AMP-activated protein kinase and cAMP-dependent protein kinase from mammary gland of lactating rat

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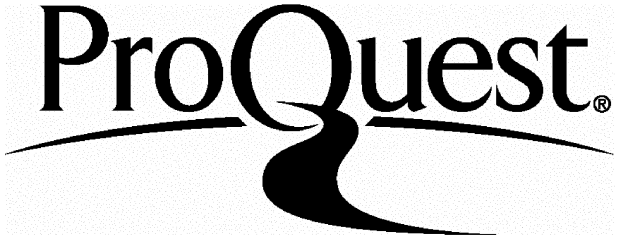

ABSTRACT OF THESIS

Acetyl-CoA carboxylase (ACC) catalyses the first committed step in fatty acid synthesis and is regulated by reversible phosphorylation and inactivation. Although cAMP-dependent protein kinase (cAMP-PK) from bovine heart can phosphorylate and inactivate ACC *in vitro*, there is now much evidence to suggest that AMP-activated protein kinase (AMP-PK) is responsible for ACC phosphorylation and inactivation *in vivo*.

AMP-PK from mammary gland was purified 3750 fold using a seven step purification, it has a native MW of 93 KDa and a possible heterodimer subunit structure with a larger catalytic subunit of 61-62 KDa. Mammary gland AMP-PK has a temperature optimum of 36^0C, pH optimum at pH 7.0, Ka for 5'AMP of 1.4μM (assayed at 200μM ATP), Ki for FSBA of 170μM (assayed at 100μM 5'AMP and 200μM ATP). AMP-PK from mammary gland is allosterically activated by 5'AMP (2-3 fold at 100μM 5'AMP) and is reversibly phosphorylated and activated by a distinct "kinase-kinase".

AMP-PK from mammary gland and liver of lactating rats have identical structural and kinetic properties indicating that they are not isozymic forms.

Partially purified kinase kinase has proven not to be cAMP-PK from mammary gland but has many properties in common with a previously described but uncharacterised ACC-kinase from lactating rat mammary gland named ACC-kinase 2.

AMP-PK appears to have a physiological role in the control of ACC activity during starvation and refeeding in the liver of virgin rats, and mammary gland of lactating rats. In both tissues changes in AMP-PK activity during starvation and refeeding closely correlate with reciprocal changes in the Vmax of ACC and with changes in plasma insulin concentration.

The catalytic subunit of cAMP-PK (C-subunit) was purified from lactating rat mammary gland and compared with C-subunit from rat and bovine heart. Despite similar molecular weight, the mammary gland and heart C-subunits appear to be different isoenzymes based on their specific activities, their substrate specificity and Cleveland mapping on SDS-PAGE.

To my wife Bev and children Alexander and Isabella.

Without their full support and understanding this thesis

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ABBREVIATIONS

Apart from those listed below, the abbreviations used through this thesis follow the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature, as detailed in the Biochemical Journal (225, pp 1-26, 1985.)

A_{280}	Absorbance at 280nm
ACC	Acetyl-CoA carboxylase
ACK-2	Acetyl-CoA carboxylase kinase 2
ACON	Acetonitrile
ACTH	Adrenocorticotropin
ADP	Adenosine 5'diphosphate
AMP	Adenosine 5'monophosphate
AMP-PK	AMP-activated protein kinase
ATP	Adenosine 5'triphosphate
ATP-CL	ATP citrate lyase
AUFS	Absorbance unit full scale
BSA	Bovine serum albumin
BZ	Benzamidine
C-subunit	Catalytic subunit of cAMP-PK
cAMP	cyclic 3'5' adenosine monophosphate
cAMP-PK	Cyclic-AMP dependent protein kinase
cDNA	Complementary deoxyribonucleic acid
CNBr	Cyanogen bromide

DAB	3,3' diamino benzidine tetrahydrochloride
DTT	Dithiothreitol
EDTA	Ethylene-di-amine-tetra-acetic acid
EGTA	Ethylene-glycol-bis(ß-amino-ethyl ether) N,N,N',N'-tetra-acetic acid
ELISA	Enzyme linked immuno sorbent assay
FF	Fast flow
FPLC	Fast protein liquid chromatography
FSBA	Fluorosulphonyl-benzoyl adenosine
GLUT-4	Glucose transporter type 4
HDL	High density lipoprotein
HMG-CoA	Hydroxymethyl glutaryl CoA
HMG-CoAR	HMG-CoA reductase
HPLC	High performance liquid chromatolgraphy
HR	High resolution
HSA	Human serum albumin
HSL	Hormone sensitive lipase
IBMX	3-isobutyl-1-methyl-xanthine
IDL	Intermediate density lipoprotein
KLH	Keyhole-limpet haemocyanin
KPi buffer	Buffer containing K_2HPO_4 and KH_2PO_4 in ratio 1 : 1
LDL	Low density lipoprotein
MBS	3-maleimidobenzoic acid N-hydroxysuccinimide ester
MG	Mammary gland
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NaPPi	Sodium pyrophosphate
PBS	Phosphate-buffered saline
PCA	Perchloric acid
PDH	Pyruvate dehydrogenase

PEG_{6000}	Poly (ethylene) glycol (MW 6000)
PKC	Protein kinase C (Ca^{2+}-phospholipid-dependent protein kinase)
PKI	Specific peptide inhibitor of cAMP-PK
PMSF	Phenylmethylsulphonyl fluoride
PP-1	Protein phosphatase 1
PP-2A	Protein phosphatase 2A
PP-2C	Protein phosphatase 2C
SBTI	Soya bean trypsin inhibitor
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SAMS	His-Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg
SEM	Standard error of the mean
SSMS	His-Met-Arg-Ser-Ser-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg
TCA	Trichloroacetic acid
TEMED	tetramethylethylenediamine
TFA	Trifluoroacetic acid
TLCK	Nα-p-tosyl-L-lysine chloromethyl ketone
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
VLDL	Very low density lipoprotein
%C	Percentage (by weight) of the crosslinker relative to the total monomer in SDS-PAGE
%T	Total concentration (in grams per 100ml) of acrylamide and bis-acrylamide in SDS-PAGE

Note: The dimensions of chromatography columns are quoted in brackets after the column name and represent internal diameter in mm (first number) and height of column bed in cm (second number).

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CHAPTER ONE

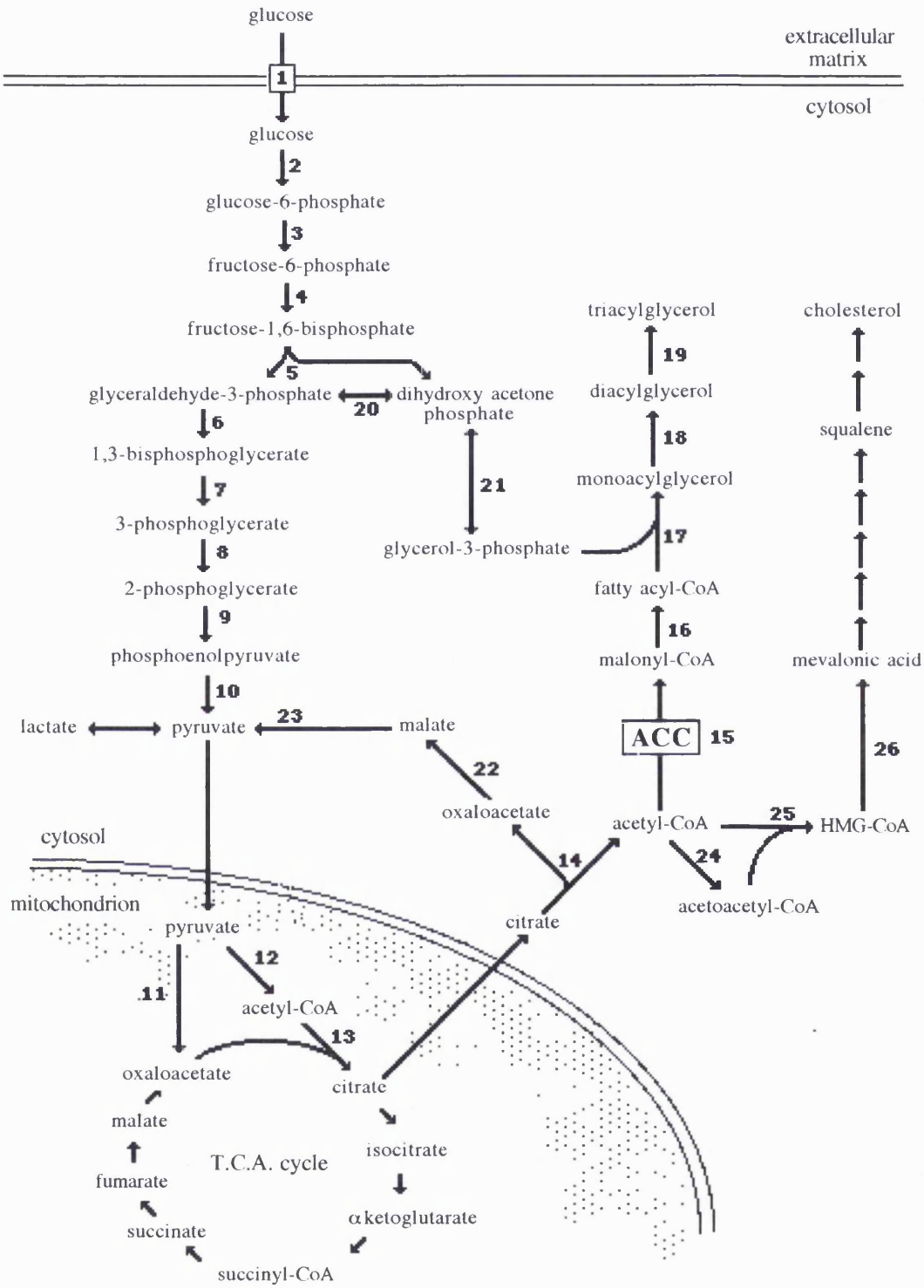
Introduction

1.1. Biosynthesis of fatty acids in mammals

The three major lipogenic tissues in mammals are the liver, mammary gland (during lactation) and white adipose tissue (Hardie, D.G., 1989). Brown adipose tissue also exhibits high rates of lipogenesis under certain conditions (McCormack, J.G. and Denton, R.M., 1977) but this tissue is only found in significant quantities in hibernating and neonatal mammals (Cooney, G.J. and Newsholme, E.A., 1984). The main role of white adipose tissue is to store fatty acids in the form of neutral triacylglycerols, either synthesized *de novo* within the tissue or taken up from the plasma, and to mobilise them when necessary. In liver, dietary fatty acids and fatty acids synthesized *de novo* are esterified to triacylglycerol and secreted with cholesterol and cholesterol ester into the blood in the form of very low density lipoprotein (VLDL) particles. The major lipogenic function of the mammary gland during lactation is to synthesise and secrete triacylglycerol in milk.

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 occurring fatty acids in animal tissues are palmitate (16:0) and oleate (18:1). The biosynthesis of fatty acid occurs almost exclusively in the cytosol, and elongation beyond sixteen carbons and desaturation are carried out by enzymes on the cytosolic face of the endoplasmic reticulum (Stryer, L., 1988).

Fig.1.1. Major pathway of lipid synthesis in mammals



(**1**) Glucose transporter; (**2**) Hexokinase; (**3**) Phosphoglucoisomerase; (**4**) 6-Phosphofructo-1-kinase; (**5**) Aldolase; (**6**) Glyceraldehyde 3-phosphate dehydrogenase; (**7**) Phosphoglycerate kinase; (**8**) Phosphoglyceromutase; (**9**) Enolase; (**1 0**) Pyruvate kinase; (**1 1**) Pyruvate carboxylase; (**1 2**) Pyruvate dehydrogenase; (**1 3**) Citrate synthase; (**1 4**) ATP-citrate lyase; (**1 5**) Acetyl-CoA carboxylase; (**1 6**) Fatty acid synthase; (**1 7**) Glycerol-3-phosphate acyltransferase; (**1 8**) Lysophosphatidate acyltransferase; (**1 9**) Diacylglycerol acyltransferase; (**2 0**) Triosephosphate isomerase; (**2 1**) Glycerol-3-phosphate dehydrogenase; (**2 2**) NAD+-linked malate dehydrogenase; (**2 3**) NADP+-linked malate dehydrogenase (malic enzyme); (**2 4**) Acetoacetyl-CoA thiolase; (**2 5**) HMG-CoA synthase; (**2 6**) HMG-CoA reductase.

In white adipose tissue and lactating mammary gland, the major precursor for *de novo* fatty acid synthesis is glucose (Fig.1.1.). Glucose is taken into a cell by a glucose transporter. Once inside the cell, glucose rapidly undergoes phosphorylation by hexokinase producing glucose 6-phosphate. Glucose 6-phosphate can be utilised in three different ways; as a precursor for glycogen synthesis (in liver and muscle), as a substrate in the pentose phosphate pathway in which it is oxidised to generate the NADPH needed for fatty acid synthesis, or in glycolysis to produce 2 molecules of pyruvate. The net conversion of glucose into pyruvate is:

glucose + $2P_i$ + 2ADP + $2NAD^+$ → 2pyruvate + 2ATP + 2NADH + $2H^+$ + $2H_2O$.

The pyruvate produced by glycolysis or from lactate (primarily in liver by lactate dehydrogenase) is then transferred to the mitochondrion matrix (Fig.1.1.). The pyruvate in mitochondria is converted to acetyl-CoA by pyruvate dehydrogenase (PDH). As acetyl-CoA cannot cross the inner mitochondrial membrane, it must first be converted to citrate by citrate synthase. Citrate is then exported via a protein carrier, and converted back to acetyl-CoA and oxaloacetate in the cytosol by ATP-citrate lyase (ATP-CL). Oxaloacetate is converted in the cytosol to malate and then to pyruvate (by NAD^+-linked malate dehydrogenase and malic enzyme, respectively) producing more of NADPH for fatty acid synthesis (Stryer, L., 1988).

The first committed step for fatty acid synthesis is the production of malonyl-CoA by carboxylation of acetyl-CoA (Fig.1.1.). This reaction utilises ATP and is catalysed by a biotin-containing enzyme, acetyl-CoA carboxylase (ACC). The condensation of acetyl units from malonyl-CoA and their reduction by NADPH is catalysed by the multi-enzyme complex fatty acid synthase. The elongation is usually terminated after seven elongation cycles by thioesterase yielding palmitate (16:0) (Stryer, L., 1988). The mammary gland enzyme complex is unique in that it contains the medium chain thioesterase which terminates elongation earlier after 3-5 cycles to produce a mixture of C-8 to C-12 fatty acids (Libertini, L.J. and Smith, S., 1978). These short and medium chain fatty acids have lower melting points, and may be important in the maintenance of milk fluidity (Munday, M.R. and Hardie, D.G., 1987).

1.2.Potential sites of regulation of fatty acid synthesis

The precusor for fatty acid synthesis in lactating rat mammary gland and adipose tissue is predominantly glucose, whereas liver fatty acid synthesis utilises mostly lactate (Hardie, D.G. et al., 1984). There are a number of potential regulatory steps in the pathway of glucose conversion to fatty acid. These include: the active transport of glucose into the cell, the regulation of glycolysis by 6-phosphofructo-1-kinase, the oxidative decarboxylation of pyruvate in mitochondria by pyruvate dehydrogenase and the first step committed to the synthesis of fatty acids catalysed by acetyl-CoA carboxylase (Fig.1.1.). Each of these proteins has been implicated as a site of metabolic regulation in some of the tissues (Newsholme, E.A. and Start, C., 1981), each has been shown to be sensitive to the action of insulin and has the potential to be regulated via phosphorylation / de-phosphorylation (for review, see Munday, M.R. and Hardie, D.G., 1987).

1.3. Regulation of fatty acid synthesis in the liver

The major precursor for fatty acid synthesis in the liver is lactate and not glucose (Hardie, D.G., 1989). Lactate is derived from anaerobic muscle metabolism or glucose metabolism in intestinal mucosa (Nicholls, T.J. et al., 1983). In rat liver neither glucose nor glycogen are utilised significantly as a precursor for fatty acid synthesis (Geelen, M.J.H. and Hendriks, G.A., 1984). This is partially due to a higher priority for glycogen repletion than *de novo* fatty acid synthesis in the liver. In animals fed on a high protein diet amino acids can also be utilised by the liver as a precursor for fatty acid synthesis, and especially ketogenic amino acids which are broken down to acetyl-CoA and cannot be used in gluconeogenesis (Stryer, L., 1988).

Fatty acyl-CoA synthesized *de novo* in the liver are then esterified to glycerol to form triacylglycerol or esterified to cholesterol to form cholesteryl ester. Triacylglycerol and cholesteryl ester, together with non-esterified cholesterol are packed into very low density lipoprotein (VLDL) and secreted into the blood stream to supply triacylglycerol and cholesterol to the peripheral tissues (for review see Schaefer, E.J., 1988).

In the liver the glucose transporter, hexokinase or 6-phospho-fructo-1-kinase have only minor importance in the control of fatty acid synthesis because lactate is the major precursor. The two important enzymes that regulate liver fatty acid synthesis are the mitochondrial enzyme complex pyruvate dehydrogenase (PDH) and cytosolic enzyme acetyl-CoA carboxylase (ACC; Sugden, M. et al., 1993).

Prolonged starvation of rats caused inactivation of PDH in liver (Wieland, O.H. et al., 1972). The expressed activity of hepatic PDH decreased by 69% and 88% in response to 6 hour and 48 hour starvation, respectively. This correlated well with the inhibition of lipogenesis. These changes in PDH activity can be attributed to the phosphorylation and inactivation of PDH, because total PDH activity (on addition of exogenous PDH phosphatase) did not change (Holness, M.J. and Sugden, M.C., 1990). Refeeding animals with chow diet reversed the effects of starvation on PDH (Holness, M.J. et al., 1988).

In response to a prolonged period of starvation, ACC in rat liver became phosphorylated and inactivated (Thampy, K.G. and Wakil, S.J., 1988).

Insulin stimulates the rate of fatty acid synthesis in isolated hepatocytes by 40% (Holland, R. and Hardie, D.G., 1985). Glucagon inhibits the rate of fatty acid synthesis by 50% in isolated hepatocytes (Holland, R. et al., 1984). Glucagon inhibits glycolysis, but is unlikely to be a significant factor for the inhibition of fatty acid synthesis in the liver because the major precursor for fatty acid synthesis in this tissue is extrahepatic lactate (Hardie, D.G. et al., 1984). Hardie and colleagues proposed that glucagon, through elevating intracellular cAMP concentration, activates cAMP-

dependent protein kinase (cAMP-PK) that in turn phosphorylates and inactivates ACC. Although cAMP-PK from skeletal muscle or bovine heart phosphorylated ACC *in vitro* and caused a 13% decrease in Vmax and 2 fold increase in the Ka for its allosteric activator, citrate (Brownsey, R.W. and Hardie, D.G., 1980; Munday, M.R. et al., 1988), this phosphorylation does not occur *in vivo* (Davies, S.P. et al., 1992) or in isolated hepatocytes (Sim, A.T.R. and Hardie, D.G. 1988). Glucagon in isolated hepatocytes does cause a decrease in ACC activity by 50% (Holland, R., et al., 1984) but increases phosphorylation only on the site phosphorylated *in vitro* by AMP-activated protein kinase (AMP-PK) and not cAMP-PK (Sim, A.T.R and Hardie, D.G., 1988). Cohen and Hardie proposed that activated cAMP-PK in the liver, in response to glucagon, does not directly inhibit ACC but through phosphorylation and inactivation of protein phosphatase-2A which acts on this enzyme (Cohen, P. and Hardie, D.G., 1991).

1.4. Regulation of the Fatty acid synthesis in adipose tissue

The major precursor for *de novo* fatty acid synthesis in adipose tissue is glucose. Fatty acid synthesis and lipolysis in this tissue is under strict hormonal control. In isolated rat adipocytes insulin stimulates fatty acid synthesis from glucose by 25 fold. The stimulatory effect of insulin is inhibited by glucagon and adrenaline (Haystead, T.A.J. and Hardie, D.G., 1988). The dramatic effect of insulin is likely to be due to the stimulation of the glucose transporter, pyruvate dehydrogenase and acetyl-CoA carboxylase. The adipose tissue possesses predominantly glucose transporter type 4 (GLUT-4). GLUT-4 is a highly insulin-responsive glucose transporter. In rat adipocytes, insulin produces an approximate 20-30 fold increase in glucose transport (Gould, G.W. and Holman, G.D., 1993). Insulin-stimulated glucose uptake in adipocytes involves translocation of insulin-responsive glucose transporters (GLUT-4) from the intracellular pool to the plasma membrane and enhancement of their intrinsic activity (Cushman, S.W. and Wardzala, L.J., 1980). Reversal of insulin stimulation

leads to translocation of GLUT-4 from the outer membrane back to an intracellular pool (Karnieli, E. et al., 1981). Under normal circumstances insulin promotes dephosphorylation of GLUT-4 (Reusch, J.E.-B. et al., 1993). Phosphorylation of GLUT-4 in intact adipocytes decreases its intrinsic activity but does not affect insulin-stimulated translocation to the plasma membrane (Reusch, J.E.-B. et al., 1993). *In vitro* phosphorylation of GLUT-4 by cAMP-PK reduced the intrinsic activity of the transporter by 35% (Reusch, J.E.-B. et al., 1993).

In response to 48 hour starvation or alloxan-induced diabetes, PDH activity in white adipose tissue was inhibited (Stansbie, D. et al., 1976). Insulin causes activation and dephosphorylation of PDH in isolated adipocytes (Hughes, W.A. et al., 1980) and this appears to be via stimulation of PDH phosphatase (Denton, R.M. and Hughes, W.A., 1978).

ACC activity increases two fold in epididymal fat-pads when incubated for 30 minutes with insulin (Halestrap, A.P. and Denton, R.M., 1973). In keeping with their antagonism of insulin stimulated lipogenesis, adrenaline and glucagon both increase phosphorylation and inactivation of ACC in isolated adipocytes (Holland, R. et al., 1984; Holland, R. et al., 1985; Haystead, T.A.J. et al., 1990).

At the same time that insulin stimulates fatty acid synthesis in adipose tissue, it inhibits lipolysis. This breakdown of triacylglycerol is catalysed by hormone sensitive lipase (HSL) which is activated by adrenaline, glucagon and ACTH. These hormones cause receptor mediated increases in intracellular cAMP that activate cAMP-PK which in turn phosphorylates and activates HSL (for review see, Yeaman, S.J., 1990). The antilipolytic action of insulin is probably mediated by its activation of cAMP phosphodiesterase and the resulting decrease of cAMP (Degerman, E. et al.,1990).

Adipose tissue from rats fed on a high fat diet (30-40% w/w of lipids in diet) almost totally lose their capacity to convert glucose to fatty acid (Denton, R.M. and Pogson, C.I. 1979). Adipose tissue taken from European humans synthesise little or no fatty

acid from glucose and this is probably due to the high fat diet consumed (Denton, R.M. and Pogson, C.I. 1979).

1.5. Regulation of fatty acid synthesis in mammary gland

In lactating rat mammary gland glucose is the most important precursor of fatty acid synthesis. Rat milk has a relatively high fat content (10.3% w/v lipids; Williamson, D.H. et al., 1984) and at peak lactation a rat produces around 40ml of milk and that puts considerable strain on the mammary gland for triacylglycerol production. Mammary gland has very active lipoprotein lipase (Hamosh, M. et al., 1970) and nearly half of the demands are made from circulating lipoprotein from both dietary and hepatic origin (Hawkins, R.A. and Williamson, D.H., 1972). The remaining half is synthesised *de novo* within the mammary gland. Expressed per gram wet weight of tissue, the rate of fatty acid synthesis in the mammary gland at peak lactation is higher than that observed in any other tissue in either lactating or non-lactating rats (Munday, M.R. and Hardie, D.G., 1987).

The high rate of lipogenesis in lactating mammary gland produces a large demand for substrate, predominantly glucose from blood. At peak lactation the mammary gland utilises 30mmol of glucose per day (Williamson, D.H. and Robinson, A.M., 1977) of which 3 mmol is oxidised completely (Katz, J. and Wals, P.A., 1972), about 7 mmol is used for lactose synthesis (Carrick, D.J. and Kuhn, N., 1978), and the rest is used primarily for fatty acid and triglyceride synthesis (Williamson, D.H., 1980). This glucose intake is five times higher than the intake of the liver of virgin rats the same size (Robinson, A.M., et al., 1978).

To meet this high demand for glucose by the lactating mammary gland, a lactating rat has a higher dietary intake and a decrease in utilisation of glucose by the white adipose tissue (Williamson, D.H., 1980). Also the mammary gland needs to be able to respond rapidly to the change in the availability of glucose in the circulation in order to maintain

maternal carbohydrate balance. For that reason the rate of mammary gland lipogenesis is stringently regulated according to the dietary status of the rat. In response to starvation, the lactating rat shuts down both mammary gland lipogenesis (Robinson, A.M., et al., 1978) and milk production (Brosnan, M.E. et al., 1982; Sampson, D.A. and Jansen, G.R., 1985). This mechanism which favours the survival of the mother rather than the litter, is a sensible choice given the rat's high fertility.

The fatty acid composition of the milk fat resembles the fatty acid composition of the rat diet (Garton, G.A., 1963; Grigor, M.R. and Warren, S.M., 1980). A high-fat diet given through lactation considerably decreases the rate of *de novo* fatty acid synthesis in rat mammary gland (Agius, L. et al., 1980; Grigor, M.R. and Warren, S.M., 1980; Munday, M.R. and Williamson, D.H., 1987), because the lactating rat switches from *de novo* synthesis to utilising those fatty acids of dietary origin.

The response of the lactating rat to food withdrawal is rapid. After a period of starvation for only 6 hours lipogenesis in the mammary gland is inhibited 88% (Jones, R.G. et al., 1984b). There are a number of potential regulatory steps in the pathway of glucose conversion to fatty acid in the lactating mammary gland at which inhibition in response to starvation could be effected. These include: the active transport of glucose into the cell, the regulation of glycolysis by 6-phosphofructo-1-kinase, the oxidative decarboxylation of pyruvate in mitochondria by pyruvate dehydrogenase and the first step committed to the synthesis of fatty acids catalysed by acetyl-CoA carboxylase. In mammary gland in response to starvation glucose transport is depressed (Threadgold, L.C. and Kuhn, N.J., 1984), as are the activities of hexokinase, 6-phosphofructo-1-kinase (Jones, R.G. et al., 1984), pyruvate dehydrogenase (Baxter, M.A. and Coore, H.G., 1978) and acetyl-CoA carboxylase (McNeillie, E.M. and Zammit, V.A., 1982; Munday, M.R. and Williamson, D.H., 1982).

The regulation of these steps in the mammary gland shows asynchronous control. Within the first 6 hours of starvation, glucose uptake, fatty acid synthesis and PDH activity in the mammary gland were inhibited by 82, 87 and 80% respectively, but ACC

activity did not change significantly (Hagopian, K. et al., 1991). This suggests greater importance of PDH than ACC in the regulation of glucose utilisation in the early stages of starvation in mammary gland (Hagopian, K. et al., 1991). The role of ACC became more important in prolonged starvation (>6hour). In response to 24 hour starvation total and expressed ACC activity decreased by approximately 50% (McNeillie, E.M. and Zammit, V.A., 1982; Munday, M.R. and Williamson, D.H., 1982; Hagopian, K. et al., 1991). The decrease in expressed activity has been shown to be due to phosphorylation and inactivation of ACC (Munday, M.R. and Hardie, D.G., 1986). The refractory nature of ACC inhibition in mammary gland during starvation may represent a "locking down" of the already decreased flux from glucose to fatty acid. This is then accentuated by the concomitant increase in citrate concentration during 6-24 hour starvation (due to inhibition of ACC) that inhibits 6-phosphofructo-1-kinase and therefore further inhibits glycolysis and glucose utilisation (Hagopian, K. et al., 1991).

1.5.1. Hormonal regulation of mammary gland lipogenesis during lactation

The lactating rat mammary gland is a highly insulin sensitive and insulin responsive tissue (Burnol, A.-F. et al., 1983; Jones, R.G. et al., 1984a). The lactating mammary gland is rich in insulin receptors (Flint, D.J., 1982). The number of insulin receptors on lactating mammary epithelial cells rises during lactation (Flint, D.J., 1982) while the numbers of receptors on peripheral adipocytes is unchanged (Flint, D.J. et al., 1979). Lactating rats have a 50% lower plasma insulin concentration than virgin or non-lactating rats (Robinson, A.M. et al., 1978) despite their much higher food intake. This would ensure that during lactation white adipose tissue is less responsive to the lowered plasma insulin concentration, while mammary gland retains its responsiveness with its higher concentration of insulin receptors. This leads to a tissue-specific direction of glucose to the mammary gland.

Due to the decrease in insulin concentration the glucagon / insulin ratio in lactation is increased. This causes increased action of glucagon on white adipose tissue which leads to a decrease in fatty acid synthesis and increase in lipolysis. As mammary gland tissue does not possess glucagon receptors (Robson, N.A. et al., 1984), the increased ratio of glucagon does not have any effect on this tissue. The increased glucagon / insulin ratio during lactation together with the insensitivity of mammary gland to glucagon and increased number of insulin receptors in mammary gland tissue, appear to be the main reason for directing the glucose towards the mammary gland at the expense of peripheral adipose tissue.

The evidence that insulin is the hormone responsible for the activation of mammary gland lipogenesis came from dietary and physiological manipulation of lactating rats *in vivo*. Withdrawal of food for 24 hours results in a 98% inhibition of mammary gland lipogenesis which is rapidly reversed by re-feeding for 2 hours (on normal chow diet). These changes correlate with changes in plasma insulin concentration (Robinson, A.M. et al., 1978). The short-term insulin deficiency (caused by administration of streptozotocin) mimics the effect of starvation on mammary lipogenesis (Robinson, A.M. et al., 1978) and insulin administration can partially reverse the inhibition caused by starvation (Munday, M.R. and Williamson, D.H., 1981).

In liver and white adipose tissue, glucagon, and glucagon and adrenaline respectively, are antagonistic to insulin stimulated lipogenesis. However, rat mammary gland has no glucagon receptors (Robson, N.A. et al., 1984) so this hormone cannot be responsible for antagonising insulin effects. The mammary gland does possess a functional cAMP signalling system: $ß_2$-adrenergic receptors (Clegg, R.A. and Mullaney, I., 1985) functionally coupled to adenylate cyclase in the plasma membrane (Bar, H.P., 1973), cAMP-dependent protein kinase (Munday, M.R. and Hardie, D.G., 1984) and phosphodiesterase (Mullaney, I. and Clegg, R.A., 1984). Mammary gland acini do respond *in vitro* to ß-adrenergic agonists and phosphodiesterase inhibitors with a 20-fold increase in cAMP levels (Clegg, R.A. and Mullaney, I., 1985). This leads to

cAMP-dependent kinase activation (Clegg, R.A. and Ottey, K.A., 1990) but the rate of lipogenesis is not affected (Clegg, R.A. et al., 1986). Thus lipogenesis is not inhibited by glucagon or adrenaline in the rat mammary gland *in vivo* (Bussman, L.E. et al., 1984; Jones, R.G. et al., 1984b) or isolated mammary acini *in vitro* (Williamson, D.H. et al., 1983; Jones, R.G. et al., 1984a).

Prolactin appears to have a role in the longer-term control of lipogenic enzyme concentrations (Field, B. and Coore, H.G., 1976; McNeillie, E.M. and Zammit, V.A., 1982) through the regulation of protein synthesis (Williamson, D.H. et al., 1984) and gene expression (Vonderhaar, B.K., 1987), and there is evidence that it mediates the insulin resistance of adipose tissue which occurs during lactation (Ros, M. et al., 1990).

Other hormonal signals e.g. thyroxine and corticosteroids play no role in the acute regulation of mammary lipogenesis, they are involved in the initiation and maintenance of lactogenesis and mammary gland development during pregnancy and early lactation (Topper, Y.J. and Freeman, C.S., 1980).

Whilst any of the regulatory steps mentioned previously may be important in the regulation of lipogenesis, ACC is the enzyme which catalyses the first step that irreversibly commits the substrate to fatty acid synthesis. All three major lipogenic tissues have in common some degree of regulation of the fatty acid synthesis pathway by ACC, and the discussion herein focuses mainly on ACC and its regulation.

1.6.Acetyl-CoA Carboxylase

1.6.1.Structure and function

Acetyl-CoA carboxylase (ACC) is a cytosolic biotinyl enzyme, whose activity was first described by Wakil in 1958. ACC catalyses the first committed step in the synthesis of fatty acids from acetyl-CoA, namely the carboxylation of acetyl-CoA to malonyl-CoA. The overall reaction catalysed by ACC is the sum of two partial reactions:

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

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

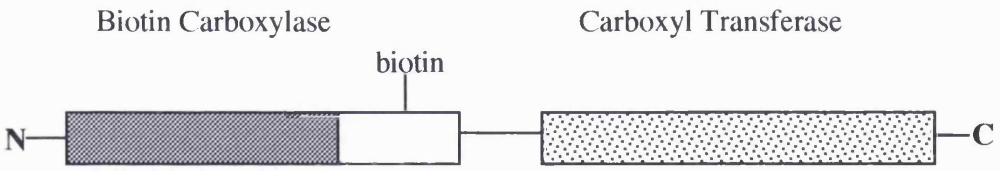
The catalytic sites for each half reaction occur on the same polypeptide chain (Lane, M.D. et al., 1974). The protomeric form of the enzyme is a homodimer (Gregolin, C. et al., 1966) and contains one mole of biotin per subunit. The cDNAs for the enzyme from chicken (Takai, T. et al., 1988) and rat liver (López-Casillas, F. et al., 1988) have been sequenced and the primary amino acid sequences deduced. Each subunit contains 2345 amino acids with a calculated MW 265 220 for rat enzyme (López-Casillas, F. et al., 1988). This is reasonably consistent with the MW determined for purified ACC subunits from rat liver (Song, S.C. and Kim, K.-H., 1981) and rat mammary gland (Ahmad, F. et al., 1978) by SDS-PAGE of about 260 KDa. An isozymic form of ACC of 280 KDa has been found in heart and skeletal muscle where it appears to be the major form of ACC (Thampy, K.G., 1989; Bianchi, A. et al., 1990). This 280 KDa form also co-expresses (although only to the small degree) with the major 265 KDa form in rat liver, mammary gland and brown adipose tissue (Bianchi, A. et al., 1990). In the fed rat, white adipose tissue contains only the 265 KDa form of ACC (Bianchi, A. et al., 1990). The 280 KDa form has a higher Km for acetyl-CoA and higher Ka for citrate (allosteric activator) than the 265 KDa form (Bianchi, A. et al., 1990). One explanation for differences in their Ka for citrate is that they are phosphorylated to a different degree. Bianchi et al. (1990) speculate that the 280 KDa form predominates in tissue with very little fatty acid synthesis (cardiac and skeletal muscle) and may have a

role in the regulation of ß-oxidation. This could be achieved via the production of malonyl-CoA which inhibits carnitine palmitoyl transferase-I (CPT 1; EC 2.3.1.21) a component of the carnitine shuttle which has been shown to be responsible for the transfer of long-chain acyl groups into the mitochondrion for ß-oxidation in rat liver (McGarry, J.D. et al., 1978).

The active ACC protomer can polymerise *in vitro* to a more active polymer in the presence of a high concentration of citrate. The polymers are formed of up to 30 protomers in linear array (Gregolin, C. et al., 1966; Ahmad, F. et al., 1978). Formation of the polymer is also encouraged *in vivo* by insulin (Borthwick, A.C. et al., 1987) possibly through dephosphorylation of inactive ACC protomer or increased phosphorylation of ACC at Ser 29 or site "I", see section 1.6.4.4.).

The domain structure of ACC (Fig.1.2.) was deduced by the homology of regions of primary sequence with that of other enzymes. The N terminal has a strong homology

Fig.1.2. Schematic representation of the structure of Acetyl-CoA Carboxylase from vertebrates



Schematic structure of vertebrate ACC based on homologies with carbamoyl phosphate synthetase, propionyl-CoA carboxylase and the location of covalently bound biotin. Modified from Hardie, D. G. (1989).

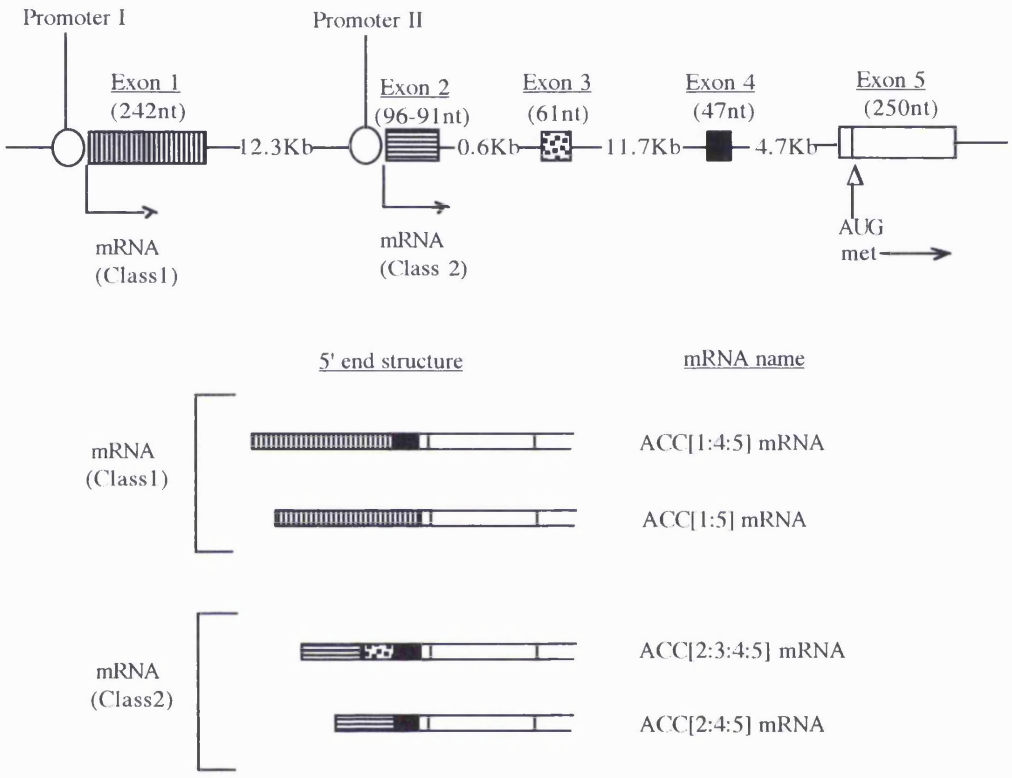
with carbamoyl phosphate synthetase from yeast and rat (López-Casillas, F. et al., 1988) which catalyses a reaction very similar to the first reaction of ACC (i). This region probably therefore represents the biotin carboxylase domain and contains ATP

and HCO_3^- binding sites. The amino acid residues 783-786 (for rat ACC) and 784-787 (for chicken ACC) Val-Met-Lys-Met which are found to bind ^{14}C-biotin (Bai, D.-H. et al., 1989) represent a variation of the normal conserved tetrapeptide sequence for biotin binding sites (Ala-Met-Lys-Met; Samols, D. et al., 1988). The biotin prosthetic group attaches to the ε-NH_2 group of the lysine residue in this sequence. This region probably corresponds to a carboxyl carrier domain. A weak homology exists between the C-terminal of ACC and the ß-subunit of propionyl-CoA carboxylase (the carboxyl transferase subunit; López-Casillas, F. et al., 1988). For this reason it was reasonable to assume that the C-terminal region represents the carboxyl transferase domain.

There is only one copy of the ACC gene per haploid set of chromosomes (López-Casillas, F. et al., 1988) but several forms of ACC mRNA with different 5'-untranslated regions exist in mammary gland (López-Casillas, F. et al., 1989), liver and adipose tissue (López-Casillas, F. and Kim, K.-H., 1989). The generation of different forms of the mRNA is the result of the selective use of two promoters and differential splicing of five different exons (Fig.1.3.). These five exons contain a total of 645 nucleotides and they are scattered over a 50-kilobase-pair genomic DNA region (Luo, X. et al., 1989).

The transcription of the two promoters differs according to tissue and nutritional state. Class 1 ACC mRNA are transcribed from inducible promoter 1 (inducible by diet) and their 5'-end leading sequences are provided by exon 1. Class 2 ACC mRNA are transcribed from the constitutively expressed promoter 2 (expressed under normal conditions) and their leading sequences are derived from exon 2. Promoter 2 does not contain TATA or CAAT boxes (promoter 1 does), but five G/C motifs whose sequences are typical for the transcriptional factor SP1 binding site. This promoter for expression additionally requires enhancer elements which is unusual for an "in house-keeping" gene (Luo, X. and Kim, K.-H., 1990). Class 1 ACC mRNA transcripts had a 6-9 fold better translational efficiency than class 2 transcripts (López-Casillas, F and Kim, K-H., 1991).

Fig.1.3. The structure of the 5'end of the rat ACC gene and the different classes of ACC mRNA produced



The upper panel shows the rat genomic map covering the first five exons of the ACC gene (rectangular boxes), whose sizes, as well as the sizes of introns separating them are also shown. The position of the two ACC gene promoters (elipsoid boxes) are also indicated. The position of the AUG codon of the ACC open reading frame is indicated by a vertical line across exon 5. Two promoters generate two primary transcripts, which are then differentially spliced to produce several different ACC mRNAs. The lower panel represents the structures of the 5' end of those different types of rat ACC mRNA. Class 1 mRNA are generated from the primary transcript initiated by promoter 1, and Class 2 by promoter 2. The names of mRNA are derived from exons composing their 5' untranslated end. The exons composing the 5' untranslated end of those mRNA can also be identified by matching the patterns from the lower and upper panel. Modified from López-Casillas, F., et al. (1991).

1.6.2. Long term regulation

The long term regulation of ACC via enzyme concentration in the cell is achieved through the control of synthesis and degradation of ACC. The rise of ACC concentration that occurs in liver upon refeeding after prolonged starvation can be prevented by inhibiting protein synthesis with puromycin or by preventing DNA transcription with actinomycin D (Hicks, S.E. et al., 1965).

Table 1.1. Changes in degradation and synthesis of ACC during starvation and refeeding in rat liver

	Total ACC (arbitrary units)	$t_{1/2}$ for degradation (hours)	Relative rate of synthesis
Control	1.0	59	1.0
48h fasted	0.5	31	0.5
72h refed (fat free diet)	2.7	55	4.0

Total ACC was measured by immunotitration, and degradation and synthesis was estimated by ^{3}H-Leucine incorporation (from Nakanishi, S and Numa, S., 1970).

Nakanishi and Numa (1970) have shown that the amount of immunotitrable ACC in rat liver is more than halved after 48 hours of fasting and increased nearly 3-fold above controls when the animals were refed a fat free diet for 72 hours. This was due to simultaneous changes in the rate of degradation and synthesis of ACC (Table 1.1.). Lactation results in a decrease in adipose tissue ACC activity (Sinnett-Smith, P.A. et al., 1980) and a rise in both the activity and amount of rat mammary ACC (Mackall,

J.C. and Lane, M.D., 1977), this is accompanied by a corresponding change in mRNA for ACC in the two tissues (López-Casillas, F. et al., 1991).

Prolactin plays a major part in the long term control of ACC during lactation. Lowering the serum prolactin level by bromocriptine treatment or litter removal led to a decrease in the rate of lipogenesis in mammary gland and an increase in the rate of lipogenesis in adipose tissue (Williamson, D.H., 1980; Vernon, R.G. and Flint, D.J., 1983). This is accompanied by a decrease of mammary gland mRNA for ACC and increased mRNA in adipose tissue (Barber, M.C. et al., 1992) and by increased activation status of adipose ACC (Barber, M.C. et al., 1992). Growth hormone also plays a role in the long term regulation of ACC during lactation. Lowering serum growth hormone by itself does not have any significant effect on ACC, but potentiates the decrease of mammary gland ACC mRNA concentration caused by lowered prolactin (but has no effect on adipose tissue ACC).Thus, prolactin has a major and growth hormone has a minor role in the long term regulation of ACC activity during lactation (Barber, M.C. et al., 1992). Changes in mammary ACC activity in response to prolactin and growth hormone are primarily due to alterations in gene transcription, whereas adaptation in adipose tissue involves both changes in gene transcription and activation status (Barber, M.C. et al., 1992).

In non lactating rats triiodothyronine and insulin play a major role in the long term regulation of ACC. In rats fed on a high fat diet (corn oil feeding) the transcription rate of ACC in liver decreases (Katsurada, A. et al., 1990). In diabetic rats the transcription rate, mRNA and enzyme induction in liver are very low. After insulin treatment the transcription rate in diabetic rats increases 2 fold (to normal levels) within 2 hours (Katsurada, A. et al., 1990). Fructose feeding also increases the transcription rate and mRNA for ACC. Triiodothyronine treatment increases the mRNA and enzyme levels in livers of diabetic and normal rats and somehow increases the transcriptional rate only in diabetic rats (Katsurada, A. et al., 1990). Only triiodothyronine treatment increases the

mRNA and enzyme levels even in normal rats. Triiodothyronine appears also to stimulate mRNA stability (Katsurada, A. et al., 1990).

The regulation of ACC gene transcription is not fully understood, but under normal conditions Wistar rats fed on standard chow diet expressed only a basal level of promoter 1 in white adipose tissue and promoter 2 in the liver. Starvation led to the virtual disappearance of transcriptional products from those promoters. Refeeding of rats with a fat-free diet led to activation of promoter 1 in adipose tissue, and promoter 1 and 2 in liver (López-Casillas, F. et al., 1991). Streptozotocin-induced diabetes led to suppression of both promoters in liver and white adipose tissue. Administration of insulin only induces promoter 1 in adipose tissue and has no effect in liver. In rat mammary gland during lactation only promoter 2 is active and its activity is increased during lactation reaching a plateau by day 7 (López-Casillas, F. et al., 1991). At the same time in liver only promoter 2 is active, and in adipose tissue all ACC gene activity is completely shut off (López-Casillas, F. et al., 1991). This indicates that by some mechanism promoters 1 and 2 are controlled by insulin and prolactin respectively. It would thus appear that during lactation, prolactin is capable of switching on the mammary promoter 2 and switching off the white adipose tissue promoter 1 (Barber, M.C. et al., 1992). The later effect must be indirect, as adipocytes appear to lack prolactin receptors (Vernon, R.G. and Flint, D.J., 1983). The effect of prolactin on promoter 1 in white adipose tissue during lactation can be indirect, through the lower insulin concentration observed during lactation and it may inhibit the insulin stimulation of promoter 1 in this tissue.

1.6.3. Short term regulation

Due to the very long half-life of ACC (>24 hours, see Table 1.1.), allosteric effectors and phosphorylation play a major role in its short-term regulation.

1.6.3.1 Allosteric modification

In vertebrates, but not in fungi, or plants, ACC is dramatically activated by citrate (Hardie, D.G., 1989). *In vitro* allosteric activation of ACC is achieved by 2-10 mM concentration of citrate (Martin, D.B. and Vagelos, P.R., 1962; Moss, J. and Lane, M.D., 1971). In most vertebrates citrate is the immediate precusor of cytoplasmic acetyl-CoA, and can be regarded as a "feed-forward activator" (Hardie, D.G., 1989).

There is some evidence to support this *in vivo* . The concentration of citrate required for the half maximal activation (Ka citrate) of mammary gland ACC is 1-2mM (for fed rats) and the intracellular concentration varies between 0.2 and 0.5mM (Williamson, D.H. et al., 1975; Robinson, A.M. and Williamson, D.H., 1977a). Nishkori, K. et al. (1973) reported a rapid rise in citrate concentration, ACC activity and fatty acid synthesis in the liver of starved rats in response to refeeding. There cannot be conclusive evidence for close correlation between citrate concentration and ACC activity *in vivo* because the level of citrate mediated allosteric activation of ACC is also dependent on the phosphorylation state of ACC. For example, prolonged starvation of lactating rats increases mammary gland citrate concentrations over a time course during which ACC is markedly inhibited (Hagopian, K. et al., 1991). During starvation, rat mammary gland ACC is phosphorylated and inactivated with Vmax down 60% and Ka for citrate raised from 2mM to 4mM (100%) which prevents increased citrate concentration from activating ACC. Similarly, a ten fold increased citrate level in epididymal fat pads caused by the administration of fluoroacetate (which is metabolised to fluorocitrate a potent inhibitor of aconitase) did not increase ACC activity (Brownsey, R.W. et al., 1977). This can be explained by the fact that ACC was already phosphorylated and inactivated, and that fluoride is a potent inhibitor of protein phosphatases which act on ACC.

ACC activity is inhibited by fatty acyl-CoA thioesters (Bortz, W.M. and Lynen, F., 1963; Lunzer, M.A. et al., 1977; Ogiwara, H. et al., 1978). Arachidonyl-CoA, stearoyl-CoA and palmitoyl-CoA exhibited the lowest Ki (concentration of inhibitor

which achieved 50% inhibition; <1nM, 1.2nM and 6.5nM respectively) in *in vitro* inhibition of pure ACC (Nikawa, J. et al., 1979). The CoA thioesters of saturated fatty acids with 16-20 carbon atoms inhibit ACC more effectively than those of saturated fatty acid of shorter or longer chain length. The CoA thioesters of unsaturated fatty acids are less inhibitory than those of saturated fatty acids of corresponding chain length (Nikawa, J. et al., 1979). This would suggest that fatty acyl CoA is an end product inhibitor of ACC.

An inverse relationship between the rate of fatty acid synthesis and intracellular levels of fatty acyl-CoA esters has been demonstrated in rat epididymal fat pads treated with insulin (Denton, R.M. and Halperin, M.L., 1968; Saggerson, E.D. and Greenbaum, A.L., 1970) and in the livers of rats starved and refed on a fat-free diet (Nishkori, K. et al., 1973). The role of fatty acyl-CoA esters *in vivo* is extremely difficult to resolve because the true free intracellular levels are very difficult to determine. They are modulated by fatty acid binding proteins (Lunzer, M.A. et al., 1977) and compartmentalisation within the cell. It is estimated that a total fatty acid concentration of 50 - 150 μM is reduced to a free concentration of 1.5 - 15 μM (Brownsey, R.W. and Denton, R.M., 1987; Lunzer, M.A. et al., 1977). The Ki of palmitoyl-CoA for ACC has been reported as < 10nM (Ogiwara, H. et al., 1978; Nikawa, J. et al., 1979). This would suggest that free fatty acids are always at inhibitory levels and thus probably not important physiological mediators. Their role is probably to modulate the ACC response to the allosteric activator, citrate. *In vitro* inhibition of ACC by fatty acyl-CoA esters is competitive with respect to citrate (Goodridge, A.G.J., 1972; Halestrap, A.P. and Denton, R.M., 1974; Ogivara, H. et al., 1978).

The physiological significance of other potential regulators remains to be established. Those regulators include:

(1) activation *in vitro* by GTP and other guanidine nucleotides (Witters, L. A. et al, 1981; Beuchler, K. F. and Gibson, D. M., 1984), polyphosphoinositides especially phosphatidyl inositol 4,5-bisphosphate (Heger, H.W. and Peter, H.W.,

1977; Blytt, H.J. and Kim, K.-H., 1982), small MW substances (Saltiel, A. R. et al., 1983; Haystead, T.A.J. and Hardie, D. G., 1986), an autocrine factor tentatively identified as an oligosaccharide (Witters, L. A. et al., 1988), an ADP-ribosilation (Witters, L.A. and McDermott, J.M., 1986), and a 75KDa protein activator through protein-protein interaction (Quayle, K.A. et al., 1993)

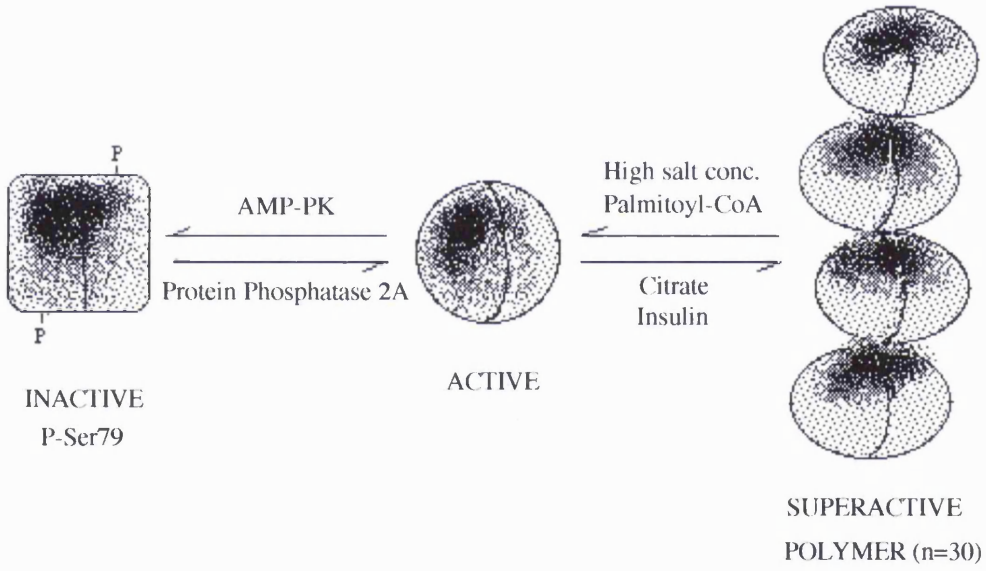
(2) inactivation *in vitro* by a small MW inactivator (Borthwick, A.C. et al., 1990) and by CoA (Moule, K. et al., 1992) although the latter observation is in contrast with previous reports (Yeh, L.-A. and Kim, K.-H., 1980; Yeh, L.-A. et al., 1981).

1.6.3.2. ACC polymerisation

ACC polymerises *in vitro* to form a linear array of up to 30 protomers in length (Ahmad, F. et al., 1978). *In vitro* polymerisation can be induced by citrate and depolymerisation by fatty acyl-CoA esters (Fig.1.4.; Hardie, D.G., 1980; Numa, S. and Tanabe, T., 1984; Brownsey, R.W. and Denton, R.M., 1985). High NaCl concentration also causes disaggregation and for this reason it is used in the purification of ACC by avidin-Sepharose affinity chromatography to shift ACC towards the protomeric form which can then bind to the avidin-Sepharose column. The filamentous form of ACC is more active than the protomeric dimer, but there is evidence that activation by citrate precedes polymerisation (Beaty, N.B. and Lane, M.D., 1983). There is evidence that polymerisation of ACC maybe a consequence of ACC activation. ACC activation by limited trypsinolysis (which removes the major inhibitory phosphorylation site at the N terminus) induces ACC polymerisation (Iritani, N. et al., 1969). Polymerisation is also induced *in vitro* by dephosphorylation (Thampy, K.G. and Wakil, S.J., 1988). Polymerisation may be a way of stabilising ACC activity since Tanaka et al. (1977) reported that the polymeric form of ACC is less susceptible to proteolysis, and Munday, M.R. (unpublished) has observed that this form is resistant to inhibitory phosphorylation. The possible significance of polymerisation *in vivo* was indicated by Ashcraft, B.A. et al. (1980) who reported that fat feeding or starvation,

both of which decrease lipogenesis and ACC activity, causes a decrease in the amount of polymeric ACC (measured via avidin insensitivity) in chick liver. Also Borthwick, A.C. et al. (1987) reported that insulin-treated white adipose tissue which has an elevated ACC activity has more highly polymerised ACC (measured via Superose 6 gel filtration chromatography) than the same tissue treated with isoprenaline (ß-agonist) which antagonises the insulin stimulation of ACC activity.

Fig.1.4. Scheme of protomer-polymer transition of ACC



1.6.3.3. Compartmentalisation of ACC

Allred and co-workers have published several reports describing the regulation of liver ACC by the translocation of an inactive form of the enzyme from the mitochondrial outer membrane to the cytosol, where upon the enzyme became active (Allred, J.B. and Roman-Lopez, C.R., 1988). The mitochondrial enzyme can be mobilised and activated

under lipogenic conditions apparently by an insulin-dependent process. The proportion of active cytosolic to inactive mitochondrial form of ACC decreases on fasting (Allred, J.B. et al., 1985) and increases on refeeding (Roman-Lopez, C.R. et al., 1989). Short term insulin deficiency induced by alloxan increases the amount of inactive ACC in the mitochondrial outer membrane pool (Roman-Lopez, C.R. and Allred, J.B., 1987). Increased ACC activity observed in the liver of genetically obese Zucker rats was due, primarily to a shift in the subcellular distribution towards an active cytosolic form of ACC (Allred, J.B. et al.,1989).

It must be noted however that Moir, A.M.B. and Zammit, V.A. (1990) were unable to observe any translocation from, or existence of, a mitochondrial pool of ACC in response to starvation and refeeding in rat liver.

1.6.4. 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, H. and Lowenstein, J.M., 1972) and from observations that crude preparations of rat liver ACC became inactivated in a time-dependent fashion when incubated with MgATP (Carlson, C.A. and Kim, K.-H., 1973,1974). Phosphorylation and inactivation of ACC in isolated adipocytes caused by glucagon and adrenaline was first reported by Brownsey, R.W. et al. (1979) and in rat hepatocytes caused by glucagon (Holland, R. et al., 1984). These early reports showed that phosphorylation and dephosphorylation of ACC was probably a mode of acute regulation *in vivo* . Purified ACC has been shown to be phosphorylated by a number of protein kinases *in vitro* (Table 1.2.). Four kinases which phosphorylated ACC *in vitro* and produced some degree of inactivation were : cAMP-dependent protein kinase (cAMP-PK) from bovine heart, Acetyl-CoA carboxylase kinase 2 (ACK-2) from lactating rat mammary gland, AMP-activated

Table 1.2. Protein kinases which phosphorylate ACC *in vitro*

Protein kinase	Effect on ACC activity	Reference
cAMP-PK	V_{max} 13% ↓ K_a 234% ↑	Hardie and Cohen, 1978; Hardie and Guy, 1980.
ACK-2	V_{max} 20% ↓ K_a 155% ↑	Hardie and Cohen, 1978; Munday and Hardie, 1984.
AMP-PK	V_{max} 81% ↓ K_a 280% ↑	Carling and Hardie, 1986; Munday et al, 1988.
PKC	V_{max} 14% ↓ K_a 35%↑	Hardie et al, 1986.
Ca^{2+} / CAM dependent multiprotein kinase	no effect	Hardie et al, 1986.
Casein kinase I	no effect	Munday and Hardie, 1984.
Casein kinase II	no effect	Munday and Hardie, 1984.

The well characterised protein kinases that phosphorylate ACC *in vitro* and their effects on ACC activity, and corresponding references are shown. cAMP-dependent protein kinase (cAMP-PK) catalytic sub-unit used in both references was from bovine heart, acetyl-CoA carboxylase kinase 2 (ACK2) was from rat mammary gland and AMP-activated protein kinase (AMP-PK) from rat liver. Ca^{2+} / calmodulin dependent multiprotein kinase (C^{2+}/CAM dependent multiprotein kinase) was from rabbit skeletal muscle and protein kinase C (PKC) was from rat brain. Casein kinase I and II were purified from lactating rat mammary gland.

protein kinase (AMP-PK) from rat liver and Ca^{2+} and phospholipid dependent protein kinase (PKC) from rat brain (Table 1.2.). Casein kinase I and II purified from lactating rat mammary gland phosphorylated ACC *in vitro* but without any effect on ACC activity (Munday, M.R. and Hardie, D.G., 1984). Ca^{2+}/calmodulin dependent

multiprotein kinase purified from rabbit skeletal muscle also phosphorylated *in vitro* ACC without any effect on ACC activity (Hardie, D.G. et al., 1986).

There were also other reports of novel kinases that phosphorylated and inactivated ACC *in vitro*. Shiao, M.-S. et al. (1981) described a kinase with MW of 160 KDa which incorporated 2 to 2.5 mol of phosphate per mol of ACC subunit and led to a rapid decrease of ACC activity. Lent, B. and Kim, K-H. (1982) described an ACC protein kinase from rat liver which incorporated 0.9 mol of phosphate per mol of ACC subunit and produced an 80% drop in ACC activity measured at 2mM citrate concentration. In their purification of this kinase the first two steps are almost identical to the purification of AMP-activated protein kinase (AMP-PK) described by Carling, D. et al. (1989). Although they claimed that their preparation contained a single polypeptide MW of 170 KDa, no evidence was presented that this corresponded to the kinase. Also their purification factor of 260 fold (with only two chromatography columns) seems insufficient to yield a homogeneous preparation. Lent, B. and Kim, K.-H. (1983) also claimed that this kinase was activated by phosphorylation by cAMP-PK and even when fully active required CoA for the inactivation of ACC. This kinase has been studied further by Jamil, H. and Madsen, N.B. (1987). They reported that this kinase produced an 82% fall in the activity of ACC at 10mM citrate and incorporated 0.45 mol of phosphate per mol of ACC subunit. It also increases the Ka for citrate from 1.4 mM to 2.1 mM.

Ramarkrishna, S. and Benjamin, W.B. (1985) described a multifunctional protein kinase (MFPK, a glycogen synthase kinase 3 like kinase) that phosphorylated and increased the Vmax of ACC. They also published a contradictory report that MFPK phosphorylates and inactivates ACC (Ramakrishna, S. and Benjamin, W.B., 1988). Unfortunately no other reports clarifying the effects of this kinase on ACC have been published.

Borthwick, A.C. et al. (1990) reported that partially purified insulin stimulated protein kinase (ISPK) from insulin stimulated epididymal fat-pads phosphorylated and activated ACC by diminishing the inhibition of ACC by low MW inhibitor.

1.6.4.1. The phosphorylation sites on ACC

ACC contains several phosphorylation sites that have been identified following *in vitro* phosphorylation by the different kinases shown in Table 1.2. or from ACC purified from ^{32}P-labelled hepatocytes and adipocytes (Haystead, T.A.J. and Hardie, D.G., 1988; Haystead, T.A.J. et al., 1988; Munday, M.R. et al., 1988; Sim, A.T.R. and Hardie, D.G., 1988). These sites are shown in Fig.1.5. Six of the eight phosphorylation sites occur in two clusters in the first 100 amino acids of the amino terminus of ACC. The remaining two sites Ser-1200 and Ser-1215 in the rat liver ACC sequence (López-Casillas, F. et al., 1988) are situated in the hinge region of ACC between the two catalytic domains.

1.6.4.2. The role of phosphorylation of Ser-77 and Ser-79 in the regulation of ACC activity

Only phosphorylation of Serine 77 and 79 on ACC directly correlates with inactivation of ACC (Munday, M.R. et al., 1988) and removal of these sites by limited trypsinolysis completely restores the activity of ACC even though Serine 1200 and 1215 remain fully phosphorylated (Davies, S.P. et al., 1990). Phosphorylation of Ser-77 by cAMP-PK, ACK2 or PK-C *in vitro* produces only a small drop in the Vmax of ACC. Only phosphorylation of Ser-79 (which is exclusively phosphorylated by AMP-activated protein kinase) led to a large enough decrease in Vmax (81%) to account for the Vmax decreases that are observed *in vivo* in response to hormonal and physiological manipulations. Ser-79 and not Ser-77 contains phosphate *in vivo* (Sim, A.T.R and

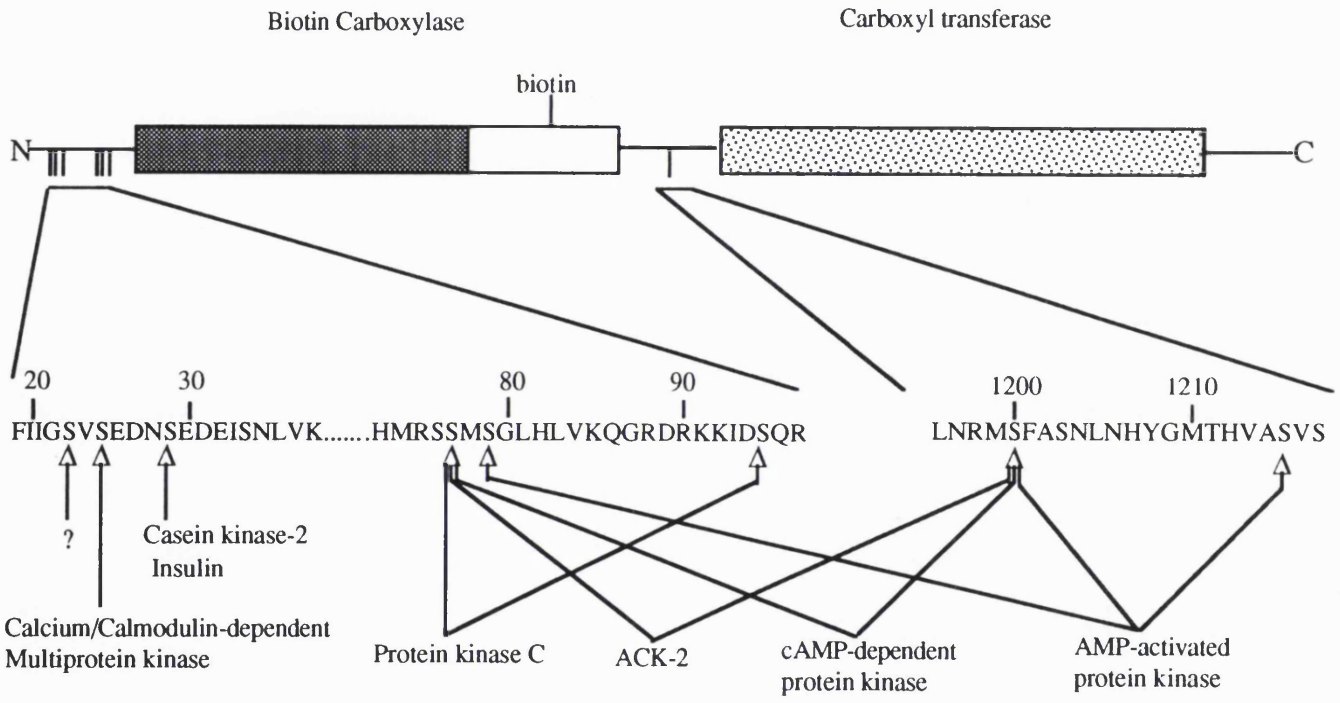
Hardie, D.G., 1988; Davies, S.P. et al., 1992). The reason for a small decrease in Vmax after phosphorylation of Ser-77 is probably due to its proximity to the real regulatory site Ser-79 (Davies, S.P. et al., 1992).

1.6.4.3. Other phosphorylation sites

Phosphorylation of purified ACC by Casein kinase I and II from rat liver and lactating mammary glands does not affect the kinetic properties of ACC (Tipper, J.P. et al., 1983; Munday, M.R. and Hardie, D.G., 1984). Casein kinase II phosphorylated Ser-29 on ACC which is also phosphorylated in intact adipocytes in response to insulin (Haystead, T.A.J. et al., 1988). Phosphorylation of purified ACC by Ca^{2+} calmodulin-dependent multiprotein kinase does not have any effect on ACC kinetic parameters (Hardie, D.G. et al., 1986). This phosphorylation occurs at Ser-25 of rat ACC (Haystead, T.A.J. et al., 1988) and although it has no effects on ACC activity, this site does contain phosphate in intact cells.

Protein kinase C also phosphorylated pure ACC *in vitro* causing a small decrease of Vmax (Hardie, D.G. et al., 1986). In this case, phosphorylation occurs on two sites, Ser-77 and Ser-95 (Haystead, T.A.J. and Hardie, D.G., 1988) of rat ACC. By comparison with the effects of cAMP-PK and ACK-2 on ACC activity and the sites that these phosphorylate (Fig.1.5.) it is reasonable to assume that it is phosphorylation of Ser-77 by PKC that produces this small drop in Vmax (13-20%). Despite the fact that PKC phosphorylated ACC *in vitro*, phosphorylation of those two residues does not occur in intact cells, even after stimulation by phorbol esters, known activators of PKC (Haystead, T.A.J. and Hardie, D.G., 1988).

Fig.1.5. Phosphorylation Sites on Acetyl-CoA Carboxylase (from rat)



Location of phosphorylation sites on acetyl-CoA carboxylase.The question mark indicates serine residue which is found phosphorylated in intact adypocytes after treatment with insulin and epidermal grwt factor (Haystead,T.A,J.,Campbell,D.G. and Hardie,D.G. 1988). Modified from Hardie,D.G, Carling,D. and Sim,A.T.R. (1989).

1.6.4.4. Role of insulin in phosphorylation of ACC

The role of insulin in the control of the phosphate content of ACC seems paradoxical. Insulin activates ACC, but there is still some uncertainty regarding the role of phosphorylation in this activation. Increased phosphorylation of ACC after insulin treatment has been documented in adipocytes. This was first observed by Brownsey and Denton (1982) who reported increased phosphate in a specific tryptic peptide (the "I" site) in response to insulin and has been confirmed by several other reports. In isolated adipocytes or hepatocytes ACC is both activated and phosphorylated within the 15 minutes of addition of insulin or epidermal growth factor (EGF) (Holland, R. and Hardie, D.G., 1985; Haystead, T.A.J. et al., 1988a,b; Halestrap, A.P. and Denton, R.M., 1973). At least three laboratories have reported a single peptide whose phosphorylation increased in response to insulin in adipocytes and hepatocytes (Holland, R. and Hardie, D.G., 1985; Brownsey, R.W. and Denton, R.M., 1982; Witters, L.A. et al., 1983), but because of differences in methodology it has not been clear that these peptides are the same. It has also been in dispute whether the increased phosphorylation causes the activation of the ACC, because the effect of insulin on enzyme activity does not persist during enzyme purification, although the phosphorylation state is preserved (Brownsey, R.W. and Denton, R.M., 1982; Witters, L.A. et al., 1983). Haystead, T.A.J. and Hardie, D.G. (1986) found that addition of purified protein phosphatase to extracts of insulin-treated adipocytes caused dephosphorylation of the insulin sensitive site without reversing the effect of insulin on ACC activity. Increased phosphorylation of ACC in response to insulin does not prove that insulin stimulated dephosphorylation is not important in regulating the ACC activity. ACC is phosphorylated on multiple sites and reciprocal changes in the phosphorylation state of sites in the enzyme might occur, as observed for ATP-citrate lyase (Ramakrishna, S. et al., 1989) and glycogen synthase (Lawrence, J.C.Jr. et al., 1986) in fat cells. Recent results indicate that ACC is dephosphorylated by insulin in

liver cells. Mabrouk, G.M. et al. (1990) purified ACC from freeze-clamped livers of control and insulin-treated rats and found that insulin increases activity from 0.8 to 2.0 units/mg, while decreasing phosphate content by approximately 20 % (from 8.5 mol to 7 mol of Pi/mol of ACC subunit). This is a relatively small percentage change, but it could be significant in view of the complex pattern of phosphorylation of the ACC. Witters, L.A. et al. (1988) found that insulin decreased by approximately 40% the amount of 32P in ACC in 32P-labelled Fao Reuber hepatoma cells. The insulin effect on activity, which represents a threefold increase, persisted following purification of the enzyme, as would be expected if change in activity was due to change in phosphorylation.

Quantitation by HPLC separation of proteolytically derived peptides of sites whose phosphorylation was increased in the presence of insulin, reveal two sites on ACC. One, which accounts for most increase in response to insulin corresponds to the site Ser-29 on ACC phosphorylated *in vitro* by Casein kinase II, and a minor site is phosphorylated only to a small extent (Haystead, T.A.J. et al., 1988). As Ser-29 phosphorylation by Casein kinase II *in vitro* does not have any effect on kinetic parameters of ACC it is reasonable to assume that increased phosphorylation of ACC at this residue (probably Casein kinase II) does not cause direct changes in ACC activity. The prevalent view is that an increase in phosphorylation of ACC in response to insulin does not directly activate the enzyme (Lawrence, J.C.Jr., 1992).

An additional indication that a possible mode of insulin action on ACC is through increased dephosphorylation came from the observation that okadaic acid treatment of rat epididymal fat-pads and isolated adipocytes abolished the 2-3 fold activation observed in insulin treated tissue. Okadaic acid (a protein phosphatases 1 and 2A inhibitor) increased the overall phosphorylation of ACC including the "I" site peptide and the peptide containing the inhibitory Ser-79 (Rutter, G.A. et al., 1991). They concluded that extensive phosphorylation of "inhibitory site" is capable of overriding any possible activatory effect of phosphorylation of site "I", but a much simpler

explanation would be that insulin acts on ACC through increased activity of protein phosphatase and that the observed increase of phosphorylation of site "I" does not have any significant physiological effect.

However, phosphorylation of site "I" may have an indirect role in regulation of ACC activity. Sommercorn, J. et al. (1987) proposed that phosphorylation of Ser-29 enhanced the dephosphorylation of site Ser-79. Borthwick, A.C. et al. (1990) reported that in isolated epididymal fat-pads insulin stimulates phosphorylation of ACC on site "I" by insulin stimulated protein kinase (ISPK). Phosphorylation of purified ACC by partially purified ISPK appears to activate ACC by blocking the effect of a low MW inhibitor. Although the authors explanation is that phosphorylation of ACC by ISPK leads to increased activity by diminishing the inhibition by low MW inhibitor, this also can be explained by direct phosphorylation of inhibitor by ISPK.

1.7. The role of AMP-PK and cAMP-PK in regulation of ACC *in vivo*

Although cAMP-PK from bovine heart can phosphorylate and inactivate ACC *in vitro* (Munday, M.R. et al., 1988), there is now much evidence to suggest that the protein kinase responsible for the phosphorylation and inactivation of ACC *in vivo* is AMP-PK. In isolated rat hepatocytes in response to glucagon (Sim, A.T.R. and Hardie, D.G., 1988) and in isolated adipocytes in response to adrenaline (Haystead, T.A.J. et al., 1990) increased phosphorylation of Ser-79 of ACC (the site phosphorylated *in vitro* by AMP-PK) and not Ser-77 (the site phosphorylated by cAMP-PK *in vitro*) was observed. From experiments *in vitro* with pure ACC phosphorylated by AMP-PK (from rat liver) and cAMP-PK (from bovine heart) phosphorylation of Ser-79 and Ser-77 appears mutually exclusive (Munday, M.R. et al., 1988). The sequence around Ser-77 on ACC which is phosphorylated *in vitro* by cAMP-PK contains only one arginine residue at position -2 preceding the phosphorylation site. A typical recognition site for cAMP-PK contains two arginines in position -2 and -3 (Taylor, S.S. et al., 1990) and

replacement of either of those arginines in a synthetic peptide substrate increases the Km by nearly three orders of magnitude (Kemp, B.E. et al., 1977). Therefore Ser-77 on ACC is likely to be a poor substrate for cAMP-PK and such phosphorylation is unlikely to occur *in vivo*.

Davies, S.P. et al. (1992) have shown that purified ACC from rat liver sampled without freeze-clamping has decreased activity and increased phosphate content in Ser-79, Ser-1200 and Ser-1215, the three sites phosphorylated *in vitro* by AMP-PK. This correlated with an increase in AMP-PK activity and a large increase in AMP and decrease in ATP (probably caused by hypoxia during the removal of the liver without freeze-clamping; Davies, S.P. et al., 1992).

The extent of ACC Vmax inhibition *in vivo* can only be matched *in vitro* by phosphorylation of ACC by AMP-PK and not by cAMP-PK (Munday, M.R. et al., 1988).

In isolated hepatocytes (Sim, A.T.R. and Hardie, D.G., 1988), isolated adipocytes (Haystead, T.A.J. et al., 1990) and in studies with rat liver (Davies, S.P. et al., 1992) it was found that under no conditions did Ser-77 contain phosphate. There is, hence considerable evidence that AMP-PK is responsible for the phosphorylation and inactivation of ACC *in vivo*.

1.8. AMP-activated protein kinase (AMP-PK)

AMP-PK, first described as acetyl-CoA carboxylase kinase-3, was discovered in rat liver by Dave Carling when Hardie's group were looking for a cAMP-independent kinase that would phosphorylate and inactivate ACC and account for the changes in enzyme V_{max} observed after physiological manipulations (Carling, D. and Hardie, D.G., 1986). Acetyl-CoA carboxylase kinase-3 was found to phosphorylate and almost totally inactivate not only ACC but also HMG-CoA reductase, and like the HMG-CoA

reductase kinase activity previously described (Beg, Z.H. et al. in 1979) it was activated by AMP. Acetyl-COA carboxylase kinase-3 and the previously described HMG-CoA reductase kinase are identical (Carling, D. et al., 1989) and this activity has been named AMP-activated protein kinase (Carling, D. et al., 1987,1989).

AMP-PK purified from rat liver is a serine kinase with MW of 63 KDa (on SDS-PAGE) and a native MW of 100 ± 30 KDa according to gel filtration (Carling, D. et al., 1989). AMP-PK is activated by 5'AMP (Ka 1.4μM at 0.2mM ATP) and is phosphorylated and activated by a distinct "kinase-kinase", and inactivated by dephosphorylation by protein phosphatases. The "kinase-kinase" seems to be activated by nmolar concentrations of palmitoyl-CoA (Carling, D. et al., 1987).

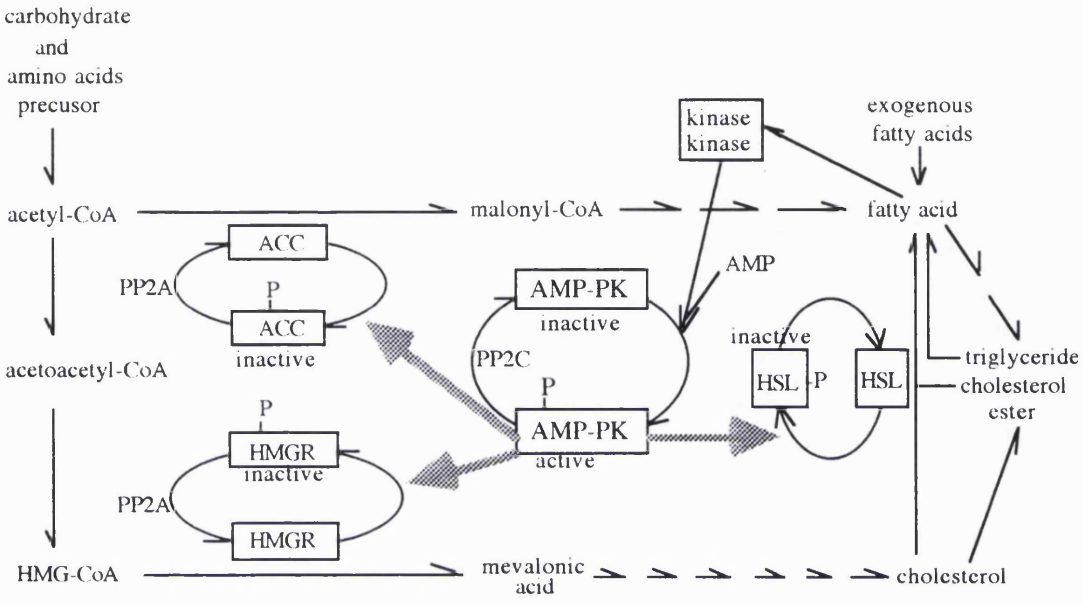
1.8.1. Physiological targets of AMP-PK

AMP-PK plays a central role in the regulation of fatty acid and cholesterol metabolism (for review see Hardie, D.G., 1992). The three enzymes which catalyse regulatory steps in fatty acid synthesis (acetyl-CoA carboxylase), cholesterol synthesis (HMG-CoA reductase), and breakdown of triacylglycerols and cholesterol esters (hormone-sensitive lipase) are regulated by AMP-PK (Fig.1.6.; Hardie, D.G. et al., 1989).

Phosphorylation of ACC on Ser-79 by AMP-PK causes direct inactivation and the crucial role of this phosphorylation in the regulation of ACC *in vivo* has already been discussed in section 1.7.

HMG-CoA reductase (HMG-CoAR) is regulated *in vivo* by reversible phosphorylation (for review see Zammit, V.A. and Easom, R.A., 1987). AMP-PK phosphorylates HMG-CoAR on Ser-871 *in vitro* and inactivates the enzyme (Clarke, P.R. and Hardie, D.G., 1990a). When rats are killed and the liver removed by dissection (without cold-clamping), HMG-CoAR becomes highly phosphorylated and inactivated (Easom, R.A. and Zammit, V.A., 1984a). This is due to its increased phosphorylation on Ser-871 by

Fig.1.6. Physiological role of the AMP-PK cascade



The diagram shows the role of AMP-PK in the regulation of the cell level of free fatty acids and cholesterol by the control of acetyl-CoA carboxylase (ACC), HMG-CoA reductase (HMGR) and hormone-sensitive lipase/cholesterol esterase (HSL). The hormone-sensitive lipase is present in tissues whose function is the mobilisation of stored lipids, and is absent from liver because liver does not contain stored lipids. Modified from Hardie, D.G (1992) and Hardie, D.G. et al. (1989).

AMP-PK which has been activated by AMP generated by hypoxia that this method of tissue sampling causes.

Increased AMP concentration in hepatocytes induced by incubation with fructose causes a similar increased phosphorylation of Ser-871 and inactivation of HMG-CoAR (Gillespie, J.G. and Hardie, D.G., 1992). Although protein kinase C (PKC) and calmodulin-dependent multiprotein kinase also phosphorylate Ser-871 and cause inactivation of HMG-CoAR *in vitro* (Clarke, P.R. and Hardie, D.G., 1990b) there is evidence from experiments using Ca^{2+} mobilising agents in isolated hepatocytes that

this does not occur in intact cells (Zammit, V.A. and Caldwell, A.M., 1990); Zammit, V.A. and Caldwell, A.M., 1991). At present AMP-PK appears to be the only protein kinase which is responsible for the physiological regulation of HMG-CoAR (Hardie, D.G., 1992).

Table 1.3. AMP-PK substrate specificity

Phosphorylation substrate	Phosphorylation sites	Phosphorylation relative rate	Phosphorylation effect
HMG-CoA reductase	Ser: 871	--	inhibition
ACC	Ser: 79, 1200, 1215	100	Vmax 90% ↓
HSL	Ser: 565	40	indirect inhibition
Phos.Kinase	Ser: 1018, 1020	62	none
Glycogen Synthase	Ser: 7	73	?
ATP-CL	unknown	5	none ?
ApoB100	unknown	<5	?

Phosphorylation of hormone sensitive lipase (HSL) by AMP-PK on Ser-565 blocks phosphorylation on Ser- 563 and activation of the enzyme by cAMP-PK (Garton, A.J. and Yeaman, S.J., 1990; Garton, A.J. et al., 1989). Since HSL appears to be essentially inactive *in vivo* unless it is phosphorylated by cAMP-PK (Nilsson, N.O. et al., 1980), this means that activation of AMP-PK will inhibit the release of fatty acids and cholesterol from cytosolic stores. The end result is similar to that of

phosphorylation and inactivation of ACC and HMG-CoAR, ie. reduction of cholesterol and unesterified fatty acids in the cell (Hardie, D.G., 1992).

The physiological role of the phosphorylation Ser-7 of glycogen synthase by AMP-PK is not clear. The phosphorylation of glycogen synthase at Ser-7 occurs *in vivo* (Poulter, L. et al., 1988) and is known to inactivate the enzyme via an increase in the Ka for the allosteric activator, glucose-6-phosphate (Picton, C. et al., 1982). However, several other protein kinases phosphorylate this same residue *in vitro* so at present is not clear which kinase is responsible *in vivo*. It is possible that AMP-PK could inactivate glycogen synthase when the energy status of the liver or muscle is low (eg. due to anoxia) thus preventing a flux of energy towards storage as glycogen until the energy status normalises.

AMP-PK phosphorylates ATP-citrate lyase (ATP-CL) *in vitro* (Carling, D. and Hardie, D.G., 1989). Phosphorylation of ATP-CL from lactating rat mammary gland *in vitro* by cAMP-PK, PKC or Ca2+/calmodulin dependent protein kinase occurs on the same serine residue and does not cause any change in enzyme activity (Hardie, D.G. et al., 1986). In isolated hepatocytes insulin and glucagon, which have opposite effects on the rate of lipogenesis, both cause increased phosphorylation of ATP-CL at the same serine residue as that phosphorylated by cAMP-PK *in vitro* (Hardie, D.G. et al., 1986) and in neither case does the degree of phosphorylation exceed 0.1 - 0.15 mol/mol subunit nor does ATP-CL activity change (Alexander, M.C. et al., 1982). At present, it appears that phosphorylation does not play a role in the regulation of ATP-CL activity.

AMP-PK also phosphorylates ApoB-100 *in vitro*, a VLDL structural protein (Takhar, S., 1992). The phosphorylation appears to be at different sites from those phosphorylated by cAMP-PK but their identity is unknown. Whether such phosphorylation occurs *in vivo* and its possible physiological role is unclear.

There were recent reports that implicated AMP-PK in phosphorylation and inactivation of diacylglycerol kinase II from rat brain *in vitro* (Chen, Q. et al., 1990; Chen, Q. et al., 1991) but the physiological significance of this finding is unclear at present.

1.8.2. Substrate specificity and consensus sequence:

The identification of Ser-79 on rat ACC as a major AMP-PK phosphorylation site led to the development of an AMP-PK assay using a specific peptide substrate (SAMS). The AMP-PK specific substrate (SAMS peptide) has the sequence based on the sequence around serine-79 on rat ACC (His-Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg) with the serine-77 (phosphorylated by other protein kinases) replaced by alanine which cannot be phosphorylated (Davies, S.P., et al., 1989).

From comparisons of the known amino acid sequences of sites phosphorylated by AMP-PK on native proteins a consensus recognition sequence for AMP-PK was proposed: a hydrophobic residue (methionine, leucine, isoleucine or valine) at position 1 on the N-terminal side of the phosphorylated serine (position -1) and position 4/5 on the C-terminal side (position +4/+5), with at least one arginine at position -2, -3 or -4 (Carling, D. and Hardie, D.G., 1989). Unfortunately, the sequences of the phosphorylation sites of rat HMG-CoA reductase (Ser-871) and rat ACC (Ser-1215) do not meet those requirements (see Table 1.4.). Very recently Weekes, J. et al. (1993) proposed a new consensus sequence which underlined the importance of a hydrophobic residue (M,V,L,I) at position -5 and +4 and at least one basic residue (R,K,H) at position -2, -3 or -4. The importance of these residues were established using a series of synthetic peptides. Weekes, J. et al. (1993) also showed that AMP-PK can phosphorylate threonine, but not tyrosine in the substituted SAMS peptide (SAMT and SAMY respectively). Even this proposed amendment does not explain all the sequences recognised by AMP-PK, particularly those sequences surrounding the phosphorylation sites of phosphorylase kinase. From the Table 1.4. it also appears that AMP-PK has a

Table. 1.4. Sequences of phosphorylation sites phosphorylated by AMP-PK

AMP-PK substrate	Sequence of phosphorylation sites	References
SAMS peptide	HMRSAMSGLHLV	Davies,S.P., Carling,D. and Hardie,D.G., 1989
Rat ACC (Ser-79)	HMRSSMSGLHLV	Davies,S.P., Sim,A.T.R. and Hardie,D.G. (1990)
Chicken ACC (Ser-80)	HMRPSMSGLHLV	Hardie,D.G., Carling,D. and Sim,A.T.R. (1989)
Rat ACC (Ser-1215)	GMTHVASVSDVL	Davies,S.P., Sim,A.T.R. and Hardie,D.G. (1990)
Rat HSL (Ser-565)	SMRRSVSEAALA	Hardie,D.G., Carling,D. and Sim,A.T.R. (1989)
Bovine HSL (Ser-?)	PMRRSVSEAALA	Garton,A.J. et al. (1989)
Rabbit muscle Phos.Kinase (α, Ser-1020)	FRRLSISTES	Carling,D. and Hardie,D.G. (1989)
Rabbit muscle Phos.Kinase (α, Ser-1018)	VEFRRLSISTES	Carling,D. and Hardie,D.G. (1989)
Rabbit muscle GS (Ser-7)	PLSRTLSVSSLP	Carling,D. and Hardie,D.G. (1989)
Rat ACC (Ser-1200)	PTLNRMSFASNL	Davies,S.P., Sim,A.T.R. and Hardie,D.G. (1990)
Chicken ACC (Ser-1193)	SHPNRMSFSSNL	Munday,M.R. et al. (1988)
Rat HMG-CoA R (Ser-871)	HMVHNRSKINLQ	Clarke,P.R. and Hardie,D.G. (1990)

The amino acid sequence around sites phosphorylated by AMP-PK is presented using one letter amino acid code. The phosphorylated serine is represented by a bold and underlined letter (**S**). Hydrophobic residues (methionine, leucine, isoleucine or valine) at position -1 and +4/+5 are represented with the appropriate letter in bold print and basic residues (usually arginine) required N terminally to the phosphorylated serine are underlined.

preference for Arg in position -4, and that the motif: S-X-S plays some role (where X represents preferably one hydrophobic residue). S-X-S is a ß-turn motif (Davies, S.P. et al., 1989), which suggests that secondary structure also plays some importance in substrate recognition by AMP-PK.

1.8.3. Tissue distribution of AMP-PK

AMP-PK is found in all lipid metabolising tissues. Rat liver and lactating mammary gland, are very active in fatty acid and cholesterol synthesis and express the highest AMP-PK activity (Fig.1.7; Davies, S.P. et al., 1990).

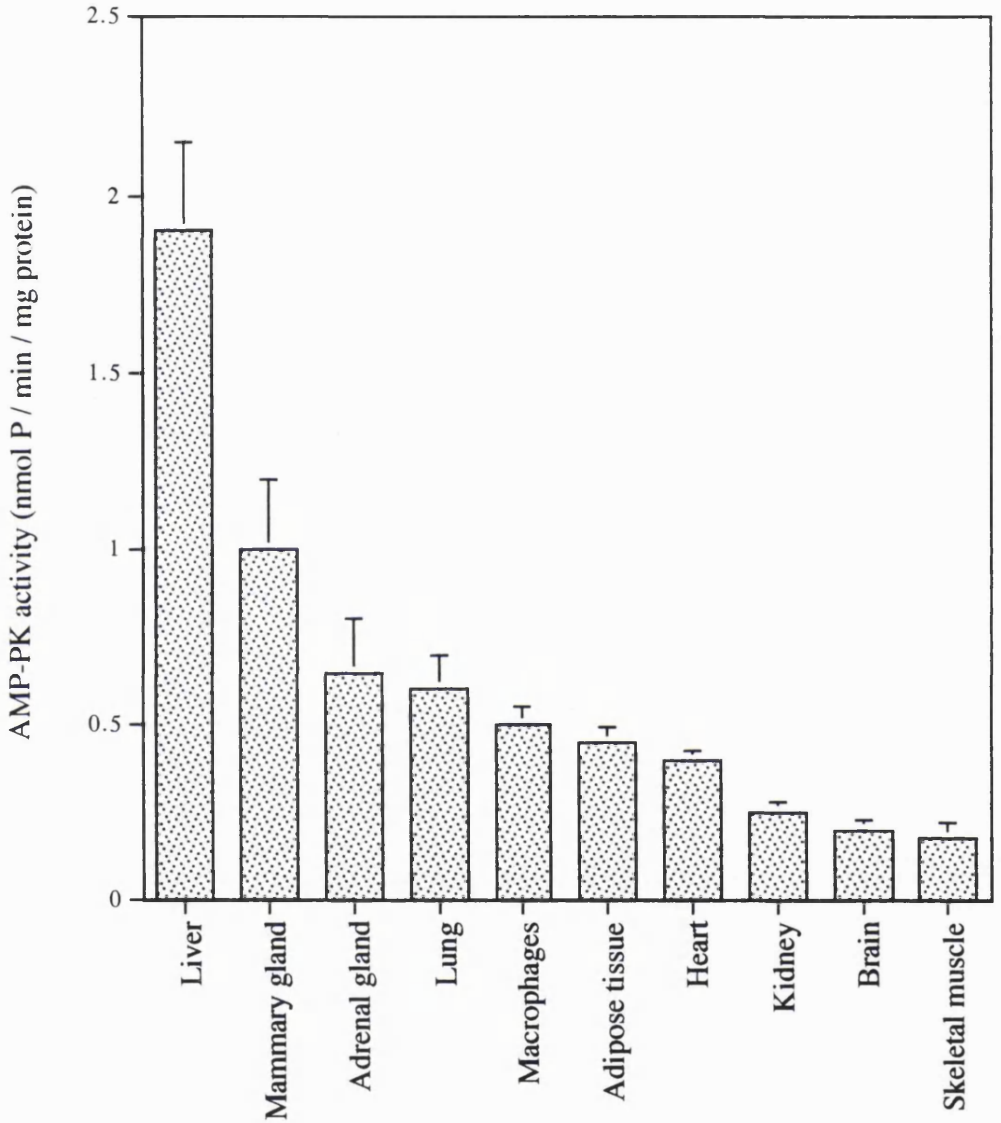
1.8.4. Regulation of AMP-PK by 5'AMP

5'AMP allosterically activates AMP-PK and produces an increase in Vmax (3-7 fold). At an ATP concentration in the assay of 0.2mM the Ka for AMP is 1.4μM. ATP is an antagonist to AMP at the AMP-binding site and when AMP-PK was assayed at more physiological concentrations of ATP (2mM) the Ka for 5'AMP shifts to 14μM (Carling, D. et al., 1989). When hepatocytes are starved of oxygen, the AMP level rises dramatically due to the action of adenylate kinase, which ensures that any small drop in the ATP levels are accompanied by a much larger increase in AMP (Vincent, M.F. et al., 1982). Activation of AMP-PK by AMP may therefore be a mechanism for switching off biosynthetic pathways (fatty acid and cholesterol synthesis) when ATP is limiting.

1.8.5. Regulation of AMP-PK by phosphorylation

The first evidence that AMP-PK is regulated by phosphorylation came from Ingebritsen, who showed that purified protein phosphatase will inactivate HMG-CoAR

Fig.1.7. Tissue distribution of AMP-activated protein kinase



AMP-PK activity was measured in post-mitochondrial crude extracts from various rat tissues following precipitation by ammonium sulphate. Results shown are means of 4 independent experiments. SEM are represented by vertical bars. Modified from Davies, S.P. et al. (1990).

kinase, and that reactivation is achieved by incubation with MgATP and a fraction which appears to be separable from the kinase itself (Ingebritsen, T.S. et al., 1981). This dephosphorylation and inactivation by protein phosphatases and phosphorylation and activation by distinct kinase-kinase has been cofirmed for AMP-PK (Carling, D. et al., 1987). This phosphorylation and activation is stimulated by AMP (Moore, F. et al., 1991; Hardie, D.G., 1992) and it seems likely that AMP causes conformational changes in AMP-PK which activates the enzyme and exposes the phosphorylation site for the kinase-kinase. It is also possible that kinase-kinase is by itself activated by 5'AMP (Moore, F. et al., 1991). The kinase-kinase itself is stimulated by sub μM concentrations of palmitoyl-CoA (Carling, D. et al., 1987; Davies, S.P. et al., 1989).

1.8.6. Protein phosphatases acting on the AMP-PK cascade

Protein phosphatases 1, 2A and 2C account for the majority of the dephosphorylation of cytosolic proteins phosphorylated on serine residues (Cohen, P., 1989). In experiments with crude cell extracts protein phosphatase 2A and 2C accounted for most of the protein phosphatase activity against ACC, HMG-CoA reductase and AMP-PK, with only a small contribution by protein phosphatase 1 (Clarke, P.R. et al., 1991; Haystead, T.A.J. et al., 1990). Results from experiments with intact adipocytes and hepatocytes using okadaic acid (a potent inhibitor of protein phosphatases 1 and 2A but not 2C) suggested that dephosphorylation of HMG-CoA reductase and ACC is primarily due to protein phosphatase-2A (Zammit, V.A. and Caldwell, A.M., 1990; Moore, F. et al., 1991; Haystead, T.A.J. et al., 1989). The phosphorylation of AMP-PK in intact hepatocytes was not affected by okadaic acid suggesting that the protein phosphatase responsible for dephosphorylation of AMP-PK is protein phosphatase-2C (Moore, F. et al., 1991).

1.8.7. Regulation of the AMP-PK cascade

The inhibition of the AMP-PK cascade and activation of ACC and HMG-CoAR may be controlled by insulin. In rat hepatoma cells and intact livers, insulin causes a dephosphorylation of ACC accompanied by increased Vmax and decreased Ka for citrate (Witters, L.A. et al., 1988; Witters, L.A. and Watts, T.D., 1988; Mabrouk, G.M. et al., 1990). These activity changes are consistent with the expected kinetic effects of dephosphorylation of Ser-79, the AMP-PK phosphorylation site, on ACC. In parallel experiments, insulin inactivated AMP-PK in hepatoma cells (Witters, L.A. and Kemp, B.E., 1992). These changes in AMP-PK activity in response to insulin are stable and survive high dilution prior to assay, suggesting that they are mediated by covalent modification. The exact mechanism of insulin action at present is unknown it may involve increased activity of PP-2A and 2C, or direct or indirect inactivation of kinase-kinase.

In liver and white adipose tissue, glucagon, and glucagon and adrenaline respectively, are antagonistic to insulin stimulated lipogenesis. Glucagon inhibits the rate of fatty acid synthesis by 50% in isolated hepatocytes accompanied by a 50% decrease in ACC activity (Holland, R. et al., 1984). This ACC inactivation results from increased phosphorylation of Ser-79 on ACC (the site phosphorylated *in vitro* only by AMP-PK; Sim, A.T.R. and Hardie, D.G., 1988). In isolated adipocytes adrenaline and isoproterenol (ß-agonist) cause inactivation of ACC and an increase in phosphorylation of Ser-79 on ACC (Haystead, T.A.J. et al., 1990). Both these agents act via ß-adrenergic receptor mediated increase of cytosolic cAMP concentration. Treatment of adipocytes with various agents which activate cAMP-dependent protein kinase (cAMP-PK) by receptor independent mechanisms (forskolin, cAMP analogues, isobutylmethylxanthine) also produced inactivation of ACC and increased phosphorylation of Ser-79 (Haystead, T.A.J. et al., 1990). As cAMP-PK activation in these tissues led to phosphorylation of ACC at a site solely phosphorylated by AMP-

PK, this raised the possibility that cAMP-PK is involved somehow in regulation of the AMP-PK cascade. However, AMP-PK purified from rat liver does not change activity if preincubated with cAMP-PK and Mg/ATP suggesting that cAMP-PK is not a "kinase-kinase" (Davies, S.P. et al., 1989).

Cohen and Hardie proposed that activated cAMP-PK in the liver, in response to glucagon, and in white adipose tissue in response to adrenaline, does not directly inhibit ACC, but may possibly act via phosphorylation and inactivation of protein phosphatase-2A which acts on this enzyme (Cohen, P. and Hardie, D.G., 1991). PP2A inhibition would allow AMP-PK to phosphorylate and inactivate ACC and HMG-CoAR without antagonism. This hypothesis gains support from the observed insulin/adrenaline control of protein phosphatase-1 involved in glycogen metabolism (PP1G) by phosphorylation of the G binding subunit on two different sites producing opposite effects (see Dent, P. et al., 1990) and that activity of protein phosphatase 2A (PP2A) also appears to be regulated by phosphorylation on multiple residues including threonine (Guo, H. and Damuni, Z., 1993) and tyrosine (Chen., J. et al., 1992).

Hardie, D.G. (1992) speculates that the AMP/ATP ratio presents a positive signal that regulates AMP-PK and switches on the AMP-PK cascade when high. In hepatocytes, the internal AMP concentration was transiently raised (with a drop in ATP concentration) by a high concentration of fructose in the medium (20mM). Fructose is rapidly converted in cells to triose-phosphate in a reaction which utilises ATP, and thus transiently raises the AMP/ATP ratio. This led as expected to a transient rise in AMP-PK activity, followed by a decrease in the expressed/total ratio of ACC activity. The increase in AMP-PK activity appeared to be due to phosphorylation and not allosteric effects because assays were performed at high dilution and at a saturated level of 5'AMP (Moore, F. et al., 1991). If the energy status of the cell decreases dramatically, (as in anoxia) the concentration of AMP rises rapidly (Faupel, R.P et al., 1972; Ottey, K.A. et al., 1989) and AMP-PK is allosterically activated immediately, and made

susceptible to phosphorylation and activation by kinase kinase. This leads to switching off of the energy consuming processes of fatty acid and cholesterol synthesis.

1.9. Acetyl-CoA Carboxylase kinase 2 (ACK-2)

ACK-2 was originally identified as a contaminant of ACC preparations purified from rabbit mammary gland (Hardie, D.G. and Cohen, P., 1978; Hardie, D.G. and Guy, P.S., 1980) and has been partially purified from lactating rat mammary gland (Munday, M.R. and Hardie, D.G., 1984). Although ACK-2 phosphorylated the same sites on ACC as cAMP-PK (from bovine heart) it has a different substrate specificity and more rapidly phosphorylated Ser-1200 on ACC (Munday, M.R. et al. 1988). ACK-2 has a MW of 76 KDa by gel filtration, it is insensitive to the specific protein inhibitor of cAMP-PK (at a concentration of 100U/ml), and it is insensitive to heparin at a concentration of 20μg/ml (at which concentration mammary gland casein kinase I and II were inhibited more than 80%). At a high concentration of heparin (100μg/ml) ACK-2 was inhibited by 75% compared to 40% inhibition of cAMP-PK (Munday, M.R. and Hardie, D.G., 1984). The true physiological role and substrates of ACK-2 are still as yet unclear.

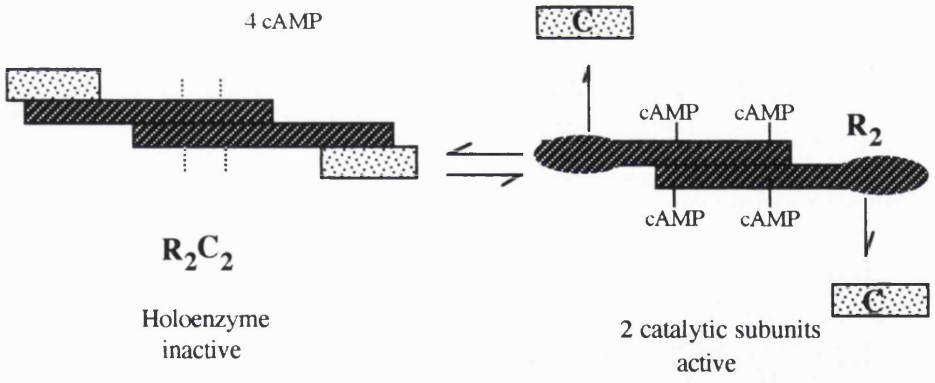
1.10. Cyclic AMP-dependent protein kinase (cAMP-PK)

Cyclic AMP-dependent protein kinase (EC 2.7.1.37) (cAMP-PK) is the major intracellular receptor for the second messenger cAMP. The enzyme exists as an inactive tetramer consisting of two regulatory and two catalytic subunits (Carlson, G.M. et al., 1979). The holoenzyme is activated by an elevated level of intracellular cAMP with a Ka for cAMP of approximately 20μM (Corbin, J.D. et al., 1975). Each regulatory subunit has two cAMP binding sites. The cAMP binds to them and this leads to a decrease in the affinity of the regulatory subunit for the catalytic subunit by

approximately four orders of magnitude and results in the dissociation of free catalytic subunit. The reaction is reversible (Fig.1.8.).

In most tissues cAMP-PK holoenzyme occurs in two major isoforms, termed type I and type II, according to the which type of regulatory subunit they contain. R I and R II subunits were assigned according to their order of elution from DEAE-cellulose (Corbin, J.D. et al., 1975). The R subunits, R I and R II each occur in two isoforms (RIα and RIβ; RIIα and RIIβ) (Clegg, C.H. et al., 1988).The RI does not have an autophosphorylation site and is more readily dissociated from the catalytic subunit in the presence of high salt concentration (Corbin, J.D. et al., 1975).

Fig.1.8. Activation of cAMP-dependent protein kinase by cAMP



The regulatory subunit of type I has a high affinity binding site for MgATP (Hoffman, R. et al., 1975). It has been shown (Ringheim, G.E. and Taylor, S.S., 1990) that binding of MgATP prevents dissociation of the type I isozyme at physiological salt concentrations. Without MgATP, a significant activation could occur in the absence of

cAMP due to salt induced dissociation of the complex. Ringheim and Taylor also suggested that MgATP accelerates the reassociation of the holoenzyme thus facilitating termination of the activation process when cAMP is removed.

The RII readily undergoes autophosphorylation. The presence of phosphate in the Ser-95 autophosphorylation site of the type II regulatory subunit (Takio, K. et al., 1982, 1983) results in a slower rate of reassociation with the catalytic subunit (Rosen, O.M. et al., 1975).

It has been generally assumed that catalytic subunits (C-subunits) from different tissues and different holoenzymes are identical (Bechtel, P.J. et al., 1977; Krebs, E.G. and Beavo, J.A., 1979; Flockhart, D.A. and Corbin, J.D., 1982; Nairn, A.C. et al., 1985) largely because C-subunits from all sources studied have had the same MW around 40 KDa (Carlson, G.M. et al., 1979), similar tryptic peptide maps (Zoller, M.J. et al., 1979) and similar catalytic properties. They can also reassociate with both types of regulatory subunits (Nimmo, G.A. and Cohen, P., 1977; Corbin, J.D. et al., 1975). The C-subunit is inhibited by a specific heat and acid stable protein, purified from skeletal muscle and named Walsh inhibitor (Walsh, D.A. and Ashby, C.D., 1973). The Walsh inhibitor, a 75-amino acid residue protein has been sequenced (Scott, J.D. et al., 1985a) and shown to contain at its N terminus a pseudophosphorylation site (in which alanine replaces serine residue) which is similar to the R I-subunit. A synthetic twenty amino acid peptide containing the sequence of the active fragment of the Walsh inhibitor is now widely used instead of the original protein ("Sigma" P-0300 protein kinase inhibitor; PKI; Cheung, H.C., et al., 1985). The Walsh inhibitor binds to the catalytic subunit in the presence of Mg/ATP with a high affinity (Ki 0.2nM; Scott, J.D. et al., 1985a). The synthetic peptide (PKI) also has a high affinity for the catalytic subunit (Ki 2.3 nM; Scott, J.D. et al., 1985b).

1.10.1. Substrate specificity of the catalytic subunit of cAMP-PK

C-subunits phosphorylate proteins and peptides which typically contain two basic residues, usually arginine, in position -2 and -3 preceding the phosphorylation site (Taylor, S.S. et al., 1990). Replacement of either of those arginines in synthetic peptide substrates is sufficient to increase the Km by nearly three orders of magnitude (Kemp, B.E. et al., 1977).

cAMP-PK has a very broad substrate specificity *in vitro* and *in vivo.* Discussion in this section is limited to some of the known metabolic intracellular targets for cAMP-PK and the effect their phosphorylation has on metabolism. The first physiological substrate to be identified was phosphorylase kinase (Walsh, D.A. et al., 1968). Phosphorylation by cAMP-PK activates phosphorylase kinase which in turn phosphorylates and converts glycogen phosphorylase to its active form (phosphorylase a). This is one of the mechanisms by which the hormones adrenaline and glucagon stimulate glycogenolysis in skeletal muscle and liver respectively, by elevating the intracellular cAMP concentration (Cohen, P. and Hardie, D.G., 1991).

Glycogen synthase (GS), the rate-limiting enzyme in glycogen synthesis, was the second substrate for cAMP-PK to be identified (Schlender, K.K. et al., 1969). Phosphorylation of GS from skeletal muscle or liver by cAMP-PK *in vitro* is accompanied by a decrease in GS activity (Schlender, K.K. et al., 1969; Killilea, D. and Whelan, W.J., 1976). There is now considerable evidence that inhibition of GS by cAMP-PK *in vivo* is not by direct phosphorylation but via the indirect phosphorylation and inactivation of protein phosphatase-1 which dephosphorylates GS (reviewed in Cohen, P. and Hardie, D.G., 1991).

Hormone-sensitive lipase (HSL) is a rate-limiting enzyme in control of lipolysis. Phosphorylation of HSL by cAMP-PK *in vitro* leads to its activation. In isolated adipocytes lipolytic hormones: adrenaline and glucagon lead via cAMP-PK mediated

phosphorylation to activation of HSL (for review see, Stralfors, P. and Belfrage, P., 1984; Yeaman, S.J., 1990).

1.10.2. Heterogeneity of catalytic subunt of cAMP-PK

The first evidence of heterogeneity of the catalytic subunit of cAMP-PK emerged when Peters, K.A. et al. (1977) separated three isoforms according to their isoelectric point. Two forms of the C subunit termed C_A and C_B have been separated chromatographically using a cation exchanger (Kinzel, V. et al., 1987). Two genes coding for the C-subunit have been identified (Uhler, M.D. et al., 1986a; Uhler, M.D. et al., 1986b) and have been designated Cα and Cβ. Both are found in all tissues but Cβ is particularly abundant in brain tissue. A third form of C-subunit named Cγ was isolated from testicular tissue (Beebe, S.J. et al., 1990). Cγ has an 83% amino acid homology with Cα and 79% with Cβ. This, together with a tissue specific expression of the Cγ, suggests that Cγ may be also functionally distinct from Cα and Cβ. Recently a further form of Cβ subunit was described by Weimann, S. et al. (1991) termed $C\beta_2$ which has MW of 46 KDa but is different from Cβ only in the 5'end of coding and non coding cDNA.

1.11. Aims of the present Work

The aim of this work was to purify and characterise AMP-PK and cAMP-PK catalytic subunit from mammary gland and to gain a better understanding of the physiological role of those enzymes in the regulation of acetyl-CoA carboxylase by phosphorylation / dephosphorylation during fasting and refeeding in the lactating mammary gland.

CHAPTER TWO

Materials and Methods

2.1.1. Animals

Albino rats (Wistar) were raised in cages and kept in a windowless room, with a 12h-light / 12h-dark cycle (light from 8^{00}h). Water and standard chow diet (52% digestible carbohydrates, 16% protein, 2% lipid and 30% non-digestible residue, by wt.) were given freely unless indicated otherwise. The female rats were mated at 200g body weight, and used in experimental procedures at peak lactation, 10-15 days *post partum* (litters varied from 8-12 rats).

Short term insulin deficiency was induced by the injection of streptozotocin (50mg/kg body weight in 10mM sodium citrate pH 4,5) into the tail vein 2 hours before sampling (Schein, P.S. et al., 1971).

Experimental procedures were carried out between 9^{30} and 10^{00}h, and unless otherwise stated, rats were anaesthetised with a non-recovery dose of pentobarbital (60mg/kg of body weight) administered by intraperitoneal injection. Dissection was started after signs of deep anaesthesia occurred. Mammary tissue was quickly and carefully freed from the skin and abdominal wall with the circulation intact, and then freeze clamped. Liver tissue was freeze-clamped *in situ*. The freeze-clamped tissues were powdered and stored in liquid nitrogen (-196°C). When the tissue was used for enzyme purification it was often from stunned and cervically-dislocated rats.

Rabbits (Dutch) were raised and kept in a windowless room with water, food and movement unrestricted. The light cycle was the same as for rats.

Bleeding or injection of the antigen into the rabbit was always performed between 10^{00}-12^{00} hours. Injection of the antigen was intramuscular into the hind legs, or multiple sub-cutaneous injections when complete Freund`s adjuvant was used. A booster injection was given every 60 days. Rabbits were bled 3 to 4 weeks after each injection. Each time the rabbit was bled from a vein along the outer edge of its ear, and around 20 ml of blood was collected. Blood was allowed to clot overnight in the fridge and then spun down (15 min, 3000 g) and plasma separated into 1 ml aliquots and stored at -20°C.

2.1.2. Apparatus

FPLC, PhastSystem™ and columns for FPLC (Mono Q, Mono S, Mono P, Superose 6, Superose 12) were from Pharmacia. Ultracentrifuge L8-M, midspeed centrifuge J2-HS, rotors for them (JA-14, JA-20, JA-21,TY-45, TY-65), scintillation counter LS 5000 CE were from Beckman. Slab systems for SDS-PAGE were Mighty Small II (SE250) from Hoefer and LKB Vertical electrophoresis system 2001. Power pack ECPS 3000/150, and Ultraspec II were also from LKB. Gel drying systems were LKB Gel Slab Drier GSD-4 and Hoefer Easy Breeze. Tracemaster 20 Automatic TLC Linear Analyser from Berthold and GyroVap from Howe were also used. Prep Cell, Rotophor and TransBlot™ were from Biorad and GE 200 SixPac gel eluter from Hoefer.

2.1.3 Chemicals

All standard laboratory reagents were of analytical grade or purer and obtained from Sigma Chemical Company Ltd. (UK), BDH Chemicals Ltd. (UK), Aldrich Fine Chemicals (UK) or Boehringer Mannheim (Germany).

2.1.4. Radiochemicals

($\gamma^{32}P$) ATP, ($\alpha^{32}P$) 8-Azido-ATP, (^{14}C) FSBA, (^{14}C) $NaHCO_3$ and (^{3}H) H_2O were obtained from ICN Radiochemicals (USA) or Amersham International (UK).

2.1.5. Kinase substrates and inhibitors

Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) and specific cAMP-dependent Protein Kinase Inhibitor (Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp) were obtained from Sigma Chemical Company Ltd. (UK).

Peptide SSMS (His-Met-Arg-Ser-Ser-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg) based on the sequence around the major phosphorylation site on ACC, and used to assay AMP-PK, cAMP-PK and ACK2, were synthesised and purified by Dr. I.Toth and colleagues, using an ABI peptide synthesiser in the School of Pharmacy, University of London (UK). Peptide SAMS (His-Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg) has the serine which is phosphorylated by cAMP-PK replaced by alanine which cannot be phosphorylated and is a specific substrate for AMP-PK. This peptide was a generous gift from Dr.D.Carling (Hammersmith Hospital). Both peptides SAMS and SSMS have added two C terminal arginine residues to facilitate binding to the Phospho-cellulose paper used in assay.

2.1.6. Protease inhibitors

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

2μg/ml SBTI (Soya-bean trypsin inhibitor) inhibits trypsin,

1mM Benzamidine inhibits peptidase,

1mM TLCK (Nα-p-tosyl-L-lysine chloro-methyl ketone) inhibits papain and trypsin,

1mM TPCK (N-tosyl-L-phenylalanine chloro-methyl ketone) inhibits chymotrypsin,

1mM EDTA (Ethylene-di-amine-tetra-acetic acid) inhibits metalloproteases,

1mM EGTA (Ethylene-glycol-bis(ß-amino-ethyl ether) N,N,N',N'-tetra-acetic acid) inhibits metalloproteases,

1mM PMSF (Phenyl-methyl-sulfonyl fluoride) inhibitor of serine proteases (e.g. elastase, trypsin, chymotrypsin). Because PMSF is not appreciably soluble or stable in water a 1M stock solution was made with anhydrous ethyl alcohol and diluted to its final concentration in an aqueous buffer just prior to use.

2.2. Preparation of Avidin -Sepharose

2.2.1. Buffers used in CNBr activation of Sepharose and coupling of ligand:

Coupling buffer:	10mM NaPi buffer pH 7.0 (5mM NaH_2PO_3, 5mM Na_2HPO_3 pH 7.0)
Blocking buffer:	1M ethanolamine/HCl pH 7.0
Monomerisation buffer:	3M guanidine/HCl in 0.2M KCl pH 1.5
Glycine Buffer:	0.1M glycine/HCl pH 2.0
Column buffer:	20mM Tris/HCl pH 7.5 at 4^0C 0.5M NaCl, 50mM NaF, 5mM NaPPi, 1mM EDTA, 10% (w/v) glycerol, 0.02% (w/v) NaN_3

2.2.2. Methodology of activation of Sepharose and coupling of ligand

40-50ml of Sepharose 4B-CL was washed with approximately one litre of cold ddH_2O on a Buchner funnel under vacuum suction. The gel was resuspended in

500ml 5mM NaPi buffer (made by dilution of Coupling buffer 1:1 with ddH_2O) and the pH then adjusted to 11.0 with 2M NaOH.

Activation of Sepharose with cyanogen bromide was carried out in a fume cupboard.

The gel suspension was mixed with finely ground cyanogen bromide (15g) in a large beaker. The temperature and pH of the reaction were maintained constant (temperature at 0-2^{0}C by adding crushed ice, and pH at 11.0 by adding 2M NaOH dropwise). The reaction was finished after approximately 15-20 min when the pH of the reaction stabilised. The reaction mixture, which contained activated Sepharose was then filtered over crushed ice on a Buchner funnel under vacuum suction, and washed with 700ml of Coupling buffer. The resulting CNBr activated Sepharose was scooped from the filter paper and resuspended in 125ml Coupling buffer containing 50mg of Avidin. The resuspension was transferred to a plastic bottle with a tight closure and mixed on an "end over end" mixer overnight at 4^{0}C.

The coupled avidin-Sepharose was filtered under suction and resuspended in 200ml of Blocking buffer for 2-3 hours at room temperature, to block uncoupled CNBr bridges. The coupled gel was then filtered under suction to get rid of the Blocking buffer, washed with Monomerisation buffer, also under vacuum suction, and then resuspended in 200ml of Monomerisation buffer and left overnight at room temperature. Monomerisation of avidin led to a decrease in binding affinity for ACC which is necessary in order to be able to elute ACC from the column.

The avidin-Sepharose was packed into a chromatography column and washed with Monomerisation buffer and then with five column volumes of Column buffer containing 0.8mM Biotin. This treatment blocks very high affinity sites on avidin which if untreated leads to irreversible binding of ACC. The column was then washed 3 times with alternatively 3 column volumes of glycine buffer and 3 column volumes of column buffer until the A_{280} was less than 0.05.

2.3. Purification of acetyl-CoA carboxylase

This procedure is a modification of that described by Tipper and Witters (1982)

2.3.1. Buffers

ACC Homogenisation buffer

250mM Sucrose, 50mM Tris/HCl pH 8.4 at 4^0C,
50mM NaF, 2mM NaPPi, 2mM EDTA,
2mM DTT, 2mM Benzamidine,1mM TLCK,
1mM PMSF, 4μg/ml SBTI

Avidin-Sepharose column buffer

500mM NaCl, 100mM Tris/HCl pH 7.5 at 4^0C,
50mM NaF, 2mM NaPPi, 1mM EDTA,
1mM DTT, 1mM Benzamidine, 2μg/ml SBTI,
10% (w/v) glycerol, 0.02% (w/v) NaN_3

2.3.2. Analytical purification of acetyl-CoA carboxylase

Mammary gland or liver was freeze clamped *in situ* and tissue powdered and stored in liquid nitrogen. 1g of mammary gland or 3g of liver were homogenised for 1 minute in 10 volumes of ACC homogenisation buffer using a machine driven glass/Teflon homogeniser. The homogenate was centrifuged at 4^0C for 20 minutes at 50000 g. The supernatant was filtered through glass wool and then taken to 40% ammonium sulphate saturation by addition of solid ammonium sulphate (0.243g per ml of supernatant). The suspension was kept on ice for five minutes and then centrifuged at 4^0C for 10 minutes at 50000 g. After discarding the supernatant, the pellet was instantly frozen in liquid nitrogen and stored until used at -70^0C.

The ammonium sulphate pellet was resuspended in 10ml Avidin-Sepharose column buffer, centrifuged at 4^0C for 10 minutes at 12000 g and the supernatant loaded

directly onto a 2ml avidin -Sepharose (5/2.5) column. The column was washed with 40ml of Avidin-Sepharose column buffer, then 10ml of the same buffer but without glycerol. ACC was eluted with 2mM biotin in 10ml of Avidin-Sepharose column buffer lacking glycerol (to avoid inhibition of ACC activity).

Fractions (0.5ml) were collected and assayed for protein concentration by the method described in section 2.8.1. The fraction containing most of the protein was diluted to 15μg/ml using 100mM Tris/HCl pH 7.4 at 37^{0}C and assayed immediately for ACC activity as described in section 2.3.3. At this concentration of ACC, the assay is linear over the 4 min. time course.

2.3.3. Assay of purified acetyl-CoA carboxylase

This procedure is a modification of that described by Holland et al. (1984)

ACC purified by avidin-Sepharose affinity chromatography was assayed at a concentration of 15μg/ml in 100mM Tris/HCl pH 7.4 at 37^{0}C in a total volume of 100μl containing: 0.3mM acetyl-CoA, 4mM $MgCl_2$,
8mM ATP, 1% (w/v) BSA,
20mM $NaH^{14}CO_3$ (sp.radioactivity ~ 1000 dpm/nmol)
and various concentrations of MgCl : Na citrate in a 1:1 ratio as indicated in the experiment. Blank assays contained no acetyl-CoA.

The assay was started with addition of ACC and terminated after four minutes incubation at 37^{0}C by addition of 50μl of 7% (v/v) PCA. The stopped assay was left on ice for 30 minutes and then centrifuged at 13000rpm in a bench microfuge to clarify the solution of precipitated proteins. 125μl of the supernatant was transferred to clean "Eppendorf" tubes, and dried in a rotary vacuum evaporator at 30-40^{0}C. Non incorporated ^{14}C which was released during drying was trapped in a methanol/dry ice trap.

Drying was completed when the pellets become gelatinous in appearance and pink-lilac in colour. 100μl of H_2O was added to each pellet, mixed, and followed by 1ml of scintillant "OptiPhase-Safe" (LKB)."Eppendorf" tubes were capped, mixed and left for 24 hours before counting for 10 minutes each.

2.3.4. Specific Radioactivity of $NaH^{14}CO_3$

10μl of 1/10 dilution of the 200mM $NaH^{14}CO_3$ stock solution was pipetted directly into an "Eppendorf" tube containing 1ml of scintillant (OptiPhase-Safe) and 90μl of 0.1M NaOH, mixed and counted on a ^{14}C program for one minute.The function of the NaOH was to neutralise the slightly acidic scintillation fluid.

10μl of the 1/10 diluted stock contained 200nmole

dpm / 200nmole = dpm/nmole of $NaH^{14}CO_3$

The specific radioactivity of $NaH^{14}CO_3$ solution used in assays was 1600-2200 dpm/nmol.

2.3.5. Calculation of ACC activity

One Unit of ACC activity incorporates 1μmole $NaH^{14}CO_3$ into malonyl-CoA per minute.

$$\text{ACC Activity (U)} = \frac{\text{dpm - dpm(blank)}}{\text{dpm/nmole Na-bicarbonate}} \times \frac{1}{4} \times \frac{150}{125} \times \frac{1}{\text{mg of ACC in assay}} \times \frac{1}{1000}$$

length of assay in minutes

volume taken from assay

converting nmoles to μmoles

Vmax, Hill coefficient (h) and Ka (citrate) were determined by computer. The program calculated the best fit for Vmax and Ka from initial rate velocity (v) for each citrate concentration [C] using the following equation:

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

2.3.6. Assay of acetyl-CoA carboxylase in PEG precipitated protein pellets

Powdered freeze-clamped tissue (0.1g) from liver or mammary gland was homogenised 30 sec. using a machine driven glass/Teflon homogeniser in 10 volumes of : 250mM mannitol, 100mM Tris/HCl pH 7.4 at 4^0C,
50mM NaF, 2mM NaPPi, 2mM EDTA, 1mM DTT,
1mM Benzamidine, 1mM PMSF, 2μg/ml SBTI.

The homogenate was centrifuged for 1 minute at 13000rpm on a bench-top microfuge and the supernatant was transferred to a clean "Eppendorf" tube. The supernatant was then split into two 300μl aliquots and transferred to two "Eppendorf" tubes containing 95μl of 25% w/v PEG_{6000} (to achieve a final concentration of 6% w/v PEG_{6000}). The tubes were vortex-mixed, left on ice for 2 minutes and then centrifuged for 3 minutes at 13000rpm. The supernatant was discarded and the PEG pellets were instantly frozen in liquid nitrogen and stored at -70^0C.

2.3.6.1. Assay of initial ACC activity in PEG precipitated protein pellets

For initial activity measurements, the PEG pellet was resuspended in 150μl of :

250mM mannitol, 100mM Tris/HCl pH 7.4 at 37^0C,

100mM NaF, 2mM NaPPi, 1mM EDTA, 1mM DTT,
1mM Benzamidine, 2μg/ml SBTI.

A 10 μl aliquot of resuspended 6% PEG pellet was assayed at 0.5 and 10mM citrate concentration as described in section 2.3.3.

2.3.6.2. Assay of total ACC activity in PEG precipitated protein pellets

For total activity measurement, the PEG pellet was re suspended in 150μl of :

250mM mannitol, 100mM Tris/HCl pH 7.4 at 37^{0}C,
20mM $MgCl_2$, 20mM sodium citrate, 1mM EDTA,
1mM DTT, 1mM Benzamidine, 2μg/ml SBTI,
20 U/ml catalytic subunit of PP2A.

The resuspended pellet was then preincubated for 30 minutes at 37^{0}C and then 10μl assayed at 20mM citrate concentration as described in section 2.3.3.

The 6% PEG pellet contained more than 90% of the ACC activity observed in the crude homogenate.

Initial and total ACC activity was expressed in mU/g wet weight of tissue and calculated by formula in section 2.3.5. with mg of ACC replaced with mg of wet weight of tissue.

2.4. Specific radioactivity of [γ-^{32}P] ATP

To calculate the specific radioactivity of [γ-^{32}P] ATP 5μl of 20mM MgCl2/1mM [γ-^{32}P] ATP stock solution was mixed with 4ml of "OptiPhase-Safe" scintillant cocktail and counted on a scintillation counter for 1 minute. 1mM ATP contains 1nmol ATP/μl .

$$\text{specific radioactivity of ATP (cpm/nmol)} = \frac{\text{cpm } (5\mu\text{l of 1mM } [\gamma-{}^{32}\text{P}]\text{ATP})}{\text{5 nmols of ATP in } 5\mu\text{l}}$$

2.5. Assay of cAMP-dependent protein kinase

This procedure is a modification of that described by Roskoski, J.R. (1983)

Liver or mammary gland (0.1g) that had been freeze-clamped and powdered under liquid N_2 were homogenised with 30 strokes of a hand-held glass/Teflon homogeniser in 10 volumes of :

10mM KPi buffer (5mM KH_2PO_3, 5mM K_2HPO_3) pH 6.8,
1mM EDTA, 1mM DTT, 1mM Benzamidine,
1mM PMSF, 1mM IBMX, 4μg/ml SBTI.

The homogenate was diluted 1/10 in 10mg/ml BSA and assayed immediately for initial and total cAMP-PK activity. 5μl of diluted homogenate or purified cAMP-PK were incubated for 4 minutes at 37^{0}C in a total volume of 25μl containing:

150μM Kemptide, 10mM Hepes pH 7.0,
50mM NaF, 1mM NaPPi, 200μM EDTA,
4mM $MgCl_2$, 200μM [γ-^{32}P] ATP
(sp. radioactivity (2-4 x 10^6 cpm/nmol),

in the presence and absence of 100U of PKI and the presence and absence of 10μM cAMP.

At the end of the 4 minute assay, the reaction was stopped by pipetting a 20μl aliquot onto a P81 phospho-cellulose paper square (2cm^2) and immersing the paper into ice-cold 75mM phosphoric acid. The phospho-cellulose paper was washed three times in phosphoric acid and twice in ddH_2O. The paper square with bound ^{32}P-labelled peptide was then immersed in 4ml "OptiPhase-Safe" scintillant cocktail and counted on a scintillation counter for 3 minutes.

cAMP-PK activity was measured as the linear incorporation of ^{32}P into Kemptide in the absence of PKI once the rate in the presence of PKI had been subtracted. Initial activity was that measured in the absence of cAMP. Total activity was that measured in the presence of cAMP. The activity ratio of cAMP-PK was expressed as the ratio of Initial/Total activity.

One Unit of cAMP-PK activity represented the incorporation of 1nmol of ^{32}P into peptide per minute.

2.6. Assay of AMP-activated protein kinase

Liver or mammary gland (0.1g) freeze-clamped and powdered under liquid N_2 were homogenised with 30 strokes of a hand-held glass/Teflon homogeniser in 10 volumes of: 100mM Tris/HCl pH 7.2 at 4^0C,
250mM mannitol, 50mM NaF, 1mM EDTA,
1mM DTT, 1mM PMSF, 2μg/ml SBTI.

The homogenate was diluted 1/10 in 10mg/ml BSA. 5μl of diluted homogenate or partially purified AMP-PK were assayed for AMP-PK activity in a total volume of 25μl containing:

10mM Hepes pH 7.0, 50mM NaF, 200μM EDTA, 4mM $MgCl_2$,
200μM [γ-^{32}P] ATP (sp. radioactivity 2-4 x 10^6 cpm/nmol),

in the presence or absence of 100μM SAMS peptide.

The assay was incubated for 4 minutes at 37^0C and then terminated by pipetting a 20μl aliquot onto a P81 phospho-cellulose paper square ($2cm^2$) and immersing the paper into ice-cold 75mM phosphoric acid. The phospho-cellulose paper was washed three times in phosphoric acid and twice in ddH_2O. The paper square with bound ^{32}P-labelled peptide was then immersed in 4ml "OptiPhase-Safe" scintillant cocktail and counted on a scintillation counter for 3 minutes.

AMP-PK activity was measured as the linear incorporation of ^{32}P into SAMS peptide once the rate in the absence of peptide had been subtracted. One Unit of AMP-PK activity represents the incorporation of 1nmol of ^{32}P into SAMS peptide per minute.

2.7. Assay of ACK-2 activity

Partially purified ACK-2 was assayed at 37^0C in 25μl of incubation mixture containing:

10mM Hepes pH 7.0, 100U of PKI.,

50mM NaF, 200μM EDTA, 4mM $MgCl_2$,

200μM [γ-^{32}P] ATP (sp. radioactivity 2-4 x 10^6 cpm/nmol),

in the presence or absence of 100μM SSMS peptide. The remainder of the procedure was as described for AMP-PK in section 2.6.

100U of PKI was included in the assay because the catalytic sub-unit of cAMP-PK also phosphorylates SSMS peptide. AMP-PK activity was assayed in parallel (against the specific SAMS peptide substrate) because AMP-PK also phosphorylates SSMS and at present no AMP-PK inhibitor is known.

One Unit of ACK-2 activity represents the incorporation of 1nmol of ^{32}P into SSMS peptide per minute when incorporation into SAMS is negligible.

2.8. Measurement of protein concentration

2.8.1. Assay of protein concentration (Bradford, M.M. 1976)

The method is based on the observation that binding of Coomassie Blue G-250 dye to protein causes a shift in the absorption maximum of the dye from 465 to 595nm. The binding is achieved through ionic interaction between the dye and basic amino acids (thus the need for acidic conditions), and there is evidence of van der Waals and

hydrophobic interaction as well. When the concentration of dye in the assay is in excess, the increase in absorbance at 595nm is directly proportional to the amount of protein in the protein-dye complex.

The Coomassie reagent used in the assay contained 30mg Coomassie Blue G-250 in 100ml of absolute alcohol, 55ml conc. phosphoric acid and 845ml H_2O.

For each batch of Coomassie reagent a calibration curve of protein concentration versus absorbance at 595nm was constructed, using as a standard 1-8μg BSA per assay (using an accurately made stock solution of 200μg/ml BSA in ddH_2O). The 200μg/ml BSA stock solution was made by dissolving a few crystals of double crystallised BSA in ddH_2O until the A_{280} was 0.13 against ddH2O as a blank.

Protein concentration was assayed by mixing 100μl of sample (or appropriate dilution of sample) and 1ml of Coomassie reagent. After five minutes the assay was transferred to a plastic cuvette and absorbance at 595nm measured on an Ultraspec II spectrophotometer. A blank containing 100μl of buffer instead of sample was subtracted from the reading. When the protein concentration was very high, protein was diluted to achieve an absorbance reading in the linear range of the calibration curve and then the result was multiplied by the dilution factor.

The protein-dye complex gave a stable reading lasting one hour after mixing.

2.8.2. Protein detection by absorbance at 280nm

This method was used routinely to manually monitor protein concentration changes during liquid chromatography (or automatically on FPLC). The method relies on the ability of aromatic amino acids and peptide bonds to absorb ultraviolet light at 280nm. There is a direct relation between the absorbance at this wavelength and the concentration of protein, but the method is not highly accurate because of the possibility of interference by other constituents in the buffer.

2.9. Electrophoresis of protein in polyacrylamide gels

Use of polyacrylamide gels in electrophoresis of protein (and DNA) was a revolutionary achievement. Electrophoresis gels and buffers can be chosen to provide separation on the basis of charge, size or a combination of both.

Polyacrylamide gels are formed by co-polymerisation of acrylamide and bis-acrylamide. Polymerisation is initiated by TEMED (tetramethylethylenediamine) and ammonium persulphate, or by riboflavin and UV light (photochemical polymerisation). The ammonium persulphate yields persulphate free radicals which, in turn, activate the TEMED. The TEMED acts as an electron carrier to activate the acrylamide monomer to a free radical. The activated monomer then reacts with unactivated monomer to begin the polymer chain elongation. During polymerisation, polymer chains are randomly cross-linked by bis-acrylamide or other cross-linkers: N,N'-bis-acrylylcystamine (BAC), N,N'-diallyltartardiamide (DATD) or N,N'-dihydroxyethylene-bis-acrylamide (DHEBA) producing a "web" polymer whose porosity depends on the total acrylamide content (%T) and the percentage of cross-linker (%C).

The %T value represents the total concentration of both monomers (acrylamide and bis-acrylamide) in grams per 100ml and %C is the percentage (by weight) of the crosslinker relative to the total monomer.

Proteins or peptides can be incorporated and immobilised inside gel matrices if included during polymerisation. This has proved useful in the immobilisation of enzyme substrates for subsequent reaction with an enzyme separated on the gel (Hutchroft, J.E. et al., 1991).

2.9.1. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This procedure was based on the method described by Laemmli, U.K. (1970) and employs a discontinuous Tris-Glycine buffer system with 0.1% (w/v) SDS. The strong ionic detergent (SDS) binds to proteins, denatures them, and gives them uniform negative charge (which is only dependent on the size of the protein and masks the original charge of the protein). When an electric field is applied, negatively charged protein-SDS complexes migrate toward the anode. Because the ratio of mass/charge for all protein-SDS complexes are essentially the same, protein migration through the polyacrylamide gel depends on the size of protein and size of the pores in the matrix. Proteins with smaller MW migrate faster and those with MW smaller than 5KDa cannot be separated because they migrate with the dye front.

Solutions:

30% Acrylamide solution (30% T, 2.7% C): 29.2g Acrylamide, 0.8g bis-acrylamide per 100ml in ddH_2O, filtered and stored at 4^0C.

Resolving gel buffer: 1.5M Tris/HCl pH 8.8 at 25^0C, and stored at 4^0C.

Stacking gel buffer: 0.5M Tris/HCl pH 6.8 at 25^0C and stored at 4^0C.

Reservoir buffer (10x) : 0.25M Tris, 1.92M Glycine, 1% (w/v) SDS.
30.3g Tris, 144g Glycine, 10g SDS dissolved in one litre of ddH_2O. The pH of the solution should be around 8.3. This was stored at room temperature and diluted 10 fold to normal strength just prior use.

5x sample buffer : 0.414M Tris/HCl pH 6.8 at 25^0C, 66.66% (w/v) glycerol, 13.33% (w/v) SDS, 0.033% (w/v) Bromophenol blue.
5.033g Tris dissolved in 25ml of ddH_2O, taken to pH 6.8 with HCl at 25^0C, 66.66g glycerol, 13.33g SDS, 33mg Bromophenol blue. Make up to 100ml with ddH_2O and store at room temperature.

Casting of resolving gel:

Glass plates were thoroughly washed and assembled in a gel casting stand. After checking for water tightness the gel was cast. The volume of resolving gel required was 4ml for the mini gel system (5 x 8cm) and 30 ml for the standard system (15 x 15cm). Different percentage resolving gel solutions were prepared as follows:

% of total Acrylamide	5%	7.5%	10%	12.5%	15%
Resolving gel buffer	7.5ml	7.5ml	7.5ml	7.5ml	7.5ml
10% (w/v) SDS	0.3ml	0.3ml	0.3ml	0.3ml	0.3ml
de-gassed ddH_2O	17.2ml	14.7ml	12.2ml	9.7ml	7.2ml
30% (w/v) Acrylamide	5ml	7.5ml	10ml	12.5ml	15ml

The resolving gel solution was degassed and polymerisation was initiated by the addition of 15μl TEMED and 75μl of 10% (w/v) ammonium persulphate per 30ml gel (for 4ml mini gel 2.5μl TEMED and 10μl of 10% (w/v) ammonium persulphate were used). The gel was cast immediately and overlaid with a thin layer of isobutanol. The isobutanol layer gives a flat meniscus and stops oxygen entering the gel which would inhibit polymerisation. Polymerisation time was approximately one hour. After that the isobutanol and non polymerised acrylamide were washed away with ddH_2O and the stacking gel cast.

Casting of stacking gel:

10ml of stacking gel for a standard 15 x1 5cm gel (4ml for two mini gels) was prepared according to the following recipe:

% of total Acrylamide	3% (w/v)	4.5% (w/v)	6% (w/v)
Resolving gel buffer	2.5ml	2.5ml	2.5ml
10% (w/v) SDS	0.1ml	0.1ml	0.1ml
de-gassed ddH_2O	6.4ml	5.9ml	5.4ml
30% (w/v) Acrylamide	1ml	1.5ml	2ml

This was degassed and polymerisation initiated by the addition of 10μl TEMED and 50μl of 10% (w/v) ammonium persulphate per 10ml gel. The gel was cast immediately and a teflon comb of appropriate sized wells was inserted. After the initial stage of polymerisation (15-20 minutes), the comb was removed and the wells were washed with ddH_2O. The gel was assembled in the electrophoretic system, and the wells were filled with reservoir buffer (1x) and left to fully polymerise for at least two hours.

Sample preparation:

The 5x sample buffer was boiled prior to use and ß-mercaptoethanol (in ratio 3:1 sample buffer: ß-mercaptoethanol) was added. This was added to the sample in the ratio 1:4 sample buffer : sample to give a final concentration of 0.0625M Tris, 10% (w/v) glycerol, 2% (w/v) SDS and 0.025% (w/v) Bromophenol Blue. The sample was then heated for 5 minutes at 100^0C or 45 minutes at 65^0C and loaded into the sample wells of the gel. The maximum volume loaded into each well was 25μl for the mini gel system and 200μl for a standard size gel.

Running conditions:

SDS-PAGE was run at constant current (with maximum voltage and power setting) using an ECPS 3000/150 power pack (LKB). For a single standard size gel (15 x 15cm and thickness 1mm) the current was set at 30mA through the stacking gel (until

the tracking dye reached the resolving gel) and at 45mA thereafter. For a single mini gel 13mA was used for the 7.5% gels and 25mA for the 15% gels. The approximate time of the run was 4-6h for standard and 1-1.5h for mini gel. Gels were then stained for proteins, and the proteins eluted or transferred as described in section 2.14.1.

2.9.2. Discontinuous Native-PAGE

This procedure was based on the method described by Ornstein, L. and Davis, B.J. (1964). Procedures and buffers were as described in the Laemmli method (section 2.9.1.), only with SDS omitted. A gel of lower acrylamide concentration (2.5%T stacking and 5%T resolving gel) was used and the run was extended (around 4 hours for mini gel).

The principle is similar to that of SDS-PAGE with the exception that the mass/charge ratio is specific for each protein. Some proteins with positive charge would not enter the gel, but the majority of proteins have natural negative charge at the pH used and penetrate and migrate in the gel according to their native size (and shape) and the porosity of the gel. An advantage of this system over SDS-PAGE is that the protein can be separated in its active (native) form. The disadvantages are very poor resolution and that some proteins aggregate because of the change of pH between the stacking and resolving gel.

2.9.3. Continuous Native-PAGE

This procedure was based on the method described by McLellan, T. (1982). In the continuous system the same buffer (43mM Imidazole, 35mM Hepes pH 7.4) is used in both chambers and through the gel so that aggregation of proteins is minimised, but resolution suffers further because the stacking of proteins is only due to the change in acrylamide concentration between the stacking and resolving gel.

2.9.4. Blue Native-PAGE

This procedure was based on the method described by Schägger, H. and von Jagow, G. (1991), which utilises the shift in charge of native proteins due to the binding of the Coomassie Blue G-250 dye. The speed of migration through the polyacrylamide gel depends on the size and shape of the negatively charged protein-dye complex and its overall charge. The overall charge of the protein-dye complex depends on the surface characteristic of the protein (availability of binding sites for dye and overall charge of protein itself). The stacking of proteins is achieved by a change in the acrylamide concentration between the stacking and resolving gel. Proteins are maintained in their native (active) form through Blue Native PAGE. Acrylamide concentrations used were similar to those described for the SDS-PAGE system and the buffer used in both chambers and through the gel was 43mM Imidazole, 35mM Hepes pH 7.4. To the top reservoir buffer (Anode buffer) 0.005% (w/v) of Coomassie Blue G-250 was added. The gel does not need staining or destaining and active proteins can be eluted.

2.10. Peptide mapping by limited proteolysis in SDS-PAGE "Cleveland mapping"

This procedure based on the method described by Cleveland, D.W.et al. (1977), was used to compare the structures of related proteins with similar molecular weights. The method uses SDS tolerant proteases for partial digestion of proteins in the gel slices (already denatured and separated by the first SDS-PAGE). This eliminates the need for electro-elution of proteins from the gel before digestion which can result in poor recovery. The protein fingerprints (pattern of peptide bands which is characteristic for each protein and protease used) were generated in a second SDS-PAGE with proteolytic digestion and electrophoretic separation of peptides occurring at the same time. The exact pattern of a protein fingerprint is a result of the substrate specificity

of the protease, the length of the digestion period and number of available sites for cleavage on the protein substrate. The degree of similarity of the proteins can be calculated by analysis of the number of the peptide fragments with identical mobility (Calvert, R. and Gratzer, W.B., 1978).

Procedure:

After SDS-PAGE (section 2.9.1.) the gel was temporarily stained by Cooper Stain (section 2.13.4.), protein bands of interest were cut out, destained (section 2.13.4.), and equilibrated in two changes (15 minutes each) of buffer containing:

125mM Tris/HCl pH 6.8 at 25^{0}C,

1mM EDTA, 0.1% (w/v) SDS,

0.01% (w/v) Bromophenol Blue (tracking dye).

After equilibration the gel slices were immediately used or stored, frozen at -20^{0}C.

A second SDS-polyacrylamide gel was cast with a 4cm long (for mini-gel) resolving gel (15% T) and longer than usual stacking gel (6% T) (section 2.9.1.) with the addition of 1mM EDTA to all solutions (to prevent non-specific metallo-proteases). When polymerisation was completed gel slices (cut into 1mm^2 squares) were loaded into the wells and overlaid with protease diluted in buffer containing:

125mM Tris/HCl pH 6.8 at 25^{0}C,

20% (w/v) glycerol, 0.1% (w/v) SDS, 1mM EDTA,

0.01% (w/v) m-Cresol purple (tracking dye).

The proteases routinely used were:

50ng/lane Papain (P3125, Sigma),

50ng/lane Chymotrypsin (TLCK treated - C3142, Sigma),

50ng/lane Trypsin (TPCK treated - T8642, Sigma),

50ng/lane Endoproteinase Glu-C (V-8 protease - P2922, Sigma)

Protease stocks (25mg/ml) were stored at -20^{0}C (Chymotrypsin, Trypsin and V-8 protease) or 4^{0}C (Papain) in 50μl aliquots and defrosted and diluted (1/1000) just prior to use. Protease diluted in the above buffer was layed on top of the gel slices in each well. The protease solution fills the gaps around the gel slices without mixing (because of the presence of 20% (w/v) glycerol) and can be observed (due to the yellow colour of the tracking dye at this pH).

The SDS-PAGE was run at 8mA constant current through the stacking gel and then the current stopped for 30 minutes just before the tracking dye reached the resolving gel. After partial digestion which occurred at the previous stage, electrophoresis was run at 20-25mA through the resolving gel.

After the the run, peptides in the gel were stained (described in section 2.13.)

2.11. Labelling of proteins

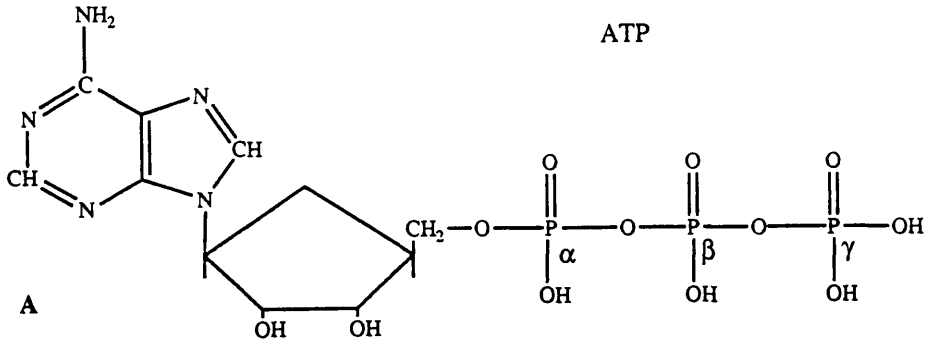
2.11.1. [^{14}C]FSBA labelling of AMP-PK

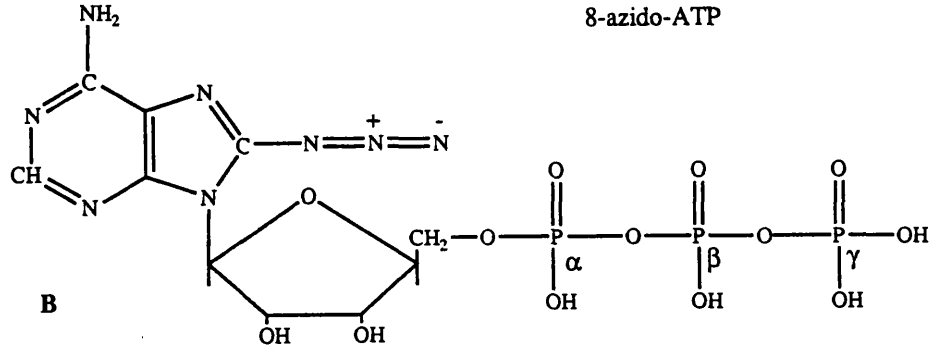
This procedure was based on that described by Harlow, K.W. and Switzer, R.L. (1990). 5'-(p-fluorosulfonylbenzoyl)-(^{14}C)adenosine (Fig.2.1.C) is an ATP (Fig.2.1.A) analogue which is commonly used as a probe for nucleoside binding sites. Its fluorosulfonylbenzoyl moiety reacts covalently with lysine or cysteine in an ATP-binding site.

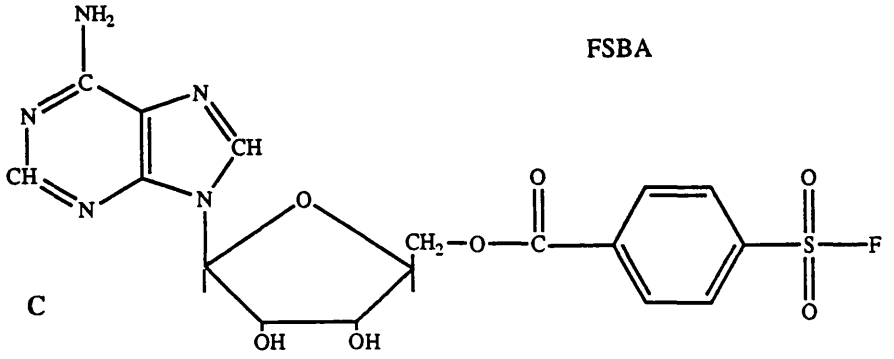
AMP-PK was incubated with Fluorosulphonylbenzoyl-(^{14}C)adenosine at 4^{0}C in 100μl incubation mixture containing :

20mM Hepes pH 7.0, 10% (w/v) glycerol,
0.004% (w/v) Brij 35, 1mM NaPPi,
5mM DTT, 0.2mM EDTA,
200μM (^{14}C)FSBA (5mCi / nmol).

Fig.2.1. Structure of ATP (A) and ATP analogues: [α^{32}P] 8-azidoATP (B) and 5'-(p-fluorosulfonylbenzoyl)-(^{14}C)adenosine (FSBA) (C)





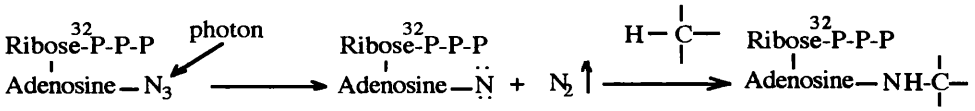


The reaction was stopped after 2h by adding 25µl of SDS-PAGE sample buffer (3 vol. of 5X sample buffer / 1 volume of β-mercaptoethanol, described in section 2.9.1.) and boiling for 3 minutes. The labelled AMP-PK was then run on SDS-PAGE as described in section 2.9.1. The gel was stained as described in section 2.13., incubated for 5 minutes in "Amplify™" (from Amersham, UK) and then dried as described in section 2.12. Autoradiography of the dried gel was as described in section 2.12.

2.11.2 [α^{32}P] 8-azido ATP labelling of AMP-PK

This procedure was based on that described by Knight, K.L. and McEntee, K. (1985) and Julin, D.A. and Lehman, I.R. (1987). The [α^{32}P] 8-azidoATP (Fig.2.1.B) is an ATP (Fig.2.1.A) photoaffinity analogue which binds to ATP-binding sites, and in the absence of light, totally mimic ATP. The 8-azido group converts to a nitrene upon photolysis and if photolyzed while reversibly bound to proteins it may form an irreversible, covalent bond by a nitrene insertion reaction (Fig.2.2).

Fig.2.2. Photolysis of [α^{32}P] 8-azidoATP



Photolysis of [α^{32}P] 8-azidoATP leads to formation of a very reactive nitrene group, which reacts by nitrene insertion with any C-H bond in its proximity.

The [α^{32}P] 8-azidoATP (specific radioactivity 7.5mCi/nmol) was stored in methanol in a dark container at -70^{0}C. Because of photosensitivity of 8-azido ATP, all handling and pipetting of 8-azido ATP was carried out in a dark room with dark red illumination. The AMP-PK (usually stored in the presence of 5mM DTT) was diluted 50 fold with: 50mM Hepes pH 7.5, 50mM NaCl to reduce the concentration of DTT to 0.1mM and then concentrated down to the original volume by centrifugation at 3000rpm in a Microcon10 concentrator (with membrane cut-off point for proteins with MW larger than 10KDa). Just prior to labelling, $MnCl_2$ was added to a final concentration of 8mM.

The labelling was performed in wells on an ELISA plate laid on ice. To each well 2.5µl of [α^{32}P] 8-azido ATP (≈5µCi) was added to make a final concentration of 25µM 8-azidoATP. 5-10 minutes later (after most of the methanol had evaporated) 20µl of AMP-PK was added, mixed, and left for 15 minutes on ice. The labelling mixture was then photolysed by exposure to a bench UV lamp for 4 minutes at 256nm. The reaction was stopped by the addition of 20µl of 100mM DTT/ 20mM EDTA or 5µl of sample buffer (3 volume of 5X SDS-PAGE sample buffer / 1 volume 1M DTT). Radio-labelled enzyme in the sample buffer was heated for 30min at 60^{0}C and run on 7.5% SDS-PAGE, as described in section 2.9.1.

2.11.3. [γ^{32}P]ATP labelling of autophosphorylation sites on AMP-PK, cAMP-PK and ACK2

The enzymes were incubated at 37^{0}C for 1 hour in 25μl of buffer containing:

50mM Hepes pH 7.4, 10% (w/v) glycerol, 50mM NaF,
5mM NaPPi, 1mM EDTA, 1mM DTT, 1mM Benzamidine,
2µg/ml SBTI, 4mM $MgCl_2$,
200μM [γ-^{32}P] ATP (sp. radioactivity 6-9 x 10^5 cpm/nmol),

The reaction was terminated by the addition of 5µl of sample buffer (3 volume of 5X SDS-PAGE sample buffer / 1 volume 1M DTT). Radio-labelled enzyme in the sample buffer was heated for 30min at 60^0C and run on 7.5% SDS-PAGE as described in section 2.9.1.

2.12. Detection of radioactive proteins and peptides after PAGE

Direct autoradiography of ^{32}P-labelled proteins after PAGE was carried out by contact exposing the dried gel with ßmax film (Amersham) in an autoradiography cassette at room temperature for different durations of times (1-10 days).

For fluorography of ^{14}C-labelled proteins, the gel was incubated for 5 minutes in Amplify™ (Amersham) and dried on filter paper with clear film as a top layer, which was removed after the gel was dry. The Hyperfilm-MP (Amersham) was pre-flashed using photographic flash light and Wratten 6B filter. The pre-flashed film was then exposed directly to gel in an autoradiography cassette at -70^0C for different durations of time (1-4 months).Both types of film were developed under dark red illumination in LX-24 developer and fixed using FX-40 fixer (Kodak).

2.13. Protein staining in PAGE

The staining of proteins inside acrylamide gels required stain which will penetrate the gel, bind to the protein but not the matrix. The most widely used are Coomassie Blue R-250, Coomassie Blue G-250, Silver Stain and Copper stain.

2.13.1. Protein staining by Coomassie Blue R-250

This procedure was based on that described by Meyer, T.S. and Lambert, B.L. (1965) and Patel, K. et al. (1987). Coomassie staining requires acidic conditions to

enhance the ionic interaction between the dye and basic amino acids. Staining by this method yields a low background with maximal sensitivity of 40ng (per protein band in 10 lane mini-gel). Due to the requirement of basic amino acids for binding of dye, some proteins, which are too acidic are not stained.

Fixing and staining of the gel are done simultaneously by immersing the gel for 30 minutes into:
0.25% (w/v) Coomassie Blue R-250, 40% (v/v) methanol, 10% (v/v) Acetic acid.

Destaining of the gel until the background was clear was achieved by gently shaking the gel in destaining solution (10% (v/v) methanol, 10% (v/v) Acetic acid) with several changes of solution. A piece of sponge was placed to the destainer with the gel to encourage destaining of the gel by lowering free dye concentration in the destainer.

2.13.2. Protein staining by Coomassie Blue G-250

This procedure was based on that described by Neuhoff, V. et al. (1988) and Diezel, W. (1972) and has a staining sensitivity of 0.25ng (per band on a mini gel) and approaches the sensitivity of silver staining, but with a clear background. This is due to the colloidal properties of Coomassie Blue G-250, the inclusion of 20% (v/v) methanol (which favours the formation of protein-Coomassie blue G-250 complexes) and the presence of ammonium sulphate (which shifts the dye towards its colloidal form which cannot penetrate the gel.

The method has a clear advantage of very short staining times (few minutes) if the concentration of 8% (w/v) ammonium sulphate was used, and does not need destaining. To achieve maximum sensitivity of staining, the gel was stained for a prolonged period of 15-24h and a concentration of 15% (w/v) ammonium sulphate was used.

Staining solution contains: 80ml of solution A + 20ml of methanol

Solution A contains: 8 or 15% (w/v) ammonium sulphate,
2% (v/v) phosphoric acid,
0.1% (w/v) Coomassie Blue G-250.

Solution A is stable for a prolonged period, but should be agitated prior to use. A fresh Staining solution was prepared each time and discharged after use.

After fixing, the gel was immersed for 5 minutes in 20% (v/v) methanol, 2% (v/v) phosphoric acid, 8% (w/v) ammonium sulphate and then transferred to the staining solution. Fast staining was used to visualise protein bands that needed to be cut out of the gels, prolonged staining overnight was used to visualise low concentration protein bands. After staining, gels were briefly washed with 25% v/v methanol in ddH_2O and dried. The gels stained by this method can be stained additionally by silver stain after prior destaining by 50% (v/v) methanol, 10% (v/v) acetic acid.

2.13.3. Protein staining by silver stain

This procedure was based on methods described by: Merril, C.R. et al. (1981), Blum, H. et al. (1987) and Rabilloud, T. et al. (1988) and can detect protein as low as 0.05ng per band in mini gels. The method is extremely sensitive, but one drawback is that it yields high backgrounds (compared to other methods) and is not specific for proteins (some proteins are not stained by this method and the method also stains DNA).

The silver staining method is based on the binding of sensitising agents to protein by an ion exchange related mechanism. Sensitising agents belong in two groups:

- sulphiding agents (DTT, sodium thiosulphate, etc.) which produce sulphide ions which in reaction with silver ions produce silver sulphide nuclei;

- reducing agents (sodium phosphite, sodium hypophosphite, sodium dithionite, etc.) which reduce silver ions to form metallic silver nuclei.

The silver sulphide and metallic silver nuclei act as catalytic centres for the reduction of silver. The reducing agents produce less background staining especially with thiosulphate in the developer solution.

After fixing, the gel was incubated for 5 minutes, three times with 10% ethanol then washed for 5 minutes, twice with ddH_2O. The gel was sensitised in the Sensitising solution:

0.02g sodium thiosulphate per 100ml ddH_2O

or 0.025g sodium dithionite per 100ml ddH_2O

The gel was rinsed three times with ddH_2O and immersed for 30 minutes into 100ml silver nitrate solution containing: 0.2% (w/v) silver nitrate and 10μl of 37% (w/v) formaldehyde. The gel was rinsed three times with ddH_2O and immersed into 100ml of developer solution containing:

4% (w/v) Na_2CO_3, 100μl of 20mM sodium thiosulphate,

60μl of 37% (w/v) formaldehyde.

The developing was stopped by adding 3.5ml glacial acetic acid directly to the developer and left on the shaker for another 10 minutes. After washing for 30 minutes in ddH_2O the gel was dried.

Silver staining can be reversed by incubating the gel for a few seconds in a 1% (v/v) solution of H_2O_2.

2.13.4. Protein staining by Copper staining

This procedure was based on the method described by Lee, C. et al. (1987) and is only applicable for SDS-PAGE. The clear advantage over the other methods is speed (bands are visible in 5 minutes) and reversibility (proteins are fixed in the gel in a

reversible manner and can be easily eluted from gel slices at any time following staining with recovery greater than 90%). This is superior to the other two non-fixative methods (Higgins, R.C. and Dahmus, M.E., 1979) that use staining with 2.5M KCl which can detect 5μg of protein per band and staining with 4M sodium-acetate which can detect 1μg of protein per band on a mini gel, because it is more sensitive and can detect 10ng of protein per band on a mini gel. The staining is more sensitive than Coomassie Blue R-250 (80ng of protein per band), and yields negative staining with clear protein bands on a semi-opaque whitish-blue background.

The principle is based on the fact that Cu^{2+} and Tris-SDS make a Cu^{2+}-Tris-SDS complex which precipitates and is bluish in colour. In protein bands the SDS is tightly bound in the SDS-protein complex and not available for complexing with Cu^{2+} and Tris and for that reason the protein bands do not stain.

After electrophoresis, the SDS gel was briefly washed (15-20 seconds) in ddH_2O and incubated in 100ml of 0.3M cupric chloride ($CuCl_2$) in ddH_2O. The gel was rocked gently in this solution for five minutes at room temperature and washed for a two minutes in ddH_2O (to remove excess reagent). The gel stained this way can be stored in ddH_2O for a year without defusing of the protein bands, but cannot be dried because the colour fades.

To elute proteins from a gel stained in this way, the gel slices which contained proteins of interest were cut out and destained by three changes (10 minutes each change) of buffer containing:

125mM Tris/HCl pH 6.8 at 25^0C,

250mM EDTA.

After destaining, the gel slices were equilibrated by incubation in two changes (15 minutes each) of buffer containing:

125mM Tris/HCl pH 6.8 at 25^0C,

0.1% (w/v) SDS.

After equilibration gel slices were stored frozen at -20^{0}C and were used to elute the protein of interest by gel elutor or for peptide mapping by limited proteolysis in SDS-PAGE as described in section 2.10.

2.14. Protein blotting

Protein blotting involves the electrophoretic transfer of proteins, separated after PAGE, onto a synthetic membrane support (Nitrocellulose, PVDF, Nylon etc.) which immobilises them. Detection of immobilised proteins is then possible by total protein staining methods or immuno-detection of a specific protein by the "Western" method.

2.14.1. Transfer and immobilisation of proteins on PVDF or Nitrocellulose membrane after SDS-PAGE

The procedure was based on the method described by Towbin, H.et al. (1979). After SDS-PAGE, the gel was quickly rinsed in the transfer buffer: 25mM Tris, 192mM glycine, 10% (v/v) methanol pH 8.8 at 4^{0}C. PVDF membrane was cut to size, then wetted in methanol for 2-3 seconds, rinsed with ddH_2O and soaked in transfer buffer. Two sheets of blotting paper and fibre-pads were also soaked in the transfer buffer. A mask was cut out from plastic sheet to cover areas of the gel not to be transferred and thus focus a transfer field on the area required. To get rid of air bubbles between the membrane and the gel, a Pasteur pipette was used as a "rolling pin". The gel holder was assembled in the following order (from anode side):

(-) cathode-fibre pad-filter paper-gel-mask-PVDF-filter paper-fibre pad- anode (+)

and fully immersed in a TransBlot™ Transfer cell (Bio-Rad) filled with transfer buffer. The electrophoretic transfer was run for 3 hours at 60V and 0.2A. Pre-stained MW markers were run on end lanes of the gel, so that transfer efficiency could be checked.

2.15. Staining of immobilised proteins on PVDF or Nitrocellulose membrane

The immobilisation of proteins on PVDF, Nitrocellulose or Nylon matrix facilitates the use of more sensitive detection systems which cannot be used on a polyacrylamide gel. One common application is the "Western" detection system. This system uses an antibody to recognise a particular protein and multiplies the detection response by coupling the antibody to an enzyme or uses second radioactivly labelled anti-antibody-antibody.

2.15.1. Temporary staining by Ponceau S

Some of the most commonly used stains in PAGE (Coomassie Blue) cannot be successfully used on membranes because they have a high affinity for both protein and matrix. The opposite case is staining with Ponceau S.

After blotting, the PVDF or Nitrocellulose membrane was immersed in Ponceau S solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) for one to two minutes and then destained by washing in ddH_2O. Proteins appear as red bands on a pink background. Staining is temporary and can be washed away by washing in ddH_2O. Ponceau S images can be preserved by rapid drying.

2.15.2. Staining by Amido Black

This procedure was based on Towbin, H. et al. (1979). After blotting, the PVDF or Nitrocellulose membrane was immersed in a solution of Amido Black (0.1% (w/v) Amido Black in 25% (v/v) isopropanol and 10% (v/v) acetic acid) for one to two minutes and then destained in 25% (v/v) iso-propanol, 10% (v/v) acetic acid. Protein bands stain black. After rinsing in ddH_2O the membrane was dried.

2.16. Production of polyclonal antibodies

2.16.1. Coupling of peptide to Keyhole Limpet Haemocyanin

5mg of a synthetic peptide based on the known sequence of AMP-PK: Asn-Val-Leu-(Thr)-Asp-Ala-Gln-Met-Asn-Cys (the amino acid in the bracket was an ambiguous residue, and C-terminal cysteine was added for coupling), was coupled to 1mg of Keyhole-Limpet Haemocyanin (KLH) using 3-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS). This method utilises the coupling of cysteine of the peptide to the primary amine of the carrier protein KLH.

A 1ml of KLH solution (1mg/ml KLH in phosphate-buffered saline pH 6.0) was stirred vigorously on a magnetic stirrer. 50μl of dimethyl sulphoxide (DMSO) containing 1.5mg MBS was added under the surface of the KLH solution and stirred for another 30 minutes.

Using a 20ml Sephadex G-25 desalting column (equilibrated in PBS pH 7.4) KLH-MBS conjugates were separated from unconjugated MBS and DMSO. The KLH-MBS conjugates elute immediately after the void volume and are a cloudy, greyish colour. 500μl of phosphate-buffered saline pH 7.4 (PBS) containing 5mg of the peptide was added to KLH-MBS conjugates, mixed vigorously by vortexing and left for one hour stirred on magnetic stirrer.

Peptide-KLH conjugates diluted to 1mg/ml with PBS pH 7.0 were stored at -20^{0}C.

PBS buffer:

8g NaCl, 0.2g KCl, 0.2g KH_2PO_4,

1.15 Na_2HPO_4, pH 7.0 in 1l ddH_2O

2.16.2. Injecting antigen and raising the polyclonal antibodies

750μl of KLH-peptide conjugate (equivalent to 750μg) was mixed with 300μl of Freund's complete adjuvant. The mixture was vortexed thoroughly, and emulsified by pumping in and out through a narrow gauge needle. The suspension was immediately injected into a rabbit through the same narrow gauge needle in multiple subcutaneous sites. This produces a faster primary response and generates antibodies with a higher titre. Two weeks later the animal received a first booster injection of 350μl of KLH-peptide conjugate (350μg) mixed with 350μl of Freund's incomplete adjuvant, and bled 10 days after that. The booster injection was administered regularly at six week intervals followed by consecutive bleeding ten days later. 30ml of blood was bled each time from the rabbit's ear to produce 15-20ml of serum (section 2.1.1.). The antibody titre against peptide was tested using the "ELISA" technique (section 2.16.3.).

2.16.3. "ELISA" (Enzyme Linked Immuno Sorbent Assay)

This procedure was based on the method described by Stephenson, F.A. and Duggan, M.J. (1991) and was used to measure the response of raised polyclonal antibodies to antigen. The method is based on multiplication of the signal response by using a biotinylated second antibody (anti-rabbit Ig antibody) which is raised in a different animal species (donkey) against the first antibody (from rabbit). The further multiplication of the signal is achieved by the addition of streptavidin with the enzyme horseradish peroxidase covalently attached. Streptavidin has a high affinity for biotin (dissociation constant 10^{-15}M) and therefore binds to the biotinylated antibody. The horseradish peroxidase produces a soluble orange coloured product from colourless substrate o-phenylenediamine dihydrochloride in the presence of H_2O_2. The intensity of colour produced is proportional to the amount of the first antibody.

Coating of ELISA plate with peptide:

The ELISA plate has 96 wells with a maximum holding capacity of 200μl. Fresh bicarbonate buffer was made by mixing: 10 ml of 50mM $NaHCO_3$ with 1ml of 0.1M NaOH. The stock AMP-PK peptide (1mg/ml in phosphate buffered saline, stored at -20^{0}C) was defrosted and diluted with bicarbonate buffer 1000 fold (for each ELISA plate 5μl of stock peptide diluted with 5ml of buffer). 100μl of 1μg/ml peptide in bicarbonate buffer was added to each well in alternate rows and the plate was wrapped in aluminium foil and left overnight at 4^{0}C. Rows without peptide serve as blanks in the assay.

Washing ELISA plate:

Fresh Blocking buffer was made by dissolving 1g of gelatin in 400ml of hot PBS buffer. The gelatine in the buffer coats the exposed plastic surfaces preventing non-specific binding of the antibodies onto plastic.

The peptide was removed from the wells of ELISA plate. Using a multi-channel pipette each well was washed three times with 200μl of Blocking buffer. The last wash was left in each well with the plate enclosed in a container for 45 minutes at 37^{0}C. The wells were emptied and dried by tapping the ELISA plate face down onto a clean paper towel.

Incubation with rabbit antibody:

A serial dilution of the serum containing polyclonal anti-peptide antibodies was made in PBS/gelatine buffer with dilution factor 3.33 between each two consecutive steps. Using a Gilson pipette and starting with the most diluted antibody concentration duplicate 95μl aliquots were transferred to a peptide-coated well and a non-coated blank well. The ELISA plate with antibodies in PBS/gelatin buffer was left (enclosed in a container) for 60 minutes at 37^{0}C. The contents of each well were then removed and using a multi-channel pipette each well was washed five times with 200μl of

Blocking buffer. The last wash was left in each well with the plate enclosed in a container for 15 minutes at 37^0C. The wells were emptied and dried by tapping the ELISA plate face down onto a clean paper towel.

Incubation with biotinylated anti-rabbit antibody:

A 750 fold dilution of biotinylated anti-rabbit antibodies (from donkey) in PBS/gelatine buffer was made. Using a multi-channel pipette 100μl of this dilution was added to each well. The ELISA plate with anti-rabbit antibodies in PBS/gelatine buffer was left (enclosed in a container) for 90 minutes at 37^0C. The wells were emptied and dried by tapping the ELISA plate face down onto a clean paper towel. Using a multi-channel pipette each well was washed four times with 200μl of PBS/gelatine buffer. The last wash was left in each well with the plate enclosed in a container for 15 minutes at 37^0C. The wells were emptied and dried by tapping the ELISA plate face down onto a clean paper towel.

Incubation with Streptavidin -Horseradish Peroxidase:

A 1000 fold dilution of streptavidin - horseradish peroxidase in PBS/gelatine buffer was made. Using a multi-channel pipette 100μl of this dilution was added to each well. The ELISA plate with streptavidin - horseradish peroxidase in PBS/gelatine buffer was left (enclosed in a container) for 45 minutes at 37^0C. The wells were emptied and dried by tapping the ELISA plate face down onto a clean paper towel. Using a multi-channel pipette each well was washed three times with 200μl of PBS/gelatine buffer and once with PBS buffer (without gelatine). The wells were emptied and dried by tapping the ELISA plate face down onto a clean paper towel.

Developing and stopping colour reaction:

The developer (pH 5.0) contained:

20mM citric acid, 50mM Na_2HPO_4,

4mM o-phenylenediamine dihydrochloride.

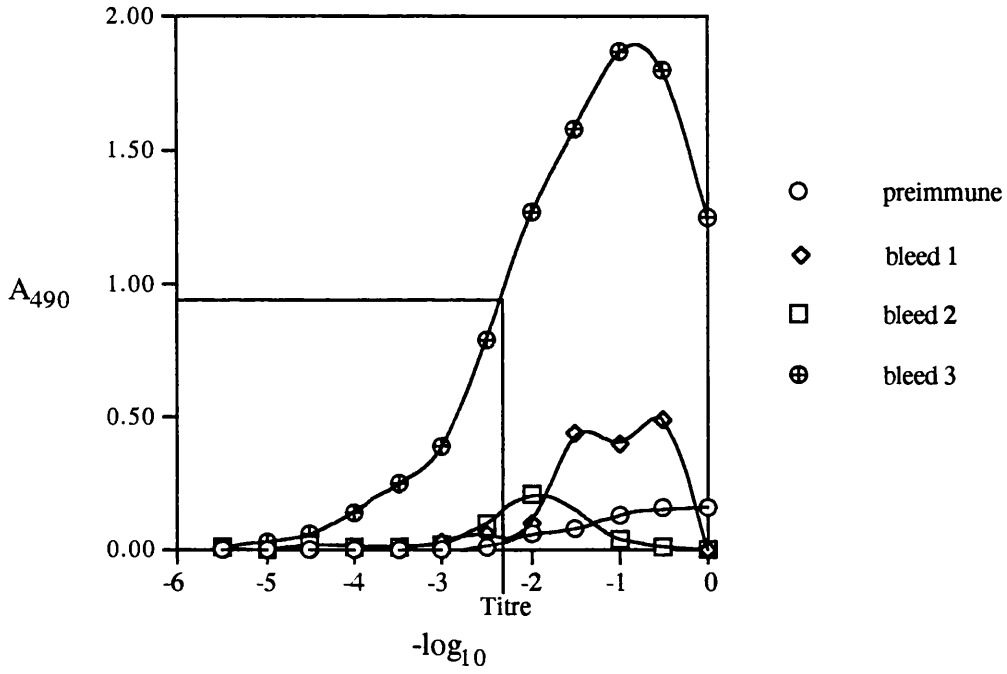
The developing solution is light sensitive and the container was wrapped in aluminium foil. Just before use 1μl of H_2O_2 was added per 10ml developer.

Using a multi-channel pipette 100μl of developer was added to each well. The reaction produces a yellowish hue. To stop the reaction 50μl of 20% (v/v) H_2SO_4 was added to each well using a multi-channel pipette. The colour immediately changed to orange. Using Minireader II (Dynatech Lab. Inc.), the A_{490} was measured for each well and after subtracting background readings (values from wells without peptide) the A_{490} was plotted against $-\log_{10}$ of antibody dilution factor (Fig.2.3). The titre represents the dilution factor at which the colour reaction reached its half maximal value. The higher the titre the more specific and more concentrated the antibodies. Serum with antibodies that show high titre can be used in "Western blot Analysis" (section 2.17.).

2.17. Detection of protein after blotting by specific antibodies "Western Blot"

This procedure was based on that described by Burnette, W.N. (1981) and utilises specific antibodies to detect the protein of interest. Proteins which were to be detected were already resolved by SDS-PAGE (section 2.9.1.) and immobilised by blotting (section 2.14.1.) onto Nitrocellulose or PVDF membranes. Detection was achieved using the same principle as in the ELISA assay. The second antibody was covalently attached to an enzyme which produces an insoluble coloured product from a soluble substrate. The insoluble product immediately precipitates in areas with enzyme-Ab"-Ab'-protein complexes. The colour, solubility of the product and sensitivity of the reaction depends on the substrate and enzyme chosen. The enzyme can be horseradish peroxidase, alkaline phospatase, glucose oxidase or ß-galactosidase with many specific substrates for each enzyme. We used horseradish peroxidase with DAB (3,3' Diamino Benzidine Tetrahydrochloride) and 4-Chloro-1-Naphthol as substrates.

Fig.2.3. ELISA results of four consecutive bleeds from a rabbit immunised with peptide derived from AMP-PK sequence



A Dutch rabbit was immunised with peptide, derived from known AMP-PK sequence, with a booster immunisation at regular intervals. Bleeding was done 10 days after each immunisation, blood was allowed to clott and sera were separated and stored at -20^{0}C. Pre-immune serum and sera from all bleeds were tested by ELISA for immune response against the peptide. Using Minireader II (Dynatech Lab. Inc.), the A_{490} was measured for each well and after subtracting background readings (values from wells without peptide) the A_{490} was plotted against $-\log_{10}$ of antibody dilution factor. The titre represents the dilution factor value at which the colour reaction reached its half maximal value. The higher the titre the more specific and more concentrated the antibodies. Bleeds 1 and 2 do not show a big response and they are very similar to pre-immune serum. The bleed 3 shows a good response with the titre 235. From this data, serum (bleed 3) with a dilution factor between 10 and 30 was chosen for "Western blot" against AMP-PK from mammary gland.

Blocking with Tris/BSA/Tween buffer:

The protein blotted Nitrocellulose sheet (8x5cm) was immersed in 25ml of Tris/BSA/Tween buffer:

50mM Tris/HCl pH 7.4 at 37^{0}C, 0.9% (w/v) NaCl,

10% (w/v) BSA, 0.1% (v/v) Tween

and left shaking gently (enclosed in a container) for 60 minutes at 37^{0}C. This blocks exposed Nitrocellulose matrix and stops non-specific binding of antibodies to it.

Incubation with rabbit antibody:

The membrane was immersed in 10 ml of antibodies diluted in Tris/BSA buffer:

50mM Tris/HCl pH 7.4 at 37^{0}C,

0.9% (w/v) NaCl, 10% (w/v) BSA,

and left shaking gently for 60 minutes at 37^{0}C. The antibodies were diluted to one tenth of their titre.

Wash with Tris/BSA/Tween buffer:

The Nitrocellulose was washed four times by shaking gently for 10 minutes with 50ml of Tris/BSA/Tween buffer at 37^{0}C.

Incubation with anti-rabbit Ab-peroxidase conjugates:

The membrane was immersed in 10 ml of 1/1000 dilution of anti-rabbit antibodies-horseradish peroxidase conjugate in Tris/BSA buffer and left shaking gently for 90 minutes at 37^{0}C. Using the second antibody-enzyme conjugate diminishes the need for the streptavidin-biotin step, but leads to some loss of sensitivity.

Wash with Tris/BSA/Tween buffer:

The Nitrocellulose was washed four times by shaking gently for 15 minutes with 50ml of Tris/BSA/Tween buffer at 37^{0}C. In the last wash BSA and Tween were omitted.

Developing and stopping colour reaction:

The developer contained:

50ml of 50mM Tris/ 0.9% (w/v) NaCl buffer pH 7.4 at 25^0C,

1.5ml of 1% (w/v) solution of $CoCl_2$,

25mg of DAB (3,3' diamino benzidine tetrahydrochloride)

The developing solution is light sensitive and the container was wrapped in aluminium foil. Just before use, 25μl of H_2O_2 was added to the developer . The Nitrocellulose was immersed in the developer and left with the container covered with aluminium foil. When the desired intensity of colour was achieved, the reaction was stopped by washing the Nitrocellulose extensively with ddH_2O. The DAB is one of the most sensitive substrates for horseradish peroxidase. The product of the reaction is intensive brown in colour and non soluble in both water and alcohol.

Instead of DAB, 4-Chloro-1-Naphthol (0.6mg/ml) was also used as a substrate.The product of the reaction with this substrate is blue-black in colour and non soluble in water. This reaction is less sensitive and the colours fade with storage.

2.18. Renaturation of proteins after separation by SDS-PAGE

Some denatured proteins slowly refold and after a period of time regain some activity. Only a small percentage of native structure was regained, due to the formation of non-soluble aggregates of semi-folded proteins, oxidation and mismatching of disulphide bonds. Also it was observed that partly-denatured proteins, if fully denatured (by 6M Guanidine/HCl or 8M Urea in presence of 100mM DTT) and allowed to renature, regain much higher activity than the control which was renatured from partly-denatured proteins (Celenza, J.L. and Carlson, M., 1991). Some groups of enzymes called Chaperonins naturally assist refolding (Mendoza, J.A. et al., 1992). The enzyme Protein Disulphide-Isomerase increases the percentage of recovered activity

by accelerating the rate of formation and breakage of disulphide bridges, and that way allows the protein to achieve the most favourable energy state and regain activity (Tang, J.G. et al., 1988). The presence of substrate and reducing agent (DTT) protects enzymes against oxidation of their active sites (Tandon, S and Horowitz, P.M., 1989). The non-ionic detergents above the micellar concentration (Zardeneta, G. and Horowitz, P.M., 1992) , $PEG_{3000-6000}$ (Cleland, J.L. et al., 1992) , pH 7.0 or below, low temperature (Brems, D.N., 1988) and low protein concentration (Mendoza, J.A. et al., 1991) prevent or slow down hydrophobic interaction of semi-unfolded proteins and this way prevent the aggregation.

2.18.1. Renaturation of kinases immobilised on PVDF or Nitrocellulose membranes after SDS-PAGE

This procedure was based on the methods described by Celenza, J.L. and Carlson, M. (1986) and Ferrell, J.E.Jr. and Martin, G.S. (1989) and utilises spontaneous refolding of denatured proteins and the fact that most kinases autophosphorylate when incubated with Mg and ATP.

Denaturation:

Protein resolved by SDS-PAGE was blotted onto a PVDF membrane with methanol omitted from the transfer buffer. The PVDF membrane was immersed in 50ml of Guanidine buffer for 60 minutes at room temperature. Guanidine buffer contains:

7M Guanidine/HCl pH 8.3 at 25^0C,

50mM Tris, 50mM DTT, 2mM EDTA

Renaturation:

The membrane was then transferred to a container with 50ml of:

100mM NaCl, 50mM Tris/HCl pH 7.5 at 4^0C, 2mM DTT,

2mM EDTA, 1% (w/v) BSA, 0.1% (w/v) Nonidet P-40.

Renaturation was allowed to proceed at 4^0C with three changes of this buffer during a period of 12-18 hours. Before incubation with Mg and ATP, any accessible site on PVDF membrane was blocked by 5% (w/v) BSA in 50ml of 100mM NaCl, 30mM Tris/HCl pH 7.5 at 25^0C, 2mM DTT, 2mM EDTA, with gentle shaking for 60 minutes at room temperature.

Autophosphorylation:

Autophosphorylation of renaturated kinase was carried out by immersing the PVDF membrane in 5ml of Mg/ATP incubation buffer containing:

30mM Tris/HCl pH 7.5 at 25^0C, 10mM $MgCl_2$, 2mM $MnCl_2$,

50μCi/ml of [γ-^{32}P] ATP (3000-6000Ci/mmol).

The reaction was stopped after 30 minutes by washing the membrane in six consecutive washes (10 minutes each) of 30mM Tris/HCl pH 7.5 at 25^0C. To reduce the radioactivity background, the membrane was washed in 1M KOH for 10 minutes at room temperature. The membrane was rinsed several times with 10% (v/v) acetic acid, then dried and autoradiographed (as described in section 2.12.).

2.18.2. Renaturing of kinases in substrate-polyacrylamide gel after SDS-PAGE

This procedure was based on the method described by Hutchcroft, J.E. et al. (1991). It was observed that only a small percentage of protein kinase regains activity after refolding, due to mismatching (and oxidation) of disulphide bonds and forming aggregates. Instead of relying on autophosphorylation, detection of low activity is possible by inclusion of the peptide substrate in the resolving acrylamide gel during polymerisation. The peptide substrate does not electrophorese from the gel during electrophoresis suggesting that some of it may be covalently attached to the acrylamide polymers. The refolding of fully unfolded proteins (by 8M urea, 100mM DTT) was encouraged by the presence of substrate, non-ionic detergents (Brij 35)

above micellar concentration, pH 7.0, low temperature (4^0C) and a reducing agent (DTT).

The synthetic peptide "SSMS" or Kemptide was added to the polymerisation mixture of the resolving gel to a final concentration of 1mg/ml. The 7.5% T resolving gel and 4.5% T stacking gel were cast as described in section 2.9.1. The protein samples were electrophoresed at 15mA constant current through the gel. After the run the resolving gel was separated from the stacking gel and washed twice for 5 minutes with ddH_2O. The water was replaced with two changes of 20ml of: 50mM Hepes pH 7.0, 50mM DTT, 25% (v/v) 2-propanol, 0.5% (v/v) Brij 35 each for 30 minutes.

This buffer was then replaced with 20ml of : 50mM Hepes pH 7.0, 50mM DTT, 8M urea, 1% (v/v) Brij 35 and the gel was shaken for 30 minutes at room temperature.

Renaturation was carried out at 4^0C in two changes of 20ml of: 50mM Hepes pH 7.0, 150mM NaCl, 0.1% (v/v) Brij 35 each for 30 minutes. The phosphorylation of immobilised peptide was achieved by incubation of the gel with renatured proteins for 30 minutes in 20ml of incubation mixture containing:

50mM Hepes pH 7.0, 150mM NaCl, 0.1% (v/v) Brij 35,
10mM $MnCl_2$, 0.2mCi [γ^{32}P] ATP (3000-6000Ci/mmol).

After 30 minutes 100μl of 20mM ATP and 100μl of 20mM 5'AMP were added to the incubation mixture to a final concentration of 100μM in respect to AMP and ATP and left for another 30 minutes on an orbital shaker. Phosphorylation was stopped by immersing the gel in 50ml of 75mM phosphoric acid. The phosphoric acid was changed 4 times (15 minutes each wash), the gel was briefly washed with ddH2O (for 5 minutes) and stained with Coomassie Blue R-250 as described in section 2.13.1. The "SSMS" peptide and Kemptide do not stain with this method and therefore only separated protein bands were detected.

Autoradiography (as described in section 2.12.) is used to match phosphorylated regions of immobilised peptide substrate to renatured protein bands in the gel.

CHAPTER THREE

Purification and characterisation of AMP-activated protein kinase from lactating rat mammary gland

3.1. Purification of the AMP-activated protein kinase from lactating rat mammary gland

Female Wistar rats were used at peak lactation, 10-15 days *post partum*. They were sacrificed by stunning and cervical dislocation. Mammary glands were rapidly removed, washed in ice cold homogenisation buffer and then freeze-clamped in wafer thin slices. They were powdered and stored in liquid nitrogen until used. Powdered mammary glands (50g) were homogenised using a domestic blender (six thirty second bursts at top speed) in 400ml of ice cold homogenisation buffer:

50mM Tris/HCl (pH 7.4 at 4^0C),
250mM Sucrose, 50mM NaF, 5mM NaPPi,
5mM DTT, 1mM EDTA, 4 μg/ml SBTI,
2mM Benzamidine, 0.2 mM TLCK, 0.2mM PMSF .

In all other purification steps (except in the Phospho-cellulose step where NaPPi was omitted) the AMP-PK purification buffer was :

50mM Tris/HCl (pH 7.4 at 4^0C), 10% (w/v) Glycerol,
50mM NaF, 5mM NaPPi, 1mM DTT, 1mM EDTA,
2μg/ml SBTI, 1mM Benzamidine, 0.02% (w/v) Brij 35.

The homogenate was centrifuged at 15000g and 4^0C for 15 min to remove crude particles. The supernatant was filtered through glass wool and 0.0533 volumes of 50% (w/v) PEG $_{6000}$ was added slowly and stirred at 4^0C for 15 min. This 2.5% (w/v) PEG suspension was centrifuged at 20000g and 4^0C for 20 min and the supernatant was retained and filtered through glass wool to remove floating fat. The PEG concentration in the supernatant was adjusted to 6% (w/v) PEG by adding 0.0747 vol. of 50% (w/v) PEG_{6000} with continuous stirring for 20 min at 4^0C. The 6% (w/v) PEG_{6000} suspension was centrifuged at 20000g for 20 min. The supernatant was discarded and the pellet used immediately or stored at -20^0C until used.

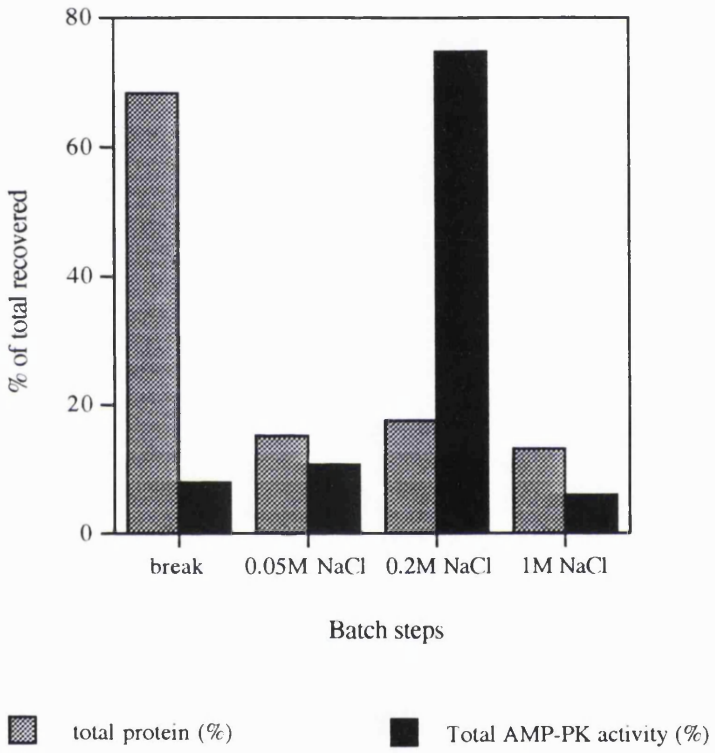
The polymer precipitation with PEG_{6000} was chosen because it does not separate sub-units or cofactors from proteins. PEG also has stabilising effects on proteins, and a re-suspended PEG pellet does not need desalting or high dilution before loading onto the chromatography column. At this stage AMP-PK and ACC co-purified in the 2.5-6% w/v PEG_{6000} cut. It was estimated that all AMP-PK activity in the crude homogenate prepared from lactating rat mammary gland was precipitated by the 2.5-6% (w/v) PEG_{6000} step (Table 3.1.).

3.1.1. Separation by DEAE-Sepharose FF (fast flow) chromatography

The 2.5-6% (w/v) PEG_{6000} pellet was re-suspended in AMP-PK purification buffer (1/5 volume of original homogenate), spun at 100000g for 1h at 4^0C in an ultracentrifuge and the supernatant applied to a DEAE Sepharose FF column (10/50) that was connected to an FPLC and had been equilibrated in AMP-PK purification buffer containing 50mM NaCl.

DEAE-Sepharose FF contains weak anion exchanger groups DEAE (Diethylaminoethyl) attached by an ether linkage to the monosaccharide units of the highly cross-linked Sepharose (chemically modified agarose gel). DEAE-Sepharose FF was chosen for a first anion-exchange column because of its high protein binding

Fig.3.1. Elution of AMP-activated protein kinase from DEAE-Sepharose FF using stepwise increases in NaCl concentration



The 2.5-6% (w/v) PEG_{6000} pellet was resuspended in AMP-PK purification buffer and loaded onto DEAE-Sepharose 6 FF column (10/50) connected to an FPLC. After washing the column, AMP-PK was eluted by step batch elution with NaCl. All steps were collected and protein concentration and AMP-PK activity were measured as described in Materials and Methods (sections 2.8.1 and 2.6 respectively).

capacity (110 mg HSA/ml of gel), high speed (due to high cross-linkage of matrix) and the relatively low salt concentration needed for AMP-PK elution (due to the weak anion exchanger group). The latter was an advantage in the next step of purification, since dialysis could be replaced by dilution for removing the salt, hence speeding up the purification.

AMP-PK was step-eluted from DEAE by increasing the salt concentration step-wise (50-200mM NaCl) in the AMP-PK purification buffer. In preliminary experiments the first salt step 0-50mM NaCl removed a significant amount of protein but not a significant amount of AMP-PK (Fig.3.1.). More than 75% of the total recovered AMP-PK activity, and less than 18% of the total recovered protein, was eluted in the 50-200mM NaCl step. The 0-50mM NaCl step eluted only 12% of the activity but nearly the same amount of protein as the 50-200mM NaCl step (Fig.3.1.).

Step-elution was used in this first column chromatography step because at this stage of purification the protein load on the column was too great to achieve good resolution with a salt gradient. Also the speed which was used (8 ml/min, for load and wash, and 4 ml/min for elution) would be too high to achieve a good separation on a salt gradient. The high speed was essential for the early steps in the purification of AMP-PK, because at these stages the mammary AMP-PK appears unstable (a rapid loss of AMP-PK activity occured if the preparation was left for a long period on DEAE-Sepharose).

3.1.2. Affinity purification by Blue-Sepharose 6-FF (fast flow)

The Blue Sepharose 6-FF contains as an active group, the reactive triazine-based textile dye, Cibacron Blue F3G-A. This active group is covalently attached by the triazine coupling method to the Sepharose 6 (a type of chemicaly modified and highly cross-linked 6% agarose gel). A wide range of proteins bind to the column by means

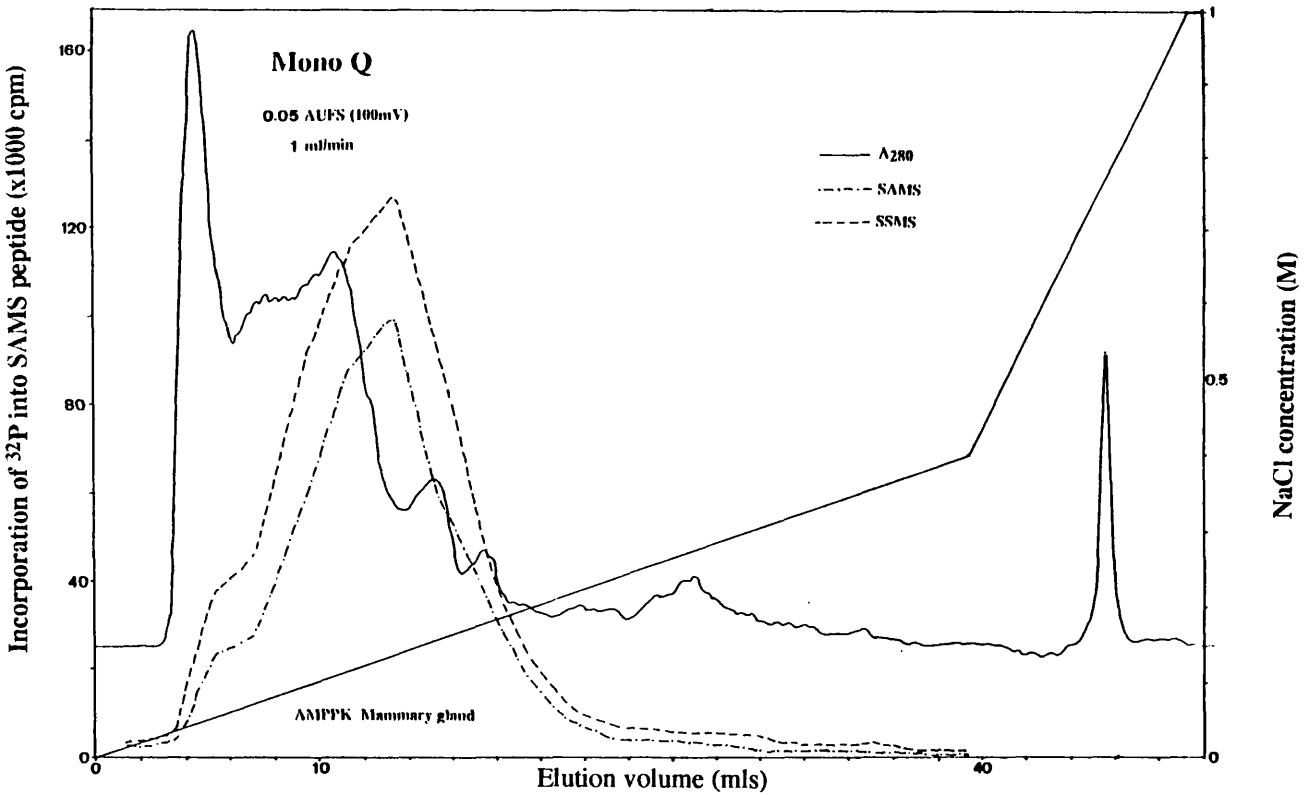
of electrostatic and/or hydrophobic interactions with the dye which is both aromatic and anionic in nature. The column has a high binding capacity, at least 16 mg of HSA per ml of gel. The dye structure also mimics purine nucleotides and binds strongly enzymes requiring adenylyl-containing cofactors (Easterday, R. L. and Easterday, I. 1974; Thompson, S. T. et al., 1975). The binding of AMP-PK to the column is particulary strong because AMP-PK contains both, an AMP and an ATP binding site.

The AMP-PK eluted from DEAE-Sepharose in 200mM NaCl was directly loaded onto a Blue Sepharose 6FF column (20/20) connected to an FPLC that had been previously equilibrated in AMP-PK purification buffer containing 200mM NaCl. The typical volume of the loaded protein was 100ml, and was loaded using a 50ml superloop (twice). The loading, washing and eluting speed was 3 ml/min. After loading and washing until a stable base line was achieved (typically 200 - 300 ml), AMP-PK was eluted with a salt gradient from 0.2-1.5M NaCl (over 300ml). The breakthrough and all fractions (10ml each) were collected and assayed for AMP-PK activity as described in Materials and Methods (section 2.6). The breakthrough did not contain a significant amount of AMP-PK activity. The peak of AMP-PK activity (eluted at 950mM NaCl concentration) was pooled and dialysed overnight at 4^0C into AMP-PK purification buffer containing 50mM NaCl. An illustration of a similar Blue Sepharose profile using slightly different settings is shown in Fig.3.13.A. The gradient part of this figure is identical to the procedure described above.

3.1.3. Mono Q anion exchange chromatography

Mono Q is a strong anion exchange FPLC column, with quaternary amines as charged groups covalently attached to the MonoBeads™ matrix. The physical properties of the matrix allow use of high pressure during the chromatographic run (5MPa). The narrow particle distribution ($10 \pm 0.03\mu$m) of the column yields high reproducibility and maximal resolution.

Fig.3.2. AMP-PK purification profile on Mono Q HR 5/5



AMP-PK from rat mammary glands eluted from Blue Sepharose 6FF was dialysed overnight into AMP-PK purification buffer (with 50mM NaCl), then filtered through a 0.2 μm filter and loaded onto a Mono Q HR 5/5 column which had been equilibrated in AMP-PK purification buffer (containing 50mM NaCl). Loading, washing and elution speed was 1ml/min. Maximal pressure was set to 4 MPa, absorbance sensitivity to 0.05 AUFS (100mV) and chart-recorder speed to 0.5cm/ml. After washing non bound protein from the column with AMP-PK purification buffer (containing 50mM NaCl), until a stable base line was achieved, NaCl concentration was lowered to 0. AMP-PK was eluted by a 0-400mM NaCl gradient over 40ml. 1ml fractions were collected and 5μl from each fraction were assayed for 10min at 37^{0}C for AMP-PK activity using the specific AMP-PK assay described in chapter 2. In parallel, similar assays were performed using the SSMS peptide instead of SAMS. The results, after subtracting the blanks (without peptide in assay) were plotted together with A_{280}.

The maximal binding capacity for a 5/5 column is 60 mg of HSA. The maximal recommended load for MonoQ 5/5 is 25mg of protein per column run. The typical load onto a Mono Q 5/5 column in purification of AMP-PK from mammary glands was around 7mg of protein (the load never exceeded 10mg of protein per run).

Dialysed AMP-PK (post Blue Sepharose) was filtered through a 0.2 μm filter and loaded onto a MonoQ 5/5 column, which had been equilibrated in AMP-PK purification buffer (containing 50mM NaCl). The loading volume was typically 80ml. Loading, washing and elution speed were 1ml/min. After achieving a stable base line, the NaCl concentration was dropped to 0, and the AMP-PK was eluted with a linear 0-400mM NaCl gradient over 40ml (Fig.3.2.). The breakthrough and all fractions (1ml each) were collected and tested for AMP-PK activity using SAMS peptide (as described in Materials and Methods section 2.6.). They were also tested for other SSMS kinases (SSMS peptide, which has the native sequence of the major regulatory phosphorylation site on ACC, can be phosphorylated also with cAMP-PK, PKC and ACK-2). The protein peak which was eluted at 800mM NaCl was devoid of any activity (not shown in figure). The peak of AMP-PK activity from mammary glands eluted at 150mM NaCl. The peaks of phosphorylation of SAMS and of SSMS peptide showed an indentical position on the elution profile, with identical shoulders. There were only two peaks of SSMS or SAMS activity in fractions assayed across the whole gradient. The exact coincidence of peaks of SSMS and SAMS activity (150mM NaCl) and absence of any other peaks of their activity indicate, that at this stage of purification, the preparation does not contain other kinases which can phosphorylate SSMS and not SAMS peptide (cAMP-PK, PKC or ACK-2). The AMP-PK peak appeared relatively broad (60 - 250mM NaCl) and with a shoulder. One can speculate, that this shoulder could represent a dephosphorylated and less active form of AMP-PK with a slight charge difference due to absence of phosphate. The fractions (fractions 9-16. in Fig.3.2.) containing the peak of AMP-PK activity (around 150mM NaCl) were pooled, and dialysed against a storage buffer :

50mM Hepes/NaOH pH 7.4 , 50% (w/v) glycerol,
50mM NaF, 5mM NaPPi, 1mM EDTA, 1mM EGTA,
5mM DTT, 4μg/ml SBTI, 1mM Benzamidine,

then concentrated to 1ml with a Centricon 30 concentrator (with membrane MW cut-off point of 30 KDa) and stored at -20^{0}C. AMP-PK, stored at this stage, retained more than 55% of its initial activity after 90 days stored at -20^{0}C (Fig.3.5.).

3.1.4. Selecting the second affinity column

Affinity purification of kinases can be achieved by their interaction with specific ligands (eg. substrates, substrate analogues, cofactors or inhibitors) covalently attached to a gel matrix.

A substrate based affinity column with SAMS peptide attached to activated resin, did not purify liver AMP-PK (Dr. D. Carling-personal communication). One could speculate that the coupling of such a short peptide to the column restricted the access of AMP-PK to the substrate. For that reason this was not attempted for mammary AMP-PK. Other AMP-PK substrates eg. ACC and HMG-CoAR are too unstable to couple to gels. Histone is a possibility but Histones are so basic that Histone-Sepharose columns bind many other proteins as anion-exchanger.

AMP-PK has two nucleotide binding sites, for AMP and ATP. Affinity purification based on selected nucleotides can be a good purification step. Initial trial affinity purification was performed with: 5'ATP-agarose (A4793, Sigma), 5'AMP-agarose (A5523, Sigma) and Phospho-Cellulose P11 (Whatman).

5'ATP-agarose has 5'ATP as an active group attached through ribose hydroxyls via a 22 atom spacer arm to an epoxy activated, cross-linked, 4% agarose.

5'AMP-agarose has 5'AMP as an active group attached through the N-6 in the adenine ring via an 11 atom spacer arm to a cyanogen bromide activated, cross-linked, 4% agarose.

Phospho-Cellulose P11 has orthophosphoric acid as an active group ether linked to a fibrous cellulose. The orthophosphoric group presumably mimics the phosphate configuration in ATP.

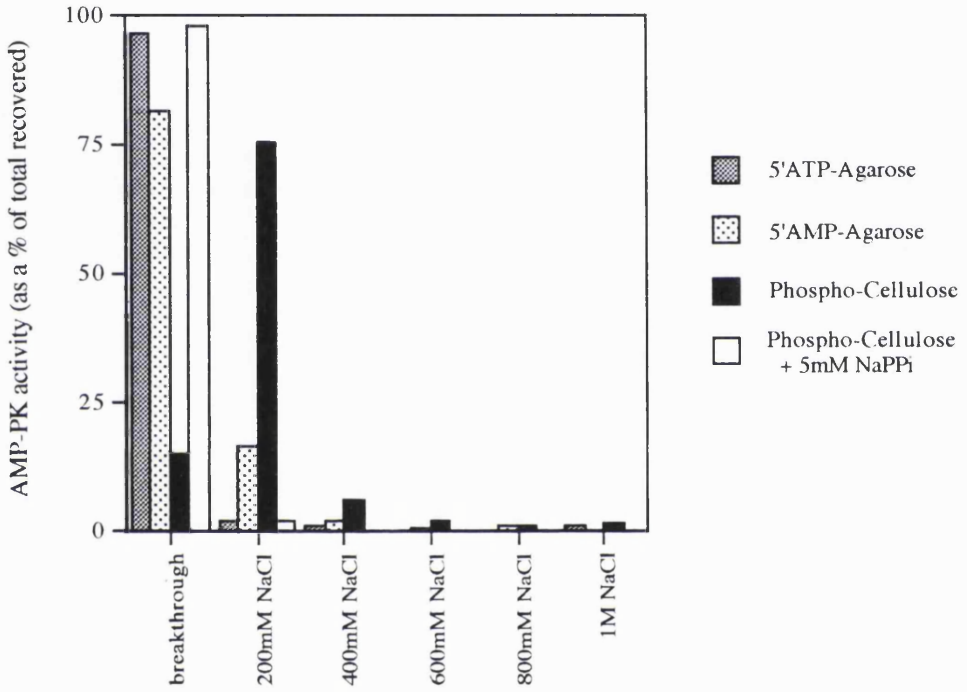
Post Mono Q AMP-PK was dialysed into AMP-PK purification buffer without sodium pyrophosphate. The AMP-PK was then split into three pools (500μl each) and each loaded onto 1ml mini columns of 5'ATP-agarose, 5'AMP-agarose or Phospho-Cellulose. Batch step chromatography (with 200mM NaCl steps) was run at 4^0C. The breakthrough and each batch step elution were collected and tested for AMP-PK activity using SAMS as a substrate (Fig.3.3.).

5'ATP-agarose did not bind AMP-PK as 97% of activity remained in the breakthrough (Fig.3.3.). This may be due to the type of ATP attachment to the spacer, or a need for ATP/Mg^{2+} complex formation and fitting into the AMP-PK ATP-binding site (di-valent metal cations were not used in the AMP-PK purification buffer due to the possible presence of metaloproteases).

5'AMP-agarose shows a low capacity for binding of AMP-PK since 80% of the activity remained in the breakthrough and 17% eluted with 200mM NaCl (Fig.3.3.). This is possibly due to the type of AMP attachment to the spacer or that the AMP binding site may already be occupied by AMP.

The Phospho-Cellulose column produced the best results. Only 16% of AMP-PK remained in the breakthrough and 76% of the activity was eluted from the column with 200mM NaCl (Fig.3.3.). The presence of 5mM NaPPi in the AMP-PK buffer totally inhibited binding of AMP-PK to the Phospho-Cellulose column since 98% of AMP-PK activity remained in the breakthrough (Fig.3.3.). For this reason NaPPi (although

Fig.3.3 Trial purification of AMP-PK on 5'ATP-Agarose, 5'AMP-Agarose and Phospho-Cellulose P11



Post Mono Q AMP-PK from mammary gland was dialysed into AMP-PK purification buffer without NaPPi and loaded onto 1ml 5'ATP-Agarose (Sigma A-4793), 5'AMP-Agarose (Sigma A-5523) and Phospho-Cellulose P11 (Whatman) columns. The breakthroughs were collected and batch step elution was carried out using 200mM NaCl steps until 1M NaCl. AMP-PK activity was measured as described in Materials and Methods (section 2.6.). Results were expressed as a % of total recovered AMP-PK activity.

necessary for the inhibition of protein phosphatases) was ommited during purification using the Phospho-Cellulose column.

3.1.4.1. Using Phospho-Cellulose P11 as an affinity step

Phospho-Cellulose P11 (Whatman) was activated according to the instructions with consecutive 5min treatments in 0.5M NaOH and 0.5M HCl, equilibrated and stored in 0.5M KPi buffer (pH 7.0). Phospho-cellulose P11 is a bi-functional (strong above pH7 and weak below pH 7) cation exchanger with ester-linked orthophosphoric acid. At pH above 7.0, the weakly acidic group undergoes a second ionisation and the column becomes a strong binder. The orthophosphate group interacts with ATP binding sites on proteins. Because AMP-PK at running conditions (pH 7.4) is negatively charged (binds to DEAE and Mono Q anion exchanger) binding to Phospho-Cellulose is achieved through weak interaction of the orthophosphate and AMP and ATP binding sites.

Phospho-Cellulose was packed into an FPLC column (10/3), connected to the FPLC and equilibrated in AMP-PK purification buffer lacking NaPPi. Before loading onto Phospho-Cellulose, AMP-PK (post Mono Q) was dialysed for 2h at 4^0C against two changes of AMP-PK purification buffer lacking NaPPi. Loading and washing of Phospho-Cellulose was carried out at 1ml/min. The volume loaded was typically 2 - 8ml, and washing of unbound proteins was performed until a stable base line was achieved (typically 10 - 15ml).

AMP-PK was eluted at 0.5 ml/min by step batch elution with NaCl (Fig.3.14.) and each batch fraction (6ml each) assayed for AMP-PK activity. Most of the recovered AMP-PK activity (72%) was in the 50-200mM NaCl step. The 0-50mM NaCl and 200-1000mM NaCl steps contained 11 and 12 % of recovered activity respectively (Fig.3.15.).

The 50-200mM NaCl batch step elution containing AMP-PK was pooled and 1/10 volume of 50mM NaPPi was added to give a final concentration of 5mM NaPPi. The AMP-PK pool was concentrated down to 200μl using a Microcon 30 and applied to a gel-filtration column.

3.1.5. Using size exclusion chromatography for protein separation

As a final step in purification, separation according to size was used. This was chosen as a last step because of the low protein loading capacity of gel-filtration columns. Trial gel filtration using a Sephadex-G-100 (Pharmacia) column (20/70) connected to FPLC did not yield a satisfactory result because of the dilution factor that occurred, due to the size of column. The size of the column and length of time necessary for achieving good separation (overnight at 0.1ml/min) led to more than 95% loss of AMP-PK activity.

The preliminary gel filtration using Superose 6, Superose 12 and both columns in line was also performed. The Superose 12 HR 10/30 was chosen for use in purification because of higher operation pressure (maximal pressure for Superose 12 is 2.4 MPa compared to 1.2 MPa for Superose 6) which led to a higher speed during the run and a shorter running time.

3.1.5.1. Gel-filtration on Superose 12 HR 10/30 (prepacked FPLC gel-filtration column)

Superose 12 HR 10/30 is an FPLC gel filtration column packed with highly cross-linked porous agarose beads (average size of bead 10-11μm). Maximum loading capacity for this size column is 5mg, but the optimal load is 4 times less. Superose 12 was equilibrated in AMP-PK purification buffer containing 500mM NaCl (to minimise protein-protein and protein-agarose interactions) using FPLC. Running speed was 0.5ml/min and maximal pressure was set for 1.8 MPa. After achieving a stable base

line a calibration run was performed using MW standards: BSA 66 KDa (132 KDa for dimer), carbonic anhydrase 29 KDa and cytochrome C 12.4 KDa.

AMP-activated protein kinase (post Phospho-Cellulose pool concentrated down to 200μl by Centricon 30) was filtered through a 0.2μm syringe prefilter, spun down at 13000 rpm on a bench centrifuge for 5 minutes, and the clear supernatant loaded onto Superose 12 (Fig.3.16.A). The run was performed with the same settings as the calibration run. Fractions (500μl) were collected after the column dead volume and assayed for AMP-PK activity, using SAMS peptide as a substrate. The AMP-PK eluted between 132 and 66 KDa, with a calculated MW of 93 KDa for the peak of AMP-PK activity (Fig.3.16.A.). This was identical to the calculated MW for AMP-PK from mammary gland using data obtained from running samples on a Superose 6 gel filtration column (Fig.3.8.) and similar to the value of 100 ± 30 KDa for rat liver AMP-PK (Carling, D. et al., 1989). Figures 3.8. and 3.16. show the peak of AMP-PK activity and no AMP-PK activity was found in fractions outside this peak. The fractions containing AMP-PK were pooled, dialysed into storage buffer (as described earlier), concentrated down using Microcon 30 (Amicon) to 100μl and stored at -20^{0}C.

3.1.6. Other chromatography methods attempted

Purification according to size and charge using non-denaturing PAGE, and according to size by induced shift in charge using non-denaturing BLUE-PAGE was attempted (detailed methodology in chapter 2 section 2.9.2.-2.9.4.) using the post Phospho-Cellulose AMP-PK preparation, without success.

Chromatofocusing on Mono P column (10/30) was also tested with 25mM Imidazole/HCl buffer pH 7.4 as a starting buffer and Polybuffer 74/HCl (1/8 dilution, pH 4.0) as an elution buffer, using the post Phospho-Cellulose AMP-PK preparation with total loss of AMP-PK activity.

3.1.7. Purification table for AMP-PK from mammary gland

The purification table (Table 3.1.) shows a typical AMP-PK purification using mammary gland tissue from five rats at peak lactation as starting material. Using a PEG cut and a five column protocol a 3750-fold purification was achieved. This is quite comparable to a 6657-fold purification achieved using the same amount of liver material from the same animals (Table 3.2.). The specific activity of AMP-PK purified from mammary gland (131 U/mg) is quite comparable to the published specific activity of AMP-PK purified from rat liver (184 U/mg) (Carling, D. et al., 1989). The samples from each step in the purification of AMP-PK from mammary gland were run on SDS-PAGE (10% T polyacrylamide resolving gel; section 2.9.1.) and are shown in Figure 3.4. Although a 4,5-fold purification (Table 3.1) was achieved between 3% PEG_{6000} and 3-6% PEG_{6000} step, no great difference in the number of bands can be observed (Fig.3.4. lane 2 and 3 respectively). This is probably, because 3-6% PEG_{6000} step will only get rid of very low MW proteins which will not be visible on 10%T SDS-PAGE. Major enrichment in specific activity was achieved at DEAE-Sepharose, Blue-Sepharose and Superose 12 steps, 7.5, 4.4 and 5-fold respectively (Table 3.1) which correspond well with major changes in band patterns (Fig.3.4.).

Although 3750-fold purification was achieved even the most pure AMP-PK preparation (Superose 12 step) shows quite few bands of proteins (Fig.3.4. lane 9). The only band with MW corresponding to published 63 KDa MW for AMP-PK from liver (Carling, D. et al., 1989) in this lane was faint 62 KDa band and represent less than 10% of total protein. This is also obvious from the final specific activity of the preparation (0.131 μmol/min/mg using SAMS peptide) which is 10-100 fold lower than that of other well-characterised protein kinases which have been purified to homogeneity (Cohen, P., 1983; Hemmings, B.A. and Cohen, P., 1983; Payne, M.E. and Soderling, T.R., 1983; Walsh, M.P. et al., 1983; Reimann, E.M. and Beham, R.A., 1983; Lincoln, T.M., 1983).

Table 3.1. Purification table for AMP-activated protein kinase from lactating rat mammary gland

Fraction	Total protein [mg]	Total Activity [U]	Specific Activity [U/mg]	Purification [fold]	Yield [%]
Crude homogenate	4106	146	0.035	1	-
2.5% PEG supernatant	2737	152	0.055	1.57	100
2.5-6% PEG pellet	608	149	0.245	7	100
post DEAE Sepharose	65.5	120	1.83	52.3	82
post Blue Sepharose	6.4	51.2	8.00	229	35
post Mono Q	0.285	3.55	12.46	356	2.43
Post Phospho Cellulose	0.08	2.1	26.25	750	1.4
Post Superose 12	0.009	1.26	131	3750	0.86

The table shows a typical AMP-activated protein kinase purification profile using mammary tissue from five lactating rat as starting material. Tissue was freeze-clamped in wafer thin slices and powdered in liquid nitrogen, before use in purification.

Protein concentration was measured by the Bradford method (section 2.8.1.). AMP-PK activity was measured by the incorporation of ^{32}P into SAMS peptide (section 2.6.). 1 Unit (U) of AMP-PK activity represents the incorporation of 1nmol of phosphate into SAMS-peptide per minute at 37^0C.

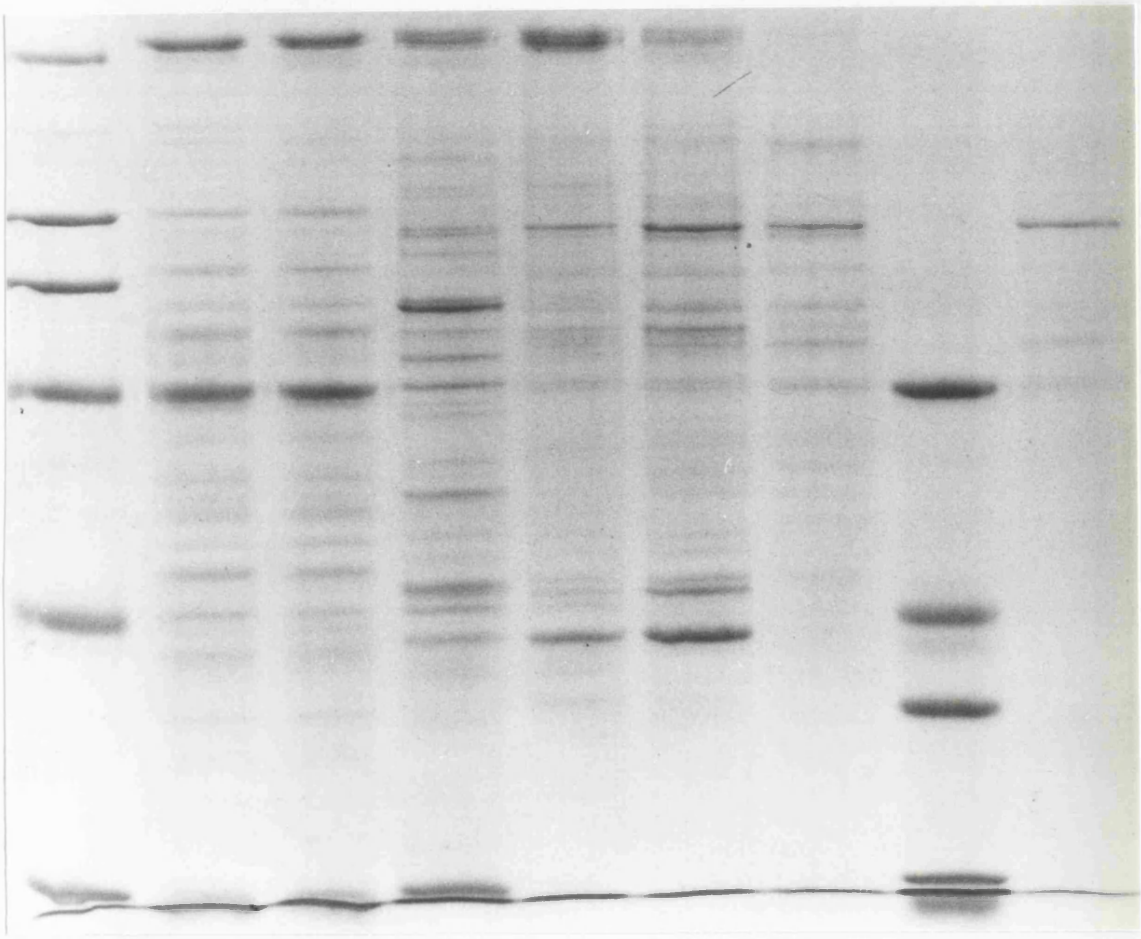
Table 3.2. Purification table for AMP-activated protein kinase from lactatng rat liver

Fraction	Total protein [mg]	Total Activity [U]	Specific Activity [U/mg]	Purification [fold]	Yield [%]
Crude homogenate	4680	327	0.069	1	100
2.5% PEG supernatant	3086	324	0.105	1.5	99
2.5-6% PEG pellet	726	304	0.419	6	93
post DEAE Sepharose	88	290	3.29	47	89
post Blue Sepharose	15.2	128	8.42	120	39
post Mono Q	0.5	18.3	36.6	523	5.6
post Phospho Cellulose	0.12	17.7	146.6	2095	5.4
post Superose 12	0.013	6.04	465.7	6657	1.8

The table shows a typical AMP-activated protein kinase purification profile using liver tissue from the same five lactating rats used for the purification described in Table 3.1. Tissue was frozen and powdered in liquid nitrogen, before use in purification.

Protein concentration was measured by the Bradford method (section 2.8.1.). AMP-PK activity was measured by the incorporation of ^{32}P into SAMS peptide (section 2.6.). 1 Unit (U) of AMP-PK activity represents the incorporation of 1nmol of phosphate into SAMS-peptide per minute at 37^0C.

Fig.3.4. SDS-PAGE of each step in the purification of AMP-PK from mammary gland



1 2 3 4 5 6 7 8 9

Samples for SDS-PAGE analysis were prepared from aliquots of pooled AMP-PK fractions from each step of purification of AMP-PK from lactating mammary gland. Samples were run on standard SDS-PAGE (10% T polyacrylamide resolving and 5% T stacking gels; as described in section 2.9.1), stained with Coomassie Blue R-250, dried and photographed (section 2.13.2).

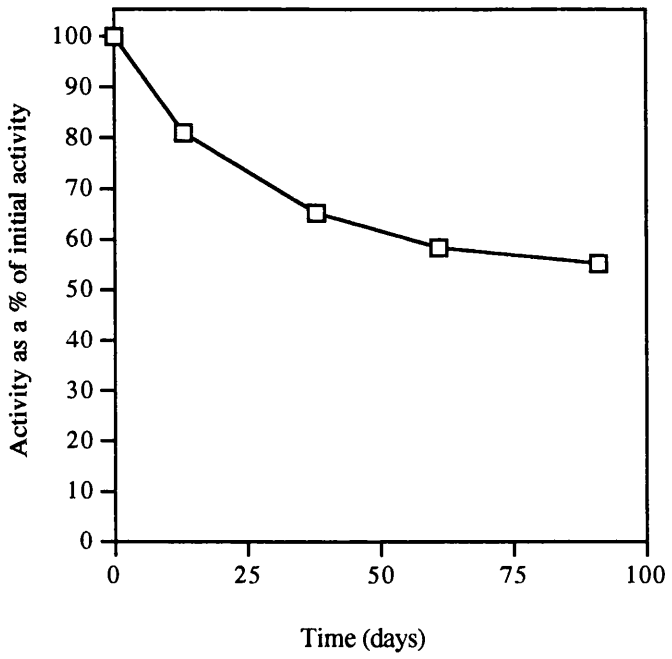
Lane 1 contains high MW SDS markers (205, 116, 97.4, 66, 45 and 29 KDa) and lane 8 low MW SDS markers (66, 45, 36, and 29 KDa). The AMP-PK purification steps are: lane 2 -post 2.5% PEG_{6000} step; lane 3 - resuspended 2.5-6% PEG_{6000} pellet; lane 4 -post DEAE Sepharose step; lane 5 -post Blue-Sepharose step; lane 6 -post Mono Q step; lane 7 -post Phospho-Cellulose step; lane 9 - post Superose 12 step.

The purification can be further optimised. The purification table clearly shows steps at which purification can be improved. The Blue Sepharose step, although producing a 4 fold purification, shows more than 50% loss in total activity. The greatest loss of activity was at the Mono Q step (more than 93%). By increasing the speed of purification, or improving the buffers to increase the stability of AMP-PK during preparation (eg. changing the detergent) or finding alternative columns can produce better purification. Comparing the final specific activity achieved, with the specific activity of kinases purified to homogeneity (as mentioned above) one can speculate that inclusion of one more step (eg. antibody column) can produce pure AMP-PK.

3.1.8. Stability of mammary gland AMP-PK preparation

In initial purification attempts the data suggested that AMP-PK purified from mammary gland was unstable and there were large losses of activity during purification and storage. This could be due to the presence of higher concentrations of proteases in mammary glands especially if the mammary gland was taken towards the end of lactation (Eto, I. and Bandy, M.D., 1990; Talhouk, R.S. et al., 1991; Talhouk, R.S. et al., 1992). For this reason a higher than normal concentration of protease inhibitors was used in the homogenisation buffer (section 2.1.6). Also all purification steps were performed using the FPLC and that shortened the overall time of purification. This yielded a higher specific activity of AMP-PK (than in initial purification attempts) and is quite comparable with purification achieved from the same amount of starting material from the livers of the same group of animals (Table 3.1, 3.2.). The AMP-PK from mammary glands purified in this way up to the post Mono Q step and stored in: 50mM Hepes pH 7.4 , 50% (w/v) glycerol, 50mM NaF, 5mM NaPPi, 1mM EDTA, 1mM EGTA, 5mM DTT, 4μg/ml SBTI and 1mM Benzamidine did retain more than 55% of its initial activity after storage for three months at -20^{0}C, providing that it was not subjected to sudden changes in temperature (Fig.3.5.).

Fig.3.5. Stability of AMP-activated protein kinase purified from rat mammary gland and stored at -20^{0}C



AMP-activated protein kinase was purified from lactating rat mammary gland as far as the Mono Q step, dialysed into a storage buffer containing 50% (w/v) glycerol, 50mM Hepes pH 7.4, 50mM NaF, 5mM NaPPi, 5mM DTT, 1mM EDTA, 1mM Benzamidine, 4μg/ml SBTI and stored at -20^{0}C. AMP-PK activity was measured at regular intervals by incorporation of radiactive phosphate into SAMS peptide (as described in section 2.6.). Results were expressed as percentage of initial activity measured directly after the purification process.

3.2. Properties of mammary gland AMP-PK

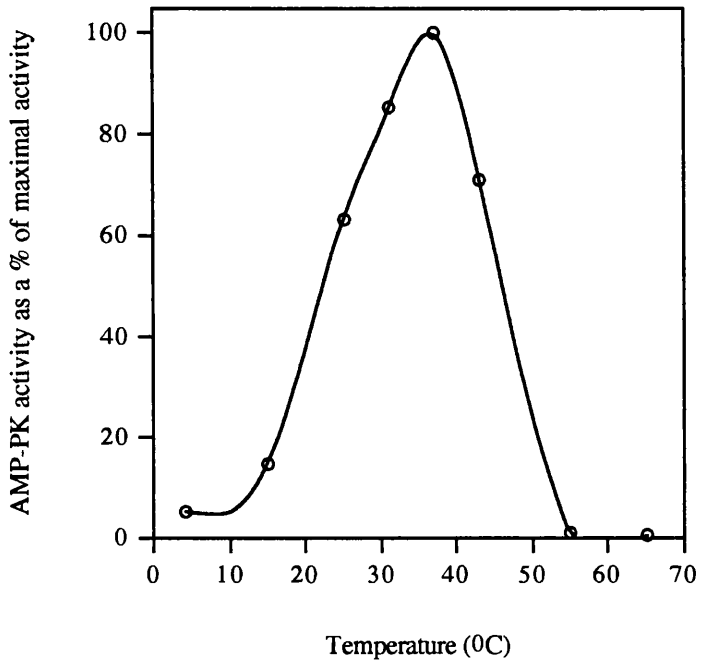
3.2.1. Temperature dependence of AMP-PK activity

AMP-PK from mammary gland purified up to the post Phospho-cellulose step was equilibrated for 10 minutes and then assayed for five minutes at temperatures ranging from 4 to 65^{0}C. Results were expressed as a percentage of maximal activity (Fig.3.6.) and show a clear optimum temperature for AMP-PK between 30 - 40^{0}C. Heating the enzyme at a temperature above 55^{0}C led to an irreversible loss of activity which could not be regained if the enzyme was subsequently equilibrated and assayed at 37^{0}C (results not shown). This presumably represents heat denaturation of the enzyme.

3.2.2. Dependence of AMP-PK activity upon pH

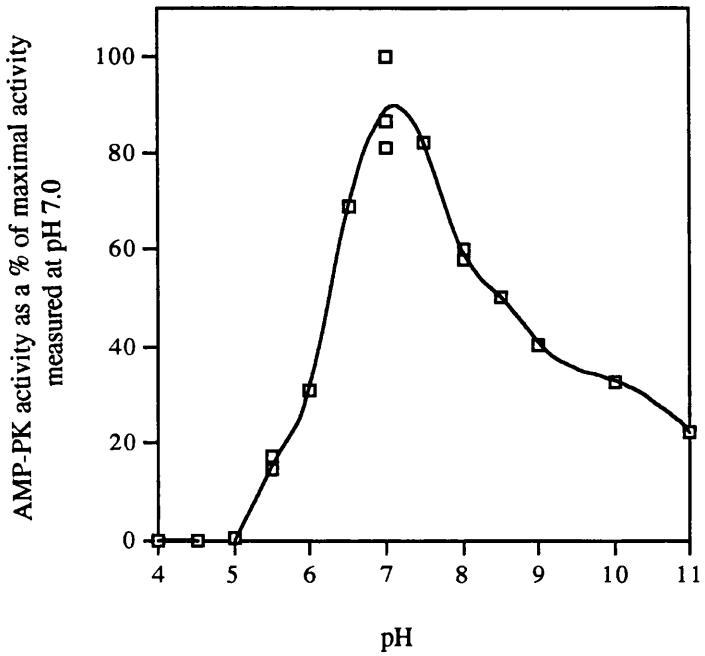
AMP-PK purified up to the post MonoQ step was assayed in different buffers across a range of pH values from 4-11 (Fig.3.7.). The maximum AMP-PK activity was observed around pH 7.0 with the optimum between pH 6.8 and 7.5 (Fig.3.7.). There was a rapid loss of activity below pH 6 and no activity at pH 5.0 and below. In basic conditions the activity decreased more slowly as pH increased. At pH 8.5 AMP-PK still retained more than 50% of its maximal activity.

Fig.3.6. Temperature dependence of AMP-PK activity from mammary gland



AMP-PK from mammary gland purified up to the post Phospho-Cellulose step was pre-incubated for 10 minutes and then assayed for five minutes at the temperatures shown above as described in section 2.6.. Results were expressed as a percentage of the maximal activity observed.

Fig.3.7. Activity of AMP-PK at different pH



AMP-PK from lactating rat mammary gland was purified to the post Mono Q step and assayed for activity at different pH values from pH 4 to pH 11 in steps of 0.5 pH units using 100mM buffers: MES (pH 4-6.6), MOPS (pH 6.5-7.5), HEPES (pH 7-8), TAPS (pH 8-9) and CAPS (pH 10-11) at 37^0C. AMP-PK activity was assayed by the incorporation of ^{32}P into SAMS peptide as described in section 2.6.. The results were expressed as a percentage of the maximal activity measured at pH 7.0 with corrections made for the possible effects of the buffer on AMP-PK activity.

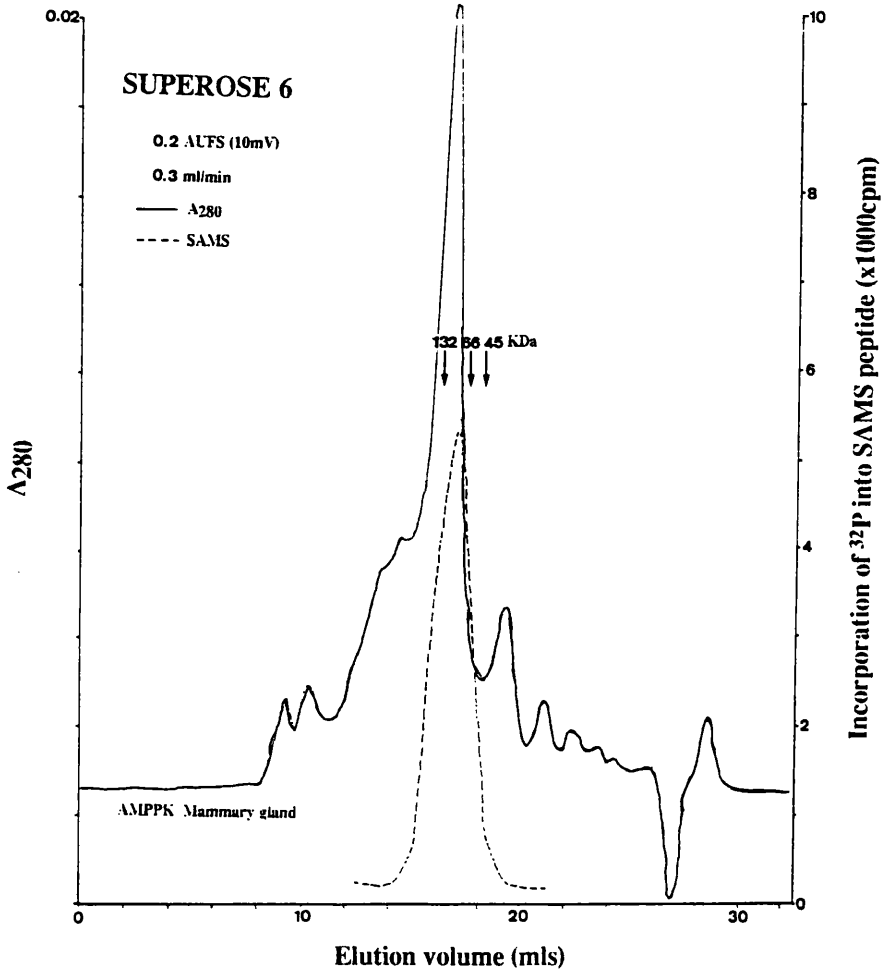
3.2.3. Molecular weight and sub-unit structure of AMP-PK

The native molecular weight (MW) of AMP-PK from the mammary gland was determined by gel filtration chromatography on Superose 6 HR 10/30 (Fig.3.8.) and on Superose 12 HR 10/30 (Fig.3.16.A). These columns were calibrated by running a set of MW standards separately: 132 KDa BSA (dimer), 66 KDa BSA (monomer), 45 KDa chicken egg albumin, 29 KDa carbonic anhydrase and 12.4 KDa cytochrome C. A calibration curve was plot of MW versus retention value (elution volume of peak activity / column dead volume) from the A_{280} peaks for each standard protein. The native MW of AMP-PK was calculated by reading the retention value for the peak of AMP-PK activity from the calibration curve for the MW standards. The calculated MW of mammary gland AMP-PK for both columns (Superose 6 and Superose 12) was 93 KDa. This is quite comparable to the published native MW obtained on Superose 12 for liver AMP-PK of 100±30 KDa (Carling, D. et al., 1989).

Incubation of post Phospho-Cellulose pool of AMP-PK with [γ-^{32}P]-ATP resulted in ^{32}P labelling of relatively few bands of protein as shown by comparison of lanes 2 and 4 in Fig.3.9. Bands were observed at 150, 62 and 48 KDa. Like all protein kinase, AMP-PK will autophosphorylate (Carling, D. et al., 1989). Therefore at least one of these bands must be the autophosphorylated AMP-PK and one might expect this to be the major ^{32}P band. The major ^{32}P band in both the post Phospho-Cellulose preparation (lanes 2 and 4) and post mono Q preparation (lanes 1 and 3) migrates with a MW of 61-62 KDa (Fig.3.9.). This MW is similar, but not identical to the published MW of liver AMP-PK of 63 KDa. These discrepancies between published MW of 63 KDa (Carling, D. et al., 1989) and 61-62 KDa obtained in this study will be addressed later.

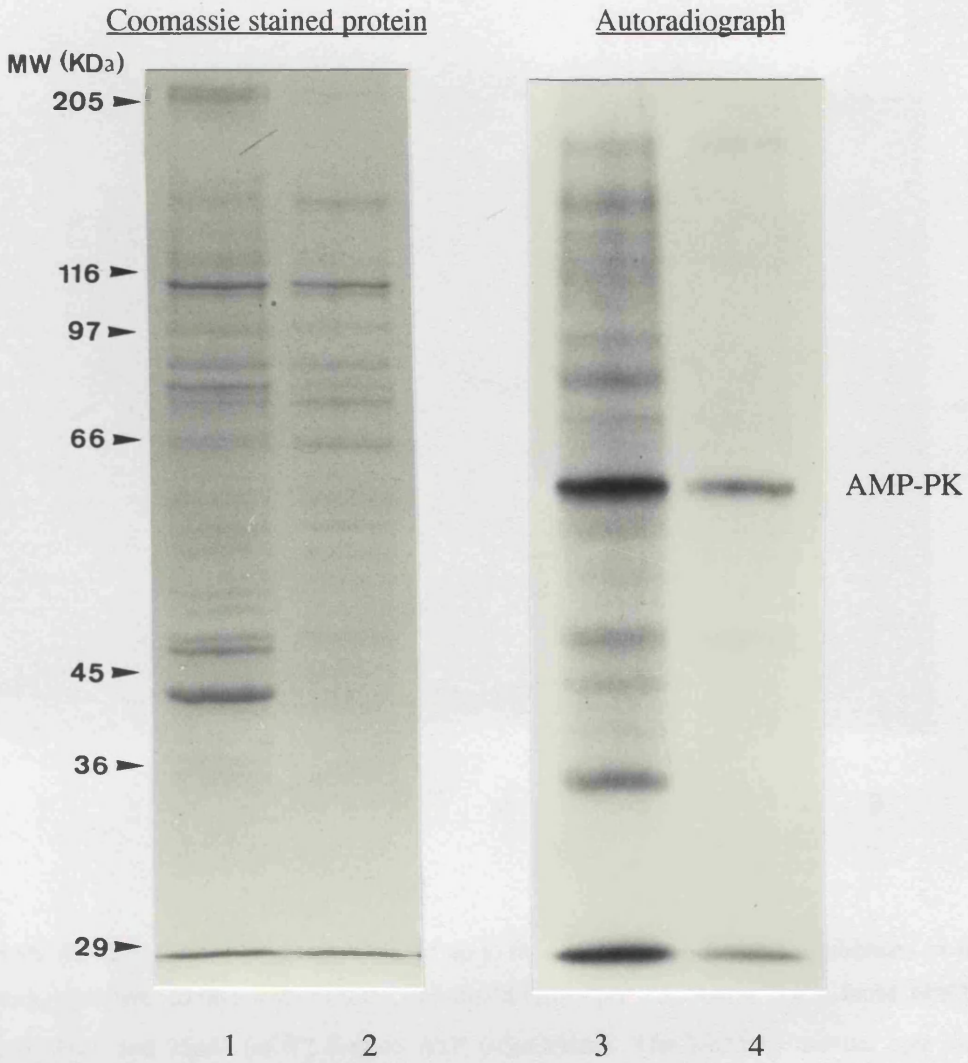
Further evidence to verify the MW of the AMP-PK catalytic subunit came from 8-azido-ATP labelling experiments (Fig.3.10.). The [α^{32}P] 8-azidoATP is an ATP

Fig.3.8. Superose 6 gel filtration of AMP-PK from rat mammary glands to determine native MW



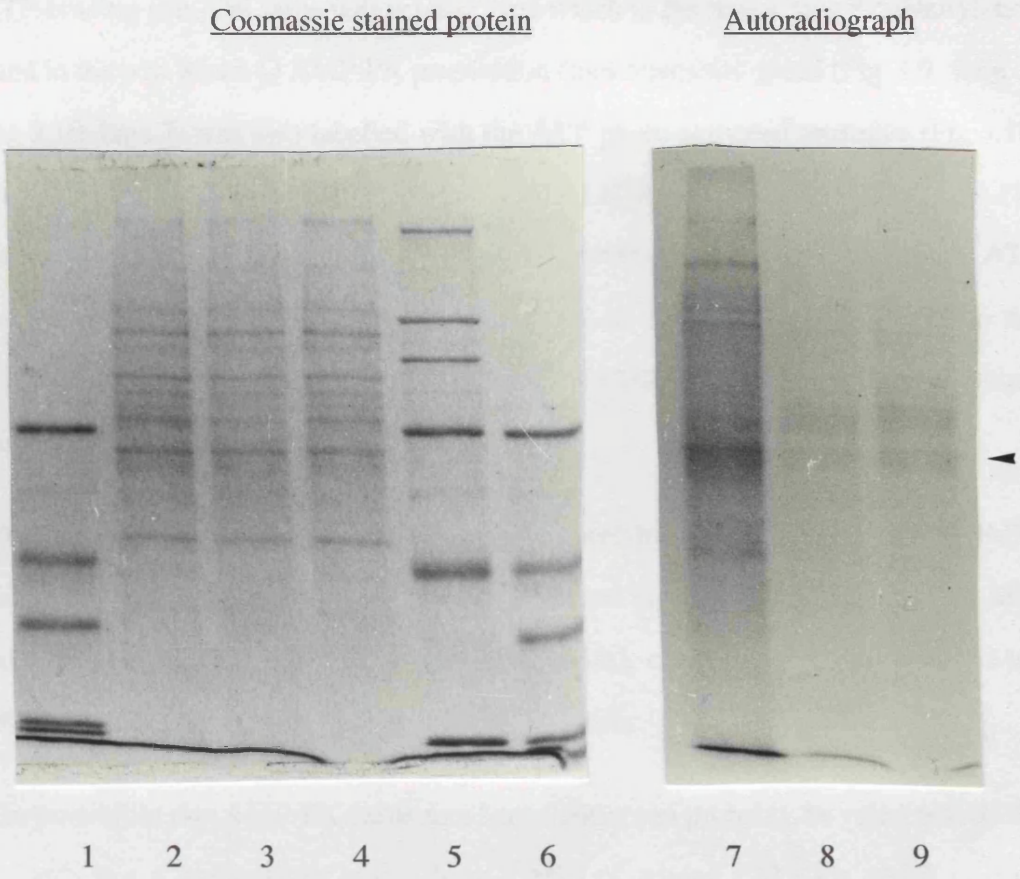
AMP-activated protein kinase from mammary glands of lactating rats was purified as far as the post Phospho-Cellulose step, dialysed into AMP-PK purification buffer containing 500mM NaCl and concentrated to 200μl using a Microcon 30 concentrator. Before loading onto Superose 6 HR 10/30 column AMP-PK was filtered through a 0.2μm prefilter and spun down for 5 minutes at 13000 rpm on a bench microfuge. The column was run at 0.3 ml/min with maximum pressure set at 1.2 MPa. The chart-recorder speed was 0.5 cm/ml and the A_{280} sensitivity was set to 0.2 AUFS (10mV). Fractions 0.5 ml were collected and 5μl of each fraction assayed for AMP-PK activity using the AMP-PK specific assay as described in section 2.6. Results were expressed as incorporation of radioactivity (^{32}P) into SAMS peptide per 5 minute of assay at 37^{0}C. A calibration run was also performed using the same buffer and settings, with BSA (132 KDa dimer, 66 KDa monomer) and chicken egg albumin (45 KDa) as MW standards.

Fig.3.9. SDS-PAGE of auto-phosphorylated AMP-PK from lactating rat mammary glands



AMP-PK from rat mammary glands, purified to the post Mono Q step (lane1) and Phospho-Cellulose step (lane 2) were incubated with 4mM $MgCl_2$ and 200μM [γP^{32}]ATP (sp. radioactivity > 10^6cpm/nmol) for 1 hour at 37^0C in total volume of 100μl. Phosphorylation was stopped by boiling with the addition of 25μl of 5x SDS sample buffer containing 25% (v/v) ß-mercaptoethanol. Standard size SDS-PAGE was performed using a 10%T acrylamide resolving gel and 5%T stacking gel (section 2.9.1.). This was subsequently stained with Coomassie Blue R-250 dried and autoradiographed (section 2.13.2, 2.12.). Lane 1 contains autophosphorylated AMP-PK purified to the post Mono Q step, and lane 2 contains autophosphorylated AMP-PK purified to the post Phospho-Cellulose step. Lanes 3 and 4 represent the autoradiograph of the gel shown in lanes 1 and 2.

Fig.3.10. 8-azido ATP labelling of AMP-PK from mammary gland



The AMP-PK from mammary glands purified up to the post MonoQ step was equilibrated in the semi-dark (with dark red illumination) at 0^0C in: 50mM Hepes pH 7.5, 50mM NaCl, 8mM $MnCl_2$, < 0.1mM DTT and 25μM [α^{32}P] 8-azido ATP (≈5μCi/lane). The labelling mixture was then photolysed by exposure to a bench UV lamp for 4 minutes at 256nm. The reaction was stopped by the addition of sample buffer (3 volume of 5X SDS-PAGE sample buffer : 1 volume 1M DTT). The sample was heated for 30min at 60^0C and run on SDS-PAGE (7.5% T acrylamide resolving and 4.5% T stacking mini gel). The gel was stained with Coomassie Blue R-250, then dried and autoradiographed as described (section 2.13.2, 2.12.).

Lanes 3 and 4 contain 8-azido labelled AMP-PK (autoradiographed in lanes 8 and 9). Lane 2 contains AMP-PK which was autophosphorylated as described for Fig.3.9. (autoradiographed in lane 7). Lanes 1, 6 contains low MW protein markers (66, 45, 36, and 29 KDa) and lane 5 high MW protein markers (205, 116, 97.4, 66, 45 and 29 KDa). The 62 KDa band is indicated by an arrow.

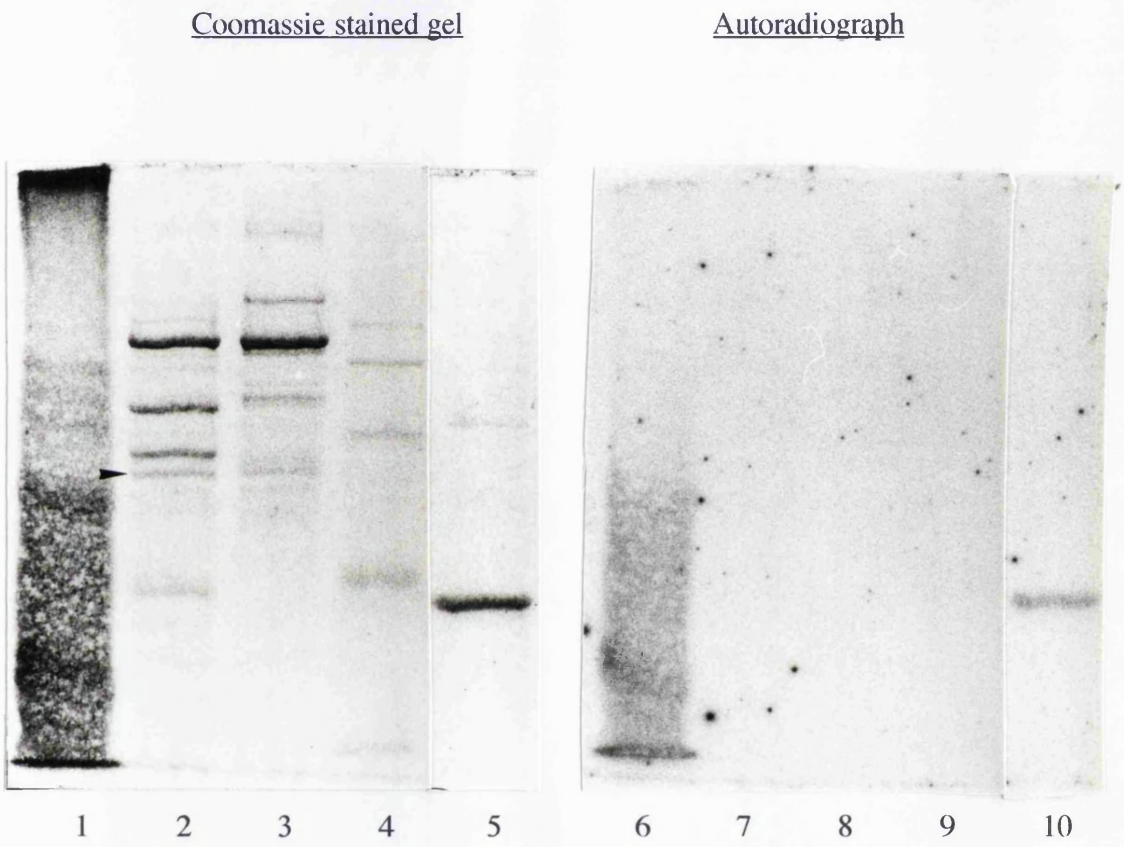
photoaffinity analogue which binds to ATP-binding sites, and in the absence of light, totally mimics ATP. Upon photolysis by UV light it forms an irreversible, covalent bond by a nitrene insertion reaction and it is commonly used for covalently labelling an ATP-binding site. The same polypeptide band which is the major autophosphorylation band in the post Mono Q AMP-PK preparation from mammary gland (Fig 3.9. lane 3, Fig 3.10. lane 7) was also labelled with the ATP photo-activated analogue (Fig.3.10. lanes 8 and 9). This strongly suggests that the 62 KDa polypeptide contains an ATP-binding site. The band was one of a number of bands that had the $\alpha^{32}P$ 8-azido ATP bound and sometimes appeared as a doublet (62-61 KDa). This may be due to the different phosphate content of AMP-PK (AMP-PK probably has multiple phosphorylation sites as discussed later).

The discrepancy between the native molecular weight of mammary gland AMP-PK calculated from gel filtration (93 KDa; Fig.3.8. and Fig.3.16.A.) and the subunit MW calculated from SDS-PAGE (Fig.3.9. and Fig.3.10.), can only be explained if AMP-PK holoenzyme exists as a multiple subunit structure.

The possibility that AMP-PK exists as a homo-dimer can probably be ruled out on the grounds that a homo-dimer would have a MW of around 120 KDa which is 30% greater than the MW calculated for the native AMP-PK from mammary gland.

An indication that the AMP-PK structure is a hetero-dimer emerged from re-naturation experiments. Renaturation of protein kinase after SDS-PAGE was described in detail in Chapter 2. In principle it is based on refolding of fully denatured protein under conditions which favour renaturation (physiological pH, presence of substrate, non-ionic detergents, low temperature, reducing agents). This works successfully for cAMP-PK catalytic subunit from bovine heart, rat heart and mammary gland (see Chapter 5). All attempts to renature the AMP-PK (61-62 KDa band) after SDS-PAGE blotted onto PVDF membrane, or inside a gel containing SSMS as a substrate as described in chapter 2) were unsuccessful. Fig.3.11 shows Coomasie stained gel from the renaturation experiment with SSMS peptide impregnated gel and autoradiograph of

Fig.3.11. Renaturation of kinases in SSMS impregnated SDS-PAGE



The AMP-PK from liver (lane 2) and mammary gland (lane 3) purified up to the post MonoQ step were run on 7.5%T SDS-PAGE containing 1mg/ml SSMS peptide polymerised in (see section 2.18.2.). Lane 5 contains as a positive control Catalytic subunit of cAMP-PK purified from bovine heart. Lane 1 contain prestained MW markers and lane 4 high MW protein markers (205, 116, 97.4, 66, 45 and 29 KDa). The 62 KDa band is indicated by an arrow. Renaturation and phosphorylation of substrate was caried as described in section 2.18.2. Lanes 6-10 represent autoradiograph of lanes 1-5.

the same gel. cAMP-PK from bovine heart (Fig.3.11. lanes 5 and 10) was chosen for positive control because its active catalytic subunit has a monomeric structure and phosphorylate SSMS peptide readily. Neither the AMP-PK from liver (Fig.3.11. lanes 2 and 6) nor from mammary gland (Fig.3.11. lanes 3 and 8) renature. This can be explained by the separation of two sub-unit polypeptides by the SDS-PAGE, one of which is the catalytic subunit of AMP-PK at 62 KDa another which is an activator. Due to their separation, they are therefore not available together to form an active enzyme after renaturation in the gel. Only when they are together in the native enzyme, AMP-PK is active. So the possible structure of AMP-PK is a hetero-dimer with one catalytic sub-unit with MW of 61-62 KDa (This polypeptide also contains ATP binding site, because the band also shows as an azido labelled band) and one or more regulatory sub-units (with mass around 30-32 KDa). The regulatory sub-unit probably contains an AMP binding site and could be labelled by using radioactive 8-azido AMP (unfortunately this AMP analogue is not commercially available).

3.2.4. Substrate specificity of AMP-PK from lactating rat mammary gland

At a concentration of 4mM $MgCl_2$ the maximal activity of AMP-PK was observed at approximately 200μM ATP with Km of 36μM (Ottey, K.A., 1992). This is much lower than the estimated cellular concentration of 2mM ATP, so normal intracellular changes in ATP concentration cannot influence enzyme activity.

The activity of AMP-PK is maximal at 2mM $MgCl_2$ concentration and at a concentration of $MgCl_2$ above 6mM activity rapidly diminishes (Ottey, K.A., 1992). That is probably due to the presence of 50mM NaF in all buffers (a necessary inhibitor of protein phosphatases) which leads to the formation of a non soluble MgF_2 precipitate.

The physiological targets of AMP-PK so far identified are three key enzymes in the regulation of the cell level of free fatty acid and cholesterol: acetyl-CoA carboxylase,

HMG-CoA reductase and hormone-sensitive lipase (Hardie, D.G., Carling, D. and Sim, A.T.R., 1989).The sites phosphorylated by AMP-PK (from rat liver) on all three enzymes mentioned above, have been identified (see section 1.8.1.). AMP-PK (from liver) also phosphorylated at an appreciable rate glycogen synthase and phosphorylase kinase and to a small extent ATP-dependent citrate lyase (ATP-CL) and ApoB-100 (Table 1.4., Carling, D. and Hardie, D.G., 1989).

Initial rates of phosphorylation of some of these substrates by mammary AMP-PK are presented in Table 3.3. and expressed as a percentage of the initial rate of phosphorylation of ACC. The table shows that for AMP-PK from mammary gland, ACC is the best substrate. This is understandable due to the fact that it is the only proven physological substrate from that list. AMP-PK phosphorylated Glycogen Synthase and Phosphorylase Kinase at 68 and 52% of the initial rate for ACC

Table 3.3. Initial rate of substrate phosphorylation by AMP-PK from mammary gland

substrate	Initial rate of phosphorylation (as % of initial rate of phosphorylation of ACC)
Acetyl-CoA Carboxylase	100
ATP-citrate lyase	15
Glycogen Synthase	68
Phosphorylase Kinase	52
ApoB100	<3
Casein	15

The initial rates of phosphorylation of substrates by the post Phospho-Cellulose preparation of AMP-PK from mammary gland are shown and are expressed relative to the phosphorylation of ACC. Substrate concentration were: ACC (0.48 mg/ml), ATP-citrate lyase (0.23 mg/ml), Glycogen Synthase (0.17 mg/ml), Phosphorylase Kinase (0.66mg/ml), ApoB100 (1mg/ml) and Casein (2mg/ml).

respectively, indicating possibility that they also represent physiological targets. AMP-PK from mammary gland also phosphorylated Casein, ATP-CL and ApoB100 but to much smaller extents (Table 3.3.). The same substrate concentrations were used in this experiment, as were used by Carling, D. and Hardie, D.G. (1989) to assay AMP-PK from rat liver, so that the results could be directly compared. The ratio of initial rates of phosphorylation for each substrate (relative to ACC phosphorylation as a 100%) by AMP-PK from lactating rat mammary gland (Table 3.3.) and rat liver (Carling, D. and Hardie, D.G. 1989) did not vary significantly. This indicates that AMP-PK purified from rat liver or lactating rat mammary gland does not have different substrate specificity.

The initial rate of phosphorylation of the SSMS peptide by AMP-PK from mammary gland or from liver is greater than the initial rate for SAMS peptide (Table 3.4.).

Table.3.4. Comparison of initial rates of incorporation of ^{32}P into 200μM SAMS or SSMS peptide substrates by AMP-PK from liver and mammary gland of lactating rat.

	Incorporation (nmol/min/mg of enzyme) of ^{32}P into		Incorporation ratio SAMS/SSMS (%)
	200μM SSMS	200μM SAMS	
AMP-PK (Liver)	86	70	81
AMP-PK (MG)	26.2	20.8	79

The initial rates of phosphorylation of SSMS and SAMS peptides by the post Phospho-Cellulose preparation of AMP-PK from rat liver and lactating rat mammary gland are measured as described in section 2.6.

SAMS peptide is phosphorylated at approximately 80 % of the rate of SSMS (Table 3.4.). This is understandable, given that the SSMS peptide has the exact sequence of the phosphorylation site on the physiological substrate ACC and that this sequence is altered in SAMS peptide by replacing Serine with Alanine. Replacement of this residue, although not crucial for recognition by AMP-PK leads to a decrease in the probability that this tetrapeptide sequence will form a ß-turn, which may be the cause of SAMS peptide being a slightly poorer substrate for AMP-PK (Davies, S.P. et al., 1989). The ratio of those initial rates for the same AMP-PK preparation vary from batch to batch of peptide. This is probably due to a variant percentage of impurity in different peptide preparations. The ratios of the initial rates of phosphorylation for both peptides (using same batches of peptides) were similar for AMP-PK purified from mammary gland and liver 79 and 81 respectively (Table 3.4.). This is another indication that mammary gland and liver AMP-PK do not have significantly different substrate specificities.

AMP-PK from mammary gland is not inhibited by 20 U/μl PKI (a specific cAMP-dependent protein kinase inhibitor containing the active sequence of Walsh Inhibitor which fully inhibits cAMP-PK from bovine heart or mammary gland at this concentration) or 20μg/ml heparin (a Casein kinase inhibitor which produces more than 80% inhibition of Casein kinase I and II at this concentration). Calphostin C (a specific protein kinase C inhibitor with IC_{50} for PKC of 50nM) at 5μM concentration had no effect on AMP-PK activity.

A specific AMP-PK inhibitor could be a useful pharmacological tool eg. in estimating changes of SSMS phosphorylation rates in tissue extracts. Many protein kinases have a specific endogenous protein or peptide inhibitors often based on pseudosubstrate sequence eg. Walsh inhibitor and regulatory domain of PKC which both have alanine replacing phosphorylating seryl residue in sequences of normal substrates (Blackshear, P.J. et al., 1988; Kemp, B.E. et al., 1991). There are indications of the

presence of an endogenous inhibitor of AMP-PK in rabbit skeletal muscles (Carling, D. - personal communication).

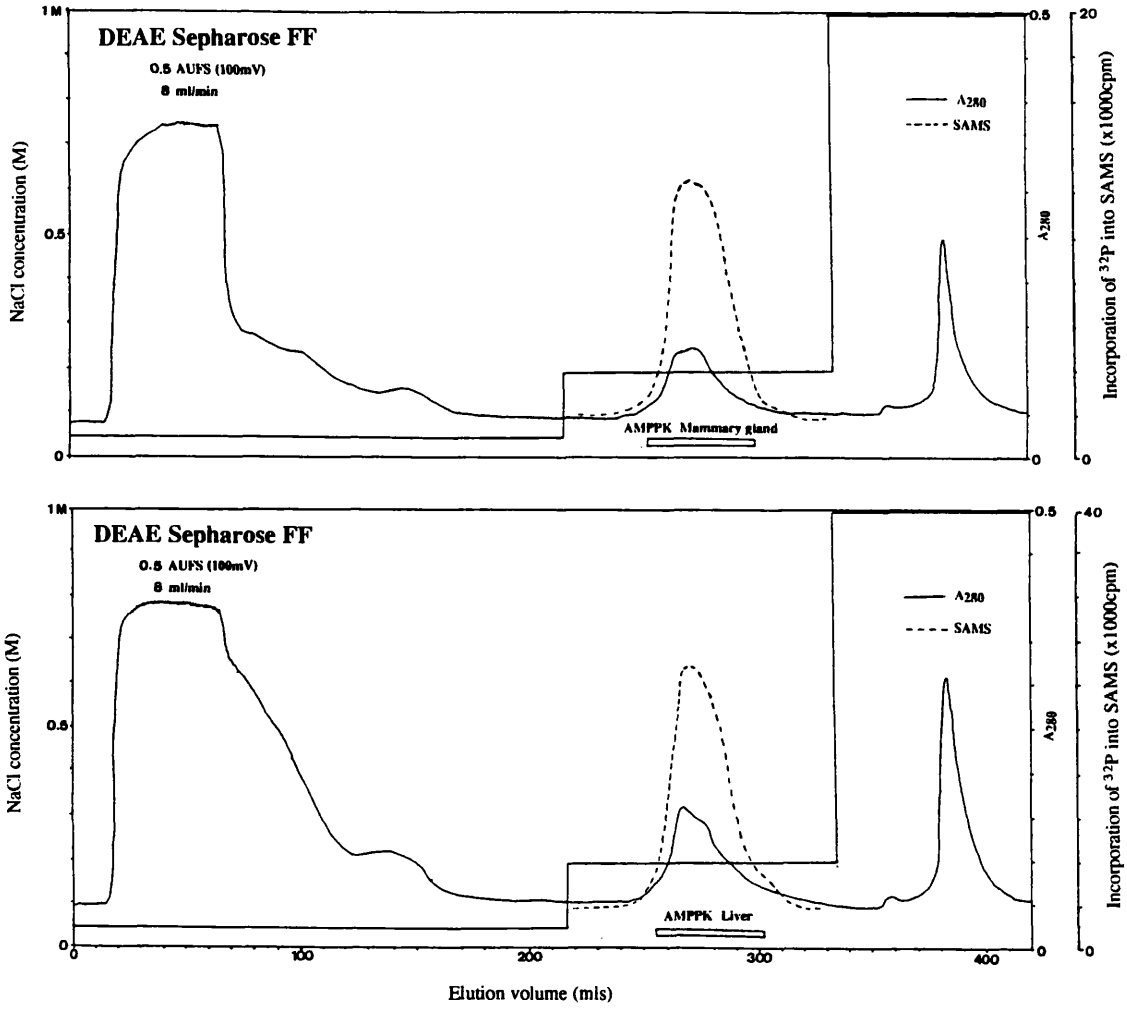
3.3. Further comparison of AMP-PK from liver and mammary gland of lactating rats

To clarify discrepancies in MW calculated for AMP-PK from SDS-PAGE (61-62KDa for mammary gland, Fig.3.9. and 3.10.; 63 KDa for rat liver, Carling, D. et al., 1989) and to make direct comparison between mammary gland and liver AMP-PK, parallel purifications from both sources were carried out using 50 g of mammary gland from rats at peak lactation and the same amount of liver from the same rats. Both tissues were freeze clamped and purification procedures were as previously described in this Chapter. During purification the same columns and same buffer were used with the same program settings on FPLC. The exception was the Phospho-Cellulose step where the column was regenerated and repackaged between the runs but with the same batch material. Comparatively similar degrees of purification were achieved from both tissues, 6657 fold for liver (Table 3.2.) and 3750 fold for mammary gland enzyme (Table 3.1.).

Purification profiles on DEAE-Sepharose (Fig.3.12) showed similar protein and activity distribution between the steps. AMP-PK activity was eluted in 50-200mM NaCl batch from both tissues. No AMP-PK activity was found outside 50-200mM NaCl step neither in profile from mammary gland nor liver of lactating rat. Pooled fraction after DEAE-Sepharose step represented 85 and 95% of total loaded activity (in mammary gland and liver respectively; Table 3.1. and 3.2.).

The Blue-Sepharose purification step also shows similar protein and activity distribution profiles (Fig.3.13.) for purification from mammary gland and liver of lactating rat. After initial batch steps, gradient was developed between 0.5M and 1.5M

Fig.3.12. DEAE-Sepharose purification profiles of AMP-PK from mammary gland and liver



The 2.5-6% PEG_{6000} pellet prepared from mammary gland or liver homogenates was resuspended in AMP-PK purification buffer and loaded onto a DEAE-Sepharose 6FF (10/50) column connected to an FPLC. The loading, washing and elution were at 8ml/min. The AUFS for A_{280} detection was 0.5 (100mV). After washing of the column, AMP-PK was eluted by step batch elution with 50-200mM NaCl. The long narrow rectangle below each AMP-PK peak of activity shows the fractions which were pooled.

Fig.3.13. Blue-Sepharose purification profiles of AMP-PK from mammary gland and liver

The post DEAE-Sepharose AMP-PK samples were directly loaded onto a Blue-Sepharose 6FF (20/20) column connected to an FPLC. The loading, washing and elution were at 3ml/min. The AUFS for A_{280} detection was 0.1 (100mV). After washing of the column, AMP-PK was eluted with a 500-1500mM NaCl gradient. Fractions were collected and assayed for AMP-PK activity using specific SAMS peptide assay as described in Chapter 2. The peaks of AMP-PK activity were pooled (long narrow rectangle) and dialysed into AMP-PK purification buffer.

Blue Sepharose 6 FF
0.1 AUFS (100mV)
3 ml/min
A280
SAMS
A
AMPPK Mammary gland
NaCl concentration (M)
Incorporation of 32P into SAMS (x1000cpm)
Blue Sepharose 6 FF
0.1 AUFS (100mV)
3 ml/min
A280
SAMS
B
AMPPK Liver
NaCl concentration (M)
Elution volume (mls)
Incorporation of 32P into SAMS (x1000cpm)

NaCl. AMP-PK activity was eluted as broad peaks in the region of 0.7-1.2M NaCl from both tissues. No activity was found outside the peaks nor in the breakthroughs. The mammary gland purification profile shows a shoulder in AMP-PK activity but this could be due to the increased probability of error in the assay due to the lower specific activity of the mammary gland enzyme (Table 3.1. and 3.2.). Both profiles (Fig.3.13.) show that the maximum of AMP-PK activity elutes at similar NaCl concentrations for preparations from liver and mammary gland (900mM and 950mM NaCl respectively).

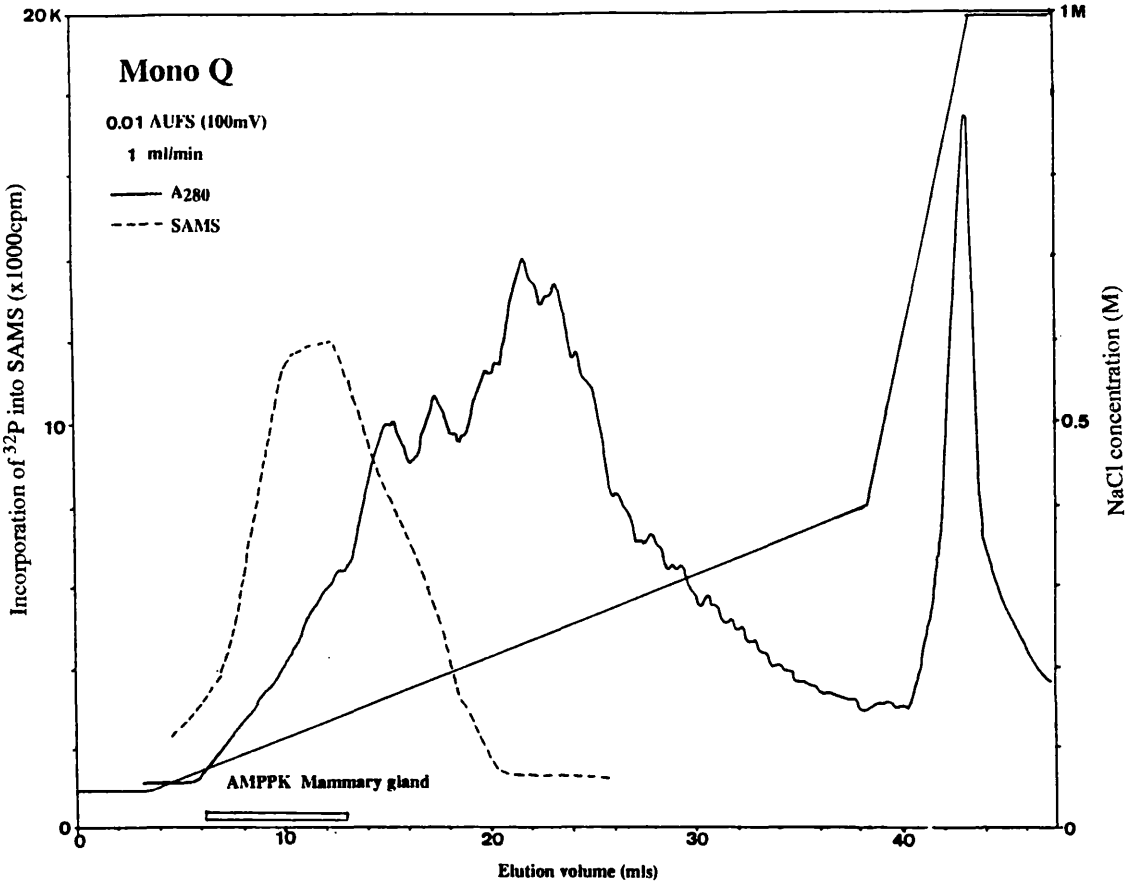
The Mono Q purification of AMP-PK from mammary gland and liver shows similar AMP-PK activity profiles (Fig.3.14.). The column was developed with gradient 50-400mM NaCl increase over 35ml. The AMP-PK activity from both tissues eluted between 50 and 250mM NaCl, with identical peaks of activity at 150mM NaCl. The specific activity of mammary gland AMP-PK is about 3 times lower than the same enzyme from liver of the same rats (Table 3.1. and 3.2.).

The batch step elution profile on Phospho-Cellulose for AMP-PK from mammary gland and liver of lactating rats shows a similar distribution pattern of AMP-PK activity (Fig.3.15.). 70 percent of the total recovered activity was in the 50-200mM NaCl batch, with the other 30% equally distributed between the breakthrough, 0-50mM and 200mM-1M NaCl steps.

Superose 12 gel filtration step produced a similar AMP-PK activity profile for both tissues (Fig.3.16.) although the liver enzyme has three times higher specific activity (Table 3.1 and 3.2). Both enzymes have the same native MW calculated from gel filtration on Superose 12 of 93 KDa.

Fig.3.14. Mono Q purification profile of AMP-PK from mammary gland and liver

The post Blue-Sepharose AMP-PK samples from mammary gland or liver were dialysed into AMP-PK purification buffer (containing 50mM NaCl) over-night and loaded onto a Mono Q HR 5/5 column connected to an FPLC. The loading, washing and elution were at 1ml/min. The AUFS for A_{280} detection was 0.01 (100mV) for mammary gland and 0.05 (100mV) for liver AMP-PK. After washing of the column with AMP-PK purification buffer containing 50mM NaCl until stable base lines were achieved, AMP-PK was eluted with 50-400mM NaCl gradient over 35 ml. Fractions were collected and 5μl assayed for the AMP-PK activity using the specific SAMS peptide as described in Chapter 2. The peaks of AMP-PK activity were pooled (long narrow rectangle) and dialysed rapidly (less than 2 hours) into AMP-PK purification buffer without NaPPi.


Mono Q
0.01 AUFS (100mV)
1 ml/min
A280
SAMS
AMPPK Mammary gland
20K
10
0
1M
0.5
0
0
10
20
30
40
Elution volume (mls)
Incorporation of 32P into SAMS (x1000cpm)
NaCl concentration (M)

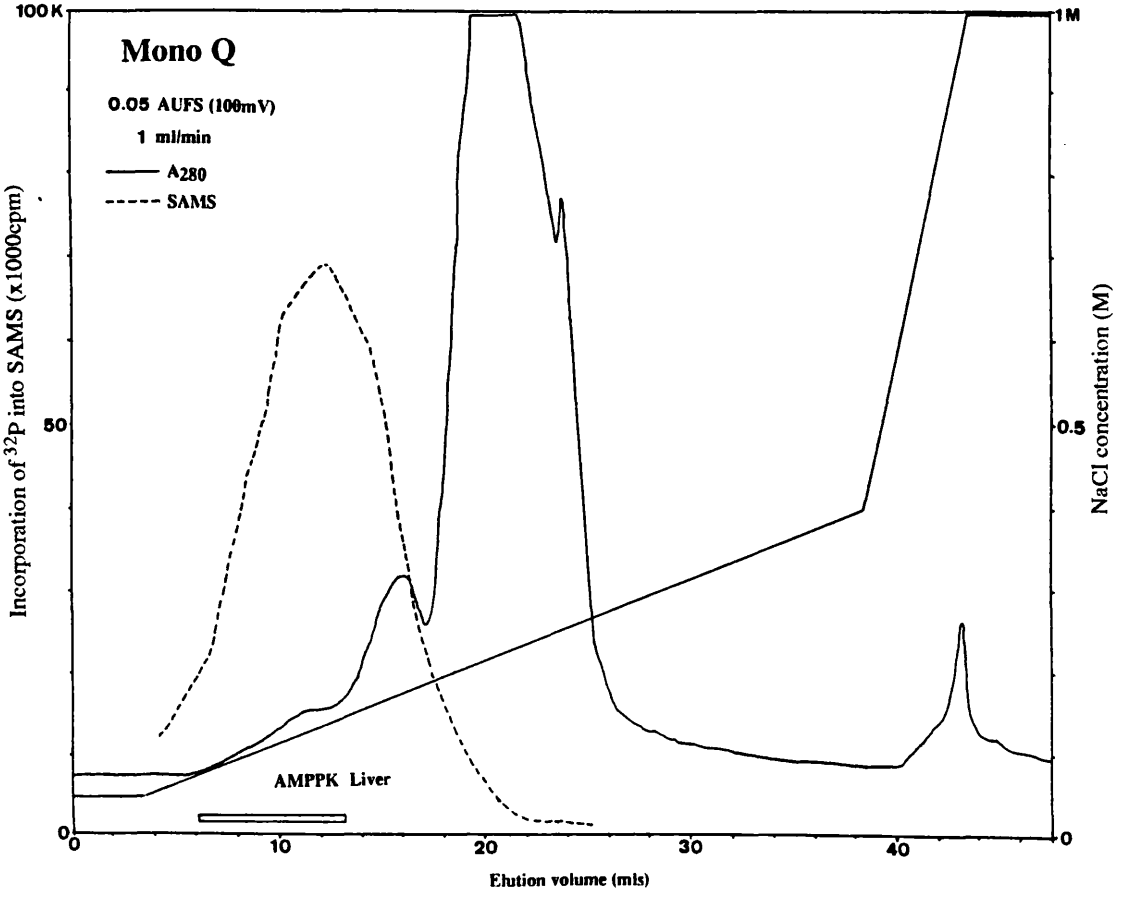
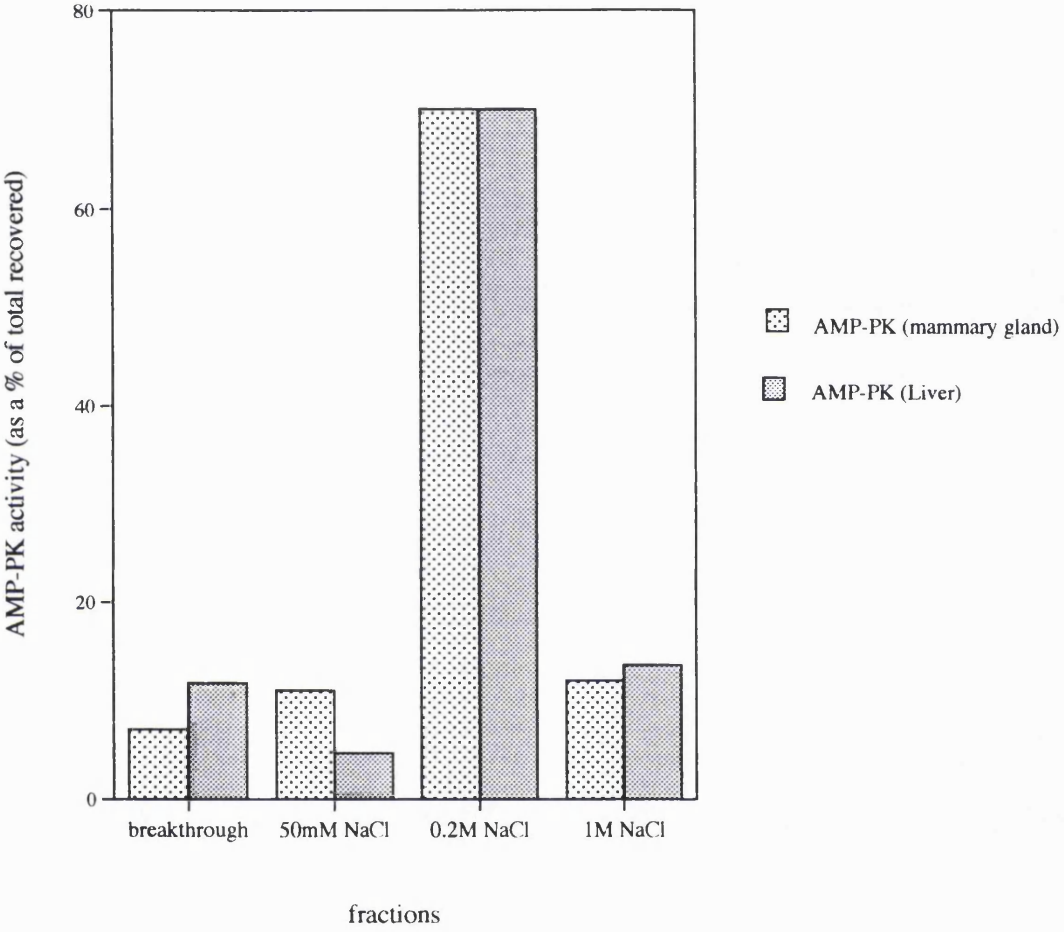
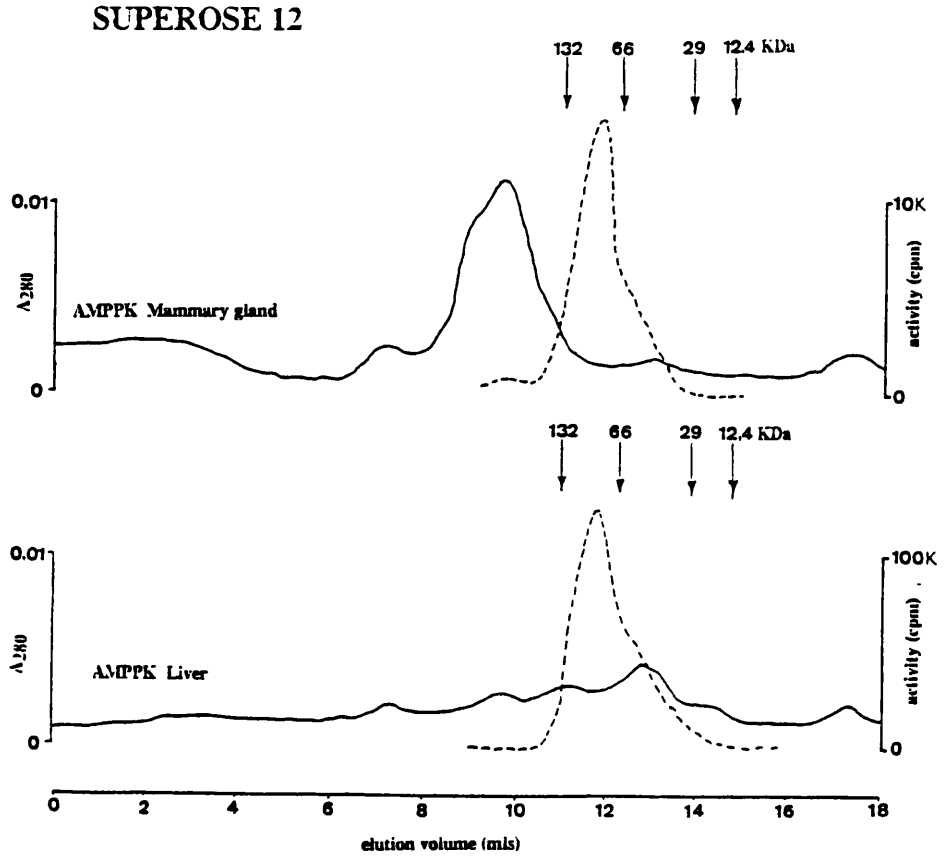

Mono Q
0.05 AUFS (100mV)
1 ml/min
A280
SAMS
AMPPK Liver
100K
50
0
1M
0.5
0
0
10
20
30
40
Elution volume (mls)
Incorporation of 32P into SAMS (x1000cpm)
NaCl concentration (M)

Fig.3.15. Phospho-Cellulose purification profile of AMP-PK from mammary gland and liver



Post Mono Q AMP-PK purified from both sources and dialysed into the AMP-PK purification buffer lacking NaPPi was loaded onto the Phospho-Cellulose (10/3) column. Loading, washing and eluting speed were 1ml/min. AMP-PK was eluted by NaCl batch step elution. Batches were pooled and assayed for protein concentration and AMP-PK activity using the specific SAMS peptide as described in chapter 2. Activity was expressed as a percentage of total recovered activity.

Fig.3.16. Superose 12 purification profile of AMP-PK from mammary gland and liver

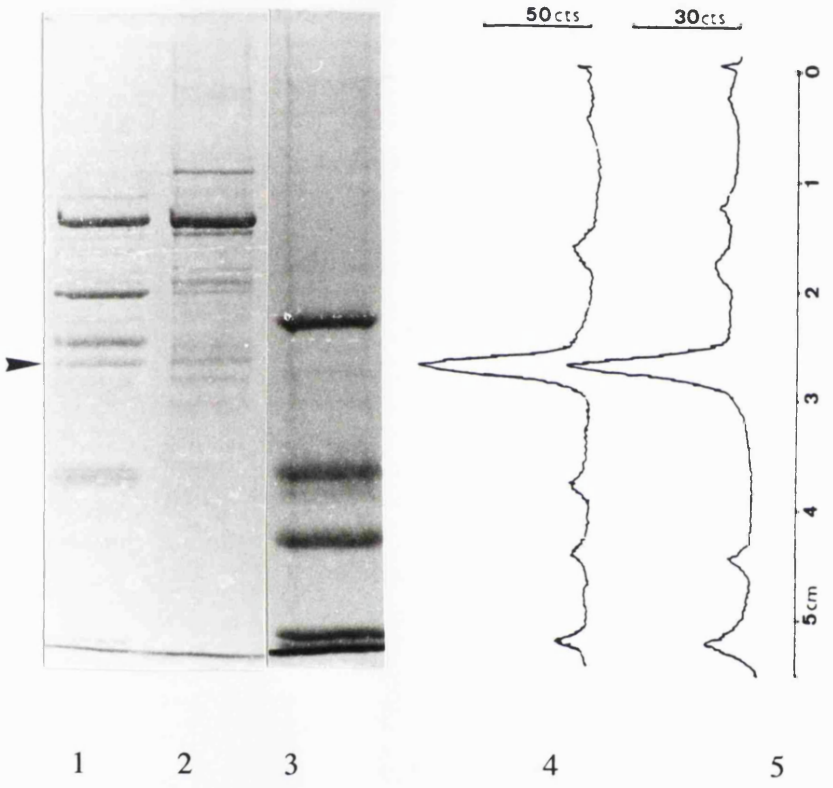


Post Phospho-Cellulose AMP-PK from both sources was dialysed into AMP-PK purification buffer containing 500mM NaCl and concentrated down to 200μl using a Microcon 30 concentrator. Before loading onto Superose 12 HR 10/30 column the AMP-PK sample was filtered through a 0.2μm prefilter and spun down for 5 minutes at 13000 rpm on a bench microfuge. The column was run at 0.5 ml/min with maximum pressure set at 1.8 MPa. The AUFS for A_{280} detection was 0.05 (10mV). Fractions (0.5ml) were collected, and 5μl of each assayed for AMP-PK activity using the specific SAMS peptide as described in Chapter 2. Results were expressed as incorporation of radioactivity (^{32}P) into SAMS peptide per 5 minute of assay at 37^{0}C. A calibration run was also performed using the same buffer and settings, with BSA (132 KDa dimer, 66 KDa monomer), Carbonic anhydrase (29 KDa) and Cytochrome C (12.4 KDa) as MW standards.

The catalytic subunits of AMP-PK from both tissues were determined as the only major autophosphorylated bands in preparations purified to the post Phospho-Cellulose step and separated by SDS-PAGE (Fig.3.17.). These autophosphorylated bands migrated to identical positions on SDS-PAGE representative of 61-62 KDa MW. Those bands are clearly visible on Coomasie stained gel and are marked by an arrow (Fig.3.16. lanes 1 and 2). The liver and mammary gland preparation clearly contains different contaminating bands. The 62KDa band (catalytic subunit of AMP-PK) represents around 10% in liver and less than 2% of total protein in mammary gland post Phospho-Cellulose preparation. This five fold difference in the relative amount is similar to the five fold differences in the specific activity (Table 3.1. and 3.2.) indicating that differences in specific activity of AMP-PK from liver and mammary gland could be solely due to the difference in their purity. The same bands were also labelled with a ^{32}P 8-azido ATP in confirmation that they were AMP-PK catalytic subunits (Fig.3.10, data not shown for the liver enzyme). The MW of AMP-PK catalytic subunit (61-62 KDa) obtained in this study is similar to the calculated MW deduced from the predicted amino acid sequence from the cDNA for AMP-PK from rat liver of non-lactating rats (62,250 Da, Carling, D. et al., 1994).

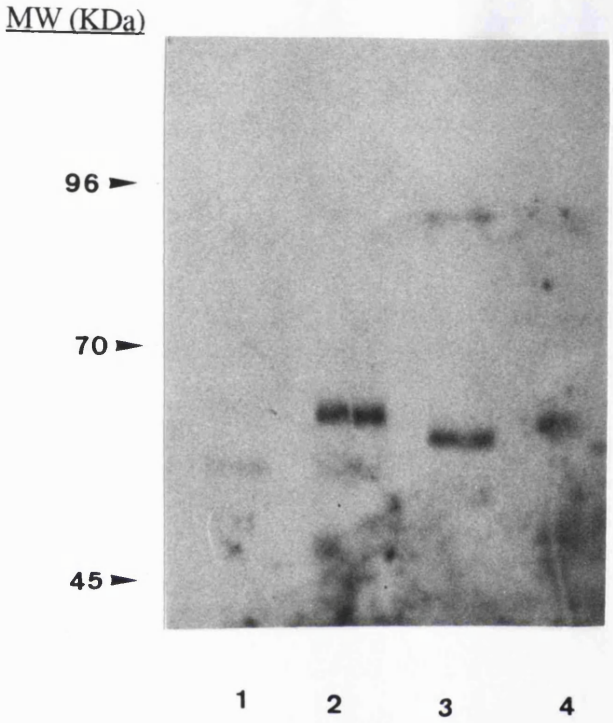
The polyclonal antibodies raised in the present study recognised the peptide against which they were raised, but did not recognise AMP-PK from liver of non-lactating rats or liver and mammary gland of lactating rats. The polyclonal antibodies, which were raised against ENVLTDAQMNC peptide as described in section 2.16. did not recognise native or SDS denatured AMP-PK from mammary gland or liver. The reason for this could be an ambiguity in part of the original sequence which could interfere in the bonding of antibodies to the unfolded AMP-PK polypeptide. The ambiguous threonine residue in the middle of the peptide sequence was proven recently to be a leucine in predicted amino acid sequence of rat liver AMP-PK (Carling, D. et al., 1994).

Fig.3.17. Autophosphorylation of AMP-PK from mammary gland and liver of lactating rat shows the same autophosphorylation band



AMP-PK from rat mammary glands and livers of lactating rats, purified to the post Phospho-Cellulose step were incubated with 4mM $MgCl_2$ and 200μM [γ P^{32}]ATP (sp. radioactivity > 10^6cpm/nmol) for 1 hour at 37^0C in a total volume of 20μl. Phosphorylation was stopped by boiling with the addition of 5μl of 5x SDS sample buffer containing 25% (v/v) ß-mercaptoethanol, as described in Chapter 2. SDS-PAGE was performed using a 10%T acrylamide resolving, and 5%T stacking, mini gel. After the run, the gel was fixed in 50% (v/v) methanol, 10% (v/v) acetic acid, then stained with Coomassie Blue R-250 and de-stained as described in Chapter 2. After destaining and drying, the gel was scaned using Bertholds Tracemaster 20 Automatic TLC Linear Analyser. Lane 3 contains low MW protein markers (66, 45, 36, and 29 KDa). Lane 2 is mammary gland AMP-PK and lane 1 is liver AMP-PK. Lanes 4 and 5 are the scans of lanes 1 and 2 respectively. The arrow indicates the 62 KDa band which was autophosphorylated in both, mammary gland and liver preparations.

Fig.3.18. Western blotting and polyclonal antibody recognition of AMP-PK from mammary gland and liver of lactating rats.



AMP-PK from the mammary gland (lane 2) and liver of lactating rats (lane 1) together with AMP-PK from the liver of male rats (purified to the post Phospho-Cellulose step, lane 4) and the inactive form of AMP-PK from rat skeletal muscles (lane3) were subjected to SDS-PAGE (7.5% T acrylamide resolving and 3.5% T stacking mini gel). After the run, the proteins from the gel were blotted onto PVDF membrane (2h at 4^0C and 30V constant voltage), and then, using the "Western blotting" technique and polyclonal antibodies against the known sequence of AMP-PK (as described in Chapter 2), the PVDF membrane with blotted proteins was screened for anti-AMP-PK peptide antibody antigens. Antibodies and AMP-PK from skeletal muscles and liver of male rats were a generous gift from Dr. Dave Carling and Dr Angela Woods.

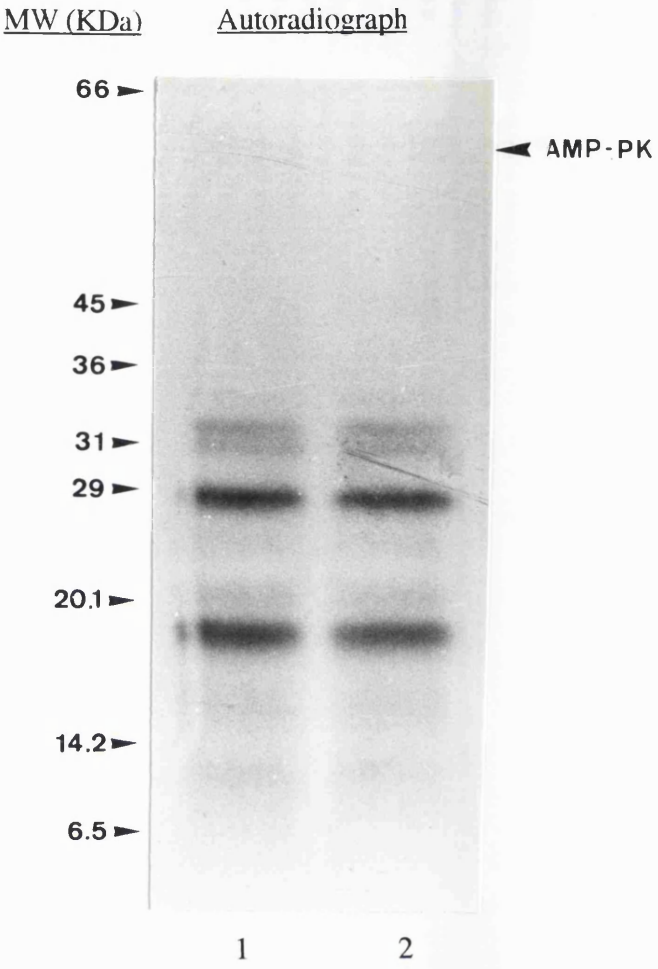
Dr. Carling and Dr. Wood raised a number of polyclonal antibodies against predicted peptide sequences from AMP-PK cDNA clone. The antibodies used in Fig.3.18. were a generous gift from Dr. Carling and the peptide sequence against which they were raised came from cDNA sequence of liver AMP-PK determined also by Dr. Carling. He and Dr. Wood generously allowed me to use one of the antibodies which recognised AMP-PK from liver of non-lactating rats. The same antibodies recognised and labelled 62 KDa SDS denatured bands of catalytic subunit of AMP-PK from mammary gland and liver of lactating rat on a Western blot (Fig.3.18. lanes 2 and 1 respectively). The Western blot also shows that AMP-PK from mammary gland or liver of lactating rat shows the same MW as an active form of AMP-PK purified from liver of non-lactating rat by Dr. Carling (Fig.3.18 lane 4). Lane 3 shows an inactive form of AMP-PK from skeletal muscle purified by Dr. Carling. All lanes show additional denaturation product which occurred after prolonged storage of the enzyme and is also recognised by antibodies (Dr. Carling - personal communication).

This is further evidence of the similarity between AMP-PK from liver and mammary gland. This is also further evidence that their catalytic subunits, which are present in both tissues in an active form, have an identical MW of 62 KDa.

The ^{32}P autophosphorylated 62 KDa bands of catalytic subunit of AMP-PK purified from both tissues after visualising by copper staining, were cut out from the gels. Bands were counted for radioactivity and the same amount of radioactivity was used in producing a V8 digest (as described in section 2.10.). The V8 digestion of autophosphorylated 62 KDa band from both tissues yielded an identical Cleveland peptide map autoradiograph (Fig.3.19.). This is another indication of a high similarity of the AMP-PK from liver and mammary gland of lactating rats.

AMP-PK from mammary gland and liver of lactating rat purified up to post mono Q step were assayed at various 5'-(p-fluorosulfonylbenzoyl)-adenosine (FSBA) concentration in the presence of 100μM 5'AMP and 200μM ATP (Fig.3.20.).

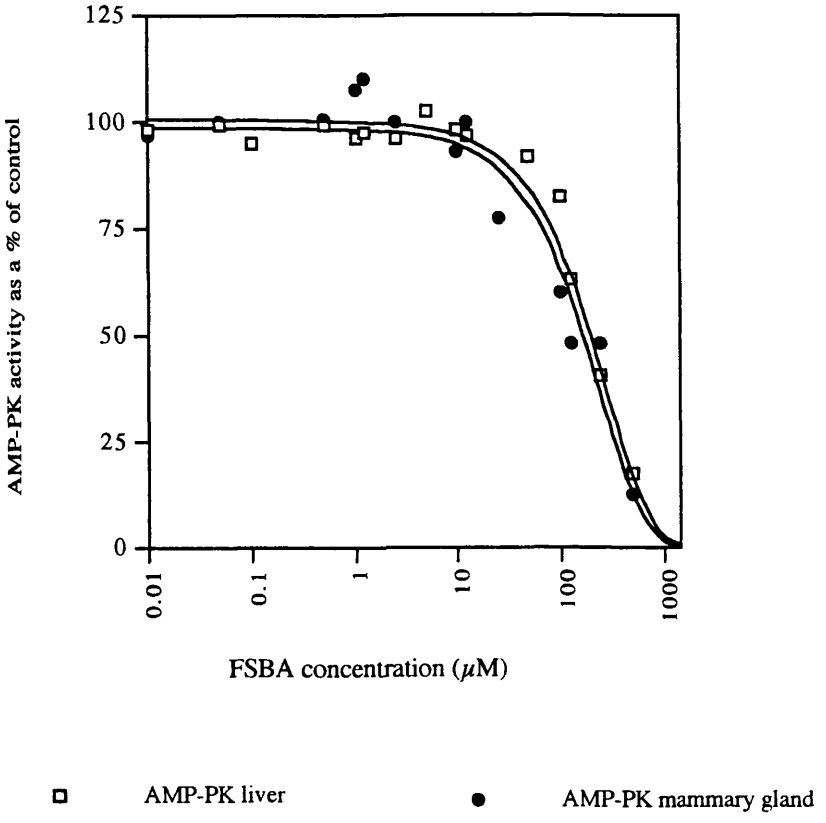
Fig.3.19. V8 digest of AMP-PK from mammary gland and liver



AMP-PK from mammary gland and liver from lactating rats was purified to the post Phospho-Cellulose step and then autophosphorylated as described (section 2.11.3.).

The autophosphorylated AMP-PK was precipitated using to chloroform/methanol, dried and resuspended in SDS-sample buffer containing 5% (v/v) ß-mercaptoethanol, boiled for 5 minutes and then run on SDS-PAGE with 7.5% T acrylamide resolving and 3% T stacking gel (section 2.9.1.). After temporary staining of gel with Copper staining method (as described in section 2.13.4.), the gel slices containing the 62KDa band were cut out, equilibrated in SDS-sample buffer (lacking ß-mercaptoethanol) and loaded onto a second SDS-PAGE (15% T acrylamide resolving and 5% T stacking gel). The wells with gel slices were overlayered with V8 protease, and standard Cleveland mapping was carried out (as described in section 2.10.). After drying, the gel was autoradiographed as described in materials and methods. Lane 1 is an autoradiograph of V8 digested AMP-PK from mammary glands and lane 2 AMP-PK from livers of lactating rats. The position of the 62 KDa AMP-PK band was marked by arrow.

Fig.3.20. AMP-PK inhibition by fluorosulphonyl-benzoyl adenosine (FSBA)



AMP-PK from mammary gland and liver of lactating rats was purified as far as the Mono Q step. AMP-PK was assayed using the specific SAMS peptide as described in Chapter 2 at the concentrations of FSBA indicated above. Results were expressed as % of control activity without FSBA. Inhibition curves were fitted by computer best fit.

5'-(p-fluorosulfonylbenzoyl)-adenosine (FSBA) is an ATP analogue which is commonly used as a probe for nucleoside binding sites. Its fluorosulfonylbenzoyl moiety reacts covalently with lysine or cysteine in an ATP-binding site. AMP-PK from both tissues were inhibited in an identical dose response by FSBA (Fig.3.20.) showing nearly identical Ki of 170 and 160μM (mammary gland and liver respectively). This is yet another indication of the similarity between AMP-PK from mammary gland and liver of lactating rat.

3.4. Conclusion

AMP-PK from mammary gland was purified 3750 fold using a seven step purification (Table 3.1.). Even the most pure AMP-PK preparation (Superose 12 step) 62 KDa band represented less than 10% of the total protein. The specific activity of AMP-PK purified from mammary gland (131 U/mg) is quite comparable to the published specific activity of AMP-PK purified from rat liver (184 U/mg) (Carling, D. et al., 1989).

The mammary gland enzyme has a temperature optimum of 36^0C (Fig.3.6.), and is irreversible inactivated at temperatures above 55^0C. The pH optimum for the mammary gland enzyme is between pH 6.8 and 7.5, with maximum activity observed at pH 7.5 and a total loss of activity at pH 5.0 and below (Fig.3.7.).

AMP-PK from mammary gland has a native MW of 93 KDa as judged by Superose 6 (Fig.3.8.) and Superose 12 (Fig.3.16.). The re-naturation experiments indicated that it has a heterodimer subunit structure with a larger subunit of 61-62 KDa containing the ATP-binding site and catalytic activity and one or more regulatory sub-units with mass around 30-32 KDa. This is further supported by findings of structural and functional homology between AMP-PK and Snf1/Snf4 protein kinase complex from yeast in which Snf1 (72 KDa) catalytic subunit requires the presence of Snf4 (36 KDa) subunit for activity (Mitchelhill, K.I. et al., 1994; Carling, D. et al., 1994)

AMP-PK from mammary gland and liver of lactating rats have remarkably similar properties. AMP-PK from mammary gland and liver of lactating rat were inhibited in an identical dose response by FSBA (an ATP analog) with Ki of 170 and 160μM respectively (when assayed in the presence of 100μM 5'AMP and 200μM ATP, Fig.3.20.). The initial rate of protein substrate phosphorylation by AMP-PK from lactating rat mammary gland (Table 3.3.) and rat liver (Carling, D. and Hardie, D.G. 1989) did not vary significantly. Phosphorylation of ACC *in vitro* by AMP-PK from mammary gland and liver produced a similar degree of inactivation of ACC (Carling, D. et al., 1987; Ottey, K.A., 1992). Also, the ratios of the initial rates of phosphorylation for SSMS and SAMS peptides were similar for AMP-PK purified from mammary gland and liver (Table 3.4.). This indicates that AMP-PK purified from rat liver or lactating rat mammary gland does not have different substrate specificity.

AMP-PK purified from the same amounts of tissues and the same group of animals, both give a similar purification profile during a five column purification (Fig.3.12.-Fig.3.16.), although the mammary gland enzyme always has lower specific activity than the liver enzyme (Table 3.1. and 3.2.). The differences in specific activity of AMP-PK from liver and mammary gland could be due solely to the difference in their purity (Fig.3.17.). AMP-PK from both tissues have the same 93 KDa native MW calculated from gel-filtration on Superose 12 (Fig.3.16.) which is quite comparable to the published native MW obtained on Superose 12 for AMP-PK from rat liver of 100±30 KDa (Carling, D. et al., 1989).

The AMP-PK from both tissues have the same subunit structure with catalytic subunit with a MW of 61-62 KDa (Fig.17.). The same 62 KDa bands were also recognised by polyclonal antibodies raised against the predicted sequence of the liver AMP-PK (Fig.3.18.). This MW is similar to the published MW of liver AMP-PK of 63 KDa (Carling, D. et al., 1989) and to the calculated MW of 62.25 KDa deduced from the predicted amino acid sequence from the cDNA for AMP-PK from rat liver of non-lactating rats (62,250 Da, Carling, D. et al., 1994). Discrepancies between the MW

obtained in this study and the MW published by Dr. Carling in 1989 are probably superficial, possibly due to the different run of MW markers. AMP-PK from liver of lactating and nonlactating rat together with AMP-PK from mammary gland of lactating rat show the same MW of 62 KDa on "Western blot" (Fig.3.18).

Cleveland mapping of ^{32}P autophosphorylated AMP-PK from mammary gland and liver of lactating rat produced an identical radioactive peptide pattern (Fig.3.19.). This, together with a remarkable similarity in structure, purification profiles and substrate specificity strongly indicates that AMP-PK from mammary gland and liver of lactating rats represents identical entities.

CHAPTER FOUR

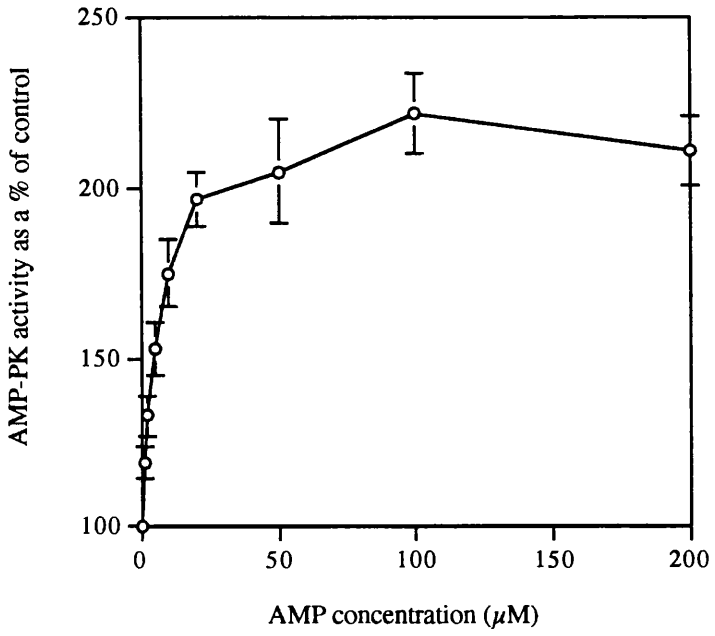
Regulation and physiology of AMP-activated protein kinase from lactating rat mammary gland

4.1. Activation of AMP-PK by 5'AMP

Mammary gland AMP-PK like the liver enzyme is activated in a concentration dependent manner by 5'AMP (Fig.4.1.). The maximum activation of two to three fold was achieved at 100μM 5'AMP with Ka of approximately 1.5μM (with 200μM ATP in the assay). This is very similar to values reported for AMP-PK from rat liver (Carling, D. et al., 1989) who reported a Ka for 5'AMP of 1.4μM in the presence of 200μM ATP. Although Ottey, K. et al. reported in 1988 a much higher Ka for 5'AMP of 0.5mM, this was later attributed by the same author to be due to a relatively crude preparation which may have contained many AMP binding proteins and thus mopping up the exogenous 5'AMP (Ottey, K., 1992).

Fig.4.2. shows AMP-PK from mammary gland purified up to the post Mono Q step and incubated at 15, 30, 60 and 120 minutes with 4mM $MgCl_2$ and 200μM γ-^{32}P ATP in the presence or absence of 200μM 5'AMP. The Coomassie stained gel (Fig.4.2.A.) shows that all lanes have an identical amount of protein loaded, and for this reason differences in density of bands on the autoradiograph are solely due to differences in incorporated ^{32}P into the protein. A number of bands in the post

Fig.4.1. 5'AMP activation of AMP-PK from rat mammary gland



AMP-activated protein kinase purified as far as the post Mono Q step was assayed as described in Chapter 2 with 5'AMP present in assay at the concentrations indicated. Activity is expressed as a percentage of the control activity (without added 5'AMP). Results represent the means of at least four separate experiments. SEM is represented by vertical bars.

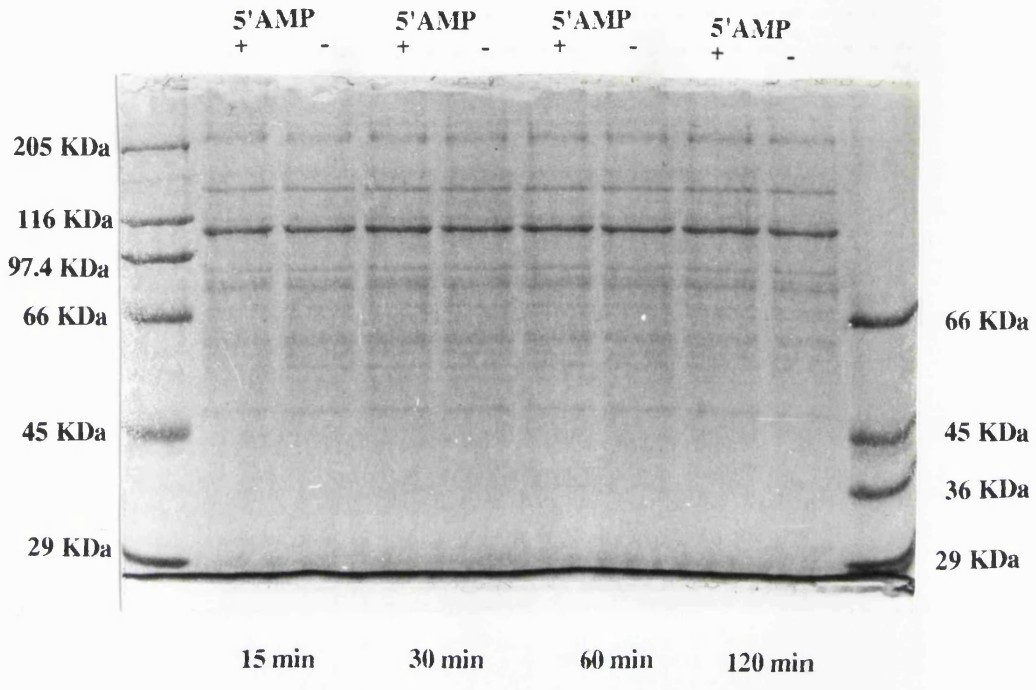
Fig.4.2. Time course of auto-phosphorylation of AMP-PK from mammary gland in the presence and absence of 200μM AMP

AMP-PK from mammary gland purified up to the post Mono Q step was incubated at 37^0C in the presence or absence of 200μM 5'AMP in: 50mM Hepes pH 7.5, 15% (w/v) glycerol, 10mM NaF, 4mM $MgCl_2$, 500μM EDTA, 500μM DTT, 500μM benzamidine, 1μg/ml SBTI, 20U/μl PKI and 200μM [γ^{32}P] ATP (sp. radioactivity > 500,000 cpm/nmol). At time points: 15, 30, 60 and 120 minutes, aliquots were taken and the reaction stopped by adding the appropriate amount of 5x SDS-sample buffer containing 25% (v/v) ß-mercaptoethanol and boiling for five minutes. The samples were run on SDS-PAGE using the mini-gel system and a 7.5% T resolving, and 3% T stacking, gel as described in Chapter 2. After the run, the gel was stained using Coomassie Blue R-250, dried onto filter paper, and autoradiographed as described in Chapter 2.

Fig.A shows The Coomassie Blue R-250 stained gel. Lanes 1 and 10 contain MW protein markers. The lanes 2,4,6 and 8 contain AMP-PK incubated in the presence of 200μM 5'AMP and lanes 3,5,7 and 9 in the absence of AMP. The time for each incubation is shown. Fig. B shows the autoradiograph of the same gel.

Coomassie Stained Proteins

A



Autoradiograph

B

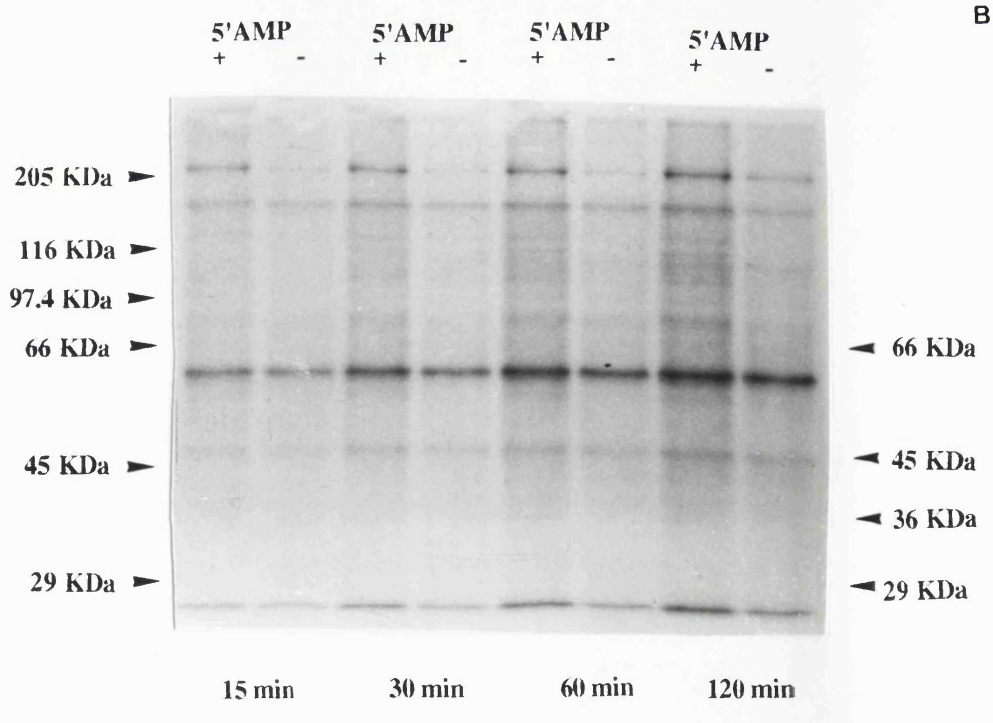


Table 4.1. Density of Autoradiographed AMP-PK bands from Fig.4.2.

Lane	Time of incubation	Density of all radiolabelled bands		Density of autoradiographed AMP-PK band	
		-200μM 5'AMP	+200μM 5'AMP	-200μM 5'AMP	+200μM 5'AMP
2,3	15 minutes	0.620	0.848	0.244	0.425
4,5	30 minutes	0.890	1.142	0.470	0.644
6,7	60 minutes	1.080	1.790	0.663	1.019
8,9	120 minutes	1.925	2.933	1.148	1.728

The autoradiograph from Fig.4.2. was scanned using a Millipore 2D scanner and the density of the AMP-PK band was compared. Values represent arbitrary units of density.

Mono Q AMP-PK preparation show a time-dependent increase in ^{32}P content (29 KDa, 48 KDa, 62 KDa, 80 KDa, 160 KDa, 210 KDa). This includes the 62 KDa AMP-PK which is the major ^{32}P band (Fig.4.2.B.). All of these bands show increased ^{32}P content in the presence of AMP (Fig.4.2.B) and this is suggestive that they are all substrates of AMP-PK.

To confirm this, the autoradiograph (Fig.4.2.B) was scanned using a Millipore 2D scanner and the data is shown in Table 4.1. The linear increase of incorporated ^{32}P with time, is similar for all bands, and the ratio of incorporated ^{32}P in the presence or absence of 5'AMP remains the same for all lanes, suggesting that all phosphorylation was carried out by AMP-PK (including its own autophosphorylation). This also suggests that the 62 KDa band increase in ^{32}P content is not due to the presence of kinase-kinase as a contaminant of the preparation. Kinase kinase phosphorylation of AMP-PK would result in the latter's activation (Carling, D. et al., 1987). To confirm

that this had not occurred, AMP-PK activity against SAMS peptide was measured at each time point (Table 4.2.). For each time point, AMP-PK in the presence of 200μM 5'AMP has about 180% of the activity of the control without added AMP (Table 4.2.). This is similar to the 150% increase in ^{32}P content of lanes preincubated in the presence of 5'AMP compared to the lanes without 5'AMP (Table 4.1.).

Preincubation with Mg/ATP did not lead to a time dependent increase in AMP-PK activity (Table 4.2.). This confirms that kinase-kinase is not present in the preparation

Table 4.2. Initial AMP-PK activity after preincubation with Mg/ATP in presence or absence of 200μM 5'AMP

Preincubation time (minutes)	- 200μM 5'AMP	+ 200μM 5'AMP	Activity ratio (+/- AMP)
15	102	175	1.71
30	98	189	1.92
60	95	167	1.75
120	96	179	1.86

AMP-PK from mammary gland purified up to the post Mono Q step was incubated at 37^{0}C in the presence or absence of 200μM 5'AMP in: 50mM Hepes pH 7.5, 15% (w/v) glycerol, 10mM NaF, 4mM $MgCl_2$, 500μM EDTA, 500μM DTT, 500μM benzamidine, 1μg/ml SBTI, 20U/μl PKI and 200μM ATP . At time points indicated above, aliquots were taken and initial AMP-PK activity was measured against SAMS peptide and expressed as a percentage of control AMP-PK activity assayed without preincubation and in the absence of 200μM 5'AMP.

at this purification step. This also confirms autophosphorylation and previous reports that autophosphorylation does not affect activity of AMP-PK (Takhar, S., 1992).

AMP activation of AMP-PK does not show time dependence (Fig.4.2., Table 4.1. and 4.2.) and does show concentration dependence (Fig.4.1.) indicating that AMP is an allosteric activator of AMP-PK.

Allosteric activation of AMP-PK by AMP is probably a mechanism for responding to pathological states, such as anoxia, by switching off biosynthetic pathways when ATP is limited. AMP-PK activation by 5'AMP is highly specific, with 5'AMP being the only one of several related nucleotides tested to be effective (Carling, D. et al., 1989). Due to the high activity of adenylate kinase, a cytosolic enzyme in hepatocytes which maintains the reaction ATP + AMP ↔ 2ADP close to equilibrium, even a small net conversion of ATP to ADP will produce a marked rise in AMP. Ligation of the blood vessels supplying the mammary gland of the lactating rat produces a rapid 6-fold increase in total AMP concentration, accompanied by a 47% decrease in the Vmax of purified ACC (Ottey, K.A. et al., 1989). This can be attributed to activation of AMP-PK and phosphorylation of ACC. Hypoxia also provides an explanation for the high state of ACC phosphorylation (and inactivation) observed by groups who do not use freeze-clamping to prepare tissue extracts. Analysis of the phosphorylation states of Ser-79, Ser-1200 and Ser-1215 in ACC from rat liver showed that phosphorylation occurs *post mortem* if the tissue is not freeze-clamped (Davies, S.P. et al., 1992.).

Manipulation (eg. fructose feeding) which raised the intracellular level of AMP by 100-fold in isolated hepatocytes produced marked inactivation of ACC and HMG-CoAR and an increase in the phosphate content of the sites known to be phosphorylated in these enzymes by AMP-PK *in vitro* (Moore, F. et al., 1991; Gillespie, J.G. and Hardie, D.G., 1992). These changes were also accompanied by an increase in AMP-PK activity.

Moore, F. et al. (1991) showed that AMP (in isolated hepatocytes) not only directly activated the AMP-PK, but enhanced its further activation by phosphorylation by "kinase-kinase".

4.2. Regulation of AMP-PK by reversible phosphorylation

As for the enzyme from the liver, AMP-PK from the mammary gland is inactivated by protein phosphatase 2A *in vitro* (see Fig.4.4). In the preparation of AMP-PK, if protein phosphatase inhibitors (5mM NaPPi and 50mM NaF) were ommited from the homogenisation buffer, AMP-PK activity was very low. When a post DEAE Sepharose preparation of AMP-PK from the mammary gland was dialysed into buffer without NaF and NaPPi overnight at 4^0C, the activity of AMP-PK decreased by 80% compared with a control sample dialysed for the same time in the presence of 5mM NaPPi and 50mM NaF (Fig.4.3.). AMP-PK activity was restored in a time dependent manner within 40 min incubation with 5mM NaPPi, 50mM NaF, 4mM $MgCl_2$ and 200μM ATP (Fig.4.3.). This suggests that covalent modification by phosphorylation was involved. As autophosphorylation of AMP-PK does not lead to an increase in activity (Fig.4.2. and Fig.4.4.), the activation observed in Fig.4.3. must be due to "kinase-kinase" present in the still relatively crude post DEAE step. Purification on Blue Sepharose separates this entity from AMP-PK because reactivation after this step is not possible.

This was similar to findings that purified protein phosphatase will inactivate the AMP-PK (from liver), and reactivation is achieved by incubation with MgATP and a fraction which appears to be separable from the kinase itself (Ingebritsen, T.S. et al., 1981). The phosphorylation and activation of AMP-PK by a distinct "kinase-kinase", appears to be AMP stimulated (Moore, F. et al., 1991). Moore, F. et al. speculate that the possible role of AMP is in substrate-mediated conformational changes in AMP-PK which would allosterically activate the enzyme and expose the phosphorylation site for

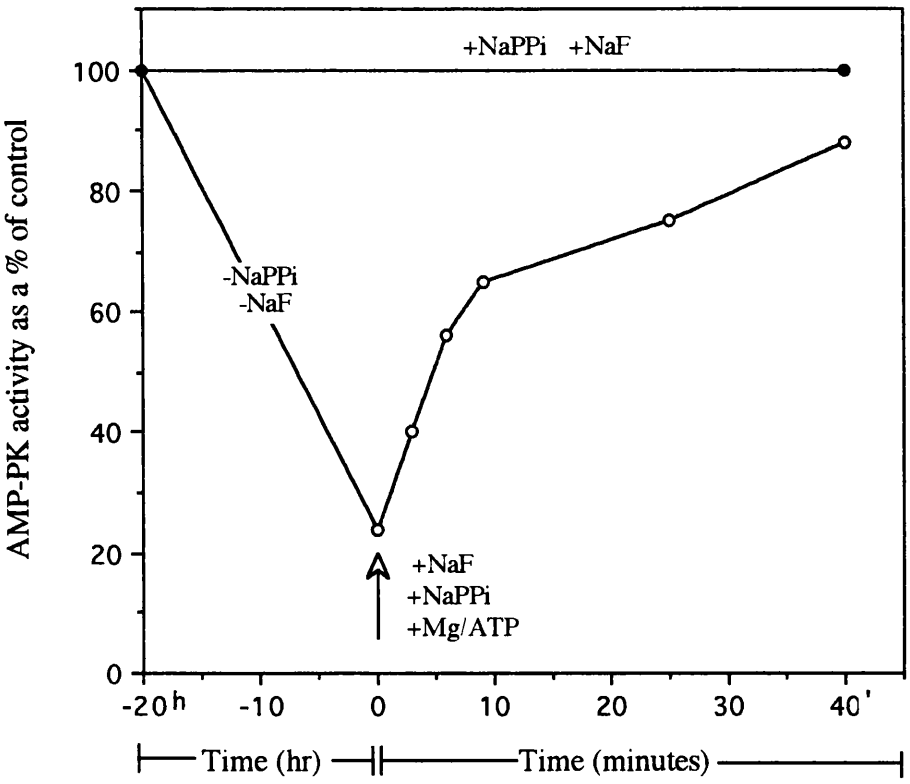
"kinase-kinase". It is also possible that "kinase-kinase" is by itself activated by 5'AMP. The "kinase-kinase" by itself appears to be stimulated by sub-micromolar concentrations of palmitoyl-CoA (Carling, D. et al., 1987; Davies, S.P. et al., 1989). Why such a small increase of palmitoyl-CoA (5-200nM) in a relatively crude post DEAE-Sepharose preparation (containing many fatty acid binding proteins) leads to stimulation of "kinase-kinase" remains a mystery. However the potential of palmitoyl-CoA to act as a feedback inhibitor of fatty acid synthesis via the activation of the AMP-PK cascade is an attractive possibility.

4.2.1. Kinase-Kinase

Lent and Kim (1983) reported that ACC kinase from rat liver which appears to be similar to the AMP-PK is activated two-fold by pretreatment with cAMP-PK (from bovine heart) in the presence of MgATP. This opened the possibility that cAMP-PK is "kinase-kinase". There was other evidence which implicated cAMP-PK in the regulation of the AMP-PK cascade: ACC is phosphorylated on Ser-79 and inactivated in response to glucagon in hepatocytes (Holland, R. et al., 1984) and glucagon and adrenaline in adipocytes (Holland, R. et al., 1985). The effect of glucagon in hepatocytes is to elevate the level of cAMP and activate cAMP-PK. Could cAMP-PK possibly phosphorylate and activate AMP-PK to cause increased phosphorylation of Ser-79 ?

Sim, A.T.R. and Hardie, D.G. (1988), showed that bovine heart cAMP-PK does not reactivate dephosphorylated liver AMP-PK. Davies, S.P. et al. (1989) showed that cAMP-PK from bovine heart does not activate purified AMP-PK, and does not affect the activation of partially purified AMP-PK by endogenous "kinase-kinase". Cohen and Hardie (1991) speculated that activated cAMP-PK in the liver, in response to glucagon, does not directly inhibit ACC, but may phosphorylate and inactivate the protein phosphatase-2A which acts on this enzyme.

Fig.4.3. Inactivation and reactivation of mammary gland AMP-PK purified to the post DEAE-Sepharose step by endogenous protein phosphatases and "kinase-kinase"



AMP-PK from mammary gland purified to the post DEAE Sepharose step was dialysed overnight at 4^{0}C into buffer in the presence (closed circles) and absence (open circles) of 5mM NaPPi and 50 mM NaF (phosphatase inhibitors). After 20h, 5mM NaPPi, 50mM NaF, 4mM $MgCl_2$ and 200μM ATP (final concentrations) were added to the sample incubated in the absence of phosphatase inhibitors. AMP-PK was assayed at the time points shown as described in Chapter 2 using the specific peptide substrate SAMS. The results were expressed as a % of the control activity at -20h.

The ability of two protein kinases, prepared from lactating rat mammary gland, to reactivate the AMP-PK was examined. These protein kinases were cAMP-PK (see Chapter 5) and acetyl-CoA carboxylase kinase-2 (ACK-2; Munday, M.R. and Hardie, D.G., 1984). The later was chosen for its known ability to phosphorylate ACC *in vitro* and both were chosen for their relatively unknown physiological function in mammary tissue.

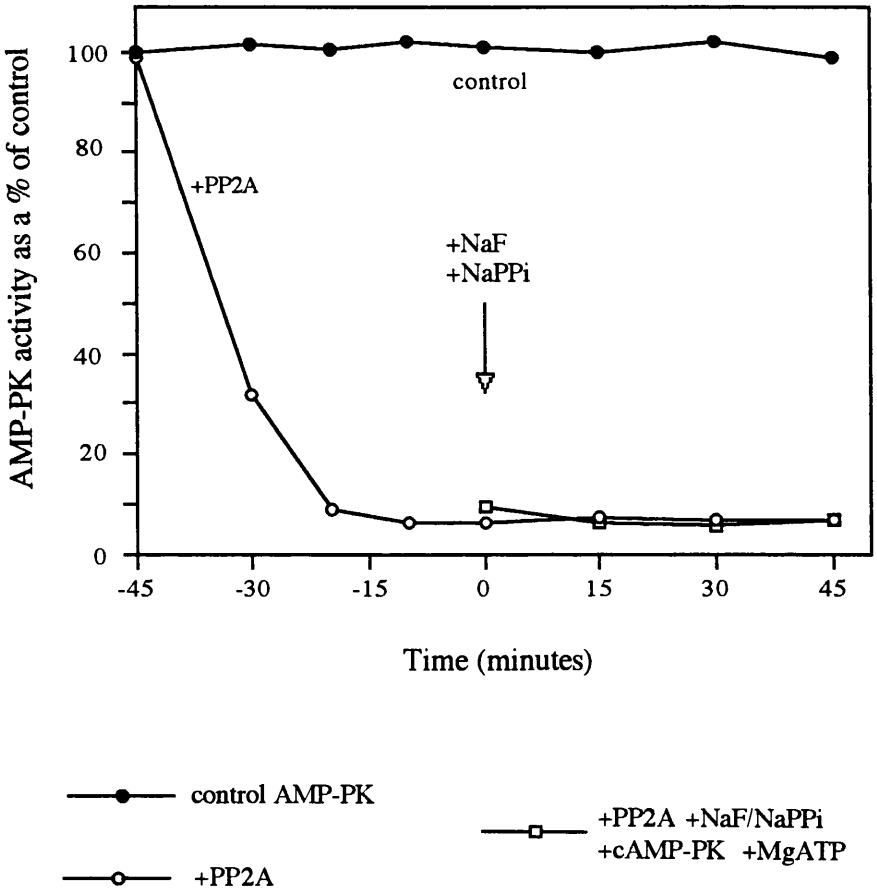
AMP-PK was purified from mammary gland up to the post Phospho-Cellulose step and dialysed into a buffer lacking phosphatase inhibitors. Incubation of this AMP-PK with purified PP-2A for 45 minutes brought about a 90% inactivation (Fig.4.4.). At the end of this incubation time, 50mM NaF and 5mM NaPPi were added to inhibit PP2A activity. Although cAMP-PK was shown to be active by its phosphorylation of the SSMS peptide substrate, it did not produce a time-dependent reactivation of the dephosphorylated AMP-PK when incubated at 37^0C for a subsequent 45 minutes in the presence of MgATP (Fig.4.4.). One can conclude that cAMP-PK from lactating rat mammary gland did not reactivate dephosphorylated mammary gland AMP-PK *in vitro* and for this reason seems unlikely to be the "kinase-kinase".

Although one cannot rule out the possibility that cAMP-PK (in liver) plays some part in the regulation of the AMP-PK cascade (upstream of kinase-kinase), this is more unlikely in mammary gland. Physiological studies described in the section 4.3. show that the activity ratio of cAMP-PK in mammary gland did not change in parallel with AMP-PK activity suggesting that cAMP-PK does not play any significant role in the regulation of the AMP-PK cascade in mammary gland *in vivo*.

4.2.2. ACK-2

ACK-2 is an ACC kinase that was originally identified and partially purified from lactating rat mammary gland (Munday, M.R and Hardie, D.G., 1984). Although ACK-2 phosphorylates ACC *in vitro* and causes an increase in the Ka citrate, the decrease in

Fig.4.4. cAMP-PK from lactating rat mammary gland does not reactivate dephosphorylated AMP-PK



AMP-PK from lactating rat mammary gland was purified to the post Phospho-Cellulose step and dialysed into 50mM Hepes purification buffer pH 7.4 lacking NaF and NaPPi. AMP-PK was incubated for 45 minutes at 37^{0}C in the presence or absence of Protein phosphatase 2A (PP2A), and aliquots were taken at the time points indicated and assayed for AMP-PK activity using the specific SAMS assay (section 2.6.). After 45 minutes, 50mM NaF and 5mM NaPPi (final concentrations) were added to both control and dephosphorylated AMP-PK to inhibit PP2A. Dephosphorylated AMP-PK was incubated with MgATP and cAMP-PK purified from mammary gland for a further 45 minutes. At the time points indicated samples were taken and assayed for AMP-PK activity. Results were expressed as a % of control AMP-PK activity which had not been subjected to dephosphorylation.

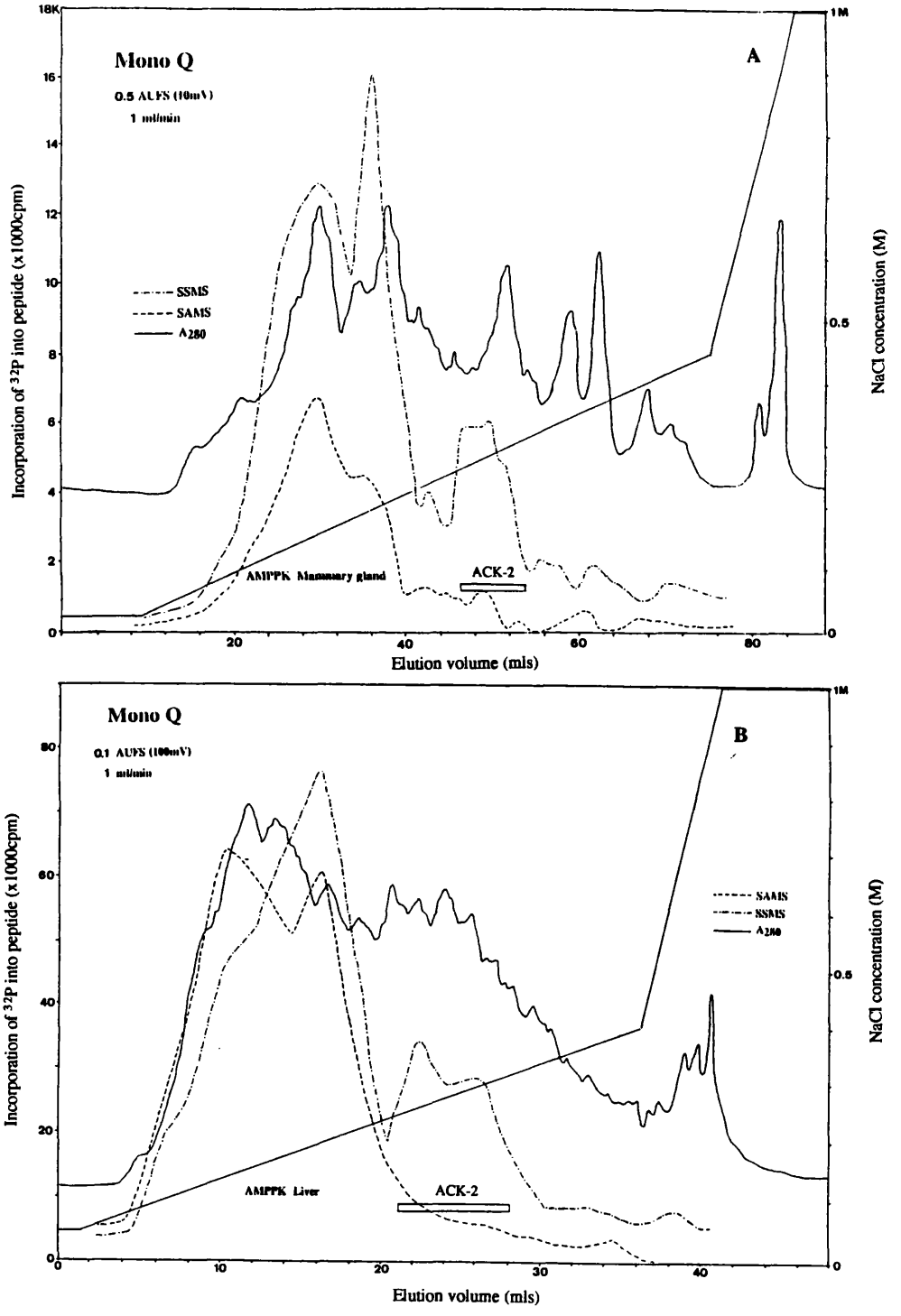
Vmax is only modest (20%; Munday, M.R. et al., 1988). As this is far below the inhibition of ACC observed *in vivo* (see Fig.4.12. and Fig.4.14.; Munday, M.R. et al., 1991) ACK-2 appears unlikely to be the protein kinase that directly regulates ACC *in vivo*. The physiological role of this protein kinase is unclear and its real physiological substrates are as yet not clearly defined. It has been shown (Munday, M.R. et al., 1988) that ACK-2 phosphorylates within the TCI peptide which contains the SSMS sequence. On the basis that it produces only a modest decrease in the Vmax it is probably phosphorylating Ser-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.

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.

If ACK-2 was "kinase-kinase" it would copurify with AMP-PK up to the post DEAE-Sepharose stage because at this stage reactivation of AMP-PK by added MgATP is still possible. For this reason the first few steps up to the post DEAE-Sepharose step of AMP-PK purification and ACK-2 purification were identical. To make a direct comparison between mammary gland and liver ACK-2, parallel purifications from both sources were carried out using 50g of mammary gland from rats at peak lactation and the same amount of liver from the same rats. Both tissues were freeze clamped and purification procedures were as previously described in Chapter 3.

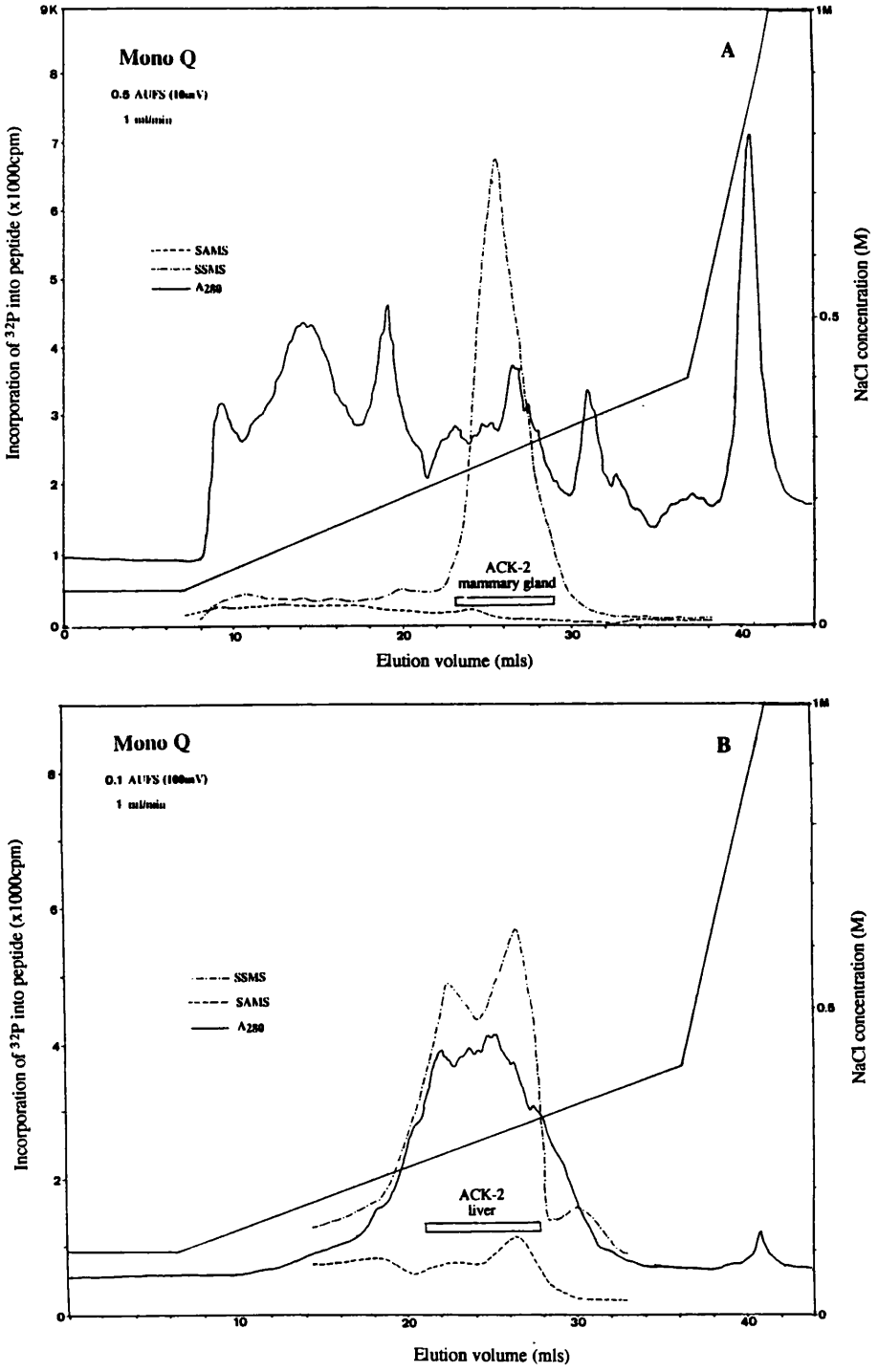
The post DEAE-Sepharose pool (0.2M NaCl batch), which was proven to contain "kinase-kinase", was dialysed into AMP-PK purification buffer (containing 50mM NaCl) and filtered through a 0.2μm filter and loaded directly onto Mono Q (HR 5/5). The column was developed by salt gradient 50mM-1M NaCl. Fractions of 500μl were collected and assayed for ACK-2 activity (SSMS) and AMP-PK (SAMS) activity. There were only two peaks of SSMS activity (Fig.4.5.). The first peak which also corresponded to SAMS activity eluted at a salt concentration identical to that previously

Fig.4.5. Mono Q profiles of separation of ACK-2 and AMP-PK from lactating rat mammary gland and liver



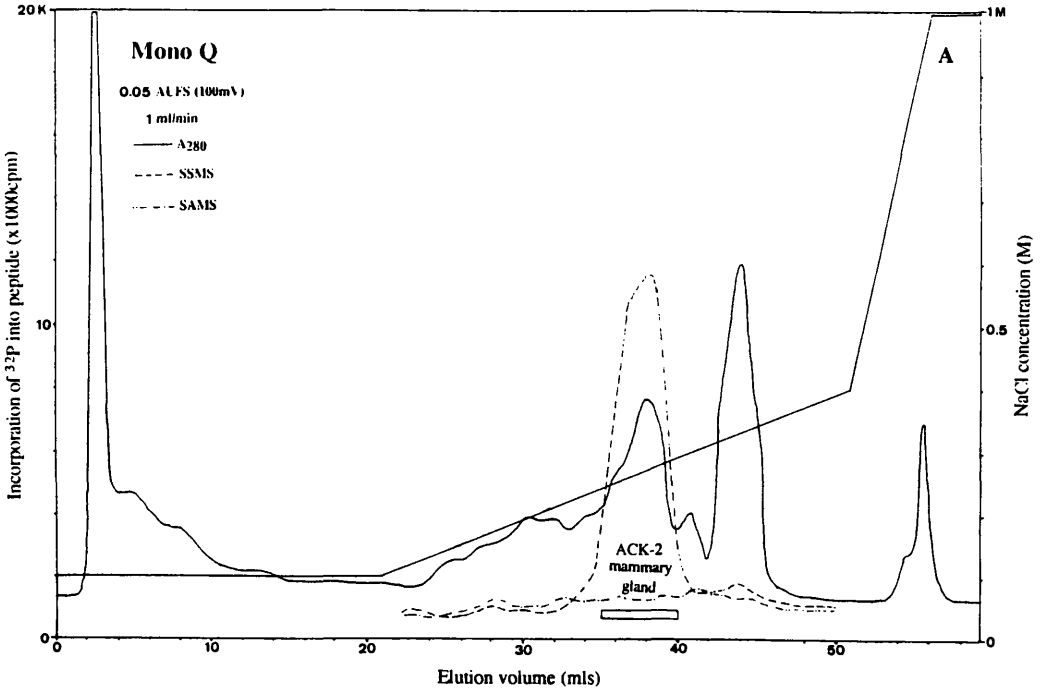
The post DEAE-Sepharose pool (0.2M NaCl batch), was dialysed into AMP-PK purification buffer (containing 50mM NaCl) and loaded onto Mono Q HR5/5 equilibrated in the same buffer (as described in text). The column was developed by salt gradient 50mM-1M NaCl. Fractions of 500μl were collected and assayed for ACK-2 activity (SSMS) and AMP-PK (SAMS) activity. Panel A represents the Mono Q profile from lactating rat mammary gland and panel B the Mono Q profile from livers of the same rats. The pooled peaks of ACK-2 activity were indicated by the long narrow rectangle.

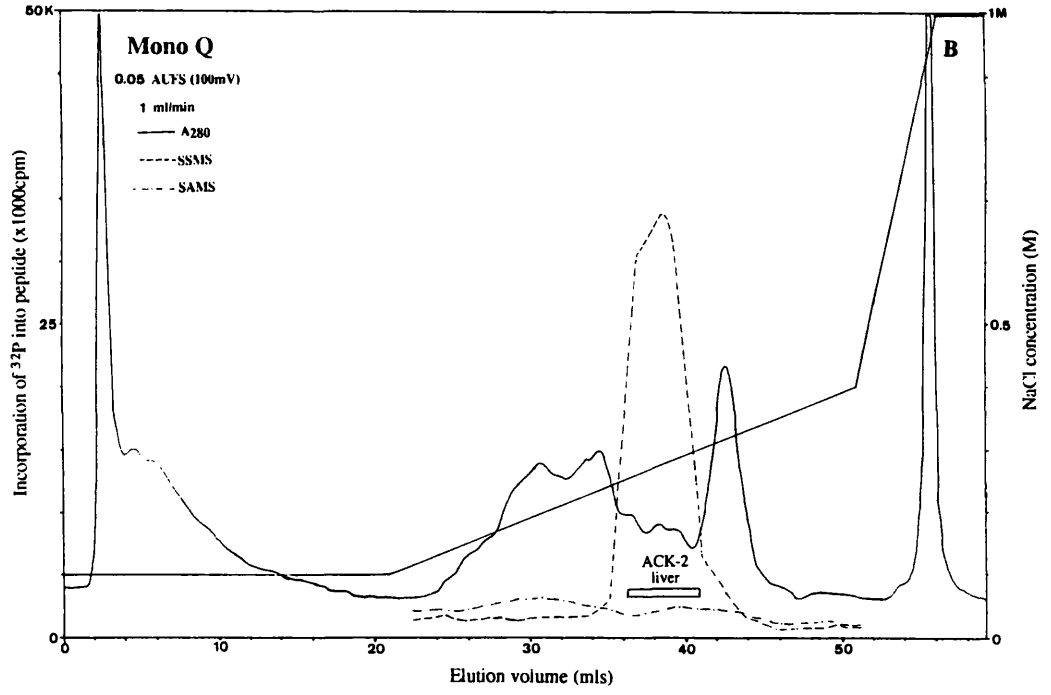
Fig.4.6. Second Mono Q profiles of separation of ACK-2 from lactating rat mammary gland and liver



The pooled fractions containing ACK-2 activity (from Mono Q run in Fig.4.5.) were dialysed and loaded onto the second Mono Q HR5/5 (as described in text). The column was developed with a salt gradient 0.1M-0.4M NaCl. Fractions of 500μl were collected and assayed for ACK-2 activity (SSMS) and AMP-PK (SAMS) activity. Panel A represents the second Mono Q profile from lactating rat mammary gland and panel B the second Mono Q profile from livers of the same rats. The pooled peaks of ACK-2 activity were indicated by the long narrow rectangle.

Fig.4.7. Mono Q profiles of separation of ACK-2 from lactating rat mammary gland and liver after Blue-Sepharose step





The break-through from Blue-Sepharose (as described in text) was loaded onto Mono Q HR5/5 equilibrated in AMP-PK purification buffer (containing 100mM NaCl). The column was developed with a salt gradient 0.1M-0.4M NaCl. Fractions of 500μl were collected and assayed for ACK-2 activity (SSMS) and AMP-PK (SAMS) activity. Panel A represents the Mono Q profile from lactating rat mammary gland and panel B the Mono Q profile from livers of the same rats. The pooled peaks of ACK-2 activity were indicated by the long narrow rectangle.

observed for AMP-PK (see Chapter 3), so it is reasonable to assume that this peak presents AMP-PK. The second peak of SSMS activity which eluted at 280mM NaCl showed very little SAMS activity. As PKI was included in the assay the C-subunit of cAMP-PK can be ruled out. Therefore this peak was tentatively ascribed (using the best criteria available) as ACK-2.

The ACK-2 peaks (from mammary gland and liver) were diluted 3-fold with AMP-PK purification buffer (to lower the salt concentration to 100mM NaCl) and reloaded onto Mono Q equilibrated in AMP-PK purification buffer (containing 100mM NaCl). A second Mono Q run was performed to remove AMP-PK impurities (due to the peak overlapping during the first run). The column was developed by salt gradient 0.1M-0.45M NaCl. Fractions of 500μl were collected and assayed for ACK-2 activity (SSMS) and AMP-PK (SAMS) activity. There was only one broad peak of SSMS activity in the preparation from lactating rat mammary gland which eluted at 280mM NaCl (Fig.4.6.A). The preparation from lactating rat liver also contained only one SSMS peak (Fig.4.6.B) which eluted at the same NaCl concentration as liver ACK-2. Both SSMS peaks were devoid of any appreciable SAMS activity.

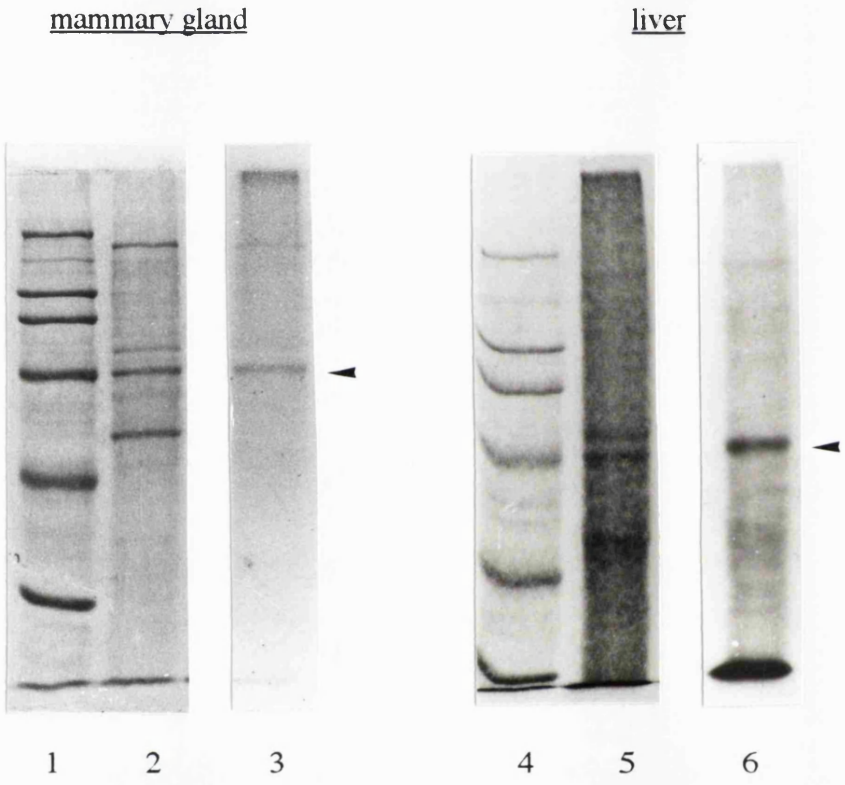
Further purification of ACK-2 from mammary gland and liver was attempted by loading the 0.2M NaCl batch elution from DEAE-Sepharose FF directly onto the Blue-Sepharose 6FF (20/20) as in the AMP-PK purification described in Chapter 3. As AMP-PK binds strongly to Blue-Sepharose (elutes at 950mM NaCl, see Chapter 3) and reactivation by incubation with MgATP after this stage is not possible, this step obviously separates kinase-kinase and AMP-PK. Batch elution was carried out with steps: break-through (containing 0.2M NaCl), 0.2-0.4M and 0.4-0.5M NaCl. A proportion of SSMS activity was found in the break through but not in the other batch eluted steps and no appreciable SAMS activity was found in either the break-through or the batch elutions.

The Blue-Sepharose break-through was diluted 2-fold with AMP-PK purification buffer (to lower the salt concentration to 100mM NaCl), filtered through a 0.2μm filter and loaded directly onto Mono Q HR 5/5 equilibrated in AMP-PK purification buffer

(containing 100mM NaCl). The column was developed with a salt gradient 0.1M-0.4M NaCl. Fractions of 500μl were collected and assayed for ACK-2 activity (SSMS) and AMP-PK (SAMS) activity. There was only one peak of SSMS activity in the preparation from lactating rat mammary gland which eluted at 280mM NaCl (Fig.4.7.A). The preparation from lactating rat liver also contained only one SSMS peak (Fig.4.7.B) which eluted at the same NaCl concentration. Both SSMS peaks were devoid of any appreciable SAMS activity. The most active fractions around each SSMS peak were pooled and concentrated using an Amicon concentrator with a PM30 membrane to a volume of 1ml, dialysed against AMP-PK storage buffer (as described in Chapter 3) and stored at -20^{0}C.

Incubation of the second post Mono Q pool of ACK-2 from lactating rat mammary gland or liver with [γ-^{32}P]-ATP resulted in ^{32}P labelling of relatively few bands of protein as shown by comparison of lane 2 with 3 and lane 5 with 6 in Fig.4.8. As all protein kinases autophosphorylate, at least one of these bands must be the autophosphorylated ACK-2 and one might expect this to be a the major ^{32}P band. The major ^{32}P band in the preparation from mammary gland (lane 3) and liver (lane 6) migrates with a MW of 68 KDa (Fig.4.8.). This MW is similar, but not identical to the published MW of rat mammary ACK-2 of 76 KDa obtained by gel filtration (Munday, M.R. and Hardie, D.G., 1984). Gel filtration of the second post Mono Q pool by Superose 12 chromatography on FPLC failed to resolve ACK-2 activity (data not shown). At present it is not possible to conclusively prove that the SSMS kinase purified and ACK-2 are the same entity. However, SSMS kinase eluted at 280mM NaCl from MonoQ from both tissues was insensitive to the specific protein inhibitor of cAMP-PK (at a concentration of 100U/ml), and was insensitive to heparin (at a concentration of 20μg/ml) which is in agreement with published data on ACK-2 (Munday, M.R. and Hardie, D.G., 1984). The SSMS-kinase from mammary gland and liver of lactating rats also appeared to be insensitive to 5'AMP (at a concentration of 100μM) and to 5μM Calphostin C (a specific protein kinase C inhibitor).

Fig.4.8. SDS-PAGE of auto-phosphorylated ACK-2 from lactating rat mammary gland and liver



ACK-2 from mammary gland (lanes 2 and 3) and liver (lanes 5 and 6) of lactating rat purified to the post second Mono Q step were incubated with 4mM $MgCl_2$ and 200μM [γP^{32}]ATP (sp. radioactivity > 10^6cpm/nmol) for 1 hour at 37^{0}C. Phosphorylation was stopped by boiling with the addition of 25μl of 5x SDS sample buffer containing 25% (v/v) ß-mercaptoethanol. Standard SDS-PAGE was performed using a 10%T acrylamide resolving gel and 5%T stacking gel (section 2.9.1.). This was subsequently stained with Coomassie Blue R-250 dried and autoradiographed (section 2.13.2, 2.12.). Lanes 1,2,4 and 5 show Coomassie stained protein markers (lane 1 and 4) and autophosphorylated ACK-2 purified from lactating rat mammary gland (lane 2) and liver (lane 5). Lanes 3 and 6 show the autoradiograph lanes 2 and 5 respectively. Lanes 1 and 4 contain high MW protein markers (205, 116, 97.4, 66, 45 and 29 KDa). The 68 KDa band is indicated by an arrow.

4.2.3. Is the ACK-2 a kinase-kinase?

The SSMS kinase eluting from Mono Q at 280mM NaCl and ascribed to ACK-2 was examined to see if it was "kinase-kinase".

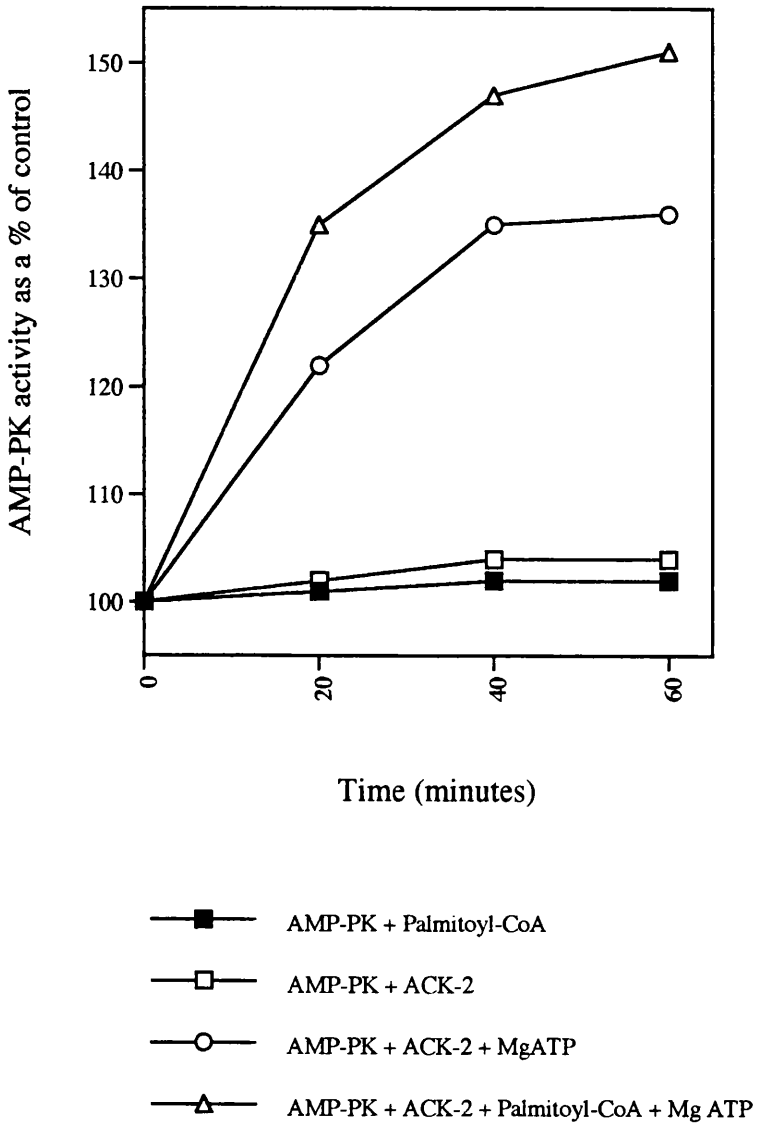
AMP-PK, purified from lactating rat mammary gland up to the post Phospho-Cellulose step and dephosphorylated by PP-2A, was incubated with ACK-2 purified from mammary gland or liver of lactating rat in the presence or absence of cold MgATP and the presence or absence of 200nM palmitoyl-CoA over a time course of 1 hour. At set times aliquots were removed from this incubation and assayed for SAMS peptide phosphorylation with [γ^{32}P] MgATP (Fig.4.9). The inclusion of palmitoyl-CoA stimulated both the rate and the final level of reactivation of AMP-PK by ACK-2 (Fig.4.9). 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.4.9).

This observation is in agreement with those made from experiments conducted on the crude DEAE preparation of AMP-PK, where reactivation only occurred in the presence of MgATP and was stimulated by palmitoyl-CoA (Carling, D. et al., 1987).

The availability of a peptide assay for ACK-2 enabled the investigation of whether the palmitoyl-CoA stimulation was a substrate (AMP-PK) mediated effect or a direct effect on ACK-2. ACK-2 purified up to the second Mono Q step was incubated at various concentrations of palmitoyl-CoA and assayed using SSMS peptide as a substrate in the presence of PKI (cAMP-PK inhibitor). Fig.4.10. clearly shows a concentration dependent increase in ACK-2 activity with maximum activation of around 147% at 200nM palmitoyl-CoA. The Ka for palmitoyl-CoA appears to be in the region of 20nM.

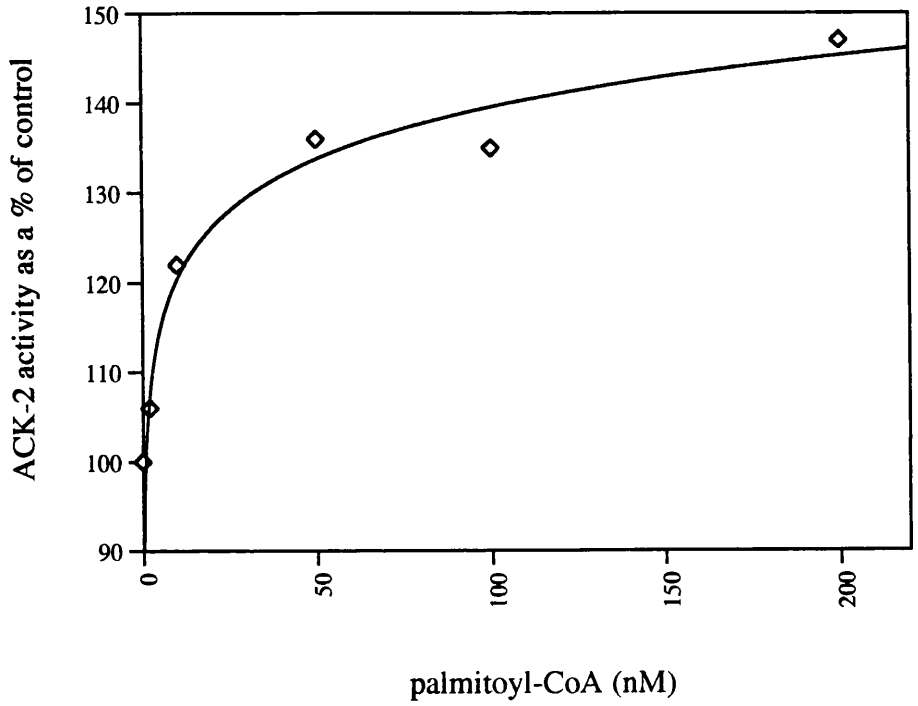
This further supports the possibility that ACK-2 and kinase-kinase are the same entity.

Fig.4.9. Reactivation of mammary gland AMP-PK with Mono Q purified ACK-2



AMP-PK was purified from lactating rat mammary gland up to post Phospho-Cellulose step (as described in Chapter 3), dephosphorylated by incubation with protein phosphatase-2A and then incubated with ACK-2 (purified up to post second Mono Q step) in the presence or absence of 200nM Palmitoyl-CoA. Control incubations of AMP-PK in the presence of 200nM Palmitoyl-CoA and MgATP (without ACK-2), and AMP-PK with ACK-2 (without 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 section 2.6.).

Fig.4.10. ACK-2 activation by palmitoyl-CoA



ACK-2 (purified from lactating rat mammary gland up to the second post Mono Q step) was assayed at various concentrations of Palmitoyl-CoA using the synthetic peptide SSMS as substrate (as described in section 2.7.) in the presence of PKI. Results were expressed as a percentage of SSMS activity without added palmitoyl-CoA.

ACK-2 is activated to a similar extent by a number of related fatty acyl-CoAs (Table 4.3.).

Table 4.3. ACK-2 activation by different fatty acyl-CoA

fatty acyl-CoA	ACK-2 activity
control	100%
200nM palmitoyl-CoA	147%
200nM stearoyl-CoA	150%
200nM oleoyl-CoA	156%

ACK-2 (purified from lactating rat mammary gland up to post second Mono Q step) was assayed in the presence of 200nM palmitoyl-CoA, stearoyl-CoA or oleoyl-CoA using the synthetic peptide SSMS as substrate (as described in section 2.7.) in the presence of PKI. Results were expressed as a percentage of SSMS activity without added fatty acyl-CoA.

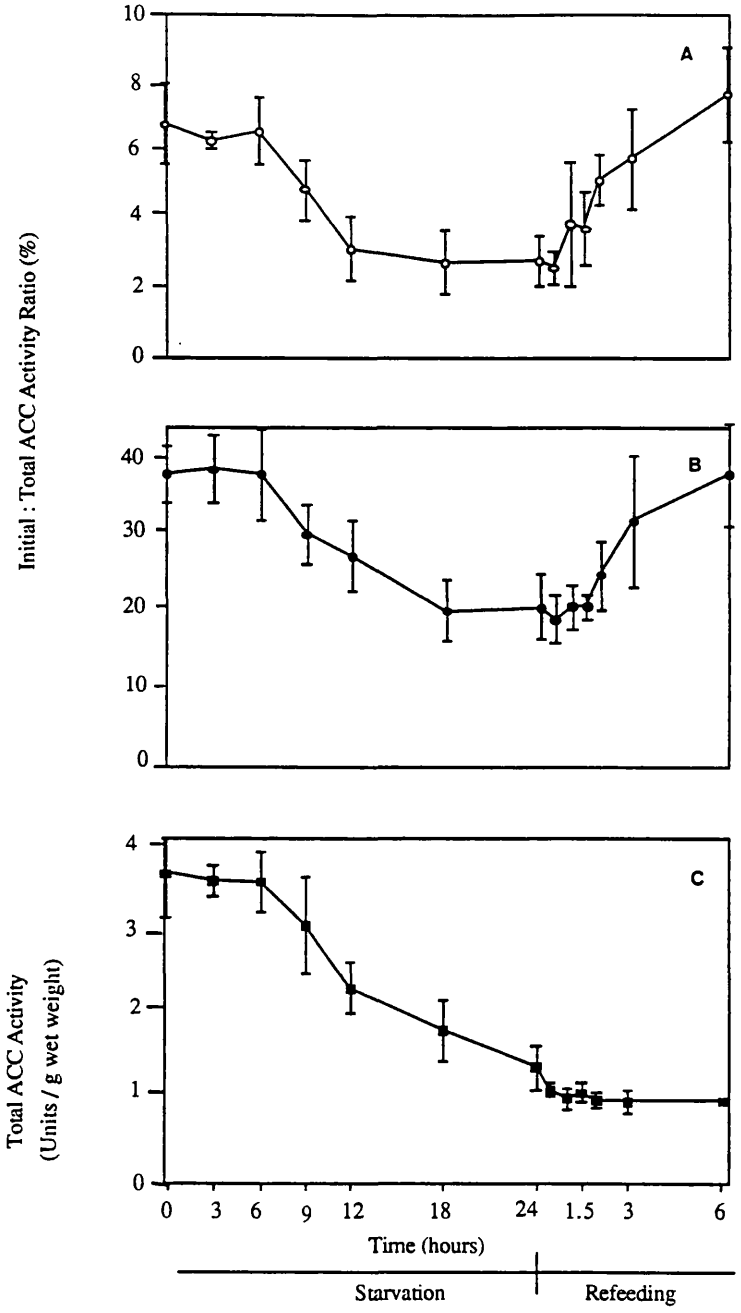
4.3. The role of AMP-PK in the regulation of ACC activity by phosphorylation during starvation and refeeding in the lactating rat mammary gland

The total activity of ACC in crude lactating rat mammary gland homogenates decreased by approximately 63% after 24 h starvation (Fig.4.11.C.). This decrease is similar to that reported by other workers for the mammary enzyme (McNeillie, E.M. and Zammit, V.A., 1982; Munday, M.R. and Williamson, D.H., 1982; Munday, M.R. and Hardie, D.G., 1986). This decrease is likely to be due to a decrease in the concentration of ACC (the "total activity" of ACC is taken to be a reasonable indication of the total concentration of the enzyme since it represents the maximum available ACC activity after extensive dephosphorylation with exogenous protein phosphatase followed by activation with the allosteric activator citrate). The decrease in ACC concentration did not occur until 9 hours of starvation, and then continued progressively over the time course of 24 hour starvation (Fig.4.11.C.). The decrease in total cytosolic ACC can be due to a decreased rate of enzyme synthesis or an increased rate of degradation.

Expressed initial : total ACC activity measured at the physiological cell citrate concentration (0.5mM citrate, Fig.4.11.A) and saturated citrate concentration (10mM citrate, Fig.4.11.B) also 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, M.R. and Williamson, D.H., 1982; McNeillie, E.M. and Zammit, V.A., 1982).

During the first 6 hours of starvation neither the amount of ACC (as judged by total ACC activity Fig 4.11.C.) nor the intrinsic activity of ACC (as judged by initial : total activity ratio Fig.4.11.A,B.) changed. The major inactivation of ACC occurred between 6 and 18 hours. At an early stage of starvation (<6h), ACC activity does not correlate well with inhibition of fatty acid synthesis. The rate of fatty acid synthesis in the mammary gland declines steadily over the first 6h of starvation until it is 87%

Fig.4.11. ACC activity during starvation and refeeding in tissue homogenates 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 Units/gram wet weight tissue (panel C). Initial ACC activities were assayed as described in chapter 2 at 0.5mM citrate (panel A) and 10mM citrate (panel B) and each were expressed as a percentage of total activity. One Unit of ACC activity represents incorporation of 1 μmol $NaH^{14}CO_3$ per minute into malonyl-CoA. Results shown are the means of at least 4 observations at each time point. SEM are represented by vertical bars.

inhibited, followed by further slower inhibition between 6 and 24h eventually being inhibited by 97%. This is more in correlation with changes in PDH activity which is inhibited by 79% by 6h starvation and 92% by 24h starvation (Hagopian, K et al., 1991) and changes in uptake of glucose by the mammary gland than in changes in ACC activity. This strongly suggests that phosphorylation and inactivation of PDH is the primary event in inhibition of lipogenesis during starvation (Hagopian, K et al., 1991) and that inactivation of ACC is not involved in short term (<6 hours) response. This is in agreement with previous reports (Munday, M.R. and Hardie, D.G., 1986; Williamson, D.H. et al., 1983). Delayed inhibition of ACC in starvation may represent a final "locking down" mechanism, 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.

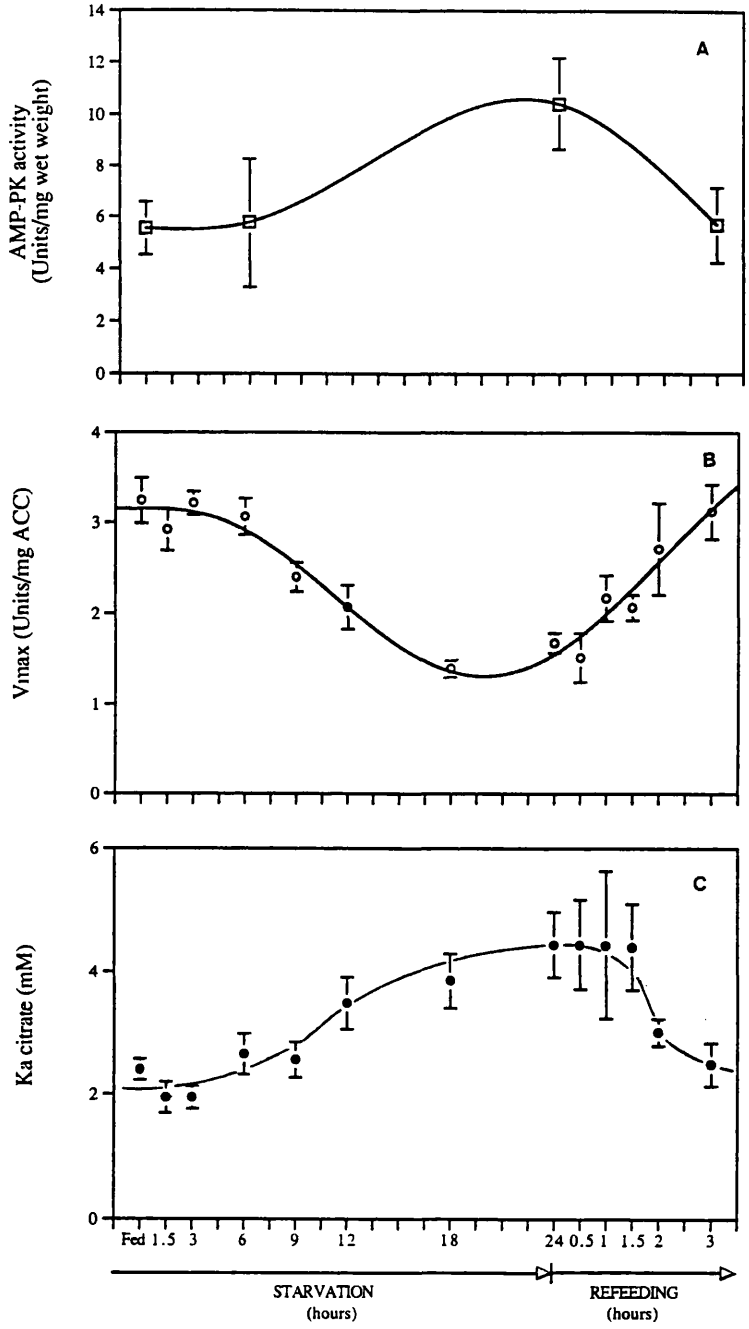
The total ACC activity did not markedly increase during 6 hours of refeeding (Fig. 4.11.C.) which indicates that the increase of ACC activity during the first 6 hours of refeeding is not due to the increased amount of ACC. After 0.5 hour refeeding there was no reactivation of ACC (Fig.4.11.A,B.). Refeeding for 1 hour caused an 18-24% reactivation of remaining ACC, which did not differ significantly from the reactivation at 1.5 hour refeeding. By 2-hour refeeding ACC initial/total activity ratio was restored to 65-77% of fed control levels, this increased to 87-92% by 3-hour refeeding and initial/total ACC activity was restored to the fed control levels by 6-hour refeeding. Changes in ACC initial/total activity ratio during refeeding correlates well with changes in lipogenesis and PDH activity (Hagopian, K. -unpublished results) suggesting more synchronous control of lipogenic enzymes during refeeding. The mechanism for the reactivation of PDH and ACC probably involves the raised plasma insulin concentration which is seen upon refeeding (Robinson, A.M. et al., 1978).

Changes in expressed ACC activity are often indicative of changes in the phosphorylation and activation state of ACC. To investigate changes in activity due to

phosphorylation, ACC was purified by avidin-Sepharose affinity chromatography (described in detail in chapter 2). This 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 Ka citrate (concentration of citrate producing half maximal activation) or Vmax (Fig.4.12.B,C). This correlates with a lack of change in initial : total activity ratio (Fig. 4.11.A,B.). By 12 hour starvation there was a 46% increase in the Ka citrate and a 36% decrease in the Vmax and these changes were significantly different from fed ACC ($P<0.05$ and $P<0.005$ respectively). The maximal increase in the Ka citrate (86%) and decrease in Vmax (69%) had occurred by 18-24 hour starvation and both were significantly different from the fed control ($P<0.025$ and $P<0.001$ respectively). There was no reactivation of purified ACC activity after 0.5 hour refeeding as reflected by no change in the Vmax or Ka citrate of the enzyme (Fig.4.12.B,C). This correlates with a lack of change in initial : total activity ratio (Fig. 4.11.A,B.). Refeeding for 2 hours decreased the Ka citrate by 32% and increased the Vmax to 84% of the fed control value such that the kinetic parameters of ACC were no longer significantly different from those of the fed control. After 3 hours of refeeding both parameters returned to within 3% of their respective initial values in the fed lactating rat. Overall this data was in agreement with the measurement of the expressed activity ratio (initial : total activity ratio in Fig.4.11.A,B) and confirmed that the observed activity changes of ACC were due to changes in its phosphorylation state. This also confirmed findings that inhibition of ACC activity during 24h starvation is the result of increased phosphorylation of the enzyme, which can be completely reversed by refeeding the rat (Munday, M.R. and Hardie, D.G., 1986).

Fig.4.12. Changes in AMP-PK activity and pure ACC activity in lactating rat mammary gland during fed-starved-refed transitions



AMP-PK activity (panel A) was assayed in crude homogenates of lactating rat mammary glands as described in Material and Methods. ACC was purified using an Avidin-Sepharose chromatography and assayed as described in chapter 2 sections 2.3.2-2.3.3. Vmax (panel B) and Ka citrate (panel C) of purified ACC were calculated by computer program, and each value represents the mean of at least four separate preparations for each time point with SEM values represented by vertical bars.

The changes in AMP-PK activity in mammary gland of lactating rat (Fig. 4.12.A.) showed a good inverse correlation with the Vmax changes and direct correlation with changes in Ka citrate of pure ACC (Fig.4.12.B and C). This is in keeping with the observed effect of *in vitro* phosphorylation of mammary ACC by mammary AMP-PK ie 86% decrease in Vmax, 88% increase in Ka citrate (Ottey, K., 1992). The AMP-PK activity did not change significantly in the first 6 hours of starvation (5% increase), but had increased significantly (88%) after 24 h starvation. Within the 3 hour refeeding period AMP-PK activity declined to within 3% of the AMP-PK activity in the fed control. This good correlation between the changes in AMP-PK and ACC activity is further proof that AMP-PK is the physiological kinase that phosphorylates and inactivates ACC in mammary gland *in vivo*. The activity ratio of cAMP-PK did not change during these physiological manipulations (as can be seen from Table 4.4.). For this reason cAMP-PK can be ruled out as a physiological ACC kinase in mammary gland and so can the possibility that it is an upstream regulatory kinase in the AMP-PK cascade.

The changes in ACC and AMP-PK activity in the lactating mammary gland are paralleled by changes in the concentration of plasma insulin which decreases approximately 64% in response to 24h starvation (Jones, R.G. et al., 1984b) and restored by refeeding (Mercer, S.W. and Williamson, D.H., 1986). However, significant decreases in plasma insulin have been reported after 6h starvation (Jones, R.G. et al., 1984b) when neither AMP-PK nor ACC exhibit any response (Table 4.4.). Preventing the release of insulin into circulation in response to refeeding by the administration of streptozotocin blocks both the inactivation of AMP-PK and reactivation of ACC (Table 4.4.). If anything, AMP-PK appears to become even more active in response to this treatment. This suggests that insulin is likely to be an important regulator of the AMP-PK cascade. The changes in AMP-PK activity in response to starvation and refeeding are stable and survive high dilution prior to assay suggesting that they are mediated either by changes in AMP-PK concentration or by

covalent modification by "kinase-kinase" and phosphatases but not by allosteric modulation by eg. AMP.

Table 4.4. Activity of ACC, AMP-PK and cAMP-PK activity ratio in lactating rat mammary gland

	ACC Vmax	AMP-PK activity	cAMP-PK activity ratio
Fed	3.24 ± 0.25	5.55 ± 1.02	0.12 ± 0.04
6h starved	3.06 ± 0.20	5.78 ± 2.48	--
24h starved	1.50 ± 0.11	10.42 ± 1.75	0.13 ± 0.05
24h starved 3h reefed	3.12 ± 0.30	5.70 ± 1.45	--
24h starved 3h Streptozotocin 3h reefed	1.22 ± 0.25	17.95 ± 2.59	--

ACC was purified using avidin-Sepharose chromatography. Values for ACC Vmax are given as μmol HCO_3 incorporated/min/mg ACC. The activities of AMP-PK and cAMP-PK were measured in crude post-mitochondrial supernatant by the incorporation of ^{32}P from [γ-^{32}P]-ATP into the specific SAMS peptide substrate in the presence of 200μM 5'AMP, and kemptide, respectively. AMP-PK activity is expressed as nmol ^{32}P incorporated into SAMS/min/g wet wt tissue. cAMP-PK activity is expressed as the ratio measured in the absence : presence 10μM cAMP. Streptozotocin was administered iv. (50mg/kg) prior to refeeding. All values are means ± SEM for at least four observations in each group.

4.4. The role of AMP-PK in the regulation of ACC activity by phosphorylation during starvation and refeeding in the liver of the virgin rat

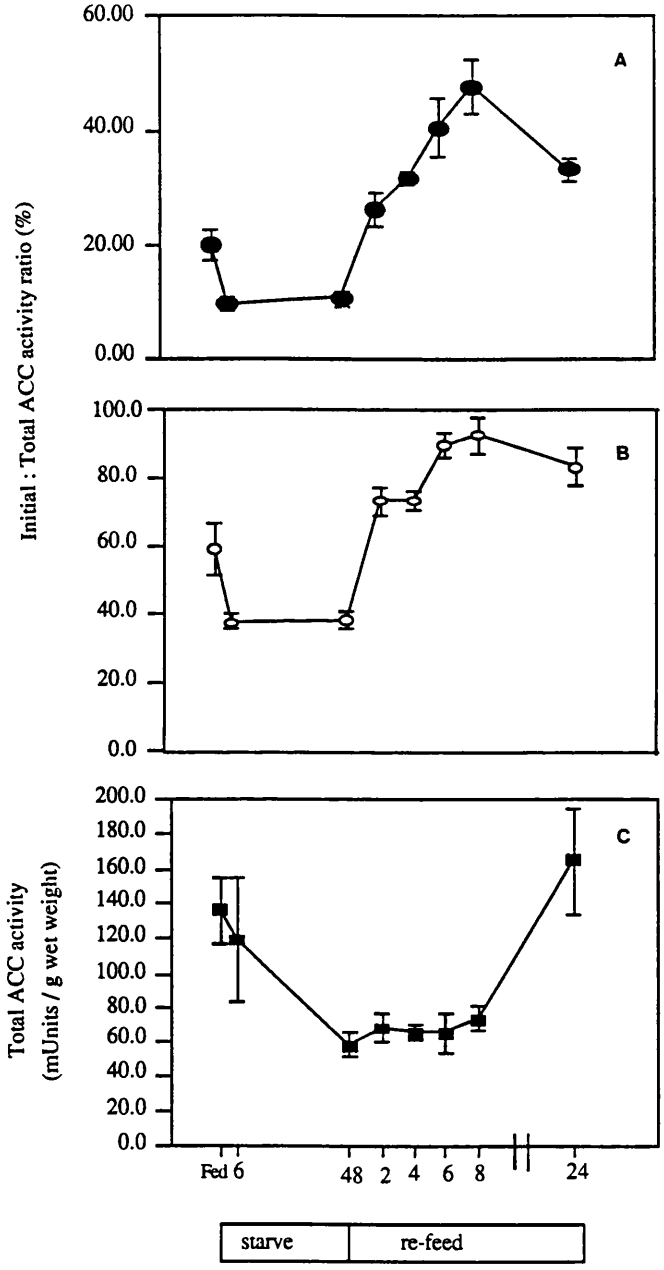
Initial and total ACC activity was measured in liver homogenates from virgin rats during the fed-starved-refed transition.

There was no significant change in "total ACC 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 (see Table 1.1.). There was however, a drop in the "total activity" of ACC of 60% by 48 hour starvation (Fig.4.13.C.). "Total activity" of ACC did not increase over the first 8 hours of refeeding, but did increase between 8 - 24 hours refeeding to a value higher than that of fed controls. These data are in agreement with previous reports that total hepatic ACC concentration does not rise within the first 8 hours, but does increase steadily over a subsequent 40 hour period of chow refeeding of 48-hour fasted rats (Nishkori, K. et al., 1973; Roman-Lopez, C.R. et al., 1989).

When the "initial activity" of ACC (measured at the physiological 0.5mM citrate, or 10mM saturating citrate concentration) was expressed as a percentage of the "total activity" (Fig.4.13.A,B.) it became 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.4.13.A,B.). Significant inactivation of hepatic ACC has previously been reported to occur within 24 hour starvation (Moir, A.M.B. and Zammit, V.A., 1990). The data in this present study suggests that inactivation occurs even more rapidly than this.

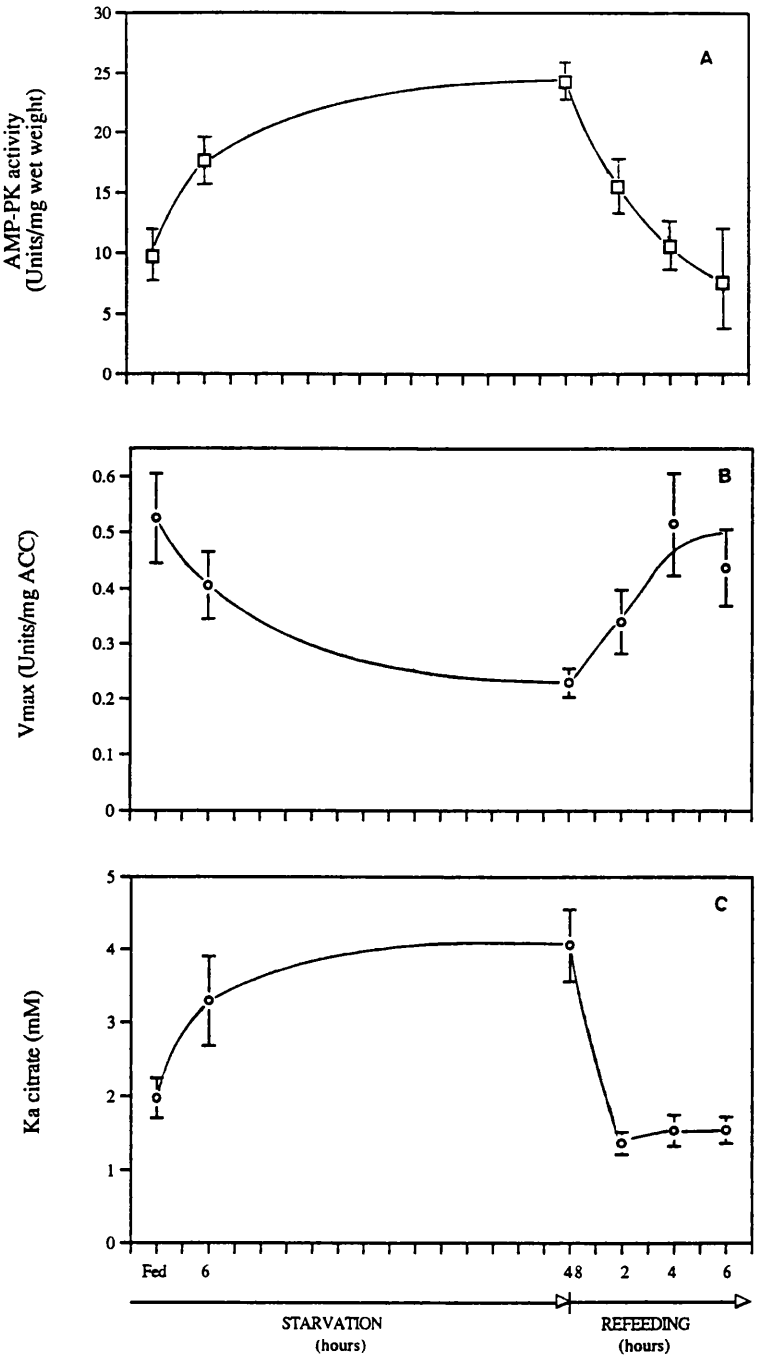
The reactivation of ACC in response to refeeding was rapid and complete within 6 - 8 hours of refeeding. Approximately 60% of this reactivation occurred within the first 2 hours of refeeding (Fig.4.13.A,B.). This reactivation was clearly observed in a part of

Fig.4.13. Changes in ACC activity during starvation and refeeding in tissue homogenates prepared from livers of virgin rats



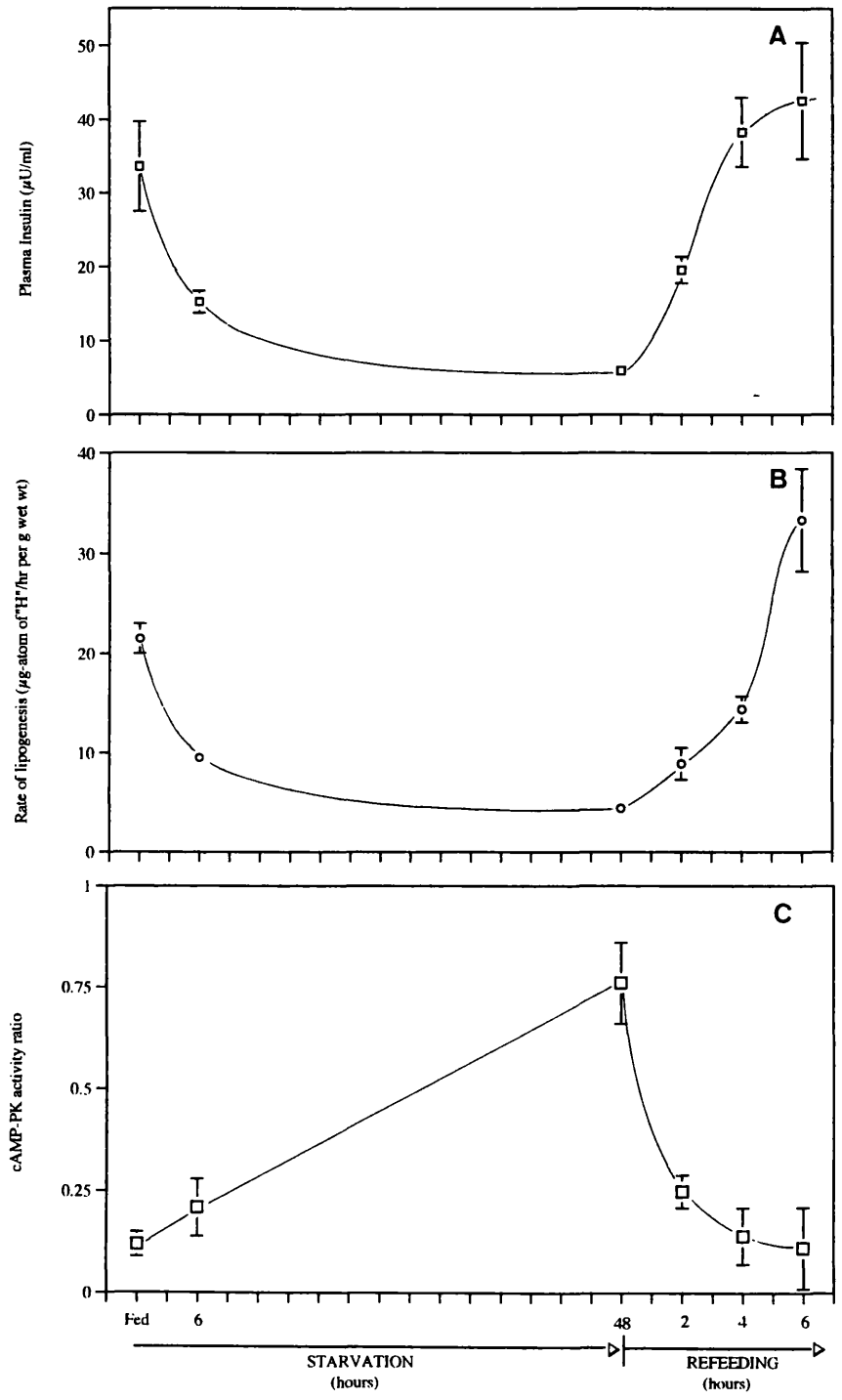
Total ACC activity was assayed in crude extracts from livers of virgin rats as described in chapter 2 and is expressed as mUnits/gram wet weight tissue (panel C). Initial ACC activities were assayed as described in chapter 2 at 0.5mM citrate (panel A) and 10mM citrate (panel B) and each were expressed as a percentage of total activity. One mUnit of ACC activity represents incorporation of 1 nmol $NaH^{14}CO_3$ per minute into malonyl-CoA. Results shown are the means of at least 4 observations at each time point. SEM values are represented by vertical bars.

Fig.4.14. Changes in AMP-PK activity and pure ACC activity in virgin rat liver during fed-starved-refed transition



AMP-PK activity (panel A) was assayed in crude rat liver homogenate as described in Material and Methods. ACC was purified using avidin-Sepharose chromatography and assayed as described in Chapter 2 sections 2.3.2-2.3.3. Vmax (panel B) and Ka citrate (panel C) of purified ACC were calculated by computer program, and each value represents the mean of at least four separate preparations for each time point with SEM values represented by vertical bars.

Fig.4.15. Changes in plasma insulin concentration, rate of lipogenesis and cAMP-PK activity ratio in virgin rat liver during the fed-starved-refed transition



Measurements of plasma insulin and rate of lipogenesis were from Munday, M.R. et al. (1991) and Sugden, M.C. et al. (1993) respectively. Insulin was expressed as μU per ml of plasma, and lipid synthesis were estimated as ^3H incorporation from ^3H_2O into tissue lipid and expressed in μmol 3H incorporated into lipid / hour / g wet weight. The cAMP-PK activity was measured in crude post-mitochondrial supernatant by the incorporation of ^{32}P from [γ -^{32}P]-ATP into kemptide and activity is expressed as the ratio measured in the absence : presence 10μM cAMP. All values are means ± SEM for at least four observations in each group.

the time course during which ACC concentration had not changed (Fig.4.13.C.). It would be reasonable to conclude from Fig.4.13. that changes in ACC activity from 0 - 6 hour starvation and from 0 - 8 hour refeeding (after 48 hour starvation) are the result of change in the intrinsic activity of the existing ACC enzyme.

To investigate changes in activity due to phosphorylation, ACC was purified by avidin-Sepharose affinity chromatography (as described in chapter 2). Purification of ACC by this technique removes any possible allosteric effects of endogenous citrate or fatty acyl-CoA present in tissue extracts, and as a consequence any differences in enzyme activity observed after purification are due solely to changes in the phosphorylation status of ACC.

Within the first 6 hours of starvation the Vmax of ACC decreased by 38% (Fig.4.14.B.) and the Ka for citrate increased dramatically by 95% (Fig.4.14.C.) and these changes were significantly different from fed control values ($P<0.01$) . There was a further decrease in Vmax and slight increase in Ka citrate in response to a further 42 hours of starvation (Fig.4.14.B and C). This supports the measurements of initial : total activity ratio which suggests that the majority of ACC inactivation occurred within the first 6 hours of starvation (Fig.4.13.A and B). After 48 hours starvation the Vmax of hepatic ACC had decreased by 53% and the Ka citrate has increased by 100% (statistically different from fed controls $P<0.005$). Both Ka and Vmax responded rapidly to refeeding and returned to fed control values within 4 hours (Fig.4.14.B and C). The speed of restoration of Ka appeared to exceed that of the Vmax and the reason for this is not clear, given that Hardie and co-workers have shown that phosphorylation of serine 79 on ACC controls both parameters (Davies, S.P. et al., 1990).

The magnitude of the decrease in Vmax for pure ACC observed after 48-hours starvation in this study (53%) strongly suggests phosphorylation by AMP-PK (which produces a Vmax decrease of 81% *in vitro*) rather than by cAMP-PK (which decreases Vmax by 13% *in vitro*; Munday, M.R. et al., 1988). The greater decrease in Vmax by AMP-PK is achieved by phosphorylation of serine-79 on the ACC polypeptide chain

compared with serine-77 phosphorylated by cAMP-PK (Munday, M.R. et al., 1988). As phosphorylation of these two sites is mutually exclusive (Munday, M.R. et al., 1988) and only serine-79 is phosphorylated when ACC is inactivated by glucagon treatment of hepatocytes (Sim, A.T.R. and Hardie, D.G., 1988; Davies, S.P. et al., 1990) this strongly indicated AMP-PK as the physiological ACC kinase.

The changes in AMP-PK activity in response to starvation and refeeding in liver of virgin rats showed a remarkable inverse correlation with the pattern of changes in pure ACC Vmax (Fig.4.14.A and B.). 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 significant and rapid decrease within 2 hours and returned to fed control levels by 4 hours (Fig.4.14.A.). These data provide further support for the physiological role of AMP-PK in regulating ACC activity *in vivo*.

The changes in ACC and AMP-PK activity also correlate well with changes in lipogenesis in virgin rat liver during 48 hour starvation and refeeding (Fig.4.15.B.). Approximately 56% inhibition of lipogenesis occurred by 6-hour starvation and eventually reach a 67% inhibition of lipogenesis by 48-hour starvation (Fig.4.15.B). Upon refeeding chow to 48-hour starved rats lipogenic inhibition rapidly diminishes and by 6 hour refeeding the lipogenesis level in liver of refed rats overtake the level of control (Fig.4.15.B.). The changes in lipogenesis, ACC and AMP-PK activity closely correlates during 48 hour starvation and the first two hours refeeding in virgin rat liver (Fig.4.15.B. and Fig.4.14.) indicating that ACC is an important control point for carbon flux into fatty acid synthesis during the period of 48-hour starvation and the first 2-hours of refeeding. These data also provide further support for the physiological role of AMP-PK in regulating lipogenesis *in vivo*.

The changes in AMP-PK activity (Fig.4.14.A) observed during starvation and refeeding 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 change in the phosphorylation and activation state of AMP-PK or a change in AMP-PK concentration.

Glucagon and insulin appear to be the main hormones involved in the regulation of lipogenesis in liver. Insulin stimulates the rate of fatty acid synthesis in isolated hepatocytes by 40% (Holland, R. and Hardie, D.G., 1985) and glucagon inhibits the rate of fatty acid synthesis by 50% in isolated hepatocytes (Holland, R. et al., 1984). There are previous reports suggesting that glucagon-stimulated phosphorylation and inactivation of ACC in isolated hepatocytes are mediated by AMP-PK phosphorylation of ACC (Sim, A.T.R. and Hardie, D.G., 1988). Although cAMP-PK does not seem to directly phosphorylate and inactivate ACC *in vivo* it has been suggested that cAMP-PK is somehow involved in the phosphorylation cascade that mediates the activation of AMP-PK by glucagon (Hardie, D.G., 1989). In the present study, however, as the activities of cAMP-PK and AMP-PK did not increase in parallel after 6-hour starvation (Fig.4.15.C. and Fig.4.14.A.) this appears unlikely. There was a small but insignificant rise in cAMP-PK activity ratio over the first 6 hours of starvation (Fig.4.15.C.), but over this time period AMP-PK activity increased 75% (Fig.4.14.A.) and purified ACC was inactivated (Fig.4.14.B,C.). Following 48 hour starvation, the activity ratio of cAMP-PK had significantly increased by 7-fold (Fig.4.15.C.). 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, H.J. et al., 1977). Upon refeeding the activity of cAMP-PK decreased rapidly and had reached fed control level within 4 hours (Fig.14.C.).

Changes in plasma insulin concentration during starvation and refeeding of virgin rats (Fig.4.15.A) showed a good correlation with changes in pure ACC Vmax (Fig.4.14.B.) and inhibition of lipogenesis (Fig.4.15.B.) indicating that insulin is a major regulator of these events.

Plasma insulin concentration during 6-hour starvation of virgin rats (Fig.4.15.A) was lowered by approximately 65%. 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 (Fig.4.15.A.), and then remained constant up until the last point assayed at 8-hours refeeding (data not shown).

Figures 4.14. and 4.15. 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.4.14.A. and Fig.4.15.A.) are almost super-imposable 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 AMP-PK (or rapid changes in AMP-PK concentration). 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.

4.7. Conclusion

AMP-PK from lactating rat mammary gland is activated, allosterically by 5'AMP, and by reversible phosphorylation. The mammary enzyme has a Ka for 5'AMP almost identical to that of liver (1.4μM for mammary gland and 1.5μM for liver) and shows a similar maximal activation by 100μM AMP (two-three fold).

As for the liver enzyme, autophosphorylation of mammary AMP-PK is without effect on its activity, and phosphorylation and activation is achieved by a distinct "kinase-kinase". Partially purified "kinase-kinase" has proven not to be cAMP-PK from mammary gland but has many properties in common with a previously described but uncharacterised ACC-kinase from lactating rat mammary gland named ACK-2. ACK-2 is known to phosphorylate the SSMS synthetic peptide substrate and we observed a

copurification of SSMS-kinase activity and kinase kinase activity through a number of steps culminating in Mono Q separation. ACK-2 phosphorylation of SSMS was palmitoyl-CoA stimulated which is a property ascribed to liver "kinase-kinase" (Carling, D. et al., 1987). It has been proposed and the AMP stimulation of AMP-PK phosphorylation by "kinase-kinase" is due to a conformational change in the AMP-PK substrate rather than an activation of "kinase-kinase" (Moore, F. et al., 1991). This is supported by our observation that ACK-2 phosphorylation of SSMS peptide was not stimulated by AMP. Hardie and coworkers have recently partially purified kinase kinase from rat liver (Weekes, J. et al., 1994) and this shows a similar elution profile on mono Q to that observed for ACK-2 from mammary gland in the present study. ACK-2 was maximally stimulated by 200nM palmitoyl-CoA with a Ka in the region of 20nM. Oleoyl- and stearoyl-CoA appeared to produce similar stimulations. Stimulation of "kinase-kinase"/ACK-2 activity by more than one form of fatty acyl-CoA could represent a general feedback inhibition by the products of fatty acid synthesis.

AMP-PK appears to have a physiological role in the control of activity of ACC during starvation and refeeding in the livers of virgin rats, and mammary glands of lactating rats. In both tissues changes in AMP-PK activity during starvation and refeeding closely correlate with reciprocal changes in the Vmax of ACC. Changes in AMP-PK activity observed during starvation and refeeding in these tissues are not due to the allosteric effect of AMP, but to changes in the concentration or phosphorylation state of AMP-PK. This may implicate "kinase-kinase" as an important regulatory step.

In lactating mammary gland administration of streptozotocin (which prevents the release of insulin into the circulation) blocks both the inactivation of AMP-PK and activation of ACC in response to refeeding starved rats (Table 4.4.). The changes in AMP-PK activity, in rat liver or lactating rat mammary gland, in response to starvation and refeeding closely and inversely correlated with changes in plasma insulin concentration. These observations suggest that insulin is likely to be an important regulator of the AMP-PK cascade *in vivo*.

CHAPTER FIVE

Catalytic subunit of cAMP-PK from mammary gland of lactating rat

5.1. Introduction

Neither glucagon nor adrenaline inhibit lipogenesis in mammary acini *in vitro* (Williamson, D.H. et al., 1983; Jones, R.G. et al., 1984a; Robson, N.A. et al., 1984). While the rat mammary gland does not possess glucagon receptors (Robson, N.A. et al., 1984) it does have a functional ß-adrenergic cAMP signalling system. The mammary gland has $ß_2$-subtype adrenergic receptors (Clegg, R.A. and Mullaney, I., 1985) functionally coupled to adenylate cyclase in the plasma membrane (Ladha, S. et al., 1985), cAMP-dependent protein kinase (Munday, M.R. and Hardie, D.G., 1984, Burchell, A. et al., 1978) and phosphodiesterase (Mullaney, I. and Clegg, R.A., 1984). Despite this, a 20-fold rise in cAMP concentration, achieved with ß-adrenergic agonists and phosphodiesterase inhibitors, in mammary acini *in vitro* did cause activation of cAMP-PK (Clegg, R.A. and Ottey, K.A., 1990), but failed to affect lipogenesis (Clegg, R.A. and Ottey, K.A., 1990; Clegg, R.A. et al., 1986).

The discrepancy between the ability of cAMP-PK from rabbit skeletal muscle or bovine heart to phosphorylate and inactivate purified mammary gland ACC *in vitro* (Hardie, D.G. and Guy, P.S., 1980; Brownsey, R.W. and Hardie, D.G., 1980; Munday, M.R. et al., 1988) and the lack of effect of increased cAMP-PK activity on the enzyme in mammary tissue *in vivo* (Clegg, R.A. and Ottey, K.A., 1990) can be partially explained by the possibility that cAMP-PK in mammary gland is a different isoenzyme form with perhaps a lower affinity for ACC (Takhar, S. and Munday, M.R., 1992). As mentioned in Chapter 1, the assumption of the invariant nature of the catalytic subunits

(C) of cAMP-PK from all sources was based largely on their molecular weight and tryptic peptide similarities (Carlson, G.M. et al., 1979; Zoller, M.J. et al., 1979).

However, there is increasing evidence for multiple isoenzyme forms of the C-subunit both between tissues and within the same tissue. At least three distinct forms of C-subunit have now been identified by chromatographic and electrophoretic techniques (Yamamura, H. et al., 1973; Peters, K.A. et al., 1977; Kübler,D. et al., 1979; Kinzel, V. et al., 1987) and three cDNA coding for separate Cα, Cβ and Cγ subunits have been identified (Uhler, M.D. et al., 1986; Showers, M.O. and Maurer, R.A., 1986; Beebe, S.J. et al., 1990). More recently, a sub-type of one of these isoenzymes (Cβ2) has been described (Weimann, S. et al., 1991). Cγ has at present only been identified in human testis. However, cDNA sequences of the mammalian C-subunit isoforms Cα and Cβ have been characterised from a number of species: mouse (Uhler, M.D. et al., 1986a; Uhler, M.D. et al., 1986b), human (Beebe, S.J. et al., 1990; Maldonado, F. and Hanks, S.K., 1988), pig (Adavani, S.R. et al., 1987), cow (Showers, M.O. and Maurer, R.A., 1986; Weimann, S. et al., 1991) and rat (Weimann, S. et al., 1991; Shuntoh, H. et al., 1992; Roth, J.S. et al., 1990). The two genes of the mouse Cα and Cβ isoforms have also been cloned (Chrivia, J.C. et al., 1988). The Cβ2-subunit which has a calculated MW of 46.1 KDa (6 KDa higher than any other mammalian C-subunits) differs from Cβ only in its N-terminal amino acid sequence (and 5'-noncoding region of its cDNA). The Cβ2 has probably been coded by the Cβ gene which seems to possess two alternatively spliced exons 1 (Weimann, S. et al., 1991). The homology between deduced amino acid sequences of different forms of mammalian C-subunit are presented in Table 5.1.

The equivalent of C-subunits of cAMP-PK have been found in a number of species including the lower eukaryotes such as *Mucor rouxii* (Paveto, C. et al., 1989), slime mold *Dictyostelium discoideum* (Mann, S.K.O. et al., 1992), aquatic fungus *Blastocladiella emersonii* (de Oliveira, J.C. et al., 1994) and yeast. Yeast *Saccharomyces cervisiae* has three isozymic forms of catalytic subunit of cAMP-PK

Table 5.1. Amino acid sequence homology between different isoforms of C-subunit of cAMP-dependent protein kinase

C-subunit isoform (species)	a.a sequence homology (%)	C-subunit isoform (species)	Reference
Cα (bovine)	93%	Cβ (bovine)	Showers,M.O. and Maurer,R.A. (1986)
Cα (mouse)	91%	Cβ (mouse)	Uhler,M.D. et al. (1986)
Cα (rat)	92%	Cβ (rat)	Shuntoh,H. et al.(1992)
Cγ (human)	83%	Cα (human)	Beebe,S.J. et al., 1990
Cγ (human)	79%	Cβ (human)	Beebe,S.J. et al., 1990
Cα (mouse)	98%	Cα (bovine)	Uhler,M.D. et al. (1986)
Cβ (rat hepatoma)	92%	Cβ (bovine)	Roth,J.S. et al. (1990)
Cβ (rat)	100%	Cβ (mouse)	Shuntoh,H., et al. (1992)
	98%	Cβ (bovine)	
	97%	Cβ (human)	

(TPK1, TPK2 and TPK3; Kuret, J. and Pflugrath, J.W., 1991). The DNA sequence for all three TPK genes has been determined (Toda, T. et al., 1987). The predicted amino acid sequences for TPK1-3 show that the carboxy terminal 320 amino acid residues have more than 75% homology to each other and more than 50% homology to the bovine C-subunit. The differences appear in composition and length of amino terminal region (Toda, T. et al., 1987). Even with only 50% amino acid homology, Cα-subunit (from mouse) can functionally replace the yeast counterpart (if expressed in yeast with all three TPK genes malfunctioning, Zoller, M.J. et al., 1991). Apart from Cβ2, mammalian C-subunit size appears to be highly conserved. The lower eukaryotes

have more variation in size of their C-subunits, for example, the yeast C-subunits, TPK1-3 all contain additional sequences at the N-terminus (Toda, T. et al., 1987), *Blastocladiella emersonii* C-subunit has a 70 amino acid extension at the N-terminus (de Oliveira, J.C. et al., 1994) and C-subunit from *Dictyostelium discoideum* has an additional 332 amino acid residues also at its N-terminus (compared to the mouse C-subunit, Veron, M. et al., 1993)

Although mammalian Cα and Cβ from the same source vary in their amino acid composition (Table 5.1.) they seem to have very similar kinetic properties and similar Km values for ATP (4μM) and Kemptide (5.6μM). However, there was no characterisation of the substrate specificities of the isoforms with respect to their physiological substrates (Olsen, S.R. and Uhler, M.D., 1989). At present there is no clear correlation between Cα and Cβ isoforms of C-subunit and the chromatographically distinguishable isoforms C_A and C_B described by Kinzel, V. et al. (1987). The latter are also different in their susceptibility to a C subunit-specific protease (Alhanaty, E. et al., 1981).

The differences in substrate specificity between catalytic subunits of cAMP-PK from mammals and yeast emerged recently (Denis, C.I. et al., 1991). Also, different specificities for peptide substrates between the isoforms CAPL-A1 and CAPL-A2 from the same organism, *Aplysia californica* (sea slug), have been described (Cheley, S. and Bayley, H., 1991). In humans the Cγ isoform of C-subunit also appears to differ from Cα and Cβ isoforms, as it is tissue specific and insensitive to inhibition by PKI (Wen, W. and Taylor, S.S., 1994).

To test the possibility that the C-subunit of cAMP-PK from the lactating rat mammary gland is a distinct tissue specific isoform of C-subunit (with different properties) from the enzyme purified from rat or bovine heart , the C-subunits from these tissues were purified.

5.2. Purification of the Catalytic subunit of cAMP-dependent protein kinase from mammary gland and heart of lactating rat

The C-subunits of cAMP-PK from mammary gland and heart of lactating rats were purified using principles of affinity elution of cAMP-PK described by Reimann, E.M. and Beham, R.A. (1983). The purification uses two chromatographic columns coupled to the FPLC. The first DEAE-Sepharose FF binds the cAMP-PK holoenzymes at a low salt concentration, and the second, Phospho-Cellulose, binds C-subunits once they are released from holoenzyme by cAMP. C-subunits were eluted from the Phospho-Cellulose column with a gradient increase in potassium phosphate. The holoenzyme binds to the DEAE-Sepharose due to its negative charge at pH 6.8 (holoenzyme pI is between pH 4.5 - 5.5). After dissociation of the regulatory subunits from the catalytic subunits caused by the presence of 10μM cAMP in the buffer, the regulatory subunits remain bound to the DEAE groups due to their high negative charge (pI of regulatory subunits is below pH 4.0), and the C-subunits are expelled from the gel due to their positive charge at pH 6.8 (pI of the C-subunit varies between pH 6.7 and 9.1; Kübler, D. et al., 1979). The C-subunits bind to the Phospho-Cellulose due to their positive charge and the presence of their ATP-binding site (see Chapter 3).

The time taken for purification of the enzyme was greatly shortened by using the two columns in series on FPLC, and in that way minimised the loss of cAMP-PK activity due to oxidation by exposure to atmospheric oxygen, and prolonged manipulation at room temperature. The use of FPLC (Fig.5.1.) increased the speed of purification and allowed the constant monitoring of eluents, resulting in increased yield and purity of preparations (Fig. 5.2. compared to Fig. 5.5.) and improved the reproducibility of the procedure to allow accurate comparison of purification profiles from separate runs.

Mammary glands and hearts were dissected, frozen, powdered and stored in liquid nitrogen as described in Chapter 2. Powdered tissue (30g) was homogenised (using a domestic blender) in 200ml of ice cold homogenisation buffer containing:

10mM KPi pH 6.8, 1mM-EDTA, 1mM-DTT,

4 μg/ml SBTI, 2mM Benzamidine,

0.2 mM TLCK, 0.2mM PMSF.

The homogenate was centrifuged at 15 000g and 4^0C for 15 min to remove crude particles. The supernatant, once filtered through glass wool, was centrifuged at 100000g and 4^0C for an additional 60 min. After the centrifugation, the filtered supernatant was loaded directly onto a DEAE-Sepharose FF column (10/20) connected to an FPLC and washed with:

55mM KPi (pH 6.8), 1mM Benzamidine,

1mM EDTA.2 μg/ml SBTI, 1mM DTT.

The loading and washing speed was 8ml/min. After achieving a stable base line, the concentration of KPi was lowered to 40mM and speed lowered to 3ml/min.

A second chromatography column, Phospho-Cellulose (10/3) equilibrated in the same buffer was connected in line with the DEAE column. The C-subunits were eluted from the DEAE column onto the Phospho-Cellulose with 50ml of:

30mM KPi (pH 6.8), 1mM EDTA, 1mM DTT,

1mM benzamidine, 2 μg/ml SBTI, 10μM cAMP.

Both columns were washed with at least an additional 4 column volumes of 30 mM KPi buffer (in which cAMP was omitted). After this step the DEAE column was disconnected and the Phospho-Cellulose column now containing bound C-subunit of cAMP-PK was washed with 60mM KPi buffer to wash away non-specifically bound proteins. After achieving a stable base line, KPi concentration was dropped to 30mM and gradient elution by increase in KPi concentration was carried out at 2ml/min (Fig.5.1.). Fractions of 500μl were collected and assayed for cAMP-PK activity as described in section 2.5. The peak of activity was pooled, concentrated using a centricon-30 concentrator (with 30 KDa MW cut off membrane), and stored in:

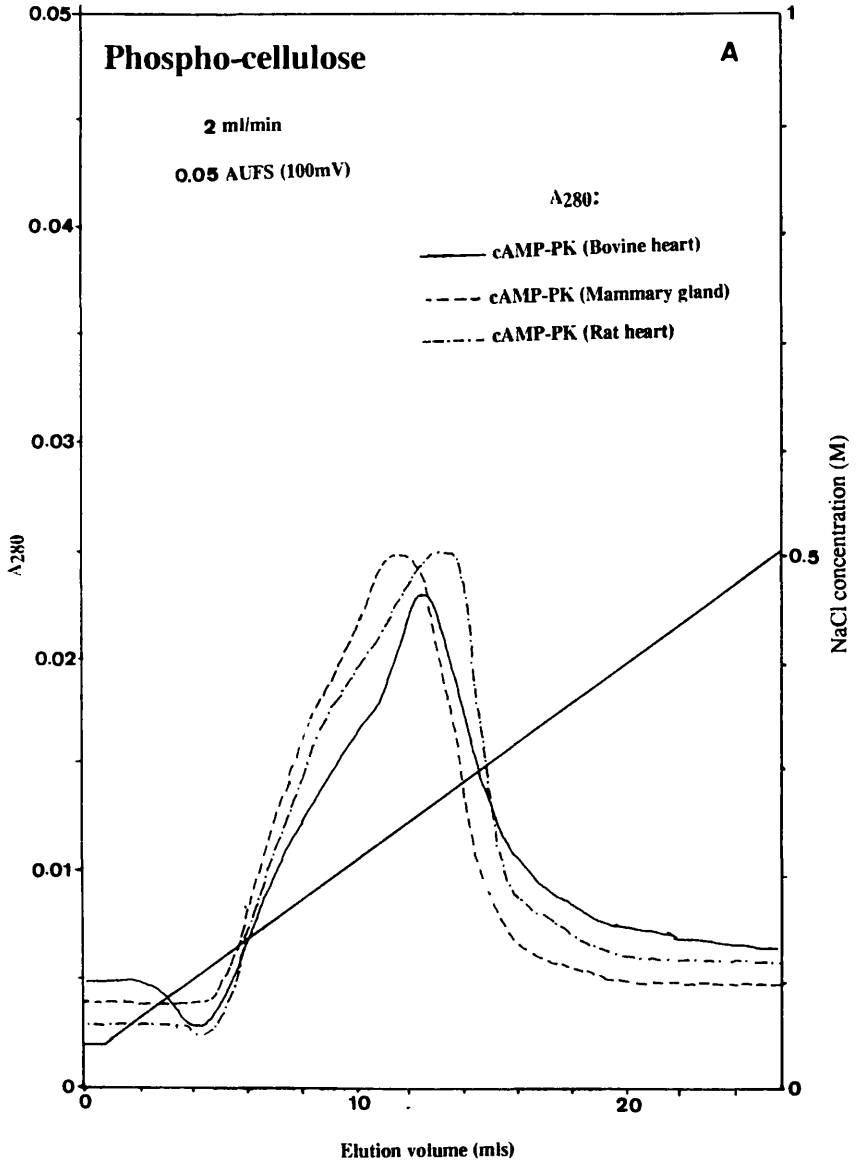
500mM KPi buffer pH 6.8, 50% (w/v) glycerol,

5mM DTT, 1mM EDTA, 1mM Benzamidine, 4μg/ml SBTI.

Fig. 5.1. Phospho-Cellulose elution profiles of C-subunits of cAMP-PK from lactating rat mammary gland, rat heart and bovine heart

C-subunits from lactating rat mammary gland and heart and bovine heart were purified as described in the text. The elution from Phospho-Cellulose is shown. Panel A represents A_{280} traces as measurements of protein elution. All three traces are on the same scale (0.05 AUFS; 100mV) and they are directly comparable.

Panel B represents the cAMP-PK activity of 5μl of each fraction assayed by incorporation of ^{32}P into 200μM SSMS for 5 minutes at 37^{O}C as described in Section 2.5. To achieve visualisation of peaks they are on a different scale (real value of each scale is represented by appropriate bars on left side of the graph).


Phospho-cellulose
A
2 ml/min
0.05 AUFS (100mV)
A280:
cAMP-PK (Bovine heart)
cAMP-PK (Mammary gland)
cAMP-PK (Rat heart)
A280
NaCl concentration (M)
Elution volume (mls)
0.05
0.04
0.03
0.02
0.01
0
1
0.5
0
10
20

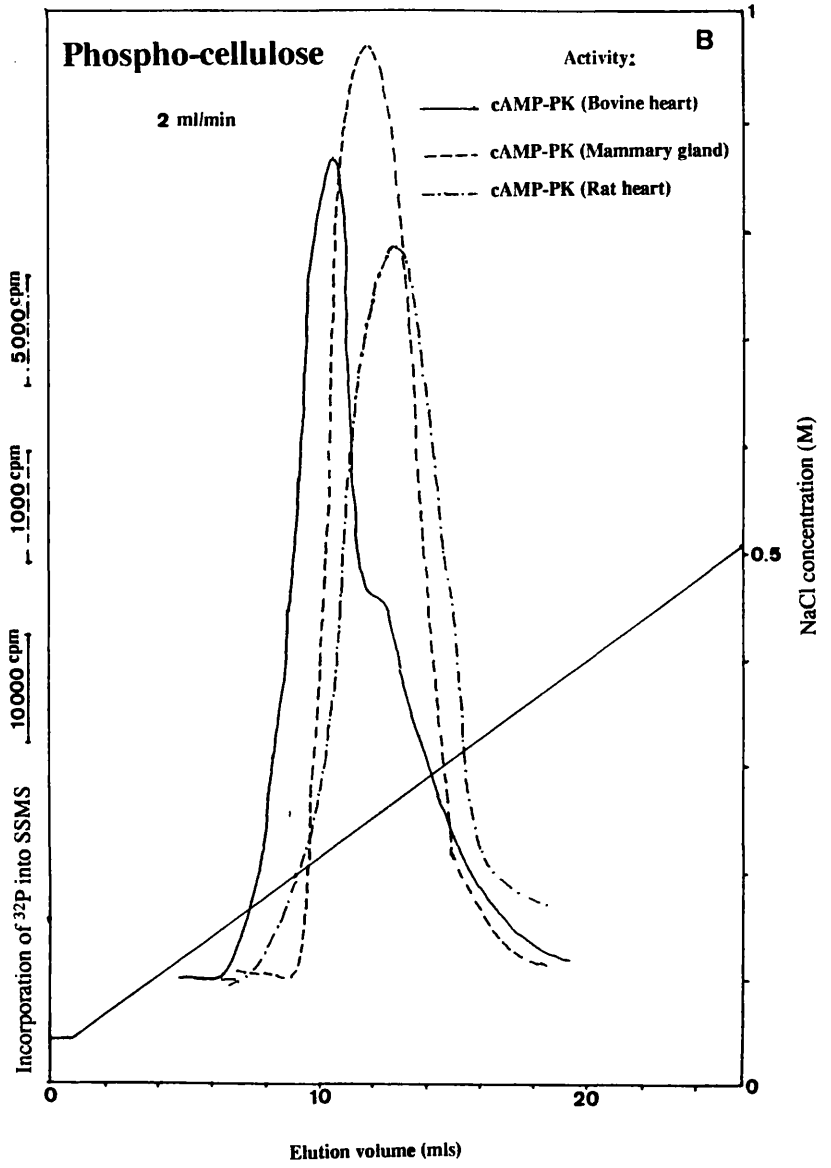
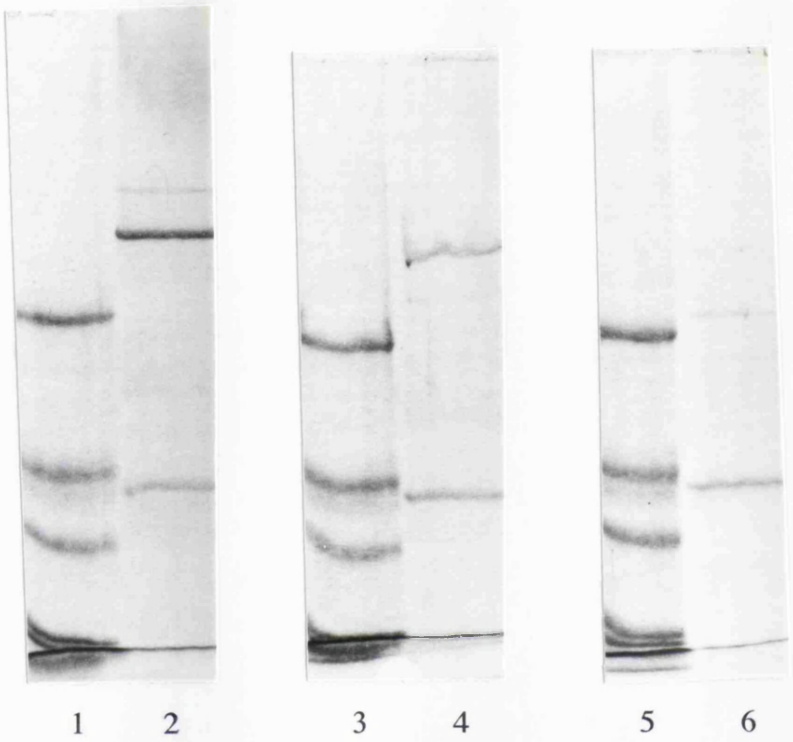

Phospho-cellulose
B
Activity:
2 ml/min
cAMP-PK (Bovine heart)
cAMP-PK (Mammary gland)
cAMP-PK (Rat heart)
5000 cpm
1000 cpm
10000 cpm
Incorporation of 32P into SSMS
NaCl concentration (M)
Elution volume (mls)
1
0.5
0
10
20

Fig.5.2. SDS-PAGE of purified C-subunits of cAMP-PK from lactating rat mammary gland, rat heart and bovine heart



C-subunits of cAMP-PK from lactating rat mammary gland and heart were purified as described in the text using DEAE-Sepharose FF and Phospho-Cellulose columns coupled in line to the FPLC. The peak fractions of activity shown in Figure 5.1 were run on separate SDS-PAGE (7.5% T acrylamide resolving and 4.5% T stacking) gels alongside low MW SDS marker proteins (66, 45, 36, 29 and 24 KDa). The gels were fixed in 50% (v/v) methanol, 10% (v/v) acetic acid, then stained with Coomassie Blue R-250, de-stained, dried and photographed, as described in Chapter 2. Lanes 1, 3 and 5 contain the low MW SDS markers. Lane 2 contains mammary gland, lane 4 rat heart and lane 6 bovine heart C-subunit of cAMP-PK.

Despite the standardisation and reproducibility of the purification procedure via the use of the same columns on FPLC it was observed that the elution profiles of C-subunits from lactating rat mammary gland, rat heart and bovine heart from phosphocellulose were not coincidental (Fig 5.1.). Peak activities eluted at 220mM NaCl for bovine heart, 245mM NaCl for lactating mammary gland, and 265mM NaCl for rat heart C-subunits. Binding of C-subunits to phosphocellulose is achieved via cationic interaction and pseudosubstrate affinity binding to the phosphate group on the column. Thus the data suggest that there may be differences with respect to overall charge, and substrate affinity of the active sites of the C-subunits from each of these tissues. Differences in the elution profiles of C-subunits from cationic columns (CM-Cellulose, Mono S HR5/5) have lead Kinzel, V. et al. (1987) to propose that there may be isozymic forms of C-subunit in bovine heart and rabbit skeletal muscle with differences in amino acid composition or covalent modification affecting their binding to the columns. Figure 5.1 also revealed that the C-subunits from these tissues differ in their specific activities when SSMS is used as a substrate. Immediately following purification the fractions containing peak activity were subjected to SDS PAGE on a 7.5% T gel (Fig. 5.2.) and it can be seen that each fraction contains a prominent 41 KDa band regardless of tissue type. The peak fractions from each tissue also contained in addition some degree of higher molecular weight contamination. For mammary tissue this was observed as a prominent bands at 100 KDa and 116 KDa, for rat heart band at 105 KDa, and for bovine heart a minor contaminant at 70 KDa. Lanes 2, 4, 6 of Figure 5.2. were scanned densitometrically to determine the proportion of 41 KDa protein, and the activity shown in Figure 5.1. was expressed as pmol ^{32}P incorporated into SSMS/ min/ mg 41 KDa band. The specific activities thus obtained were 380 for bovine heart, 371 for rat heart and 108 μmol/ min/ mg 41KDa protein for lactating rat mammary gland. While the specific activities of the C-subunits from rat heart and bovine heart are comparable, it can be seen that both are at least three times higher than that of the C-subunit from lactating rat mammary gland.

5.3. Properties of purified C-subunit of cAMP-PK from lactating rat mammary gland

5.3.1. Substrate specificity

The C-subunit of cAMP-PK is serine/threonine kinase with a broad spectrum of substrates and generally recognises the primary amino acid consensus sequence:

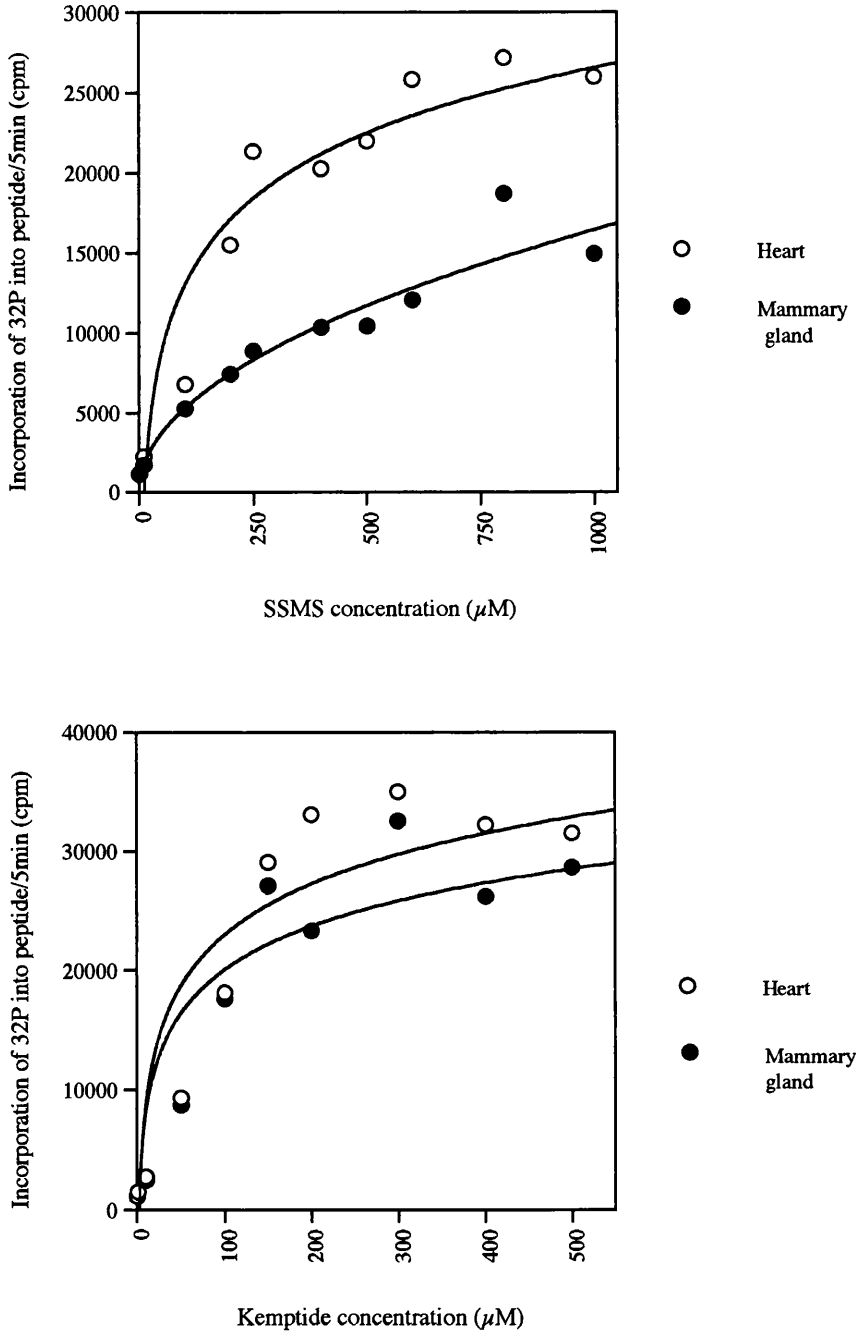
Arg-Arg-X-Ser[Thr]-Y

(where X is any small residue and Y is a large hydrophobic group, Knighton, D.R. et al., 1991; Kemp,B.E. et al., 1977). Although cAMP-PK prefers serine as a substrate for phosphorylation, its also incorporates phosphate into threonine and homoserine residues (Kemp,B.E. et al., 1977; Prorok, M. et al., 1989).

The specific activities calculated from the purification of the subunits (section 5.2.) were calculated from the relative rates of phosphorylation of the SSMS synthetic peptide based on the primary sequence of ACC. We were interested to discover whether the difference in specific activity was a general phenomenon or whether it reflected altered substrate specificities of the C-subunits. The initial rate of phosphorylation of SSMS and Kemptide was measured at a range of peptide concentrations for C-subunits from lactating rat mammary gland and rat heart (Fig.5.3.). The v against [S] plots clearly show that while the C-subunits from both tissues showed similar affinity for Kemptide, the C-subunit from mammary tissue had a lower affinity for the SSMS peptide. The data were subjected to Wilkinson analysis and Table 5.2. shows the kinetic parameters. While C-subunits have similar Vmax and Km for Kemptide and similar Vmax for SSMS regardless of the tissue source, it is clear that the C-subunit from mammary tissue has a considerably higher Km for SSMS than that of C-subunit from rat heart.

To further test substrate specificity differences between the C-subunits from mammary gland and rat heart, the initial rates of phosphorylation of a range of substrates were measured.

Fig.5.3. Activity of C-subunits of cAMP-PK from lactating rat mammary gland and heart at a range of Kemptide and SSMS peptide concentrations



C-subunits of cAMP-PK from lactating rat mammary gland and heart, were purified as described in the text and activity was assayed by the incorporation of ^{32}P from [γ-^{32}P] ATP into Kemptide or SSMS peptide at the concentrations shown as described in Chapter 2.

Table 5.2. Kinetic parameters of C-subunits of cAMP-PK from lactating rat mammary gland and heart

	rat heart	lactating rat mammary gland
Vmax Kemptide	43200 ± 5430	36700 ± 4700
Km Kemptide	107 ± 41.2	99.4 ± 40.5
Vmax SSMS	34100 ± 3440	23400 ± 4920
Km SSMS	239 ± 70.5	452 ± 209

The data shown in Fig.5.3. was subjected to Wilkinson analysis and results displayed above. Km is expressed in μM of peptide substrate and Vmax as incorporation of ^{32}P into peptide (cpm) / 5 minutes.

Initial rates of phosphorylation by purified C-subunit of cAMP-PK of a range of substrates at concentrations previously described as saturating for cAMP-PK (Munday, M.R. and Hardie, D.G., 1984), were measured for each C-subunit. Table 5.3. 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 C-subunits and comparable to those previously quoted for rabbit skeletal muscle cAMP-PK (Munday, M.R. and Hardie, D.G., 1984). Differences were observed for ATP-citrate lyase which was phosphorylated at a 7-fold faster rate by the mammary C subunit, and for ACC which was at least a 17-fold poorer substrate for the mammary enzyme, and the synthetic SSMS peptide which was phosphorylated by the mammary C-subunit at a rate three times slower than that of the cardiac C-subunit (Table 5.3.).

Table 5.3.Substrate specificity of purified C-subunits of cAMP-PK from rat heart and lactating mammary gland.

Substrate	Substrate concentration (mg/ml)	cAMP-PK C-subunit activity (relative to histone = 100%)	
		Mammary gland	Heart
Histone	0.80	100	100
ACC	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	1050	1100
SSMS	0.13	242	770

C-subunit of cAMP-PK was purified from rat heart and lactating mammary gland, and assayed, as described in Chapter 2. Results show initial velocities measured at the indicated substrate concentrations and are expressed relative to the rate of phosphorylation of histone (type II-AS).

The data in Figure 5.3. and Tables 5.2. and 5.3. explain the previous anomaly that while heart cAMP-PK phosphorylates ACC *in vitro*, there was no observed effect on ACC by raising cAMP concentration in mammary tissue. It seems fairly clear that ACC (and synthetic peptide containing the sequence around serine-77 phosphorylated by the cardiac enzyme) is a poor substrate for the C-subunit of cAMP-PK from mammary tissue. However, while the peptide SSMS is phosphorylated at 70% and 24% of the rate of Kemptide by cardiac and mammary C-subunit, respectively, ACC is

phosphorylated at only 1.5% and 0.09% of the rate of Kemptide phosphorylation by cardiac and mammary C-subunit, respectively (Table 5.3.). The shortfall in the phosphorylation rate by mammary C-subunit compared to rat heart is much greater for the sites on native ACC than it is on the synthetic peptide with the same sequence. 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 cardiac cAMP-PK lies in their ability to recognise both primary sequence and higher orders of structure.

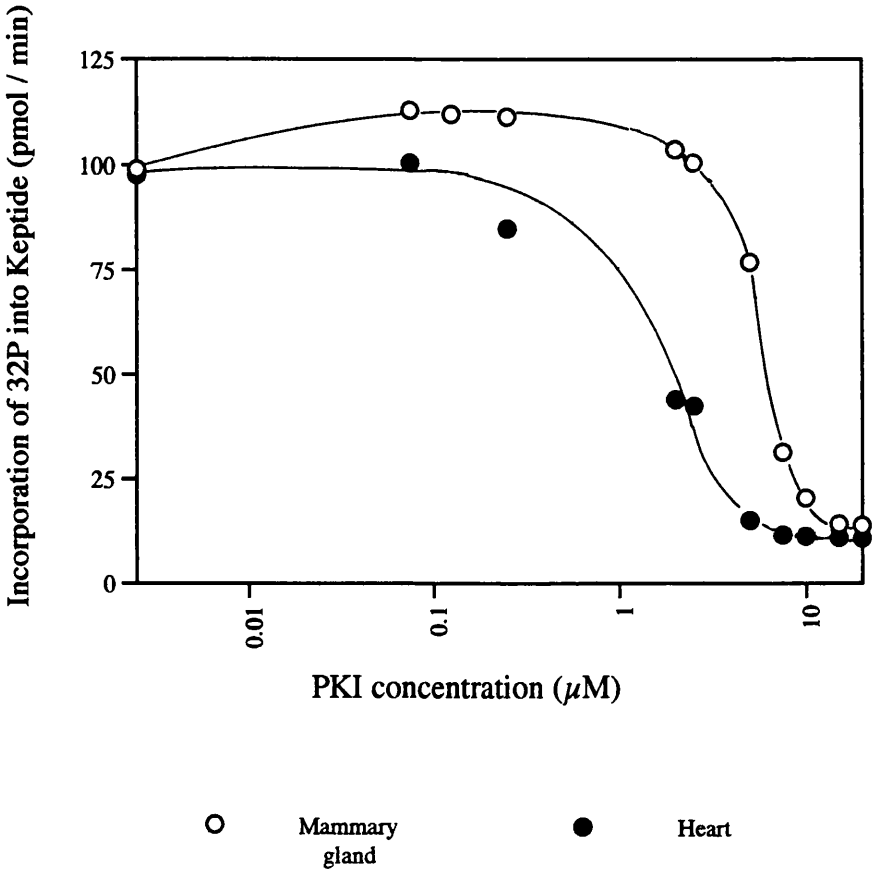
5.3.2. The C-subunit inactivation by cAMP-PK specific peptide inhibitor (PKI)

Equivalent amounts of C-subunit purified from lactating rat mammary gland and heart, as judged by their equal rates of Kemptide phosphorylation, were assayed using Kemptide as a substrate at a range of concentrations of the specific pseudosubstrate inhibitor of cAMP-PK (PKI). This peptide contains the N-terminal amino acid residues 5-24 ("the active component") of the endogenous pseudosubstrate inhibitor of cAMP-PK known as Walsh Inhibitor and has the sequence: Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp (Cheng, H.-C. et al., 1985). PKI appears to inhibit C-subunit with potency one order lower than the native Walsh inhibitor (Van Patten, S.M. et al., 1987).

The ID_{50} (concentration at which 50% inhibition is achieved) for mammary gland isozyme appears five fold higher than heart isozyme from lactating rat (Fig.5.4.). This is broadly in agreement with data published by Takhar, S. (1992) who found that the C-subunit from lactating rat mammary gland has nine fold higher ID_{50} than heart isozyme. The ID_{50} for C-subunits from lactating rat mammary gland and heart were 6.4μM and 1.3μM respectively (Fig.5.4).

This five to nine fold difference in ID_{50} between C-subunits from lactating rat mammary gland and heart may suggest that they represent different isozymes.

Fig.5.4. PKI inhibition of C-subunit from lactating rat mammary gland and heart



C-subunits from lactating rat mammary gland and heart were assayed using 150μM Kemptide as a substrate at a range of concentrations of PKI. Amount of C-subunits were adjusted to give a similar activity when assayed without PKI.

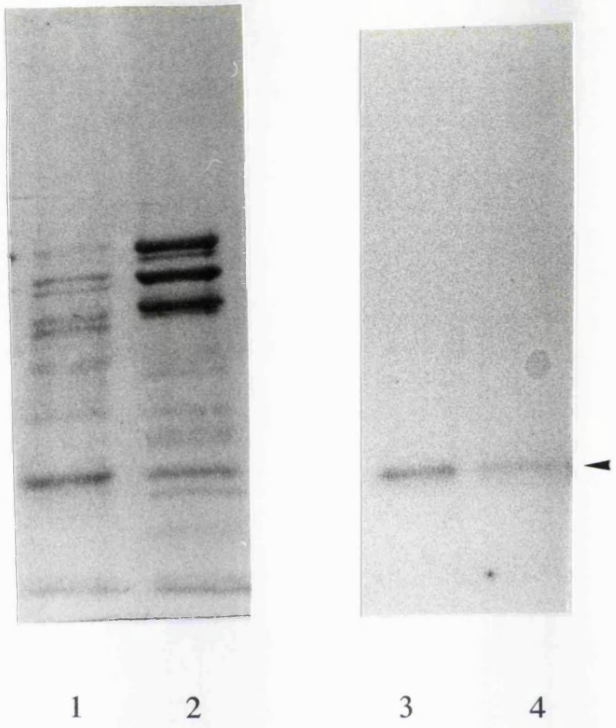
Wen, W. and Taylor, S. (1994) have shown that Arg-133 and Arg-134 of the C-subunit are important for the formation of the PKI/C-subunit complex. The Cγ-subunit which is insensitive to PKI inhibition has Arg-133 replaced with Gln (Wen, W. and Taylor, S., 1994; Beebe, S.J. et al., 1990). It is possible that the rat mammary gland C-subunit has a conservative replacement in this region eg. Arg replaced with His/Lys which would explain its decreased sensitivity to PKI inhibition.

An alternative explanation could be that the higher ID_{50} of mammary C-subunit reflects a higher proportion of inactive C-subunit in the mammary preparation. Walsh and coworkers (McPherson, J.M. et al., 1979) have demonstrated that even though PKI is a tight-binding competitive inhibitor of C-subunit, a greater concentration of PKI is required to effect an equivalent decrease in protein kinase activity for a partially denatured enzyme. This infers that PKI may bind equally well to inactive denatured C-subunit. Although the studies shown in Fig.5.4. were carried out immediately following purification, the greater instability of mammary C-subunit compared to heart may have been a contributing factor to its apparent insensitivity to PKI.

5.3.3. MW and structure

The evidence presented so far that the C-subunit of cAMP-PK from lactating rat mammary gland has a different elution profile from Phospho-Cellulose, a different substrate specificity and possibly an altered sensitivity to PKI to that of its cardiac counterparts could be explained if the C-subunit was a different isozymic form. All the known mammalian C-subunits of cAMP-PK are monomers, with the same native MW of 41KDa (except Cβ2 which has MW 46.1 KDa) and do not require any prosthetic group for their activity. This made the renaturation of C-subunit of cAMP-dependent protein kinase from mammary gland and heart of lactating rat, after the separation by SDS-Kemptide-PAGE possible (as described in section 2.18.). The partially purified

Fig.5.5. Renaturation of C-subunit of cAMP-PK from mammary gland and heart of lactating rat in Kemptide-SDS-PAGE



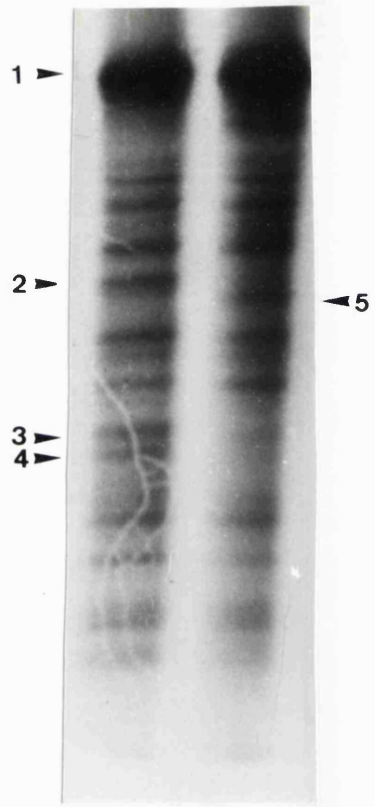
Partially purified C-subunit of cAMP-dependent protein kinase (purified using rapid purification method described by Takhar, S., 1992) from mammary gland (lane 2) and heart (lane 1) of lactating rats was subjected to SDS-PAGE on a 7.5% T mini-gel containing Kemptide (1 mg/ml). After the run the gel was subjected to denaturation in 8M urea and renatured as described in section 2.18.2. The phosphorylation of immobilised Kemptide was achieved by incubation of gel for 30 minutes in 50mM Hepes pH 7.0, 150mM NaCl, 0.1% (v/v) Brij35, 10mM $MnCl_2$ and 0.2mCi [$\gamma^{32}P$] ATP (3000-6000Ci/nmol). The phosphorylation was stopped by immersing the gel into 75mM phosphoric acid. After washing of non bound ATP for 1 hour with 4 changes of phosphoric acid , the gel was stained with Coomassie Blue R-250 (Kemptide does not stain with Coomassie stain), dried and autoradiographed as described in Chapter 2. Lanes 3 and 4 show the autoradiograph of lanes 1 and 2 respectively. The position of 41 KDa band representing the C-subunit of cAMP-PK is marked with an arrow.

C-subunits from rat heart and lactatng rat mammary gland renatured after SDS-Kemptide-PAGE (Fig.5.5.) and C-subunit from bovine heart renatured after SDS-SSMS-PAGE (Fig.3.11.) showed up as single bands of activity migrating at 41KDa for all three preparations (Fig.5.5. and Fig.3.11.). It is reasonable to assume that the C-subunits (from rat heart and mammary gland) are monomers, with the same native MW of 41KDa as the bovine counterpart.

C-subunits of cAMP-dependent protein kinase purified using the method described in this chapter were obtained as nearly pure preparations from all three sources (bovine heart, rat heart and mammary gland; Fig.5.2.). The purified C subunits of cAMP-PK from rat mammary gland and heart were indistinguishable from C-subunit from bovine heart in terms of apparent molecular weight as estimated by SDS-PAGE on 7.5% T polyacrylamide gels (MW = 41,000 Da, Fig.5.2.).

The similarity of apparent MW judged by SDS-PAGE for C-subunits from mammary gland and heart does not rule out the possibility that there are structural differences between the subunits either in terms of amino acid sequence and composition or post-translational modifications that would not be detected within the limits of accuracy of MW determination by SDS-PAGE. As a first step in addressing this question, C-subunits purified from lactating rat mammary gland and rat heart (Fig.5.2.) were autophosphorylated with [γ-^{32}P]ATP as described in section 2.11.3. The 41 KDa bands of autophosphorylated C-subunit from rat heart and lactating rat mammary gland were excised from the gel and subjected to Cleveland mapping using V8 protease as described in section 2.10. The autoradiograph of the Cleveland map shows the pattern of V8-derived C-subunit peptides containing autophosphorylated residues. Both lanes show a major ^{32}P-containing band of undigested C-subunit (Fig.5.6. arrow 1) and the approximate equal intensity of this band in either lane confirms the equal loading of C-subunit. Many of the autophosphorylated peptides co-migrate in both lanes with equal intensity of ^{32}P content, however, the maps are not identical. There are some peptides present in the map of mammary gland C-subunit (lane 1) that are not present, or much

Fig.5.6. Cleveland V8 map of autophosphorylated C-subunits from rat heart and lactating mammary gland



The C-subunits from mammary gland and heart of lactating rat were autophosphorylated as described in section 2.11.3. and run on 7.5% T mini SDS-PAGE. After temporary staining by copper (section 2.13.4.), the 41KDa bands of autophosphorylated C-subunits from rat heart and lactating mammary gland were excised and subjected to Cleveland mapping on 15% SDS-PAGE as described in section 2.10. The loading of protein into each lane was 4μg . The limited proteolysis was carried out for 15 minutes with V8-protease added in ratio 1/50. At the end of the run, the gel was fixed and autoradiographed as described in section 2.12. and the autoradiograph is shown. Lane 1 shows C-subunit from lactating rat mammary gland and lane 2 shows C-subunit from rat heart.

less pronounced, in the heart map (Fig.5.6. arrows 2, 3 and 4); and one peptide present in the heart C-subunit map appears to be absent in the mammary gland map (arrow 5).

The non-coincidence of the Cleveland V8 autophosphorylated peptide maps of C-subunit from rat heart and lactating mammary gland strongly suggests that these are different species either in terms of their amino acid sequence or in terms of their autophosphorylation state. Differences in amino acid sequence between the C-subunits could affect the sites cleaved by the V8 protease and hence account for the different pattern of peptides between the two. Alternatively, differences in the phosphate content of the autophosphorylated sites would also show up on the Cleveland map. Four autophosphorylation sites have been identified on the recombinant Cα-subunit expressed in *E.coli* and they are Ser-10, Ser-139, Thr-197 and Ser-338 (Yonemoto, W. et al., 1993). Two of them, Thr-197 and Ser-338 are stable phosphorylation sites observed in the mammalian enzyme *in vivo* (Shoji, S. et al., 1979; Shoji, S. et al., 1983). Thr-197 is very resistant to dephosphorylation *in vitro*, but Ser-338 is slowly dephosphorylated and can be autophosporylated again (Shoji, S. et al., 1979; Toner-Webb, J. et al., 1992). Autophosphorylation at Ser-10 is associated with only one of two isoelectric isoforms, C_A, as no phosphorylation at Ser-10 was ever observed for C_B (Toner-Webb, J. et al., 1992). It is possible that certain specific sites (eg. Ser-10) are unavailable or already filled with phosphate in the mammary C-subunit such that they could not be autophosphorylated and visualised in this experiment.

The phosphorylation of Thr-197 in mammalian Cα-subunit appears to be important for activity of C-subunit (Steinberg, R.A. et al., 1993). The region around this Thr (**T**) residue T(W/Y)**T**LCGTP is highly conserved between species as diversed as human, yeast, *Drosophila* and *Aplysia* (when sequences were aligned). The equivalent threonine in yeast (Thr-241) also appears to be phosphorylated (Levin, L.R. and Zoller, M.J., 1990). This Thr-residue is also important for the tight interaction beween subunits in the holoenzyme in the absence of cAMP (Levin, L.R. and Zoller, M.J., 1990). Although the Ser-338 position is far from the active site (Taylor, S.S. et al.,

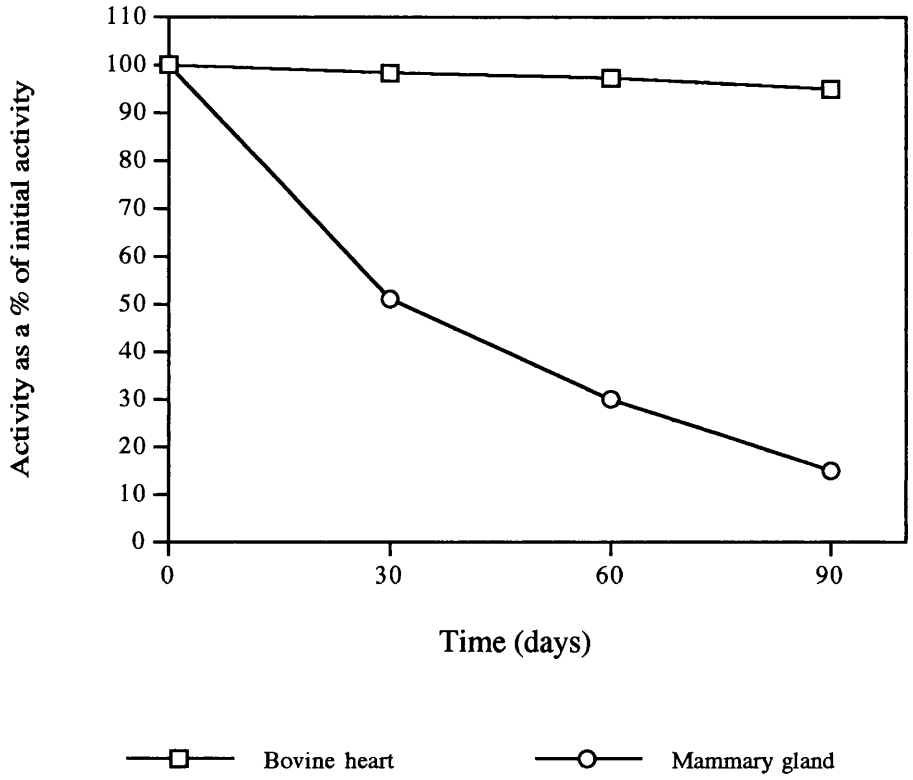
1993), one cannot rule out the importance of phosphorylation of this residue. At present, the importance of Ser-10 phosphorylation is not clear, although phosphorylation of Ser-10 does not have a major effect on C-subunit activity (Toner-Webb, J. et al., 1992).

The mammalian C-subunit is myristylated on its N-terminus (Carr, S.A. et al., 1982). Although the altered C-subunits without myristic acid have similar activity to normal enzyme (Clegg, C.H. et al., 1989) the myristylation seems to lead to a more structurally stable and temperature degradation resistant enzymes (Yonemoto, W. et al., 1993). The near proximity of the Ser-10 residue to the N-terminus could impair myristylation. However, differences in N-terminal myristylisation can be caused by differences in amino acid sequence and can lead to differences in availbility of Ser-10 site for phosphorylation. A difference in N-terminus structure can also be caused by deamidation of Asn to Asp as was shown for bovine heart C-subunit for Asn -2 (Hotz, A. et al., 1989).

5.3.4. Stability of purified C-subunit of cAMP-PK from mammary gland

When stored at -20^{0}C in: 500mM KPi buffer pH 6.8, 50% (w/v) glycerol, 5mM DTT, 1mM EDTA, 1mM Benzamidine, 4μg/ml SBTI the mammary gland enzyme appeared less stable compared to its bovine cardiac counterpart which was stable for more than a year (Fig.5.7.). During the first month of storage mammary gland C-subunit lost nearly 50% of its initial activity compared to bovine heart C-subunit which retained more than 97% of its initial activity. One possibility is that the C-subunit purified from mammary gland is de-myristylated. As myristylation seems to lead to more structurally stable and temperature degradation resistant enzymes (Yonemoto, W. et al., 1993) this would explain greater loss of activity of stored mammary gland C-subunit (compared to bovine heart C-subunit, Fig.5.7.).

Fig.5.7. Stability of C-subunits of cAMP-PK purified from lactating rat mammary gland and bovine heart stored at -20^{0}C



C-subunits of cAMP-PK purified from lactating rat mammary gland and bovine heart as described in this Chapter were dialysed into a storage buffer containing 50% (w/v) glycerol, 500mM KPi buffer pH 6.8, 5mM DTT, 1mM EDTA, 1mM Benzamidine, 4μg/ml SBTI and stored at -20^{0}C. cAMP-PK activity was measured at regular intervals by incorporation of radioactive phosphate into Kemptide peptide (as described in section 2.5.). Results were expressed as a percentage of initial activity measured directly after the purification process.

5.4. The activity of C-subunit of cAMP-PK in lactating rat mammary gland *in vivo*

Table 5.4. confirms previous observations that cAMP-PK is present in mammary tissue in appreciable amounts (Burchell, A. et al., 1978; Clegg, R.A. and Ottey, K.A. 1990; Munday, M.R. and Hardie, D.G., 1984). Using Kemptide as a substrate, both the total activity (in the presence of cAMP) and the basal activity of cAMP-PK were only about 3-fold higher in rat heart than in lactating rat mammary gland when expressed per gram wet weight tissue (Table 5.3.). In confirmation of the work of Clegg, R.A. and Ottey, K.A. (1990) and Gardner, R.A. et al. (1993), mammary cAMP-PK was activated approximately 10-fold by the addition of cAMP so that the activity ratios of around 0.1 were similar to those observed in rat heart (Table 5.4.) and

Table 5.4. cAMP-PK activity in tissue extracts of lactating rat mammary gland and rat heart

	cAMP-PK Activity (Units/g wet wt tissue)		cAMP-PK Activity Ratio
	-cAMP	+cAMP	
Fed Mammary gland	0.25 ± 0.05	1.97 ± 1.05	0.12 ± 0.04
24h-Starved Mammary gland	0.29 ± 0.06	2.53 ± 0.30	0.13 ± 0.05
Heart	0.61 ± 0.03	5.98 ± 0.43	0.10 ± 0.03

The activity of cAMP-PK was measured in tissue extracts of lactating rat mammary gland and rat heart in the absence and presence of 10μM cAMP as described in Section 2.5. The ratio of activity in the absence to the presence of cAMP is shown. Each value represents mean ± S.E.M for at least four separate experiments.

in other tissues (Corbin, J.D., 1983). The quantity and responsiveness of mammary gland cAMP-PK holoenzyme to cAMP observed here, confirms that uncoupling of the second messenger and cAMP-PK activation cannot be the reason for the lack of response of ACC to raised intracellular cAMP concentrations in mammary acini (Clegg, R.A. et al., 1986).

In response to 24 h starvation ACC is phosphorylated and inactivated in the lactating rat mammary gland, but the same time course of starvation had no effect on the activity ratio of cAMP-PK (Table 5.4.). This confirms that cAMP-PK is unlikely to be the physiologically important ACC kinase in mammary gland.

One of the possible reasons for non responsiveness of mammary gland tissue to β-adrenergic stimuli is that it has an extremely active form of cyclic nucleotide phosphodiesterase which has a total activity three orders of magnitude greater than that of adenylate cyclase (Mullaney, I. and Clegg, R.A., 1984) and can nullify the change in cAMP concentration produced by adenylate cyclase. The lack of cAMP-PK activation in starvation also makes it a very unlikely upstream activator of the AMP-PK whose activity we have observed to increase in starvation and which does appear to be the physiological ACC kinase (see Chapter 4).

5.5. Conclusion

Although significant concentrations of cAMP-PK and a responsive cAMP signalling system exist in the lactating rat mammary gland, the activity of the kinase does not change in response to food withdrawal *in vivo* and it therefore cannot regulate ACC or AMP-PK. Its responsiveness to cAMP *in vitro* means that such observations might be explained by an overactive phosphodiesterase activity that does not allow cAMP to reach sufficient concentrations to activate cAMP-PK in mammary tissue. Alternately, the lactating rat mammary gland may contain an abundance of a specific inactivating or

targetting protein that destroys or sequesters the C-subunit upon its release from the holoenzyme. A specific C-subunit protease has been identified in other epithelial cells (Alhanaty, E. and Shaltiel, S., 1979; Alhanaty, E. et al., 1981; Alhanaty, E. et al., 1984; Hemmings, B.A., 1985) and an inactivating activity that may be a similar species has been described in mammary tissue (Takhar, S., 1992). A potential "receptor" protein for C-subunit has been identified in rat liver (Clegg, R.A. and Connor, K., 1992).

The ability to increase cAMP-PK activity in acini with β-agonists and phosphodiesterase inhibitors but have no effect on ACC, despite the ability of heart and muscle cAMP-PK C-subunits to phosphorylate and inactivate ACC *in vitro*, leads to speculation that the lactating rat mammary gland C subunit is a different isoenzyme.

No kinetic differences have been observed between the four mammalian C-subunit isoenzymes so far identified. Although, Cγ has no sensitivity towards the specific PKI inhibitor because of the replacement of the critical Arg-133 with Gln in its structure (Wen, W. and Taylor, S., 1994). However, the C-subunit from mammary tissue has an altered substrate specificity and PKI sensitivity compared to C-subunit from rat heart. There are clear indicators of possible differences in their pI, and differences in their primary amino acid composition or post-translational modification.

Post translational modifications could be phosphorylations of Ser/Thr-residues which could lead to altered activity, or could be fatty acylations - myristoylation. High fatty acid content of mammary gland may facilitate different fatty acylations. If it is post translational modification, then it clearly has to be in a region that affects active site. The best candidates appears to be Thr-197 and Ser-10, because phosphorylation of the threonine residue is necessary for activity of C-subunit and phosphorylation of the serine residue could impair myristylation and this may lead to a less stable enzyme. A high level of Ser-10 phosphorylation might explain the apparent instability of C-subunit from lactating rat mammary gland compared to C-subunit from bovine heart.

C-subunit MW was estimated in this study by SDS-PAGE. Analysis using a mass-spectrometer would give more accurate measurements. Although recent quantification of lactating rat mammary gland C-subunit mRNA by Northern blotting using probes specific for Cα and Cβ showed that Cα mRNA was several fold more abundant than mRNA for Cβ (Gardner, R.A. et al., 1993) one cannot rule out the possibility that mammary C-subunit has differences in sequence outside the probing regions. For a full understanding of the differences in activity of C-subunit from mammary gland and heart, and a proof of different isozymes, a full primary sequence and screening of mammary gland cDNA libraries would be needed.

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