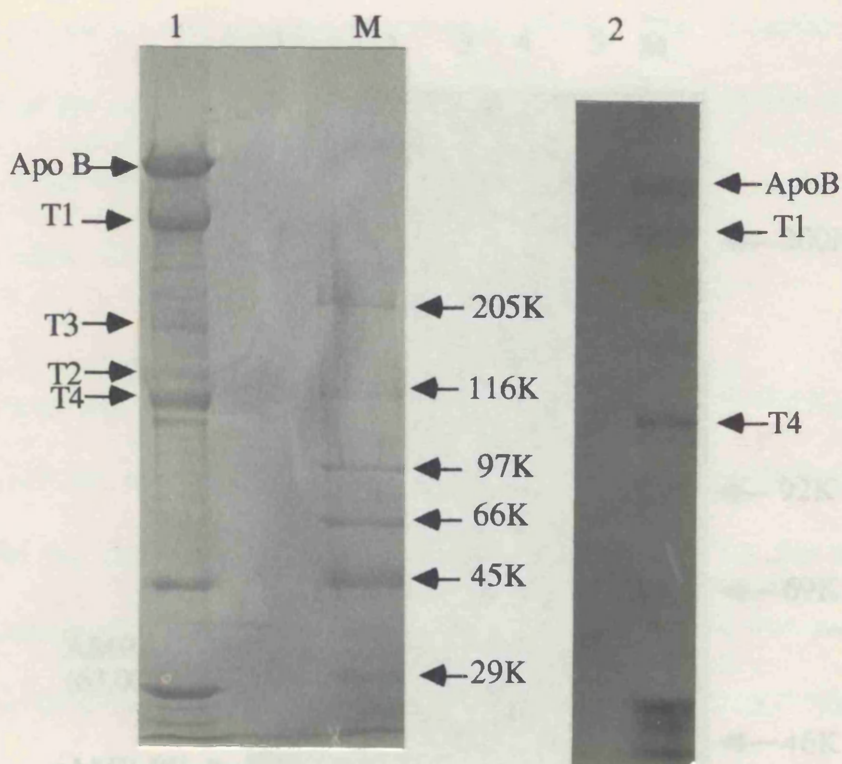


Human LDL was incubated at 37°C with [γ^{32}P] ATP and either the catalytic subunit of cAMP-PK purified from bovine heart (squares) or AMP-activated protein kinase purified from rat liver (circles) (see chapter 2 for details of purification). Aliquots were removed at the indicated time points during the incubation and precipitated with TCA. 32P incorporation was then determined as described in the Materials and Methods section. The data shown is representative of a number of such incubations carried out with different preparations.

LDL-apo B) at 37°C for 16 hours at which time the degradation was terminated by the addition of SDS-sample buffer which also delipidated the LDL particle. The SDS sample was mixed and then subjected to denaturing electrophoresis on a 10% polyacrylamide gel as described in Chapter 2, and the gel was then autoradiographed using Kodak X-omat film as described in Chapter 2.

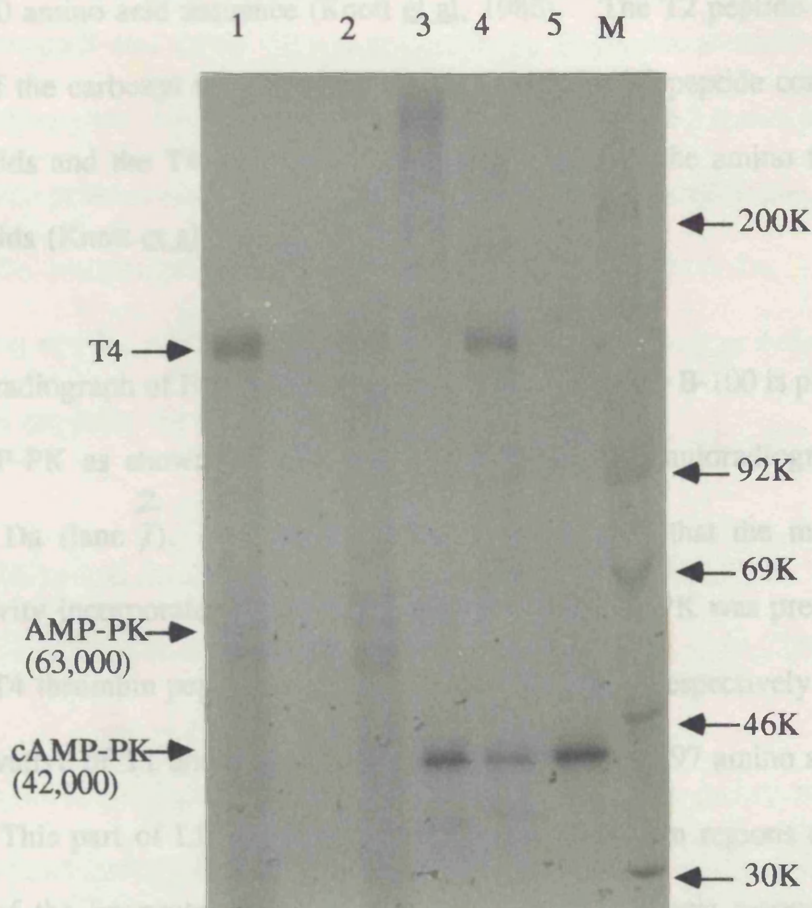
Fig. 5.3A shows a 10% SDS-polyacrylamide gel on which LDL phosphorylated by cAMP-PK was run after thrombin digestion. Undigested apo B ran with an approximate M_r of 540,000 (Lane 1). The mature protein of 4,536 amino acids has a predicted M_r of 514,000 (Knott et al, 1986) compared with the 540,000 found by SDS-gel electrophoresis (Fig. 5.3A) in this study and the 550,000 reported by Cardin et al (1984). This discrepancy is probably caused by glycosylation as 8-10% of the mass of apo B-100 is N-linked oligosaccharide (Knott et al, 1986). Thrombin digestion produced four peptides, T1, T2, T3 and T4 with apparent molecular weights (based on molecular weight markers) of 380,000 Da, 162,000 Da, 229,000 Da, 147,000 Da respectively (lane 1). These correlated with the previously reported thrombin digest products of apo B-100 by Cardin et al (1984) who suggested that T1-T4 to have molecular weights (based on molecular weight markers) of 385,000$\pm$6000 Da, 170,000$\pm$4000 Da, 238,000$\pm$4000 Da and 145,000$\pm$3000 Da respectively. The sum of the apparent molecular weights of T1 and T2 in Fig. 5.3A ($M_r \approx 542,000$) suggests that these peptides result from a specific cleavage of apo B-100 at a single site. The sum of the molecular weights of the minor fragments, T3 and T4 ($M_r \approx 376,000$ Da) suggested that these peptides probably arose from the further degradation of T1 ($M_r \approx 380,000$ Da). It is unlikely that these peptides are the

Fig. 5.3 A Thrombin Digestion and Autoradiograph of Apo B Phosphorylated by cAMP-PK



LDL-apoB-100 was prepared from human blood as described in chapter 2 and phosphorylated, using the catalytic subunit of cAMP-PK purified from bovine heart (for details of purification see chapter 2) to a stoichiometry of phosphorylation of approximately 1.0 molP/mol apoB, as described in chapter 2. Phosphorylated apoB was then incubated for 18-hours in the presence of thrombin. At the end of the 18-hour incubation samples from the incubation were boiled in SDS-sample buffer before being analysed by SDS-PAGE (lane 1) using a 10% polyacrylamide gel as described in chapter 2. The gel was then autoradiographed (lane 2) as described in chapter 2. Lane M shows the molecular weight markers.

Fig. 5.3 B Autoradiograph of Apo B-100 Phosphorylated by cAMP-PK and AMP-PK



LDL-apoB-100 was prepared from human blood as described in chapter 2 and phosphorylated using the catalytic subunit of cAMP-PK purified from bovine heart or the AMP-PK from rat liver (for details of purification see chapter 2) to a stoichiometry of phosphorylation of 1.0 and 0.4 molP/mol apoB respectively. Phosphorylated apoB was then incubated in the presence and absence of thrombin (1:10, thrombin : apoB) for 18-hours. At the end of the incubation samples from each incubation were boiled in SDS sample buffer before being analysed by SDS-PAGE and autoradiography. Lane 1 shows apoB that has been phosphorylated by AMP-PK and then subjected to thrombin digestion, lane 2 shows the AMP-PK blank, lane 3 shows the phosphorylation of the intact apoB, lane 4 shows apoB that has been phosphorylated by cAMP-PK and then subjected to thrombin digestion and lane 5 shows the cAMP-PK blank.

products of a contaminating protease in the thrombin solution, since the addition of PPACK (D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone), an inhibitor with high specificity for thrombin prevented any LDL-apoB-100 degradation (Cardin et al, 1984). The four thrombin generated peptides T1 - T4 have been mapped to the full apo B-100 amino acid sequence (Knott et al, 1986). The T2 peptide is reported to consist of the carboxyl terminal 1287 amino acids, the T3 peptide consists of 1952 amino acids and the T4 peptide is reported to consist of the amino terminal 1297 amino acids (Knott et al, 1986).

The autoradiograph of Fig. 5.3A, shows that intact LDL-apo B-100 is phosphorylated by cAMP-PK as shown by the signal generated on the autoradiographic film at 540,000 Da (lane 2). Furthermore it can also be seen that the majority of the radioactivity incorporated into LDL-apo B-100 by cAMP-PK was present in the T1 and the T4 thrombin peptides at 380,000 and 147,000 Da respectively (lane 2). T4 is a derivative of T1 and represents the amino terminal, 1297 amino acids of apo B-100. This part of LDL-apoB-100 is known to be rich in regions exposed to the surface of the lipoprotein (Fig. 5.1) and contains two classic recognition sites for cAMP-PK:

Phe - Arg - Lys - Phe - ^{606}Ser - Arg - Asn

Ile - Arg - Lys - Gly - ^{794}Ser - Lys - Asn

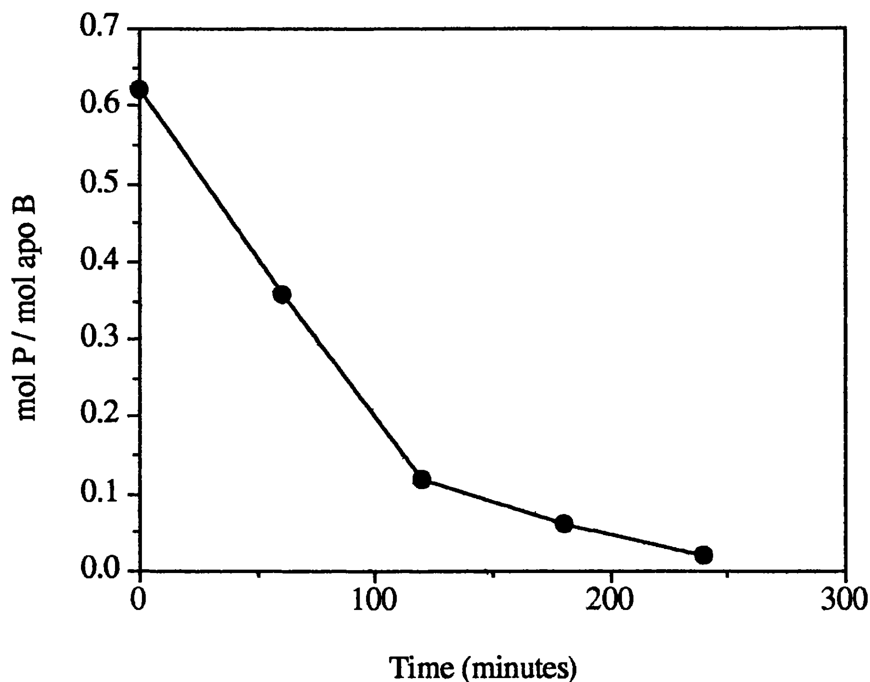
This T4 thrombin peptide also contains the site phosphorylated by AMP-PK, since all radioactivity detected subsequent to LDL phosphorylation by AMP-PK and SDS

PAGE of thrombin digests was observed in the T4 thrombin peptide (Fig. 5.3B) (lane 1). In this experiment LDL phosphorylated by cAMP-PK or AMP-PK was exhaustively digested with thrombin so that all the apo B was cleaved to the T2, T3 and T4 peptides. Autoradiography of the gel and comparison of the digests shows that all the apo B-associated ^{32}P was present in the T4 peptide whether apo B-100 had been phosphorylated by either cAMP-PK or AMP-PK. Lane 2 shows phosphorylated peptides and proteins endogenously present in the AMP-PK preparation. Lanes 3 and 5 show the autophosphorylated band of cAMP-PK at 42,000 Da. The possible recognition site for AMP-PK within the T4 peptide is difficult to define because the concensus sequence for AMP-PK is as yet unknown.

5.2.3 Analysis of the Sites Phosphorylated on LDL-Apo B-1OO by cAMP-PK and AMP-PK Using High Performance Liquid Chromatography

LDL-apo B-100 was incubated with [$\gamma^{32}P$] MgATP and either cAMP-PK or AMP-PK to give a stoichiometry of phosphorylation of 1.0molP/mol apo B and 0.4 molP/mol apo B respectively as described in Chapter 2. The phosphorylated apo B was then incubated in a 50 : 1 ratio with trypsin (w/w) at 37°C. Fig. 5.4 shows that greater than 97% of the [^{32}P] label, incorporated by either protein kinase, was released from TCA precipitable apo B-100 within 4 hours. The tryptic peptides generated were TCA soluble and this helped in their isolation and purification, prior to separation on reverse phase HPLC using a C18 column (Vydac). The radioactive peptides were separated using a gradient of acetonitrile/water in 0.1% (v/v) TFA (for full experimental details see Chapter 2).

Fig. 5.4 Release of Radiolabelled Phosphate from Apo B Using Trypsin

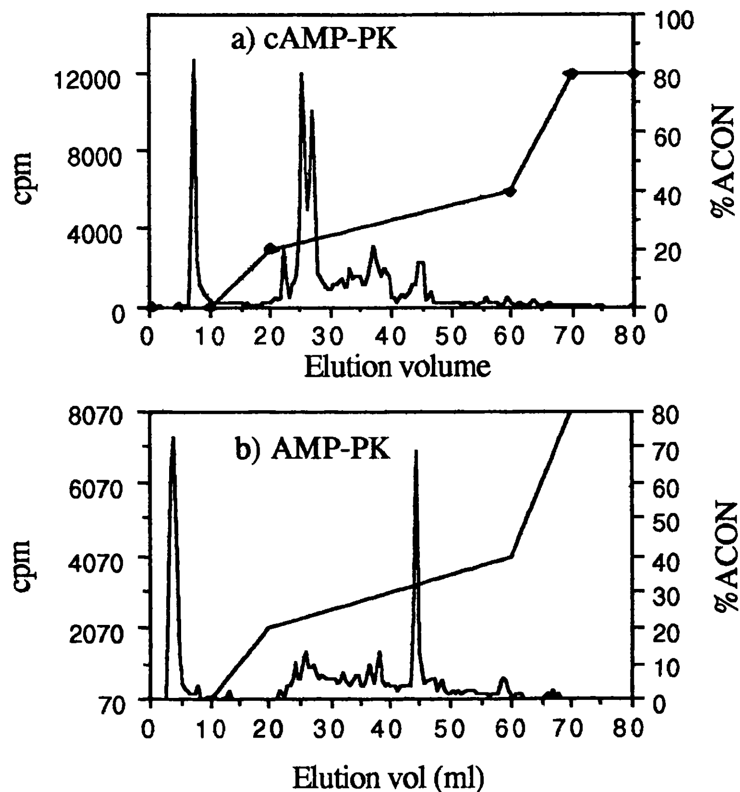


Apo B was phosphorylated using the catalytic subunit of cAMP-PK purified from bovine heart (see chapter 2 for details of purifications). The 32P labelled apoB was then incubated with trypsin (as described in the text) and at the time points indicated on the above figure aliquots were removed from the incubation and the phosphate content of apo B determined as described in chapter 2. The result shown above is representative of that obtained with a number of different preparations. ApoB-100 that had been phosphorylated by AMP-PK showed a very similar pattern of release of 32P label as that shown above for cAMP-PK.

The HPLC profiles of tryptic phosphopeptides generated from apo B-100 that had been phosphorylated by cAMP-PK or AMP-PK are shown in Fig. 5.5. The sample had been run through a Sep-pak C18 reverse phase cartridge prior to purification on HPLC in order to remove ^{32}P and unreacted radioactive ATP. On reverse phase HPLC using a C18 column both profiles showed an initial peak of radioactivity that was quite large, this was in the initial breakthrough and probably contained unreacted [$\gamma^{32}P$] ATP that did not bind to the C18 column. After the initial breakthrough peak, the main peak, a doublet, eluted from the apo B-100 that had been phosphorylated by cAMP-PK at a concentration of approximately 22% acetonitrile (Fig. 5.5a). This was preceded and succeeded by a number of minor radioactive peaks, none of which amounted to more than approximately 4% of the major peptide at 22% acetonitrile and are probably not significant. The doublet could represent two distinct peptides each with a distinct phosphorylation site and this correlates with the > 1molP/mol apo B-100 stoichiometry of phosphorylation observed with cAMP-PK (Fig. 5.2), or a single phosphorylation site in 2 peptides where one peptide is possibly an oxidised/reduced or somehow modified form of the other or an incomplete digestion product. However, the same doublet is still observed following longer exhaustive periods of digestion with trypsin, so this appears to be an unlikely explanation. No further radioactive peptides eluted after a concentration of approximately 35% acetonitrile (Fig. 5.5a).

The major radioactive tryptic peptide from the AMP-PK phosphorylated LDL-apo B-100 eluted at a concentration of approximately 32% acetonitrile (Fig. 5.5b). This major radioactive peak was preceded and succeeded by a number of smaller

Fig. 5.5 HPLC Purification of Tryptic Phosphopeptides Derived from Apo B



Apo B-100 was phosphorylated to a stoichiometry of 1.0 molP/mol apoB using the catalytic subunit of cAMP-PK purified from bovine heart (a) or to a stoichiometry of 0.4 molP/mol apoB using the AMP- activated protein kinase purified from rat liver (b) (see chapter 2 for details of purification). The trypsin generated radiolabelled peptides were eluted from a reverse phase C-18 column (Vydac) with a gradient of acetonitrile (ACON) in water containing 0.1% (v/v) TFA. 1ml fractions were collected and counted by Cerenkov.

radioactive peaks, none of which amounted to more than approximately 4% of the major peptide at 32% acetonitrile and are probably not significant. No further radiolabelled peptides were detected after a concentration of approximately 40% acetonitrile.

The major tryptic phosphopeptides from cAMP-PK phosphorylated LDL-apo B-100 eluted at 22% acetonitrile, whereas the major phosphopeptide from AMP-PK phosphorylated LDL-apo B-100 eluted at 32% acetonitrile. Therefore it would appear that cAMP-PK and AMP-PK phosphorylate apo B-100 at different sites. From the data produced by the thrombin digestion, these sites are at the amino terminus of the human LDL-apo B-100 protein. This suggestion is however, tentative at present, and is currently being confirmed by the further purification and amino acid sequencing of these radiolabelled peptides. This will determine the exact amino acid residues that are phosphorylated, and will allow their comparison with in vivo sites of phosphorylation.

5.3 A Need For Apo B Kinases?

It is clear from the above data that both cAMP-PK and AMP-PK phosphorylate apo B-100 in vitro. If either of these phosphorylations of apo B-100 are physiologically important are they important in the intrahepatic assembly and secretion of VLDL or in the extracellular uptake of LDL via receptor mediated endocytosis by LDL-receptor recognition of apo B. Davis et al (1984) have reported that incubation of VLDL

(containing ^{32}P labelled apo B), isolated from ^{32}P labelled rat hepatocytes, with freshly isolated rat serum results in a rapid dephosphorylation of apo B (incubation for 8 hours resulted in a 70% dephosphorylation). This indicates that any phosphorylations will not survive for very long in the plasma (due to high plasma phosphatase activity (Davis et al, 1984)) and suggests therefore that the role of phosphorylation, if any, is not in LDL uptake but rather in the intrahepatic assembly and secretion of VLDL. However, alkali labile phosphate measurements show that LDL does contain phosphate at about 0.6molP/mol apo B (Dr. M.R. Munday, unpublished data), therefore phosphorylation at certain sites does survive secretion into the plasma and is therefore presumably not accessible to plasma phosphatases. Furthermore, Davis et al, (1984) have shown that LDL isolated, from both rat blood and rat liver, 3 hours after injection of the rats with 10mCi of [^{32}P]orthophosphate contains ^{32}P. It is possible that this phosphorylation has a structural function and therefore there are likely to be sites on apo B that are phosphorylated by protein kinases other than cAMP-PK and AMP-PK. While LDL is almost certainly not the real substrate for an apo B kinase (it must be newly translated apo B exposed on the endoplasmic reticulum or the VLDL particle) it is the most convenient substrate because it is the most homogeneous lipoprotein preparation that contains only apo B and no other apolipoproteins.

5.4 A Novel Apo B Kinase

In order to look for other apo B kinases, a rat liver homogenate was screened for its ability to phosphorylate apo B in human LDL.

5.4.1 Purification of a Novel Apo B Kinase

Male Wistar rats were anaesthetized with a non-recovery dose of pentobarbital (60mg/kg body weight) administered by intraperitoneal injection. After induction of complete anaesthesia, the liver was rapidly dissected out and rinsed in ice-cold homogenisation buffer. The tissue was then finely chopped and homogenised in buffer containing; 0.25M sucrose, 20mM Tris HCl pH 7.2 at 4°C, 50mM NaF, 1mM NaPPi, 1mM EDTA, 1mM EGTA, 10% (w/v) glycerol, 5μg/ml SBTI, 1mM Benzamidine, 2mM PMSF and 2mM DTT, using a domestic kitchen blender, with three, thirty second bursts at top speed, and with a minute on ice in between each burst. The homogenised tissue was centrifuged at 100,000g for 60 minutes at 4°C and the supernatant filtered through glass wool.

Purification on Phosphocellulose

The supernatant was then applied to a phosphocellulose column that had been previously equilibrated in separation buffer containing; 50mM Tris HCl pH 7 at 4°C, 50mM NaF, 1mM NaPPi, 1mM EDTA, 1mM EGTA, 10% (w/v) glycerol, 5μg/ml SBTI, 1mM Benzamidine and 2mM PMSF. The phosphocellulose column was washed with separation buffer until no protein eluted from the column as judged by an A_{280} reading of less than 0.05. The column was then developed using a linear gradient of 0 to 1M NaCl, in separation buffer. Fractions of equal volume were collected and their protein concentration determined by their absorbance at 280nm.

The fractions were then assayed for their ability to phosphorylate apo B-100. An aliquot of each fraction was incubated with LDL-apo B-100, presented at a final concentration of 1mg/ml, 0.2mM [γ^{32}P] ATP, specific radioactivity of 250,000 cpm/nmol and 4mM $MgCl_2$. The assay was carried out in buffer containing 20mM Tris/Cl pH 7.1 at 37°C, 50mM NaF, 0.5mM EDTA, 1mM Benzamidine, 5μg/ml SBTI and 2mM PMSF. Assays were allowed to incubate for 60 minutes at 37°C before being terminated by the addition of 1ml of 25% (w/v) TCA. 300μg of BSA was added and the assay mixture centrifuged at 10,000g in a bench centrifuge. The precipitate was washed three times with 1ml of 25% (w/v) TCA and then counted by Cerenkov counting in a LKB-Rackbeta Scintillation Counter, as described in Chapter 2.

A number of protein kinase activity peaks were obtained and some of these proved to be extremely labile and could not be purified on subsequent identical purification steps. The results shown in Fig. 5.6 show the four peaks of protein kinase activity that could however be consistently purified, these were subsequently characterised as follows;

Peak 1, which eluted at approximately 100mM NaCl and was judged to be AMP-PK due to its activation by 200μM AMP.

Peak 2, which eluted at approximately 300mM NaCl and was judged to be cAMP-PK due to its inhibition by the specific peptide inhibitor of cAMP-PK.

Peak 3, which eluted at approximately 650mM NaCl and was judged to be casein kinase II due to its sensitivity to inhibition by the polyamine, heparin (at a concentration of heparin of 2.5μg/ml), and

Fig. 5.6 Purification of the Novel Apo B Kinase by Chromatography on Phosphocellulose

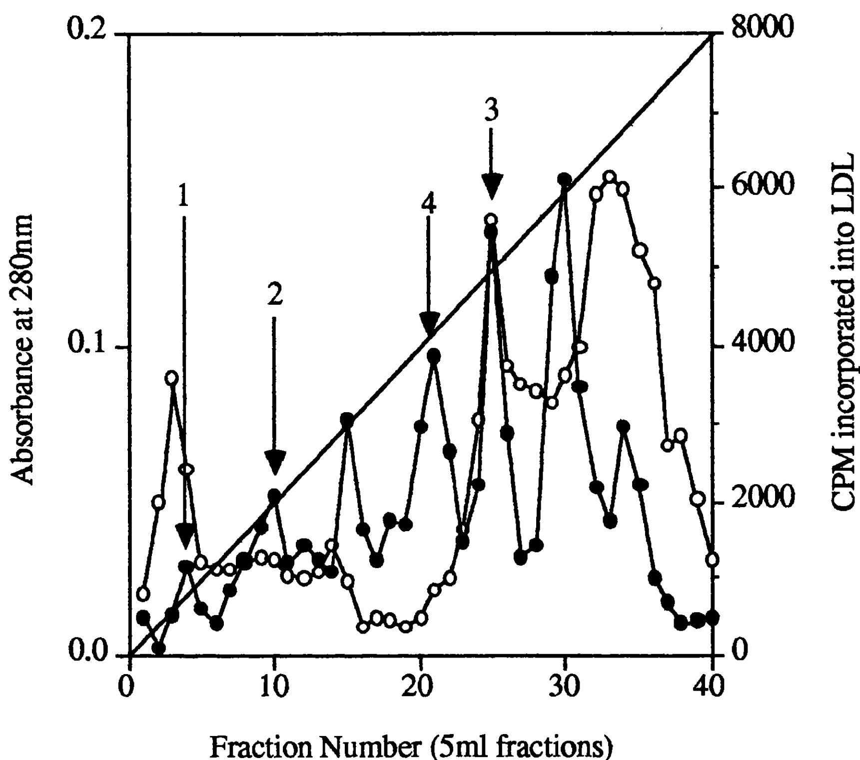


Fig. 5.6 shows the purification of the novel apo B kinase by phosphocellulose chromatography of a post 100,000g supernatant prepared from rat liver (as described in section 5.4.1). The column was developed with a linear gradient running from 0-1M NaCl as shown by the diagonal line in the above figure. The absorbance of the collected fractions at 280nm is shown by the open circles and the apo B kinase activity of each fraction is shown by the full circles. Apo B kinase activity in each fraction was assayed as described in the text.

Peak 4, which eluted at approximately 600mM NaCl and was partially characterised as a LDL-apoB protein kinase.

In subsequent purifications of the protein kinase activity in peak 4, the 100,000g rat liver supernatant was loaded onto phosphocellulose, previously equilibrated in separation buffer, containing 400mM NaCl. The supernatant was applied and the phosphocellulose column washed with separation buffer containing 400mM NaCl until no more protein eluted from the column as judged by an A_{280} reading of less than 0.05. The protein kinase activity was then batch eluted with separation buffer containing 650mM NaCl. Fractions of equal volume were collected and protein concentrations determined by absorbance at 280nm. The fractions collected were tested for apo B protein kinase activity, using the methodology described above and the results of the purification procedure are shown in Fig. 5.7. The active fractions were pooled and concentrated using an Amicon concentrator with a nominal molecular weight cut off of M_r 30,000 Da. The concentrated pool of apo B protein kinase activity was then dialysed into separation buffer containing no salt.

Purification on Q-Sepharose

This pooled peak of apo B kinase activity from phosphocellulose was then applied to a Q-sepharose column (an anion exchange column, similar to Mono-Q) which had been previously equilibrated in the same separation buffer containing no salt. The Q-Sepharose column was washed with separation buffer until no more protein eluted as judged by an absorbance A_{280} of less than 0.05. The apo B protein kinase activity

Fig. 5.7 Purification by Batch Elution of the Novel Apo B Kinase from Phosphocellulose

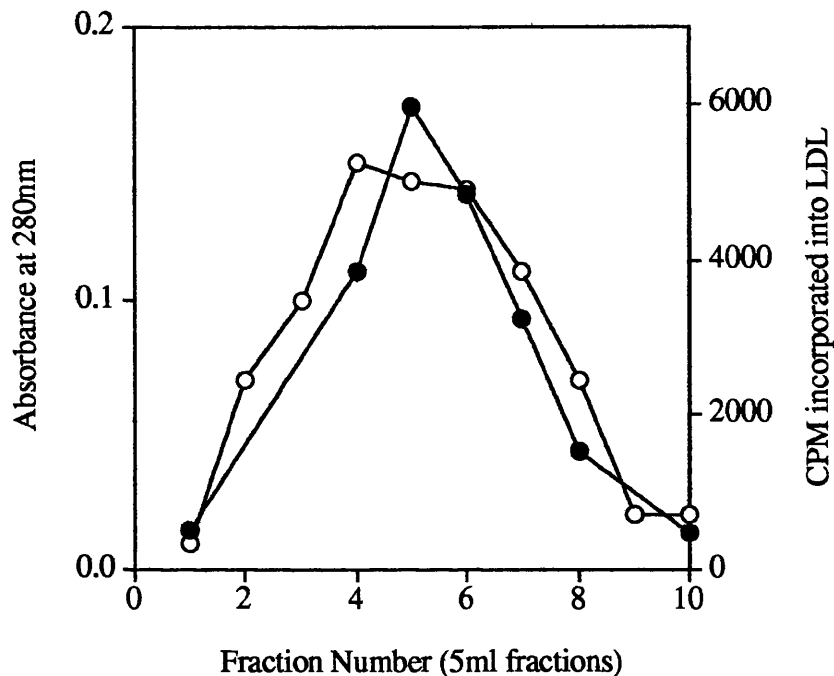


Fig. 5.7 shows the purification of the novel apo B kinase by batch elution from phosphocellulose. The column was eluted with buffer containing 0.65M NaCl as describd in the text (see section 5.4.1 for details). The absorbance of the collected fractions at 280nm is shown by the open circles and the apo B kinase activity of the the collected fractions is shown by the full circles. Apo B kinase activity was assayed as descrinbed in the text

was then eluted using a gradient running from 0 to 1M NaCl, in separation buffer, the gradient was developed over a total volume of 100ml and 4ml fractions were collected, (Fig. 5.8). The protein concentration of each fraction was measured by its absorbance at 280nm and each fraction was tested for apo B protein kinase activity, using the methodology described above.

Fig. 5.8 shows that there is an initial elution of protein as the gradient starts to develop, but there is no apo B protein kinase activity associated with this peak. This is followed by a much larger peak of protein eluting off the Q-Sepharose column at a concentration of approximately 250-320mM NaCl, this protein peak is associated with a very sharp peak of apoB protein kinase activity which is very active. There is a third protein peak that elutes at approximately 680mM NaCl but there is no apo B protein kinase activity associated with this protein peak.

The active fractions showing the greatest apo B protein kinase activity were then pooled and concentrated to a volume of less than 1ml using an Amicon concentrator (molecular weight cut off $M_r \approx 30,000$ Da). The concentrated, pooled peak was then dialysed overnight, into separation buffer containing 50% (w/v) glycerol, and the apo B protein kinase was then stored at 4°C until required for use. This partially purified preparation of the apo B protein kinase activity was able to incorporate 1mol P/mol of apoB over a 60 minute time course of phosphorylation incubated at 37°C.

Fig. 5.8 Purification of the Novel Apo B Kinase by Chromatography on Q-Sepharose

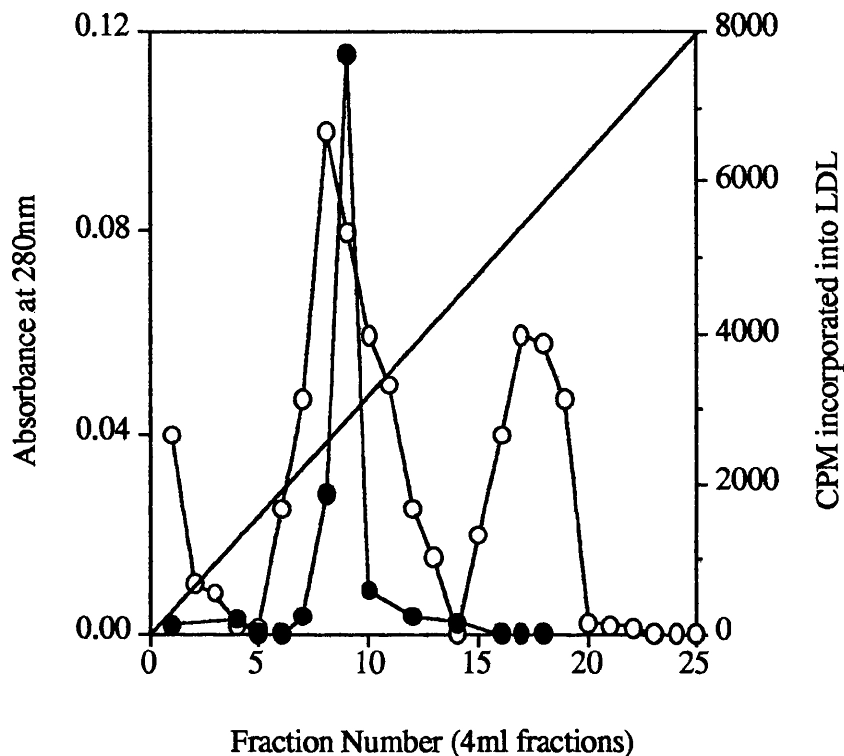
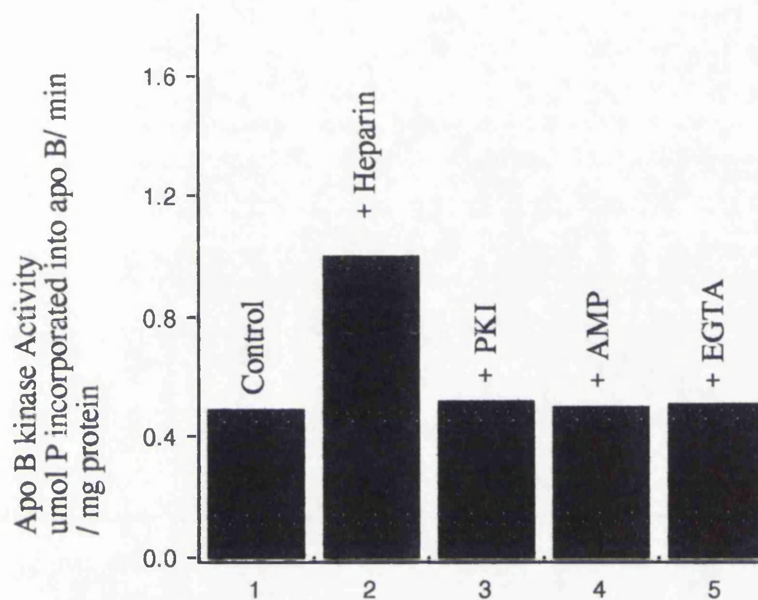


Fig. 5.8 shows the further purification of the novel apo B kinase after phosphocellulose chromatography by Q-Sepharose chromatography (see section 5.4.1 for details). The column was developed with a linear gradient running from 0-1M NaCl as shown by the diagonal line in the above figure. The absorbance of the collected fractions at 280nm is shown by the open circles and the apo B kinase activity of the collected fractions is shown by the full circles. Apo B kinase activity was assayed as described in the text

5.4.2 Sensitivity of the Apo B Kinase to Various Effectors

The sensitivity of the apo B protein kinase to a number of effectors was tested by measuring the rate of phosphorylation of apo B, using the methodology described above. The control incubation of apo B and apo B protein kinase showed an incorporation of approximately 0.5μmol P/min/mg protein, as shown in Fig. 5.9. The inclusion of the active peptide fragment of the Walsh inhibitor (an inhibitor of cAMP-PK) at a final concentration of 10μg/ml had no effect on the rate of phosphorylation of apo B by the apo B protein kinase compared to the control (Fig. 5.9). The fact that the presence of the specific peptide inhibitor of cAMP-PK had no effect on the rate of phosphorylation of apo B by apo B protein kinase strongly suggests that this apo B protein kinase activity is not cAMP-PK, since it would have been inhibited by the former if this were the case. Similarly the presence of AMP (200μm) in concentrations known to cause a 3-4 fold stimulation of purified AMP-PK has no effect on the rate of phosphorylation of apo B by the apo B protein kinase activity this suggests that this apo B protein kinase activity is not AMP-PK since the AMP stimulation is an effect quite specific to this protein kinase (see Chapter 1). It can also be seen from Fig. 5.9 that in the presence of EGTA (2mM) the level of phosphorylation of apo B by the apo B protein kinase activity is 0.46μmol P/min/mg which is very similar to the control incubation so it would seem that EGTA has no effect on the activity of the apo B protein kinase activity. The effect of EGTA is to specifically chelate divalent cations e.g. calcium, so this result suggests that the apo B protein kinase activity is not calcium dependent. Finally, heparin at a concentration of 5μg/ml produced virtually a 100% activation of apo B protein kinase activity

Fig. 5.9 Sensitivity of Apo B Kinase to Various Effectors



The sensitivity of the novel apo B protein kinase to various effectors is shown above. The novel apo B kinase was purified as far as the Q-Sepharose step (see section 5.4.1) and incubated with heparin (5ug/ml), the specific peptide inhibitor of cAMP-PK, PKI (10 ug/ml), AMP (200uM) and EGTA (2mM). The activity of the apo B kinase was then assayed as described in the text.

compared to the control, giving a rate of phosphate incorporation of approximately 1μmol/min/mg protein. This concentration of heparin has been shown to be sufficient to inhibit both casein kinases, I and II, (Munday and Hardie, 1984) so this stimulation of activity shows that the apoB protein kinase activity cannot be casein kinase II. To summarize therefore, this apoB protein kinase does not appear to be cAMP-PK, AMP-PK, a calcium dependent protein kinase or casein kinase II.

5.5 Conclusions

The two protein kinases cAMP-PK and AMP-PK, that are heavily implicated in the phosphorylation and inhibition of acetyl-CoA carboxylase in rat liver by starvation or by glucagon treatment, both phosphorylate LDL-apo B-100 in vitro. The amount of phosphate incorporated by the two protein kinases is different and the higher stoichiometry of phosphorylation achieved with cAMP-PK may be due to multisite phosphorylation, but in vitro phosphorylation does not necessarily reflect the in vivo situation. AMP-PK is known to phosphorylate ACC and HMG-CoAR, two enzymes involved in lipid metabolism therefore its phosphorylation of apo B, a key component in VLDL assembly and secretion, correlates with the idea of it being important in the regulation of proteins involved in lipid metabolism.

Human plasma low density lipoprotein contains one major apolipoprotein, apo B-100 of $M_r \approx 514,000$ Da. The apoB-100 can be cleaved using thrombin to yield a limited number of peptide fragments, termed T1 - T4. Autoradiographic analysis of [^{32}P]

labelled apo B-100 phosphorylated by cAMP-PK or AMP-PK, and then subjected to thrombin digestion revealed all of the radioactive signal to be present in the T4 peptide. This region of apoB-100 is known to be rich in regions exposed to the surface of the lipoprotein and contains two classic recognition sites of cAMP-PK at serine-606 and at serine-794. Analysis of the sites phosphorylated on apo B-100 using HPLC separation of tryptic peptides on a C18 reverse phase column yielded two different peptide profiles, with the major radioactive peaks eluting at different concentrations of acetonitrile, for apo B-100 phosphorylated by the two different protein kinases i.e. cAMP-PK and AMP-PK. These results suggest that these phosphorylations may be at different sites, but further work on the purification and sequencing of the peptides is required to confirm this. Furthermore, the physiological significance of such phosphorylations and whether they occur in vivo require further investigation. A potent apo B protein kinase activity has been identified in rat liver and has been partially purified by chromatography on phosphocellulose and Q-Sepharose. This protein kinase is not cAMP-PK, AMP-PK, Casein kinase I or II or a calcium-dependent protein kinase but it is stimulated by heparin. This protein kinase activity is as yet unidentified and may well represent a novel and potent apoB protein kinase. A fuller characterisation of this kinase, its regulation and the sites it phosphorylates on apoB may shed further light on the role of apoB phosphorylation.

The role and site of apo B phosphorylation during the assembly and secretion of lipoproteins are still unclear. On a mechanistic level it is possible that apo B phosphorylation allows apo B to dissociate from membrane lipids and affect lipid nucleation and formation of VLDL (Sparks et al, 1988). It has been suggested by

Davis et al (1984) that phosphorylated apo B may have an important function in the intracellular transport of hepatic VLDL during assembly and secretion. Phosphorylation may be a general characteristic of a specific class of amphipathic proteins and several such proteins which are known to associate with lipids to form lipid-protein aggregates similar to VLDL-apo B are known to be phosphorylated. Examples of this include, avian liver which secretes lipoproteins to transport lipid to the oviduct for egg development, two of these proteins derived from vitellogenin are phosphorylated (Deeley et al, 1975). Casein, a major milk protein, is a hydrophobic protein excreted complexed with phospholipid and triglyceride and is phosphorylated at both serine and threonine residues (Turkington and Topper, 1966), myelin basic protein another hydrophobic protein complexed with phospholipid is also phosphorylated at serine and threonine residues (Miyamoto and Kakiuchi, 1974). It has been proposed that phosphorylation of proteins in chloroplasts is necessary to provide the molecular forces required for lateral movement in membranes (Staehelin and Arntzen, 1983), and it is possible that a similar role for phosphorylated apo B exists (Davis et al, 1984). It has recently been shown that intact rat liver Golgi vesicles translocate ATP into their cisternal space and utilise it to phosphorylate certain secretory proteins, it is proposed that phosphorylation renders these proteins resistant to proteolysis (Capasso et al, 1989) and it is possible that apo B phosphorylation may serve to protect it from proteolysis during VLDL assembly, however this protective role presumably does not apply once VLDL is secreted into the plasma since it is known that there is a rapid dephosphorylation of apo B by plasma phosphatases (Davies et al, 1984). Jackson et al (1990), on the basis of ^{35}S and ^{32}P labelling studies, suggest the existence of two separate pools of hepatic apo

B-48 of which one is phosphorylated and constitutively secreted and one which is phosphorylated but whose phosphorylation is insulin-regulated to allow for chanelling into an intracellular degradative pathway, but the entire apo B-100 pool is insulin-sensitive. This suggests that insulin may be affecting apo B secretion from the liver by altering its intracellular turnover and phosphorylation. Insulin-regulated changes in apo B phosphorylation status may play a role in regulating VLDL particle size since it has been shown (Powell and Glenney, 1987) that the dephosphorylated form of lipocortin-1 had a greater affinity for phosphatidylserine liposomes than the phosphorylated form, thus the isulin-stimulated, dephosphorylated apo B-100 could result in increased lipid association and a larger VLDL particle. It has been shown that apo B-100 is secreted as a phosphoprotein and that its phosphorylation is increased in hepatocytes from hypoinsulinaemic nonketotic diabetic rats (Sparks et al, 1988), the increased apo B phosphorylation may decrease the affinity of apo B for lipids resulting in the smaller, more dense VLDL particles secreted from diabetic rat livers (Berry et al, 1981). Human liver only secretes apo B-100 therefore the observed in vitro insulin effects on apo B-100 phosphorylation and degradation may be very relevant to human apo B and VLDL metabolism. Phosphorylation of specific amino acids of apo B may be hormonally regulated, similar to many enzyme phosphorylation-dephosphorylation reactions, which may allow the co-ordination and control of the sophisticated series of events involved in the assembly of apo B with triglyceride and phospholipid and the eventual secretion of VLDL (Sparks et al, 1988). Hormonal regulation will be mediated by second messengers and is likely to involve protein kinases, cAMP-PK is known to be activated by hormones that raise cAMP concentrations e.g glucagon, there is indirect evidence that there is negative

regulation of AMP-PK by insulin (see chapter 4) and although the signals that regulate the partially characterised novel apo B kinase remain to be elucidated it is possible that this kinase may be insulin stimulated and therefore important in the hormonal regulation of VLDL assembly and/or secretion.

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