INTERACTION OF PYRIDOXAL PHOSPHATE WITH ACETYL-CoA CARBOXYLASE

By

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ABSTRACT

Acetyl-CoA carboxylase (ACC) catalyzes the conversion of acetyl-CoA to malonyl-CoA, the first committed step in *de novo* fatty acid synthesis. Two mammalian ACC isoforms exist, with many distinctive properties and differential tissue distribution. ACC isoforms exist as inactive dimers that can be allosterically activated by citrate; activation being associated with polymerization, in the case of ACC-1. Inhibition of other citrate-utilizing enzymes by pyridoxal phosphate (PLP) suggested that PLP might also inhibit ACC. This thesis describes work done to assess the interaction of PLP with ACC.

PLP was found to be a potent inhibitor of ACC, with an apparent IC_{50} of 225 μM against ACC purified from liver. The effects of PLP were seen in affinity-purified ACC, suggesting its effects are direct. PLP is effective against both ACC isoforms, but ACC-1 shows greater sensitivity to PLP than ACC-2. PLP inhibition of ACC is rapid, occurring within 1 minute of addition. Pre-treatment of ACC with citrate provided substantial protection against PLP inhibition at low inhibitor concentrations; the protection being more evident with ACC-1 than ACC-2. This suggests PLP and citrate bind to the same site on ACC. Treatment with PLP and sodium borohydride leads to irreversible ACC inhibition, confirming that PLP forms a Schiff base with ACC that can be reduced with sodium borohydride. In the absence of borohydride reduction PLP interacts reversibly with ACC. Selective labelling of ACC using [^{3}H]-borohydride indicated that PLP reacts directly with the ACC subunits.

Numerous analogs of PLP were tested for inhibitory action against ACC. From these studies it was concluded that the aldehyde and negative charges on PLP are especially important for its inhibitory action on ACC. The pyridine ring is also important, but to a lesser degree than the aldehyde or phosphate.

The ability to use PLP to determine the sequence of the citrate binding site and to provide the basis for inhibitor design is discussed.

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LIST OF ABBREVIATIONS

$A_{###}$	Absorbance at ### nanometers
ACA	1'acetoxychavicol acetate
ACC	Acetyl-CoA carboxylase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AOA	Aminooxyacetate
BH_4	Borohydride (typically the sodium salt)
â-ME	â-mercaptoethanol or 2-mercaptoethanol
BR	Bradford reagent
cAMP	Cyclic-AMP
CaMK	Calcium/Calmodulin dependent protein kinase
CK	Casein kinase
CPT-1	Carnitine Palmitoyl Transferase I
DNA	Deoxyribonucleic acid
FAS	Fatty acid synthetase
FFA	Free fatty acid
GLUT4	Glucose transporter 4
HA	Hydroxylamine
HCO_3^-	Bicarbonate
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HSL	Hormone sensitive triacylglycerol lipase
IC_{50}	Concentration of a compound required to show 50% inhibition of a target
Lys	Lysine
PDA	Piperazine diacrylamide
PDH	Pyruvate dehydrogenase
PDHP	Pyruvate dehydrogenase phosphatase
PEP	Phosphoenolpyruvate
PKA	cAMP-dependent protein kinase
PLP	Pyridoxal 5'-phosphate
REA	Radioenzymatic assay
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
S.E.M.	Standard error of the mean
Ser	Serine
TAG	Triacylglycerol
TCA	Tricarboxylic acid cycle (also known as Krebs Cycle)
VLDL	Very low density lipoproteins

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CHAPTER ONE

INTRODUCTION

1.1 PREFACE

An unfortunate but very evident fact exists: people in developed countries, especially North America, have higher rates of obesity and Type II diabetes than elsewhere in the world. And the prevalence of these conditions is on the rise. While living in developed countries does not predispose one to becoming overweight, the generally sedentary lifestyle and high calorie, high fat foods that have been and are being consumed in these countries do contribute to this phenomenon of increasing body weight and diabetic prevalence in the population.

While the primary deficit in Type I diabetes (seen most often in children) is autoimmune destruction of pancreatic β-cells (which produce insulin), the type II form of the disease is most prevalent. Type II diabetes is characterized by impaired insulin action, largely due to defects in insulin sensitivity. Type II diabetes accounts for about 90% of the cases of this disease, and being overweight or obese is strongly correlated with development of type II diabetes. In the United States in 2001, 58% of the population was overweight, and those overweight had over 7 times the risk of developing diabetes than those with normal body mass [1].

The link between diabetes and obesity is clearly important. Equally important is understanding how the body becomes overweight. This is not an easy task, as there are numerous factors which can lead to weight gain. However, since most of the increase in body mass is associated with accumulation of fat, an understanding of fat metabolism is vital.

1.2 LIPID METABOLISM

The metabolism of lipids represents a complex and highly regulated process, which has three main roles in the body. First is storage and release of energy in the form of triacylglycerols, which act as long-term energy stores and are most abundant of the lipids in the body. Second, glycerophospholipids and sphingolipids comprise the bulk of the lipid content of biological membranes, with the latter especially prevalent in nerve cells. Thirdly, the steroids, including cholesterol, represent components of biological membranes, are present in blood plasma in the form of various lipoproteins, including very low-density, low-density and high-density lipoproteins (VLDL, LDL and HDL), and can act as cellular messengers or neurotransmitters.

The intricate balance of biosynthesis and breakdown of these lipids is important in maintaining health, and imbalances can lead to cellular damage, disease and death. Therefore, controlling the rate of intake, production, utilization and breakdown of lipids is fundamental to correct operation of the body. The mechanisms by which the different classes of lipids are controlled are both similar and distinct. The metabolism of glycerophospholipids and steroids is complex and involves many integrated control systems, and will not be discussed here. However, fatty acid and triacylglycerol metabolism is a central aspect of this thesis, and knowledge of the relevant pathways and controls is important in understanding the relevance of this work.

1.2.1 Triacylglycerol Metabolism

Triacylglycerols (TAGs), composed of a glycerol backbone with three esterified fatty acid residues, make up the largest proportion of lipids in the body and are the primary long-

term energy storage supply. In times of excess caloric intake, unused food "energy" is converted into TAGs and stored, primarily in the adipocytes of adipose tissue, but also to a small degree in hepatocytes of the liver. In times of low caloric intake, these TAGs are metabolized to provide energy.

This TAG breakdown is promoted by the action of the hormone sensitive triacylglycerol lipase (HSL), so named due to its sensitivity to regulation by phosphorylation and dephosphorylation in response to hormonally controlled cyclic AMP (cAMP) levels. Catabolic hormones such as glucagon and norepinephrine increase adipose tissue cAMP concentrations, thereby allosterically activating cAMP-dependent protein kinase (PKA). PKA then phosphorylates certain enzymes, such as hormone-sensitive lipase and acetyl-CoA carboxylase (ACC). Phosphorylation of HSL activates the enzyme, and together with phosphorylation of perilipin, lead to stimulation of triacylglycerol hydrolysis. This conversion of TAG into free fatty acids (FFA), raises blood FFA levels, and stimulates β-oxidation of the fatty acids in other tissues such as liver and muscle. Before β-oxidation, the fatty acids are first esterified with coenzyme A, making acyl-CoA esters, and the acyl group is then transferred to carnitine (via CPT-I), producing acyl-carnitines. These acyl carnitines are transported across the mitochondrial membrane by the translocase associated with CPT-I. Once inside the mitochondria, the acyl group is once again transferred back to CoA (via CPT-II) and the acyl-CoA undergoes β-oxidation. In parallel with cAMP-mediated activation of lipid catabolism, phosphorylation of ACC (by PKA and other kinases) inactivates the enzyme, thus preventing fatty acid synthesis.

TAG synthesis is accomplished via a highly controlled pathway. Assuming that *de novo* lipid synthesis begins with the supply of glucose, several control points exist in the

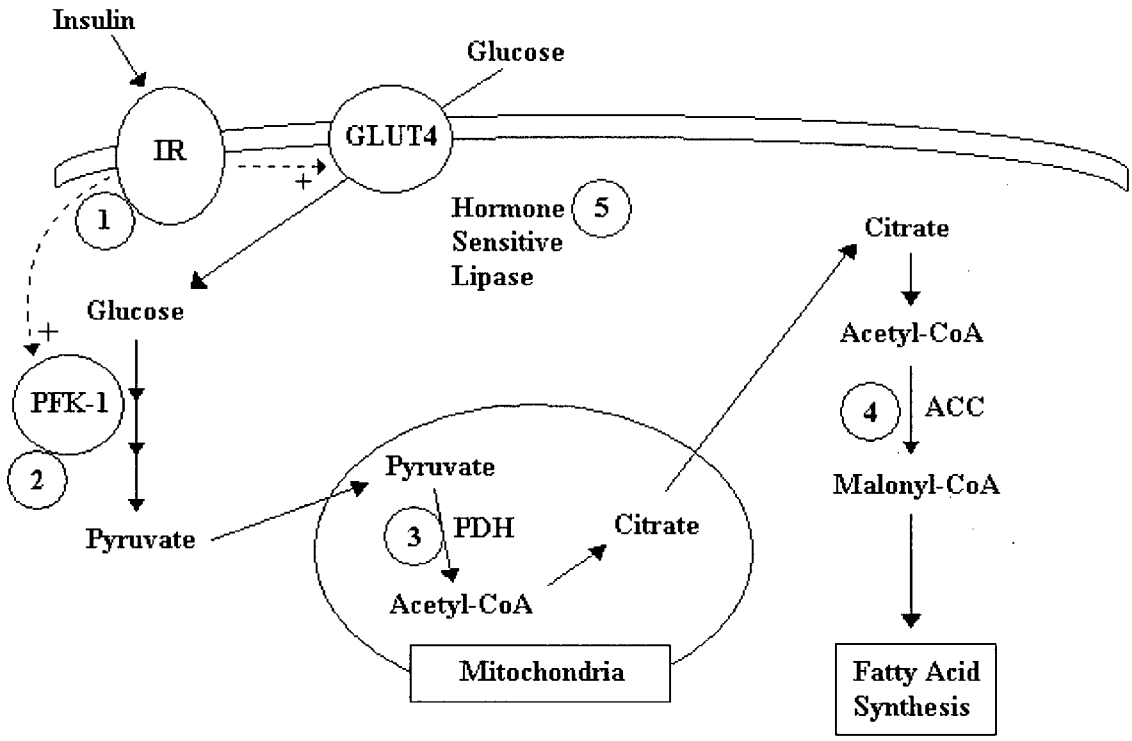
generation of cytoplasmic pyruvate, notably GLUT4, PFK-1 and pyruvate kinase. Another crucial control point is the production of acetyl-CoA from pyruvate, by pyruvate dehydrogenase (PDH). This acetyl-CoA can then enter the TCA cycle or can be converted to malonyl-CoA by ACC, arguably the first *committed* step in fatty acid biosynthesis. This malonyl-CoA product is then used as the 2-carbon substrate of fatty acid synthetase (FAS), which produces an acyl chain end-product, usually 16 carbons long (palmitate). Clearly, several control points exist in the overall pathway, and those relevant to this thesis will be discussed further.

Insulin, in many respects, antagonizes the effects of glucagon and norepinephrine. Insulin decreases cAMP levels, thus inactivating PKA and preventing both activation of HSL and inactivation of ACC. Insulin also results in activation of GLUT4, PFK-1, PDH and ACC, and although the mechanism of these activations is still not completely understood, they appear to involve a variety of processes. The first step is promotion of glucose uptake via stimulation of the insulin receptor, which activates signalling cascades resulting in translocation of intracellular vesicles from subcellular locations to the plasma membrane. These vesicles contain the GLUT4 glucose transporter (insulin sensitive), and this increase in glucose transporters on the plasma membrane leads to an increase in glucose uptake by the cell, resulting in available fuel for fat synthesis in adipose tissue. In general, regulation of GLUT4 shows many similarities to that of synaptic vesicle trafficking (For reviews, see [2-4]). The regulation of PFK-1 in response to insulin, like the other metabolic effects, is dependent on PI3-kinase activation. The generation of PIP_3 leads to activation of downstream kinases including PDK-1, PDK-2 and PKB. PFK-1 appears to be activated following phosphorylation by PKB or a related kinase. The next major control point is pyruvate

dehydrogenase (PDH). Insulin activates PDH phosphatase (PDHP), which maintains PDH in an active, dephosphorylated state, thus ensuring continued production of acetyl-CoA for fatty acid synthesis [5]. Figure 1.1 illustrates these control points.

Of considerable interest, acetyl-CoA carboxylase (ACC) represents the first committed step of fatty acid synthesis and is highly regulated. As mentioned, insulin promotes ACC activation, but the enzyme is also controlled by other phosphorylation and dephosphorylation events, as well as allosterically and through association with at least one regulator protein. These varied control mechanisms together with other control features suggest ACC is a key point in fat metabolism, and understanding of how this enzyme is regulated can provide new insight into localized and body-wide fat metabolism.

Figure 1.1 *Selected Insulin-related control points for lipid biosynthesis in adipose tissue.*



Visual representation of selected insulin-mediated control points for lipid biosynthesis in adipose tissue. The first control point is through the insulin receptor, mediating glucose uptake via GLUT4 vesicular transport up-regulation (1). Activation of PI3-kinase and production of PIP_3 result in activation of PKB (not shown). PKB (or a related kinase) then phosphorylate and activate PFK-1 (2). Insulin activation of pyruvate dehydrogenase phosphatase ensures that PDH remains active (3). Insulin decreases cellular cAMP levels, resulting in decreased activity of acetyl-CoA carboxylase (4) via inactivated PKA, and inactivation of hormone sensitive lipase (5).

1.3 ACETYL-CoA CARBOXYLASE (ACC)

1.3.1 Structure and Function of ACC

Acetyl-CoA carboxylase (EC 6.4.1.2) is a biotin-containing carboxylase, catalyzing the first committed step in *de novo* fatty acid synthesis. The catalysis occurs in two steps. The first is transfer of a carboxyl group (CO_2^-) from an activated bicarbonate substrate to a biotin prosthetic group in the enzyme, producing carboxy-biotin. This energy dependent step requires ATP, and is shown as (1). The second step involves carboxyl group transfer from carboxy-biotin to an acetyl-CoA substrate, producing the product, malonyl-CoA (2). Both of these partial reactions are blocked by pre- or co-incubation with avidin [6].

(1) ACC-biotin + ATP + HCO_3^- → ACC-carboxy-biotin + ADP + Pi

(2) ACC-carboxy-biotin + acetyl-CoA → ACC-biotin + malonyl-CoA

ACC is considered to contain 3 functional domains: the biotin carboxylase domain, the carboxyltransferase domain and a 'flexible' biotin arm which can access both distinct active sites. In most prokaryotes, ACC catalysis is carried out by a complex, with the functional catalytic domains present on two or more polypeptides. For example, in *Escherichia coli*, ACC is comprised of four polypeptides, each encoded by a distinct gene. Several groups have crystallized and determined the three-dimensional structures of these component polypeptides individually, but as of yet, no structure exists for the whole complex [7, 8].

Yeast ACC is encoded by a single gene and is highly related to other eukaryotic forms. It is expressed as a tetramer with a subunit size of ~265 kDa [9]. Animal ACC exists in two isoforms, each encoded by a separate gene on separate chromosomes, and each

comprised of a single polypeptide containing the three functional domains. The isoforms/subunits range in size from 265-280 kDa and the native proteins are dimeric under non-denaturing conditions. Unlike other ACC forms, these animal ACC's are induced to assemble into polymers of 5-20 dimers under the influence of di- and tri-carboxylic acids. As well, unlike the prokaryotic forms of ACC, no single-polypeptide form has been structurally characterized, mostly due to the large size of the protein. However, very recently, a fragment of the yeast enzyme, comprising the carboxyltransferase domain was crystallized and the structure solved [10]. The significant similarity in primary sequence between yeast and mammalian enzymes suggests that this structure may be the first step towards a complete structure for the mammalian enzyme.

1.3.2 ACC Isoforms

As mentioned, mammalian ACC exists as either of two isoforms. The smaller, (ACC-1 or ACC-α or ACC-265) at 265 kDa, was the first isoform characterized. The second, larger isoform at 280 kDa (ACC-2 or ACC-β or ACC-280) was discovered more recently, and has 142 additional N-terminal residues [11]. This isoform was first identified in heart tissue and this discovery was unexpected, since heart tissue is highly catabolic and non-lipogenic and ACC was not thought to be required in this tissue. The increased molecular weight of ACC-2 was also confusing, since no ACC isoforms were known at the time, but using immunological and other classical techniques, ACC-2 was shown to indeed be an ACC isoform found most prevalently in the heart [12], although it was shown shortly thereafter that it is also found in liver, skeletal muscle, mammary gland and brown adipose tissue [13].

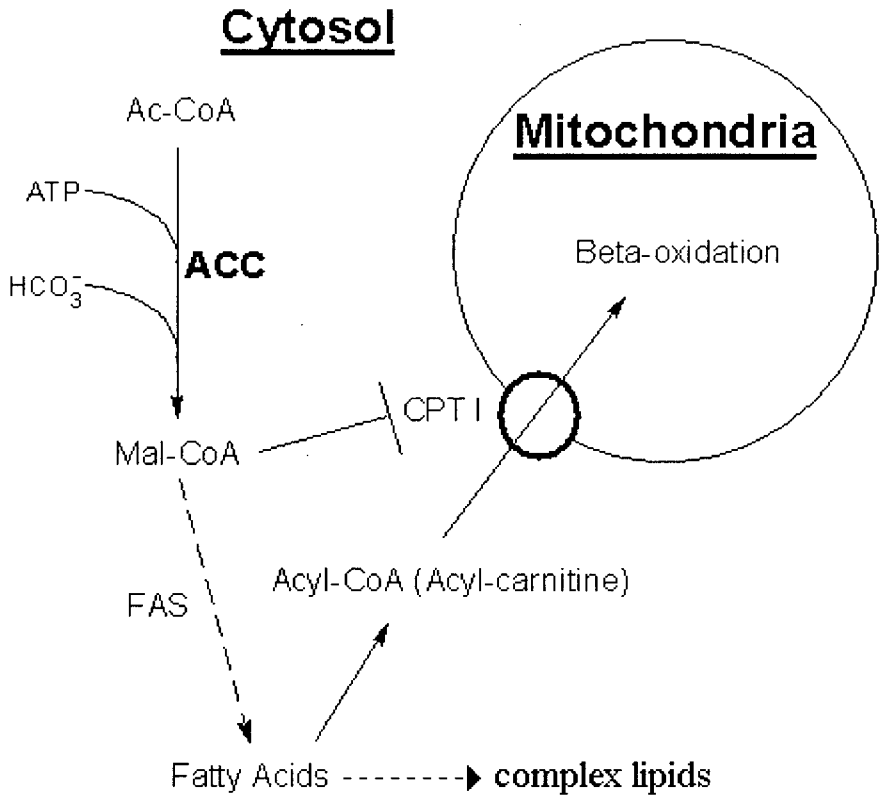
Although sharing over 60% primary sequence homology, the two isoforms show significant differential tissue distribution, as alluded to previously. In rats, ACC-1 is the sole isoform expressed in white adipose tissue, testes, bladder, uterus and placenta. In tissues such as the liver, lactating mammary and pancreas, about 75% of the expressed ACC is ACC-1, with ACC-2 as the other 25%. However, in heart and skeletal muscle, ACC-2 is the predominantly expressed isoform.

Differential distribution of protein isoforms often suggests differing roles for each isoform, and this was suspected for ACC. The metabolic nature of the tissue in which each is expressed, as well as the sequence differences between them indicates possible roles for each isoform. Since lipogenic tissues (liver, adipose) express mainly ACC-1, it has been speculated that ACC-1 is largely responsible for *de novo* fatty acid synthesis, producing malonyl-CoA for use by fatty acid synthase (FAS) to make palmitate. However, as there is little *de novo* fatty acid synthesis in heart and skeletal muscle, due to absence of FAS, the role of ACC-2, which is more highly expressed in these tissues, is unlikely to be fatty acid production. Instead, ACC-2 is believed to be involved in regulation of β-oxidation in muscle tissue. This is achieved through the action of the ACC product, malonyl-CoA. Malonyl-CoA is a potent inhibitor of carnitine palmitoyl transferase-I, which catalyzes the formation of acyl-carnitines prior to translocation into the mitochondria for β-oxidation. Some recent evidence suggests that the additional N-terminal sequence on ACC-2 is a targeting motif for the outer mitochondrial membrane, and immunofluorescence evidence shows that an N-terminal fragment of ACC-2 does localize to the mitochondrial membrane [14]. This co-localization is believed to place ACC-2 in close proximity to CPT-I, thus allowing tight control over fatty acid transport into the mitochondria. When ACC-2 is active, increased

production of malonyl-CoA may inhibit CPT-I and inhibit fatty acid oxidation. The reverse is true when ACC-2 is inactivated. One significant difference between the two ACC isoforms is their requirement for survival. Disrupting one ACC allele in diploid yeast prevents vegetative growth, suggesting that ACC is essential for survival [15]. Surprisingly, long-chain fatty acids cannot, alone, restore growth, suggesting function beyond formation of C16 (palmitate). In animals, given that two ACC isoforms exist (unlike in yeast where there is only one form of the enzyme), it was hypothesized that deletion of ACC-2, given its potential role in β-oxidation control, might not result in a lethal phenotype, whereas ACC-1 deletions likely would. Indeed, recent evidence shows that a deletion of ACC-2 in mice is non-lethal [16]. In fact, the knockout mice were more lean and consumed more food than wild-type mice, illustrating the effect that ACC-2 has on regulating fatty acid oxidation. Fat oxidation control is shown in Figure 1.2.

The important concept regarding the role of ACC in metabolism is that if the enzyme is active, then malonyl-CoA can be used to synthesize lipids and inhibit lipid oxidation; whereas if ACC is inactivated, decreased malonyl-CoA reduces lipid synthesis and relieves inhibition of carnitine palmitoyl transferase I, allowing β-oxidation of lipids to proceed. In short, active ACC contributes to net lipid production, while inactive ACC should contribute to net fat oxidation.

Figure 1.2 *ACC-Related Control of Fatty Acid Oxidation.*



1.3.3 ACC Regulation – Long Term

ACC-2 expression in muscle tissue is not altered during conditions of altered diet [13, 17], as shown using immunoprecipitation with ACC isoform-specific antibodies [13], or in streptozotocin-induced diabetes [18]. It does show repression during hibernation [19], and concomitant repression with ACC-1 in liver tissue during starvation and diabetes [20]. However, ACC-1 shows considerable variation in expression under different physiological states. For example, ACC-1 is repressed in adipose and liver tissue in rats fed high-fat diets, during starvation, and in diabetes induced by insulin deficiency [21]; conversely, the repression of ACC-1 in these conditions could be reversed by low-fat feeding, feeding, and

insulin treatment, respectively [20]. It has been suggested that several factors, including mRNA transcription and stability and protein translation are involved in varying the ACC-1 levels under repression conditions [22, 23]. Three promoters control ACC-1 gene expression. The first responds to hormonal and dietary conditions [24], with the second expressed constitutively [24] or under control of SREBP transcription factors [25], and the third induced during lactation, under influence of the STAT5 transcription factor [26, 27]. The single ACC-2 promoter is activated by transcription factors such as MyoD, Myf4 and Myf6 [28].

1.3.4 ACC Regulation – Short Term

In addition to the longer term regulation of ACC through control of gene expression, shorter term controls are also responsible for regulating the enzyme. Three different mechanisms seem to account for the short term regulation of ACC: small allosteric ligands, interacting proteins and ACC phosphorylation and dephosphorylation by several protein kinases and phosphatases. As mentioned, hormones can exert effects on ACC activity, and this process occurs very rapidly. Glucagon and epinephrine result in decreased ACC activity, coinciding with lipid oxidation, and insulin induces ACC activation, concomitant with lipid biosynthesis. These hormone controls are primarily exerted through phosphorylation events on ACC.

1.3.4.1 Allosteric Regulation of ACC

As previously indicated, animal ACC's have the ability to form polymers, under the influence of di- and tri-carboxylic acids. The polymeric form represents active ACC, while the dimeric form is inactive. This polymerization was first observed in 1962, using sucrose

density centrifugation, which indicated that ACC which had been pre-activated by citrate (the most effective carboxylate activator [12, 17, 29-31]) sedimented at a higher velocity than enzyme lacking citrate treatment [30]. Since the polymerization was complete within minutes (and difficult to study with the techniques available at the time), the depolymerization process was studied instead, due to its slower rate. This promoted the discovery that depolymerization and loss of ACC activity were positively correlated. As well, treatment of ACC dimers with avidin resulted in inactivation, while ACC polymers were resistant to avidin. This indicated either that the dimer-dimer interaction regions were in close proximity to the biotin binding region (and avidin binding blocks these regions from interacting) or that polymerization indirectly results in shielding of biotin on ACC from avidin [32]. Citrate is the best allosteric activator of ACC, but glutamate also activates ACC effectively [33]. Although the site of binding of these acids is unknown, it probably does not occur in the active sites, nor at the binding site for biotin, nor at the sites of phosphorylation, but this has not been established. Other molecules can have the reverse effect: CoA [34], fatty acyl-CoA esters [12, 31, 35], and chloride [36] all promote ACC depolymerization and deactivation. ACC is also inhibited by malonyl-CoA [12, 31] and a biotin analog, CABI (chloroacetylated biotin derivative) has also been shown to be a fairly potent inhibitor of the bacterial ACC carboxyltransferase subunit [37]. Several pentenedioic acid-based metabolites from the fungus *Gongronella butleri* have been shown to inhibit the enzyme as well [38]. Additionally, herbicides of the aryloxyphenoxypropionic acid class inhibit plant ACCs [39], possibly by acting on the carboxyltransferase domain in a eukaryotic form of the enzyme (usually present in plants lacking an insensitive prokaryotic form of ACC) [40]. Although significant information regarding their structural features and how these affect their potency

on plant ACCs is known, the effect of these herbicides on animal ACCs is not well studied. One study suggests that CoA-esters of aryloxyphenoxypropionates act as potent inhibitors of rat liver ACC [41], but these results have not been sufficiently validated to establish these herbicides as mammalian ACC inhibitors. Nonetheless, there is also insufficient evidence to rule out the inhibitory actions of these herbicides on mammalian ACC. Additionally, as with many other enzymes, ACC is inhibited by high salt and low protein concentrations as well as alkaline pH [42], although very low salt (less than 100 μM) leads to irreversible ACC aggregation and inhibition.

Surprisingly, even though ACC-2 is activated by citrate [12, 17, 29] it does not form polymers like ACC-1 [43]. Although ACC-2 does not seem to form homodimers, some evidence exists for the formation of heterodimers with ACC-1 [13, 44]. This suggests the two isoforms may be co-regulated, through a form of direct linkage, at least in some tissues.

1.3.5.2 Regulation of ACC by a Protein Regulator

Experiments with size exclusion and ion-exchange chromatography revealed two proteins which affected the activity of ACC, one in liver [45] and one in adipose tissue [46]. These are 75 and 130 kDa, respectively, but any similarity between the properties of these proteins has not been elucidated. In the absence of these proteins, the sensitivity of ACC to citrate, is significantly lower than when they are present. Since polymerization is partially preceded by citrate binding, it is possible that these regulator proteins somehow act to facilitate ACC polymerization or citrate binding, but the exact mechanism of their action remains unclear.

1.3.5.3 ACC Phosphorylation

The phosphorylation events that regulate ACC activity are numerous, complicated and still not completely defined. As mentioned earlier, ACC is activated by insulin and inhibited by glucagon and epinephrine. How these hormones exert their effects is through distinct phosphorylation events on ACC by different protein kinases, thus altering the activity of the enzyme. Most evidence concerning identity of specific sites relates to ACC-1, with sites on ACC-2 still not defined. Since ACC phosphorylation does not represent an important component of this thesis, only a brief discussion of the process will be included.

Several inactivating phosphorylation sites and kinases for ACC-1 have been elucidated, as well as sites with an unknown or no clear effect on ACC activity. These are detailed in Table 1.1. Phosphorylation of the 'I-peptide' is insulin-dependent, but neither the phosphorylating kinase nor the phosphorylation site has been characterized. AMPK phosphorylates ACC at several sites, but site-directed mutagenesis has shown an inhibitory effect is only seen through phosphorylation of Ser-79 on ACC-1 [47]. PKA phosphorylates ACC only on serine residues [43], and on ACC-1, these have been determined to be Ser-77 and Ser-1200. Mutagenesis has shown that PKA-mediated inactivation of ACC is a result of phosphorylation of Ser-1200 [47]. PKA phosphorylates ACC-2 much more efficiently than ACC-1 [43, 44]. CaMK shows similar substrate sequence specificity as AMPK, but CaMK phosphorylates ACC-1 at Ser-25, and this does not seem to affect ACC activity *in vitro* [43, 48]. Casein kinase 2 (CK2) phosphorylates ACC-1 at Ser-29, but this has no apparent effect on ACC activity directly. Finally, PKC has been shown to phosphorylate ACC-1 at Ser-77 and Ser-95, with some indication that PKC-mediated phosphorylation of Ser-77 leads to ACC activation. This contrasting result with PKA phosphorylation of the same Ser-77 could be

due to impure kinase preparation, and the role of PKC phosphorylation of this site remains undetermined.

Table 1.1 Acetyl-CoA Carboxylase-1 Phosphorylation. [1]

Phosphorylation Site(s)	Phosphorylating Kinase	Kinase Control [2]	Effect of Phosphorylation	Reference
Ser-25	CaMKII	Ca^{2+}/CaM, Insulin (all +)	None	[49, 50]
Ser-29	CK2	?	None	[48-50]
Ser-77	PKC	DAG, PS, Ca^{2+} (all +)	?	[48, 49, 51]
	PKA	Catecholamines, cAMP (both +) Insulin (-)	Possible inhibition	[48, 49, 51, 52]
Ser-79	AMPK	AMP (+) Insulin (-)	Inhibition	[49, 52]
Ser-95	PKC	DAG, PS, Ca^{2+} (all +)	?	[49, 51]
Ser-1200	AMPK	AMP (+) Insulin (-)	Possible Inhibition	[49, 52]
	PKA	Catecholamines, cAMP (both +) Insulin (-)	Inhibition	
Ser-1215	AMPK	AMP (+) Insulin (-)	Possible Inhibition	[52]
'I-peptide'	'I-peptide kinase'	Insulin (+)	Possible activation	[51, 53]

[1] Adapted from [54].

[2] Activating signals are given by a (+) and inhibitory signals by a (-).

1.4 PYRIDOXAL 5'-PHOSPHATE (PLP)

Although several compounds, both endogenous and exogenous, can inhibit acetyl-CoA carboxylase, some of these, especially exogenous inhibitors are either generally not physiologically relevant to the mammalian enzyme, or limited in their use. Since ACC represents an important control point for lipid metabolism, it is a potential target for pharmacologic inhibitors, whether these be synthesized or naturally derived. Additionally, it is of great interest to fully understand endogenous compounds and mechanisms that regulate ACC activity, since these can offer insight into drug design for targeted ACC inhibition.

Pyridoxal phosphate (PLP), the phosphorylated and most common form of vitamin B_6 *in vivo*, has previously been shown to inhibit enzymes using citrate as a substrate or as an allosteric activator of enzyme activity. Given these previous results and the understanding that citrate acts allosterically on ACC, it seemed possible that PLP might act on ACC.

1.4.1 Pyridoxal Phosphate Structure and Function

Pyridoxal 5'-phosphate (pyridoxal phosphate or PLP) is the biologically active form of vitamin B_6 (pyridoxine). Vitamin B_6 functions as a coenzyme in over 100 enzymatic reactions, involved in amino acid, lipid, carbohydrate and neurotransmitter metabolism. The major forms of vitamin B_6 in mammalian tissues and fluids are: pyridoxine, pyridoxal, pyridoxamine and their phosphorylated derivatives, pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate, as well as the end product of vitamin B_6 metabolism, 4-pyridoxic acid. The structures of these vitamers (one of two or more related chemical substances that fulfill the same specific vitamin function) are shown in Figure 1.3. PLP contains a pyridine ring with a reactive aldehyde para to the ring nitrogen, at position $C_{4'}$. A

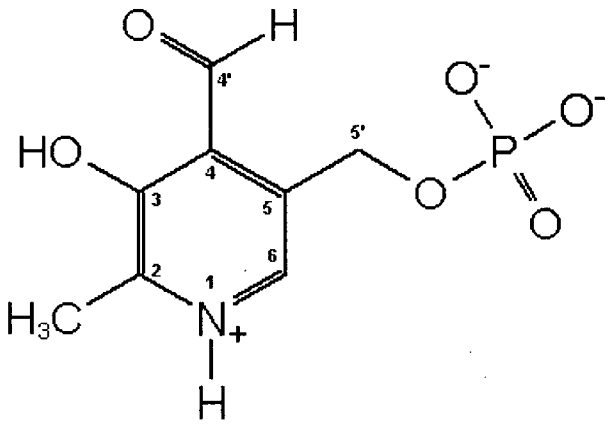
methyl group is ortho to the ring nitrogen at position $C_{2'}$ and a hydroxyl is meta, at $O_{3'}$. Also meta is a carbon bound to a phosphate group, at $C_{5'}$. These structural features are believed to be important for the role of PLP as an inhibitor of certain enzymes, as will be discussed later.

PLP cannot be biosynthesized by mammals, and must be obtained from the diet. Major sources include certain vegetables, beans, fish, seeds and nuts. Vitamin B_6 is typically found in the diet as pyridoxal, pyridoxamine and pyridoxine [55]. It is passively absorbed by the intestine and delivered to the liver, where it is converted to it's active form, PLP. For most cells, PLP cannot freely cross the cell membrane and must be hydrolyzed to pyridoxal by alkaline phosphatases. Pyridoxal can pass through the cell membrane or be transported into the cell, whereupon pyridoxal kinases phosphorylate pyridoxal back to PLP [56].

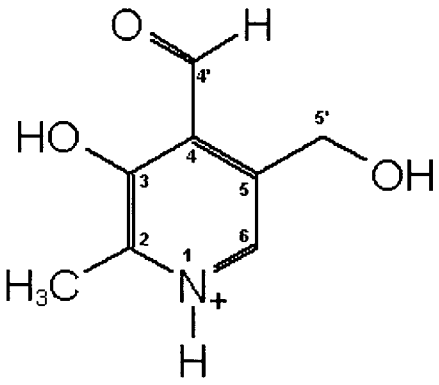
Vitamin B_6 deficiency is rare in developed countries, but this condition could pose a risk for cardiovascular disease since vitamins B_6 and B_{12} are cofactors of enzymes used in the breakdown of homocysteine. Some preliminary evidence exists showing that hyperhomocysteinemia results in negative outcomes for patients with high plasma levels of homocysteine with regards to developing cardiovascular disease and risk of death for those with coronary heart disease [57, 58]. It is widely recommended that the diet include sources of vitamins B_6, although it is currently not clear if there are long-term effects (on plasma vitamin B_6 concentrations) of daily pyridoxine supplementation, as used for treating diseases such as cystathioninuria and homocystinuria, seizures, and carpal tunnel syndrome [59].

As mentioned, once in the cell, PLP acts as a cofactor in many enzymatic reactions. Most familiar is its role in transaminases, common in amino acid metabolism. The binding of PLP to an enzyme is through its reactive aldehyde residue to an ε-amino group of an enzymatic lysine residue, forming an "internal" aldimine.

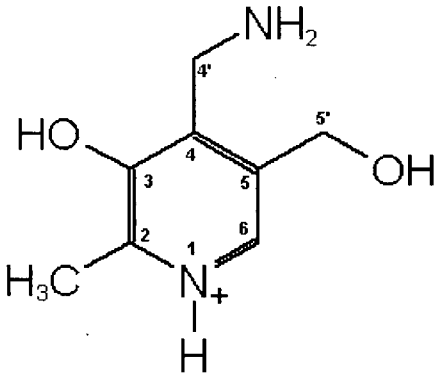
Figure 1.3 *Structures of B_6 vitamers.*



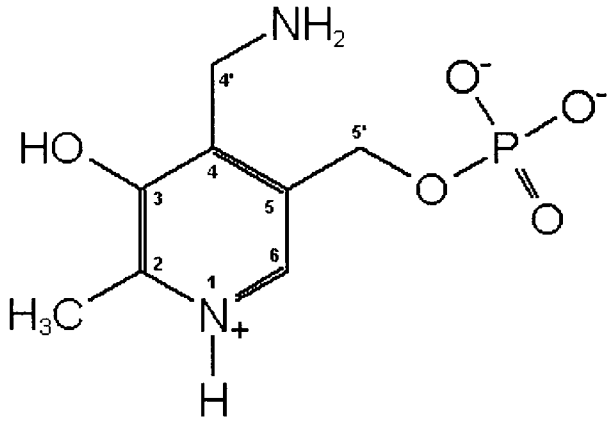
Pyridoxal Phosphate



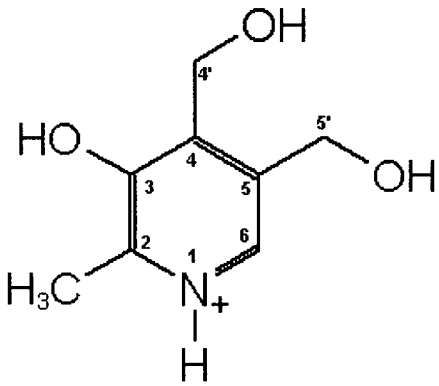
Pyridoxal



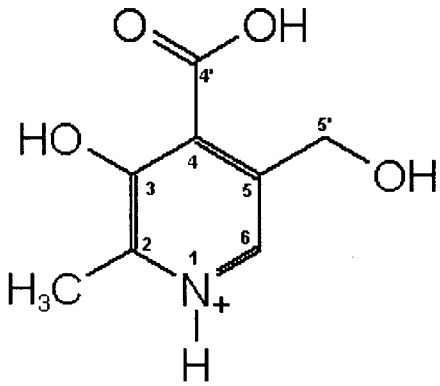
Pyridoxamine



Pyridoxamine Phosphate



Pyridoxine



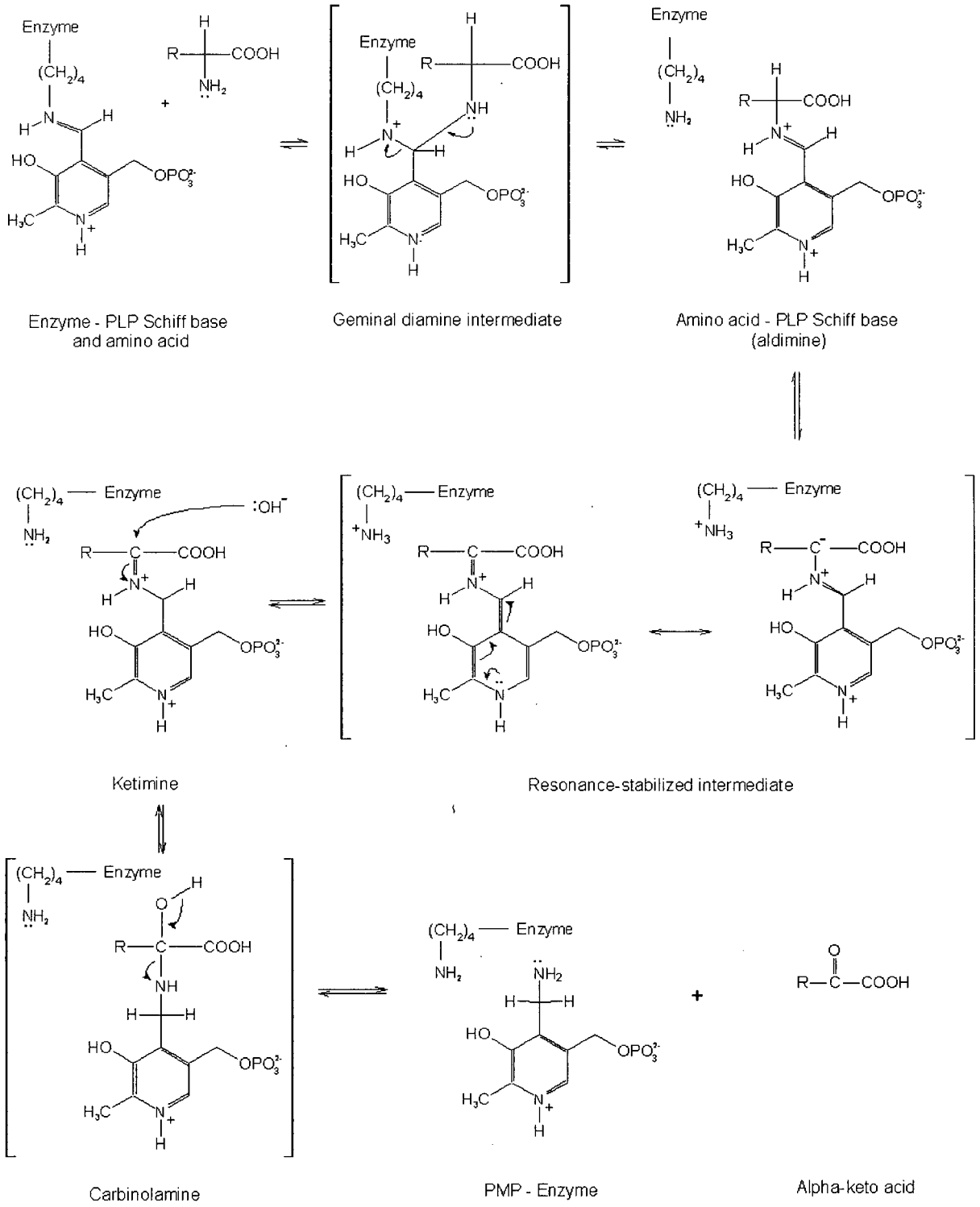
4-Pyridoxic Acid

To illustrate the principle of PLP-mediated catalysis, a transamination reaction will be described, as illustrated in Figure 1.4. Incoming amine-containing substrates react with the labile double bond, forming an "external" aldimine linkage to PLP. A proton on the substrate is removed by a reactive group on the enzyme (often the ε-amino group to which PLP was first linked with), leading to a tautomerization of PLP. A reactive molecule then interacts with the bound amine substrate, and this leads to hydrolysis of the substrate, leaving pyridoxamine phosphate. The second substrate, lacking an amino group then reacts with the reactive amine, and the reverse reactions mentioned above occur, regenerating PLP and producing a new product containing an amine and one lacking an amine, hence 'transamination'. In general PLP acts as an "electron sink", storing electrons from cleaved substrate bonds, and dispensing them to allow the formation of new linkages with incoming protons or secondary substrates [60].

1.4.2 Experimental Use of PLP

While PLP is naturally used as a cofactor in enzymes, it can also be used experimentally for numerous purposes. These range from using it as a measure of vitamin B_6 absorption and usage [55, 61, 62], determining reactive lysine residues and PLP/substrate/ allosteric binding sites of various enzymes [63-71], measuring transport of metabolites across membranes [72-75], and for determining enzymatic activity [76, 77].

Figure 1.4 *PLP-dependent Transamination Mechanism.*[1]



[1] Adapted from [78].

1.4.2.1 Measuring PLP in the Body

Although PLP is the most important of the vitamin B_6 vitamers, and required for survival, only minute amounts are present in blood plasma, or free in the cytoplasm of cells. This presents a challenge for diagnostic determination of PLP concentration, as would be required for estimating the clinical status of an individual. Typically, all measurements made thus far have been with blood plasma, due both to ease of procurement, as well as greater PLP abundance in plasma than as a free molecule in the cell.

Several methods have been developed for estimating PLP concentration in plasma. These include microbiological, enzymatic and chemical methods, as well as HPLC-based approaches [61]. The most common assays use either activated apo-tyrosine decarboxylase, which requires PLP to convert [1-^{14}C]tyrosine to $^{14}CO_2$, which is then counted for radioactivity, or a ^{3}H radioenzymatic assay (REA) which also uses PLP-dependent tyrosine decarboxylase and HPLC [79]. Unfortunately, these methods typically are complex, non-homogeneous and require radioactive reagents and specialized equipment, which often makes them inappropriate for clinical diagnosis, since the techniques and equipment are expensive and not widely available. However, some new, non-radioactive approaches are emerging which are both faster, less complicated, comparatively accurate and less toxic than those currently employed. One such approach uses the apo-form of PLP-dependent, recombinant homocysteine α,γ-lyase cloned from *Trichomonas vaginalis* [62]. This enzymatic method is simple, rapid, sensitive, specific and requires only small volumes of plasma.

The range of techniques used for the assay of PLP have yielded very variable results as illustrated by a comparison of reports of plasma PLP levels [61]. Early work, prior to 1990, showed similar plasma PLP concentrations, but results since then have generally

differed from the earlier data. Whether this is due to improved sensitivity of the techniques, or more specific procedures is unknown. Clearly though, additional work must be done in order to develop a simple, rapid and accurate method for determining plasma PLP concentration, since this would provide a very useful diagnostic tool, both in the laboratory and in a clinical setting.

1.4.2.2 PLP and Reactive Lysine Residues

Because PLP interacts with enzymes through a Schiff base linkage to ε-amino groups of lysine residues, this feature can be exploited experimentally to determine which lysine residues account for enzymatic functionality, either through use of PLP as a natural cofactor, or through its reaction with enzymes that depend upon catalytic lysines even if they function without a PLP cofactor. The key step in this procedure is the reduction of the aldimine linking PLP and the enzyme lysine using the common reducing agent, sodium borohydride (BH_4). The now covalently linked PLP-lysine can then be determined by sequencing the labeled peptide after protein digestion, using either peptide sequencing with traditional N- or C-terminal solution approaches, or more recently using mass spectrometry. PLP binding is also measured using spectrophotometric and spectrofluorometric methods, since borohydride reduction shifts the absorbance and emission maximum of PLP, giving a diagnostic signal. Numerous reports of PLP used in these ways are found in the literature. For example, the important PLP-binding lysine residues and novel PLP-binding motifs in two PLP-dependent aminomutases from two different bacterial species were determined using PLP reduction with BH_4, followed by HPLC and mass spectrometry [63, 67]. The interaction of PLP with the heme-containing protein cystathionine β-synthase showed how PLP-dependent catalysis

proceeded by localizing the lysine residue to which PLP binds [65]. PLP has also been used to determine the reactive lysine residues in enzymes lacking a natural PLP cofactor, by adding it exogenously. An essential lysine residue in the substrate-binding site of retinal oxidase was determined using PLP [68], as was a lysine believed to localize near the NADP binding site of glutathione reductase [64].

1.4.2.3 PLP and Metabolite Transport Measurements

PLP has been successfully used to determine important residues in metabolite transporters, as well as to measure the kinetics of such transport. PLP seems to be most effective as an inhibitor of transporters of di- and tri-carboxylic acids such as oxaloacetate [75], α-ketoglutarate [73] and citrate [72, 74]. Implications from such studies is that PLP is a very effective inhibitor, showing IC_{50} values in the range of 200-500 μM, and complete inhibition at concentrations above about 5 mM. Why PLP interacts so specifically with these particular transporters is not completely understood, but it has been suggested that that since citrate is highly negatively charged, its binding site would be expected to have positively charged amino acids (such as lysines), allowing charge stabilization and ionic interactions. Since PLP also contains a negatively charged phosphate group and an amine-reactive aldehyde, it should also interact favourably with the citrate binding sites on these enzymes [69, 70]. In most of these studies PLP inhibition was concentration dependent, and PLP inhibition could be successfully blocked using specific substrates.

1.4.2.4 PLP and Enzymatic Activity Determination

One particularly interesting use of PLP was to show that a gain-of-function mutant of *Escherichia. coli* was due to an uncompensated lysine residue. This lysine showed substrate-protectable inhibition by PLP of the sugar phosphate transporter UhpT. This also showed that the ability of the mutant to utilize a new phosphoenolpyruvate (PEP) substrate was due to the interaction of the lysine residue with the carboxyl group of PEP [66]. Similarly, PLP has been used to determine the activity of both succinic semialdehyde dehydrogenase [76], and succinic semialdehyde reductase [77].

1.5 THESIS INVESTIGATION

Although it is firmly established that citrate acts as an allosteric activator of both ACC-1 and ACC-2, its exact binding location has remained a mystery. Determination of the citrate binding site would be highly beneficial in understanding how citrate can induce polymerization of ACC-1, and result in activation of both isoforms. The current progress in structural studies of ACC combined with citrate binding site information would allow a much better understanding of ACC function and regulation.

As mentioned, PLP can act as an inhibitor of di- and tri-carboxylate transporters, competitively interacting at the active site, although why PLP acted in this fashion was not explained [72-75]. As described in other reports, PLP was probably effective because it can provide a combination of a highly negatively charged phosphate group and a reactive aldehyde. The phosphate group would mimic the negative charges of the carboxylate substrates, and the aldehyde would enable an interaction with an active site lysine residue, providing additional stability. Studies of the allosteric citrate binding site of rabbit muscle phosphofructokinase (PFK) utilized PLP as a key component [69-71]. These suggested that the rationalization for using PLP was that since citrate contains three negatively charged moieties, the citrate binding site is likely to have multiple positive charges. If one of these was due to a lysine residue, it could potentially interact with PLP. This was shown to be the case, as evidenced by citrate protection of PLP inhibition. Using sodium borohydride, [^{3}H]pyridoxal phosphate was reduced, and the covalently labelled protein digested with trypsin. The peptides were separated by HPLC and labelled peptides isolated. One peptide contained 70% of the radioactivity recovered, and was sequenced using Edman degradation.

Since ACC is also allosterically activated by citrate, it was hypothesized that perhaps PLP would show a similar response with ACC as it did with PFK. The major motivation for considering examination of PLP effects on ACC was that it might provide a means to localize the elusive citrate binding site of ACC. Furthermore, positive results with PLP would open up the possibility of structure-function studies to determine the important structural aspects of PLP that enabled ACC inhibition.

This thesis addresses two of these concepts. In Chapter 3, characterization of the effects of PLP on ACC is given. In Chapter 4 the effects of PLP analogs are examined and how the different moieties of PLP account for its specificity to ACC and other enzymes. Finally, Chapter 5 concludes the thesis, and also provides some insight into future work, including a brief discussion of the use of PLP in determining the citrate binding site of ACC.

CHAPTER TWO

EXPERIMENTAL PROCEDURES

2.1 MATERIALS

Male Wistar rats were obtained from the University of British Columbia animal care facility at weights ranging from 120-250 g. All animal procedures were carried out within the guidelines of the Canadian Council of Animal Care and with the approval of the UBC Committee on Animal Care. Rats were maintained on a 12 hour light/12 hour dark cycle at a controlled temperature with free access to water and laboratory rat chow.

Solvents and laboratory chemicals were mostly obtained from Fisher Scientific or BDH Chemicals Canada Ltd. HEPES, glycine, EDTA, EGTA, PMSF, ATP, PLP (& analogs), glutathione, Pepstatin A and BSA were from Sigma-Aldrich. ICN supplied fatty-acid free BSA, and Roche supplied coenzyme A. Tris and MOPS were from USB (Amersham). Peptides International supplied the protease inhibitor leupeptin. Streptavidin-HRP, [^{14}C]-$KHCO_3$, pre-stained SDS-PAGE high molecular weight Rainbow Markers and ACS scintillation fluid were from Amersham Pharmacia Biotech. PVDF membrane and Ultrafree-4 and Ultrafree-15 centrifugal filters (Biomax-30) were from Millipore. SDS-PAGE reagents and Affigel-10 were from BioRad. Hen egg-white avidin was from Canadian Inovatech, and anti-phosphorylated ACC (S79) antibody was supplied by Upstate Biotech. [^{3}H]-$NaBH_4$ was from Perkin Elmer Life Sciences and SoftLink™ Soft Release Avidin Resin was from Promega.

2.2 METHODS

2.2.1 Tissue Preparation

2.2.1.1 Isolation

Rats were killed by CO_2 asphyxiation usually between 9:00 and 10:00 AM, and liver was immediately removed onto ice, with skeletal muscle and heart immediately frozen in liquid nitrogen. Adipose tissue was not chilled or frozen immediately, but instead was incubated in pre-gassed (O_2:CO_2;95:5) Krebs-Henseleit buffer (25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$ and 11 mM glucose) pre-warmed to 37°C. This buffer was made up freshly each day, gassed while at 4 °C then warmed to 37 °C.

2.2.1.2 Adipose Tissue Incubation

Epididymal and perirenal fat pads were dissected into smaller pieces (50-100 mg) and rinsed once with warmed, pre-gassed Krebs-Henseleit buffer, then incubated with gentle shaking in fresh buffer for approximately 30 minutes to counteract the effects of any residual hormones. Following incubation, the pieces were blotted to remove excess buffer and then frozen in liquid nitrogen.

2.2.1.3 Homogenization

Liver was homogenized in 3-7 volumes of homogenization buffer using a fitted Teflon-glass Potter-Elvehjem homogenizer kept on ice. The buffer contained 20 mM MOPS (pH 7.2), 250 mM sucrose, 2mM EDTA, 2 mM EGTA, 2.5 mM benzamidine HCl, 3 μM Pepstatin A, 5 μM leupeptin, 2.5 mM glutathione and 0.5 mM PMSF. Following freezing in

liquid nitrogen, skeletal muscle, heart and adipose tissue were powdered and to the powder was added 4 volumes (muscle), 9 volumes (heart) and 5 volumes (adipose) of homogenization buffer. These three tissues were homogenized using a Polytron homogenizer and as with liver, all homogenization steps were carried out on ice.

2.2.2 Purification of Acetyl-CoA Carboxylase

Tissues were isolated and homogenized as described above and the homogenate was then centrifuged for 90 seconds at 1000 rpm to pellet cell debris. The supernatant was filtered through glass wool to help remove any residual floating fat and centrifuged for 20-30 minutes at 12000 rpm (11000 x *g*) to pellet larger organelles, microsomes and other cellular components. Following this, the supernatant was again filtered through glass wool and subjected to ultracentrifugation at 55000 rpm (215000 x *g*) for 60-90 minutes. All centrifugation steps except the first 90 second spin (room temperature) were performed at 4°C. The ultracentrifuged supernatant was then treated with ammonium sulphate to precipitate ACC.

2.2.2.1 Ammonium Sulphate Precipitation

Powdered ammonium sulphate was slowly added to the high speed supernatant on ice to give a final saturation of 40%. The mixture was stirred on ice for a minimum of one hour, then the precipitate recovered by centrifugation at 14000 rpm (15000 x *g*) for 20-30 minutes. For samples used primarily in activity assays, the pellet was resuspended in homogenization buffer lacking protease inhibitors. For samples to be subsequently applied to an avidin affinity column, the pellet was resuspended in HB buffer (50 mM Tris, 500 mM KCl, 2 mM

EDTA, 2 mM EGTA, 0.02% (v/v) sodium azide). Samples of ACC recovered by ammonium sulphate precipitation were typically stored at either -20 °C (short-term) or -80 °C (long-term) until use or further purification. If further purification was required, samples were thawed and centrifuged at 13200 rpm (16000 x *g*) for 3 minutes to clarify the enzyme sample. Any enzyme dilutions required for further purification were made at this point.

2.2.2.2 Avidin Affinity Chromatography

Due to the high affinity of biotin to tetrameric avidin (~ 10^{-15} M), reversal of this binding can only be achieved using denaturing conditions such as boiling in SDS buffer containing β-mercaptoethanol. These conditions destroy ACC activity and thus tetrameric avidin cannot be used to recover active enzyme. However, using a monomerized form of avidin, and saturating the high affinity sites with free biotin, the affinity of the avidin-biotin interaction can be lowered and ACC desorbed under mild conditions – simply by adding free biotin. This allows the "lower affinity" biotin binding sites on the avidin to be used in affinity purification of biotin-containing proteins, such as ACC.

SoftLink™ Soft Release Avidin Resin from Promega was used according to the manufacturer's instructions. Briefly this involved washing the column with HB buffer followed by binding of free biotin to the reactive sites on the resin using 5 mM biotin in HB buffer. The column was then "stripped" of biotin bound to the lower affinity sites using 10% acetic acid, followed by washing in HB buffer. Following this, a diluted and briefly centrifuged sample of the resuspended ammonium sulphate pellet was added to the column and either incubated on the column, or mixed with the resin under shaking for a minimum of one hour, but usually for two to three hours, to ensure complete binding of ACC. Unbound

proteins were then washed from the column with HB buffer until the absorbance of the washing buffer, monitored at 280 nm was constant and very low (typically less than 0.01 A_{280}). ACC was then eluted using HB buffer containing 5 mM biotin and the eluate was concentrated using the "aquacide" method. This involved pouring the sample of ACC into a dialysis sac and incubating the sac in a container of powdered polyethylene glycol to extract moisture from the dialysis sac. Alternatively, ACC samples were concentrated with Ultrafree (4 or 15) centrifugal filter units (Millipore). The flow-through "Void" fraction was also retained and concentrated using the same method as the ACC eluate. The avidin affinity column was regenerated with 10% acetic acid and washed with PBS containing 0.02% (w/v) sodium azide for storage.

2.2.3 ACC Activity Assays

The [^{14}C]-HCO_3 fixation method [30, 80] was used to determine ACC activity. The first step involved pre-incubating the enzyme for 30 minutes at 37°C, to allow ACC activation/polymerization. The pre-incubation buffer consisted of homogenization buffer containing 10 or 20 mM sodium citrate (allosterically activates ACC), 2 mg/mL BSA and the ACC sample. 50 mM sodium glutamate was used instead of citrate in some instances.

Following pre-incubation, a 50 µL aliquot of the activated ACC was added to 450 µL pre-warmed (37 °C) assay buffer (50 mM HEPES pH 7.2, 10 mM MgSO4, 0.5 mM EDTA, 5 mM ATP, 7.5 mM glutathione, 2 mg/mL BSA, 150 µM acetyl-CoA and 7.5 mM [^{14}C]-$KHCO_3$). Assay time varied by tissue and experiment, but all assays were terminated by the addition of 200 µL of 2M HCl. Following a brief centrifugation at 13200 rpm {16000 x *g*} for one minute, 600 µL of the assay mixture was transferred to a liquid scintillation vial and

evaporated under a stream of air, to remove unincorporated [^{14}C]-$KHCO_3$. The remaining dried material contains [^{14}C]-malonyl-CoA, which is acid stable and non-volatile. Each scintillation vial was then shaken for at least one hour with 400 µL of added water, followed by addition of 4 mL of ACS liquid scintillation fluid (Amersham). Samples were counted using a Beckman LS6000IC Scintillation Counter. To determine the substrate specific activity 10 µL of the assay buffer was added to 4 mL of ACS fluid containing 200 µL of 2-phenylethylamine (a CO_2-trapping compound). The specific activity of the assay buffer was determined in triplicate for each experiment. One unit of ACC activity is defined as the amount of ACC needed to convert one µmole of acetyl-CoA to malonyl-CoA in one minute.

2.2.4 Inhibition Assays

Inhibition of ACC by various compounds was assessed by two protocols. The first involved treating ACC samples with pre-incubation buffer lacking sodium citrate, but containing the desired inhibitor, typically for 30 minutes at either 25 or 37 °C. This was followed by addition of citrate and incubation for a further 30 minutes at 37 °C. Incubation with assay buffer then followed as described above. The second variation was the reverse of the first and involved pre-incubating first with citrate (30 minute, 37 °C), then adding the inhibitor and incubating for a further 30 minutes at 37 °C prior to assaying (as above).

2.2.5 Sodium Borohydride Reductions

Reduction of potential aldimine bonds between pyridoxal 5'-phosphate and ACC using sodium borohydride was carried out using one of two methods. In the first method, using unlabelled borohydride, ACC was pre-incubated (30 minute, 25 °C) with PLP. Sodium

borohydride (1 to 10 mM) was then added and the incubation continued for a further 15 minutes at 25 °C. Unreacted PLP and borohydride were then removed by two wash steps in which ACC was diluted by adding 10 mL of homogenization buffer (containing or lacking 20 mM sodium citrate), and then concentrated at 4 °C using a 15 mL Biomax-50 centrifugal filter unit (Millipore). The centrifugations were performed according to the manufacturer's instructions, which involved centrifugation at 8000 x *g* in an SS-34 Sorval fixed angle rotor and a Sorval 5CB refrigerated centrifuge. Following washing and concentration, ACC activity assay were carried out as described previously, using 50 µL of concentrated enzyme sample. Appropriate controls lacking PLP, borohydride, and citrate were also performed.

The second method of ACC reduction was carried out using [^{3}H]-$NaBH_4$ in order to test for ACC protein-labelling. The enzyme was reacted with tetrameric avidin beads for 45 minutes at 4 °C, followed by thorough washing. The beads were then treated with 0.5 mM PLP for 30 minutes at 25 oC, followed by addition of 1 mM BH4 for 15 minutes. The beads were again thoroughly washed and the bound proteins ready for elution by heating in Sample loading buffer, as described below.

2.2.6 SDS-PAGE

Using the procedure of Laemmli [81], discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins of interest. Gels run solely to observe ACC running patterns, such as in an ammonium sulphate preparation, were typically 3% stacking and 5% separating acrylamide, using piperazine diacrylamide as the crosslinker instead of bis-acrylamide, to increase gel strength. Run times were typically 1.5 hours at 175 volts and 50 milliamps. Following electrophoresis, gels were

fixed, stained with Coomassie blue, de-stained, stabilized with glycerol and placed between two sheets of cellulose film and dried at room temperature.

To separate ACC from other proteins following treatment with PLP and [^{3}H]-$NaBH_4$, 4-15% gradient acrylamide mini-gels were employed (BioRad). Prior to electrophoresis, samples were heated for 10 minutes at 95 °C in SDS loading buffer (125 mM Tris (pH 6.8), 10% (w/v) SDS, 20% (w/v) sucrose, 0.02% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol). Molecular weight of the protein bands was estimated by co-loading pre-stained high molecular weight Rainbow markers on each gel (Amersham). Gel run times were typically 2-3 hours at 135 volts and about 20-30 milliamps. Gels were fixed, stained with Coomassie blue, de-stained, stabilized with glycerol and placed between two sheets of cellulose film and dried at room temperature. Quantitation of incorporated [^{3}H] into proteins due to PLP reduction by [^{3}H]-$NaBH_4$ was determined similar to the method of Brownsey and Denton [53]. Briefly, stained bands were cut from the gel and digested in 30% H_2O_2 at 80 °C for 3 hours and then evaporated to dryness. The dried residue was then dissolved in 200 µL 2M HCl, 4 mL of ACS liquid scintillation fluid added, and counts determined.

2.2.7 PLP Spectrophotometry

Because free PLP exhibits a significantly different absorbance maximum than the borohydride-reduced adduct, spectrophotometric analysis of PLP covering wavelengths from 200 to 600 nm provides a useful analytical tool. Scans of free and reduced PLP, as well as free BH_4 and lysine were used as controls for PLP conformation. Measurements were made in acid, water and base initially, and at neutral pH in subsequent observations. All measurements were made using a Perkin Elmer Lambda 35 spectrophotometer.

CHAPTER THREE

PLP EFFECTS ON ACC

3.1 RATIONALE

Tri-carboxylic acids such as citrate and isocitrate [12, 17, 29-31] and di-carboxylic acids such as glutamate [33] are well known to promote polymerization of ACC-1 and activation of both ACC isoforms. As discussed in Chapter 1, PLP has been shown to interact at the citrate binding site of several proteins, including phosphofructokinase. The major goal of the work described in this chapter was to test the hypothesis that PLP can interact at the allosteric citrate binding site of ACC. Because PLP interferes with effects of citrate on PFK [69-71], as well as inhibiting tri- and di-carboxylate transporters, it was also speculated that PLP would have inhibitory effects on ACC. Given the differences in citrate sensitivity of ACC isoforms, it was speculated that PLP may show different effects in ACC prepared from different tissues. In this chapter I describe effects of citrate and PLP on ACC isoforms and the ability of citrate and other analogs to protect ACC against potential effects of PLP.

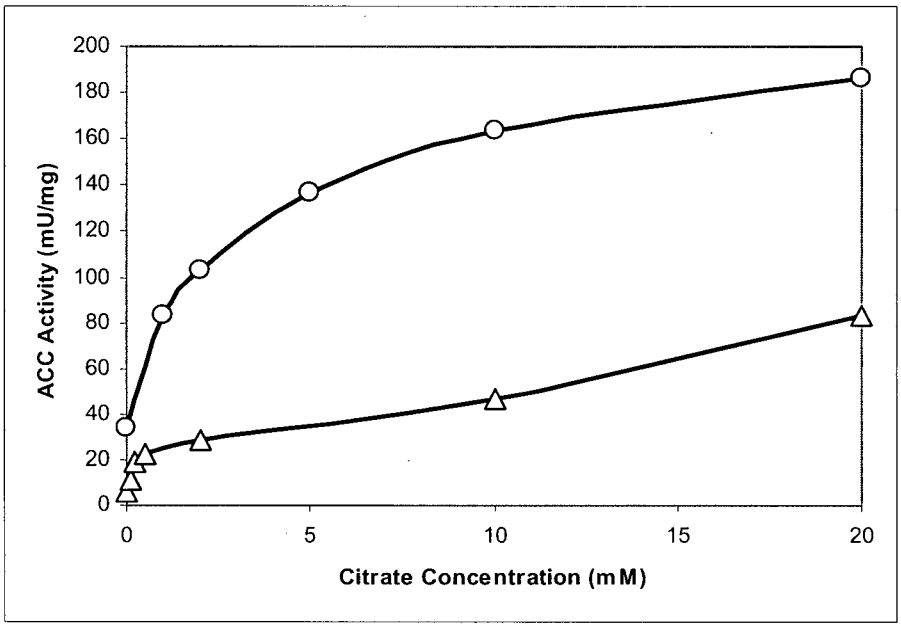
3.2 RESULTS AND DISCUSSION

3.2.1 ACC Activation by Citrate

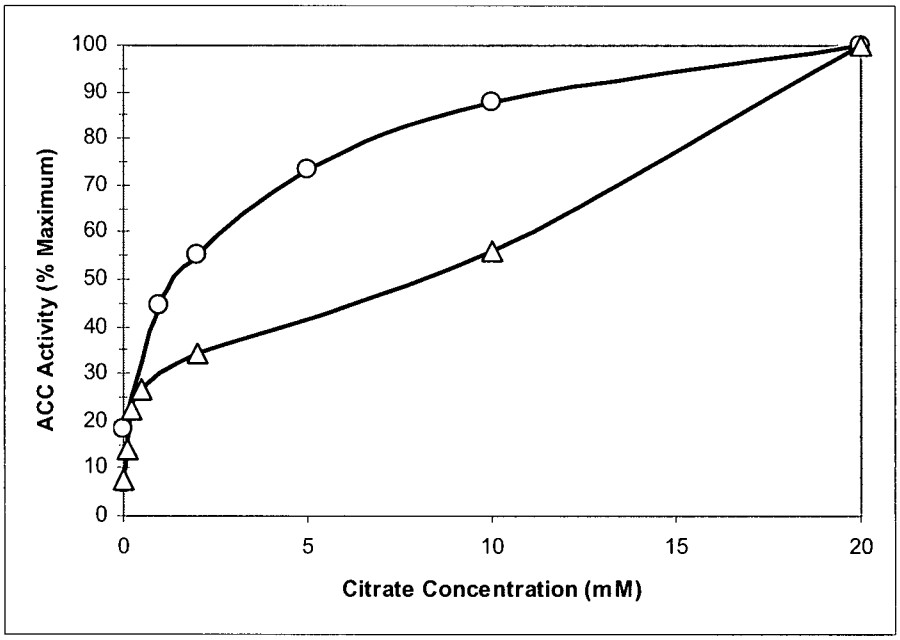
Citrate treatment results in rapid activation of ACC-1 and ACC-2, and, at least in the case of ACC-1, activation is accompanied by polymerization [30]. ACC activation is concentration-dependent, and typically exhibits maximal effect at citrate concentrations around 10-20 mM. Liver typically contains the largest amount of ACC per gram of wet weight tissue (on the order of 200-300 mU/g), followed next by adipose tissue (100-150 mU/g). Both heart and skeletal muscle contain much smaller amounts of ACC than do either liver or fat tissue (usually less than 10 mU/g). The same rank order of activities also apply when ACC activity is expressed as mU/mg protein recovered following ammonium sulphate precipitation. This is illustrated in Figure 3.1, where liver contains more units per milligram than a comparable preparation from muscle. This figure also shows that ACC from both tissues exhibits maximal activation at around 20 mM citrate (no significant further activation occurs with 30 or 40 mM citrate, results not shown). Finally, the sensitivity of ACC to citrate is much higher in extracts from liver (initial steepness of liver curves), largely due to the significant amount of ACC-1 present in this tissue. Over a larger number of experiments, K_a values for citrate with liver/fat ACC are indeed lower than that of ACC from heart or skeletal muscle.

Figure 3.1 *Activation of ACC by Citrate.*

A



B



Preparations of ACC purified by ammonium sulphate precipitation from liver (o) and skeletal muscle (Δ), were incubated with citrate and assayed as described. ACC activity is expressed as mU/mg of protein (A) or as % maximum activity (B). Results for liver are expressed as the mean ± S.E.M. of three experiments, while skeletal muscle data represents a single experiment typical of 3 experiments.

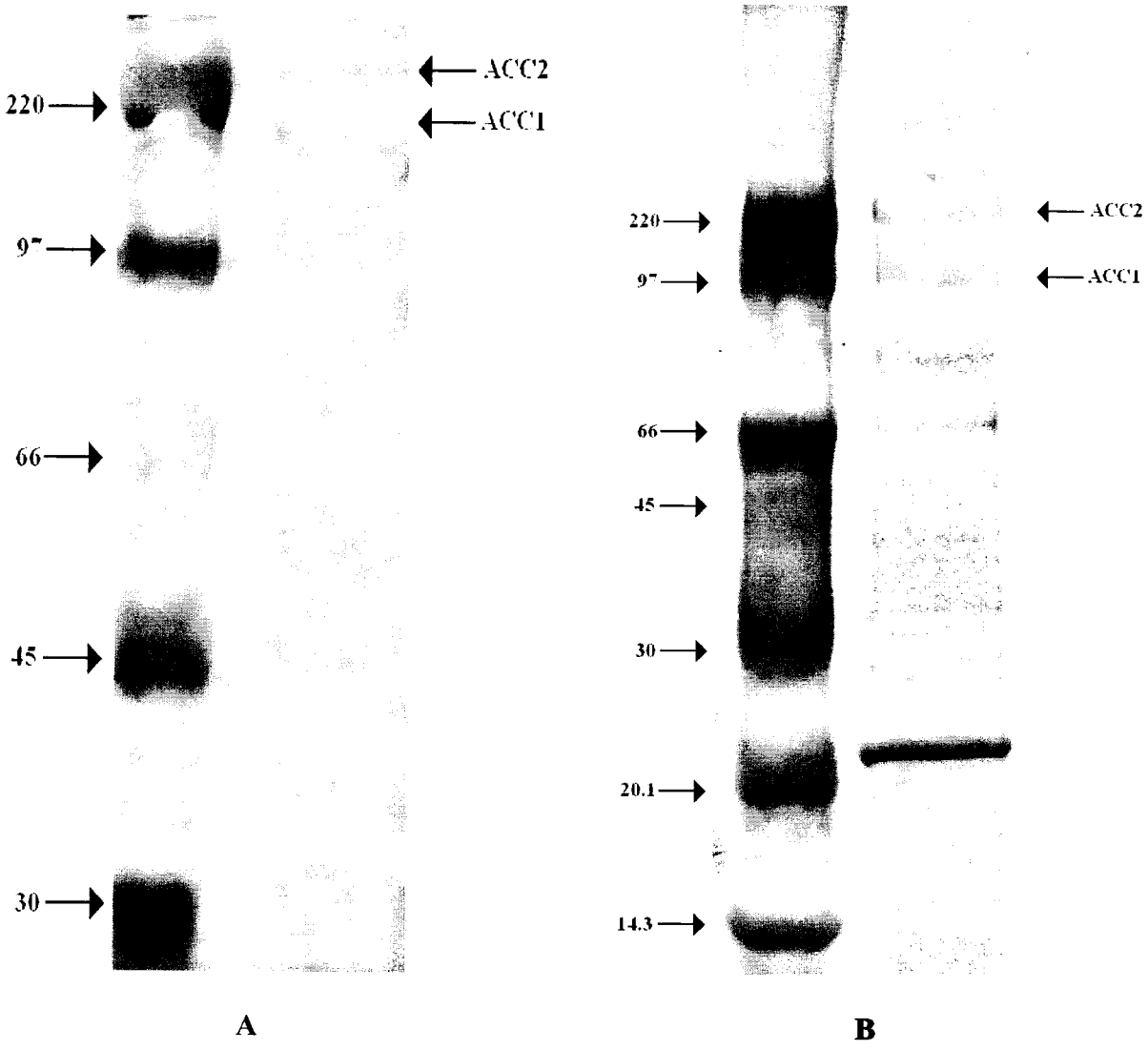
3.2.2 ACC Detection by Electrophoresis

Confirmation and quantitation of ACC in enzyme preparations is usually accomplished by activity assays. However, certain conditions, such as enzyme inactivation, or detection of enzyme in degraded preparations can be difficult using an assay of catalytic activity. Furthermore, the purification of ACC may be easily followed using other methods. Electrophoresis can overcome some of the ACC assay limitations, due mainly to staining sensitivity, and visualization of protein content of a given enzyme preparation.

Typical results of electrophoresis of ACC is given in Figure 3.2. In part (A), a liver ACC ammonium sulphate precipitation sample is run alongside molecular size markers (using a 5% separating gel). In part (B), a sample of another liver ACC ammonium sulphate precipitate was purified over tetrameric avidin beads and electrophoresed (on a 4-15% gradient gel). Although both preparations show ACC 1 and ACC2 bands at approximately the same spot on the gel (relative to the markers), the other bands which stain are different. This is partly due to the differences in run time between the two, as well as differences in the type of gel used and the purification steps. It is important to note that these gels are presented to illustrate the ability to detect the two isoforms by electrophoretic separation, following different purification steps, and for different end purposes. The gel in part (A) was used for visualization purposes only, while the gel in part (B) was used to detect bound PLP, as described in 3.2.9. Western blot analysis can also be performed, but such an analysis is not useful in this thesis and are not described.

Note that the apparent sizes of ACC1 and ACC2 as seen on these gels do not correspond with known values from cDNA sequencing. The reasons for this are due to technical limitations as well as other as yet unknown causes.

Figure 3.2 *ACC Detection by Electrophoresis and Coomassie Blue Staining.*



Liver ACC prepared by ammonium sulphate precipitation was run on a 3%/5% stack/separating acrylamide gel using a PDA cross-linker, for 1.5 hours at 175 volts and 50 milliamps, alongside molecular weight standards (A). A separate liver ammonium sulphate preparation was purified using tetrameric avidin beads and eluted using Sample loading buffer prior to running alongside molecular weight standards on a 4-15% gradient acrylamide gel at 135 volts and 30 milliamps (B). Size standards are indicated as are the positions of ACC1 and ACC2 bands.

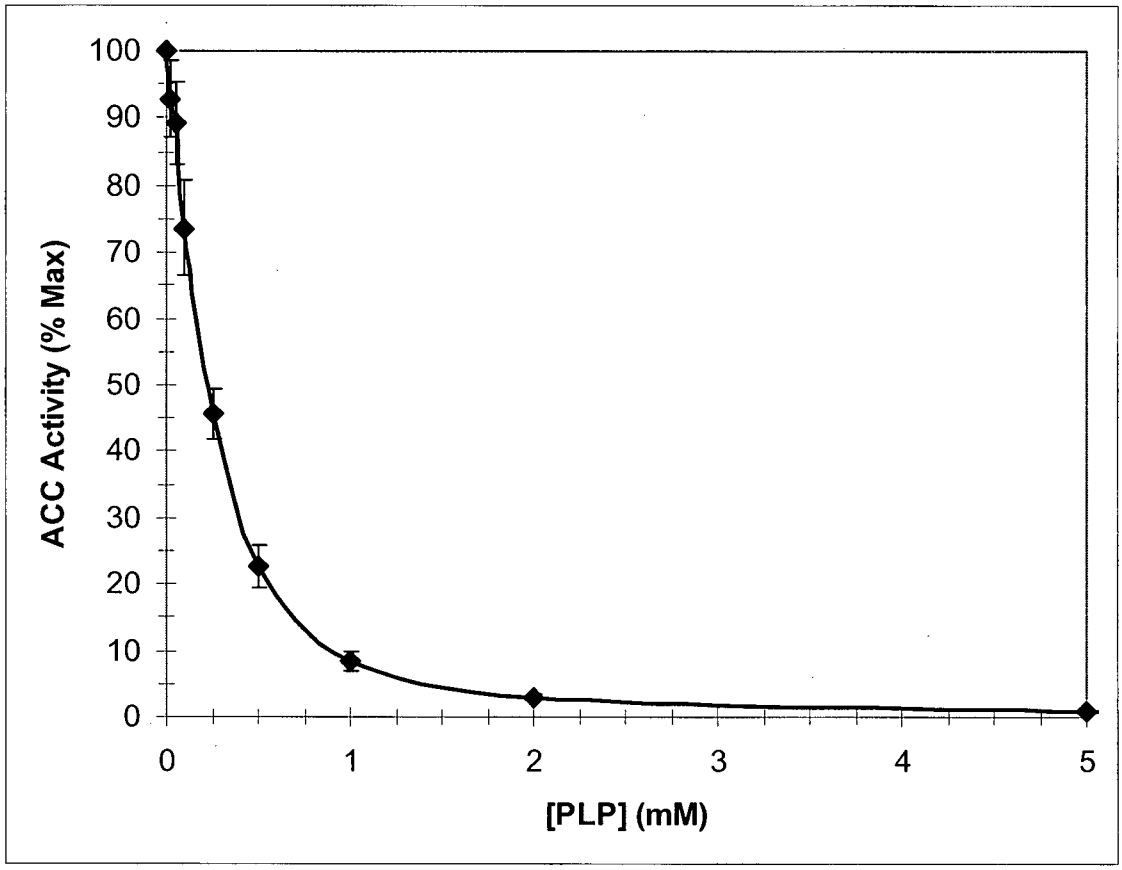
3.2.3 Inhibitory Effect of PLP on ACC

It has been shown that PLP can inhibit phosphofructokinase in a concentration-dependent manner, with an IC_{50} of about 200μM and complete inhibition at around 2 mM, when reduced with sodium borohydride [71]. Similar inhibitory potency was observed in studies of tricarboxylate transporters [72-75] suggesting that PLP might be examined for potential inhibitory effects on ACC over this concentration range. The results of such experiments on liver ACC are shown in Figure 3.3. In this experiment, ACC was partially purified from rat liver and then treated with PLP before citrate activation of the enzyme.

Clearly, PLP has a strong, concentration-dependent inhibitory effect on liver ACC, with an IC_{50} value around 225 μM, and 90% inhibition of enzyme activity seen at PLP concentrations less than 1 mM. Also evident from Figure 3.3 is that 10 mM citrate was unable to reverse the effects of PLP at concentrations as low as 25 μM.

While PLP shows strong inhibitory effects on liver ACC, would it be expected to show similar responses in ACC from other tissue types? Since liver contains both ACC-1 and ACC-2 in significant amounts (3:1 ratio, respectively) it might be concluded that both isoforms are being affect by PLP. However, as shown in Figure 3.1, ACC-2 (from skeletal muscle) is not as citrate sensitive as ACC-1, and this citrate sensitivity could be reflected in ACC-2 sensitivity to PLP inhibition. Experiments were therefore carried out to test the effect of PLP treatment on various ACC preparations and results are shown in Figure 3.4.

Figure 3.3 *PLP Inhibition of Liver ACC.*

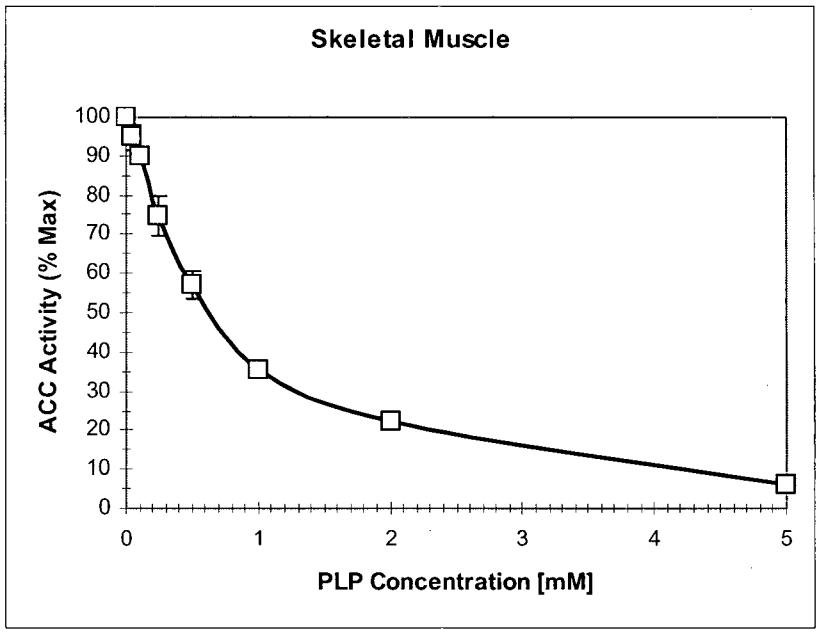


ACC partially purified by ammonium sulphate precipitation of liver was incubated with the PLP concentrations shown for 30 minutes, followed by a 30 minute incubation with 10 mM citrate to activate ACC. Samples were then assayed as described. Results are expressed relative to the maximum activity obtained in each individual experiment (no added PLP) and represent the mean ± S.E.M. for 6 separate ACC and PLP preparations. Some error bars fall within the symbols.

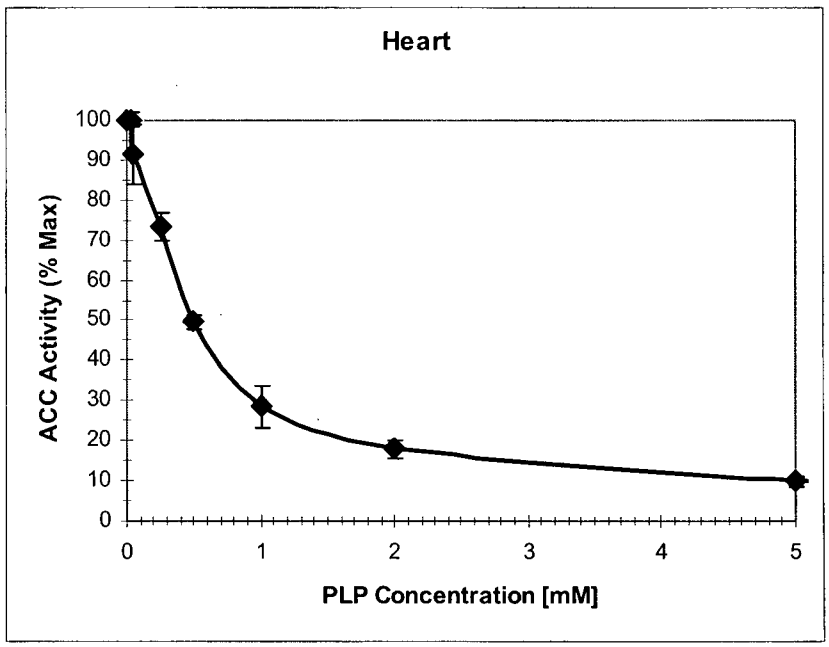
Figure 3.4 *PLP Inhibition of Various ACC Preparations.*

Tissue preparations of skeletal muscle (A, €), heart (B, ♦), liver (C, o) and adipose (D, Δ) ACC purified by ammonium sulphate were treated with PLP for 30 minutes, followed by activation with citrate for 30 minutes and subsequent assay for enzyme activity (all at 37 °C). For comparative purposes, (A) to (D) are combined in (E), with the same symbols used to represent each tissue type as in (A) to (D). Results for (A) and (C) are expressed as the mean ± S.E.M. for 3 and 6 separate experiments, respectively. Results from (B) are expressed as the mean ± range of 2 separate experiments. (D) represents a single experiment. Results are represented as the percentage of the maximum obtained in each individual experiment (no added PLP). Some error bars fall within the symbols.

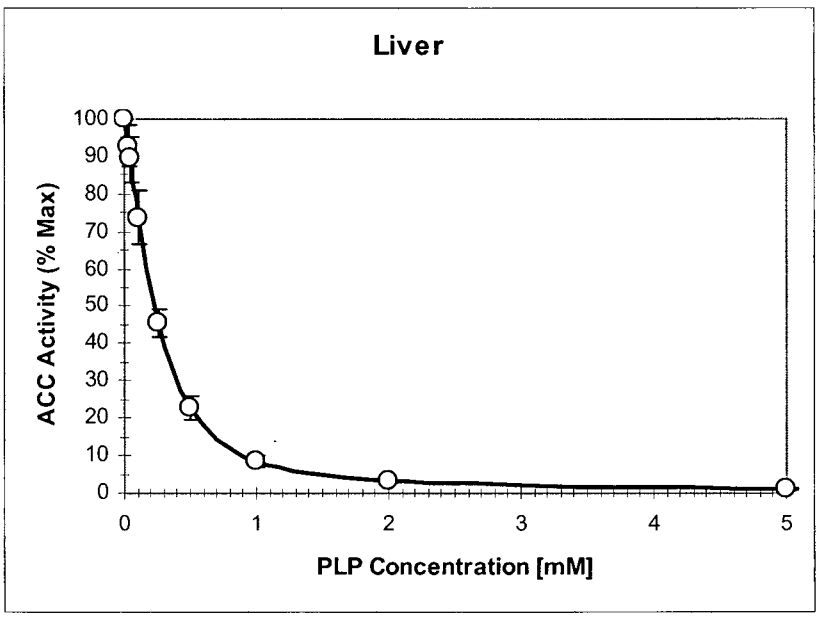
A



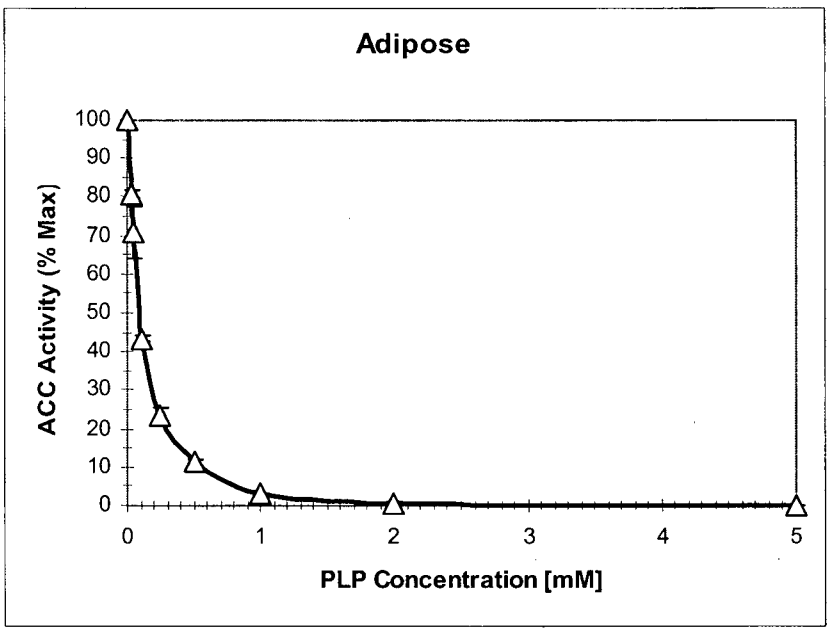
B



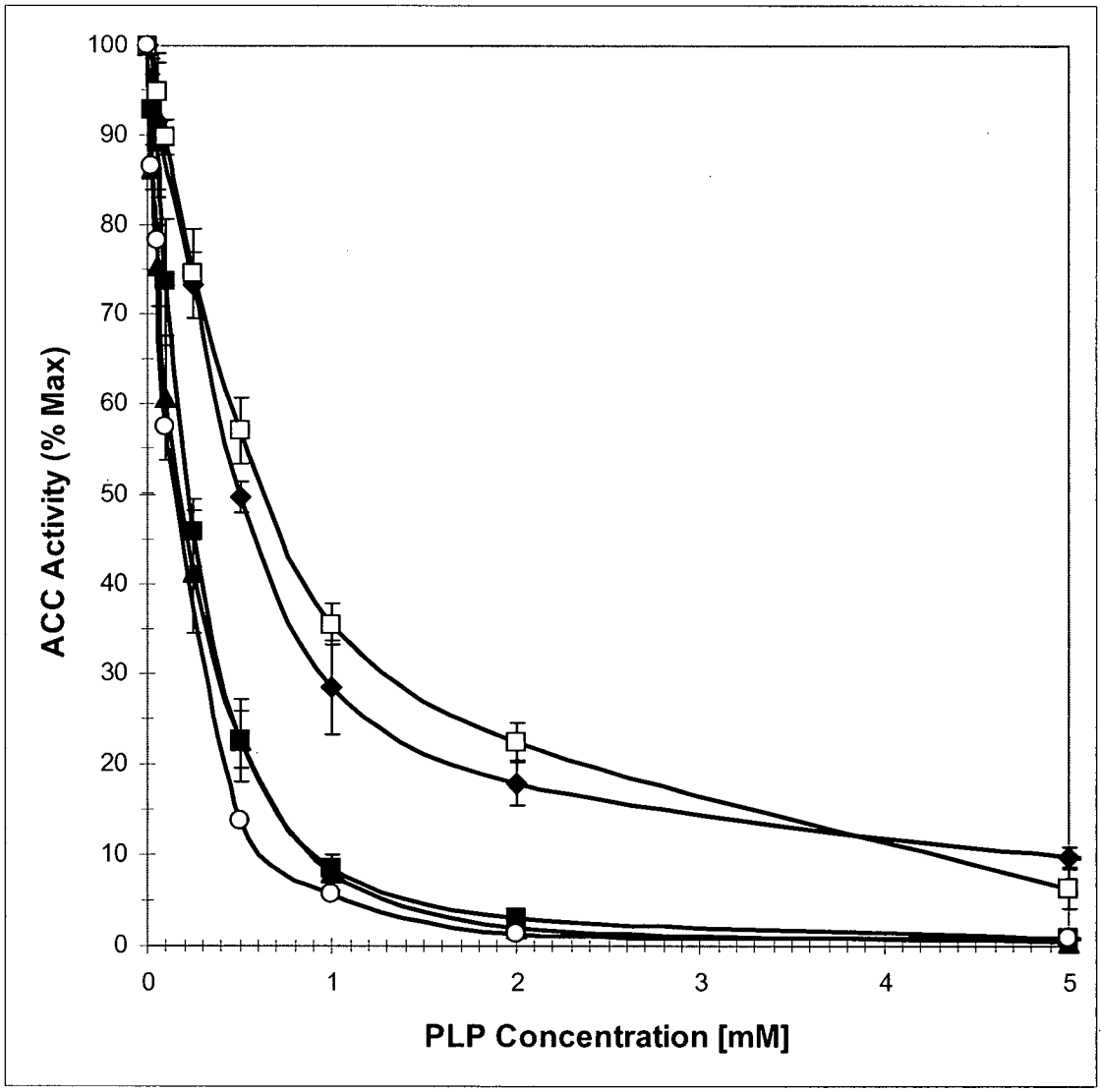
C


Liver
ACC Activity (% Max)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
PLP Concentration [mM]

D


Adipose
ACC Activity (% Max)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
PLP Concentration [mM]

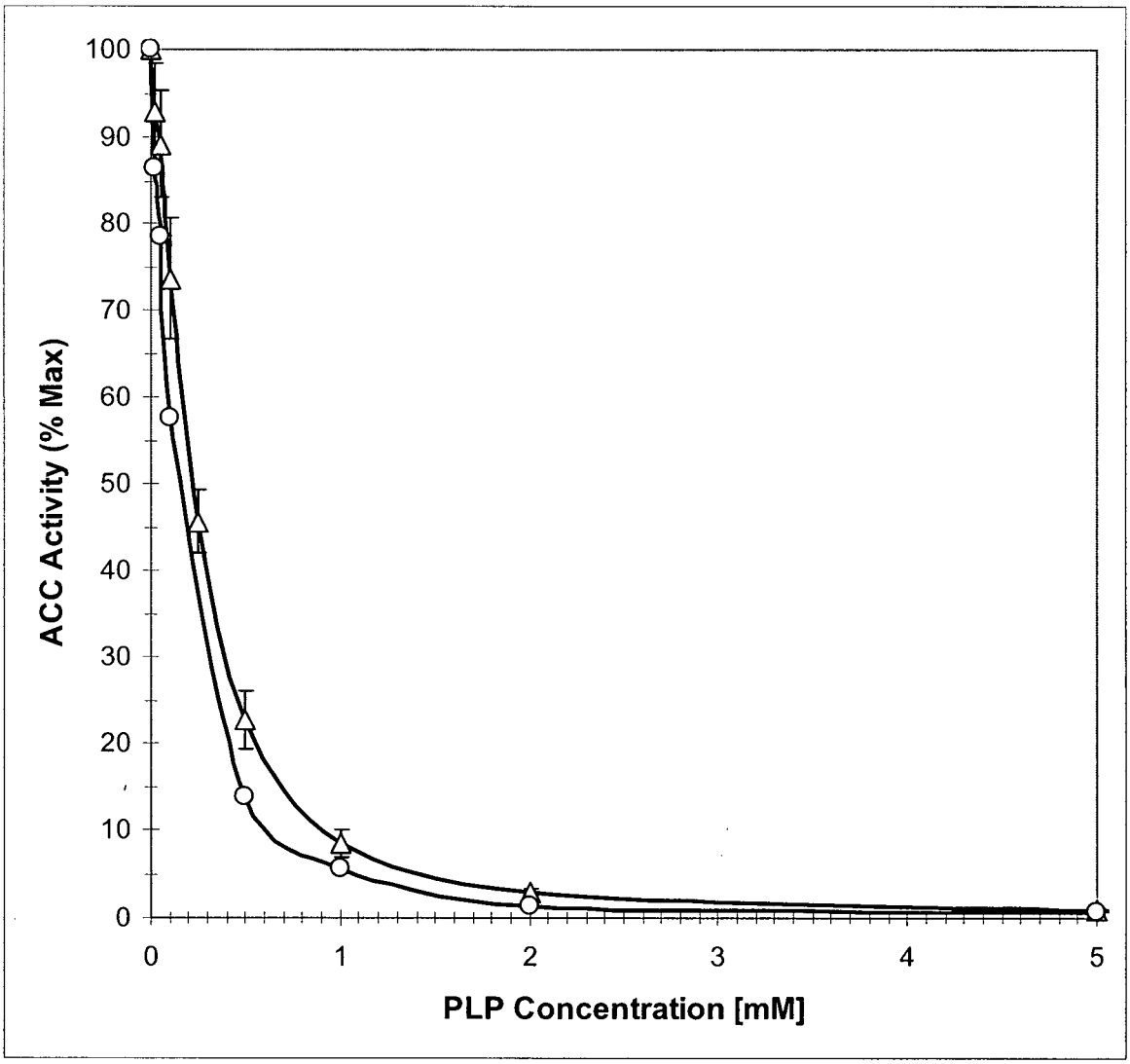
E


ACC Activity (% Max)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
PLP Concentration [mM]

ACC from oxidative tissues (muscle and heart) generally shows less sensitivity to PLP than does enzyme from lipogenic tissues (liver and adipose). Comparison of the curves in Figure 3.4 shows that skeletal muscle ACC (Figure 3.4A) shows the least PLP sensitivity of all tissue types tested, with an IC_{50} around 630 μM, compared to around 225 μM or less for liver and adipose tissue (Figure 3.4C and 3.4D). Heart tissue, which contains slightly more ACC-1 than does skeletal muscle, shows PLP sensitivity between liver and skeletal muscle, with an IC_{50} value near 500 μM (Figure 3.4B). Also significant is the observation that even at high concentrations of PLP (2-5 mM) skeletal muscle and heart ACC, unlike fat and liver ACC, show considerable residual activity. In fact, unlike other tissues, heart ACC is not completely inhibited even at PLP concentrations in excess of 7 mM.

These experiments therefore provide the first evidence for PLP action on ACC, and illustrates at least two important points. First, ACC from all tissue types examined did respond to PLP and all isoforms are inhibited by this compound, even at relatively low concentrations. Second, the ACC isoform composition of the tissues is reflected in the overall sensitivity of the enzyme to PLP. More specifically, tissues containing a greater proportion of ACC-2 seem to show less sensitivity to PLP than those tissue expressing more ACC-1. Whether this is due directly to the difference in citrate sensitivity between the two isoforms or whether other proteins mediate the interaction between PLP and ACC in different tissue preparations is not evident from this experiment. However, as shown in Figure 3.5, the response of liver ACC purified by ammonium sulphate and more highly purified by avidin affinity chromatography show little difference in response to PLP. This suggests that in liver at least, direct binding of PLP to ACC is likely, and that the response of ACC to PLP is not mediated by another interacting protein.

Figure 3.5 *PLP Inhibits Highly Purified ACC.*



Preparations of liver ACC purified by ammonium sulphate (Δ) or avidin-affinity chromatography (o) were treated with PLP for 30 minutes, followed by activation with 10 mM citrate for 30 minutes and subsequent assay for enzyme activity (all at 37 °C). Results for ammonium sulphate purified ACC are expressed as the mean ± S.E.M. for 6 separate experiments, while those for avidin purified ACC are for a single experiment. Results are represented as the percentage of the maximum obtained in each individual experiment (no added PLP). Some error bars fall within the symbols.

3.2.4 Time Dependence of PLP Inhibition

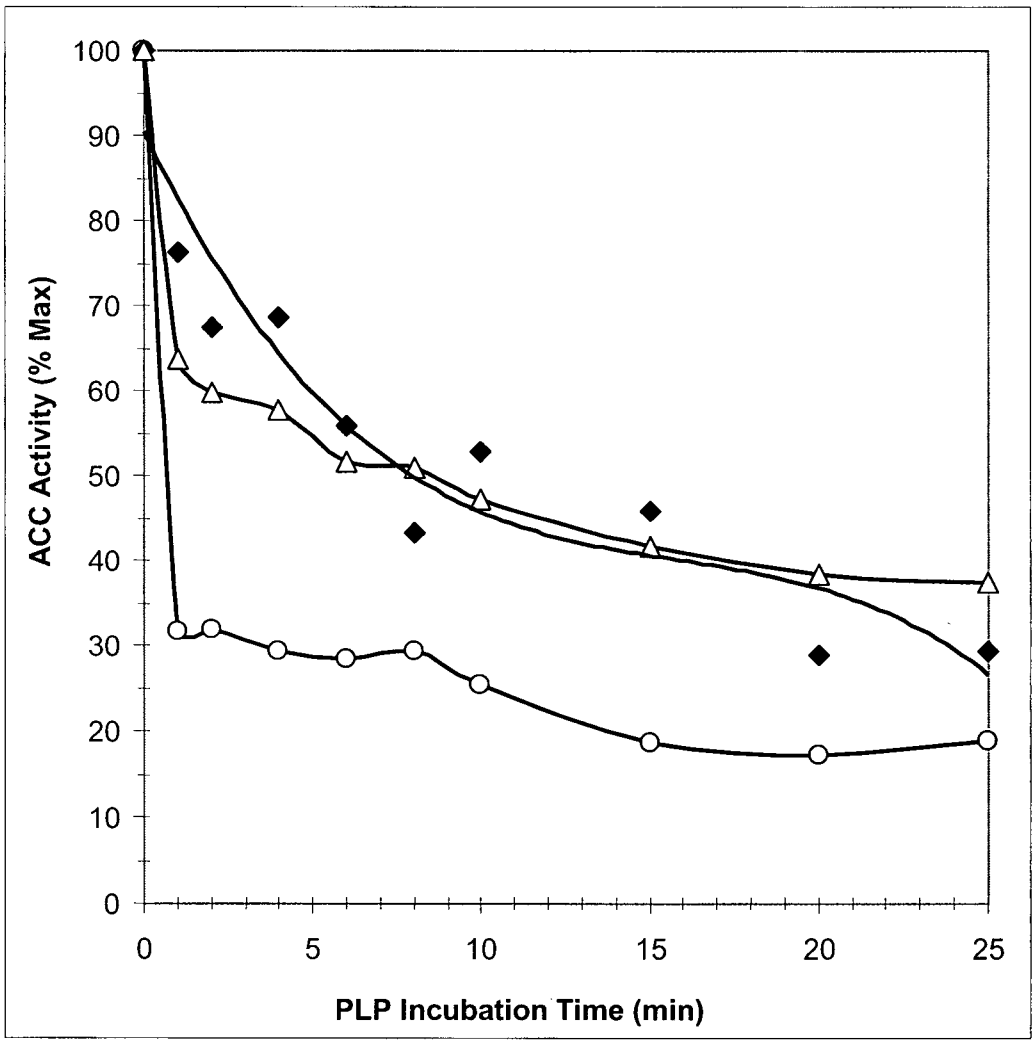
Regardless of whether PLP is acting at the citrate binding site, or another distinct site, certain aspects of its inhibition on ACC can be explored. One of these is the time dependence of inhibition; specifically, how fast does PLP inhibit ACC? This question was addressed by incubating ACC with PLP, followed by a citrate incubation and assay. The results of such experiments are given in Figures 3.6 , 3.7 and 3.8.

Figure 3.6 describes the effects of ACC incubated 0 to 25 minutes with 500 μM PLP, prior to citrate addition and subsequent assay. ACC activity loss with PLP incubation appeared to occur in two distinct phases; an initial rapid loss of activity (< 2 minutes of PLP incubation), followed by a slower, continuing decline in ACC activity (2 to 25 minutes of PLP incubation). Interestingly, liver ACC preparations showed the most extensive initial, very rapid decrease in ACC activity (~ 70% loss), followed by a linear, gradual decrease in activity as PLP incubation time increased. In contrast, heart and skeletal muscle ACC seem to show a less extensive initial rapid loss of activity (25-35% loss); followed by a further gradual decrease in activity with increasing incubation time, in a roughly linear fashion.

Conclusions from this experiment must be drawn cautiously because only one trial of each tissue type was analyzed. Also, since these results occur for ACC incubated with PLP, then followed by citrate incubation, the effective incubation time with PLP is actually the time given, plus the 30 minutes of citrate incubation. Additionally, whether the liver, heart and skeletal muscle ACC responses truly differ and whether this difference can be attributed to the isoform composition differences between the two tissues is not certain from this data. However, given the observation that muscle ACC in previous experiments appears to give significantly different results than ACC in liver, heart or adipose tissue, the isoform

differences cannot be ignored as a possible explanation of the differences in this experiment. Additional experiments were therefore carried out to focus on shorter times of PLP treatment.

Figure 3.6 *Time Dependence of PLP Inhibition of ACC – Long-Term.*



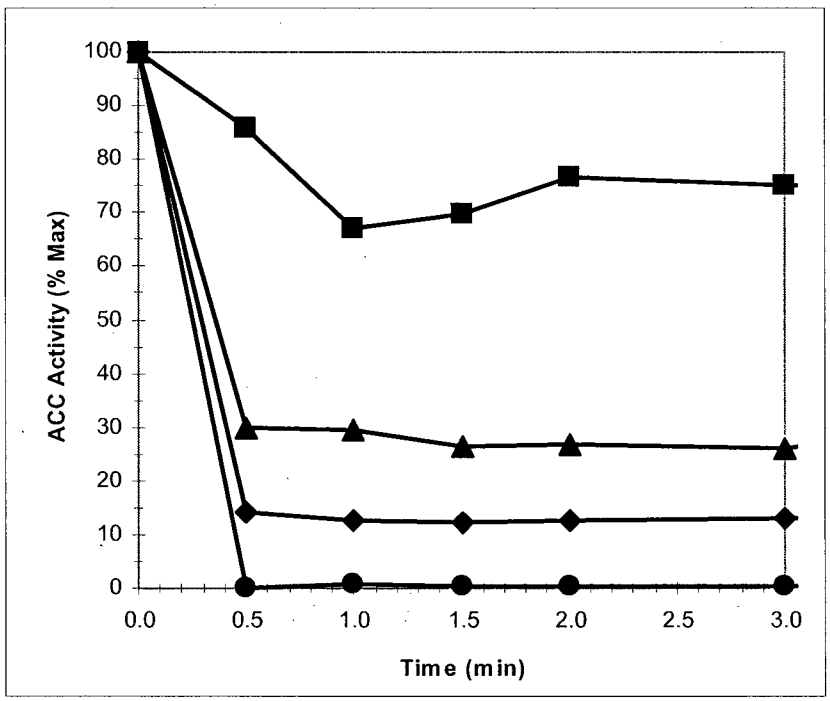
Preparations of ACC partially purified by ammonium sulphate precipitation of liver (o), muscle (♦) and heart (Δ), were incubated with 500 μM PLP for the indicated time (1 to 25 minutes), followed by a further incubation of 30 minutes with 20 mM citrate and subsequent assay. Results are all of single experiments and are represented as the percentage of the maximum obtained in each individual experiment (no added PLP).

In Figure 3.7, the short-term PLP incubation effects on ACC inhibition were examined for liver, skeletal muscle and heart ACC. In all of the short incubations, the effects of PLP are seen within 30 to 60 seconds, with very little subsequent inhibition of ACC seen from 1 to 4 minutes, indicating that PLP's effect on ACC is indeed very rapid. As well, the lack of variation in activity after the 30 second time point in most samples indicates that following the initial inhibition, PLP does not cause further inhibition, at least in the short term. The greatest difference between tissues is evident between liver (Figure 3.7A) and skeletal muscle (Figure 3.7C), with liver ACC activity remaining largely constant after the initial inhibition. Comparison of the results at 500 μM show that for liver, the results correlate very well with those shown in Figure 3.6 (also 500 μM) (both ~ 30% inhibition). Short-term skeletal muscle and heart (Figure 3.7B) inhibition levels do not correlate as well to long-term data as compared to liver, but are not considerably different. As mentioned previously, since these results represent only single experiments, some variability is to be expected.

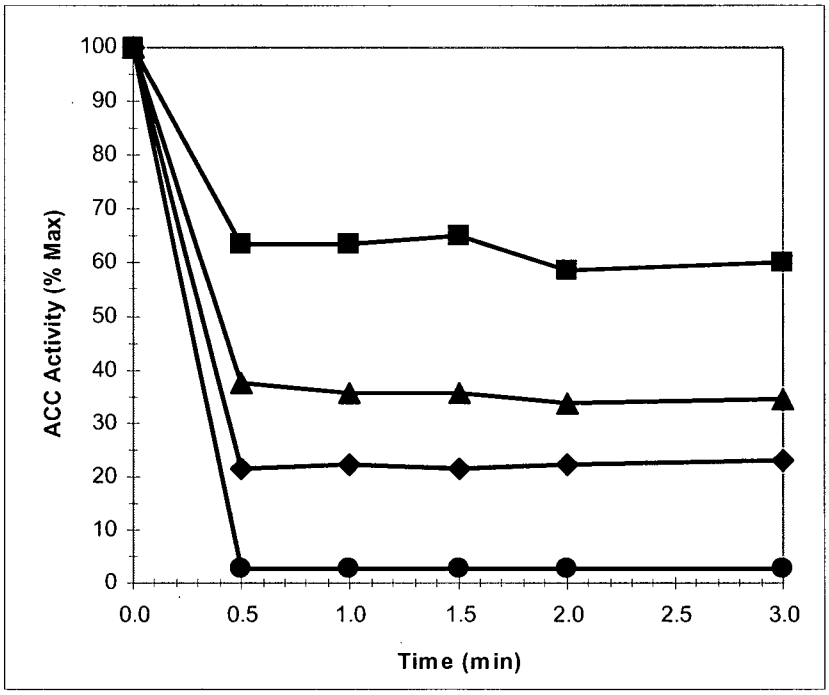
Figure 3.7 *Time Dependence of PLP Inhibition of Liver, Heart and Skeletal Muscle ACC.*

ACC partially purified by ammonium sulphate precipitation of liver (A), heart (B) and skeletal muscle (C) was incubated with PLP for 30 seconds to 4 minutes, followed by 30 minutes of 20 mM citrate incubation and subsequent assay. PLP concentrations tested were 100 μM (€), 500 μM (Δ), 1 mM (♦) and 5 mM (•). Results are all of single experiments and are represented as the percentage of the maximum obtained in each individual experiment (no added PLP).

A



B


ACC Activity (% Max)
100
90
80
70
60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (min)

C

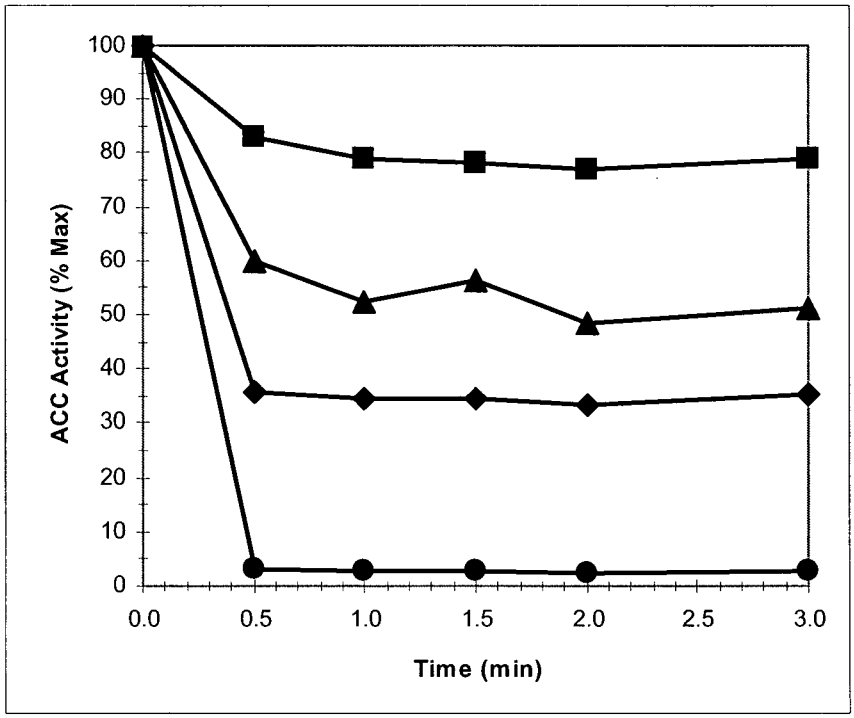
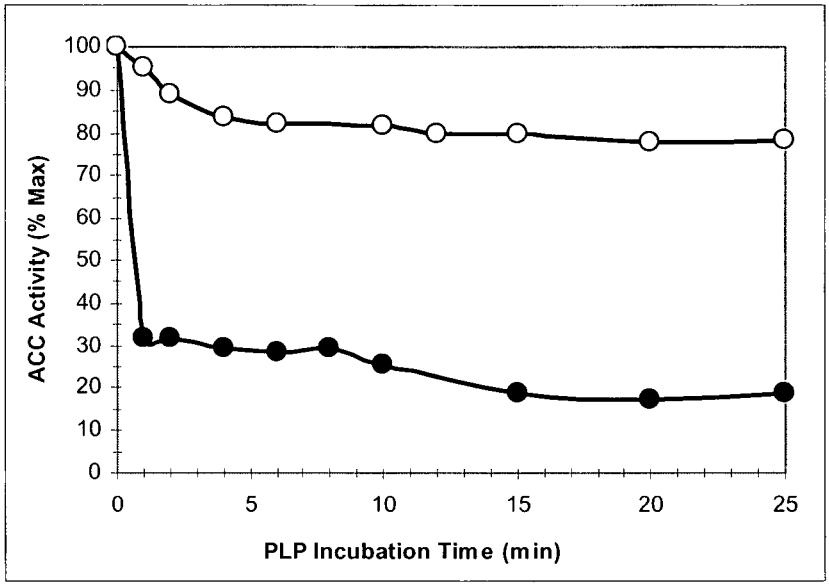
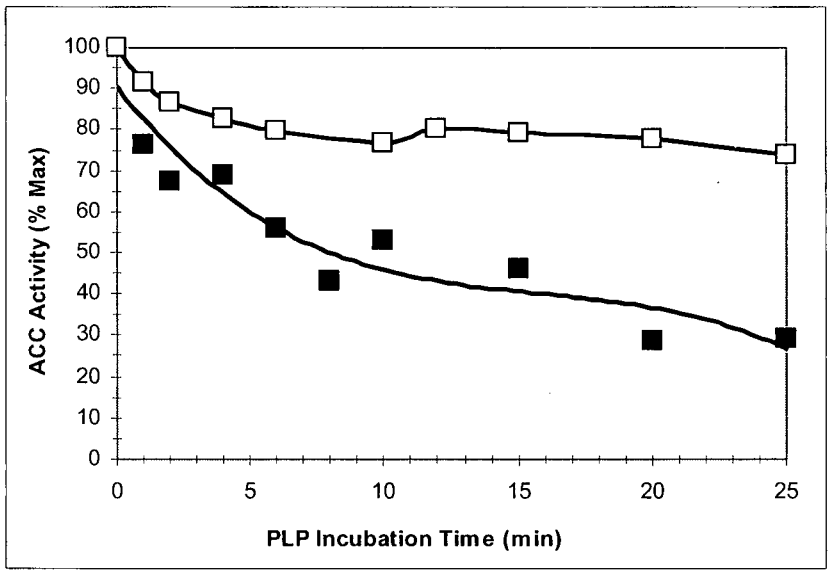

ACC Activity (% Max)
100
90
80
70
60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (min)

Figure 3.8 *Effect of Citrate Pre-Incubation on Time Dependence of PLP Inhibition of Liver and Skeletal Muscle ACC.*

A



B



ACC partially purified by ammonium sulphate precipitation of liver (A) and skeletal muscle (B) was incubated with 20 mM citrate for 30 minutes, followed by incubation with PLP for 1 to 25 minutes, and subsequent assay (open symbols). Results shown by filled symbols represent the same curves as are shown in Figure 3.6, and have ACC incubated first with 0.5 mM PLP, followed by 30 minute incubation with 20 mM citrate. PLP concentrations tested were 500 μM. Results are all of single experiments and are represented as the percentage of the maximum obtained in each individual experiment (no added PLP).

In Figures 3.8 the effect of citrate pre-incubation on PLP inhibition time was explored in liver (Figure 3.8A) and muscle (Figure 3.8B), respectively. These results are somewhat surprising, since the absolute degree of protection against PLP inhibition offered by citrate is very similar between the two tissues, somewhat contrary to the results shown in Figure 3.9 (see 3.2.5). However, citrate does offer better protection against PLP in liver compared to muscle, which supports the results in Figure 3.9. It is also interesting that the rate of PLP inhibition of ACC for both liver and muscle is slowed significantly with citrate pre-incubation, with maximal inhibition seen at 5 minutes, compared to 30 seconds without citrate pre-incubation. This suggests that citrate somehow prevents PLP inhibition of ACC. Whether this is simply due to citrate physically blocking the binding site, or due to a conformational change induced by citrate binding is unclear. An important distinction with these experiments and those shown in Figure 3.7 is that in Figure 3.8, ACC was treated for 30 minutes with citrate, and then incubated with PLP. This means that PLP incubation time with ACC is actually the time shown (i.e. there is no additional 30 minutes due to subsequent citrate incubation).

Another interesting result from Figures 3.7 and 3.8 confirms the concept that ACC-2 is less sensitive to PLP than is ACC-1. More precisely, at lower PLP concentrations, muscle ACC does not show the same level of inhibition seen in liver and heart. For example, in Figure 3.7A it can be seen that with 1 mM PLP, liver is inhibited to about 15% residual activity and heart to about 20% (Figure 3.7B), while muscle shows less inhibition, at around 35% residual ACC activity (Figure 3.7C). This result agrees with that seen in Figure 3.4.

3.2.5 Citrate Protection Against PLP Inhibition of ACC

The experiments described so far provide evidence that PLP does inhibit ACC, but do not conclusively show that PLP interacts at the citrate binding site. Although there is evidence that PLP does interact at the allosteric citrate binding site of PFK [69-71], and at the active site of tricarboxylate transporters [72-74], there is no guarantee that PLP acts at the allosteric citrate binding site on ACC. Definitive proof of this is not easily obtained. In the longer-term it will be important to attempt studies such as those used with enzymes containing known citrate binding sites [69-74]. In those cases, PLP has been covalently linked to the enzyme, the enzyme digested and the PLP-labelled peptide sequenced so that the PLP and citrate binding sites could be compared. Since in ACC the citrate binding site is unknown, a comparison between the PLP and citrate binding sites cannot be performed. Instead, other experimental data must be obtained, such that convincing evidence exists to support the idea that PLP is in fact binding at the citrate binding site on ACC.

One common approach used to address this problem is to use protection experiments, in which one molecule binding at the site of interest can protect the enzyme from the inhibitory or stimulatory effects of another molecule potentially binding at the same site. In this event, citrate might offer some protection to ACC against inhibition by PLP if the two molecules indeed bind at the same site on ACC. Using this approach, ACC preparations were pre-treated with citrate, prior to incubation with PLP. If citrate binding to ACC could prevent PLP binding, ACC activity should remain higher than in assays where ACC was treated with PLP first. The results of such experiments are shown in Figure 3.9.

Citrate offers significant protection to ACC against PLP inhibition, especially at low PLP concentrations for liver ACC (Figure 3.9A) and to a lesser degree for heart ACC (Figure

3.9B). Conversely, in muscle tissue (Figure 3.9C), citrate does not seem to offer any substantial protective effect at any of the concentrations tested. These data provide further support for the idea that PLP and citrate might compete at the same binding site and also further support the fact that ACC from different tissues (likely resultant from the different isoform expression) responds differently to both citrate and PLP.

A comparison of IC_{50} values, as well as the concentrations of PLP required to inhibit ACC to a level of 10 and 90% under the conditions used in the experiment shown in Figure 3.9 is given in Table 3.1.

Table 3.1 *Effect of ACC Pre-Incubation With Citrate on PLP Inhibition.*

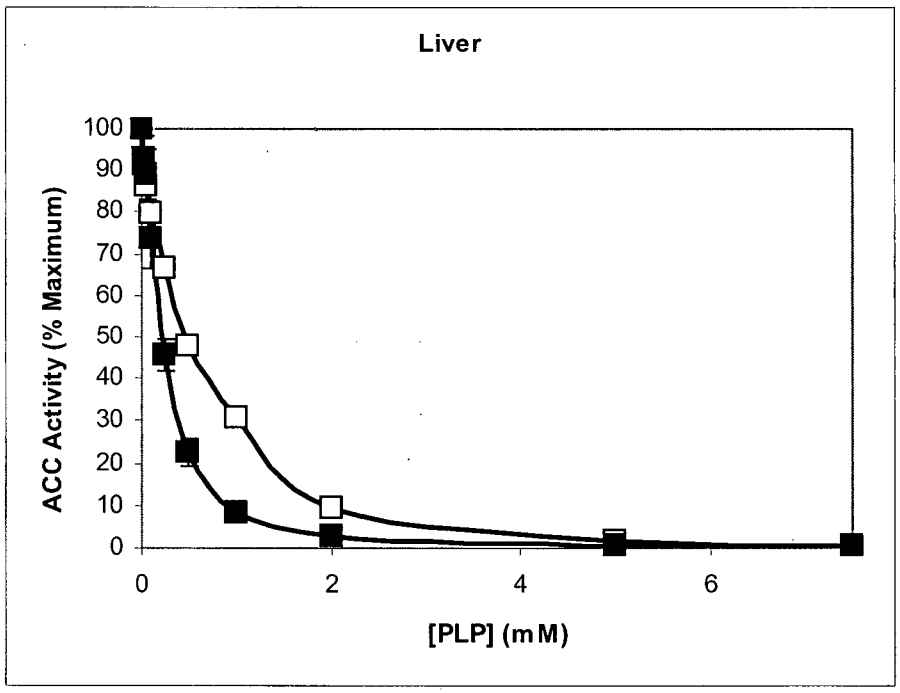
Data for PLP inhibition of ACC is represented as IC_{50} and the concentration of PLP required to show 10 and 90 % inhibition of ACC for the experiments performed as described in Figure 3.9. All results are given as PLP concentrations in millimolar (mM) amounts. 'PLP First' refers to treatment of ACC with PLP prior to citrate activation, while 'Citrate First' implies ACC pre-incubation with citrate before PLP incubation.

Tissue	10% Inhibition		IC_{50} for PLP		90% Inhibition	
	PLP First	Citrate First	PLP First	Citrate First	PLP First	Citrate First
Liver	0.04	0.03	0.22	0.48	0.90	2.0
Heart	0.07	0.22	0.50	1.08	4.90	7.20
Sk. Muscle	0.10	0.08	0.63	0.77	3.60	4.15

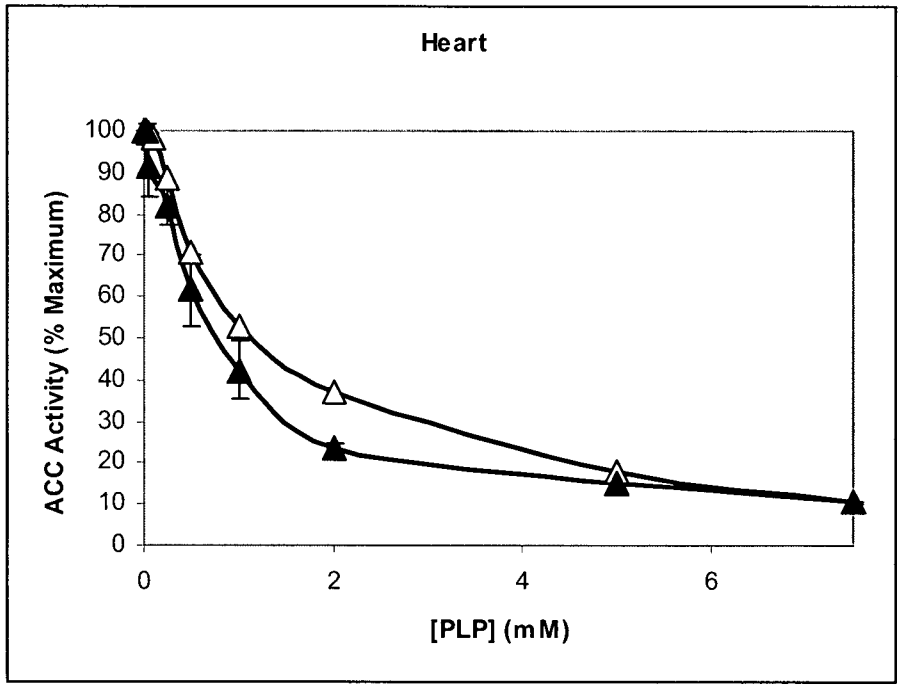
Figure 3.9 *Effect of Citrate Pre-Incubation on PLP Inhibition of ACC.*

ACC was prepared by ammonium sulphate precipitation for liver (A, squares), heart (B, triangles) and skeletal muscle (C, circles). In one set of experiments (open symbols) ACC was treatment first with citrate (10 mM for 30 minutes at 37 °C), followed by a further incubation with PLP (30 minutes at 37 °C at concentrations show) and subsequent enzyme assay. In another set of experiments (filled symbols) ACC was treated first with PLP, then with citrate under the same conditions as the other experiment set. Results for (A) and (C) for filled symbols are expressed as the mean ± S.E.M. for 6 and 3 separate experiments, respectively, while filled-symbol results in (B) are expressed as the mean ± the range for two separate experiments. Open circles in (C) are expressed as mean ± S.E.M. for 3 separate experiments, while those in (A) are the mean ± range for two experiments. Open symbols in (B) are for a single experiment. Some error bars fall within the symbols. Results are represented as the percentage of the maximum obtained in each individual experiment (no added PLP).

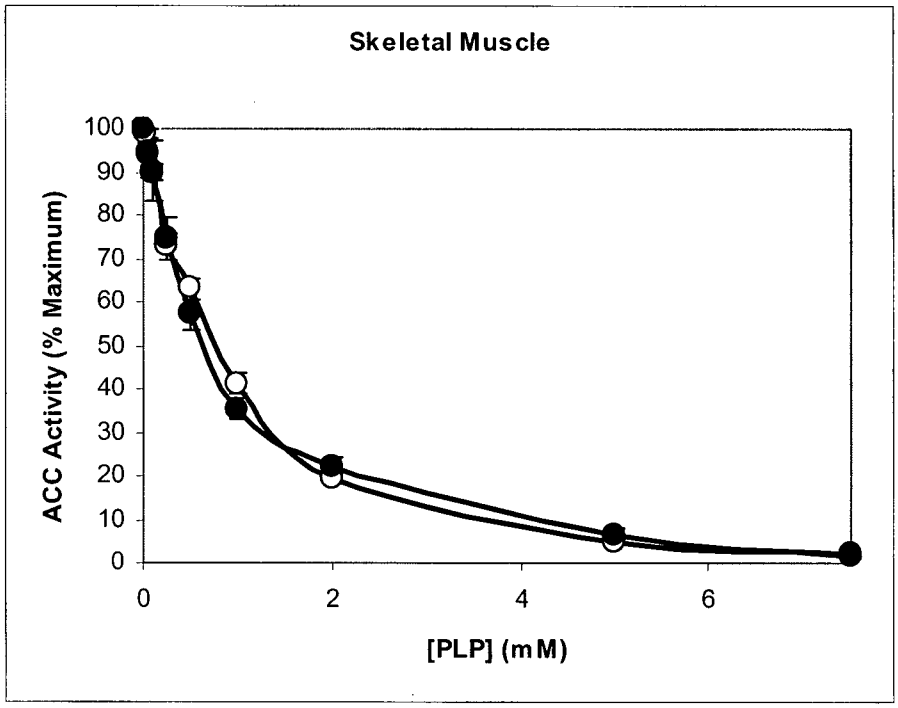
A



B


Heart
ACC Activity (% Maximum)
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
[PLP] (mM)

C


Skeletal Muscle
ACC Activity (% Maximum)
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
[PLP] (mM)

Several interesting results are evident from the data in Table 3.1. First, as expected, liver ACC shows the strongest affinity for PLP, with or without prior citrate treatment. In fact, the concentration of PLP required to inhibit ACC at the levels indicated ranges from 2.5 to 7 fold lower for liver ACC compared to heart and muscle ACC. Secondly, liver ACC also seems to show the best protection against PLP by citrate pre-incubation, especially at the 50 and 90 % inhibition levels. Third, citrate pre-treatment is least effective in skeletal muscle ACC (of the three tissues examined), with the best citrate protection on muscle being a 22 % increase in PLP concentration at IC_{50}.

Importantly, these experiments cannot show definitively that PLP and citrate are binding at the same site. Although citrate does protect ACC from PLP inhibition somewhat at lower concentrations in liver and heart, it does not totally block PLP inhibition at higher concentrations in these tissues, or to any degree in muscle. There are several possible reasons for this. The first could be that PLP can bind much more strongly to the enzyme than can citrate, possibly through it's highly reactive aldehyde, forming a Schiff base. This would imply that even if citrate does bind to ACC, PLP's favourable binding characteristics would cause it to "knock out" citrate and take its place in the binding site. A second possibility is that PLP is actually binding to a site distinct from the citrate binding site, and this somehow causes inactivation of the enzyme. In this scenario, PLP would likely cause a conformational change in ACC such that citrate could not correctly bind and induce enzyme activation, or which prevents citrate activation of the enzyme by some other means, such as affecting substrate binding, or the flexible biotin arm. Previous evidence with other enzymes and proteins would suggest that PLP is binding to the same site as citrate on ACC, but the second

concept cannot be ruled out at this point. Further work which will be essential for determining the identity of the PLP/citrate binding site is discussed later.

Additionally, it would be tempting to assume that citrate induced polymerization of ACC-1 would account for the loss of rapid PLP effect and protection against PLP on citrate pre-treated liver ACC (and to a lesser degree heart ACC), by somehow preventing PLP binding. However, given the observation that ACC-2 does not appear to form polymers [43], and citrate treatment also slows down PLP inhibition of ACC in muscle (Figure 3.8B), albeit less significantly than in liver, it seems that this hypothesis is unlikely and polymerization state of ACC does not significantly affect PLP inhibition. Further work needs to be done to clarify this issue.

An additional experiment was performed, with the goal of establishing the minimum concentration of citrate required to regain maximal ACC activity for a given concentration of PLP. More specifically, following a PLP incubation, various concentrations of citrate were added to the ACC/PLP mixture for 30 minutes and subsequently assayed. The results of such experiments with liver ACC are shown in Figure 3.10.

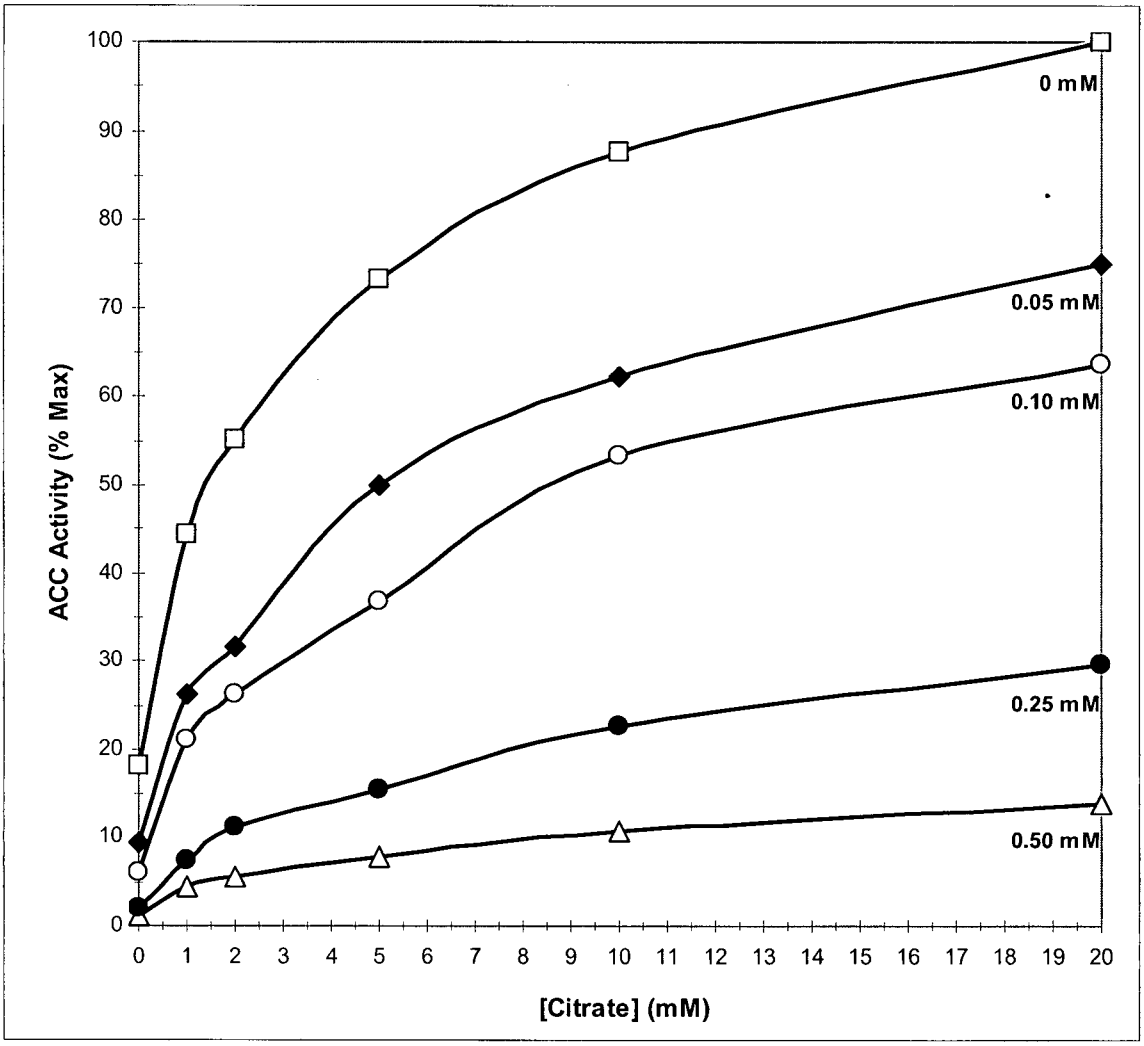
The information in Figure 3.10 is interesting mainly because it shows that as the PLP concentration increases, citrate is less able to overcome the inhibitory effects. For example, comparison of 50 and 500 μM PLP treatment shows that even at maximal citrate (20 mM), only approximately 12% ACC activity is recovered (at 500 μM PLP), while inhibition caused by 50 μM PLP can be recovered approximately 60% by maximal citrate treatment. This has at least two possible implications. First, although strong inhibition of ACC by PLP can be accomplished, and significant loss of enzyme activity can occur even at low PLP

concentrations, there is likely an equilibrium taking place between PLP bound to ACC and free in solution.

This view is supported by previous work which showed that PLP binding to succinic semialdehyde dehydrogenase was reversible and reached equilibrium [76]. Since an equilibrium state is probably reached, it is logical that at low PLP concentrations the amount of ACC with PLP bound is going to be smaller than at higher PLP concentrations. Coinciding with this is the fact that as the citrate concentration increases, citrate is better able to compete for binding sites on ACC, regardless of PLP's greater binding efficiency; simply put, there is just too much citrate present for PLP to compete for all of the binding sites on ACC. However, as the PLP concentration increases, it can in a sense 'saturate' the binding sites, and prevent citrate from binding. Nonetheless, even at lower PLP concentrations (50 μM), citrate treatment cannot recover full enzyme activity, indicating that PLP does show preferential binding to ACC compared to citrate.

Based on these results, it might be instructive to examine effects of even higher concentrations of citrate. Although ACC activation, per se, is not further increased above 20 mM citrate, it is possible that the ability to overcome PLP inhibition might show continued dose-dependency above 20 mM citrate. Such experiments are ongoing.

Figure 3.10 *Citrate Activation Curves at Constant PLP Concentrations.*



ACC prepared from rat liver by ammonium sulphate precipitation was incubated with PLP at 0 mM (€) (control), 0.050 mM (♦), 0.10 mM (o), 0.25 mM (•) and 0.50 mM (Δ) for 30 minutes each, followed by a 30 minute incubation with varying concentrations of citrate as shown, with a subsequent assay for ACC activity (all at 37 °C). Results are represented as the percentage of the maximum obtained with no added PLP and 20 mM citrate. Results are typical for 2 experiments.

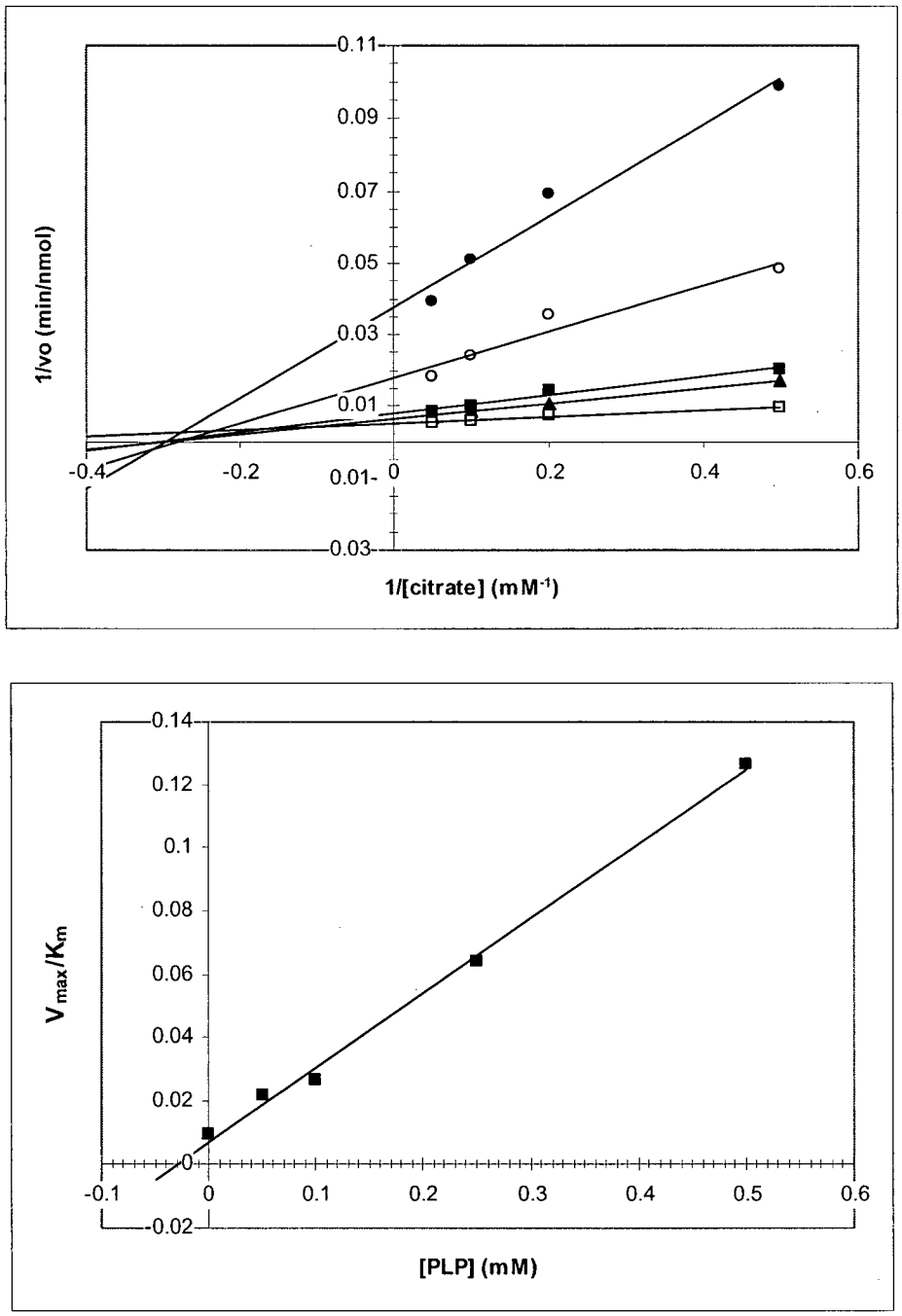
Additional information can be taken from the data shown in Figure 3.10. Since the curves shown can be represented in terms of reaction rate against PLP concentration, a double reciprocal plot (Lineweaver-Burk) can allow a determination of the inhibition type seen using PLP. Further manipulation of this plot, by taking the slopes of the lines and plotting them against the concentration of PLP at which they occur, can give an estimate of the apparent K_i for PLP on ACC. Results of such manipulations are shown in Figure 3.11.

As shown in Figure 3.11A, curves of inverse reaction rate against the inverse citrate concentration, for 5 different concentrations of PLP suggest that the type of inhibition exhibited by PLP on ACC is most likely *mixed*. Both V_{max} and K_m values change with variations in the concentration of PLP, and the curves intersect to the left of the vertical axis, consistent with mixed inhibition.

Figure 3.11B shows the result of plotting the slopes from the curves in Figure 3.11A against the PLP concentrations at which these slopes occur. The curve generated is linear and the intercept of this curve with the x-axis is an estimate of the $-K_i$ value for PLP on ACC. This value is estimated to be about 30 μM.

Care must be taken with these plots, since they are the result of only two experiments, and more importantly, PLP and citrate are not substrates for ACC. Kinetics of allosteric activators are considerably more complex than with substrates, and defining kinetic effects of an inhibitor of an allosteric activator is equally challenging. Figure 3.11 is presented only as introductory example of further kinetic analysis which will be required in order to define more precisely the interaction of PLP with ACC.

Figure 3.11 *Double Reciprocal Plot and Ki Determination.*



Data from the curves in Figure 3.10 was re-plotted as the reciprocal of the rate ($min \cdot nmol^{-1}$) against the inverse citrate concentration (mM^{-1})(Lineweaver-Burk) in (A) and as the slopes of the curves in (A) against the concentration of PLP at which these slopes occur (B). The x-intercept in (B) represents $-K_i$. The curves in (A) are generated from an average of two experiments.

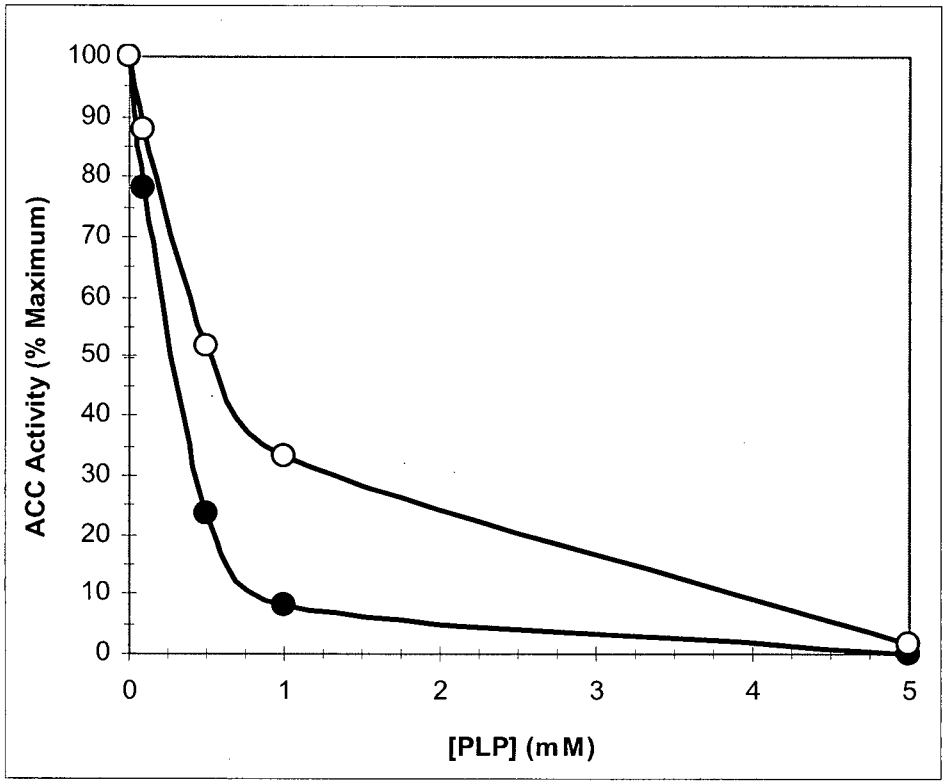
3.2.6 Effect of PLP on ACC Activated by Glutamate

In view of the evidence that glutamate also causes allosteric activation of ACC coupled with changes in enzyme polymerization [33], the effects of PLP on ACC activated by glutamate were examined. The results of these experiments are shown in Figure 3.12. Figures 3.12A and 3.12B show that PLP inhibits glutamate activation of liver and muscle ACC in a similar manner to the citrate activation. Also evident from Figures 3.12C and 3.12D is that pre-incubation with glutamate affords ACC approximately the same level of protection against PLP inhibition as does citrate pre-incubation of the enzyme. Comparison of the IC_{50} values of PLP against ACC alone and ACC pre-incubated with 10 mM citrate or 50 mM glutamate are given in Table 3.2 A general trend in this data is that 50 mM glutamate yields a higher IC_{50} for PLP compared to 10 mM citrate. As previously described, glutamate shares a structure similar to citrate, with two carboxyl groups, and since there is no additive activating effect with citrate and glutamate, there is a high likelihood that both citrate and glutamate bind at the same site [33].

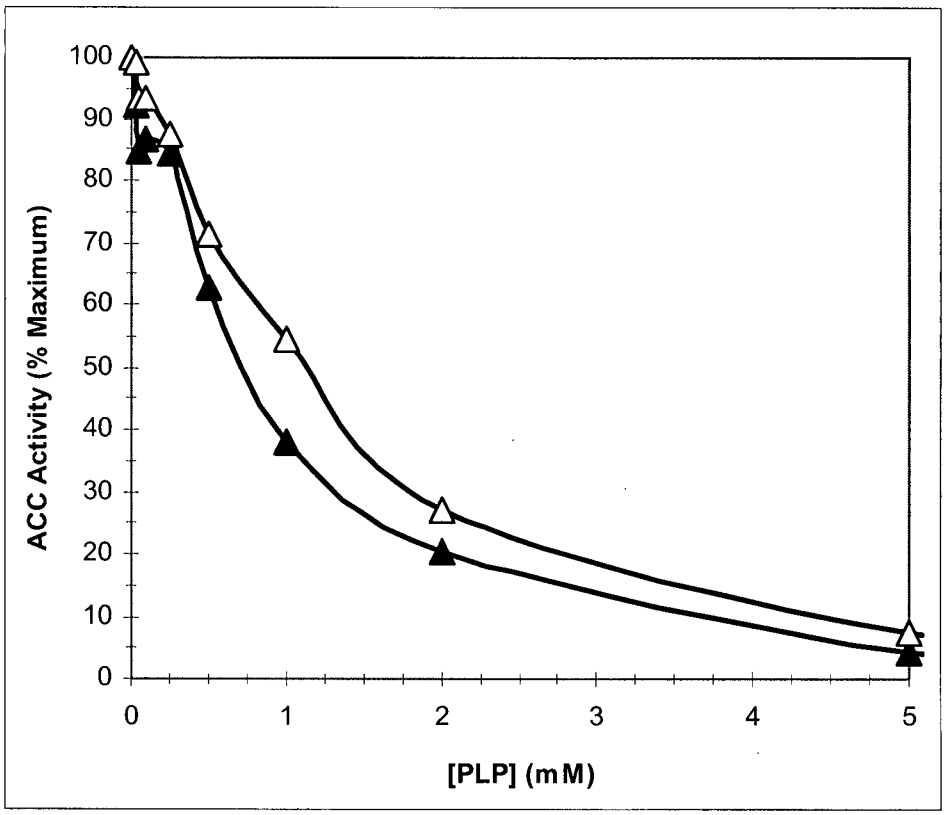
Figure 3.12 *Effect of PLP on ACC Activated by Glutamate.*

ACC purified by ammonium sulphate precipitation from liver (circles) and skeletal muscle (triangles) was incubated with PLP for 30 minutes followed by a 30 minute incubation with 50 mM glutamate or 10 mM citrate, with subsequent assay for ACC activity (filled symbols). Alternatively, the PLP and glutamate/citrate incubations were reversed (open symbols). (A) represents liver ACC with glutamate activation, (B) is skeletal muscle with glutamate activation, (C) is liver and skeletal muscle activated with citrate, and (D) is liver and muscle activated with glutamate. Results in (A and B) are for single experiments each, and those in (C) are as described for Figure 3.9A and 3.9C. (C) and D are presented for comparative purposes between citrate (C) and glutamate (D) activation of liver and skeletal muscle ACC. Results are represented as the percentage of the maximum obtained in each individual experiment (no added PLP).

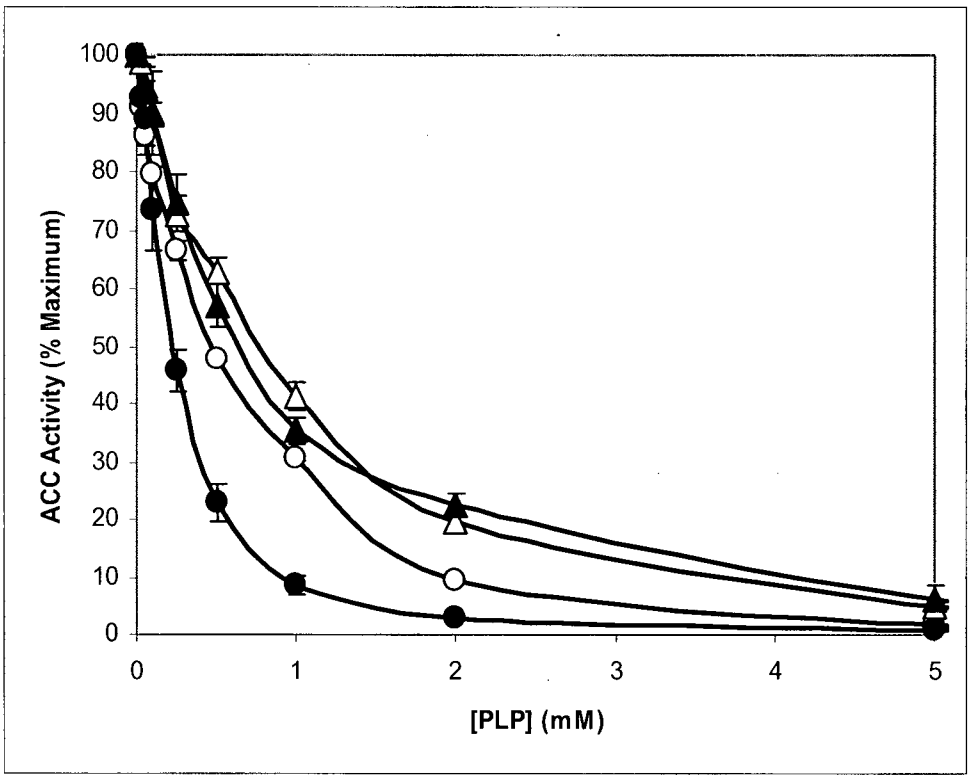
A


ACC Activity (% Maximum)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
[PLP] (mM)

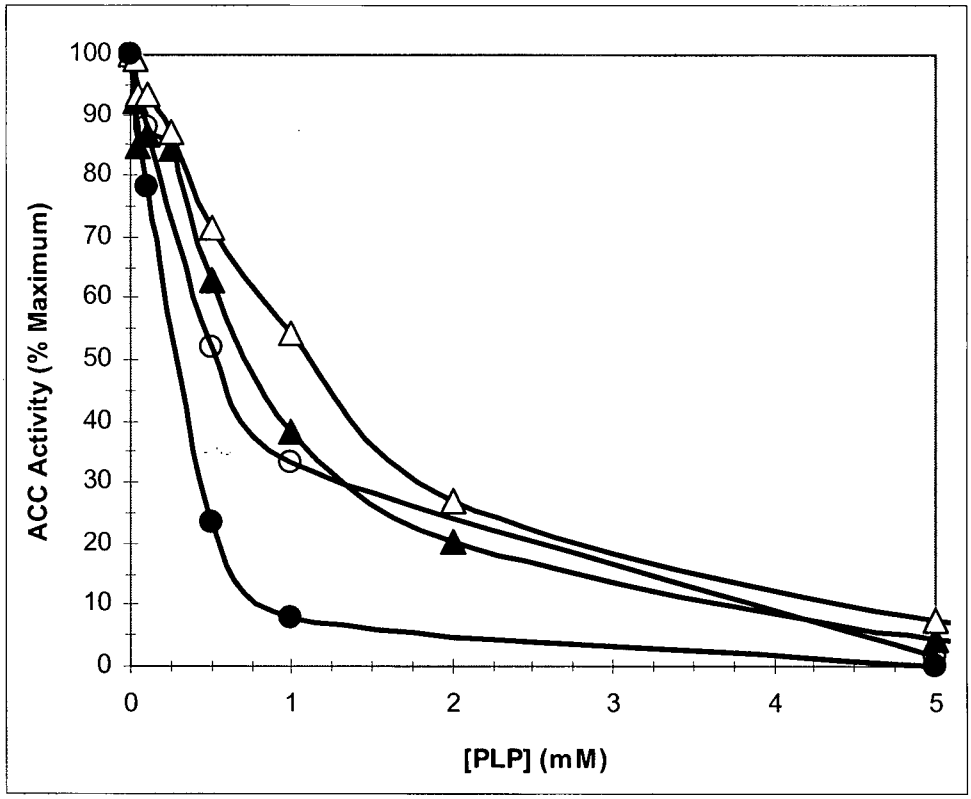
B


ACC Activity (% Maximum)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
[PLP] (mM)

C


ACC Activity (% Maximum)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
[PLP] (mM)

D


ACC Activity (% Maximum)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
[PLP] (mM)

The similarity of PLP inhibition of ACC when activating with citrate and glutamate suggests that both of these activators is binding to the same site. If they were binding to different sites, and PLP interacted at the binding site of one or the other, the results obtained would be expected to differ. The similarity of the inhibition curves provide some additional evidence that these activators likely bind at a common site on ACC, and that both provide some protection from inhibition by PLP.

Table 3.2 *Comparison of ACC Pre-Incubation With Citrate and Glutamate on PLP Inhibition.*

Data for PLP inhibition of ACC is represented as IC_{50} for ACC prepared by ammonium sulphate precipitation and pre-incubated with 10 mM citrate or 50 mM glutamate, for the experiments shown in Figures 3.9 and 3.12, respectively.

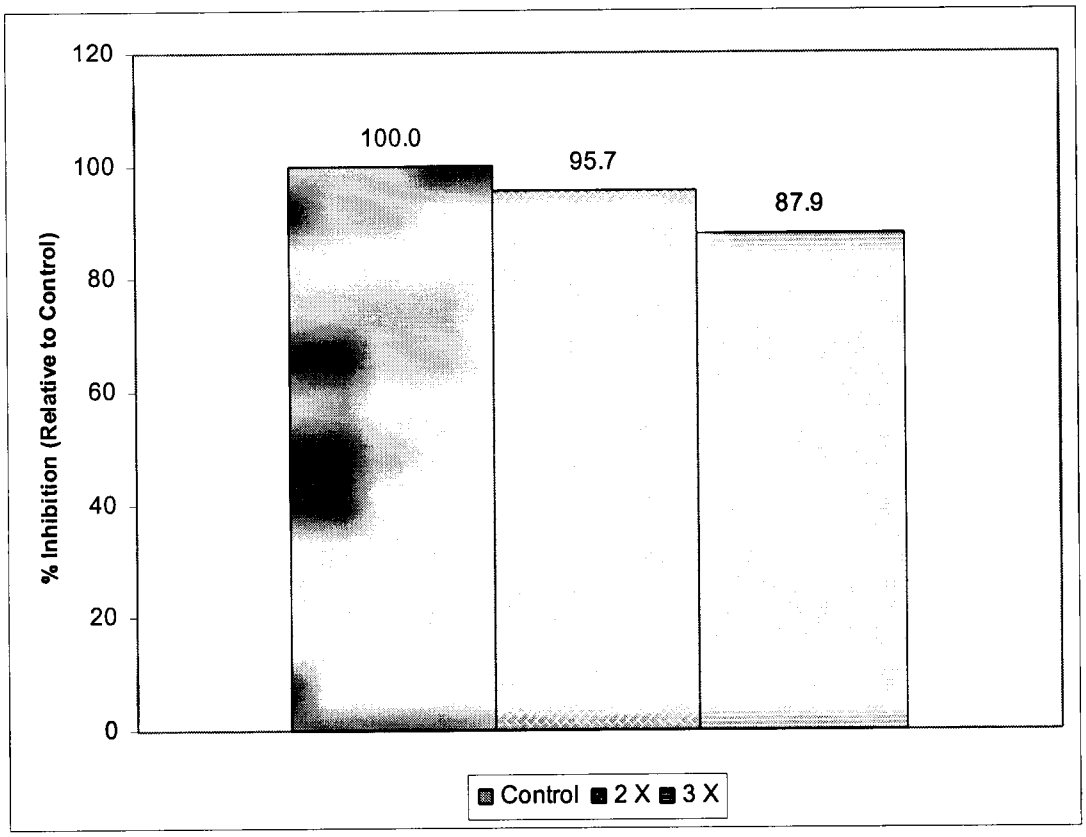
Tissue	**IC_{50} for PLP**			
	Citrate after PLP	**Glutamate after PLP**	**Citrate before PLP**	**Glutamate before PLP**
Liver	220 μM	295 μM	480 μM	530 μM
Sk. Muscle	630 μM	700 μM	770 μM	1100 μM

3.2.7 Potential Substrate Protection Against PLP Inhibition of ACC

Although the experiments described previously cannot definitively show that PLP and citrate are indeed binding at the same site on ACC, it is possible to show, in principle, that PLP is not causing inhibition of ACC by interfering with substrate binding or catalysis. By increasing the amount of substrate present in the assay, ACC is guaranteed to be saturated with substrate. If PLP binds reversibly at or near one of the substrate binding sites, the increased substrate concentration should show some protection against PLP, since substrate would impair the ability of PLP to bind to ACC, simply through physical hindrance. The effects of doubling and tripling the substrate concentration in the ACC assay, with regard to PLP inhibition of ACC, is shown in Figure 3.13.

The data in Figure 3.13 clearly indicates that doubling and tripling the substrate concentration in the ACC assay buffer gave little protective effect to ACC against PLP inhibition. Although this information is based only on one experiment, it suggests that PLP is not binding at or near the active site of ACC. Additional studies need to be done to clarify this result, and a further modification of this experiment currently being examined is to pre-treat ACC with its substrates (individually) and see if incubation prior to PLP treatment affords any protection against inhibition.

Figure 3.13 *Effect of Increasing ACC Substrates on PLP Inhibition of ACC.*



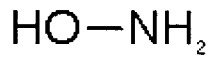
ACC purified by ammonium sulphate precipitation of liver was treated with 0.5 mM PLP 30 minutes at 25 °C in the dark, followed by a 30 minute incubation with 10 mM citrate and subsequent activity assay. The assay buffer contained either the normal concentration of substrates (control), twice the normal amount or three times the normal amount of substrates, as indicated. Results are represented as % inhibition, relative to the control (normal assay buffer), for single experiments.

3.2.8 Effect of PLP Reactive Compounds on PLP-Inhibited ACC

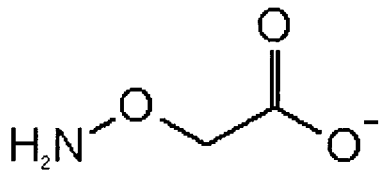
While pyridoxal phosphate has been shown to bind to many different proteins, it has also been shown to establish an equilibrium between the bound and free states [76]. This implies that PLP is not permanently bound to the target protein, and under certain circumstances can be removed. If PLP is indeed reacting to form a Schiff base with free amino group(s) of ACC, then it should be possible to prevent or reverse this effect with compounds that provide amino groups to react with both free PLP and PLP attached to proteins through a Schiff base. Two such PLP-reactive compounds are hydroxylamine (HA) and aminooxyacetate (AOA), whose structures are shown in Figure 3.14.

Because HA and AOA both contain terminal amines, they react with free PLP, forming Schiff base products. HA and AOA thereby compete with the ε-amino group of protein lysyl residues to which PLP normally forms a Schiff base. As well, since PLP is present in a bound and unbound equilibrium, removal of free PLP should induce bound PLP to detach from any proteins to maintain the bound:free equilibrium. In fact, AOA is an effective inhibitor of transaminases and is used to achieve transaminase inhibition, both on purified enzymes *in vitro* and in intact cells [82]. With ACC, loss of PLP binding should relieve inhibition caused by PLP, and activity of the enzyme should be regained when treated with either HA or AOA. The results of experiments testing this idea are shown in Figure 3.15.

Figure 3.14 *Structures of PLP-Reactive Compounds.*



Hydroxylamine

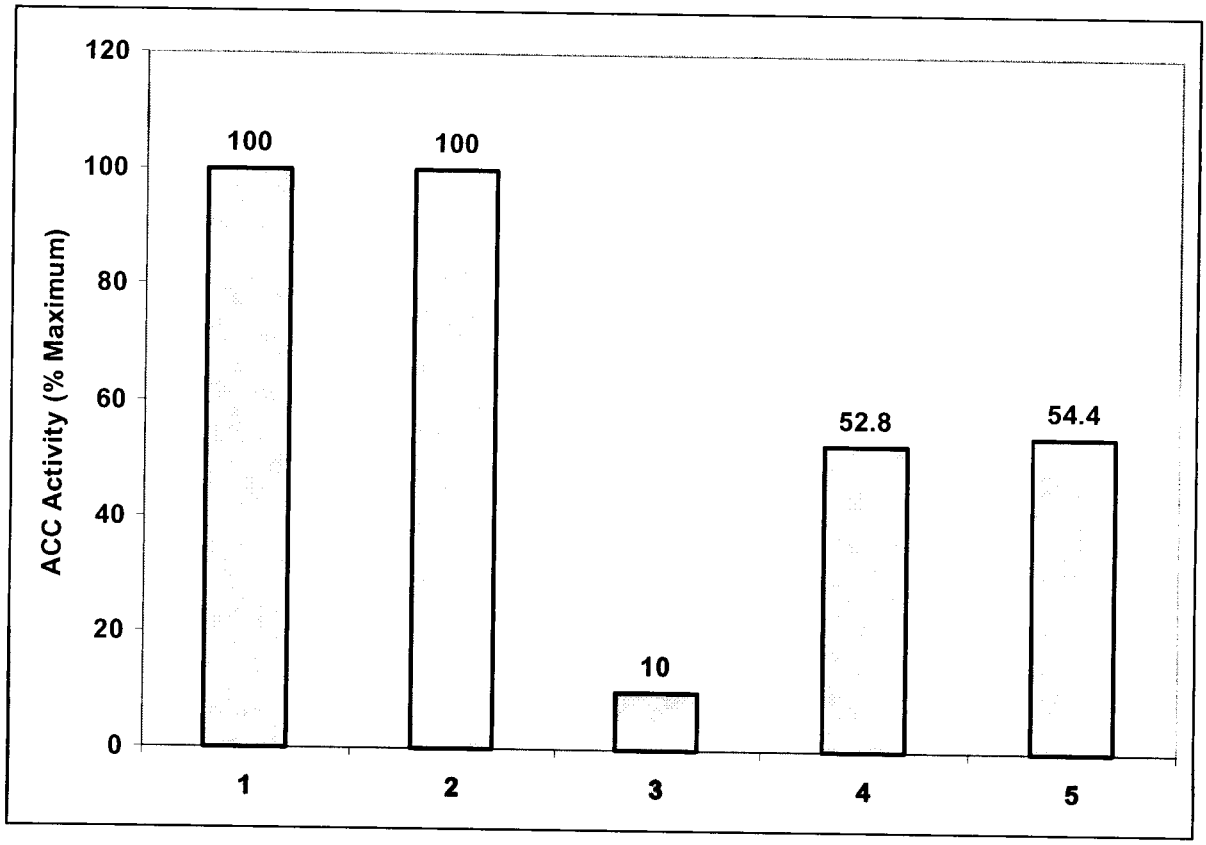


Aminooxyacetate

From Figure 3.15 it is evident that although neither HA or AOA affects the maximum activity of ACC when incubated with the enzyme alone, enzyme previously treated and inhibited by PLP shows recovery of activity when reacted with both HA and AOA. This indicates that both of these compounds are capable of reacting with PLP and in so doing, release some of the inhibition on ACC induced by PLP. This is consistent with previous results using these compounds with other enzymes labelled with or endogenously containing a PLP co-factor [65, 76, 77]. As shown, equal concentrations of PLP and either HA or AOA results in approximately 43% recovery of ACC activity. A summary of the data in Figure 3.15 as well as additional experiments is shown in Table 3.3.

Reducing the concentration of HA and AOA 10-fold resulted in no recovery of ACC activity. As well, higher concentrations of hydroxylamine did not improve recovery of ACC activity, while aminooxyacetate at concentrations above 2 mM showed very poor recovery of ACC activity. An additional experiment using 100 μM PLP showed a similar percentage recovery of ACC activity with 1 mM HA and AOA (compared to Figure 3.15), but less recovery when using 100 μM concentrations of these compounds (Table 3.3). This is interesting, because it suggests that equal concentrations of PLP and either of HA and AOA is not sufficient to recover similar amounts of ACC activity. It seems as though a minimum concentration of 1 mM HA and AOA is required in order to achieve acceptable recovery of ACC activity following PLP inhibition.

Figure 3.15 *Effect of Hydroxylamine and Aminooxyacetate on PLP-treated ACC Activity.*



ACC, partially purified by ammonium sulphate precipitation of liver extracts, was treated for 30 minutes with 1 mM PLP, followed by 30 minute incubation with either 1 mM hydroxylamine (HA) or 1 mM aminooxyacetate (AOA), with a subsequent 10 mM citrate activation and ACC activity assay. (1) and (2) are ACC treated with only HA and AOA, respectively. (3) is ACC treated with only PLP. (4) and (5) are ACC treated with PLP followed by HA or AOA treatment, respectively. Results are expressed as the percentage of the maximum obtained in the absence of PLP, HA or AOA. Results are for single experiments.

The key understanding that arises from these experiments is that PLP does seem to exist in an equilibrium with bound and unbound forms. Addition of HA and AOA can remove free PLP and promote at least some de-inhibition of ACC, by removal of PLP. Results with HA and AOA also provide a possible explanation for the fact that citrate can induce recovery of some ACC activity when added following PLP incubation. Addition of enough citrate, especially at low PLP concentrations can force some of the bound PLP into solution, thus relieving some inhibition of ACC.

3.2.9 Effect of PLP Reduction by Sodium Borohydride

As shown in Figure 3.15 and described elsewhere, PLP seems to exist in an equilibrium between bound and unbound forms [76]. Using this rationale, if ACC treated with PLP were dialyzed or washed thoroughly, it is reasonable to expect that since free PLP would be washed away, some PLP bound to ACC would dissociate, thus relieving inhibition on the enzyme. This hypothesis was tested using an incubation with 0.5 mM PLP followed by incubation with some samples with sodium borohydride at concentrations of 0.5 mM, 1.0 mM and 2.5 mM. Duplicates of these samples were either desalted or assayed directly. Desalting used a buffer containing 20 mM citrate and was performed using 15 mL Biomax-50 centrifugal filter units (Millipore), with multiple buffer changes to ensure complete washing of ACC. Washed enzyme was then concentrated in the same unit and assayed directly for ACC activity. The results of this experiment are shown in Figure 3.16.

Table 3.3 *Effect of Hydroxylamine and Aminooxyacetate on PLP-treated ACC Activity.*

ACC, partially purified by ammonium sulphate precipitation of liver extracts, was treated for 30 minutes with PLP at the concentrations shown, followed by 30 minute incubation with either hydroxylamine (HA) or aminooxyacetate (AOA) at the concentrations shown. The enzyme was then incubated with 10 mM citrate and then subsequently assayed. (% Activity Recovered) refers to the percentage of total ACC activity (of control) recovered following treatment with either HA or AOA. Results are for single experiments. Some results are also presented in Figure 3.15.

[PLP] (mM)	[HA] (mM)	[AOA] (mM)	% Activity Recovered
0.1	1.0	-	44
0.1	-	1.0	26
0.1	0.1	-	27
0.1	-	0.1	31
1	1.0	-	43
1	-	1.0	44
1	0.1	-	0
1	-	0.1	0
1	10	-	36
1	-	6	4

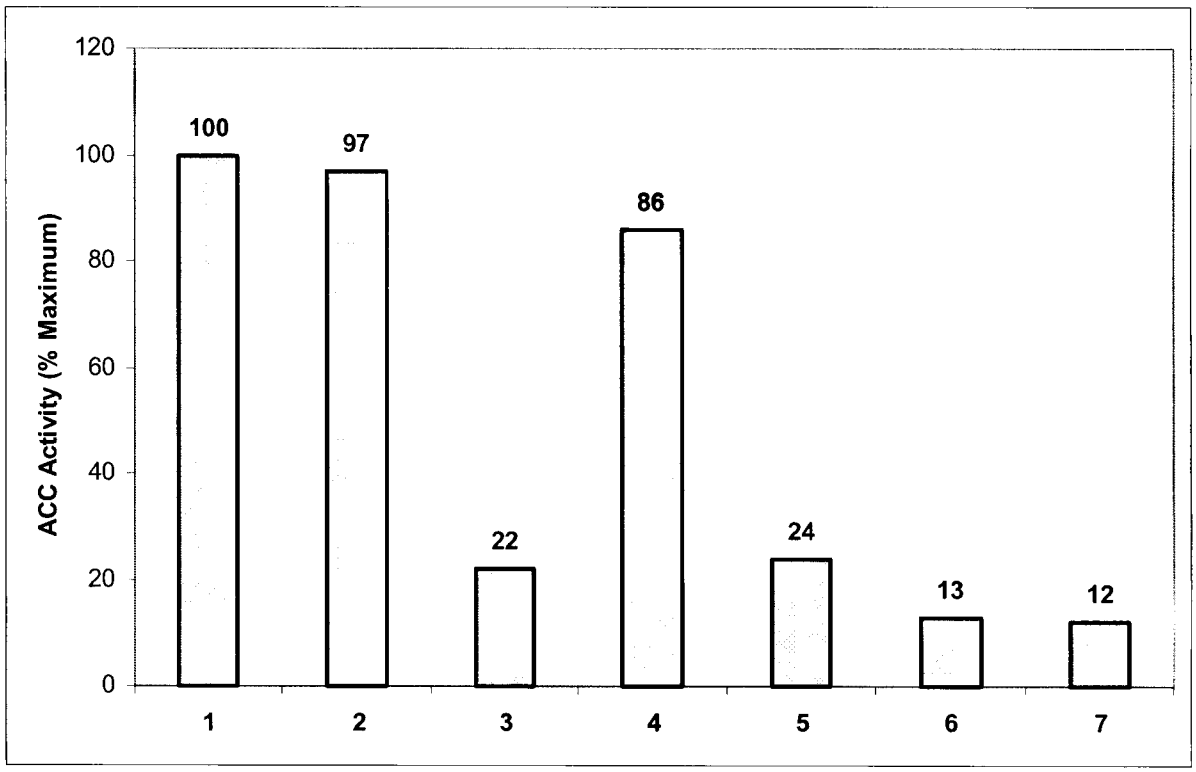
Results of this experiment lead to several conclusions. First, it supports the hypothesis described above, that PLP interacts reversibly with ACC and the removal of PLP by washing PLP-labelled ACC can result in recovery of enzyme activity (Figure 3.16 lanes 3 and 4). Second, it shows that reduction of PLP-treated ACC with sodium borohydride can render ACC irreversibly inhibited, thereby preventing loss of PLP inhibition during subsequent washing, even in buffer containing citrate (Figure 3.16 lanes 5-7). This experiment also shows that borohydride reduction of ACC-PLP can lead to the same or greater inhibition as with ACC- PLP which is unreduced and unwashed. Finally, it illustrates that increasing the BH_4:PLP ratio beyond 5:1 does little to improve ACC inhibition. In fact, a 2:1 ratio works about equally well, suggesting that at those concentrations, BH_4 can adequately react with most, if not all, PLP present as Schiff bases with ACC.

Looking ahead to future work, one particularly important aspect of the experiment shown in Figure 3.16 is the observation that PLP is indeed forming a Schiff base with ACC, and borohydride reduction allows a covalent linkage between PLP and ACC to be formed which is resistant to washing. This is an important consideration, since covalent coupling of PLP has been used by others to determine binding sites and essential lysines; specifically by proteolytic digestion and analysis of PLP-labelled peptides. Thus, showing that this technique works with ACC is key to further experiments used to characterize the PLP binding site, which is likely also the citrate binding site on ACC.

An initial example of this work on PLP is shown in Figure 3.17. This figure clearly shows that under the conditions used, PLP can be successfully linked to ACC, and through use of radioactive borohydride, its incorporation can be followed. The segment with the highest radioactive counts represents the single band on an SDS-PAGE gel corresponding to

ACC. Additional work regarding the level of PLP incorporation and the appropriate concentrations to use is currently ongoing.

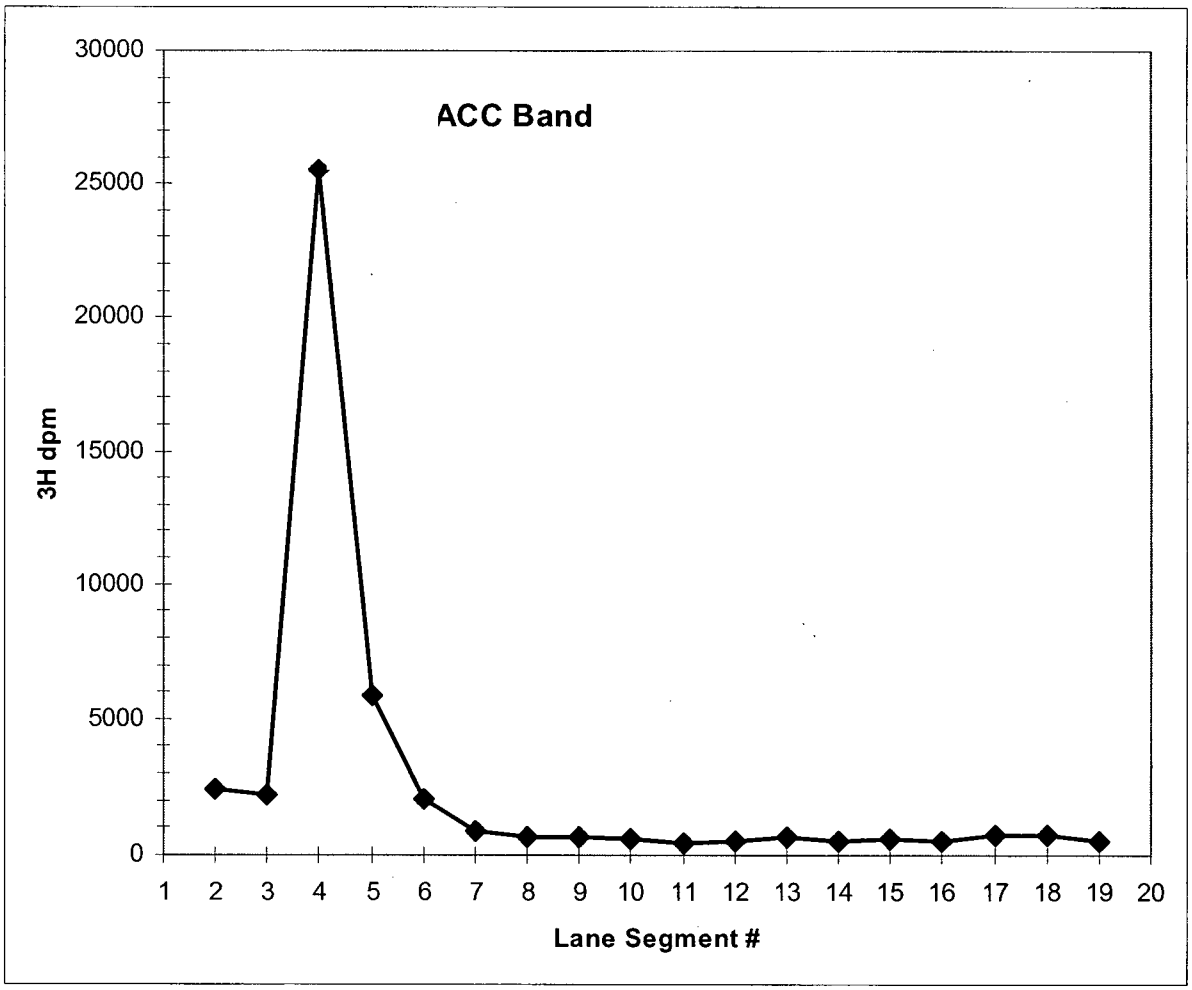
Figure 3.16 *Effect of Sodium Borohydride Reduction of PLP-Labelled ACC.*



ACC partially purified by ammonium sulphate precipitation of liver extracts was allowed to react with 0.5 mM PLP for 30 minutes followed by a 20 minute incubation with varying borohydride concentrations. Samples were then washed with buffer containing 20 mM citrate using Biomax-50 centrifugal filter units and assayed for ACC activity. Results are represented as % activity of ACC treated with only 20 mM citrate (1). (2) is ACC treated with 0.5 mM borohydride; (3), ACC treated with 0.5 mM PLP without washing, (4) ACC treated with 0.5 mM PLP with washing, (5), (6) and (7) are ACC treated with 0.5 mM PLP followed by 0.5 mM, 1.0 mM and 2.5 mM borohydride and then washing, respectively. (2), (4) and (5) are the average of two experiments, with all other results coming from single experiments.

Figure 3.17 *PLP/BH_4 Incorporation in ACC.*

ACC purified by ammonium sulphate precipitation of liver was incubated with tetrameric avidin beads for 45 minutes at 4 °C. The beads were then washed thoroughly with buffer and incubated with 5 mM PLP for 20 minutes at 25 °C in the dark, followed by reaction with 15 mM [3H]-BH_4 for 10 minutes at 25 °C. The beads were then again washed thoroughly with buffer, followed by digestion and SDS-PAGE on a 4.5% gel. Following electrophoresis, the gel was stained with Coomassie Blue and dried. The gel lane was then sliced into segments and each segment digested and subjected to scintillation counting as described in Methods.



3.2.10 Spectrophotometric Analysis of PLP Reduction

It is well established that free PLP has an absorbance maximum around 395 nm. Significantly, when reduced by borohydride, the PLP wavelength maximum shifts from 395 nm to around 325 nm, characteristic of the formation of a phospho-pyridoxyl-lysyl group, with an extinction coefficient of 9710 M^{-1} cm^{-1} at 325 nm [77]. As a complementary method to studying PLP interaction with ACC, spectrophotometric methods were therefore employed to exploit the absorbance characteristics of PLP.

In order to first test the feasibility of using spectrophotometric detection to monitor the binding state of PLP, the absorbance of PLP was studied when free in solution as well as following reduction with borohydride in the presence of lysine. Following this, tests with PLP combined with proteins were performed. Spectra from both experiments are shown in Figure 3.18.

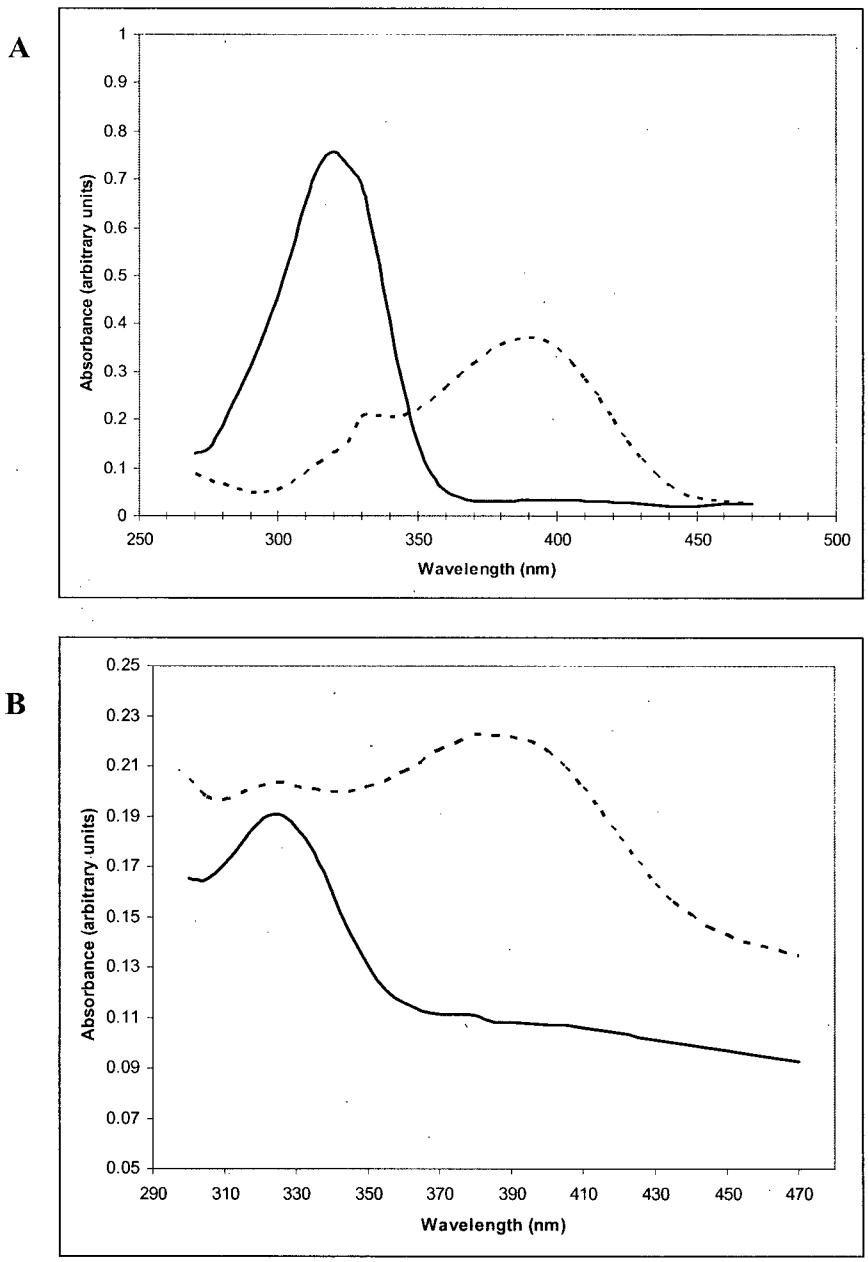
In Figure 3.18A, two curves are shown. One is the spectrum of a solution containing 5 μM PLP and 5 μM lysine; the second is that of PLP/lysine combined with BH_4, all at 10 μM. Both spectra were measured at neutral pH. The key point to observe in this figure is that PLP bound to lysine exhibits the expected absorbance maximum at about 390 nm, while the spectrum is markedly shifted and has a sharper absorbance maximum near 330 nm, following reduction by sodium borohydride. Because PLP shows the same spectrum in the absence or presence of lysine (but not borohydride) this also shows that borohydride treatment of a PLP-lysine mixture leads to the shift of the absorbance maximum due to reaction of the reversible Schiff base to produce a permanent covalent PLP-lysyl compound. In Figure 3.18B, the spectrum of partially purified liver ACC in the presence of 5 μM PLP is shown alongside that of ACC and 5 μM PLP following the addition of 5 μM BH_4. As expected, protein + PLP

shows an absorbance maximum at 390 nm, and addition of borohydride to the protein/PLP mix results in reduction of the PLP-protein Schiff base and causes a shift in the absorbance maximum from 395 nm to 325 nm.

The power of these experiments lies in the fact that a detectable change in PLP absorbance can be measured even at low 5 μM concentrations (7.5 nmoles of PLP in the cuvette during measurement), both with free lysine and more importantly, with proteins. The ability to detect a PLP signal at low concentrations is an important consideration, since this method may be applied to tracking and purification of ACC peptides labelled with PLP. The sensitivity of the instrumentation available makes using a spectrophotometric approach to monitor PLP labelling an attractive and viable method. It is important to note that although ACC is present in the sample treated with PLP and BH_4 in Figure 3.18B, the preparation is still far from homogeneous and it is most likely that PLP is also reacting with other proteins in this sample. Further work using avidin affinity purified ACC will be required to show definitively that PLP-labelling of ACC can be followed spectrophotometrically.

Figure 3.18 *Spectrophotometric Analysis of PLP Reduction by BH_4 With Lysine & Proteins.*

In (A), PLP (5 μM) was incubated with lysine (5 μM) for 35 minutes at 25 °C, and then the absorbance spectrum measured (- - -), or 10 μM PLP and lysine were reacted for 35 minutes at ambient temperature and then reacted for 10 minutes with 10 μM BH_4, and the absorbance spectrum recorded ( ). In (B) a similar approach was taken, except lysine was replaced with ammonium sulfate purified liver ACC, with PLP and BH_4 present at 5 μM. All spectrophotometric measurements were made at 37 °C.



3.3 SUMMARY

Based on evidence that PLP can bind to citrate active and/or allosteric sites and thereby inhibit a variety of enzymes [64, 69-77], we initially argued that PLP might provide a useful probe for the citrate binding site of ACC. PLP strongly inhibited ACC, with particular potency against enzyme from tissues containing predominantly ACC-1 (adipose, liver, heart). Skeletal muscle ACC (largely ACC-2) seemed less sensitive to PLP inhibition.

PLP inhibition of ACC was rapid, with near maximal ACC inhibition for a given PLP concentration seen within 30 seconds for liver tissue. Heart and muscle ACC showed slower response to PLP, but short term inhibition of ACC occurred rapidly, even in these tissues.

Binding of PLP to the citrate-binding site of ACC was supported by at least partial protection against PLP inhibition using citrate/glutamate pre-activation. Citrate/glutamate afforded slight protection at high PLP concentrations, and greater protection at low PLP concentrations. ACC from liver/heart shows greater protection than ACC from muscle.

Treatment of PLP-labelled ACC with compounds known to react with PLP, via Schiff base formation, resulted in recovery of ACC activity (approximately 50%). HA and AOA (at 1 mM) achieved the best recovery of ACC activity, against both 100 μM and 1 mM PLP.

Treatment of PLP-labelled ACC with sodium borohydride resulted in covalent and irreversible linkage of PLP to ACC, so that PLP could no longer be removed and ACC no longer re-activated by washing. Washing of borohydride-treated PLP/ACC revealed inhibition similar to or greater than that seen simply by adding PLP with no reduction, indicating that BH_4 treatment was reducing PLP bound to ACC in its inhibitory position.

Spectrophotometric analysis of borohydride reduction of PLP produced characteristic changes in absorbance maximum, and might be used to follow PLP-labelled ACC peptides.

CHAPTER FOUR

PLP ANALOGS ACCOUNT FOR PLP SPECIFICITY

4.1 RATIONALE

Previous work has demonstrated the utility of PLP as an enzyme inhibitor [72-75], and as a probe for the binding site of citrate or other anionic molecules [69-71, 76, 77] or reactive lysine residues [63-68]. It has been speculated that the main reason that PLP will bind to certain sites, notably citrate binding sites, is due to the presence of positively charged residues at these sites, which ordinarily allow for stabilization of the negative charges on citrate. While an attractive idea, conclusive evidence for this is really only available in the case of PLP binding to the active site of transaminases (for which crystal structures are known). Some studies have examined several PLP analogs for functionality, and although these point towards the importance of several moieties on PLP, they do not comprehensively define the portions of PLP that account for its greater effectiveness than its analogs in these studies [71, 74, 76, 77].

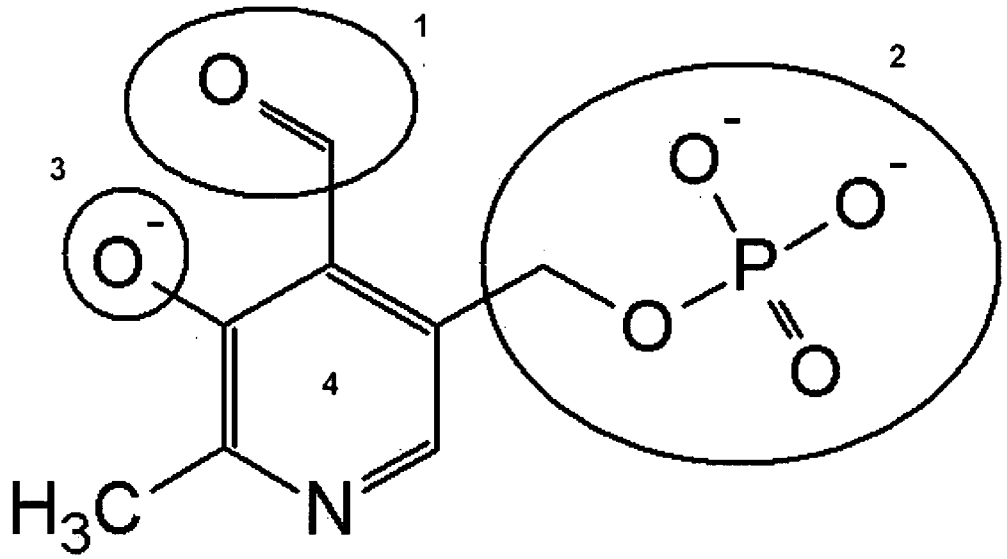
The goal of the work reported in this chapter was to perform an analysis of the effects of a range of different PLP analogs, as well as other compounds containing similar moieties, in an attempt to understand which portions of PLP account for its effect on ACC. This was achieved by performing inhibition assays of ACC.

4.2 RESULTS AND DISCUSSION

4.2.1 Possible PLP Moieties That Could Account For ACC Inhibition

As shown in Figure 4.1, there are several possible moieties on PLP that could account for both its specificity of binding, as well as the efficiency and strength of binding to ACC and other proteins. These include one or more of four moieties, as designated in Figure 4.1: (1) the reactive aldehyde at position 4', (2) the anionic phosphate group at position 5', (3) the ring hydroxyl group at position 3 (which should be ionized under most conditions tested), and (4), the pyridine ring itself. These four moieties and the role they play in PLP binding will be considered and discussed below. The methyl group on the pyridine ring of PLP is expected to be non-reactive and is not considered in the following analyses.

Figure 4.1 *Possible PLP Moieties Accounting For Specificity and Binding Efficiency.*



Structural characteristics on PLP which might account for its binding specificity and efficiency with ACC. (1) is the reactive aldehyde, (2) is the phosphate group, (3) is the hydroxyl group and (4) is the pyridine ring.

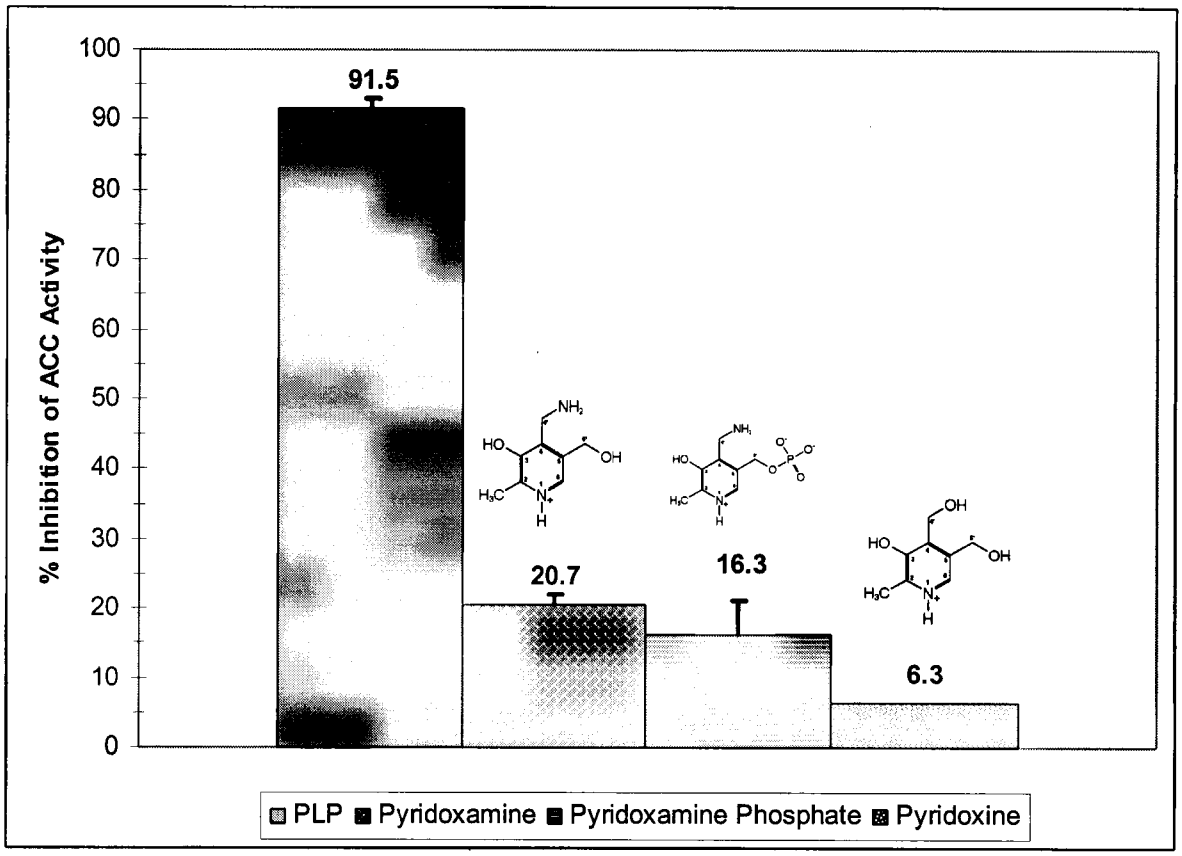
4.2.2 PLP's Reactive Aldehyde

The aldehyde at position 4' on PLP is the reactive component of the molecule which forms the Schiff base with lysine residues on proteins. Since this moiety is essential in forming the Schiff base, enzymes which use PLP as a cofactor are not expected to function as efficiently using analogs lacking this aldehyde. However, when PLP is able to inhibit other enzymes, notably those with a citrate binding site, the absence of this aldehyde may or may not affect this inhibitory capacity. Two previous studies have shown that replacing the aldehyde with an amino group (pyridoxamine phosphate) resulted in loss of inhibition against succinic semialdehyde dehydrogenase [76] and succinic semialdehyde reductase [77].

In an effort to see if the aldehyde group accounted for some of the inhibitory action of PLP on ACC, several compounds which are similar to PLP were tested for their inhibitory action on ACC. These include pyridoxamine, pyridoxamine phosphate, and pyridoxine. The structures of these three compounds are shown in Figure 1.3.

Since PLP shows very strong inhibition of ACC at a concentration of 1 mM (approximately 90% inhibition – 10% residual activity) this concentration was chosen as the standard to assess the effects of analogs in this and other sections. Inhibition at concentrations higher than 1 mM were not considered, since these concentrations are not physiologically relevant in most cases, nor achievable pharmacologically, in general. All experiments were performed using ammonium sulphate purified liver ACC, since this preparation typically showed the best response to PLP inhibition and was most easily obtained. Results of treating ACC with 1 mM of pyridoxamine, pyridoxamine phosphate and pyridoxine are compared with 1 mM PLP inhibition in Figure 4.2

Figure 4.2 *Effect of Aldehyde-Lacking PLP Analogs.*



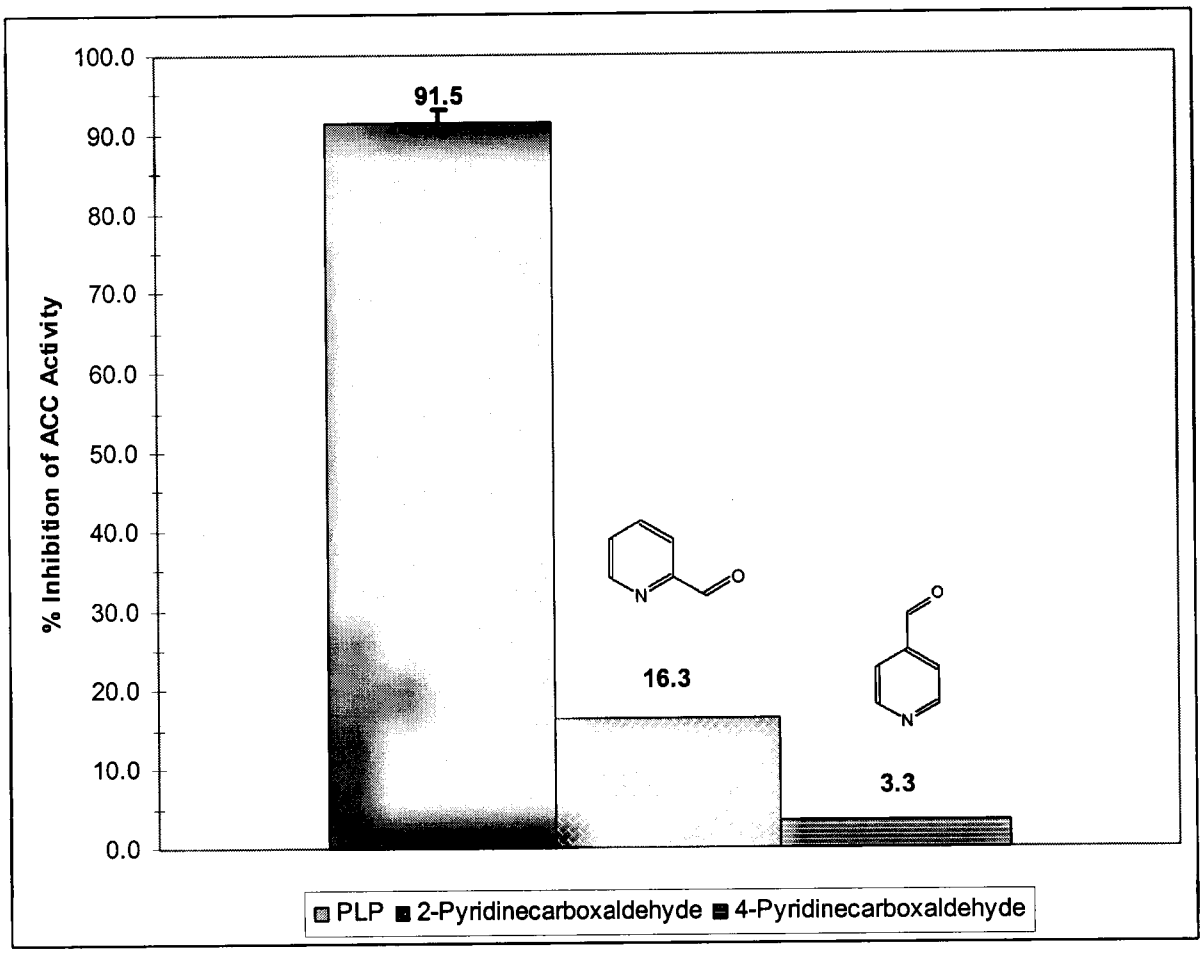
ACC partially purified by ammonium sulphate precipitation of liver was treated with PLP or the indicated analogs at concentrations of 1 mM for 30 minutes, followed by a 30 minute incubation with 10 mM citrate and subsequent activity assay. Results are represented as % inhibition, compared to a sample of ACC treated with 10 mM citrate alone (0% inhibition) for each separate experiment. Results for PLP, pyridoxamine and pyridoxamine phosphate are given as the mean ± SEM for 6, 2 and 2 experiments, respectively. Pyridoxine results are for a single experiment.

As can be seen in Figure 4.2, PLP analogs lacking the aldehyde show very poor inhibition of ACC. The strongest of the three tested, pyridoxamine, shows only 21% inhibition of ACC, compared to the 92% inhibition with PLP itself. These results indicate that PLP's aldehyde group is important for its inhibitory action on ACC. The result best showing this is pyridoxamine phosphate, since this compound has an amine replacing the aldehyde, but with the phosphate group and phenolic oxygen still intact. Pyridoxamine phosphate shows very poor inhibition, at only 16%, suggesting that the aldehyde on PLP is binding to a nearby lysine residue on the enzyme, or that the amino group in pyridoxamine was repelled by the positively charged residues in the binding site.

The results of pyridoxamine and pyridoxine have to be analyzed carefully, because they both lack not only the aldehyde, but the phosphate group as well. From these results it is interesting that these two compounds, which differ at the group off of position 4', show reasonable difference in inhibitory action on ACC. Pyridoxamine shows 14% better inhibition that pyridoxine, which perhaps indicates that the amino group on pyridoxamine is better able to hydrogen bond or at least deal with the high positive charges hypothesized to surround the citrate/PLP binding site, than is the hydroxyl group of pyridoxine.

Two additional compounds, both with a pyridine ring and an aldehyde, show that the aldehyde alone is not sufficient for inhibition. Both 2- and 4-pyridinecarboxaldehyde poorly inhibit ACC, as shown in Figure 4.3. The structures of these molecules is given in Figure 4.10. Surprisingly, of the two compounds, 4-pyridinecarboxaldehyde, which more closely resembles PLP, shows less inhibition of ACC than does 2-pyridinecarboxaldehyde. This also confirms that the aldehyde and pyridine ring *alone* are *not* sufficient to account for ACC inhibition, regardless of the position of the aldehyde group on the ring.

Figure 4.3 *Effect of Pyridinecarboxaldehydes on ACC.*



ACC partially purified by ammonium sulphate precipitation of liver was treated with PLP or the indicated analogs at concentrations of 1 mM for 30 minutes, followed by a 30 minute incubation with 10 mM citrate and subsequent activity assay. Results are represented as % of ACC inhibition, compared to a sample only treated with citrate (0% inhibition) for each separate experiment. Results for PLP are given as the mean ± SEM for 6 experiments, while those for the two analogs are for single experiments each.

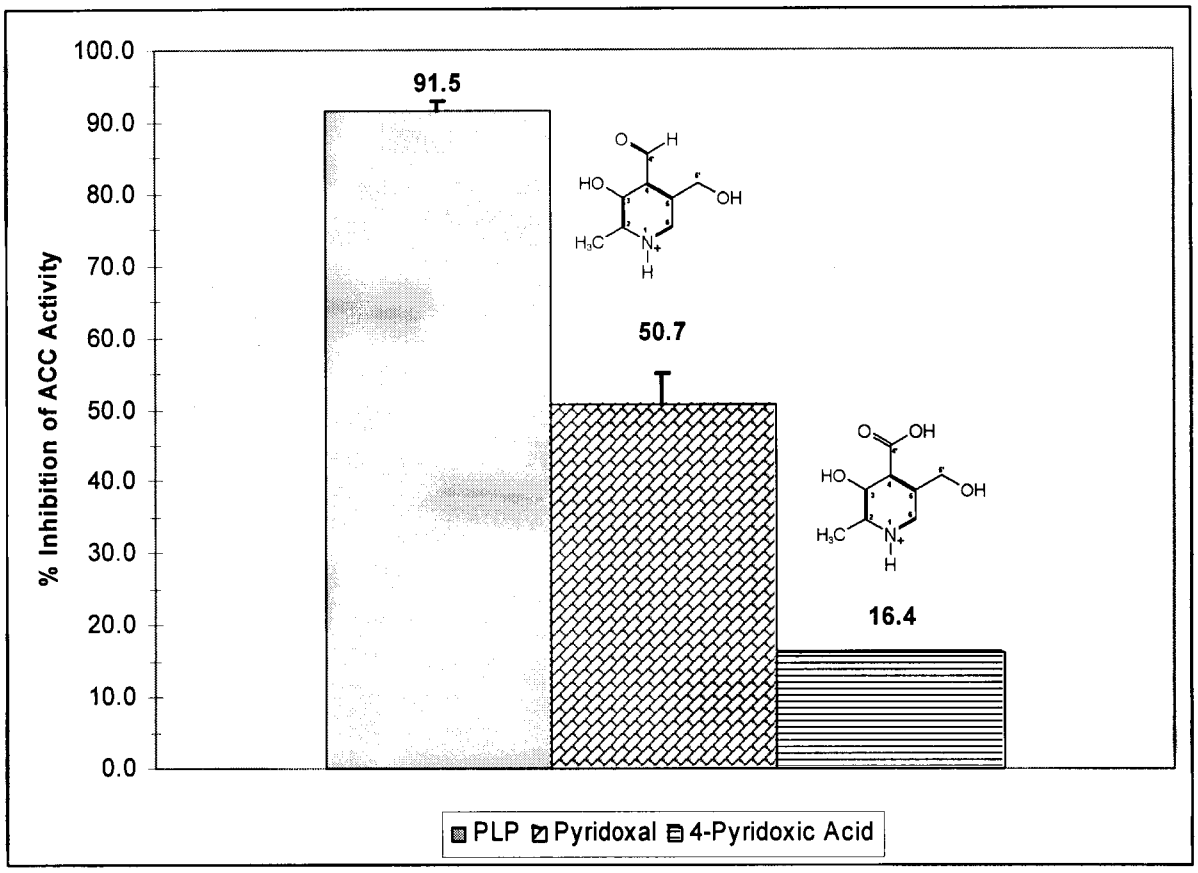
4.2.3 PLP's Phosphate Group

Although the aldehyde group on PLP may contribute a significant portion of the specificity and efficacy of PLP, it is not the only component that plays a role. Several studies have previously shown that pyridoxal can only weakly, if at all substitute for PLP as an inhibitor [71, 76, 77]. In order to verify the effect of pyridoxal on ACC, similar inhibition assays to those testing the effect of the PLP aldehyde were performed, using pyridoxal and another analog, 4-pyridoxic acid. The structures of these compounds are shown in Figure 1.3, and the result of the assays is given in Figure 4.4.

As can be seen in Figure 4.4, pyridoxal inhibits ACC at a reasonable level, but is still far less effective than an equal concentration of PLP. 4-pyridoxic acid shows poor inhibition characteristics similar to other analogs such as pyridoxamine and pyridoxamine phosphate.

The results from these experiment are interesting, because they show that loss of a phosphate group on pyridoxal does not decrease the inhibitory properties against ACC by nearly as much as a similar analog such as pyridoxamine. This clearly indicates that it is indeed the aldehyde group on PLP which plays the most important role in its binding and inhibitory characteristics against ACC. Analogs lacking both the aldehyde and the phosphate group, such as pyridoxine and 4-pyridoxic acid, show very poor inhibition, and even pyridoxamine phosphate, which contains the phosphate group, shows only minor improvements over the other two. Thus although the phosphate group may play a minor role in determining PLP specificity and efficacy, it is not the primary moiety accounting for PLP's inhibitory action on ACC.

Figure 4.4 *Effect of Phosphate-Lacking PLP Analogs.*



ACC partially purified by ammonium sulphate precipitation of liver was treated with PLP or the indicated analogs at concentrations of 1 mM for 30 minutes, followed by a 30 minute incubation with 10 mM citrate and subsequent activity assay. Results are represented as % of ACC inhibition, compared to a sample only treated with citrate (0% inhibition) for each separate experiment. Results for PLP and pyridoxal are given as the mean ± SEM for 6 and 2 experiments, respectively. 4-Pyridoxic acid results are for a single experiment.

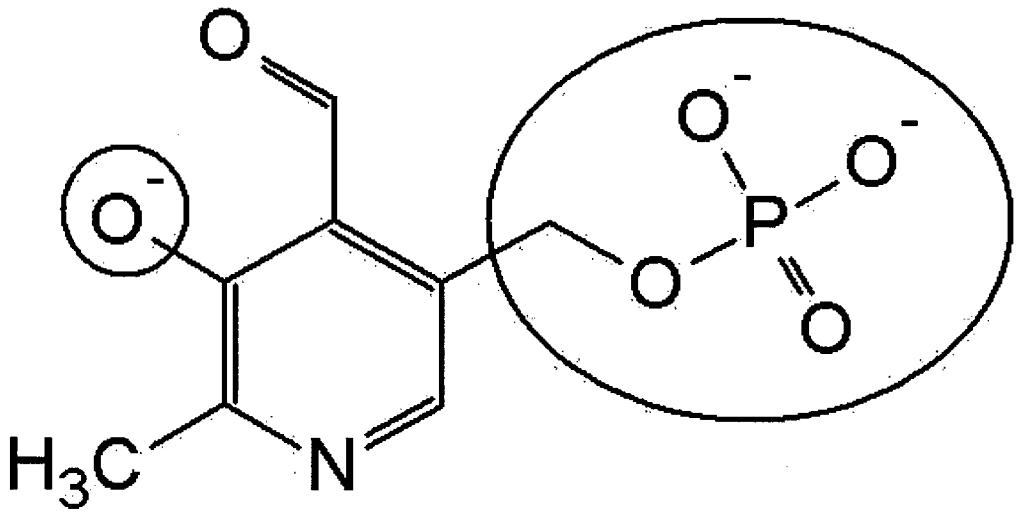
4.2.4 PLP's Anionic Poles and Pyridine Ring

Direct testing of the efficacy of the hydroxyl group on PLP is very difficult, since analogs lacking this group are not commercially available, and considerable expense and time would be required to synthesize them. However, since both ends of the PLP molecule can be negatively charged (the hydroxyl and the phosphate), it seemed relevant to examine if PLP's spaced negative charges could account for some inhibition of ACC. This can be rationalized by comparing the structures of PLP and citrate, as shown in Figure 4.5. Since both molecules have spaced negative charges (5 carbons apart in citrate and 6 carbon equivalents in PLP), these could account for the ability of PLP to enter the citrate binding site and thus position itself to form a Schiff base with the enzyme.

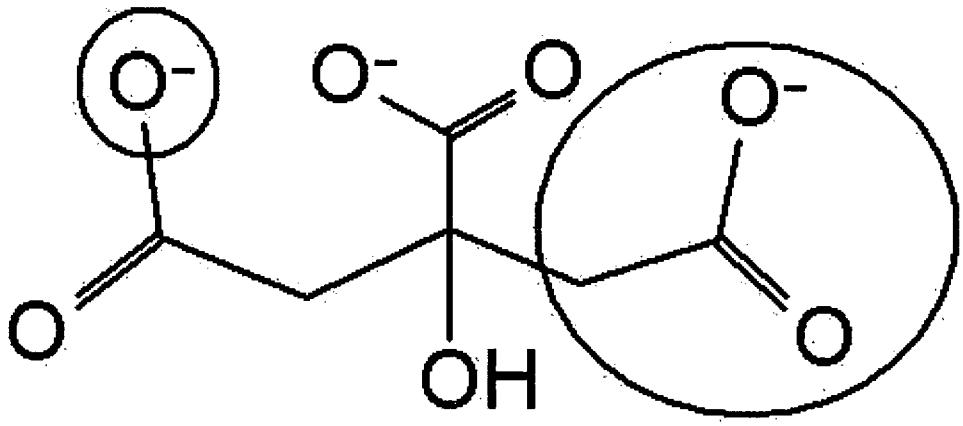
In order to test the importance of the spaced negative charges, several compounds were used, each with slightly different spacing or orientation of negatively charged groups. The most common group used to produce a negative charge were carboxyl groups, since these are also present on citrate and maintain the most structural similarity to the natural ligand, as well as generally being negatively charged at neutral pH.

Compounds with separated negative charges best thought to resemble PLP were pyridine rings with two carboxylic acid moieties. The results of ACC treatment with these compounds is shown in Figure 4.6, and their structures in Figure 4.10.

Figure 4.5 *Comparison of PLP and Citrate Structures.*

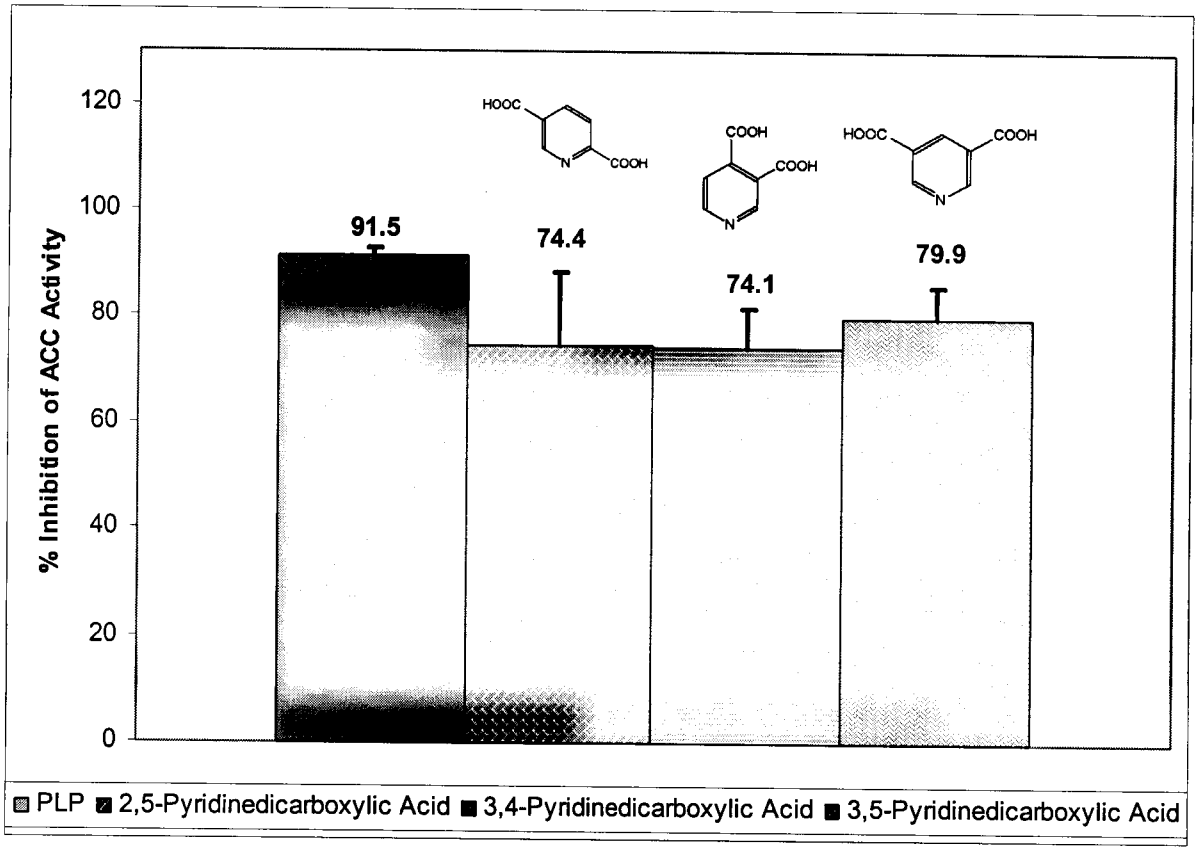


PLP



Citrate

Figure 4.6 *Effect of Pyridinedicarboxylic Acids on ACC.*



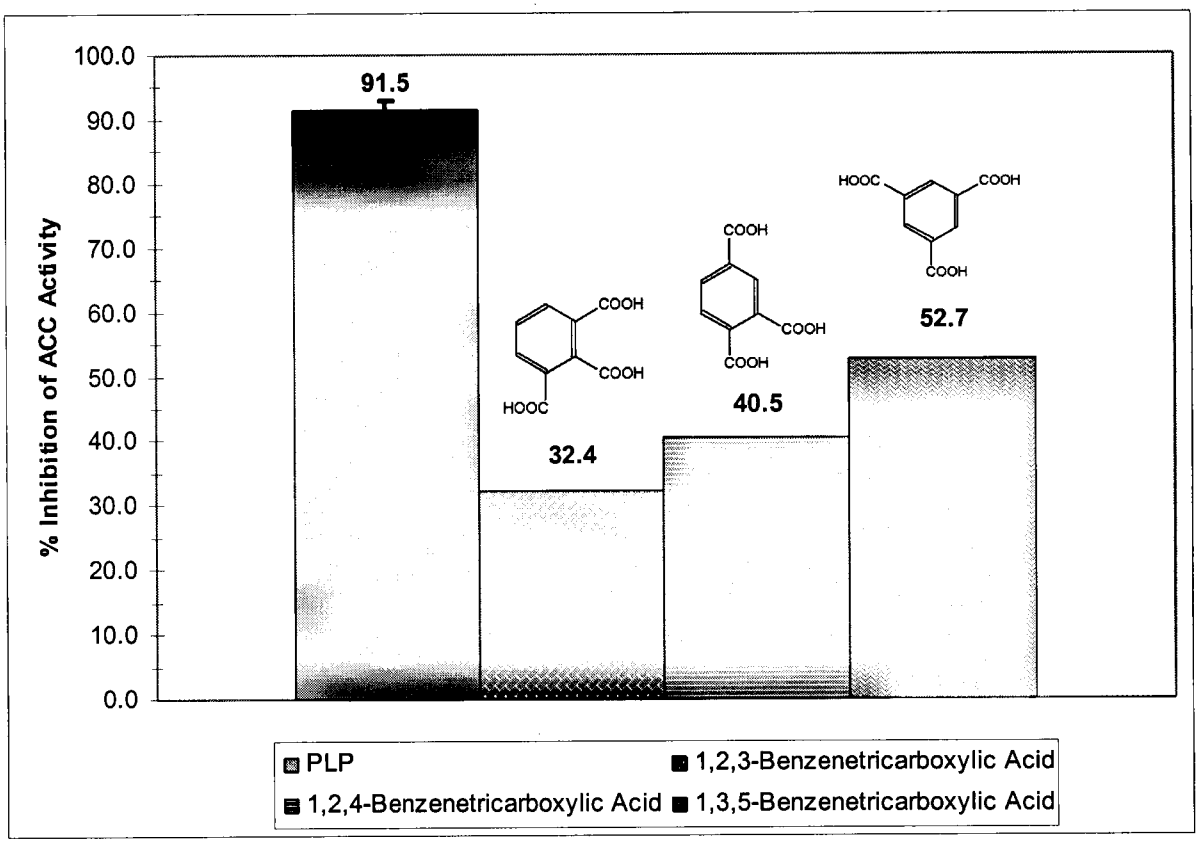
ACC partially purified by ammonium sulphate precipitation of liver was treated with PLP or the indicated analogs at concentrations of 1 mM for 30 minutes, followed by a 30 minute incubation with 10 mM citrate and subsequent activity assay. Results are represented as % of ACC inhibition, compared to a sample only treated with citrate (0% inhibition) for each separate experiment. Results for PLP, 2, 5-, 3, 4-, and 3, 5-pyridinedicarboxylic acids are given as the mean ± SEM for 6, 2, 4 and 4 experiments, respectively.

As shown in Figure 4.6, all three pyridinedicarboxylic acids tested appear to inhibit ACC very well at 1 mM concentrations; far better in fact than any other analogs tested. This result is interesting because it indicates that while the aldehyde portion of PLP may account for strong binding, hence citrate cannot adequately remove PLP at higher concentrations, it is also likely that the negative charges on PLP allow it to enter the citrate binding site, due to favourable ionic interactions with positively charged residues in the binding site.

In addition, the difference in location on the ring of the carboxylic acid groups appears to be rather unimportant, although the 3,5-pyridinedicarboxylic acid form seems to show slightly better ACC inhibition than the other two. This is likely due to favourable and similar charge placement on the pyridine ring relative to citrate and PLP, whereas the others differ slightly. Having 5 carbon equivalents separating the negative charges appears to be best (matching with citrate), while 4 and 6 equivalents between negative charges show no real difference. Further, the second carboxyl group of this set of analogs appears to be better for binding to ACC than the corresponding hydroxyl group on PLP.

The pyridine ring in both PLP and the three pyridinedicarboxylic acids could play a role in PLP binding to ACC. The ring itself is fundamental for PLP-dependent catalysis, but it's role in PLP acting as an inhibitor is unclear. In order to ascertain the function of the pyridine ring, three benzenetricarboxylic acids were tested for ACC inhibition. These compounds lack the ring nitrogen atom, but are still aromatic, and thus fairly similar in overall size to the pyridinedicarboxylic acids tested previously. The results of these experiments are shown in Figure 4.7, and the structures of these compounds in Figure 4.10.

Figure 4.7 *Effect of Benzenetricarboxylic Acids on ACC.*



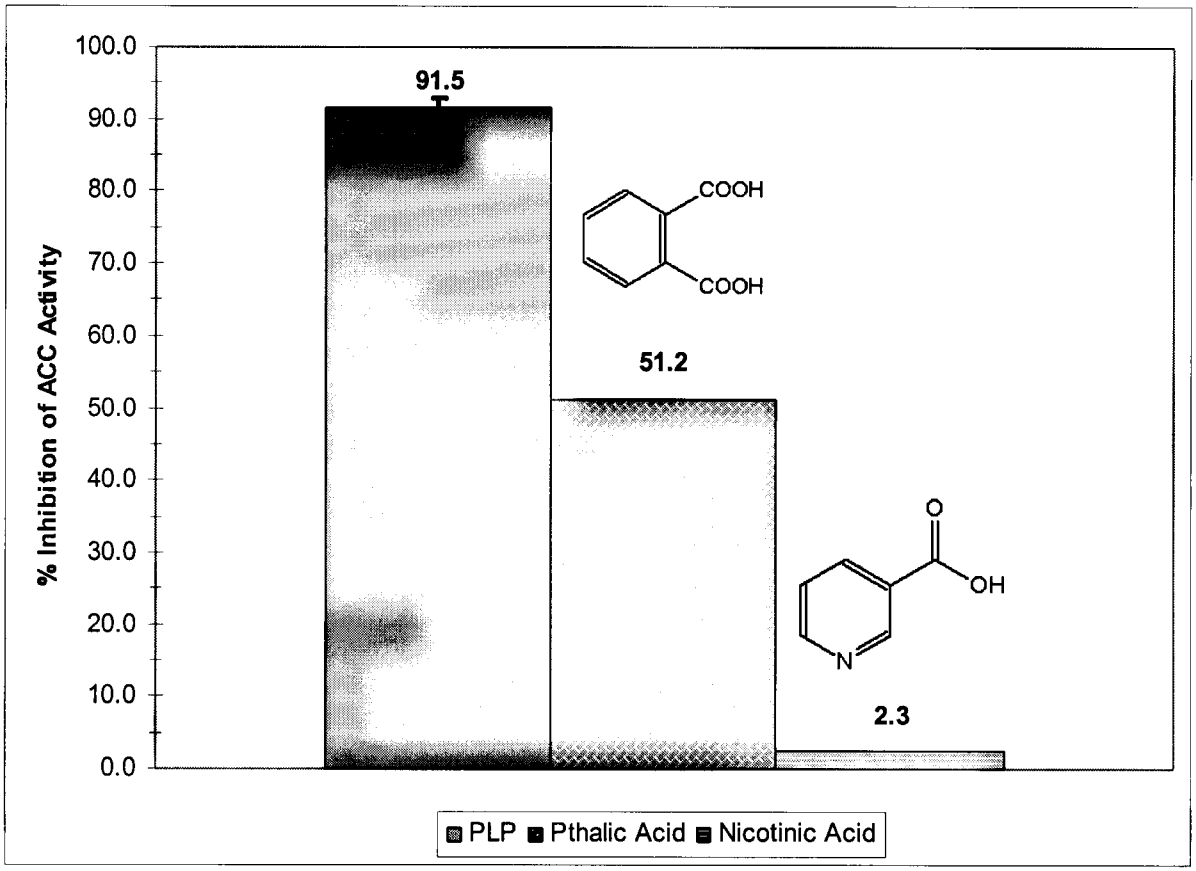
ACC partially purified by ammonium sulphate precipitation of liver was treated with PLP or the indicated analogs at concentrations of 1 mM for 30 minutes, followed by a 30 minute incubation with 10 mM citrate and subsequent activity assay. Results are represented as % of ACC inhibition, compared to a sample only treated with citrate (0% inhibition) for each separate experiment. Results for PLP are given as the mean ± SEM for 6 experiments, while the 3 analog results are for single experiments.

From Figure 4.7 it is evident that all three benzenetricarboxylic acids inhibit ACC to a fairly substantial degree at 1 mM (30-50%). Interestingly, even the best of the three benzenetricarboxylates, the 1,3,5-form, is not as effective at inhibiting ACC as even the least effective of the pyridinedicarboxylic acids, 3,4-pyridinedicarboxylic acid (greater than 70% inhibition). This can be interpreted to mean that although the negative charge placement on the ring is important, it is possible that either the pyridine ring itself is required to account for the additional effective inhibition shown by the pyridinedicarboxylic acids, or the additional carboxylic acid group on the benzenes negatively affects the inhibition properties of these compounds, relative to the pyridines.

It is interesting too, that for 1,3,5-benzenetricarboxylic acid, the negative charges are equally 5 carbon equivalents apart, while the 1,2,3-form does not have this same spacing, and the 1,2,4-form has only one of these 5 carbon equivalents. This suggests that the placement of the negative charges could play a role with the benzene compounds, although this appeared not to be the case with the pyridinedicarboxylic acids.

In an attempt to clarify these ambiguities, two additional compounds were tested. These include a benzenedicarboxylic acid (phthalic acid) and a pyridinecarboxylic acid (nicotinic acid or niacin). The structures of these compounds are given in Figure 4.11 and the results of the experiments are shown in Figure 4.8.

Figure 4.8 *Effect of Phthalic Acid and Nicotinic Acid on ACC.*



ACC partially purified by ammonium sulphate precipitation of liver was treated with PLP or the indicated analogs at concentrations of 1 mM for 30 minutes, followed by a 30 minute incubation with 10 mM citrate and subsequent activity assay. Results are represented as % of ACC inhibition, compared to a sample only treated with citrate (0% inhibition) for each separate experiment. Results for PLP are given as the mean ± SEM for 6 experiments, while the 2 analog results are for single experiments.

The results of the experiments shown in Figure 4.8 illustrate three points, and help to address the uncertainties described above. First, since phthalic acid is 1,2-benzenedicarboxylic acid (with 4 carbon equivalents between negative charges), and it shows an ACC inhibition level comparable to 1,3,5-benzenetricarboxylic acid (~ 50%) this indicates that the third carboxylic acid groups at positions 3 and 4 of 1,2,3- and 1,2,4-benzenetricarboxylic acids, respectively, do not account for any increase in inhibitory activity. In fact, these third carboxylic acids appear to decrease the inhibition of those two benzenetricarboxylic acids relative to phthalic acid. Secondly, it shows that having 5 carbon equivalents between negatively charged groups is not absolutely essential, at least on benzene rings and when only two negatively charged groups are present. Introduction of the third negatively charged group appears to decrease inhibitory effect, especially if that group is directly adjacent on the ring to one of the others. Perhaps some charge repulsion is occurring in these molecules, which distorts the ring structure or the ability to interact with the positively charged residues of the citrate binding site. Finally, this result shows that having two carboxylic acids on a benzene ring does not produce the same level of ACC inhibition as a pyridinedicarboxylic acid. This suggests that the pyridine ring may be contributing to the inhibitory effect of the pyridinedicarboxylic acids and PLP.

The observation that nicotinic acid (3-pyridinecarboxylic acid) shows almost no inhibition of ACC also indicates that a pyridine ring with only one negatively charged substituent is ineffective, in sharp contrast to a pyridine ring with two carboxylic acid moieties. Thus it appears that a combination of a pyridine ring, with two negatively charged groups accounts for substantial efficacy of PLP inhibition of ACC. Significantly, the second

carboxyl group may confer a stronger influence than the ring hydroxyl of PLP, which is probably only partially ionized at neutral pH.

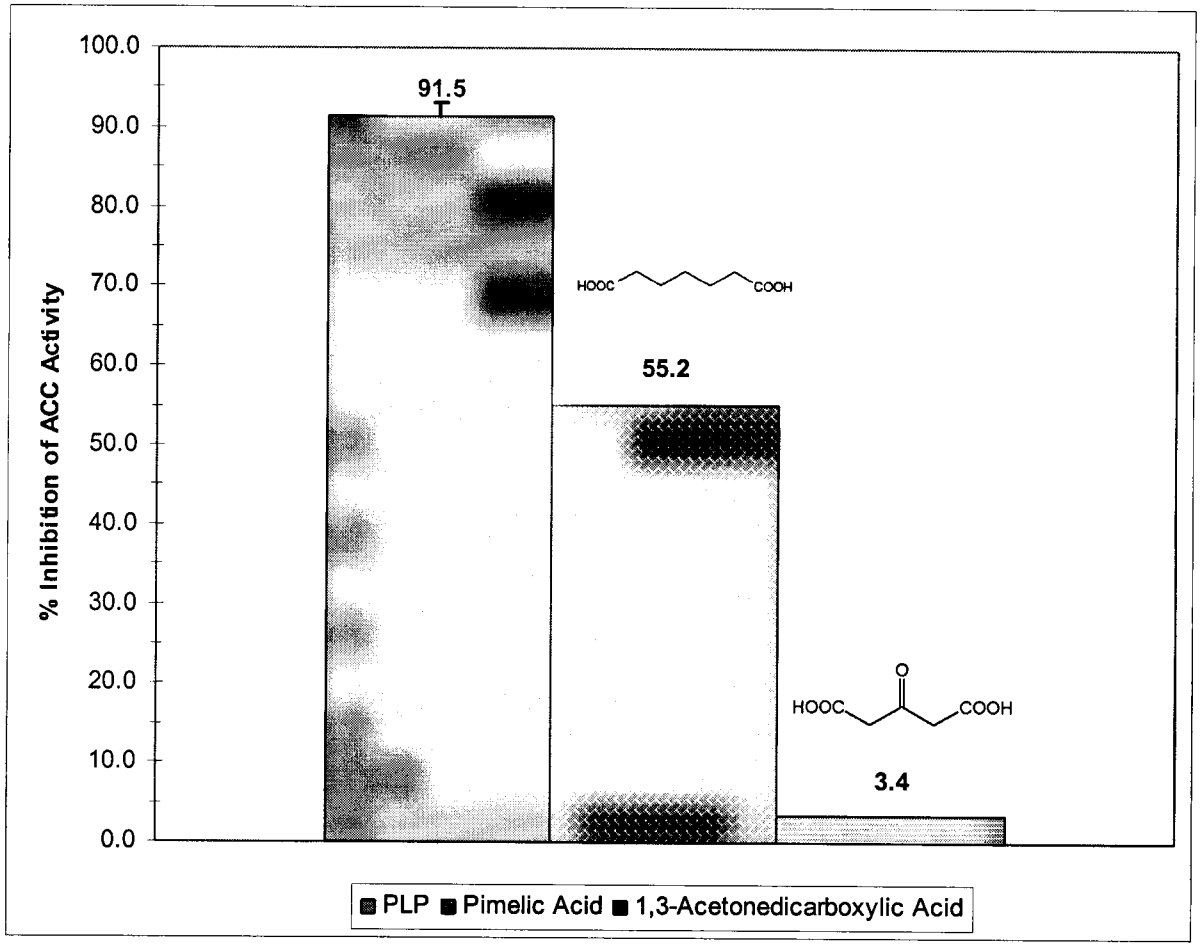
4.2.5 Non-Aromatic PLP Analogs

All of the analogs tested so far have had either a benzene ring or a pyridine ring as the central unit. Although the results to date seem to indicate that a pyridine ring, as opposed to a benzene ring, is beneficial for conferring ACC inhibitory characteristics to these molecules, these experiments have not addressed whether a ring structure is needed for ACC inhibition.

To address this issue, several molecules were tested for ACC inhibitory capability. These molecules either did not contain rings at all, or had rings which were separate from the negatively charged groups on the molecule. These molecules included: pimelic acid (a straight chain, saturated, dicarboxylic acid), 1,3-acetonedicarboxylic acid (a dicarboxylic acid with a carbonyl group between the two carboxylic acids), benzylmalonic acid, and butylmalonic acid. The structures are shown in Figure 4.11, and the results in Figure 4.9.

From Figure 4.9 (compared to Figure 4.7), it is clear that pimelic acid inhibits ACC slightly better than 1,3,5-benzenetricarboxylic acid and phthalic acid. Benzylmalonic acid and butylmalonic acid did not inhibit ACC at all (data not shown). This indicates the two carboxyl groups alone provide a substantial inhibitory potential, provided the two negatively charged groups are spaced apart. For pimelic acid the spacing is by 5 carbons, and inhibition was evident, whereas essentially no spacing is present between the carboxyl groups of the malonic acid species. Furthermore, pimelic acid is a less effective inhibitor than either PLP or the pyridinedicarboxylates, demonstrating that the pyridine ring does still contribute to inhibition of ACC.

Figure 4.9 *Effect of Non-Ring PLP Analogs on ACC.*



ACC partially purified by ammonium sulphate precipitation of liver was treated with PLP or the indicated analogs at concentrations of 1 mM for 30 minutes, followed by a 30 minute incubation with 10 mM citrate and subsequent activity assay. Results are represented as % of ACC inhibition, compared to a sample only treated with citrate (0% inhibition) for each separate experiment. Results for PLP are given as the mean ± SEM for 6 experiments, while the 2 analog results are for single experiments.

Figure 4.10 *Structures of Some of the PLP Analogs Tested.*

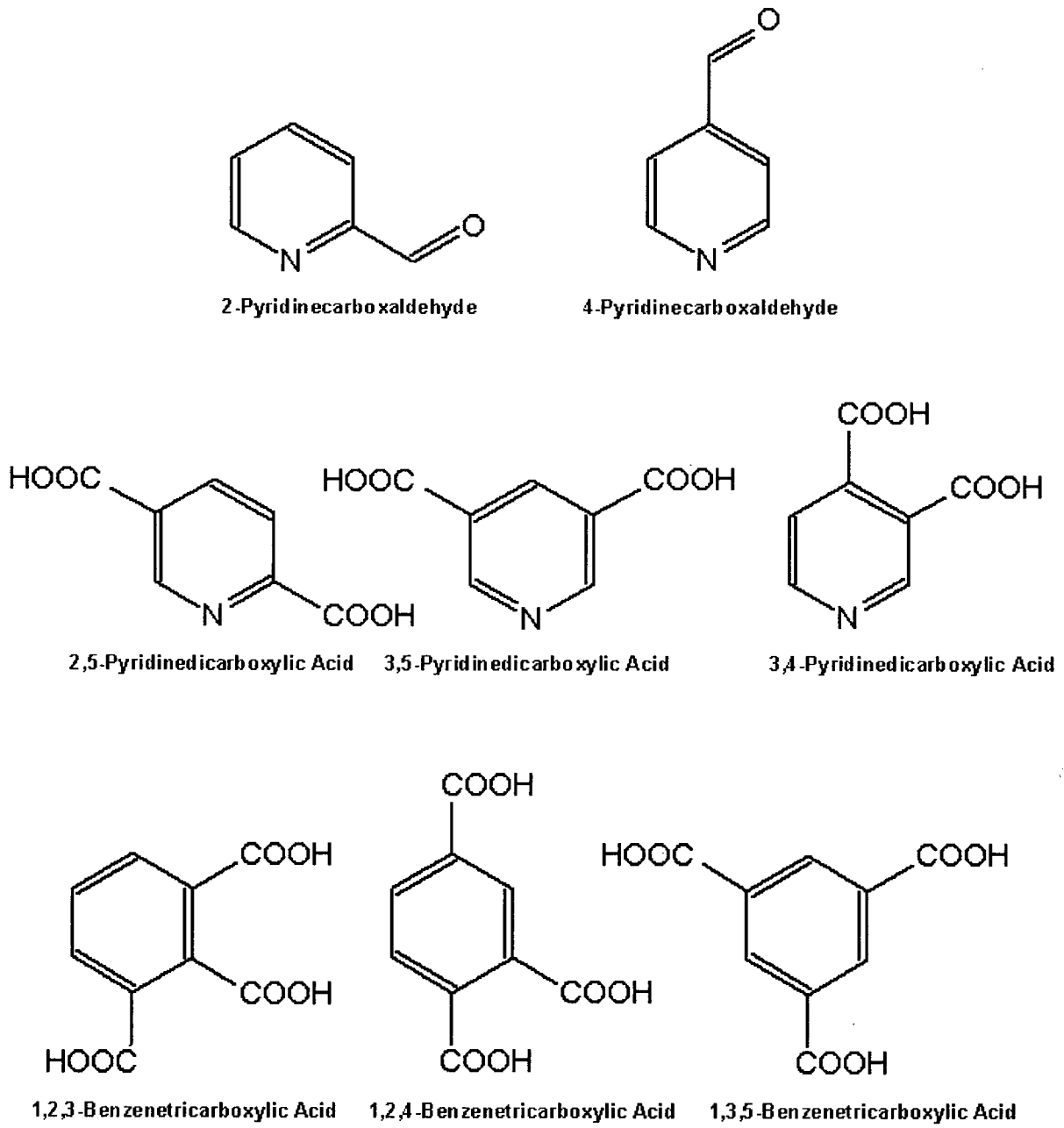
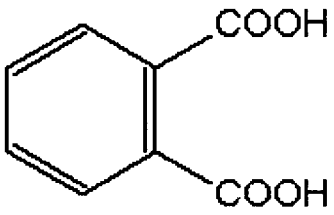
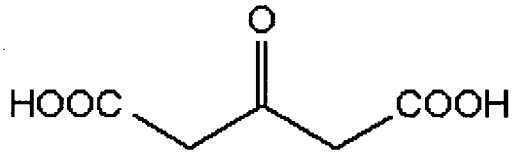


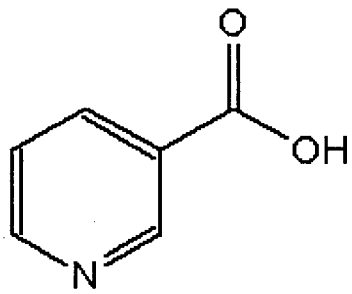
Figure 4.11 *Structures of Some of the PLP Analogs Tested.*



Pthalic Acid

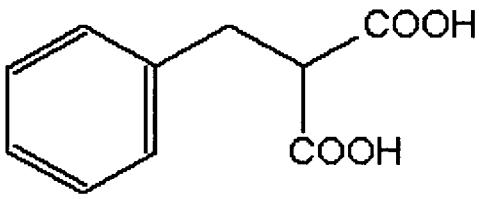


1,3-Acetonedicarboxylic Acid

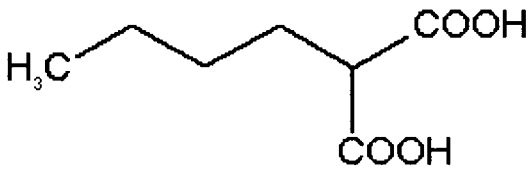


Nicotinic Acid

Pimelic Acid



Benzylmalonic Acid



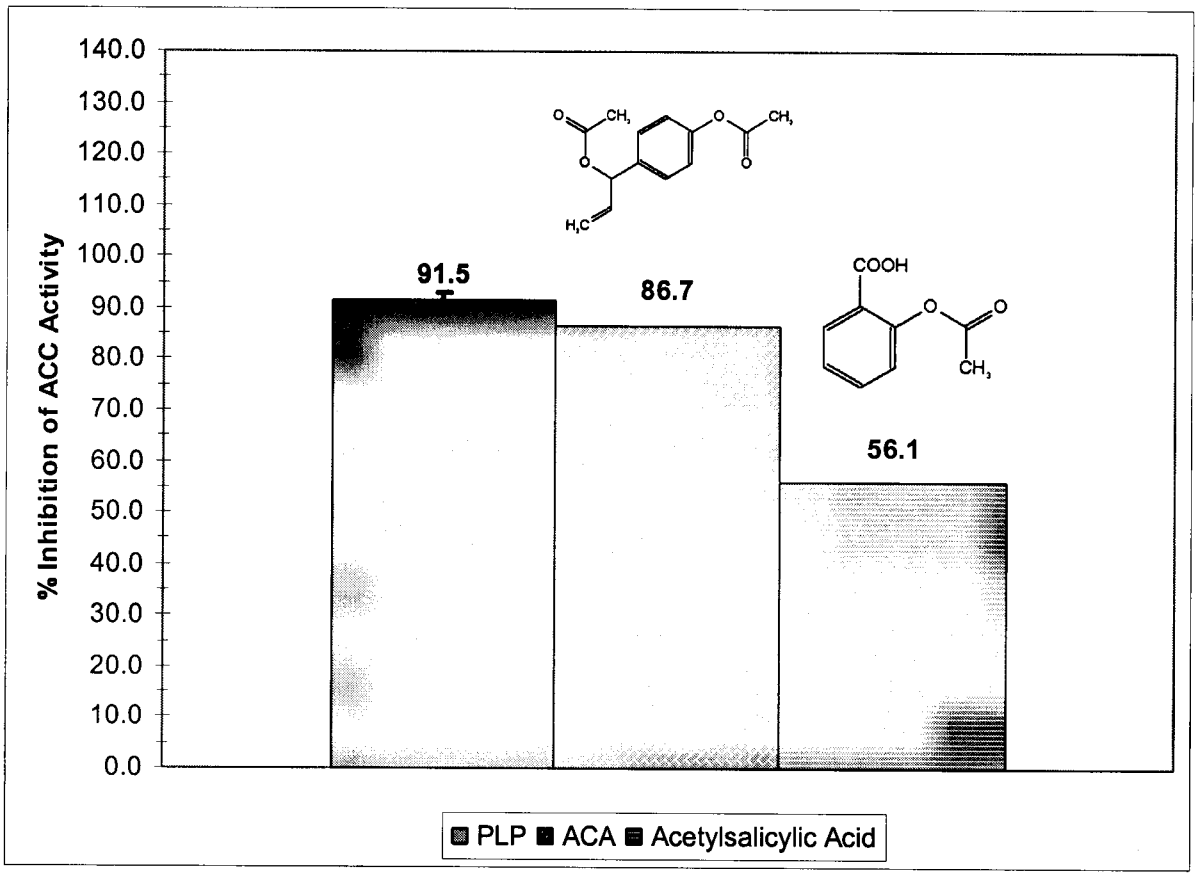
Buylmalonic Acid

4.2.5 Use of ACA and an Analog

Upon the advice of a colleague, an additional compound, which was thought to bear structural similarity to PLP, was tested for ACC inhibitory properties. This compound, 1'acetoxychavicol acetate (ACA) is isolated from the rhizomes of *Languas galanga* (Zingiberaceae), a common plant in Thailand. It has been shown in several studies to inhibit xanthine oxidase, and prevent tumours in several tissue types in rats [83]. Although the available evidence did not strictly suggest any activity with regard to inhibiting ACC, it was tested like the other analogs. In fact, ACA did cause substantial ACC inhibition, and so one of its analogs, acetylsalicylic acid (aspirin) was also tested for ACC inhibition. The results for both compounds are shown in Figure 4.12 and the structures in Figure 4.13.

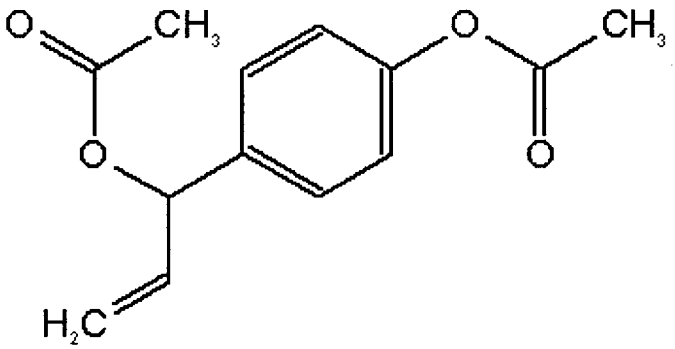
The data in Figure 4.12 is very interesting, because ACA appears to be almost as potent an inhibitor of liver ACC as is PLP. This is a potentially important result, because although there is some structural similarity between the two compounds, how ACA would favourably interact with ACC is not clear. Notably, there are no groups with negative charge present on ACA, although the two ester functionalities could potentially hydrogen bond with residues in the citrate binding site. Nonetheless, this molecule does inhibit ACC with high potency. This raises the question as to whether this compound is also acting at the citrate binding site, or if it is inhibiting ACC through other means. Unfortunately, due to limited supply of this compound, inhibition of ACC following citrate activation of the enzyme could not be performed, thus precluding an examination of citrate protection of ACC against ACA.. This might be an interesting candidate compound to consider in future work. It might also be interesting to test the effects of some of the compounds already examined, in which the carboxyl groups are esterified, as in ACA.

Figure 4.12 *Effect of ACA and Acetylsalicylic Acid on ACC.*

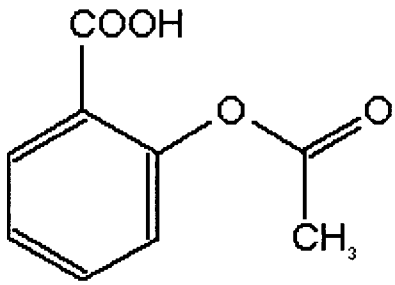


ACC partially purified by ammonium sulphate precipitation of liver was treated with PLP or the indicated analogs at concentrations of 1 mM for 30 minutes, followed by a 30 minute incubation with 10 mM citrate and subsequent activity assay. Results are represented as % of ACC inhibition, compared to a sample only treated with citrate (0% inhibition) for each separate experiment. Results for PLP are given as the mean ± SEM for 6 experiments, while the 2 analog results are for single experiments.

Figure 4.13 *Structures of ACA and Acetylsalicylic Acid.*



1'-acetoxychavicol acetate (ACA)



Acetylsalicylic Acid

Interestingly, aspirin also shows reasonable inhibition of ACC at 1 mM, although this is about the same level as other PLP analogs tested, such as phthalic acid and 1,3,5-benzenetricarboxylic acid. The carboxylic acid and benzene ring structure may account for this action.

In order to allow easier comparison of PLP analogs, their structural components and their effect on ACC, this information is presented in Table 4.1.

Table 4.1A *Summary of PLP Analogs and Their Effects on ACC*

PLP analogs are listed, as well as pertinent structural information of each. The % inhibition of an ammonium sulphate purified liver ACC preparation is also given, and this value represents the results presented in the other figures in this chapter. In each of the structural columns a filled circle indicates the presence of this moiety on the compound, and a "-" indicates this feature is not present. A number in brackets following a filled circle indicates how many of each feature is present in the molecule, if there is more than one.

Compound	Aldehyde	Phosphate	(-) Charged Groups	Pryidine Ring	Benzene Ring	% Inhibition of ACC
PLP			(2)		-	91.5
Pyridoxal		-			-	50.7
Pyridoxamine	-	-	(2)		-	20.7
Pyridoxamine Phosphate	-		(2)		-	16.3
Pyridoxine	-	-	(3)		-	6.3
4-Pyridoxic Acid	-	-	(3)		-	16.4
2-Pyridinecaboxaldehyde		-	-		-	16.3
4-Pyridinecaboxaldehyde		-	-		-	3.3

Table 4.1B *Summary of PLP Analogs and Their Effects on ACC*

PLP analogs are listed, as well as pertinent structural information of each. The % inhibition of an ammonium sulphate purified liver ACC preparation is also given, and this value represents the results presented in the other figures in this chapter. In each of the structural columns a filled circle indicates the presence of this moiety on the compound, and a "-" indicates this feature is not present. A number in brackets following a filled circle indicates how many of each feature is present in the molecule, if there is more than one.

Compound	Aldehyde	Phosphate	(-) Charged Groups	Pryidine Ring	Benzene Ring	% Inhibition of ACC
2,5-Pyridinedicarboxylic Acid	-	-	(2)		-	74.4
3,4-Pyridinedicarboxylic Acid	-	-	(2)		-	74.1
3,5-Pyridinedicarboxylic Acid	-	-	(2)		-	79.9
1,2,3-Bezenetricarboxylic Acid	-	-	(3)	-		32.4
1,2,4-Bezenetricarboxylic Acid	-	-	(3)	-		40.5
1,3,5-Bezenetricarboxylic Acid	-	-	(3)	-		52.7
Phthalic Acid	-	-	(2)	-		51.2
Pimelic Acid	-	-	(2)	-	-	55.2

Table 4.1C *Summary of PLP Analogs and Their Effects on ACC*

PLP analogs are listed, as well as pertinent structural information of each. The % inhibition of an ammonium sulphate purified liver ACC preparation is also given, and this value represents the results presented in the other figures in this chapter. In each of the structural columns a filled circle indicates the presence of this moiety on the compound, and a "-" indicates this feature is not present. A number in brackets following a filled circle indicates how many of each feature is present in the molecule, if there is more than one.

Compound	Aldehyde	Phosphate	(-) Charged Groups	Pryidine Ring	Benzene Ring	% Inhibition of ACC
Benzylmalonic Acid	-	-	(2)	-		0.0
Butylmalonic Acid	-	-	(2)	-	-	0.0
1,3-Acetonedicarboxylic Acid	-	-	(2)	-	-	3.4
Nicotinic Acid	-	-			-	2.3
1'Acetoxychavicol acetate (ACA)	-	-	-	-		86.7
Acetylsalicylic Acid (Aspirin)	-	-		-		56.1

4.3 SUMMARY

From the experiments presented in this chapter, it becomes clear that PLP contains multiple structural components which regulate and affect its ability to inhibit ACC. Since it contains all of the favourable components of its analogs, PLP inhibits ACC better than any of the analogs tested. Individually, the phosphate, aldehyde and ring all contribute to the inhibitory characteristics of PLP. The most important moiety appears to be the aldehyde at position 4', since removal of this component substantially reduces the ability of these compounds to inhibit ACC. The phosphate group at position 5' was not as potent as the aldehyde group. Minimally, a benzene or pyridine ring with two carboxyl groups is rather effective as an ACC inhibitor. A pyridine ring with only one carboxyl group is not effective, but in general, compounds containing a pyridine ring showed stronger inhibition of ACC than compounds with a benzene ring. Compounds containing two carboxyl groups are probably better at binding to ACC than the combination of phosphate and the ring hydroxyl of PLP, but direct testing of this hypothesis requires an additional PLP analog to be synthesized. The unusual compound ACA gives very high ACC inhibition at 1 mM, although little structural similarity between ACA and PLP makes ACA's method of ACC inhibition a little unclear. Conceivably, the carbonyl group could be effective binding determinants even if esterified.

CHAPTER FIVE

CONCLUSIONS & FUTURE GOALS

Previous work has shown that PLP can bind to allosteric citrate binding sites on phosphofructokinase [69-71], and that it can inhibit both PFK and citrate transport proteins [72-75]. Using this information, it was hypothesized that PLP might also influence ACC activity by binding to the allosteric citrate binding site. The information presented in this thesis suggests that PLP is a potent inhibitor of acetyl-CoA carboxylase, with an IC_{50} around 225 μM. On balance, based on the evidence obtained thus far, it is likely that PLP does indeed bind to the citrate binding site, but further work will be required to establish this more definitely. Experiments aimed at using citrate as a competitive inhibitor of PLP, notably by adding citrate first to pre-activate ACC, did show substantial protection against PLP, especially when PLP concentrations were below 2 mM and citrate was used at higher concentrations (20 mM). A more extensive dose-response matrix (varying both citrate and PLP concentrations) is possibly warranted. Tentatively, then, given that citrate treatment of liver ACC preparations affords some protection against PLP, it is concluded that PLP is indeed binding to the citrate binding site on ACC.

Why PLP should have such strong affinity for this site is likely due to several of its structural features. Since citrate is highly negatively charged, it's binding site should contain positively charge amino acid residues, at least one of which could be a lysine, such as in phosphofructokinase [69]. Since the aldehyde on PLP can form a Schiff base with the ε-amino group of a lysine, and the phosphate and hydroxyl groups can mimic the negatively charged carboxylic acids on citrate, PLP should be able to (a) bind in a stable complex and (b)

form a temporary covalent linkage with ACC through the Schiff base. It is also possible that the pyridine ring structure can interact with aromatic amino acid side chains adjacent or in the citrate binding site, adding additional stabilization not seen with citrate.

An interesting result reported previously found that although citrate protected PFK against inhibition by PLP, it did not significantly protect the enzyme from PLP incorporation. This was speculated to be a result of either many partial interactions at various ε-amino groups, or a reaction at a particular group made more reactive upon citrate binding to PFK [70]. Whether such a situation is occurring in ACC is currently unknown, but would be important to investigate. This could be achieved in a number of ways, but the simplest is to have sufficient enzyme to monitor the incorporation of PLP on ACC by reduction with borohydride. The amount of PLP incorporated could then be determined by spectrophotometric analysis of the change in absorbance at 325 nm, characteristic of the reduced Schiff base, or by using tritiated borohydride and measuring the counts incorporated. This second approach has been attempted, although initial results were inconclusive, mainly due to imprecision in the procedure, and have thus not been included in this thesis. More recent attempts using tetrameric avidin agarose beads have been more successful, but are still not refined enough for conclusive results, as will be discussed below. Initial tritium incorporation suggested a large excess of PLP binding to ACC, but as mentioned, these results are not reliable, and more recent results show far less tritium incorporation, indicating much less PLP is actually binding to ACC. Typical results reported previously show about a 1 or 2:1 PLP:enzyme ratio [70], although one report found a huge PLP excess of up to 40:1 of PLP:PFK [71].

Use of structural analogs has pointed towards the aldehyde of PLP being crucial for ACC inhibition. The phosphate and the negative charges are also important. An interesting result not reported in this thesis is the observation that treating ACC with PLP followed by either 3,4- or 3,5-pyridinedicarboxylic acid does not produce the same level of ACC inhibition if the enzyme is treated first with the analog and then with PLP. This suggests that the analog binding first is somehow altering the structure of ACC sufficiently to promote additional PLP inhibition. This also suggests that the hypothesis that citrate alters the conformation of PFK such that it can bind more PLP may also hold true in ACC, although in this case the analog may promote both more PLP binding and greater PLP inhibition of ACC. This will again require further tests with promising analogs and PLP, as well as measurements of PLP incorporation.

As mentioned, PLP has been successfully used to determine the sequence of the citrate binding site in PFK, as well as active sites in other proteins. This is a key goal for studies with ACC as well. As shown in Figure 3.16, PLP can be covalently linked to ACC, using sodium borohydride reduction. Refinements in the procedure for determining PLP incorporation are still ongoing. Knowing this, we are using a similar approach to produce labelled peptides. Following the procedure detailed in Figure 3.16, instead of digesting the beads in Sample Loading Buffer, ACC is instead digested with trypsin and the peptides produced are analyzed by spectrophotometry for a reduced Schiff base signal at 325 nm. The spectrophotometric analysis has not been successful, although this was due to technical problems with a batch of beads. These procedures will be refined in the near future. Upon successful spectrophotometric determination of a reduced Schiff base signal in the peptide mix, these will be purified on a reverse-phase HPLC column and monitored for absorbance at

325 nm. The isolated peptide(s) can then hopefully be sequenced using mass spectrometry. Ideally, one peptide will be produced, and upon sequencing will yield the identity of several of the residues in the citrate binding site.

Although three-dimensional structures of mammalian ACC are not yet known, primary sequence homology between mammalian and prokaryotic forms with known structures could enable identification of additional residues in the citrate binding site, and help end the mystery of where citrate binds on ACC, and allow new insight as to how this binding causes shifts in ACC activity and polymerization state in mammalian ACC.

So even if PLP is not a relevant endogenous ACC regulator (additional work will be needed to observe the effects of PLP on *in vivo* lipid synthesis), it could prove to be a useful tool in determining the location of citrate binding on ACC. At the very least it can be used as a way to study additional kinetic and polymerization properties of ACC. For example, questions regarding the polymerization state of PLP-labelled ACC still need to be answered, as this would be very useful in coming to understand if PLP can reverse the polymerization effects of citrate, and thus lower ACC activity. Such work could be accomplished using both size exclusion chromatography and sucrose density centrifugation. These experiments are currently ongoing.

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