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Identification and characterization of acetyl CoA carboxylase as a glycoprotein

Bowers, Diana Fullen, Ph.D.
The Ohio State University, 1991
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IDENTIFICATION AND CHARACTERIZATION
OF ACETYL COA CARBOXYLASE AS A GLYCOPEPTIDE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Diana Fullen Bowers, B.S., M.S.

* * * * *

The Ohio State University
1991

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To Phil, Jessica, and Martha
ACKNOWLEDGEMENTS

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PUBLICATIONS


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Major Field: Food Science and Technology
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LIST OF ABBREVIATIONS

ACC Acetyl CoA Carboxylase
ATP Adenosine triphosphate
ATP-CL ATP citrate lyase
BIS N,N′-Methylene-bis-acrylamide
BME β-mercaptoethanol
cAMP Cyclic adenosine monophosphate
C Celsius
CoA Coenzyme A
cpm Counts per minute
dpm Disintegrations per minute
EDTA Ethylene-diaminetetraacetic acid
g Grams
GC/MS Gas Chromatography/Mass Spectrometry
GPI Glycosylated phosphatidylinositol
HCl Hydrochloric acid
HRP Horseradish peroxidase
IU International units
IS Internal standard
kDa Kilodalton
kg Kilograms
M Molar

xii
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MeOH</td>
<td>Methyl alcohol</td>
</tr>
<tr>
<td>μl</td>
<td>Microliters</td>
</tr>
<tr>
<td>mg</td>
<td>Milligrams</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliters</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>Mr</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N</td>
<td>Normal</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PI</td>
<td>Protease inhibitors</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion chromatogram</td>
</tr>
<tr>
<td>TLCK</td>
<td>Na-p-Tosyl-L-lysine chloromethyl ketone</td>
</tr>
<tr>
<td>TMS</td>
<td>Trimethylsilane</td>
</tr>
<tr>
<td>TPCK</td>
<td>N-Tosyl-L-phenylalanine chloromethyl ketone</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>wt</td>
<td>Weight</td>
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CHAPTER 1
INTRODUCTION

Over the past two decades the role of carbohydrates as regulators of metabolic function via their covalent attachment to proteins has been the subject of extensive investigation. Carbohydrate or glycan side chains attached to polypeptides have been associated with modulation of the physicochemical properties of proteins, stabilization against proteolysis, proteolytic processing of precursor glycoproteins to an active form, and modulation of the biological activity of the protein (1). In addition, carbohydrate side chains have been implicated as essential in the mechanism by which cells recognize other cells, and may function to direct the intracellular transport of proteins for membrane insertion, secretion, or export (1-3). Elucidation of the role of covalently attached oligosaccharides continues as an area of active research, as it is now well established that in fact, most biological proteins are glycoproteins (2,4).

Improved methods of isolation and purification in carbohydrate chemistry have led to the identification and characterization of several types of oligosaccharide side
chains, and their linkages to glycoproteins (5). Oligosaccharides may be glycosidically linked to a protein via the nitrogen moiety of asparagine, or via the oxygen moiety of a hydroxyl group of serine, threonine, hydroxylysine or hydroxyproline (2,4,6). \(N\)-linked oligosaccharides, which are more abundant in nature, form when an \(N\)-acetylglucosamine residue bonds to the nitrogen of the amide group of asparagine on the protein backbone. The three major classes of \(N\)-linked oligosaccharides are identified as high mannose, complex, or hybrid, based on their sugar composition (4). One to thirty or more glycan chains may be present on any given glycoprotein (6), with the total carbohydrate content ranging from 1% to more than 85% of the total molecular weight (2,6). The principal sugars found in human glycoproteins include galactose, glucose, mannose, fucose, \(N\)-acetylated neuraminic acid, \(N\)-acetylgalactosamine, and \(N\)-acetylglucosamine.

In a recent review (7), Low describes various mechanisms by which membrane associated proteins may be anchored in the membrane lipid bilayer. The review includes extensive data describing a covalent linkage between a phosphatidylinositol molecule, having its 1,2-diacylglycerol moiety embedded in the lipid bilayer, and an intervening glycan structure attached to the \(C\)-terminal amino acid of a protein. This type of membrane anchor has been identified for several glycoproteins, including the mammalian tissue enzymes
alkaline phosphatase and 5′-nucleotidase (7-9).

**Statement of the Problem**

Acetyl CoA carboxylase (ACC) catalyzes the carboxylation of acetyl-CoA to malonyl-CoA in the first committed reaction of de novo fatty acid biosynthesis (10). Recent studies indicate that the enzyme, previously thought to be associated with only the soluble portion of homogenized cells, is also found in association with the outer mitochondrial membrane of rat liver cells (11-14). Allred et al. (11), have shown the mitochondrial fraction of ACC in rat liver to be relatively inactive as compared to the cytoplasmic form, and that the distribution of the total quantity of enzyme between the cytoplasm and mitochondria is dietary dependent. With total quantity of the enzyme remaining unchanged, fasting caused a shift in the subcellular distribution to the mitochondrial fraction, whereas refeeding was associated with an increase in the cytoplasmic fraction.

Subsequent work from the same laboratory using an alloxan-induced diabetic rat model showed that the diabetic state also produced a shift from the cytoplasmic to the mitochondrial form of ACC, while total enzyme quantity remained unchanged (12). This study indicated that insulin may be a regulatory factor in the mobilization of the inactive mitochondrial form to the active cytoplasmic form of the enzyme. Data suggests that the mitochondrial fraction
of the enzyme represents an inactive storage form, which is released and activated under lipogenic conditions (13).

Further studies by Allred et al. (14), comparing homozygous obese (fa/fa) Zucker rats to homozygous lean (Fa/Fa) littermates also supported the above findings. Results showed that regardless of diet or genetic obesity, there was no change in total enzyme quantity per unit weight of liver. The increase in the active cytoplasmic enzyme in the obese Zucker rat was, as in the above previously described studies, found to be due to a change in subcellular distribution of the enzyme.

Discovery that the distribution of ACC between the mitochondria and cytosol could be altered without a change in total enzyme quantity led to an area of inquiry of how the protein was attached to the outer mitochondrial membrane, and what physiological factors could induce its release. Preliminary studies were undertaken to first determine if ACC was indeed a glycoprotein. Samples of purified enzyme were subjected to trimethylsilane (TMS) derivatization and, along with sugar standards, analyzed using gas chromatography. Results showed that peaks from the purified enzyme eluted simultaneously with several sugar standards, indicating that ACC may indeed be a glycoprotein. Thus, further studies were undertaken using the hypothesis that ACC was a glycoprotein, bound to the outer mitochondrial membrane via a phosphatidylinositol-glycan linkage, as described by Low (7).
Significance of the Study

Prior to the work of Allred et al., ACC was known to be associated primarily in the cytoplasmic compartment of cells (10), although enzyme activity has also been reported in association with the microsomal fraction of rat liver and adipose tissue (15,16). The total quantity of cytosolic enzyme, however, was thought to be regulated by enzyme synthesis and degradation alone (17,18). Discovery of the mitochondrial form of ACC and its change in subcellular distribution due to diet (11), in alloxan-induced diabetes (12), and in genetic obesity (14), has introduced another important regulatory mechanism for this enzyme. These changes in the subcellular distribution of ACC characterize the enzyme as ambiquitous, and represent a form of metabolic regulation which occurs in an intermediate time frame, with maximal activity and quantity of the cytosolic form occurring after a lag time of 8 hours in the liver of refed rats (19).

Glycan moieties of glycoproteins are known to be involved in proteolytic processing of precursor glycoproteins, and also to mediate the activity of glycoproteins (1). Therefore, the identification of ACC as a glycoprotein along with the chemical characterization of its glycan moiety, represent important first steps in elucidating the function and metabolic role of the mitochondrial form of this enzyme. This work may also aid in identifying the mechanism by which the inactive mitochondrial form of the enzyme is attached to the
outer mitochondrial membrane, and factors which cause its release and activation.
CHAPTER II
REVIEW OF LITERATURE

The Metabolic Role of Acetyl CoA Carboxylase

De novo fatty acid biosynthesis occurs in the soluble (cytosolic) fraction of cells (10). Many tissues are capable of fatty acid biosynthesis including liver, kidney, brain, lung, mammary gland, and adipose tissue (20). The process involves the enzyme systems ATP citrate lyase, acetyl-CoA carboxylase, and fatty acid synthetase, which function in sequence to produce free palmitate; a sixteen carbon fatty acid. Acetyl-CoA, produced when ATP citrate lyase cleaves extramitochondrial citrate, serves as the substrate for the process, with NADPH, ATP, magnesium (or manganese), and HCO$_3^-$ as substrates (20).

Acetyl CoA carboxylase (EC 6.4.1.2) catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA. Because this reaction is the first committed step in fatty acid biosynthesis, ACC is considered to be the regulatory enzyme for this process (10). The reaction is not reversible and occurs as a two step process. The first step requires ATP and magnesium and involves the carboxylation of the biotin moiety of ACC using HCO$_3^-$ as the CO$_2$ donor. A carboxybiotin
intermediate is formed from the first step of the reaction. In the second step, the activated carboxyl group is transferred from the carboxybiotin intermediate to the methyl carbon of acetyl-CoA, thus forming malonyl-CoA (10).

The newly synthesized malonyl-CoA is then linked to an acyl carrier protein (ACP), and through a condensation reaction with acetyl-ACP, fatty acid chain elongation begins. After the initial condensation, the fatty acid chain is reduced, dehydrated, and again reduced, before combining with another molecule of malonyl-ACP. Chain elongation takes place on the fatty acid synthetase complex (20).

The Metabolic Regulation of Acetyl CoA Carboxylase

The activity of ACC is regulated by metabolic effectors (21-26), covalent modification (26-29), subcellular distribution (11-14), and changes in the rates of enzyme synthesis and degradation (17,18). Metabolic effectors and covalent modification alter the enzyme activity on a short term basis via changes in catalytic efficiency, whereas synthesis and degradation provide long-term regulation of the enzyme. The more recently discovered regulatory mechanism of subcellular distribution is reported to occur in an intermediate time frame (19).

Allosteric activation of acetyl Co-A carboxylase is induced by the metabolic effector citrate (21-24,26). Citrate activation involves a conformational change in the enzyme
near the biotin prosthetic group (21), which is thought to increase the reactivity of the carboxybiotin enzyme intermediate. This conformational change induces polymerization of an inactive protomeric form of the enzyme into an active filamentous form, composed of 10-20 protomers (22). Large amounts of citrate (5-20 mM) are required for the in vitro activation of ACC (23). However, the intracellular concentration of citrate is substantially lower (.3mM), and remains relatively stable over a variety of metabolic states, including fasting and refeeding (30).

The phosphorylation state of the enzyme is thought to account for the paradox between the amount of citrate required for in vitro vs. in vivo activation of the enzyme (31). Thampy and Wakil (32) have recently isolated a citrate-independent form of ACC from rapidly freeze-clamped rat liver, and have identified it as the dephosphorylated form of ACC. This work showed that ACC isolated from rapidly freeze-clamped livers showed high activity (3.5 units/mg) in the absence of citrate and low phosphate content (5.0 mol of phosphate/mol of subunit). Conversely, ACC isolated from unfrozen liver or liver kept in ice-cold sucrose solution for 10 minutes and then freeze-clamped, showed low activity (0.3 units/mg) and high phosphate content (7-8 mole of phosphate/mol of subunit). The citrate concentration to achieve half-maximal activity in the later preparations was 1.6 mM, well above the physiological range of 0.3 mM. The
authors suggested that ACC undergoes rapid phosphorylation with subsequent inactivation after excision of the liver, thus requiring high concentrations of citrate for in vitro activation. Therefore, it appears that under lipogenic conditions, the efficiency of the physiologic concentration of citrate in activating ACC is dependent on the phosphorylation state of the enzyme (31).

The enzyme is allosterically inhibited by long chain fatty acyl CoA derivatives (24-26). Early work by Pande and Mead (25) suggested that the in vitro inhibition of several different enzyme activities by free long chain fatty acids was due to the detergent properties of the various fatty acid inhibitors. However, it was later proposed by Goodridge (24) that fatty acyl-CoA derivatives inhibited fatty acid synthesis by directly inhibiting ACC or by inhibiting the mitochondrial citrate carrier, thereby reducing ACC activity caused by citrate.

Oglivara et al. (33) showed that inhibition of ACC by long-chain fatty acyl-CoA esters is accompanied by enzyme depolymerization from the active filamentous form to the inactive protomeric form of the enzyme. It is thought that the two allosteric control mechanisms of fatty acid synthesis, citrate induced substrate activation and long-chain fatty acyl-CoA mediated feedback inhibition, function as a dual mechanism in vivo (33). Changes between the inactive protomeric and active polymeric forms of the enzyme
appear to be the mechanism through which allosteric effectors influence activity (34).

Acetyl CoA carboxylase is also regulated on a short term basis by covalent modification via phosphorylation and dephosphorylation (26-29,31). Because the efficacy of the metabolic effectors citrate and long-chain fatty acyl-CoA esters in influencing ACC activity is intimately linked to the enzyme's phosphorylation state, it is thought that the allosteric and covalent modification mechanisms complement each other for maximum control of ACC activity (26,31). The phosphorylation state of the enzyme serves to alter the conformation of ACC in such a way as to allow maximal sensitivity to either activation by citrate or inhibition by palmitoyl-CoA (26,31).

Initial work by Carlson and Kim (27) showed that partially purified ACC was inactivated by ATP in a reaction that was both time- and temperature-dependent. They proposed that phosphorylation of the enzyme was carried out by an ATP-dependent kinase, and its reactivation by dephosphorylation was mediated via a magnesium-dependent phosphatase. Subsequent work from the same laboratory (28) showed that phosphorylation of the enzyme occurred on a subunit of the carboxylase molecule itself. Allred et al. (29) using purified ACC from liver of fasted-refed rats showed that an endogenous protein kinase was present in the purified preparation, which catalyzed the transfer of radioactive
phosphate from \([r^{-32}P]}\)ATP to ACC. This transfer was accompanied by a decrease in enzyme activity, which was not further enhanced by the addition of either cyclic-AMP or purified cyclic-AMP-dependent protein kinase catalytic subunit.

The phosphorylation state of ACC is regulated by the hormones insulin and glucagon (35,36). Witters et al. (35) showed that insulin promoted rapid activation of ACC in Fao Reuber hepatoma cell lysates. The insulin-stimulated enzyme showed a marked decrease in total enzyme phosphorylation coincident with activation, as well as an increase in citrate-independent and -dependent activity. Insulin may induce dephosphorylation of ACC by inhibiting the activity of protein kinase(s) or stimulating the activity of protein phosphatase(s). Holland et al. (36) found that in isolated rat hepatocytes, glucagon caused a 50% decrease in fatty acid synthesis. This decrease was entirely attributed to phosphorylation of ACC by cAMP-dependent protein kinase, with resultant inactivation.

Seven regulatory phosphorylation sites have been identified for ACC (31). Six of the sites are localized on the first 100 amino acid residues from the NH\(_2\) terminus, while the seventh is located at the serine-1200 site. Munday et al. (37) examined three major sites phosphorylated on ACC by three different protein kinases shown to inactivate the enzyme: cAMP-dependent protein kinase, acetyl-CoA carboxylase
kinase-2, and AMP-activated protein kinase. Each protein kinase phosphorylated two of three major sites. Phosphorylation of site 3 was primarily responsible for the large decrease in $V_{max}$ produced by the AMP-activated protein kinase, while phosphorylation of site 1 by cAMP-dependent protein kinase and ACC kinase-2 may be responsible for the increase in the $K_s$ for citrate as well as for a more modest decrease in the $V_{max}$.

Subcellular distribution or physical alteration, has recently been elucidated as a mechanism of ACC regulation (11-14). Determination of enzyme quantity based on biotin content rather than activity, through the use of [$^{14}$C]methyl avidin, was instrumental in the discovery and characterization of the relatively inactive mitochondrial form of the enzyme (11,38). By incubating SDS-denatured proteins of subcellular fractions of rat liver with [$^{14}$C]methyl avidin, Allred and Roman-Lopez (13) were able to identify two mitochondrial forms of ACC, both of which were precipitated by antibody to the enzyme. This study showed that approximately 75% of the total ACC was associated with the mitochondria, while 3.5% was associated with the microsomal fraction. The remainder of the enzyme was present in the cytosol as intact active enzyme, or as a degradation product. The fraction of ACC associated with the mitochondria was relatively inactive as compared to the cytosolic enzyme.

Fasting (11) and acute alloxan diabetes (12) caused a
shift in the subcellular distribution of ACC from the active cytosolic form to the inactive mitochondrial form, while total enzyme quantity remained unchanged. Those studies indicated that insulin may be involved in the mobilization and activation of the enzyme (12). Later work using a genetically obese rat model (14) demonstrated a relative shift in the enzyme from the inactive mitochondrial form to the active cytosolic form.

Using fasted-refed rats, Roman-Lopez et al. (19) found maximal activity and quantity of ACC to occur after a lag time of about 8 hours (19). Allred and Roman-Lopez suggest that the inactive form of the enzyme associated with the mitochondrial membrane represents a storage form which can be released and activated under lipogenic conditions (13). This hypothesis is supported by the observations of Spence et al. (39) in studies of the effects of insulin on the activity, rate of synthesis, and amount of translatable mRNA coding for ACC and HMG-CoA reductase. They found that when insulin was added to the culture medium of adult-rat hepatocytes maintained in primary culture, there was a rapid increase in ACC activity within 1-2 hours, with maximal activity occurring after six hours. The increase in activity was observed before the increases in the rate of synthesis and the amount of translatable mRNA were apparent. This observation suggested the involvement of post-translational modification of ACC, leading to the initial increase in
activity.

Synthesis and degradation of ACC represent long-term control mechanisms, with changes occurring in a 24 to 60 hour time frame (17-18,40-41). Like phosphorylation and subcellular distribution, the rates of synthesis and degradation are also under hormonal control (17-18,40-41). Early work by Majerus and Kilburn (17) attributed the changes in cytosolic ACC activity after dietary alteration to changes in the enzyme content of liver rather than from activation or inhibition of the preformed enzyme. They found a 5- to 10-fold increase in the rate of enzyme synthesis after fat-free feeding of previously fasted rats. Degradation rates (t%_f) for ACC in rats fed a fat-free diet or fasted were 48 and 18 hours respectively.

Nakanishi and Numa (18) likewise attributed decreases in the enzyme activity of fasted and diabetic rats to be due to a decrease in enzyme quantity related to synthesis and degradation rates. Synthesis of rat liver ACC was decreased 1.9-fold and 1.7-fold by fasting and diabetes, respectively. Fat-free refeeding produced a 4-fold increase in the rate of enzyme synthesis. Degradation rates for the enzyme in normal, refed, and diabetic rats were found to be 59, 55, and 59 hours respectively. Fasted rats showed an increased rate of ACC degradation of 31 hours. The authors concluded that the decrease in carboxylase quantity in fasted animals was due to a diminished rate of enzyme synthesis and to accelerated
degradation. Later work by Katz and Ick (40) and Giffhorn and Katz (41) also found an increase in enzyme activity in primary cultures of rat hepatocytes treated with increasing concentrations of insulin or glucose. The authors suggested that this increased activity was due to induction of the enzyme rather than to allosteric effects or interconversion. The rate of enzyme degradation in these studies remained essentially unchanged.

Previous study of the rates of ACC synthesis and degradation has been complicated by the inability to measure enzyme quantity directly. In vitro measurement of enzyme activity was extrapolated to represent a measure of in vivo enzyme quantity. The recently described method of measuring ACC quantity based on biotin content rather than activity (38), along with consideration to the previously unknown control mechanism of subcellular distribution (11-14) will allow more direct and accurate study of ACC synthesis and degradation.

The two other key enzymes involved in de novo fatty acid biosynthesis, ATP citrate lyase and fatty acid synthetase, have been found to be regulated by mechanisms which parallel those of ACC (41-45). ATP-citrate lyase (EC 4.1.3.8) cleaves extramitochondrial citrate to produce acetyl-CoA and oxaloacetate. The acetyl-CoA then becomes the substrate needed for ACC to produce malonyl-CoA. Acetyl CoA carboxylase and ATP-CL demonstrated a parallel induction in primary
hepatocyte cultures incubated with increasing concentrations of glucose and/or insulin (41), indicating a co-ordinate long-term regulation. The two enzymes also show similar increases in phosphorylation within rat epididymal adipose tissue exposed to insulin (42). Like ACC, ATP-CL is also regulated by subcellular distribution (43-44). The substrates for ATP-CL (CoA, citrate, and ATP) were found to accelerate the release of the non-cytosolic fraction from the mitochondrial membrane (43). Because there is no known mechanism for the transport of CoA into or out of the mitochondria, the association of ATP-CL with the mitochondrial membrane may be advantageous for efficient production of cytosolic acetyl-CoA (43). Like ACC, the relative amounts of mitochondrial and cytosolic forms of ATP-CL also vary with the animals nutritional state (44). Using digitonin fractionation of isolated hepatocytes, Cornell et al. (44) were able to show a 10 to 12 fold increase in ATP-CL activity in rats that were fasted then refed a fat-free diet, as opposed to rats that were fasted or fed ad libitum. The addition of 20 μM CoA to the digitonin fractionation medium caused all of the ATP-CL to be released from the cells into the cytosol. Conversely, decreasing the cellular free CoA, resulted in an increase in the bound fraction of ATP citrate lyase.

Fatty acid synthetase also shows similar regulatory patterns to those of ACC (45). Porter and Swenson (45) have
shown that both enzymes are induced by fasting and refeeding of animals. Insulin stimulated the induction of the enzymes on the order of 10- to 30-fold, which was attributed to a change in the quantity of translatable mRNA, possibly due to a change in the rate of transcription of genes coding for the enzymes.

The Metabolic Role of Glycoprotein Glycans

Many types of proteins, including blood plasma proteins, enzymes, and hormones, are now known to have covalently-linked oligosaccharide side chains (2). Glycan side chains linked to proteins mediate several diverse biological functions (1-3,46). The functions served by the glycan moiety of glycoproteins include: 1) maintenance of protein conformation and solubility, 2) proteolytic processing and stabilization of the polypeptide against uncontrolled proteolysis, 3) mediation of biological activity, 4) intracellular sorting and externalization of glycoproteins, and 5) embryonic development and differentiation (1).

The specific functions of glycan side chains have been elucidated through the use of selective inhibitors of protein glycosylation, including the antibiotic tunicamycin, sugar analogs, and glycosidase inhibitors (46). These inhibitors serve to either alter the oligosaccharide structures, or allow the synthesis of proteins devoid of the oligosaccharide moieties. In addition, several glycosidases of defined
specificity have been useful in identifying the structure and function of the glycan moieties (6). Exoglycosidases, including neurominidases and galactosidases, act at positions external to the oligosaccharide chain. Their sequential use removes terminal N-acetylneuraminic acid residues and subterminal galactose residues from most glycoproteins. Endoglycosidases cleave the oligosaccharides at specific N-acetylgalactosamine residues near the polypeptide backbone. Thereby, large oligosaccharide chains may be isolated for structural analysis. Endoglycosidase F acts on both high-mannose and complex oligosaccharides, while endoglycosidase H acts on high-mannose oligosaccharides.

**Structural Aspects of Glycoproteins**

The principal monosaccharides identified as components of human glycoproteins include the neutral sugars L-fucose, D-galactose, D-glucose, D-mannose; the amino sugars N-acetylgalactosamine and N-acetylgalactosamine; and the acidic sugar N-acetyl- (or glycolyl-) neuraminic acid (NANA) (4). Glucose is found infrequently in mature glycoproteins and appears to be confined mainly to collagens (2), while the stereoisomers of glucose, galactose and mannose, are more commonly found in several different glycoprotein types. Fucose is structurally similar to galactose except that a hydrogen atom replaces one hydroxyl group. (Figure 1) Fucose may be external in both N- and O- linked glycoproteins, or

Figure 1. Sugars found in glycoproteins.*
linked to the $N$-acetylglucosamine residue attached to asparagine in $N$-linked oligosaccharides.

In the amino sugars $N$-acetylglucosamine and $N$-acetylgalactosamine, an aminoacetyl group replaces one of the hydroxyl groups of the native sugar. $N$-acetylgalactosamine attaches to serine or threonine in $O$-linked glycoproteins, while $N$-acetylglucosamine is the sugar moiety that attaches to asparagine in $N$-linked glycoproteins. Because neuraminic acid contains both an aminoacetyl and a carboxyl group, its structure is quite different from those of the other monosaccharides. The acidity of this molecule is responsible for the distinctive chemical and physical properties of the polymers which contain it (2). It is often the terminal sugar in both $N$- and $O$-linked glycoproteins.

Glycan chains typically contain up to 15 sugar units of various combinations (2). One to thirty or more glycan chains may be present on any given glycoprotein (6), with the total carbohydrate content ranging from 1% to more than 85% of the total molecular weight (2,4,6). Sugar polymers on a polypeptide backbone can combine in many ways to form extensive branching, creating diverse conformations in glycoproteins (2).

Studies with asparaginyl-linked glycoproteins have identified three basic types of oligosaccharide structural patterns; high mannose, complex, and hybrid (4). (Figure 2) The inner core of the three types is a common feature, and
High-Mannose
\[
\begin{align*}
\text{Man}\alpha1&\rightarrow 2, \text{Man}\alpha1\rightarrow 6 \quad \text{Man}\alpha1\rightarrow 6 \\
\text{Man}\alpha1&\rightarrow 2, \text{Man}\alpha1\rightarrow 3 \quad \text{Man}\beta1\rightarrow 4, \text{NAcGlu}\beta1\rightarrow 4, \text{NAcGlu-Asn} \\
\text{Man}\alpha1&\rightarrow 2, \text{Man}\alpha1\rightarrow 2, \text{Man}\alpha1\rightarrow 3
\end{align*}
\]

Hybrid
\[
\begin{align*}
\text{Man}\alpha1&\rightarrow 6 \quad \text{Man}\alpha1\rightarrow 6 \\
\text{Man}\alpha1&\rightarrow 3 \quad \text{Man}\beta1\rightarrow 4, \text{NAcGlu}\beta1\rightarrow 4, \text{NAcGlu-Asn} \\
\text{Gal}\beta1&\rightarrow 4, \text{NAcGlu}\beta1\rightarrow 2, \text{Man}\alpha1\rightarrow 3
\end{align*}
\]

Complex

Biantennary
\[
\begin{align*}
\text{NANA}\alpha2&\rightarrow 6, \text{Gal}\beta1\rightarrow 4, \text{NAcGlu}\beta1\rightarrow 2, \text{Man}\alpha1\rightarrow 6 \\
\text{NANA}\alpha2&\rightarrow 6, \text{Gal}\beta1\rightarrow 4, \text{NAcGlu}\beta1\rightarrow 2, \text{Man}\alpha1\rightarrow 6 (\text{Fuc}\alpha1\rightarrow 6)
\end{align*}
\]

Triantennary
\[
\begin{align*}
\text{NANA}\alpha2&\rightarrow 6, \text{Gal}\beta1\rightarrow 4, \text{NAcGlu}\beta1\rightarrow 6 \\
\text{NANA}\alpha2&\rightarrow 6, \text{Gal}\beta1\rightarrow 4, \text{NAcGlu}\beta1\rightarrow 2 \quad \text{Man}\beta1\rightarrow 4, \text{NAcGlu}\beta1\rightarrow 4, \text{NAcGlu-Asn} \\
\text{NANA}\alpha2&\rightarrow 6, \text{Gal}\beta1\rightarrow 4, \text{NAcGlu}\beta1\rightarrow 2, \text{Man}\alpha1\rightarrow 6 (\text{Fuc}\alpha1\rightarrow 6)
\end{align*}
\]

Tetraantennary
\[
\begin{align*}
\text{NANA}\alpha2&\rightarrow 6, \text{Gal}\beta1\rightarrow 4, \text{NAcGlu}\beta1\rightarrow 6 \\
\text{NANA}\alpha2&\rightarrow 6, \text{Gal}\beta1\rightarrow 4, \text{NAcGlu}\beta1\rightarrow 2 \quad \text{Man}\beta1\rightarrow 4, \text{NAcGlu}\beta1\rightarrow 4, \text{NAcGlu-Asn} \\
\text{NANA}\alpha2&\rightarrow 6, \text{Gal}\beta1\rightarrow 4, \text{NAcGlu}\beta1\rightarrow 2, \text{Man}\alpha1\rightarrow 6 (\text{Fuc}\alpha1\rightarrow 6)
\end{align*}
\]


Figure 2. Types of asparagine-linked oligosaccharides.*
is comprised of two covalently bonded N-acetylglucosamine residues, and three mannose residues. One N-acetylglucosamine residue is attached to asparagine, while the other is attached to a mannose residue from which two other mannose residues branch (4). This common inner core structure may be explained by the fact that the three types share an initial common mechanism of biosynthesis.

The high mannose glycan structures contain only two types of monosaccharide units, mannose and N-acetylglucosamine. Three to eleven mannose residues are found in the peripheral region of the oligosaccharide (4,5). In contrast, complex glycan structures may contain galactose, mannose, L-fucose, N-acetylglucosamine, and/or sialic acid in the peripheral region. One or two complex oligosaccharide chains may be attached to each of the two outer mannose residues of the inner core. Complex glycans can therefore be further classified as biantennary, triantennary, or tetraantennary. (Figure 2) Hybrid type structures contain oligosaccharides of both the high mannose and complex types in the peripheral region.

Glycosylation occurs at specific sites on the protein molecule, and is species-, cell-, and tissue-specific (5). The type of covalent bond that links the oligosaccharide structure to the peptide plays a major role in the structural uniqueness of each glycoprotein (4). Three types of glycopeptide bonds have been identified, and are
differentiated based on the attachment of a given sugar to a specific functional group of an amino acid. The bond types include N-glycosidic bonds, O-glycosidic bonds, and S-glycosidic bonds (4). Some glycoproteins contain both N- and O-glycosidic linkages.

N-glycosidic bonds are formed between the N-acetylglucosamine residue of the glycan inner core and the amide group of asparagine. (Figure 3a) This is the most commonly occurring bond in glycoproteins. The bond is formed on the polypeptide around the tripeptide sequence Asn-X-Ser(Thr), with X representing any amino acid with the possible exceptions of proline or aspartate (47). The presence of this tripeptide sequence on the polypeptide is highly suggestive of sugar modification. Approximately one-third of the tripeptide sequences of Asn-X-Ser(Thr) on native polypeptides are glycosylated (6). A specific amino acid sequence on the N-terminal side of asparagine has not been identified. The N-glycosidic bond is stable to mild alkaline conditions (4), which serves to distinguish this linkage from common O-glycosidic linkages.

Three O-glycosidic linkages between sugar moieties and an oxygen moiety of a hydroxyl group on serine, threonine, hydroxylysine or hydroxyproline residues have been identified (2,4,6). The most common involves a linkage between N-acetylgalactosamine and serine or threonine, in a tripeptide sequence of Asn-Y-Ser(Thr) (figure 3b). In this case, Y is

Figure 3. Types of monosaccharide-amino acid linkages.*
an amino acid other than aspartate (6). As with N-glycosidic linkages, although this series can occur several times in any polypeptide, not all such sequences are glycosylated. Rather, glycosylation of a particular serine or threonine residue is based on the protein conformation surrounding the tripeptide sequence as it emerges through the endoplasmic reticulum (6). This bond is characteristic of mucin-type chains, and is found in submaxillary glycoproteins, bovine cartilage, antifreeze glycoprotein, immunoglobulin A, and the β-subunit of human chorionic gonadotropin (4). The bond readily undergoes cleavage by β-elimination under alkaline conditions (4).

A second type of O-glycosidic linkage involves sugars other than N-acetylgalactosamine, namely xylose, mannose, galactose, and fucose, forming the glycosidic bond (figure 3c). This linkage is characteristic of proteoglycans, and is also alkali-labile. The third and least common type of O-glycosidic linkage involves bonding between galactose and hydroxylysine or hydroxyproline (figure 3d,e). This bond is found in various collagens, basement membranes, and the lens capsule. In contrast to the first two types of O-glycosidic linkages, this bond is alkali-stable (4).

When the glycan chain is bonded via the thiol group of cysteine, an S-glycosidic bond is formed. This linkage is rare, and involves either a glucose or galactose residue from the glycan chain. It has been identified in human urinary
glycopeptide, and human erythrocyte glycopeptide (4).

A fundamental difference in \( N \)- and \( O \)-linked glycoproteins is their biosynthetic pathways (6,48-49). The sugars of \( O \)-glycosidically linked glycans are built up directly onto the polypeptide backbone through stepwise donations of sugar moieties from nucleotide sugars, namely UDP-\( N \)-acetylgalactosamine, UDP-galactose, and CMP-\( N \)-acetylneuraminic acid (48). Membrane bound glycoprotein glycosyltransferases catalyze these sugar transfers, with specific transferases catalyzing one specific type of linkage. The addition of the first inner sugar residues occurs during translation of the protein in the endoplasmic reticulum (6). Terminal sugars such as \( N \)-acetylneuraminic acid are added on the Golgi apparatus.

In sharp contrast to the biosynthesis of \( O \)-linked glycans, \( N \)-linked glycans are first assembled on a pyrophosphoryl-dolichol backbone and then transferred \textit{en bloc} to asparagine residues of acceptor apoglycoproteins during their synthesis on membrane-bound polyribosomes (48). Once transferred, the glycan chain undergoes processing to create either the high-mannose, complex, or hybrid form.

Dolichol is a polyisoprenol compound which is synthesized in the cytosol from mevalonate. It is a long hydrocarbon, having 17 to 20 repeating isoprenoid units. Dolichol must first be phosphorylated to form dolichol phosphate (Dol-P) before oligosaccharide chain elongation can begin (48-49).
Cellular levels of Dol-P appear to be rate limiting in glycoprotein formation (6). Rip and Carroll (49) found the highest concentrations of dolichol to be in the Golgi, however, the rough endoplasmic reticulum contained the largest absolute amounts. These findings were consistent with the important role of dolichol as an intermediate in asparagine-linked glycoprotein synthesis.

Once phosphorylated, Dol-P undergoes a transfer reaction with UDP-N-acetylglucosamine (UDP-GlcNAc) to form GlcNAc-P-P-Dol, the key lipid which serves as an acceptor for other sugars in N-linked glycan assembly. This reaction occurs in the membranes of the endoplasmic reticulum, and is blocked by the antibiotic tunicamycin (46). Subsequent steps represent the formation of the inner core of asparagine-linked glycans and include: 1) the addition of a second GlcNAc residue using UDP-GlcNAc as the donor, 2) the addition of five mannose residues using GDP-mannose as the donor, 3) the addition of four more mannose residues using Dol-P-mannose as the donor, and lastly, 4) the addition of three peripheral glucose residues donated by Dol-P-Glc (6,48). The oligosaccharide is then transferred to an asparagine residue of an acceptor protein emerging from the luminal surface of the membrane of the endoplasmic reticulum (6). The transfer is catalyzed by an 'oligosaccharide transferase' which will recognize any glycolipid with the general structure R-(GlcNAc)$_2$-P-P-Dol (6).
After transfer, the oligosaccharide undergoes processing to create either the high-mannose, complex, or hybrid forms (6,48). High-mannose chains are formed by the removal of the terminal glucose residues by glucosidase I, followed by removal of the next two glucose residues by glucosidase II. Following glucose removal, up to four more mannose residues may also be removed. The formation of high-mannose glycans takes place in the endoplasmic reticulum (6).

Processing continues from this point on the Golgi apparatus for the formation of complex oligosaccharides. The four external mannose residues are removed, followed by the addition of a GlcNAc residue onto one of the inner core mannose residues. Next, another mannose residue is removed, reducing the total number of mannose residues to three, which are confined to the inner core of the glycan chain. (Figure 2) To the second branched inner core mannose residue, another GlcNAc moiety is added. The final steps involve the sequential addition of fucose, galactose, and N-acetylneuraminic acid onto the GlcNAc moieties by specific transferases. Hybrid oligosaccharides, also formed on the Golgi apparatus, typically have one complex oligosaccharide chain attached to one of the inner core mannose residues, while only mannose residues are attached to the second inner core mannose residue. (Figure 2)
Mechanisms of Protein Linkages to Membranes

Proteins are attached to the lipid bilayer of biological membranes through either binding to the polar surface, or by penetrating the bilayer to some extent (7). (Figure 4) Interaction between the hydrophobic and polar domains of the polypeptide with the hydrophobic core of the lipid bilayer occurs when proteins are embedded in the lipid bilayer. This interaction is important not only for attachment of the protein to the membrane, but also for allowing the protein to adopt its correct conformation and transmembrane orientation for proper function (7).

Many proteins associated with the lipid bilayer in both prokaryotic and eukaryotic cell membranes have also been found to have covalently attached lipid. (Figure 4d, 4e, 4f) The covalent attachment between the fatty acid and protein is via amide, thioester, or O-acyl bonds. In proteins containing a covalently attached glycosylated phosphatidylinositol (GPI) moiety (Figure 4f), considerable evidence indicates that the attached lipid is responsible for membrane anchoring (7).

The GPI membrane anchor is widely distributed among many different organisms from protozoan parasites to mammalian species (7,50). However, unlike other proteins anchored by a covalent fatty acid attachment, this anchor has not been reported in prokaryotic cells. In eukaryotic cells, a diverse array of proteins from hydrolytic enzymes to antigens to cell
a. much of the protein is buried within the lipid bilayer.
b. two hydrophilic domains are connected by a hydrophobic transmembrane domain.
c. one hydrophilic domain is connected by a hydrophobic membrane domain.
d. a covalently attached lipid anchor via a amide-link to the $\alpha$-amino group on the $N$-terminal glycine residue (e.g. NADH, cytochrome $b_5$ reductase)
e. an amide-linked fatty acid and a thioether-linked diacylglycerol attached to the $\alpha$-amino and thiol groups of the $N$-terminal cysteine, respectively (e.g. *E. coli* lipoprotein and *B. licheniformis* penicillinase)
f. a phosphatidylinositol molecule is covalently attached through an intervening glycan structure to the $C$-terminal amino acid of the protein.


Figure 4. Types of protein anchors to membranes.*
adhesion proteins have been reported to utilize the GPI anchor (50).

Proteins utilizing the GPI anchor are mainly located on the cell surface; the mechanism has yet to be reported as an anchor for intracellular locations. Low initially postulated (7) that this may simply reflect the fact that the anchoring of proteins is easier to study at the cell surface as opposed to intracellular locations. In a later report however (50), Low indicated that GPI-anchored proteins were unlikely to occur in association with intracellular organelles such as the nucleus or mitochondria because evidence suggested that the GPI anchor attachment occurs after the COOH-terminal region of the polypeptide is translocated across the membrane of the rough endoplasmic reticulum (51). GPI-anchored proteins seemed more likely to occur in association with other organelles such as lysosomes, endosomes and secretory granules due to the close temporal and topological relationship of protein translocation and the GPI anchor attachment in the lumen of the rough endoplasmic reticulum. At any rate, the involvement of phosphatidylinositol as an anchor for cytoplasmically oriented proteins has received little attention. However, two intracellular proteins, myelin basic protein and styrene oxide hydrolase, have been reported to have covalently attached phosphoinositide, although the attachment appears to involve direct linkage of a phosphorylated phosphatidylinositol to serine residues (7).
The C-terminal structure containing the membrane anchoring domain from three GPI-anchored proteins of diverse origin (Thy-1, human erythrocyte acetylcholinesterase (AChE), variant surface glycoprotein (VSG)) shows a similar composition pattern (7). They all contain ethanolamine (1 mol/mol in VSG and 2 mol/mol in Thy-1 and AChE), glucosamine (1 mol/mol), myo-inositol (1 mol/mol), and where analyzed, glycerol, phosphate, and fatty acid. In addition to glucosamine, other sugars were present including mannose (2 mol/mol) and galactose (0-8 mol/mol) in VSG, and mannose (2-3 mol/mol) and galactosamine (0-1 mol/mol) in Thy-1.

The basic structure of the anchoring domain begins with a phosphatidylinositol molecule whose 1,2-diacylglycerol moiety is embedded in the lipid bilayer, which serves as the membrane anchor (7). (Figure 5) Next a glycan chain of varied structure and composition is linked to the phosphatidylinositol molecule via a glycosidic linkage with a glucosamine residue, having a free amino group. To the non-reducing end of the glycan, a mannose-6-phosphate residue is phosphodiester-linked to the hydroxyl of an ethanolamine residue. The ethanolamine residue is then amide-linked via its amino group to the α-carboxyl of the C-terminal amino acid of the protein. This model is based on work with VSG, and is consistent with composition and degradation studies of the anchors of other proteins.

Figure 5. Proposed structure for the glycosylphosphatidylinositol membrane anchor.*
Glycoproteins in Lipid Metabolism

Enzymes involved in lipid metabolism which have been identified as glycoproteins include 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), and lipoprotein lipase. HMG-CoA reductase is the rate limiting enzyme for cholesterol biosynthesis, and is also responsible for the biosynthesis of isoprenoid compounds, ubiquinone and dolichol. The enzyme is associated with the endoplasmic reticulum and has been identified as a glycoprotein with glycan moieties of the high-mannose type (52). Because the enzyme is associated with the endoplasmic reticulum, it is reasonable to find glycan moieties of only the high-mannose type, as the protein may not migrate to the Golgi where the complex and hybrid types are formed.

Studies by Volpe and Goldberg (53) on the effect of tunicamycin on HMG-CoA reductase activity in C-6 glial cells show that the enzyme is dependent upon glycosylation for activity. The effect of tunicamycin on enzyme activity and sterol synthesis was apparent after only four hours incubation, with maximal reductions in activity (65-70%) occurring after six hours incubation. The decrease in enzyme activity was paralleled by a decrease in incorporation of \[^3\text{H}\]mannose into protein. Further support of the relationship between enzyme activity and glycosylation was demonstrated by the fact that when sufficient N-acetylglucosamine was added to overcome the effect of tunicamycin on incorporation
of mannose into glycoprotein, there was no drug effect on either enzyme activity or glycoprotein synthesis. Treatment of C-6 glial cells with tunicamycin produced no change in the activities of NADPH-cytochrome c reductase, ACC, or fatty acid synthetase.

Haro et al. (54) using rat hepatocytes incubated with \(^{35}\)Smethionine in pulse and pulse-chase experiments followed by immunoprecipitation of the HMG-CoA reductase and SDS-PAGE, found two labelled polypeptides of 104 and 180 kDa. Following treatment with endoglycosidase H, the 180 kDa reductase split into two labelled peptides of 110 and 97 kDa. The authors suggested that in addition to the 104 kDa reductase, the endoplasmic reticulum contains a dimer of two reductases (110 and 97 kDa) linked by a carbohydrate chain composed by \(N\)-acetylglucosamine residues. Because the monomeric and dimeric forms of the enzyme have different half lives (80 and 46 minutes respectively) the authors suggested that dimerization- dedimerization reactions may control the degradation of HMG-CoA reductase and cholesterol biosynthesis.

In later work by Haro et al. (55) the authors suggest that the 180 kDa form is a glycoprotein of \(N\)-linked high-mannose oligosaccharide chains. The apparent molecular mass of the carbohydrate was 16 kDa. The carbohydrate-free 164 kDa protein was found to degrade one tenth as fast as the intact glycoprotein, and degradation was not accelerated by sterols.
The authors concluded that glycosylation of the enzyme was required for rapid and controlled degradation of the protein.

Lipoprotein lipase (LPL) is the rate-limiting enzyme for the removal of lipoprotein triglycerides from the circulation (56). The enzyme is a glycoprotein with the carbohydrate content ranging from 3 to 10% by weight (56-57). Like HMG-CoA reductase, LPL requires glycosylation to be active (58-59). Studies using carbonyl cyanide m-chlorophenylhydrazone (CCCP) (59), a drug which blocks the transfer of LPL from the endoplasmic reticulum, show that in the presence of CCCP, LPL activity is completely blocked. When CCCP is removed, the activity and transport of LPL rapidly resumes. This suggests that activation of the enzyme occurs on the Golgi apparatus, and that the glycan moieties may be of the complex or hybrid types.

Work by Chan et al. (60) suggests that after activation on the Golgi apparatus and transport to the outside of the plasma membrane, LPL is bound to the plasma membrane by a phosphatidylinositol anchor. The group found that when 3T3-L1 adipocytes were treated with either insulin or a phosphoinositol-specific phospholipase C (PI-PLC), LPL activity peaked within 10 minutes and then diminished over the next 30 minutes in both cases. Treatment with cycloheximide did not inhibit the release of LPL, indicating that the release was independent of protein synthesis. The kinetics of LPL release were similar for both insulin and PI-
PLC, but were different from the release induced by heparin, in which activity of LPL peaked at 20 minutes and returned to basal levels by 40 minutes. The authors concluded that LPL appears to be anchored to the 3T3-L1 cell surface by a glycosyl-phosphatidylinositol anchor, and its rapid release by insulin may be due to activation of a glycosyl-phosphoinositol-specific phospholipase C.

Later work by Semenkovich et al. (61) supports the findings by Chan et al. that insulin induction of LPL activity is not related to synthesis of the enzyme. In 3T3-L1 adipocytes treated with insulin, LPL activity rose to 146% compared to carrier-treated cells, while mRNA levels were 109% of controls. Insulin stimulation of LPL activity was dose-dependent but changes in mRNA levels were not. The authors concluded that insulin regulation of LPL activity in 3T3-L1 adipocytes is mediated entirely at post-transcriptional and post-translational levels.

Substantial evidence supports the fact that two regulatory enzymes in lipid metabolism, HMG-CoA reductase and LPL, are glycoproteins (52-60). Evidence also supports the fact that LPL, like other membrane bound glycoproteins (7), may be anchored to the plasma membrane via a phosphatidylinositol-glycan linkage (60). Two key enzymes in lipogenesis, ACC and ATP-CL, are known to be associated with both the mitochondrial membrane and cytoplasmic cell fractions (11-14,43-44). Acetyl CoA carboxylase, herein
identified as a glycoprotein, joins the list of rate limiting enzymes in lipid metabolism which are glycoproteins. Given the association of ACC with the mitochondrial membrane, it is also reasonable to postulate that ACC, like LPL, may be anchored to the mitochondrial membrane via a glycosyl-phosphoinositol anchor.
CHAPTER III
MATERIALS AND METHODS

Animals and Diet

Liver from male Sprague Dawley rats was used to prepare the crude homogenate and purified acetyl CoA carboxylase samples. Rats were procured from the Harlan-Sprague Dawley Vendor at initial weights of 90 to 110 grams, and were pair housed in galvanized wire bottom cages. A twelve-hour light-dark cycle was maintained, with the hours of 0700 to 1900 representing the dark cycle. The dark cycle was interrupted only for animal care. Animals were fed a semi-purified high carbohydrate diet ad libitum (table 1), and had free access to water.

Chemicals and Feed Components

Methanolic HCl kits, pyridine, and trimethylchlorosilane were obtained from Alltech Associates, Inc., Deerfield, Illinois. Septa for reaction vials were obtained from Pierce, Rockford, Illinois. Silver carbonate was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin. Acetic anhydride was obtained from EM Science, Gibbstown, New Jersey. Radiochemicals were obtained from New England
Table 1. Composition of Rat Diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>grams</th>
<th>% dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>dextrose</td>
<td>11,600</td>
<td>58</td>
</tr>
<tr>
<td>corn oil</td>
<td>400</td>
<td>2</td>
</tr>
<tr>
<td>cellulose</td>
<td>2,600</td>
<td>13</td>
</tr>
<tr>
<td>vitamins</td>
<td>400</td>
<td>2</td>
</tr>
<tr>
<td>casein</td>
<td>4,200</td>
<td>21</td>
</tr>
<tr>
<td>minerals</td>
<td>800</td>
<td>4</td>
</tr>
</tbody>
</table>

*Composition of the vitamin diet supplement triturated in dextrose (g/kg): α-tocopherol (100 IU/gm) 5.0, L-ascorbic acid 45.0, choline chloride 75.0, d-calcium pantothenate 3.0, inositol 5.0, menadione 2.25, niacin 4.5, PABA (para-aminobenzoic acid) 5.0, pyridoxine HCl 1.0, riboflavin 1.0, thiamin HCl 1.0.

vitamin A acetate 900,000 units/kg, calciferol (D2) 100,000 units/kg, biotin 20 mg/kg, folic acid 90 mg/kg, vitamin B12 1.35 mg/kg.

Composition of the salt mixture USP XIV (g/kg): ammonium 0.57, cupric sulfate 0.48, ferric ammonium citrate 94.33, manganese sulfate 1.24, potassium iodide 0.25, sodium fluoride 3.13, calcium carbonate 68.6, calcium citrate 308.3, calcium biphosphate 112.8, magnesium carbonate 35.2, magnesium sulfate 38.3, potassium chloride 124.7, dibasic potassium phosphate 218.8, sodium chloride 77.1.
Nuclear, Boston, Massachusetts. The glycan chain detection kit was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Indiana. All other chemicals were purchased from Sigma Chemical Company, St. Louis, Missouri.

Ingredients of the rat diet, including dextrose, cellulose, casein, vitamins, and minerals were purchased as individual components from United States Biochemical Corporation, Cleveland, Ohio. Corn oil (Mazola) was purchased locally from a retail grocery. The vitamin mixture and corn oil were stored at 0-5°C, while the other diet components were stored at room temperature.

General Methods

Purification of Acetyl CoA Carboxylase

Livers from eight to ten fasted/refed animals were used for purification of acetyl CoA carboxylase (ACC). After sufficient time for acclimation to the semi-purified diet and the light/dark cycle, animals were single-housed and fasted for 48 hours, with free access to water. After fasting, the animals were again pair housed and refed the semi-purified diet for 48 hours. Following refeeding, animals were killed two to three hours into the dark cycle by decapitation following a stunning blow to the head. Livers were quickly removed and chilled in ice. Total liver weight was obtained, and the livers were homogenized, two to three at a time, in a total of two volumes of cold 0.3M mannitol, to which
protease inhibitors (table 2) and β-mercaptoethanol (BME) had been added to a final concentration of 1:1000. A Potter-Elvehjem homogenizer was used, and the homogenization was done in a cold room (0-5°C). The homogenate was centrifuged at 27,000 x g at 0-5°C for 10 minutes in a Sorvall RC-2 centrifuge equipped with a fixed angle rotor. The supernatant was then filtered through four layers of cheesecloth which had been prewetted with distilled water and kept on ice.

The filtered supernatant was centrifuged at 105,000 x g at 0-5°C for 60 minutes in a Beckman Model L8-55 ultracentrifuge equipped with a 40S fixed angle rotor. Following centrifugation, the supernatant (cytosol) was again filtered through four layers of cold prewetted cheesecloth and carefully measured. Ammonium persulfate was added to achieve a 35% saturation, and the solution was stirred in an ice bath for 20 minutes.

Following ammonium persulfate precipitation, the solution was centrifuged at 27,000 x g at 0-5°C for 20 minutes. The supernatant was discarded and the pellet was resolubilized to 15% of the original supernatant volume (measured after the 60 minute centrifugation at 105,000 x g) in cold KPhos/citrate buffer (.1M potassium phosphate, 25 mM citrate, pH 7.0 with PI and BME added to a concentration of 1:1000). The solution was then added to dialysis membrane having a molecular weight cutoff of 12,000 to 14,000 kDa, and dialyzed against ≈900 mls of the above KPhos/citrate buffer for four
Table 2. Composition of the Protease Inhibitors

<table>
<thead>
<tr>
<th></th>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1:</td>
<td>PMSF</td>
<td>26.0 mg</td>
</tr>
<tr>
<td></td>
<td>TLCK</td>
<td>5.4 mg</td>
</tr>
<tr>
<td></td>
<td>TPCK</td>
<td>5.4 mg</td>
</tr>
<tr>
<td>Tube 2:</td>
<td>Benzamidine</td>
<td>235 mg</td>
</tr>
<tr>
<td>Tube 3:</td>
<td>NaN₃</td>
<td>150 mg</td>
</tr>
<tr>
<td>Tube 4:</td>
<td>Pepstatin</td>
<td>1.5 mg</td>
</tr>
<tr>
<td></td>
<td>Leupeptin</td>
<td>1.5 mg</td>
</tr>
<tr>
<td></td>
<td>Trypsin Inhibitor</td>
<td>1.5 mg</td>
</tr>
</tbody>
</table>

* Mixed well with 1.5 mls of ethyl alcohol

* Mixed well with 1.5 mls of distilled water
hours at room temperature.

Following dialysis, the solution was centrifuged at 27,000 x g at 0-5°C for 10 minutes. The supernatant was carefully weighed, and a 50% solution of polyethylene glycol (PEG) was added to achieve a final concentration of 0.5% PEG. The solution was then covered with parafilm and stirred at room temperature for three hours.

Following the first PEG precipitation, the solution was centrifuged at 10,000 x g at 0-5°C for 20 minutes. The supernatant was then discarded and the pellet was again resuspended in the cold KPhos/citrate buffer using 10% of the original supernatant volume. The solution was weighed, and a 50% solution of PEG was again added to achieve a final concentration of 0.5% PEG. The solution was covered and stirred at room temperature for two hours.

Following the second PEG precipitation, the solution was again centrifuged at 10,000 x g at 0-5°C for 20 minutes. The supernatant was discarded and the pellet was resuspended in cold KPhos/citrate buffer to 5% of the original supernatant volume, and weighed. A 50% solution of PEG was again added to achieve a final concentration of 0.5% PEG, and the solution was covered with parafilm, stirred for 20 minutes at room temperature, then refrigerated overnight.

The following morning, the solution was stirred for two hours, then centrifuged at 10,000 x g at 0-5°C for 20 minutes. The supernatant was discarded, and the pellet was
resuspended in 2-3 mls of cold tris/potassium/citrate buffer (.12M tris-acetate, pH 7.8, .2M potassium acetate, 2 mM citrate), with BME added to a concentration of 1:1000. This solution was stirred for 20 minutes in an ice bath to assure complete resolubilization, and then centrifuged at 10,000 x g at 0-5°C for 10 minutes.

The supernatant was measured and added to a sepharose 6B column in the cold room (0-5°C). The column had been previously washed, and was subsequently eluted following the addition of the final resuspended pellet, with aliquots of the same cold tris/potassium/citrate buffer used to resuspend the final pellet. One ml fractions were collected in plastic tubes and analyzed for protein concentration and enzyme activity.

SDS Dissociation of Proteins

Prior to polyacrylamide gel electrophoresis (PAGE) protein samples were dissociated by adding an aliquot of sample to one or two volumes of hot (100°C) SDS mixture. composed of 3.3% SDS, 1.3% BME, and 11% sucrose, with bromophenol blue as an indicator. Diluted samples were boiled for four minutes.

Polyacrylamide Gel Electrophoresis

SDS dissociated proteins were separated through a 3% polyacrylamide stacking slab gel followed by a 5% separating
polyacrylamide slab gel (table 3). Gel electrophoresis separation was maintained at a current of 7.5 milliamperes until the dye front came to within one inch of the bottom of the plate. Gels were then removed from the plate in a solution of Towbin's buffer (62) in preparation for Western blotting. When necessary, individual gel lanes were cut from the main gel and stained for protein in a solution of coomassie blue R-250 (0.6% coomassie blue R-250, 50% MeOH, 10% acetic acid).

**Western Blot**

Following PAGE, proteins were electrophoretically transferred onto 0.45 micron nitrocellulose membrane (Bio-Rad Laboratories, Rockville Center, New York) according to the method of Towbin et al. (62) The transfer was carried out in cold Towbin's buffer at an amperage of 0.5, for three hours. Following the transfer, gels were stained for protein using the above mentioned coomassie blue R-250 solution, to assure complete transfer of proteins. The nitrocellulose membrane was allowed to dry at room temperature for at least two hours prior to blocking with 3% BSA or 1% gelatin in a solution of 10 mM tris-Cl, pH 7.6, with .9% NaCl.

**Protein Determination**

Protein concentration was determined using the dye binding assay described by Bradford (63). Bovine serum
Table 3. Composition (wt:vol) of the 3% Stacking and 5% Separating Polyacrylamide Gels.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide - 0.8% BIS</td>
<td>2.0 mls</td>
</tr>
<tr>
<td>1.5 M TrisCl, pH 6.8</td>
<td>2.0 mls</td>
</tr>
<tr>
<td>distilled H₂O</td>
<td>15.5 mls</td>
</tr>
<tr>
<td>20% SDS</td>
<td>100 μl</td>
</tr>
<tr>
<td>13% freshly made ammonium persulfate</td>
<td>100 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide - 0.8% BIS</td>
<td>6.7 mls</td>
</tr>
<tr>
<td>1.5 M TrisCl, pH 8.7</td>
<td>10.0 mls</td>
</tr>
<tr>
<td>distilled water</td>
<td>23.0 mls</td>
</tr>
<tr>
<td>20% SDS</td>
<td>200 μl</td>
</tr>
<tr>
<td>13% freshly made ammonium persulfate</td>
<td>100 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 μl</td>
</tr>
</tbody>
</table>
albumin was used to generate a standard curve and distilled water served as the blank. The absorbance of each sample was read on a Coleman spectrophotometer at a wavelength of 595 nanometers.

**Assay of Acetyl CoA Carboxylase Activity**

Acetyl CoA carboxylase activity was measured by the fixation of $[^{14}C]$bicarbonate (measured in dpm/nmole) into acid stable form (malonyl CoA), as previously described by Allred and Roehrig (64). Prior to the fixation reaction, 75 μl of 2M tris-acetate pH 8.0, was added to each fraction from the Sepharose 6B column. An aliquot of the sample (100 μl) was then added to 460 μl of the assay premix (table 4), which had been previously incubated at 37°C for five minutes. After gentle mixing, samples were returned to the 37°C water bath and incubated for one minute. The reaction was then terminated with the addition of 50 μl of 12N HCl, and samples were removed from the water bath. Sample aliquots of 200 μl were then added to scintillation vials and dried in an exhaust oven at 50°C to remove any unreacted $[^{14}C]$bicarbonate. After drying, samples were resolublized in 200 μl of 50% ethanol. Two mls of dioxane based scintillation fluid (65) was added to each sample, and radioactivity was then counted for 10 minutes for each sample, in a Packard Tri-Carb liquid scintillation spectrometer, model 1500.

Enzyme activity was determined based on the known
Table 4. Reaction mix for \([^{14}C]bi\)carbonate Assay for Acetyl CoA Carboxylase Activity

<table>
<thead>
<tr>
<th>Component</th>
<th>mM Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-acetate buffer (pH 7.75)</td>
<td></td>
</tr>
<tr>
<td>tris base</td>
<td>60.00</td>
</tr>
<tr>
<td>potassium acetate</td>
<td>100.00</td>
</tr>
<tr>
<td>Potassium citrate</td>
<td>10.08</td>
</tr>
<tr>
<td>Reduced glutathione (GSH)</td>
<td>3.00</td>
</tr>
<tr>
<td>ATP</td>
<td>1.10</td>
</tr>
<tr>
<td>Potassium bicarbonate</td>
<td>25.00</td>
</tr>
<tr>
<td>Acetyl CoA</td>
<td>0.35</td>
</tr>
<tr>
<td>Magnesium acetate</td>
<td>8.00</td>
</tr>
<tr>
<td>([^{14}C] Sodium Bicarbonate)</td>
<td>0.0025</td>
</tr>
<tr>
<td>Plus 0.6 mg/ml bovine serum albumin (BSA)</td>
<td>---</td>
</tr>
</tbody>
</table>
specific activity of $[^{14}\text{C}]{\text{bicarbonate}}$ and the counts obtained from each sample. A unit of ACC activity was equivalent to one micromole of $[^{14}\text{C}]{\text{malonyl CoA}}$ produced per minute from acetyl CoA and $[^{14}\text{C}]{\text{bicarbonate}}$.

**Experimental Protocols**

**Preparation of Sugar Standards and Purified ACC for GC/MS**

Nine sugars were analyzed by GC/MS, including fucose, galactose, galactosamine, $N$-acetylgalactosamine, glucose, glucosamine, $N$-acetylglucosamine, myo-inositol, and mannose. Mannitol was used as an internal standard (IS) in all sugar standards and enzyme analysis. Samples were prepared according to the methods of Chambers and Clamp (66). Two mg of each sugar were added to 100 ml of distilled water and thoroughly mixed. A 10 $\mu$l aliquot of each sample was then added to one of three 2 ml glass ampules along with 10 $\mu$l of IS as follows:

- ampule A: fucose, galactose, $N$-acetylglucosamine, mannitol;
- ampule B: glucose, myo-inositol, mannose, mannitol;
- ampule C: glucosamine, galactosamine, $N$-acetylgalactosamine, mannitol;

Samples were then dried using a Savant Speed Vac Concentrator, model SVC 100H. Following this, 500 $\mu$l of MeOH and 500 $\mu$l of methanolic HCl were added to each ampule and gently mixed. Nitrogen gas was then blown into each ampule
for 30 seconds, and the ampules were sealed and heated at 85°C for 18 hours. Samples were allowed to cool to room temperature and were then quantitatively transferred to eppendorf tubes using 150 μl of MeOH. Following neutralization with silver carbonate, samples underwent reacetylation for six hours, with 120 μl of acetic anhydride. Following reacetylation, samples were thoroughly mixed and centrifuged four minutes, and the supernatant was transferred to a clean reaction vial. The pellet was then resolubilized with 200 μl of MeOH, and the centrifugation and transfer process was repeated three more times. The samples were then dried on the vacuum drier, covered, and left at room temperature.

Purified ACC was prepared for GC/MS analysis as described in the general methods. Fractions from the sepharose 6B column showing enzyme activity were added to a sephadex G25 column and eluted with distilled water. Four fractions from the sephadex G25 column showing enzyme activity were combined and mixed. After the addition of 0.2 μg of mannitol, the sample was dried. The sample then underwent methanolysis, neutralization, reacetylation, and drying as described above.

**TMS Derivatization**

Dried samples were derivatized just prior to GC/MS analysis using a freshly made solution of pyridine, trimethylchlorosilane, and hexamethyldisilazane, in a 5:1:1
mixture by volume. Fifty µl of this solution was added to each sample and the samples were then thoroughly mixed and held at room temperature for 30 minutes. The resulting TMS derivatives were then extracted into 100 µl of hexane, and after gentle mixing, samples were centrifuged at 400 x g for 10 minutes. An aliquot (80 µl) of the supernatant was transferred to a clean vial, capped, and used for GC/MS analysis.

Gas Chromatography/Mass Spectrometry

GC/MS analysis was carried out using a Hewlett Packard gas chromatograph, model 5890, equipped with Hewlett Packard 5970 series mass selective detector. A 28 meter, DB-1 fused silica capillary column (J&W Scientific, Inc., Rancho Cordova, California) with an internal diameter of .25mm was used. The carrier gas was helium.

Prior to analysis, the system was autotuned (calibrated against known amounts of standards) according to the manufacturer specifications. The temperature of the injection port and transfer line was 250°C. The initial column temperature was 140°C which was held for three minutes to allow for a solvent delay, then increased at a rate of 5°C per minute to a final temperature of 250°C. Aliquots of each sample (2 µl) were injected using a Hamilton gas-tight µl syringe. A TMS blank, having been prepared the same as the sugar standards and purified ACC, was injected between each
sample, to assure that there was no column carry-over between samples.

**Streptavidin-HRP and Concanavalin A-HRP Detection of Acetyl CoA Carboxylase**

Acetyl CoA carboxylase was purified and then boiled in SDS, as previously described. Forty μl aliquots and 20 μl aliquots of the SDS boiled enzyme were loaded onto a 3% stacking/5% separating polyacrylamide gel, and electrophoretically separated. Following PAGE, proteins were transferred onto nitrocellulose membrane using the western blot procedure. After drying overnight, two lanes of the nitrocellulose membrane, each loaded with 40 μl of purified enzyme, were blocked at 37°C for two hours in 1% gelatin in 10 mM tris-Cl buffer, pH 7.6, with .9% NaCl. Following blocking, one lane was incubated at 37°C for two hours in a solution (10 mM tris-Cl, .9% NaCl, 3% BSA, pH 7.6) containing HRP-labeled concanavalin A (5 μg/ml), while the other lane was incubated similarly in a solution containing HRP-labeled streptavidin (25 μl in 30 ml). Each strip was then separately washed three times for 10 minutes each, in ≈ 50 mls of 10 mM tris-Cl, .9% NaCl buffer, pH 7.6. The color reaction for the visualization of the HRP-labeled streptavidin and concanavalin A was carried out according to the method of Hawkes, et al. (67), using 4-chloro-1-naphthol.
Detection of Acetyl CoA Carboxylase with Rabbit anti-rat Acetyl CoA Carboxylase Antibody, Visualized by Alkaline Phosphatase Color Reaction

A third strip of nitrocellulose membrane from the enzyme sample (20 μl) prepared and blocked as described under HRP detection, was incubated overnight in rabbit anti-rat ACC antibody. The membrane was then washed three times for 10 minutes each time in ≈ 50 ml of the tris-Cl/NaCl buffer, and incubated at 37°C in goat anti-rabbit IgG alkaline phosphatase for 2 hours. The membrane was again washed in tris-Cl/NaCl buffer as described above. Color was developed using a solution of nitroblue tetrazolium chloride in dimethylformamide (70% v/v) and 5-bromo-4-chloro-3-indolyl-phosphate.

Enzyme Immunoassay Detection of Sugars in Purified Acetyl CoA Carboxylase

This assay was conducted according to the procedures outlined in the glycan detection kit purchased from Boehringer Mannheim Biochemicals, Indianapolis, Indiana. Purified ACC was subjected to two different immunoassay labeling trials, one before and one after PAGE. For the first trial, purified ACC was transferred onto nitrocellulose membrane following PAGE. The hydroxyl groups of sugars in the glycan chain were then oxidized to aldehydes with sodium metaperiodate. After washing with phosphate buffered saline,
the membrane was incubated with digoxigenin-succinyl-\(\Sigma\)-amidocaproic acid hydrazide, to allow covalent attachment to the aldehydes via a hydrazide group. The membrane was then washed in tris buffered saline (TBS) and blocked. Following a second wash in TBS, the membrane was incubated with polyclonal sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase, to allow for the subsequent color reaction. The membrane was again washed in TBS, and color was developed using a solution of nitroblue tetrazolium chloride in dimethylformamide (70% v/v) and 5-bromo-4-chloro-3-indolyl-phosphate.

The second immunoassay trial involved labeling purified ACC prior to PAGE. Purified ACC was first added to a sephadex G25 column and eluted with water to remove any Tris, which interferes with digoxigenin labeling. Twenty 1 ml fractions were collected from the sephadex G25 column, and protein concentration was determined for each fraction. Fractions 5 through 10 were then dried using a Savant Speed Vac Concentrator, model SVC 100H. Fractions 9, 5, and 7, containing 48, 75, and 100 \(\mu\)g/ml of protein respectively, were used for the assay. The assay was conducted according to the procedures outlined in the kit, except that triton X-100 was added to the sodium acetate buffer (used for the initial protein dilution) to a final concentration of 0.2%. Twenty \(\mu\)l of this solution was then added to each of the three dried samples, and the samples were carefully
resolubilized. Hydroxyl groups were then oxidized to aldehydes with sodium metaperiodate, followed by an addition of sodium disulfate, to destroy any excess periodate. The aldehyde groups were then labeled with digoxigenin-succinyl-E-amidocaproic acid hydrazide. Following this incubation, 15 μl of SDS buffer was added to each sample and gently mixed. The solution was then heated at 100°C for two minutes to dissociate the proteins. Aliquots of 15 and 40 μl of each of the three samples were loaded onto a 3% stacking/5% separating polyacrylamide gel and electrophoretically separated. Following PAGE, proteins were transferred to nitrocellulose membrane, and the membrane was dried overnight. Prior to blocking the membrane, it was stained for proteins using a solution of Ponceau S solution (0.2% w/v) in trichloroacetic acid (3% w/v). After blocking, the membrane was washed with TBS and incubated with polyclonal sheep anti-digoxigenin Fab fragments. Following a second TBS wash, color was developed as described in the first trial.

Concanavalin A-Sepharose Affinity Binding of Acetyl CoA Carboxylase

Concanavalin A bound to sepharose 4B was used for affinity chromatography to bind purified ACC. The column was maintained in the cold (0-5°C) at all times. Prior to use, the column material was equilibrated with 400 mls of cold .05M tris-acetate buffer, pH 7.0, with 1M NaCl, and 3 mM
concentrations of the metal ions CaCl₂, MgCl₂, and MnCl₂. The column was then repacked with ≈ 20 mls of the equilibrated concanavalin A-sepharose 4B, and washed with cold .05M tris acetate buffer, pH 7.0, with 2 mM citrate and 3 mM concentrations of the above mentioned metal ions.

Four mls of the above cold tris-acetate/citrate buffer were added to 1.3 mls of purified ACC (980 µg protein) and gently mixed. An aliquot of 5.2 mls of this mixture (≈ 962 µg protein) was added to the column. The column was then eluted with the above cold tris-acetate/citrate buffer, and twenty 1 ml fractions were collected. These fractions are referred to as the unbound enzyme fractions.

The column buffer was then changed to .05M tris-acetate, pH 8.0, with 10 mM citrate, 0.5M NaCl, and 0.5M α-methyl-D-mannopyranoside. Twenty-six 1 ml fractions were collected. These fractions represent the bound enzyme fractions and were also analyzed for protein. In addition, aliquots of each fraction (25 µl) were also detected with streptavidin/HRP, using a dot blot technique onto nitrocellulose membrane.

**Incubation of Purified ACC with Glycopeptidase F**

This experiment was performed according to the method of Chu (68), for the purpose of confirming the type of linkage of the glycan moiety to purified ACC. A sample of the purified enzyme which was first added to a sephadex G25 column and eluted with water (as described in the enzyme
immunoassay experiment) was used. The dried sample contained 210 µg of protein. Fifty µl of 0.1 M sodium phosphate buffer, pH 8.6, containing 20 mM EDTA, 20 mM BME, and .125% SDS was added to the purified ACC. The sample was then boiled for six minutes. Nonidet buffer was then added to the SDS-boiled enzyme to a final concentration (v/v) of 6 times that of SDS: 450 µl nonidet added to 50 µl of SDS-boiled ACC. After gentle mixing, 180 µl of the sample was added to each of two tubes. To one tube, 20 µl of water was added, and to the other tube, 20 µl of glycopeptidase F was added. Both tubes were then incubated for two hours at 37°C, while the remainder of the sample (100 µl) was frozen as an unincubated control.

After incubation, 20 µl of the sodium phosphate buffer, with 4 mg of BSA, was added to each incubated sample, and 10 µl (2 mg BSA) was added to the frozen-thawed unincubated control. The three samples were then loaded onto a 5% PAGE gel (50 µl aliquots), in two sets. Following electrophoresis and transfer onto nitrocellulose membrane, one set was incubated with rabbit anti-rat ACC antibody, while the other was incubated with streptavidin-HRP. Color detection for both was as previously described.
Enzyme Purification

Protein concentration (μg/ml) and ACC activity (DPM) of fractions collected from the sepharose 6B column during enzyme purification are shown in figure 6. This figure represents a typical elution curve for both enzyme activity and protein content. For GC/MS analysis, ACC from a similar purification procedure was used. The four fractions from the sepharose 6B column having the highest enzyme activity were combined and put over a sephadex G25 column at 0-5°C. The sephadex G25 column was eluted with distilled water and one ml fractions were collected. The four fractions showing the highest protein concentration (fractions 18-21) were combined and .2 μg of the internal standard mannitol was added. The sample was then dried. After drying, the purified ACC sample was prepared for GC/MS as described in chapter III.

Gas Chromatography/Mass Spectrometry

The gas chromatogram of purified ACC is shown in figure 7. The purified enzyme contains significant amounts of mannose and galactose, identified by mass spectral analysis.
Figure 6. Protein concentration and activity of purified ACC
Figure 7. Gas chromatogram of purified ACC

A: mannose
B: fucose
C: galactose
D: mannitol (IS)
E: 6-deoxy-mannose
F: N-acetylglucosamine
G: myo-inositol
NS: non-sugar
and/or verification by co-elution with sugar standards. Composite sugar standards are shown in figures 8-10. Because the neutral sugars D-mannose, D-galactose, and D-glucose are stereoisomers displaying similar total ion chromatograms (TIC), it was difficult to differentiate between each monosaccharide in the composite standards. Therefore, additional standards were prepared by TMS derivatization only, using both unmethylated sugars (figures 11-12) as well as the methylated forms (figures 13-14), purchased from Sigma Chemical Company. Comparison of the elution times of the unmethylated sugar standards against the methylated and composite standards showed that very little, if any, of the sugars did not get methylated during sample preparation.

Other sugars identified in the purified ACC sample included trace amounts of fucose, N-acetylglucosamine, and inositol. N-acetylneuraminic acid is a terminal sugar in the peripheral regions of complex and hybrid glycan chains, which typically has the longest retention time of glycoprotein monosaccharides (70-71). This sugar was not detectable in the GC/MS analysis of purified ACC. We were also unable to identify this sugar as a standard after methanolysis and reacetylation. The difficulty encountered with identification of this sugar was attributed to inadequate reacetylation in sample preparation.

The elution profile for all of the identified sugars in the purified ACC and standards corresponded to those reported
A: fucose
B: galactose
C: N-acetylglucosamine
IS: mannitol
NS: non-sugar

Figure 8. Gas chromatogram of standard a: fucose, galactose, mannitol, N-acetylglucosamine.
A: mannose
B: glucose
C: 6-deoxy-mannose
D: myo-inositol
IS: mannitol
NS: non-sugar

Figure 9. Gas chromatogram of standard b: mannose, glucose, mannitol, myo-inositol.
A: glucofuranoside
B: glucopyranoside
C: galactoside
D: N-acetylglucosamine
E: N-acetylgalactosamine
IS: mannitol
NS: non-sugar

Figure 10. Gas chromatogram of standard c: glucosamine, galactosamine, N-acetylgalactosamine, mannitol.
A: mannose, B: galactose, C: glucose, D: composite.

Figure 11. Gas chromatogram of unmethylated neutral sugars, 9 to 16 minutes.
A: mannose, B: galactose, C: glucose, D: composite.

Figure 12. Gas chromatogram of unmethylated neutral sugars, 16 to 22 minutes.
A: mannose, B: galactose, C: glucose, D: composite.

Figure 13. Gas chromatogram of methylated neutral sugars, 9 to 16 minutes.
A: mannose, B: galactose, C: glucose, D: composite.

Figure 14. Gas chromatogram of methylated neutral sugars, 16 to 22 minutes.
for methylated TMS derivatives (69-71). For the purified ACC sample, the sugars were identified in order of retention time (RT) as mannose (RT 13.1), fucose (RT 13.21), galactose (RT 13.75), mannose (RT 13.9), galactose (RT 14.53), mannose (RT 15.0), galactose (RT 15.5), mannitol (IS) (RT 16.6), 6-deoxy-mannose (RT 17.38) N-acetylglucosamine (RT 18.78), and myo-inositol (RT 19.52). Multiple peaks for many of the sugars were common, resulting from solvent equilibrium mixtures of pyranosidic, furanosidic, and anomeric forms of the methyl glycosides (69). The presence of a characteristic multiple peak pattern having correct relative retention times and peak areas contributed to the confidence of monosaccharide identification (70). The IS mannitol eluted as predicted (69) as the hexatrimethylsilyl derivative between glucose and the hexosamines.

Neutral sugars, amino sugars, NANA, and mannitol all display characteristic abundant molecular ions in the TIC (71). The neutral sugars mannose, galactose and glucose display the molecular ion 204 as the most abundant ion. The identification of fucose in ACC was as the ring structure alpha-L-6-deoxy-galactofuranoside, a compound having the molecular ion 217 as the most abundant. The amino sugars N-acetylgalactosamine and N-acetylglucosamine display the molecular ion 173 as the most abundant, while NANA and mannitol display the molecular ions 298 and 319, respectively. Comparisons between the TICs for the sugars in
purified ACC and the corresponding MS computer matches are shown in figures 15-21. To identify neutral sugars when the confidence interval of the computer match was not definitive, the composite standards and methylated standards were utilized.

The RT of 13.1 for mannose in the purified ACC represents the primary peak for the methylated form of this monosaccharide. Identification of the peak as methylmannose is verified by the MS computer match as well as the composite (figure 9) and the methylated standards (figure 13). The mannose peak identified at RT 13.9 is verified by the methylated standard (figure 13). The mannose peak at RT 15.0 is verified by the MS computer match for the purified ACC (figure 15) and the composite standard b (figure 9). In this case, the computer match for the purified sample listed nearly equal confidence for the identification of either glucose or mannose. The compound was determined to be mannose on the basis of the composite standard, and because glucose is rarely found as a component of mature glycoproteins and is mainly confined to collagens (2).

Fucose (6-deoxy-L-galactose) is identified at RT 13.21 in the purified ACC on the basis of the MS TIC computer match. (Figure 16) Further evidence to support this identification is shown in standard a. (Figure 8)

Galactose is identified at RT 13.75, 14.53, and 15.5 in the purified ACC sample. In each case, the computer match
Figure 15. TIC matches for mannose: RT 13.1, 13.9, 15.0.
Figure 16. TIC match for fucose: RT 13.21.
Figure 17. TIC matches for galactose: RT 13.75, 14.53, 15.5.
Figure 18. TIC match for mannitol: RT 16.6.
Figure 19. TIC match for 6-deoxy mannose: RT 17.38.
<table>
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<tr>
<th>Compound</th>
<th>CAS Number</th>
<th>RT (min)</th>
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<tr>
<td>Tetradecanamide, N-t.</td>
<td>074420-89-6</td>
<td>68</td>
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<tr>
<td>Glucopyranoside, methyl</td>
<td>018434-98-3</td>
<td>62</td>
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<td>1-ISOBUTYL-1,2,3,4-T</td>
<td>029234-68-2</td>
<td>38</td>
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<tr>
<td>Quinoline, 6-methoxy</td>
<td>001078-23-0</td>
<td>35</td>
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<tr>
<td>alpha-D-Glucopyran</td>
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<td>35</td>
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<td>alpha-D-Glucopyran</td>
<td>056211-04-2</td>
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<td>054789-45-6</td>
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<tr>
<td>E-Methoxy-3-methyl-1-1</td>
<td>16</td>
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</table>

**Figure 20.** TIC match for N-acetylglucosamine: RT 18.78.
Figure 21. TIC match for inositol: RT 19.52.
shows equal or nearly equal confidence for the peak being either galactose or mannose (figure 17). The minor peak for galactose at RT 13.75 was determined to be galactose by composite standard a (figure 8). The galactose peak at RT 14.53 is unique for galactose and is verified by composite standard a (figure 8) and the methylated standard (figure 13). The final galactose peak at RT 15.5 is verified by standard a (figure 8).

The IS mannitol showed a RT of 16.6 in the purified ACC sample (figure 18). For comparison of the relative retention times of the composite standards, mannitol peaks were identified as follows: standard a, RT 16.47; standard b, RT 16.53; standard c, RT 16.52. A mannitol peak at 16.6 was also present in the unmethylated (figure 12) and methylated (figure 14) neutral sugar standards.

A small sugar peak was identified at RT 17.38, having the molecular ion 204 as the most abundant molecular ion. The computer match from the purified sample (figure 19) shows nearly equal confidence for identification of the sugar as either mannose or glucose. The sugar is identified as 6-deoxy-mannose, or rhamnose in standard b (figure 9). Although a specific standard for rhamnose was not obtained, the fact that it was identified in standard b suggests that the hydroxyl group at carbon 6 of mannose may have been removed during sample preparation.

The purified ACC sample has a small amount of N-
acetylglucosamine at RT 18.78. Identification of this sugar is verified by the MS computer match (figure 20) and by standard a (figure 8). The final sugar identified in the purified ACC sample is inositol, at RT 19.52. Identification of this sugar is verified by the MS computer match (figure 21) and by standard b (figure 9).

The presence of fucose and galactose in the purified ACC sample suggests that some of the glycan moieties of the polypeptide are either complex or hybrid in nature (4). The most abundant monosaccharide in the enzyme however, is mannose. This monosaccharide profile therefore indicates that the glycan chains are likely hybrid, having combinations of both high mannose and complex glycan chains (figure 2).

The amino sugar N-acetylgalactosamine links glycan chains to either serine or threonine in O-linked glycoproteins (figure 3). Based on composite standard c (figure 10), N-acetylgalactosamine has a RT of 18.12 minutes. This amino sugar was not identified in the purified ACC sample. However, a small amount of the amino sugar N-acetylglucosamine was detected in the purified ACC sample. This sugar forms the linkage between the glycan chain and asparagine in N-linked glycoproteins. The presence of N-acetylglucosamine in purified ACC suggests that the glycan moieties of the enzyme are N-linked (4), like those of HMG-CoA reductase, another enzyme involved in lipid metabolism (52).

Glycan moieties of N-linked glycoproteins are first
assembled on a pyrophosphoryl-dolichol backbone (48-49). The sugars are then transferred en bloc to asparagine residues of acceptor apoglycoproteins during their synthesis on membrane-bound polyribosomes (48). Once transferred, the glycan chains undergo processing to create either the high-mannose, complex, or hybrid forms. High-mannose glycans are formed in the endoplasmic reticulum, and can undergo further processing on the Golgi apparatus to form complex or hybrid glycans (72). If the glycan moieties of ACC are indeed hybrid in nature, it indicates that the enzyme migrates from the endoplasmic reticulum to the Golgi, where at least some of the glycans on the polypeptide undergo further processing.

The amino acid sequence of ACC has been identified by Lopez-Casillas et al. (73). The tripeptide sequence Asn-X-Ser(Thr) occurs seven times in the amino acid profile, where X is an amino acid other than proline or aspartate. The presence of this tripeptide sequence on a polypeptide is thought to be highly suggestive of sugar modification (47), with approximately one-third of the tripeptide sequences usually being glycosylated (6). Thus, the occurrence of this tripeptide sequence in the amino acid profile of ACC provides further support that ACC is indeed a glycoprotein. Although a specific amino acid sequence on the N-terminal side of asparagine has not been associated with glycosylation, it is interesting to note that the sequence Ser(Thr)-X-Asn occurs eight times in the amino acid sequence of ACC.
In a review relating glycosylation types to positions in the known sequences of given glycoproteins, Pollack and Atkinson (74) found that the glycosylation site with respect to the N-terminus affects the extent of oligosaccharide processing and subsequent presentation of complex or high-mannose structures in the mature glycoprotein. Complex glycans displayed a definite distribution pattern about the N-terminal region, while high-mannose glycans were rarely found in this region. Furthermore, the authors found that secreted glycoproteins usually contained only complex oligosaccharides, whereas membrane proteins contained both types.

Acetyl CoA carboxylase has been identified as having 2346 amino acids (73). The seven amino acid sequences Ser(Thr)-X-Asn of ACC begin at positions 697, 739, 821, 927, 1016, 1461, and 1531 respectively. This pattern shows that the possible sites for glycosylation on ACC occur primarily in the first half of the amino acid sequence.

Rather than being covalently glycosylated, it is possible that ACC is tightly associated with a separate glycan-containing effector which remains bound through enzyme purification. This possibility is supported by several papers describing the insulin-induced release of two low molecular weight compounds which increase ACC activity (35,75-79). These compounds have been identified as carbohydrate-phosphate substances (35,76-78) with an estimated Mr of 1400.
They are known to contain inositol and glucosamine (76-77), and are acid and heat stable (78). Their mode of action is thought to be through regulating the activity of cAMP phosphodiesterase (76-77) or by directly binding to the enzyme (79).

The factors can be released from membranes not only by insulin, but also by a phosphatidylinositol-specific phospholipase C (75-78). The release by PI-PLC suggests that the substances are bound to the membrane by a phosphatidylinositol linkage analogous to the GPI anchor described by Low (76). If indeed this carbohydrate-phosphate substance influences ACC activity by binding directly to enzyme and can remain bound through enzyme purification, it would suggest that the GPI linkage must exist intracellularly as well as on the cell surface.

The association of these low Mr effectors with ACC could be by either ionic or van der Waals bonds. In this case, the effectors could be dissociated from the enzyme by increasing the ionic strength of the solution through the addition of sodium chloride (79). The enzyme and effectors could then be isolated based on molecular weight using a sepharose G-75 column. The enzyme, having the highest molecular weight, would elute in the first few fractions, while the low Mr effector would elute later.

The presence of inositol in the purified ACC may be attributed to these carbohydrate-containing effectors, or the
inositol may originate from an ACC membrane-bound GPI anchor. As early as 1966, Margolis and Baum (80) suggested that the native ACC associated with the microsomal fraction of pigeon liver might be membrane bound. If membrane-bound via a GPI anchor, the inositol may remain tightly bound to ACC after hydrolysis from the membrane anchor and through enzyme purification, as suggested by Heger and Peter (81). These authors found that highly purified ACC from rat liver contained phospholipids, the removal of which by phospholipases A, and D, inactivated the enzyme. Incubation of the inactivated enzyme with phosphatidylinositol restored activity to 70% of the initial value. In this case, the binding interaction between phosphatidylinositol and ACC was not ionic in nature, because the reactivation needed about 30 minutes at room temperature, or 3 hours at 0°C. The authors were also unable to remove phosphatidylinositol from ACC using the detergent deoxycholate, suggesting that phosphatidylinositol must be tightly bound to the enzyme. In addition, the citrate concentration required for optimal activity of ACC was reduced when the phospholipase-treated preparation was incubated with increasing amounts of phosphatidylinositol. This tight bonding of inositol is reported for several other GPI membrane-anchored proteins, and is thought to be covalent in nature (82-85).

Lastly, a recent report by Muller and Bandlow (86) describes the first example of a mitochondrial protein with
a covalently attached phosphatidylinositol moiety acting as a membrane anchor. The protein is an amphitropic cAMP-binding protein in yeast mitochondria, which can be metabolically labeled with stearic acid and inositol. Upon hydrolysis with phospholipase D, the stearic acid label is removed, and the protein, retaining the inositol label, is released from the membrane. The protein is known to be tightly associated with the inner mitochondrial membrane, and is converted to a soluble form during incubation of isolated mitochondria with calcium and phospholipid.

Based on these reports (80-86), and the recent papers describing the association of ACC with the mitochondrial membrane (11-14) is reasonable to postulate that ACC is attached to the outer mitochondrial membrane via a GPI anchor. Upon hydrolysis from the GPI anchor, ACC would be released as the soluble (cytosolic) form, with the inositol anchoring moiety remaining tightly bound to the enzyme. This would account for the presence of inositol in the GC/MS analysis of purified ACC.

**Streptavidin-HRP Detection of Acetyl CoA Carboxylase**

The results of the incubation of nitrocellulose membrane-bound purified ACC with HRP-labeled streptavidin, followed by colorimetric detection, are shown in plate 1, lane B. The bands represent the 280 kDa cytosolic form of ACC. The lower band is lighter due to the fading of the colorimetric stain
Plate I. Photograph of nitrocellulose membrane after transfer and detection of purified ACC.

A: Protein detection with Ponceau S
B: Streptavidin-HRP detection
C: Rabbit anti-rat ACC antibody detection
D: Enzyme immunoassay detection of carbohydrate before PAGE
E: Enzyme immunoassay detection of carbohydrate after PAGE
over time. The relative amount of ACC was determined by analyzing the nitrocellulose strip immediately after staining with an LKB enhanced laser densitometer, model 2222-020. These results are shown in figure 22.

The avidin-biotin bond is the strongest non-covalent bond known in nature (87). Application of this physicochemical bond has been used to detect biotin-containing proteins in rat liver (38,87). The streptavidin used in this incubation was from *Streptomyces avidinii*, and conjugated with HRP for the purpose of colorimetric detection. One mole of streptavidin binds four moles of biotin. Thus, the results of this experiment, namely the visualization of purified ACC by streptavidin-HRP, represent the bonding of streptavidin to the biotin moieties of the purified ACC.

The upper band of the cytosolic ACC is represented by the first peak of the densitometer scan. Comparison of the two peaks indicates that the upper band is smaller, indicating less protein than the lower band. This pattern is typical for cytosolic ACC (87).

**Concanavalin A-HRP Detection of Acetyl CoA Carboxylase**

The results of the incubation of nitrocellulose membrane-bound purified ACC with HRP-labeled concanavalin A, followed by colorimetric detection, are shown in plate 2. The bands also represent the 280 kDa cytosolic form of ACC, staining at the same Mr as the protein detection with Ponceau S, and
Figure 22. Densitometer scan of purified ACC detected with streptavidin-HRP.
Plate II. Photograph of nitrocellulose membrane after transfer and ConA-HRP detection of purified ACC.
the incubations with streptavidin-HRP and rabbit anti-rat ACC antibody. Although both bands can be visualized, the top band in this case was very faint as compared to the lower band.

Concanavalin A (Con A) is a lectin used to detect carbohydrate on a variety of molecules following SDS-PAGE and transfer to nitrocellulose membrane (89,90). Concanavalin A displays a strong affinity for HRP, and the use of conjugated Con A-HRP has become widespread for the detection of glycoproteins by affino-blotting after SDS-PAGE and transfer onto a solid support (89).

The lectin is purified from Canavalia ensiformis (jack bean) and has a particular affinity for mannose, glucose, and N-acetylglucosamine residues (90). Denaturing of the glycoprotein by boiling in SDS prior to Con A incubation allows maximum accessibility of the lectin to oligosaccharide side chains. The binding affinities of Con A for a particular oligosaccharide also help elucidate which type of carbohydrate chain is present (89-91). For example, Con A binds biantennary type complex chains (figure 2) with a lower affinity than either the high-mannose or hybrid-type chains. Hence, Con A may be dissociated in situ on the nitrocellulose blot from biantennary complex chains by as little as 15 mM α-methyl glucose, whereas up to 300 mM α-methyl mannose may be necessary to dissociate Con A from a high-mannose type glycan chain (89).
The binding of Con A-HRP to purified ACC indicates that oligosaccharides are indeed associated with the enzyme. The oligosaccharides may represent either covalently linked glycans, or the previously described tightly bound low Mr effector oligosaccharide. It is unlikely, however, that an oligosaccharide which is not covalently bonded to the enzyme would remain in association with the enzyme through SDS boiling and PAGE. Moreover, this effector is not known to contain mannose, glucose, or N-acetylglucosamine, the three sugars for which Con A has the highest binding affinity.

The fact that the visualization of the top band was very faint suggests that there were either fewer glycan side chains present, or that the binding affinity was lower than for those glycans in the lower band. It is possible that if the low Mr effector oligosaccharide did remain bound through SDS boiling and PAGE, that perhaps it remains associated with the top band of the cytosolic ACC, while the glycans covalently bonded to the enzyme are associated with the lower band. Analysis of the nitrocellulose blot by laser densitometry shows no differentiation of the two bands (figure 23), indicating that an insignificant amount of Con A-HRP bound to the upper band as compared to the lower band. The presence of carbohydrate in the purified ACC preparation suggests that the actual Mr of the protein portion of the two bands is less than 280 kDa.
Figure 23. Densitometer scan of purified ACC detected with concanavalin A-HRP.
Detection of Acetyl CoA Carboxylase with Rabbit anti-rat ACC Antibody

Purified ACC was also incubated with rabbit anti-rat ACC antibody, followed by incubation with goat anti-rabbit IgG alkaline phosphatase for the purpose of color development. The complex was visualized with nitroblue tetrazolium chloride in dimethylformamide (70% v/v) and 5-bromo-4-chloro-3-indolyl-phosphate. The results are shown in plate 1, lane C.

Both bands of the purified cytosolic ACC are clearly visualized in this experiment. The binding of rabbit anti-rat ACC antibody to the 280 kDa bands provides further confirmation that the bands visualized after the Con A and enzyme immunoassay incubations indeed represent ACC.

Enzyme Immunoassay Detection of Sugars in Purified Acetyl CoA Carboxylase

The results of the enzyme immunoassay detection of sugars in purified ACC are shown in plate 1, lanes D and E. Lane D represents the labeling of ACC with digoxigenin before SDS-PAGE and transfer onto nitrocellulose membrane. Lane E represents ACC labeled with digoxigenin after SDS-PAGE and transfer onto nitrocellulose membrane.

Detection of oxidized hydroxyl groups in sugars of glycoconjugates through covalently attached digoxigenin offers higher sensitivity and specificity than the biotin-
streptavidin system (89). Although the detection limit varies with each glycoprotein, as little as 10 nanograms of carbohydrate can be detected by increasing the color development up to 60 minutes. (Color development of the ACC labeled before SDS-PAGE and transfer took about 30 minutes.) Labeling the glycoprotein after SDS-PAGE and transfer onto nitrocellulose membrane produces a slightly lower sensitivity. Although labeling before SDS-PAGE and transfer is more sensitive, if the glycoprotein has a high carbohydrate content, a significant molecular weight shift on SDS-PAGE can incur due to the attachment of the digoxigenin spacer to the glycoprotein.

The labeling of purified ACC after SDS-PAGE and transfer onto nitrocellulose membrane produced a negative stain. (Plate 1, lane E) This was attributed to the presence of phospholipid which remained bound to ACC during purification (81) and through SDS-PAGE. The enzyme was then labeled before SDS-PAGE and transfer, in a buffer containing 0.2% of the detergent triton X-100 to remove the phospholipid. Prior to labeling, the purified enzyme was added to a sephadex G25 column and eluted with water to remove any tris, which interferes with digoxigenin labeling.

The results of this label and stain (plate 1, lane D) are positive for the presence of carbohydrate in both the upper and lower 280 kDa bands. The very slight molecular weight shift indicates that there is a relatively small amount of
carbohydrate present. The carbohydrate detected likely represents covalently attached sugars, as a non-covalent association would not have remained after treatment with detergent, SDS boiling, and PAGE.

**Concanavalin A-Sepharose Affinity Binding of Acetyl CoA Carboxylase**

The results of the concanavalin A-sepharose affinity binding of purified ACC are presented in figure 24. A small amount of protein (+) was eluted from the column in fractions 13 to 18. This was attributed to a small amount of unbound protein eluted in the tris-acetate buffer solution. Protein again eluted from the column beginning with fraction 29, and corresponded nicely to the amount of ACC (△) eluted from the column. Acetyl CoA carboxylase eluted from the Con A column beginning with fraction 29, and continued through fraction 46. The buffer used to elute the bound enzyme from the column was .05M tris-acetate, pH 8.0, with 10 mM citrate, 0.5M NaCl, and 0.5M α-methyl-D-mannopyranoside.

Acetyl CoA carboxylase had to be quantitated by a method other than enzyme activity, because the presence of 0.5M NaCl in the elution buffer made the enzyme inactive (64). The relative amount of ACC was determined by blotting 25 µl of fractions 21-46 onto nitrocellulose membrane, followed by streptavidin-HRP detection as previously described. The blots were analyzed using a laser densitometer (figures 25-26).
Figure 24. Protein and ACC concentrations of the concanavalin A-sepharose affinity column effluent.
Figure 25. Densitometer scan of the concanavalin A affinity column effluent detected with streptavidin-HRP, fractions 23-34.
Figure 26. Densitometer scan of the concanavalin A affinity column effluent detected with streptavidin-HRP, fractions 35-46.
Con A-Sepharose binds the high-mannose, hybrid-, and biantennary complex-type of asparagine-linked glycan chains (90-94). (Figure 2) The biantennary-type binds with a lower affinity than the high-mannose or hybrid-type chains, whereas the triantennary complex-type asparagine-linked glycans are not bound by Con A-Sepharose (91-92). Concanavalin A has a specificity for mannose residues, and work by Ogata et al. (93) demonstrated that at least two nonsubstituted or 2-O-substituted α-mannopyranosyl residues are required for the glycan to be retained on a Con A-agarose column. In addition, terminal N-acetylglucosamine residues on the mannose α1-6 branch of the glycan inner core favor very tight binding by Con A (92). The presence of fucose on the innermost N-acetylglucosamine residue of the inner core (complex biantennary-type), does not affect the binding of glycopeptides to Con A (92).

The interaction of Con A-sepharose with glycoproteins is dependent upon time, temperature, pH, and ionic strength (94-95). Low temperatures enhance the affinity of most lectins for glycoproteins (91). Hence, the dissociation of glycoproteins from Con A by α-methylglucoside or α-methylmannoside is decreased at low (4°C) temperatures. Therefore, prolonged equilibration with the haptenic sugar may be necessary for elution of the glycoprotein at low temperatures (94).

The pH range for maximum activity of those reactions
which involve agglutination or precipitation with Con A is between 6 and 7. This pH range also favors the tetrameric form of Con A. Bishayee and Bachhawat (94) found that a pH of 8.0 achieved maximal elution of brain acid hydrolase glycoproteins from Con A-sepharose. The reaction of Con A with mannopyranosides is reported to be best between pH 4 and 8 (95). In the present study, the Con A-sepharose column was initially equilibrated, and the first twenty fractions were eluted with a .05M tris-acetate buffer at pH 7.0. For elution of the bound fractions, the pH of the tris-acetate buffer was increased to 8.0, along with an increase in the ionic strength and the additions of citrate and α-methyl-D-mannopyranoside. High ionic strength (0.5M NaCl) minimizes the electrostatic interactions between ConA and other proteins (94) and also serves to stabilize Con A by inducing the tetrameric form of the protein.

Bishayee and Bachhawat (94) also found that .13M α-methyl-D-mannoside was optimum for maximal elution of the brain acid hydrolase enzymes. Although their experiments were conducted at 4°C, columns were closed for one hour after the addition of α-methyl-D-mannopyranoside to allow for column equilibration. Purified ACC was eluted in the present study using .5M α-methyl-D-mannopyranoside. Approximately 962 μg of protein was initially added to column. Results of the protein determinations from the 46 fractions collected show that approximately 309 μg of protein (32%) was recovered.
This degree of recovery may be related to the enhanced affinity of Con A for carbohydrate at low temperatures, or because the column was not stopped for equilibration after the addition of the buffer containing α-methyl-D-mannopyranoside.

The results of this experiment show that purified ACC binds to Con A-sepharose, and can be subsequently eluted with α-methyl-D-mannopyranoside. Because Con A is a lectin known to bind carbohydrate(s), the binding of ACC to Con A indicates that carbohydrate is present in the purified enzyme. This carbohydrate may be covalently attached to ACC, or tightly associated with the enzyme as previously described. The fact that the enzyme was purified and that the buffers used were of a high ionic strength would indicate that the association of carbohydrate with ACC is covalent in nature. In accord with the previously described experiments, this experiment suggests that ACC contains asparagine-linked glycan chains which may be of the high-mannose, hybrid-, or biantennary complex-type in nature, based on the binding affinities of Con A.

**Incubation of Purified ACC with Glycopeptidase F**

The results of incubation of SDS boiled purified ACC with glycopeptidase F followed by PAGE are shown in plate 3. Lanes A,B, and C were detected by incubation with streptavidin-HRP, and represent the incubation with glycopeptidase F, the
Plate III. Nitrocellulose membrane after transfer of purified ACC treated with and without glycopeptidase F.

Lanes A, B, and C are detected with streptavidin-HRP. Lanes C, D, and E are detected with rabbit anti-rat ACC antibody.

A: incubated with glycopeptidase F.
B: incubated control.
C: unincubated control.
D: incubated with glycopeptidase F.
E: incubated control.
F: unincubated control.
unincubated control, and the incubated control, respectively. Lanes D, E, and F were detected by incubation with rabbit anti-rat ACC antibody, and also represent the incubation with glycopeptidase F, the unincubated control, and the incubated control, respectively.

Glycopeptidase F (endo-β-N-acetylglucosaminidase F) is purified from Flavobacterium meningosepticum. The enzyme cleaves glycans from the polypeptide backbone at the N-acetylglucosamine-asparagine linkage, and hydrolyses primarily high-mannose oligosaccharides, although hybrid and biantennary complex-type oligosaccharide chains are susceptible to cleavage also (91,96). Hybrid structures possessing bisecting N-acetylglucosamine residues linked β-1→4 to the core mannose are resistant to cleavage, as are triantennary and tetraantennary complex-type structures (96). Digestion is routinely carried out in 0.25 M sodium acetate buffer (pH 5-7) containing 10 mM EDTA and 10 mM BME (91).

The enzyme works best on denatured substrates. Denaturing by boiling in SDS facilitates the accessibility of glycopeptidase F to the N-acetylglucosamine-asparagine linkage by destroying the three-dimensional structure of the glycoprotein of interest (89,96). After denaturation, a nonionic detergent (such as triton X-100 or nonidet P-40) is added to the buffer in large excess of the SDS in the digestion mixture (91). The detergent serves to protect the glycopeptidase F from denaturation during the incubation
Following glycosidase digestion, an apparent reduction in the molecular size of a protein based on SDS-PAGE analysis, is often considered as evidence for the presence of oligosaccharide chains (91).

The pH of the reaction and the time and temperature of the incubation also influence the outcome of glycoprotein digestion with glycopeptidase F (68,96). The pH selects for a particular enzyme cleavage on the basis of its stability and pH optimum (96). The time of the incubation is influenced by the amounts of glycoprotein and glycopeptidase F present, and also whether or not the glycoprotein was denatured prior to the incubation. The activity of glycopeptidase F is enhanced at higher temperatures. However, at temperatures higher than 23°C, there is an apparent lack of stimulation of deglycosylation (68). This may be due to an accelerated rate of heat inactivation of glycopeptidase F at higher temperatures, so that the incubation may be best carried out at the lower temperature of 23°C rather than 37°C.

Another potential problem with glycoprotein digestion by endoglycopeptidases is that some enzymes may be contaminated with proteases (91). In this case, the change in protein size after PAGE would be the result of proteolysis rather than removal of carbohydrate. To guard against this problem, BSA is a good choice as a control because it is not a glycoprotein and therefore will have no Mr change unless the reaction is contaminated with proteases.
The results of this experiment show no visible molecular weight change in the enzyme incubated with glycopeptidase F. These negative results may be due to several factors. The buffer used, as according to Chu (68), was 0.1 M sodium phosphate, pH 8.6, containing 20 mM EDTA, 20 mM BME, and .125% SDS. However, Cummings et al. (91) reported that digestion with glycopeptidase F is routinely carried out in 0.25 M sodium acetate buffer (pH 5-7) containing 10 mM EDTA and 10 mM BME. These differences may have been significant enough to retard or prevent the cleavage of glycan chains from ACC. Another possibility is that the temperature of the incubation (37°C) inactivated the glycopeptidase F (68).

One must also consider as well that; 1) ACC is not glycosylated after all, 2) that if ACC is glycosylated, it is via an O-linkage, in which case glycopeptidase F would have no effect, or 3) that the glycan chains present in ACC are hybrid structures possessing bisecting N-acetylglucosamine residues linked β-1→4 to the core mannose, or triantennary or tetraantennary complex-type structures, all of which are resistant to cleavage by glycopeptidase F (96). Results from the previously described experiments indicate that possibilities 1 and 2 are unlikely, as they suggest that ACC is glycosylated via N-linkages. It may be however, that the glycans present on the enzyme (possibly hybrid in nature) are of the type resistant to cleavage by glycopeptidase F. In any case, repeat incubations with
glycopeptidase F, altering the buffer conditions and temperature, would be worthwhile before ruling out the possibility that the glycan chains present in purified ACC are not susceptible to this enzyme.
CHAPTER V
CONCLUSIONS

The studies presented in this paper were undertaken to identify ACC as a glycoprotein and to elucidate the nature of its glycan moieties. The evidence presented which identifies ACC as a glycoprotein includes; 1) GC/MS shows that the enzyme contains mannose, galactose, fucose, N-acetylglucosamine and inositol; 2) in purified ACC oxidized with periodate, labeled with digoxigenin-succinyl-amido caproic acid hydrazide, subjected to SDS-PAGE, and transferred to nitrocellulose membrane by Western blot, polyclonal sheep anti-digoxigenin Fab fragments bound at the same Mr as the protein which bound polyclonal rabbit anti-rat ACC antibody and streptavidin; and 3) purified ACC binds to a Con A-sepharose affinity column and is specifically released by high concentrations of α-methyl-D-mannopyranoside.

Based on the sugar composition identified by GC/MS, the glycans present are N-linked, and likely of the high-mannose and hybrid-type. The tight binding affinity of the enzyme for the Con A-sepharose column further supports the likelihood of high-mannose and hybrid-type oligosaccharides. The
presence of inositol in the purified enzyme suggests that the
mitochondrial form of the enzyme may have been initially
bound via a GPI anchor, and subsequently released in response
to an increase in insulin, produced as a result of
fasting/refeeding conditions prior to enzyme purification.

Subcellular compartmentation is a well established form
of enzyme regulation which occurs in an intermediate time­
frame. The two mitochondrial forms of acetyl CoA carboxylase
have molecular weights of 280 kDa and 250 kDa, while the
cytosolic forms have molecular weights of 280 kDa and 150
kDa. It is possible that the covalent attachment of a glycan
chain at the C-terminal of the enzyme allows a reversible
shift in the two 280 kDa forms between the mitochondria and
cytoplasm, representing an intermediate form of regulation
in response to hormonal changes. Insulin stimulated release
of the inactive mitochondrial form of the enzyme to the
active cytosolic form would explain the fact that the
activity of the enzyme increases well in advance of protein
synthesis. Insulin may also serve to regulate ACC by
stimulating the glycosylation of the enzyme on the
endoplasmic reticulum.

Glycosylation is known to control the activity and rate
of degradation of two enzymes in lipid metabolism, LPL and
HMG-CoA reductase. These effects of glycosylation were
elucidated using the antibiotic tunicamycin, a specific
inhibitor of N-glycosylation. Although the effects of
tunicamycin on ACC activity and degradation are not as yet
known, it is reasonable to postulate that as with the other
two enzymes, inhibition of ACC glycosylation would decrease
the activity and degradation of this enzyme.

The carbohydrate content of HMG-CoA reductase represents
a molecular weight of approximately 16 kilodaltons. Based on
the peak heights of the sugars found in ACC relative to the
internal standard (which represents \(\approx 2.7\) nanograms), the
enzyme contains a substantial amount of carbohydrate. Acetyl
CoA carboxylase has seven tripeptide sequences which could
potentially become glycosylated. Generally one-third of the
potential sites are actually glycosylated with
oligosaccharides of up to 15 sugar units. Assuming an average
Mr of 180 for each sugar and oligosaccharides with 15 sugar
moieties, the Mr of the carbohydrate portion of ACC could
range between 5400 and 8100, if 2 or 3 of the tripeptide
sequences were glycosylated. Hydrolysis of this amount of
carbohydrate from the polypeptide backbone would produce a
distinct change in the Mr of the enzyme after SDS-PAGE. For
this reason, it is important to repeat the experiment with
glycopeptidase F under varying conditions.

In addition to being key enzymes in the regulation of
lipid metabolism, glycoproteins also mediate lipid metabolism
at the receptor level. Multifunctional glycoprotein receptors
for insulin and the insulin-like growth factors have been
identified (97), and insulin action markedly increases the
proportion of one class of these receptors in the plasma membrane. Insulin binding to another class of these receptors appears to account for the insulin-stimulated serine phosphorylation of the insulin receptor itself, ATP-CL, and ACC. Clearly, carbohydrates are intimately involved in the regulation of lipid metabolism.

Identification of ACC as a glycoprotein opens new areas of research in the regulation of fatty acid synthesis. Further elucidation of the nature of the ACC glycan moieties, including size and position on the polypeptide backbone, and the influence deglycosylation has on enzyme function and activity, will provide considerable insight into the regulation of lipid synthesis. In addition, discovery of the nature of the association of the mitochondrial form of ACC with the mitochondrial membrane and the mechanisms by which it is released in response to insulin, will be instrumental in understanding the role of this intermediate form of ACC regulation.
REFERENCES


