Fatty acid synthesis in eukaryotes

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1. Introduction

Fatty acids fulfill several crucial roles in animals. They represent a major storage form of energy, provide an essential structural component of membranes, through direct covalent linkage they are used to modify and regulate the properties of many proteins and, as components of certain lipid signaling molecules, they perform important roles in metabolic regulation. This chapter will focus primarily on the structure, mechanism of action and regulation of the enzymes responsible for the biosynthesis of long-chain saturated fatty acids, de novo. All of the carbon atoms of fatty acids are derived from the two-carbon precursor, acetyl-CoA and until the demonstration in 1958 that CO₂ was required for the biosynthesis of fatty acids de novo, it had been assumed that the pathway utilized the enzymes of β-oxidation operating in the reverse direction (S.J. Wakil, 1958). Studies during the late 1950s and early 1960s, primarily in the laboratories of S.J. Wakil, P.R. Vagelos and E Lynen, established clearly that the thermodynamic barrier posed by condensation of two acetyl-CoA molecules is circumvented by the introduction of an energy-dependent carboxylation step that generates malonyl-CoA as a co-substrate. Much of the early progress in identifying the individual enzymes involved uniquely in the biosynthetic route was made using the Escherichia coli system and it was not until the mid-1970s that it became clear that in eukaryotes, the enzymes are covalently linked in ‘multifunctional’ polypeptides. In prokaryotes, exemplified by E. coli, more than 10 individual proteins are involved in the biosynthesis of long-chain saturated fatty acids from acetyl-CoA (Chapter 3). This system, in which each enzyme is present on a single polypeptide, became known as the ‘type II’ FAS system and the multifunctional polypeptide system, exemplified by yeast and animals, became known as the ‘type I’ FAS system. Remarkably, in animals, the catalytic components required for the entire fatty acid biosynthetic pathway are integrated into two multifunctional polypeptides, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) [1]. Both proteins are posttranslationally modified by the covalent attachment of vitamin derivatives that play essential roles as ‘swinging arms’ in the translocation of intermediates between different catalytic sites. In ACC, a biotin moiety, attached to the ε-amino group of a lysine residue, serves as a carboxyl carrier between the carboxylase and transcarboxylase catalytic domains and in the FAS, a phosphopantetheine moiety, attached to a serine hydroxyl, serves to transport substrates and the growing acyl chain through the various catalytic centers of the complex. Expression of both enzymes is regulated at the transcriptional level, in a tissue-specific manner, in response to various developmental, nutritional, and hormonal signals [2]. In addition, ACC is subject to short-term regulation by allosteric and phosphorylation

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mechanisms and is commonly regarded as the pace setting enzyme for fatty acid synthesis [3]. The only free intermediate in the entire biosynthetic pathway is malonyl-CoA; all other intermediates exist only as covalently bound acyl-enzyme complexes [1]. Recently, this metabolite has been recognized as playing a critical role in a fuel sensing and signaling mechanism that regulates food intake and energy metabolism [4,5].

2. Acetyl-CoA carboxylase

2.1. The reaction sequence

The formation of malonyl-CoA from acetyl-CoA is a two-step reaction involving first, the ATP-dependent carboxylation of the biotinyl moiety, followed by transfer of the carboxyl to an acetyl-CoA acceptor.

\[
\text{Enzyme-biotin} + \text{HCO}_3^- + \text{ATP} \overset{\text{biotin carboxylase}}{\xrightarrow{\text{Mg}^+}} \text{Enzyme-biotin-CO}_2^- + \text{ADP} + \text{Pi}
\]

\[
\text{Enzyme-biotin-CO}_2^- + \text{acetyl-CoA} \overset{\text{transcarboxylase}}{\rightarrow} \text{Enzyme-biotin} + \text{malonyl-CoA}
\]

2.2. Domain organization

The amino acid sequence and domain structure of ACC is highly conserved in eukaryotes, such that antibodies raised against animal and fungal forms cross-react with each other. The two catalytic domains, biotin carboxylase and transcarboxylase, are located in the amino- and carboxyterminal halves of the polypeptide, respectively (Fig. 1). Between these domains lies the conserved biotin-binding site motif Met–Lys–Met within the biotin

Fig. 1. Domain organization of the animal ACCα-isoform. The ACC contains 2346 residues. The precise locations of boundaries between the biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP) and transcarboxylase (TC) domains has not been established; none of the domains has been expressed as individual proteins. The locations of ATP, biotin and CoA-binding sites are shown above the map and the location of phosphorylation sites shown to influence activity are shown below the map. Adapted from Corton and Hardie [6] and Kim [7].
carboxyl carrier protein domain. The presumed ATP and HCO$_3^-$ binding sites within the biotin carboxylase domain and the acetyl-CoA-binding site, within the transcarboxylase domain, have been identified by sequence analysis but have yet to be confirmed by mutagenesis. The major differences between animal and yeast ACC are in the first 100 or so aminoterminal residues and near the center of the polypeptides, both regions that are implicated in regulation of activity of the animal form by phosphorylation.

The minimal functional unit (protomer) is a homodimer. In the presence of citrate, the inactive protomer rapidly assumes a catalytically active conformation and undergoes a slower, reversible polymerization to a long filamentous polymeric structure, ~100 Å wide and up to 5000 Å long, containing as many as 20 protomers [8]. The polymeric structure may stabilize the enzyme protomers in an active conformation. Depolymerization and inactivation of the enzyme is promoted by long-chain acyl-CoA thioesters, which are direct competitors with citrate, and malonyl-CoA.

2.3. Isoforms

Two major isoforms of ACC, α and β, have been described in animals (K.-H. Kim, 1988, 1996; S.J. Wakil, 1995) that, paradoxically, have distinctly different roles in metabolism [4]. The 265 kDa α-isofrom is the predominant form expressed in the soluble cytoplasm of lipogenic tissues such as adipose, liver and mammary gland, where it plays an essential role in providing malonyl-CoA as a carbon source for fatty acid synthesis. The 280 kDa β-isofrom, on the other hand, is expressed mainly in heart and skeletal muscle, tissues with very low lipogenic capacity, but also to some extent in liver, and is associated with the outer mitochondrial membrane. In these tissues, malonyl-CoA produced by the β-isofrom functions primarily as a negative regulator of carnitine palmitoyltransferase I and, in so doing, controls the flux of fatty acids into the mitochondria for β-oxidation (Chapter 5). The two isoforms exhibit extensive sequence similarity, the major difference being the presence of ~120 additional residues at the aminoterminus of the β-form that serve to anchor the enzyme in the outer mitochondrial membrane [7]. The presence of a third isoform, containing eight additional amino acids, four residues upstream from Ser1200, has been inferred by detection of a mRNA species containing an additional 24 nucleotides that may result from alternative splicing of the gene for the α-isofrom [9]. The mRNA encoding the longer version of the α-isofrom dominates in liver and adipose, whereas that encoding the shorter one dominates in lactating mammary gland. Activity of the two α-isofroms appears to be regulated by different phosphorylation mechanisms (Section 4.2).

3. Fatty acid synthase

3.1. The reaction sequence

The overall reaction catalyzed by the animal FAS can be summarized by the equation:

\[
\text{Acetyl-CoA} + 7\text{Malonyl-CoA} + 14\text{NADPH} + 14\text{H}^+ \rightarrow \text{Palmitic Acid} + 7\text{CO}_2 + 8\text{CoA} + 14\text{NADP}^+ + 6\text{H}_2\text{O}
\]
The pathway can be visualized as a cyclic process in which the acetyl primer undergoes a series of Claisen condensation reactions with seven malonyl extender molecules, and, following each condensation, the β-carbon of the β-ketoacyl moiety formed is completely reduced by a three-step ketoreduction–dehydration–enoylreduction process [10]. The saturated acyl chain product of one cycle becomes the primer substrate for the following cycle, so that two saturated carbon atoms are added to the primer with each turn of the cycle (Fig. 2). The final product is released as a free fatty acid by the animal FAS and as a CoA ester by the yeast complex.

3.2. The catalytic components

Several catalytic elements are required for the biosynthetic process: acyltransferases that load the primer and extender substrates onto the FAS complex; a posttranslationally phosphopantetheinylated acyl carrier protein, which translocates the various intermediates between catalytic sites; a β-ketoacyl synthase, which performs the condensation reaction; the β-ketoacylreductase, dehydrase and enoylreductase enzymes responsible
for the β-carbon processing reactions and a chain terminating enzyme responsible for product release. Although the animal and yeast FASs employ essentially the same overall reaction scheme, they exhibit minor differences in the enzymatic details and major differences in their macromolecular architecture. (1) Whereas the animal FAS utilizes the same acyltransferase for loading of both the acetyl and malonyl substrates (S. Smith, 1996, 1997), the yeast FAS uses two different enzymes, one specific for acetyl moieties, the other able to load acetyl or malonyl [12]. (2) The animal FAS releases the product as a free fatty acid, through the action of a thioesterase, which severs the bond between fatty acid and phosphopantetheine thiol (S. Smith, 1978) whereas the yeast FAS uses the same broad specificity acyltransferase responsible for substrate loading to transfer the product to a CoA acceptor, so that the product is an acyl-CoA thioester [12]. (3) The enoylreductase of the animal FAS utilizes only NADPH, but the yeast enoylreductase requires both NADPH and FMN as cofactors [1].

3.3. Domain organization

In eukaryotes, the enzymes required for fatty acid synthesis de novo are integrated into large multifunctional polypeptides that are located in the cytosol. However, the domain organization and overall molecular architecture of the animal and yeast complexes are quite different (Fig. 3). Thus, the animal FAS is a dimer of identical, 272 kDa polypeptides [10,11,13], whereas the yeast FAS contains six copies each of two different subunits, α and β, of molecular masses 208 kDa and 220 kDa, respectively (E. Schweizer, 1973).
Remarkably, the ordering of the catalytic domains in the yeast multifunctional polypeptides is quite dissimilar to that of the animal and the two multifunctional proteins appear to have evolved along quite different gene fusion pathways. A unique feature of the yeast FAS is that phosphopantetheinylation is catalyzed by a discrete domain located at the carboxyterminus of the α subunit (E. Schweizer, 2000). This endogenous phosphopantetheinyl transferase cooperates with the ACP domain of the second α subunit within the α₂β₂ protomer. Phosphopantetheinylation of the animal, prokaryotic and plant ACPs is catalyzed by separate, discrete enzymes. The phosphopantetheinyl transferase acting on the animal FAS has not yet been characterized.

Both eukaryotic forms of FAS require the oligomeric structure for activity: the animal α₂ complex has two equivalent sites for fatty acid synthesis (S.J. Wakil, 1984; S. Smith, 1995) and the yeast α₀β₀ complex has six (S.J. Wakil, 1980). Thus far the complexes have been refractory to crystallographic analysis and structural analysis has been limited to lower-resolution methods. Electron microscopy and small-angle neutron scattering studies (S.J. Wakil, 1987; A. Ikai, 1988) reveal the animal FAS as an ellipsoid structure, ~216 x 144 x 72 Å, containing two cavities, one at each end of the molecule. The two cavities may represent the two sites for fatty acid synthesis in the dimeric structure. Electron micrographic images of the complex labeled with Fab fragments derived from antibodies raised against the thioesterase domain indicate that the two carboxyterminal domains are located one at each pole of the ellipsoid. Imaging of complexes labeled by Fab fragments from antibodies raised against the region between the dehydrase and enoylreductase domains, indicate that this non-catalytic core is located near the center of the ellipsoid structure. This core region contains dimerization domains that may serve to stabilize interactions between the two subunits [13].

A more detailed 25 Å resolution structure of the yeast FAS has been computed from galleries of electron microscopic images (J. Stoops, 1996). The complex appears as a barrel-shaped structure, ~245 x 220 Å, formed by three zig-zag-shaped, α subunit pairs, each with a pair of over- and under-lying arch-shaped β subunits that cap the ends of the barrel. Thus the protomeric unit is an α₂β₂ structure. The barrel contains six cavities that likely constitute the six equivalent sites for fatty acid synthesis. Each cavity has two funnel-shaped openings of ~20 Å that may allow diffusion of substrates into, and products out of, the catalytic centers.

3.4. Chain initiation

Since the animal FASs utilize a common loading site for both the primer and chain-extender substrates, acetyl- and malonyl-CoA are mutually competitive inhibitors of each other. These FASs cannot order the sequential loading of one acetyl moiety and seven malonyl moieties but instead rely on a self-editing process, in which both substrates are rapidly exchanged between CoA ester and enzyme-bound forms [11,13]. Only when the appropriate pair of substrates is bound, that is an acetyl, or longer saturated acyl moiety, at the cysteine active site and a malonyl moiety at the phosphopantetheine site, does condensation take place. Nonproductive binding, for example when the malonyl moiety binds before the acetyl moiety, or when two acetyl moieties are bound sequentially, results in rapid translocation of the substrates back to
their CoA ester form. Paradoxically then, for the system to function efficiently, free CoA, which appears on the product side of the equation, must be available at all times (S. Smith, 1982).

The yeast FAS employs two different acyltransferases for loading of acetyl and malonyl substrates and does not rely on a self-editing mechanism to facilitate translocation of the appropriate substrates to the condensation sites [12]. Nevertheless, since these complexes terminate acyl-chain growth by transfer of the acyl moiety to a CoA acceptor, free CoA is also required for efficient operation of the pathway. Both the animal α₂ and yeast α₆β₀ FASs exhibit the expected stoichiometry for acyl chain assembly when all substrates and cofactors are present (i.e. two fatty acyl chains per α₂, six fatty acyl chains per α₆β₀). However, neither the animal (S. Smith, 1985) nor the yeast [12] FAS can be fully saturated with substrate, when only malonyl-CoA or acetyl-CoA is present. This substoichiometric binding of substrates by the yeast FAS has been referred to by Schweizer and colleagues as a negative cooperativity that ensures that the complex is not overloaded with substrates that would otherwise compete for sites required for processing the various acyl-enzyme intermediates present on the enzyme [12].

3.5. Chain termination and product specificity

The eukaryotic FASs synthesize predominantly the 16-carbon saturated product with smaller amounts of 14- and 18-carbon products. The enzymatic basis of product specificity of the animal FAS has been studied in some detail in Gordon Hammes’, and in our, laboratory. The β-ketoacyl synthase has a relatively broad chain-length specificity and is able to transfer efficiently saturated acyl moieties with 2 to 14 carbon atoms from the phosphopantetheine thiol to the active-site cysteine thiol (Fig. 4). However, longer chain-length acyl moieties are transferred between thiols with increasing difficulty.

![Fig. 4. Chain-length specificities of the interthiol transferase activity associated with the 3-ketoacyl synthase and the chain-terminating thioesterase. The interthiol transferase activity was assayed on a recombinant form of FAS in which functionality of the malonyl/acetyl transferase, ACP and thioesterase domains had been inactivated by mutation; acyl-CoAs were used as model acyl donors and pantetheine as a model acceptor [15]. Thioesterase activity was assayed on a recombinant form of the isolated thioesterase domain and acyl-CoAs were used as model substrates (S. Smith, 1978).](image)
[11]. In contrast, the chain-terminating thioesterase has very limited ability to remove acyl moieties with less than 16 carbon atoms from the phosphopantetheine thiol (S. Smith, 1978, 1991). Thus the specificities of the chain-elongating and chain-terminating enzymes complement each other perfectly, ensuring that the 16-carbon fatty acid is the major product (Fig. 4). The β-ketoacyl synthase cannot transfer incompletely reduced intermediates (β-keto- or enoyl) from the phosphopantetheine thiol to the active-site cysteine, so only the saturated intermediates are elongated (S. Smith, 1997).

Some specialized animal tissues, such as the lactating mammary gland of mammals and the preen glands of birds are able to use the FAS to produce the shorter chain-length fatty acids characteristic of milk fat and the oily secretion used for waterproofing feathers, respectively. In the mammary glands of nonruminants (J. Knudsen, 1981; S. Smith, 1981) and the preen glands of birds (P.E. Kolattukudy, 1981), formation of these unusual products is attributable to the expression of a separate, discrete 29 kDa thioesterase that is able to access and release saturated acyl chain intermediates directly from the phosphopantetheine of the FAS complex. Ruminants, on the other hand, possess a FAS with unusual properties. In these species, the acyltransferase responsible for substrate loading on the FAS has a very broad acyl chain-length specificity so that saturated C4, C6, C8 and C10 intermediates can equilibrate between enzyme-bound and CoA ester forms (J. Knudsen, 1981). In the lactating mammary gland, this pool of short chain-length acyl-CoAs can be utilized for triglyceride synthesis and milk fat production. The FASs of some specialized sebaceous glands, such as the harderian, meibomian and preen glands, produce significant amounts of odd carbon-number and methyl-branched fatty acids, primarily as a result of the availability of significant amounts of propionyl-CoA and methylmalonyl-CoA as primer and chain-extender substrates (P.E. Kolattukudy, 1978, 1987).

3.6. Interdomain communication

The 20-Å-long, phosphopantetheine ‘swinging arm’ of the animal and fungal FASs has long been accepted as a key factor in allowing each of the six catalytic centers access to the ACP domains. However, distances between some of the catalytic sites of the animal FAS have been estimated, by fluorescence energy transfer, as being considerably greater than 20 Å; for example, 48 Å between the phosphopantetheine thiol and the thioesterase active site and 40 Å between the phosphopantetheine thiol and the two nucleotide-binding domains (P.E. Kolattukudy, 1985; G.G. Hammes, 1986). These observations suggest that cooperation between the acyl carrier domain and the catalytic domains during fatty acid biosynthesis may involve extensive conformational changes within the complex. Such conformational changes may be mediated in part through the presence of flexible linker regions between the catalytic domains.

Although the dimeric form of the animal FAS contains two sites for fatty acid synthesis, the monomeric form is incapable of transferring substrates from the CoA ester form to the phosphopantetheine thiol and cannot catalyze the condensation reaction. In 1991, the demonstration by J. Stoops and S.J. Wakil that the cysteine active-site thiol could be cross-linked to the phosphopantetheine thiol of the opposite subunit by dibromopropanone inspired the proposal that the two subunits of the animal
FAS are positioned in a fully extended antiparallel orientation, such that two centers for fatty acid synthesis are formed at the subunit interface. The arrangement of domains in the multifunctional polypeptide is consistent with a model in which each center for fatty acid synthesis requires cooperation of the three aminoterminal domains of one subunit (β-ketoacyl synthase, malonyl/acyetyl transferase and dehydrase) with the four carboxyterminal domains of the other subunit (enoylreductase, β-ketoacylreductase, acyl carrier protein and thioesterase) [10,11,13]. The development, in our laboratory, of procedures for the production of recombinant animal FAS dimers containing different mutations on each subunit has afforded a unique opportunity to test this model (S. Smith, 1998). Several of our findings cannot be explained adequately by the prevailing model [16]. First, in vitro mutant complementation analyses have revealed that the β-ketoacyl synthase and malonyl/acyetyltransferase can cooperate functionally with the acyl carrier protein domains of both subunits, although the intrasubunit functional interaction is less efficient than is the intersubunit interaction. Second, mutant complementation analysis also indicates that the β-hydroxy dehydration reaction is catalyzed exclusively by cooperation between the dehydrase and acyl carrier protein domains associated with the same subunit.

Third, the results of a thorough reinvestigation of the specificity of dibromopropanone interaction with the animal FAS are inconsistent with the original interpretation that cross-linking of active-site cysteine and phosphopantetheine thiols by this reagent occurs exclusively intersubunit. Thus, treatment of animal FAS dimers with dibromopropanone generates three new molecular species with decreased electrophoretic mobilities; none of these species is formed by fatty acid synthase mutant dimers lacking either the active-site cysteine of the β-ketoacyl synthase domain (Cys161Ala FAS mutant) or the phosphopantetheine thiol of the acyl carrier protein domain (Ser2151Ala FAS mutant). When dimers carrying one or both mutations on one or both subunits were treated with dibromopropanone and analyzed by a combination of sodium dodecyl sulfate/polyacrylamide gel electrophoresis, Western blotting, gel filtration and matrix-assisted laser desorption mass spectrometry, the two slowest moving of the three cross-linked species were identified as doubly and singly cross-linked dimers, respectively, whereas the fastest moving species was identified as originating from internally cross-linked subunits. This internally cross-linked species accounted for as much as 35% of the total cross-linked species [17].

On the other hand, certain features of the original model are supported by results of the mutant complementation studies. Thus, the substrate loading and condensation reactions are catalyzed most efficiently by cooperation of the β-ketoacyl synthase and malonyl/acyetyltransferase domains with the ACP domain of the opposite subunit and the β-carbon processing and chain-terminating reactions are catalyzed by cooperation of the phosphopantetheine moiety with catalytic domains associated with the same subunit [16].

Perhaps the most important implication of these recent findings is that the structural organization of the animal FAS must permit head-to-tail interactions between domains located on opposite subunits and must allow for functional interactions between domains located distantly on the same subunit. These requirements could be met by a modified head-to-tail model in which the two ACP domains, at each of the subunit 'tails', have
access to the β-ketoacyl synthase and malonyl/acetyltransferase domains at the 'heads' of either subunit (Fig. 5, top). Although such a mechanism would require a remarkable degree of flexibility within the FAS polypeptides, to ensure productive interactions both inter- and intrasubunit, it cannot be formally excluded at this time.

An alternative model has been envisaged that requires much less conformational flexibility in the subunits (Fig. 5, bottom). In this model, the β-ketoacyl synthase and malonyl/acetyltransferase domains of both subunits are located near the center of the
dimer, where they can freely access the ACP domains of both subunits, and the domains responsible for the β-carbon processing reactions are located at opposite ends of the dimer and therefore have access to only one ACP domain. Thus the model retains the original concept of head-to-tail oriented subunits, but also allows for head-to-tail interactions between domains of the same subunit.

4. Short-term regulation of fatty acid synthesis

Fatty acids, esterified in triglycerides, represent the major form of stored energy in animals. A relatively small proportion of excess caloric intake in the form of carbohydrate is stored as glycogen, most is converted to fat via the de novo lipogenic pathway. Thus the flux of substrate through the pathway is high in animals that have ingested high amounts of carbohydrate and is low during periods of fasting. In most animals, the liver and adipose tissue are the major sites of fatty acid biosynthesis, although the pathway is also of vital importance in other tissues during certain stages of development. In animals having access to a regular daily supply of food, fatty acid synthesis in the liver varies dramatically during the diurnal cycle. In the laboratory rat fed ad libitum, the peak in hepatic lipogenesis occurs during the feeding period, in the dark phase of the daily cycle, and is paralleled by changes in the activity of ACC (N. Iritani, 1985, 1990). Untreated diabetic rats do not exhibit this diurnal rhythm in lipogenic activity and ACC activity remains low throughout each cycle; administration of insulin restores the normal diurnal rhythm. However, most investigations into the role of hormones and nutrients in the regulation of lipogenesis have not studied these diurnal changes, but instead have focused on models in which the animals are first subjected to the stress of a prolonged fast of one or two days, prior to reintroduction of a food supply.

4.1. Regulation of substrate supply for fatty acid synthesis

Following a period of fasting, the initial stimulus for activation of the lipogenic pathway appears to be the influx of dietary carbohydrate. Glucose stimulates secretion of insulin from pancreatic β-cells and, as the ratio of insulin to glucagon increases in the blood, the activities of several enzymes in the glycolytic and lipogenic pathways are elevated, while the activities of key gluconeogenic enzymes are decreased (Fig. 6). These early changes in activity are brought about primarily by changes in the catalytic efficiency of key enzymes, whereas the longer-term responses can also involve changes in enzyme concentration (Section 5.4).

The resulting increased flux of pyruvate into the mitochondria and the subsequent activation of pyruvate dehydrogenase causes a rapid increase in the production and export of citrate via the tricarboxylate anion carrier. The elevated concentration of cytosolic citrate can simultaneously provide acetyl-CoA, the carbon source for fatty acid synthesis, through the action of ATP: citrate lyase, and stimulate the conversion of acetyl-CoA to malonyl-CoA, by activation of ACC. The reducing equivalents required for fatty acid synthesis are provided largely through the action of malic enzyme and
the two pentose phosphate cycle enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The supply of NADPH by these enzymes appears to be largely dependent on its rate of utilization and is unlikely to be limiting in the lipogenic pathway.

The two pancreatic hormones, insulin and glucagon exert essentially opposite effects on energy metabolism. Thus, during the intervals between feeding and during prolonged periods of fasting, when the ratio of insulin to glucagon decreases in the blood, the activities of key enzymes in the glycolytic and lipogenic pathways are decreased, while the activities of key gluconeogenic enzymes are increased resulting in a reversal of the process described above.

4.2. Regulation of acetyl-CoA carboxylase α activity by reversible phosphorylation

ACC preparations purified from lipogenic tissues contain up to eight phosphate residues per subunit. Six of the phosphorylation sites are located within the first 100 aminoterminal residues, the other two near the center of the polypeptide (Fig. 2). Mild, limited proteolysis of ACC results in cleavage of the highly phosphorylated aminoterminal
domain and full activation of the enzyme in the absence of citrate. Unless special precautions are observed to prevent activation of protein kinases during tissue disruption, ACC preparations typically are highly phosphorylated and require addition of supraphysiological concentrations of citrate (1–5 mM) for full activity, regardless of the nutritional status of the animal (N.B. Madsen, 1987; S.J. Wakil, 1988 and D.G. Hardie, 1992). However, when livers of fed animals are freeze-clamped prior to isolation of the enzyme, preparations are obtained that have low phosphate content and can be fully activated by physiological concentrations of citrate (~0.2 mM). On the other hand, the enzyme purified from freeze-clamped livers of fasted animals has a high phosphate content and requires high added citrate concentrations for full activity. A plethora of kinases has been implicated in the phosphorylation of the enzyme (Fig. 2) but, despite the flurry of activity that occurred in this field in the 1980s and early 1990s, there is still no clear consensus as to which of them is physiologically the most important in regulating ACC activity. Two kinases, one dependent on cAMP for activity (cAMPK) the other dependent on 5'-AMP (AMPK) can inactivate ACC by phosphorylation in vitro and have been considered the strongest candidates [6,7]. The cAMPK phosphorylates ACC at Ser77 and Ser1200 and causes primarily an increase in the $K_a$ for citrate and a slight reduction in $V_{max}$, whereas the AMPK phosphorylates mainly at Ser79, Ser1200 and, to a lesser extent, at Ser1215 and produces a large decrease in $V_{max}$. The AMPK has been considered as a particularly attractive candidate, since it is also able to phosphorylate and inactivate 3-hydroxy-3-methylglutaryl coenzyme-A reductase, and so potentially is in a position to coordinately regulate both the fatty acid and sterol biosynthetic pathways [6]. Treatment in vitro with cAMPK and AMPK of recombinant ACCs, mutated in one or more of the potential phosphorylation sites, indicated that when only Ser79 is available as a phosphorylation target, AMPK is able to inactivate ACC and when only Ser1200 is available, cAMPK, but not AMPK is able to inactivate ACC [7]. Thus, the critical target for cAMPK appears to be Ser1200 and for AMPK, Ser79. Strong arguments have been made supporting roles for both cAMPK and AMPK as regulators of ACC activity. During fasting, glucagon secretion raises the intracellular concentration of cAMP, which potentially can result in phosphorylation and inactivation of ACC by cAMPK, and indeed glucagon treatment of hepatocytes increases phosphorylation at Ser1200 (D.G. Hardie, 1988). Similarly, during fasting, as ATP production falls into deficit and AMP concentrations rise, AMPK could be activated, resulting in phosphorylation and inactivation of ACC. Indeed not only is AMPK activated directly by AMP, but so is an AMPK kinase that activates AMPK by phosphorylation, whereas an AMPK phosphatase that inactivates AMPK is inhibited by AMP (Fig. 6). Nevertheless, some puzzling observations remain unresolved. First, a report from Ki-Han Kim’s laboratory in 1990 concluded that the α-isoform of ACC containing eight additional residues near Ser1200 is not phosphorylated at this site by the cAMPK. However, the same report documented that it is precisely this putative cAMPK-resistant, longer isoform that dominates in liver, the major site of lipogenesis and a tissue that is responsive to glucagon; this finding casts serious doubt on the role of the cAMPK in regulation of ACC activity. Second, although the diurnal rhythm in the activation state of ACC correlates nicely with variations in the phosphate content at position Ser79, AMPK activity remains at the same level throughout the light and dark cycles [6]. Furthermore,
no reduction in AMPK activity is observed when either hepatocytes or pancreatic β-cell lines are exposed to high levels of glucose. The possibility that it is in fact a phosphatase with specificity for Ser79 of ACC that is the regulated enzyme has yet to be resolved.

Collectively, these observations indicate that the catalytic activity of ACC is determined by a complex interplay between citrate-induced conformational changes and phosphorylation events. However, the question as to exactly how the covalent modification and conformational changes in ACC bring about changes in activity has not yet been addressed at the structural level.

4.3. Malonyl-CoA, fuel sensing and appetite control

Within the last several years there has grown an increasing awareness of the important role played by malonyl-CoA as a fuel sensing and signaling molecule [4]. Of 28 intermediates formed between acetyl-CoA and palmitic acid, malonyl-CoA is the only true free intermediate and, as a regulator of CPTI, is uniquely positioned to control both the rate of biosynthesis and degradation of fatty acids.

Malonyl-CoA formed in liver is utilized primarily as a substrate for fatty acid synthesis, but at the same time, as an inhibitor of carnitine palmitoyltransferase I, it controls entry of fatty acids into the mitochondria for oxidation (J.D. McGarry, 1980) (Fig. 6). Thus, following feeding, when excess dietary carbohydrate is being converted to fat and tissue malonyl-CoA levels are elevated, activity of carnitine palmitoyltransferase I is inhibited and flux of fatty acids into the mitochondria is prevented [4]. This control mechanism ensures that the fatty acid biosynthetic and oxidation pathways are not simultaneously activated in the same tissue, thus avoiding the establishment of a futile cycle. Conversely, during periods of fasting hepatic malonyl-CoA levels are lowered, flux of substrate through the fatty acid biosynthetic pathway is halted and the block in entry of fatty acids into the mitochondria is removed.

Recent studies have revealed a previously unsuspected correlation between intracellular concentration of malonyl-CoA and appetite control [5]. As anticipated, mice treated with cerulenin, an inhibitor of the β-ketoacyl synthase activity of the FAS (Chapter 3), exhibit decreased rates of hepatic lipogenesis and elevated intracellular malonyl-CoA. However, these animals also cease feeding, maintain normal activity, exhibit a dramatic loss of adipose tissue mass and suffer a major reduction in body weight. Treatment with 5-(tetradecyloxy)-2-furoic acid, an inhibitor of ACC, also results in decreased fatty acid synthesis but has no effect on feeding behavior. Indeed, administration of 5-(tetradecyloxy)-2-furoic acid restores normal feeding behavior in mice treated with a FAS inhibitor, indicating that it is the elevated intracellular concentration of malonyl-CoA that triggers loss of appetite. The attenuation effect is observed when the compounds are administered intracerebroventriculally, implicating the central nervous system as the site of action of the inhibitors. This conclusion is further supported by the observation that expression of the hypothalamic neuropeptide Y, an appetite stimulant, is dramatically reduced in mice treated with an inhibitor of FAS. Since both ACC and FAS are expressed in hypothalamic neurons, it appears likely that the malonyl-CoA concentration sensing mechanism may be directly ‘hard-wired’ to the appetite control center in the hypothalamus. Under normal conditions, the rise in tissue malonyl-CoA
concentration following feeding may provide a 'satiety signal' indicating that excess caloric intake is now being channeled into fat stores, so that neuropeptide Y production is turned off and feeding ceases. Thus the condition induced by treatment with FAS inhibitors likely mimics the normal fed state so that the animal is deceived into a prolonged period of fasting for the duration of the treatment.

Perhaps it would appear paradoxical that in mice treated with FAS inhibitors, where food intake is dramatically reduced and intracellular malonyl-CoA levels rise in the lipogenic tissues, fatty acid oxidation provides the major source of energy and adipose mass is reduced. The answer to this puzzle likely is attributable to unique metabolic control mechanisms operative in muscle, the main site of fatty acid oxidation during physical activity [4]. The activity of the ACCβ-form and the concentration of malonyl-CoA in skeletal muscle decreases within seconds of the initiation of exercise. The mechanism by which activity of the β-form is regulated in muscle appears to involve both allosteric regulation by citrate and reversible phosphorylation, since activity of AMPK is elevated during muscle contraction. The mechanism of disposal of malonyl-CoA produced in muscle by ACCβ is not well understood. Most likely malonyl-CoA is utilized by a cytosolic malonyl-CoA decarboxylase, since genetic defects in this enzyme produce a phenotype characteristic of mitochondrial fatty acid oxidation disorders (S.J. Gould, 1999). Almost certainly, in view of the scarcity of FAS in muscle, malonyl-CoA is not utilized for fatty acid synthesis, so that administration of the FAS inhibitors is unlikely to impact significantly the concentration of malonyl-CoA in muscle. The regulation of malonyl-CoA levels by different mechanisms in muscle and lipogenic tissues allows for use of fatty acids as metabolic fuel in muscle, even when malonyl-CoA levels may be high in liver, as in the case of animals treated with FAS inhibitors.

5. Regulation of the intracellular concentration of lipogenic enzymes

5.1. Strategies and methodology

The intracellular concentrations of lipogenic enzymes potentially can be altered through changes in their rate of synthesis or degradation. Thus changes in the rates of either transcription or translation, as well as changes in the stability of specific mRNAs and their encoded proteins need to be examined when evaluating factors that may regulate changes in enzyme concentration. In reality, the decrease in concentration of ACC and FAS that occurs during relatively long-term fasting appears to be due to both a cessation in transcription of their genes and a decrease in the half-life of the proteins themselves. On refeeding of animals that have been subjected to long-term fasting, transcription of ACC and FAS is activated and the half-life of the proteins is lengthened until a new steady state concentration of enzymes is established. During differentiation of preadipocytes into mature adipocytes, in vitro, the increase in the intracellular concentration of lipogenic enzymes can be attributed to both stabilization of their mRNAs and increased rate of transcription of their genes. In recent years investigators have focused primarily on factors that influence the rate of gene transcription, so that relatively few details are known as to how changes in the stability of the mRNAs and proteins are brought about.
Cell culture systems are widely used to study the regulation of gene transcription, as these cells can usually be induced to take up and express heterologous reporter gene constructs and can be manipulated to mimic changes in the hormonal and nutritional environment that occur in intact animals. The most commonly used cell culture systems are primary hepatocytes and hepatoma cell lines, as models for studying the effects of hormones and nutrients on expression of lipogenic enzymes in the liver, and 3T3-L1 and 30AS cells, as adipocyte models. These adipocyte lines are derived from fibroblasts which, at confluence, can be modulated to differentiate and accumulate fat droplets. Upon differentiation of these preadipocytes, lipogenic enzymes accumulate to levels similar to that characteristic of normal adipose tissue, so that these cell lines are commonly used to study gene transcription in both differentiating and mature adipocytes. However, the differentiating preadipocyte culture system does not mimic all of the characteristics of intact adipose tissue and the responsiveness of hepatoma cells to hormones and nutrients is typically muted, compared to that of hepatocytes or the liver of whole animals. Therefore, caution needs to be exercised in interpreting results obtained with these model systems, particularly when corroborating evidence from in vivo experiments is lacking. The use of transgenic animals affords a powerful model system for evaluating the roles of putative cis-regulatory and trans-acting proteins in mediating tissue-specific gene expression in response to nutritional status.

Regulation of gene transcription involves interaction of DNA regulatory sequences, termed cis-acting elements, that are usually found in the 5'-flanking regions of genes, with nuclear proteins, termed trans-acting factors. Understanding the mechanism of transcriptional regulation of a gene, therefore, requires the identification of both the cis-acting elements in the gene and the trans-acting factors in the nuclei that bind to them as well as elucidation of the signaling events that lead to changes in the interaction between the cis elements and trans factors.

5.2. The acetyl-CoA carboxylase promoter

The two major isoforms of ACC, α and β, which exhibit about 80% amino acid sequence similarity, are encoded by separate genes that map to chromosomes 17q21 and 12q23.1, respectively. The α-isofrom is expressed predominantly in tissues that exhibit high rates of fatty acid synthesis, such as liver and adipose tissue, whereas the β-isoform is expressed mainly in heart and skeletal muscle, and to a lesser extent in liver. Transcription of the α gene is under the control of two promoters, designated as PI and PII that are separated by 12.3 kbp. Five different species of mRNA, all of which contain the same base sequence in the coding region but differ in the 5'-untranslated region, are generated by the alternative splicing of the first four exons [18].

The usage of α gene promoters is tissue-specific (Fig. 7). Thus, only promoter PI is active in adipose tissue and promoter PII in mammary gland, whereas both promoters are active in liver. PII, which is active in all tissues at least at a low level, is sometimes described as a 'housekeeping promoter', although it contains a strong enhancer element and several regulatory cis-elements that confer responsiveness to external stimuli. A third ACCα promoter, PIII, has been described in sheep that gives rise to an N-terminal variant of ACCα that exhibits tissue-restricted mode of expression (M.C. Barber, 2001).
The choice of gene promoter usage also seems to vary with animal species and developmental status. Thus, when animals are fasted and refed a high-carbohydrate, low-fat diet, transcripts from both PI and PII promoters are elevated in the liver of 12 days old chicks, whereas mainly the transcript from the PI promoter-driven transcript is induced in adult rats; similarly, changes in the thyroid hormone level affect both PI and PII promoter activity in chickens but only PI promoter activity in adult rats. The ACC\(\beta\) gene is also transcribed by two promoters, PI\(\beta\) and PII\(\beta\), that are located immediately upstream of the first and second exons, respectively. However, the promoter regions of the \(\alpha\) and \(\beta\) genes exhibit little sequence similarity as the ACC\(\beta\) promoter region contains cis-acting elements that bind muscle-specific transcription factors (K.-S. Kim, 2001).

5.3. The fatty acid synthase promoter

FAS genomic clones containing the 5'-flanking DNA have been isolated and partially characterized from rat, goose, chicken and human. The FAS gene reportedly is transcribed from two promoters in humans [19], but from only one in other species. Promoter I, which contains recognizable TATA and CAAT boxes, controls transcription from an initiation site near the beginning of exon 1, whereas promoter II, which contains
neither a TATA nor CAAT box, controls transcription from two sites, one in intron
1, the other 49 nt upstream of the ATG start codon in exon 2. Transient transfection
experiments with FAS-reporter gene chimeras indicate that promoter I is about 15
times stronger than promoter II and that the presence of promoter II attenuates the
transcriptional activity of promoter I. Based on these results, a model has been proposed
(S.J. Wakil, 1996) in which intronic promoter II exerts a roadblock for RNA polymerase
II that has initiated transcription from promoter I. Promoter II of the human FAS gene,
which has not yet been well characterized, may play a role in low-level constitutive
expression of FAS, whereas promoter I may be preferred for rapid transcription, under
demand for high lipogenic rates.

The rat and goose genes exhibit 90 and 61% identity, respectively, with the human
FAS gene in the promoter region containing the TATA box, an inverted CCAAT box
and a functional Sp1-binding site. In common with the human FAS gene, the rat gene
contains a long first intron that exerts a negative effect on promoter activity. The negative
regulatory element has been mapped to a region between +405 to +1083 in the rat gene.
Sequence elements within this region, which are capable of binding a variety of nuclear
proteins, also confer a negative effect on transcription via a heterologous promoter, in an
orientation-dependent manner. However, the results of run-on assays with nuclei derived
from tissues that express FAS at either high or low levels indicate that the different rates
of transcription can be accounted for by differences in the extent of initiation, rather
than by operation of a transcriptional pausing mechanism, as proposed for the human
FAS gene (S. Smith, 1997). The sequence of the first intronic region is poorly conserved
between rat and human genes and the physiological significance, if any, of the use of an
intronic promoter in the human, but not in the rat, FAS gene remains to be demonstrated.

Table 1 summarizes various cis-acting elements that have been identified in the FAS
and ACC promoters and the trans-acting factors that bind to them.

5.4. Transcriptional regulation of lipogenic gene expression in response to dietary
carbohydrate

The adaptive response of gene expression to nutrient availability is vital to the survival
of all organisms. In animals, the response to availability of dietary carbohydrate
is mediated both by effects of glucose metabolism itself and by secondary effects
on hormone secretion. Thus, glucose entering the blood stream provides the initial
stimulus for the synthesis and secretion of insulin, which in turn promotes the entry
of glucose into cells and its subsequent metabolism. Insulin release at the onset of
feeding also stimulates the conversion of thyroxine into the more potent hormone
3,5,3'-triiodothyronine (T3) and may increase the level of T3 receptors in the liver. Thus
the feeding stimulus activates both insulin and T3-mediated signaling pathways, both of
which have the effect of increasing lipogenesis [20].

The initial, rapid hepatic response to carbohydrate intake involves activation of
key glycolytic and lipogenic enzyme activities (Fig. 6). The long-term response is
accompanied by an increased enzyme production, primarily as a result of increased
gene transcription. Conversely, glucagon production is elevated during fasting, resulting
in down-regulation of glycolytic and lipogenic enzymes. The involvement of insulin,
<table>
<thead>
<tr>
<th>Consensus cis-acting motif</th>
<th>Trans-acting factor</th>
<th>Effect on transcription</th>
<th>Location in rat FAS promoter</th>
<th>Location in ACCα promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td>TATA box (TTTAAAT)</td>
<td>TBP</td>
<td>Initiates transcription</td>
<td>-33/-26</td>
<td>Not present</td>
</tr>
<tr>
<td>E-Box (CANNTG)</td>
<td>USF and other bHLH-LZ family members</td>
<td>Mediates the insulin response in FAS and ACCα PI promoter</td>
<td>-60/-65, -326/-332</td>
<td>-931/-936 (Rat, PII); -109/-114 (Sheep, PI)</td>
</tr>
<tr>
<td>Inverted CAAT box (ATTGGCC)</td>
<td>NF-Y</td>
<td>Activates the basal transcription; required for conferring sterol response to FAS promoter; mediates cAMP response in FAS promoter</td>
<td>-98/-92, -516/-498</td>
<td>Not identified</td>
</tr>
<tr>
<td>GC-Box (G/TG/AAGGCG/TG/AG/AG/T)</td>
<td>Sp1 family members</td>
<td>Activates transcription; participates in conferring sterol response to ACC and FAS promoters; mediates glucose response in ACCα PII promoter</td>
<td>-91/-83, -168/-160, -226/-218, -242/-284, -482/-474 and -557/-549</td>
<td>-254/-242, -329/-317 (Rat, PII)</td>
</tr>
<tr>
<td>Sterol response element (TCACNCCAC)</td>
<td>SREBP family members</td>
<td>Mediates the sterol response; mediates the nutritional response</td>
<td>-73/-43, -150/-141</td>
<td>-273/-256, -977/-967 (Rat, PII)</td>
</tr>
<tr>
<td>Thyroid hormone response element (A/GGTT/AC/AAN₁/GGGT/AC/AA)</td>
<td>TR/RXR and/or LXR/RXR</td>
<td>Mediates the thyroid hormone response</td>
<td>-120/-125, -669/-650</td>
<td>-108/-82 (Chicken, PII)</td>
</tr>
<tr>
<td>Carbohydrate response element (CAÎGN/CGTG)</td>
<td>ChoRF/USF</td>
<td>Mediates the carbohydrate response in FAS and ACCα PI promoter</td>
<td>-7214/-7190</td>
<td>-126/-102 (Rat, PI)</td>
</tr>
</tbody>
</table>

TBP, TATA box binding protein; USF and bHLH-LZ, upstream stimulatory factor and basic helix-loop-helix leucine zipper; NF-Y, nuclear factor Y; Sp1, stimulatory protein I; SREBP, sterol-response element binding protein; TR/RXR, a heterodimer consisting of thyroid hormone receptor and retinoid receptor; LXR/RXR, a heterodimer consisting of liver receptor X and retinoid receptor; ChoRF, carbohydrate-response element binding factor. Consensus cis-motifs are given in 5' to 3' orientation and N represents any nucleotide. It should be noted that many variations of the consensus motif are functional in various genes.
glucagon and T3 in the regulation of lipogenic gene expression has been appreciated for some time, but only recently have carbohydrates themselves been implicated in this process. The identity of the intracellular signaling molecule produced by the metabolism of glucose has not yet been determined. One candidate is glucose-6-phosphate, since intracellular concentration of this metabolite varies in parallel with ACC and FAS mRNA concentrations in liver and adipose tissue, in response to dietary carbohydrate (P. Ferre, 1997). Another is xylulose-5-phosphate, an intermediate in the pentose phosphate cycle, since this metabolite can mimic the effect of hyperglycemia on glucose-regulated genes, without causing an elevation of glucose-6-phosphate [21].

5.4.1. The role of SREBPks
The effects of glucose, insulin and glucagon on transcription of lipogenic enzymes involves recruitment of a number of transcriptional activators, including SREBP, USF, NF-Y and Sp1. Pioneering work in the Goldstein and Brown laboratories established that SREBPs belong to the basic helix–loop–helix leucine zipper family of transcription factors and are involved in the regulation of the biosynthesis of both cholesterol and fatty acids [22] (Chapter 15). These DNA-binding proteins form dimers that can recognize both the direct repeat sterol regulatory element 5’-TCACNCCAC-3’ and the inverted repeat E-box, 5’-CANNTG-3’ [23].

The SREBPs are synthesized as ~1150 amino acid precursor proteins, bound to the endoplasmic reticulum and nuclear membrane, that undergo a sequential two-step proteolytic cleavage under low cellular sterol concentrations. This proteolytic cleavage results in the release of the aminoterminal section of SREBP which enters the nucleus and activates the transcription of genes involved in cholesterol and fatty acid synthesis, by binding to regulatory elements within their promoter regions. There are three major SREBP isoforms encoded by two different genes. The single SREBP-2 isoform produced by the SREBP-2 gene is believed to function primarily in the maintenance of cholesterol homeostasis [23]. The SREBP-1 gene can be transcribed from two different promoters, generating two isoforms, SREBP-1c and SREBP-1a, the latter having 29 additional amino acids at the aminoterminus. The additional aminoterminal residues of SREBP-1a carry a binding site for NF-Y (also known as CBP) and imparts a strong promoter enhancing effect to this isoform; SREBP-1c, on the other hand, is a relatively weaker transcriptional activator, unless additional sequences are present in the promoter region that can recruit co-activators such as NF-Y and Sp1. The relative levels of SREBP-1a and 1c mRNAs vary significantly in different tissues. In tissues active in lipogenesis, such as liver and adipose tissue, the SREBP-1c mRNA is more abundant, whereas in spleen and most cultured cell lines, the SREBP-1a mRNA predominates.

The results of various in vivo and in vitro studies indicate that SREBPs play a major role in the regulation of lipogenic gene expression. For example, transcription of SREBP-1c, ACC and FAS genes is shut off during fasting of normal animals and turned on following refeeding a high-carbohydrate diet. However, transgenic animals carrying a disrupted SREBP-1 gene fail to modulate transcription of the lipogenic genes in response to these changes in nutritional status (H. Shimano, 1999). Over-expression of SREBP-1a or 1c in cultured preadipocytes or in transgenic animal liver activates transcription of lipogenic genes [24]. Transgenic animals over-expressing SREBP-1c in
adipose cells develop insulin-resistant hyperglycemia, accumulate triglycerides in the liver and serum, and fail to down-regulate hepatic lipogenic gene expression in response to fasting. SREBP-1c also has been shown to be involved in the insulin-induced expression of the glucokinase gene [25,26]. The observation that insulin activates the expression of the SREBP-1c gene in primary hepatocytes whereas glucagon and cAMP have the opposite effect suggests that SREBP could mediate some of the effects of insulin on lipogenic gene expression in the liver, indirectly, by increasing glucose flux through glucokinase, and/or directly, by activation of lipogenic gene expression (Fig. 8).

5.4.2. The role of SREBP co-activators

As relatively weak transcriptional activators, SREBPs typically operate synergistically with other general transcriptional activators such as Sp1, NF-Y and cAMP-response element binding protein (T.F. Osborne, 2000). However, between different promoters, there are significant differences in the identity of these co-regulators and in the position of their binding sites relative to the position and number of SREBP-binding sites. These differences may be attributable, at least in part, to the differential recruitment of SREBP isoforms having different co-regulatory factor requirements. Sp1 may function synergistically with SREBP in mediating the glucose/insulin activation of both the FAS and ACCα genes. Increased binding of Sp1 to GC-rich regions between nucleotides −329/−317 and −254/−242 of the ACCα II promoter has been observed in 30A5 adipocytes exposed to glucose. Disruption of either Sp1-binding site by mutation eliminates glucose responsiveness of the ACCα II promoter. The increased binding of Sp1 has been attributed to a glucose-mediated induction of protein phosphatase I in the nucleus, which dephosphorylates Sp1, generating a form that binds more effectively to the promoter (K.-H. Kim, 1996).

NF-Y, a heterotrimeric transcription factor that binds to the inverted CCAAT-box motif of the FAS promoter, also appears to play a role as an activator of basal transcription of this gene. The cAMP-mediated down-regulation of FAS transcription

Fig. 8. Central role of SREBP-1c in the regulation of lipogenesis. Influx of glucose leads to increased secretion of insulin by pancreatic β-cells. Insulin and glucose induce lipogenic gene expression by increasing synthesis of SREBP-1c, whereas glucagon and fatty acids exert negative effect by decreasing SREBP-1c synthesis. Arrows carrying + and − symbol indicate positive and negative effect, respectively, on expression of SREBP-1c by corresponding external stimuli. The question mark indicates that direct activation of SREBP-1c expression by glucose has not been demonstrated definitively.
that occurs on fasting appears to involve the interaction of NF-Y with the inverted CCAAT-box, although the mechanistic details have not yet been worked out [27].

5.4.3. The role of USFs
The ubiquitous DNA-binding proteins USF1 and USF2 were initially proposed as the key mediators of the glucose/insulin-response in lipogenic genes. USF1 and USF2, members of a basic helix–loop–helix leucine zipper family of transcription factors, bind to the E-box sequence motif, CANNTG. Both transient transfection experiments in cell culture and the use of transgenic animal models have implicated two USF-binding sites in the glucose/insulin responsiveness of the FAS gene, one at −65, the other at −332 [28]. The USF-binding site at position −65 in the rat FAS promoter is overlapped by an SREBP-binding site. Although this region of the gene can bind USF and SREBP independently, studies with transgenic mice suggest that it is the binding of USF at this site that is more important in mediating the fasting/refeeding response. Thus, it appears that it is SREBP binding at the second site, −150 to −141, that is essential in modulating transcription of the FAS gene. The nucleotide sequence in this region is a motif that is highly conserved in rat, human and goose FAS promoters. An E-box located at −114 of the ACCα I promoter that binds USF1 and 2, but not SREBP-1, has also been implicated in the insulin responsiveness of the ovine gene [29]. As yet, it is unclear as to whether, or how, SREBPs and USFs interact in modulating transcription of the FAS and ACC genes in response to fasting and refeeding. Quite possibly, the USF proteins act synergistically with SREBP, as do other co-activators such as Sp1 and NF-Y.

5.4.4. The role of carbohydrate-response elements
Additional sequence elements have been identified within the ACC and FAS genes that play a role in modulation of transcription in response to glucose, independent of insulin. These sequences, termed glucose-response elements, or carbohydrate-response elements, consist of two E-box half-sites related to the sequence 5′-CACG, in either direct or inverted orientation and separated by 7 or 9 nucleotides, and are similar to those identified as carbohydrate-response elements in the pyruvate kinase and S14 genes (H.C. Towle, 2000). The carbohydrate-response elements, which are located at −126 to −102 in the rat ACCα I promoter, and at −7240 to −7190 in the rat FAS gene, bind both USF and an additional, as yet unidentified trans-acting factor [30,31]. This factor, termed a carbohydrate-response factor, presumably is linked to the metabolism of glucose by an as yet unidentified signaling pathway.

5.4.5. Signaling pathways
Although it is well established that genes involved in glucose catabolism and lipid biosynthesis are up-regulated by high glucose and insulin concentrations in both liver and adipose tissues, the signaling pathway from glucose/insulin to the transcriptional machinery is not clearly understood. The binding of insulin to its receptor activates the associated tyrosine kinase and results in autophosphorylation of the receptor. The signal is then propagated by two main routes: the insulin receptor substrates/phosphatidylinositol 3-kinase pathway and the Ras/mitogen-activated protein kinase pathway. It is the phosphatidylinositol 3-kinase signaling pathway that has been implicated in
the regulation of FAS transcription by insulin, although the identity of the participants remains to be established. The AMPK which is activated under various stress conditions due to a rise in the cellular AMP to ATP ratio, plays a significant role in the regulation of glucose-activated genes including FAS and ACC. Glucose-mediated transcriptional activation of FAS and ACC genes is inhibited by activated AMPK possibly due to inhibition of a yet to be identified activator or activation of an unidentified repressor. However, AMPK plays no role in the induction of glucose-activated gene expression since basal AMPK activity is not reduced by high levels of glucose in the cell [32]. A similar situation may be operative in the regulation of ACC activity by reversible phosphorylation. Here too, clear evidence implicates AMPK in the down-regulation (dephosphorylation), but not in the up-regulation (phosphorylation) of ACC activity.

5.4.6. The role of thyroid hormone
Thyroid hormone plays a significant role in regulation of expression of lipogenic enzymes in response to changes in nutritional status. T3 stimulates transcription of ACC and FAS in a tissue-specific manner, exerting its strongest effect in liver. In chick embryo hepatocytes, the rate of lipogenesis and the levels of lipogenic enzymes are low until the newly hatched chicks are fed and transcription of the lipogenic enzymes is activated. The induction of lipogenic enzymes can be simulated in vitro by treatment of chick embryo hepatocytes with T3. The mechanism of thyroid hormone-induced gene expression involves activation of the thyroid hormone receptor to form a heterodimer with the retinoid X receptor (RXR) or liver X receptor (LXR), which in turn binds to the consensus thyroid hormone-response element (Table 1) and activates the transcription. Treatment of chick embryo hepatocytes with T3 results in the stimulation of the promoter II-driven transcript of the ACCα gene, whereas in Hep G2 cells, T3 increases transcription from the human FAS promoter I, but has no effect on promoter II (S.J. Wakil, 1998; F.B. Hillgartner, 2001). The T3 response is mediated by the consensus thyroid hormone-response element half-site sequence, arranged as direct repeats on the noncoding strand of ACCα promoter II and the coding strand of FAS promoter I. The FAS promoter I also harbors an additional thyroid hormone-response element motif having only one half-site of consensus sequence. The amount of T3-receptor/RXR heterodimers that binds to these thyroid hormone-response elements is increased in the presence of T3. As yet, the details of the mechanism by which T3 regulates ACCα and FAS transcription remain unclear. F.B. Hillgartner and colleagues (2001) have proposed that T3 regulates ACCα transcription by a novel mechanism involving changes in the composition of nuclear receptor complexes bound to thyroid hormone-response element. Heterodimeric complexes containing LXR/RXR ensure basal level of ACCα expression whereas the complexes containing LXR/RXR and T3-receptor/RXR mediate T3-induced ACCα expression. Insulin also contributes to the T3-mediated activation of lipogenic gene expression by facilitating the conversion of thyroxin to T3 and increasing the level of nuclear T3 receptor.

5.4.7. Down-regulation during fasting
The down-regulation of transcription of the ACC and FAS genes that accompanies fasting is most likely initiated by a change in the relative amounts of glucagon and
insulin produced by the pancreas that results in decreased transcription of SREBP-1, phosphorylation of Sp1, and withdrawal of the carbohydrate-response element-mediated stimulatory effect and down-regulation of T3 production. The elevation of intracellular cAMP concentration that results from increased glucagon production also plays a role in the down-regulation process. However, the cis-acting elements and trans-acting factors involved appear to be different for ACC and FAS. NF-Y binding at an inverted CCAAT-box has been implicated in the case of the FAS promoter and AP-2 binding at variants of well-characterized cAMP-response element in the ACCα PII promoter. Phosphorylation of AP-2 by cAMPK facilitates the binding of AP-2 to the cAMP-response element [33]. Further investigation is required to determine the precise mechanism whereby cAMP exerts its effects on these trans-acting factors. The cis-elements and the trans-factors that confer cAMP responsiveness to the ACCα PII promoter still remain to be identified.

5.4.8. Summary
In summary, a tentative model for the transcriptional regulation of the ACC and FAS genes, in response to fasting and refeeding a carbohydrate-rich diet, can be envisaged in which the carbohydrate-response factor, together with SREBP, play central roles and Sp1, NF-Y and USF function as co-activators of SREBP. However, experimental validation of this model is incomplete in that it remains unclear as to exactly how functionality of the putative co-activators NF-Y and USF is modulated. For example, binding of NF-Y to the inverted CCAAT-box of the FAS gene is not altered either in cultured hepatoma cells treated with cAMP, or in the livers of fasted animals, although FAS transcription is down-regulated in both situations. Similarly, binding of USF to the E-box at −68/−52 of the rat FAS gene is unaltered in the livers of refed animals as well as in cultured hepatoma or adipose cells treated with insulin/glucose, although this interaction appears essential for mediating the fasting/refeeding response. Possibly, covalent modification of these co-activators of SREBP may alter their functionality within the transcriptional initiation complex, but experimental evidence is lacking at present.

5.5. Transcriptional regulation of lipogenic gene expression in response to dietary polyunsaturated fatty acids
Dietary fish oils containing n-3 fatty acids, such as eicosapentaenoic acid and docosahexaenoic acid, decrease blood triglyceride concentrations in hypertriglycemic patients and are considered to have protective effects against cardiovascular diseases. Moreover, polyunsaturated fatty acids, when fed to rodents inhibit hepatic de novo lipogenesis, triglyceride secretion, and increase activities of enzymes involved in fatty acid oxidation. These effects on lipid metabolism are due to activation or suppression of transcription of key metabolic enzymes. Thus, polyunsaturated fatty acids inhibit the transcription of the FAS and ACC genes and activate the transcription of genes encoding acyl-CoA oxidase and cytochrome P450 4A2, enzymes that are involved in peroxisomal and mitochondrial fatty acid oxidation, respectively (S.D. Clarke, 1994). The transcriptional activation of genes encoding fatty acid oxidation enzymes is mediated by a nuclear peroxisome proliferator-activated receptor (Chapter 5). However, these receptors are not involved in
transcriptional regulation of the genes encoding lipogenic enzymes. The mechanism of
down-regulation of lipogenic enzyme expression appears to be mediated, at least in part,
through SREBP-1, since feeding of dietary fish oil to mice results in a decrease of the
hepatic mRNA for SREBP-1, but not in that for SREBP-2 [34,35].

5.6. Transcriptional regulation during development

Fatty acids are important components of membrane phospholipids and are essential for
phosphatidylcholine biosynthesis in proliferating cells. Nevertheless, transcription of
ACC and FAS genes is not regulated by cell-cycling, but is regulated by certain growth
factors such as colony stimulating factor 1 (S. Jackowski, 2000). The transcription of
ACC and FAS genes is also regulated during the differentiation and development of
several tissues. For example, in mammary glands, expression of lipogenic enzymes is
increased during late pregnancy and early lactation, in preparation for the production
of milk fat. In brain, FAS expression is elevated during the myelination phase of
development, but is low in mature animals (P.R. Vagelos, 1972, 1973). In the lung, FAS
activity increases during late gestation, in preparation for the synthesis of the palmitoyl
component of lung surfactant that is required for air breathing (L.W. Gonzales, 1999).

Our knowledge of the tissue-specific mechanisms regulating most of these processes
is, as yet, scant. Perhaps the most extensively studied differentiation model is that of
the preadipocyte, in which the maturation process is characterized by the coordinate
induction of the lipogenic enzymes and the accumulation of intracellular triglycerides
(Chapter 10). Paradoxically, cAMP, which is implicated in the down-regulation of
lipogenic enzyme gene expression during fasting, is required as a positive effector in
the transcriptional activation of lipogenic enzymes in both the developing lung and
the differentiating preadipocyte. In culture, preadipocytes must first be sensitized by
exposure to cAMP before they will respond to insulin and turn on the expression of
lipogenic enzymes (K.-H. Kim, 1991). In the case of ACC, this effect appears to be
mediated primarily through the PII promoter. However, it still remains to be established
whether this synergistic interaction between insulin and cAMP occurs in vivo or is
specific to differentiation of 30A5 cells in culture.

6. Future directions

A major obstacle in understanding how the enzymes of the multifunctional polypeptide
system cooperate to effect the biosynthesis of fatty acids is the absence of a detailed
three-dimensional structure for either ACC or FAS. Crystal structures of many of the
individual type II FAS enzymes have been obtained in recent years and have provided
valuable information about the various catalytic mechanisms that likely operate in their
type I counterparts. However, the most interesting aspects of the type I FASs clearly
concern the overall architecture of the complex. The intriguing question as to how
an ACP domain of the animal FAS is able to make functional contacts with eight
catalytic domains, six on the same subunit and two on the companion subunit, can
only be answered by generating high-quality images of the entire 540 kDa dimer,
either by X-ray crystallography or high-resolution electron microscopy. Similarly, a
detailed structural analysis of the animal ACC is required in order to understand how
reversible phosphorylation and the conformational changes induced by citrate modulate
the activity of this enzyme.

Substantial progress has been made during the last 5 years in identifying the various
trans-acting factors that regulate transcription of the ACC and FAS genes in response
to nutritional stimuli and we are likely to see many of the remaining details of these
processes elaborated in the near future.

Arguably, the most exciting recent discovery in the field of fatty acid biosynthesis
is that both ACC and fatty acid synthase may constitute legitimate targets for the
development of therapeutic agents for the treatment of obesity and cancer. The potential
usefulness of inhibitors of the two enzyme complexes rests largely on their ability
to modulate intracellular malonyl-CoA concentrations. Cerulenin (2,3-epoxy, 4-oxo,
7,10-dienoyl, dodecylamide), the fatty acid synthase inhibitor that, when administered
to mice, causes loss of appetite and reduction in adipose mass, is too unstable for
therapeutic use, but a chemically stable inhibitor has been synthesized (C75; 3-carboxy-
4-octyl-2-methylenebutyrolactone) that is equally effective in suppressing appetite and
inducing weight loss [36]. Both cerulenin and C75 are also potent inhibitors of
DNA replication in many types of tumors that characteristically overexpress fatty
acid synthase. The toxicity of these compounds appears to be due to their ability
to inhibit fatty acid synthesis and cause an elevation in intracellular malonyl-CoA
concentration that ultimately results in apoptosis of the tumor cell. Tumor cells
exposed to these inhibitors are unable to synthesize or oxidize fatty acids, since
the elevated malonyl-CoA concentration inhibits CPT1 and blocks entry of fatty acids
into the mitochondria. The effects of cerulenin can be mimicked by the combined
administration of 5-(tetradecyloxy)-2-furoic acid and etomoxir, inhibitors of ACC
and carnitine palmitoyltransferase I, respectively, supporting the theory that it is the
inhibition of both fatty acid synthesis and fatty acid oxidation that triggers the apoptotic
process (E.P. Kuhajda, 2001). The exact mechanism by which apoptosis is initiated by
the disruption in fatty acid metabolism is not yet completely understood. One possibility
is that fatty acids are diverted away from mitochondrial oxidation into ceramide
synthesis, a situation that is known to induce apoptosis. Alternatively, the process
could involve interaction of carnitine palmitoyltransferase I with Bcl-2, a mitochondrial
membrane protein that regulates programmed cell death.

In another ground-breaking study, ACCθ isoform knockout mice were generated and
found to have a higher-than-normal rate of fatty acid oxidation and lower amounts of
adipose fat (S.J. Wakil, 2001). The absence of malonyl-CoA in muscle tissue appears
to free the carnitine palmitoyltransferase from its normal down-regulation control
mechanism and lead to efficient oxidation of fatty acids that have been mobilized from
adipose tissue. Most surprisingly, the knockout mice accumulate 50% less adipose fat,
despite consuming 20% more food than wild-type mice!

Although further study is needed to carefully evaluate the long-term consequences
of administration of inhibitors of ACC and fatty acid synthase, undoubtedly these
findings will stimulate searches for new inhibitors that could offer novel therapies for
the treatment of both obesity, its associated disorders, and of cancer.
Abbreviations

ACC  acetyl-CoA carboxylase
FAS  fatty acid synthase
ACP  acyl carrier protein
cAMPK  cAMP-dependent protein kinase
AMPK  AMP-dependent protein kinase
T3   3,5,3'-triiodothyronine
SREBP  sterol-response element binding protein
USF  upstream stimulatory factor
NF-Y  nuclear factor Y
Spl  stimulatory protein 1
RXR  retinoid X receptor
LXR  liver X receptor

References

acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. J. Biol. Chem. 273, 25537–25540.