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CONTROL MECHANISMS FOR FATTY ACID SYNTHESIS IN *MYCOBACTERIUM SMEGMATIS*

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CONTENTS

John Simon Guggenheim Fellow 1975–1976.

I. Introduction: Comparative Aspects of Fatty Acid Synthesis

Enzyme systems for synthesizing long-chain fatty acids occur in the vast majority of cells. They provide the paraffinic moieties of phospholipids and related amphipathic molecules that in turn are requisite components of the universal plasma membrane and the membrane envelopes of organelles. There are few exceptions to this generalization. Some mycoplasma strains, bacterial mutants that occur naturally, have lost the biosynthetic machinery for fatty acid synthesis. However, such organisms will not grow in the absence of added fatty acids (1). In some of the highly specialized halobacteria the phospholipids contain the branched-chain phytyl group instead of straight acyl chains. In these bacteria the enzyme system for long-chain fatty acid synthesis may still be present, but it is possible that they are normally repressed (2).

Wherever fatty acid synthesis occurs, the operating chemical mechanism is always the same (3). Acetyl and malonyl groups activated in the form of thioesters of acyl carrier protein (ACP) condense to β-ketoacyl thioester in a concerted process driven by decarboxylation of the free malonyl carboxyl group (4). Three subsequent reactions serve to convert the β-carbonyl oxygen to methylene by way of β-hydroxyacyl- and α,β-enoyl intermediates. This sequence (Table I, reactions 1–7) affords the elongated saturated product with retention of the thioester function. Repeti-

TABLE I

Reaction Sequence for Fatty Acid Synthesis in Multienzyme Complexes

Reaction 1. Acyl transacylation consists of three experimentally separable events: (*a*) acyl transfer to a serine residue, (*b*) acyl transfer to 4-phosphopantetheine-SH of the "ACP" region, and (*c*) acyl transfer to a SH group of condensing enzyme. Reaction *c* is usually written as a component of reaction 3.

Reaction 6. In most synthetates NADPH is the specific electron donor for both β-ketoacyl- and α,β-enoyl reduction. In some instances, NADH is the more effective or specific reductant for α,β-enoyl reduction.

Reaction 7. In all animal tissue synthetases the synthetic process is terminated by "deacylases" which transfer completed acyl chains to water, while in microbial synthetases acyl chains are transferred to CoASH. In some microbial synthetases, the catalytic regions for malonyl transacylation (reaction 2) and long-chain transacylation (reaction 7*b*) are the same, while in animal synthetases malonyl transacylation and deacylation are distinct.

1. Acyl transacylation
   a. $CH_3CO\text{-}SCoA + enz(Ser\text{-}OH) \rightleftarrows CH_3CO\text{-}(\text{-}OSer)\text{-}enz + CoASH$
   b. $CH_3CO\text{-}(O\text{-}Ser)\text{-}enz + ACP\text{-}enz \rightleftarrows CH_3CO\text{-}S\text{-}ACP\text{-}enz\text{-}(Ser\text{-}OH)$
   c. $CH_3CO\text{-}S\text{-}ACP\text{-}enz + HS\text{-}enz_{cond} \rightleftarrows CH_3CO\text{-}S\text{-}enz_{cond} + ACP\text{-}enz$
2. Malanonyl transacylation
   $HOOCCH_2COS\text{-}CoA + ACP\text{-}enz \rightleftarrows HOOCCH_2COS\text{-}ACP\text{-}enz + CoASH$
3. Condensation (β-ketoacyl-ACP-enz synthetase)
   $HOOCCH_2COS\text{-}ACP\text{-}enz + CH_3Co\text{-}S\text{-}enz_{cond} \rightleftarrows CO_2 + HS\text{-}enz_{cond} + CH_3COCH_2COS\text{-}ACP\text{-}enz$
4. β-Ketoacyl reduction
   $CH_3COCH_2COS\text{-}ACP\text{-}enz + NADPH + H^+ \rightleftarrows CH_3CHOHCH_2CO\text{-}S\text{-}ACP\text{-}enz + NADP$
5. Dehydration (β-hydroxyacyl dehydratase)
   $CH_3CHOHCH_2COS\text{-}ACP\text{-}enz \rightleftarrows H_2O + CH_3CH{=}CH\cdot CO\text{-}SACP\text{-}enz$
6. Enoyl reduction (2,3-*trans*-enoylacyl reductase)
   $CH_3CH{=}CHCO\text{-}S\text{-}ACP\text{-}enz + H^+ + NADPH \rightleftarrows CH_3CH_2CH_2CO\text{-}S\text{-}ACP\text{-}enz + NADP(NAD)$ (NADH)
7. Long-chain transacylase or deacylase
   a. $CH_3CH_2CH_2CO\text{-}S\text{-}ACP\text{-}enz + enz(Ser\text{-}OH) \rightleftarrows CH_3CH_2CH_2CO\text{-}(OSer\text{-}enz) + ACP\text{-}enz$
   b. $CH_3CH_2CH_2CO\text{-}(OSer\text{-}enz) + CoASH \rightleftarrows CH_3(CH_2)_nCO\text{-}SCoA, + enz(Ser\text{-}OH)$
      $H_2O \qquad CH_3(CH_2)_nCOO^-$

tion of the same set of reactions six or seven more times yields palmitate and stearate, respectively. At the end of each round (condensation, first reduction, dehydration, and second reduction) the resulting saturated acyl chain returns from protein-linked 4-phosphopantetheine to condensing enzyme (β-ketoacyl-ACP synthetase) for addition of another malonyl unit. In this manner the activated carboxyl function at the growing end of the acyl chain is preserved until the chain reaches the appropriate length. In all nonaggregated or type II fatty acid synthetase systems (see below), fatty acid synthesis ends at this stage, the long acyl chains remaining attached to ACP. The ACP derivatives can then enter directly into lipid formation by transacylation (5). On the other hand, synthetases that are multienzyme complexes (type I) discharge completed acyl chains by transacylation either to water (6, 7) or to coenzyme A (8). Such transacylase activities are integral parts of multienzyme complexes. Apart from these differences in the termination events, the sources of reductant may also vary. Some synthetases use NADPH effectively for both the first and the second reduction step; in others only the β-ketoacyl thioester reduction is NADPH specific, NADH serving more effectively in the enoyl thioester reduction (9). Only the yeast synthetase is known to employ reduced FMN as a direct electron donor for enoyl reduction (8).

While the covalent chemistry of fatty acid synthesis appears essentially invariant for all forms of life—the differences noted are mechanistically trivial—this uniformity does not extend to the macromolecular structure and organization of the participating enzyme activities. There are two fundamentally different types of enzyme system that carry out fatty acid synthesis. In the more conventional systems, the various steps are catalyzed by separable enzymes that act individually, but constitute a single functional system. Such nonaggregated or type II synthetases occur in many bacteria and plants, but apparently not in animal tissues. Since in type II systems ACP is also a separate entity (10–12) synthetases in this category consist of a total of seven individual proteins. By contrast, the catalysts for fatty acid synthesis in all animal tissues, in many eukaryotic microorganisms, and in some bacteria are polyfunctional entities known as multienzyme complexes (type I). This terminology was introduced after the successful isolation of a

fatty acid synthetase from yeast that could be purified to homogeneity without loss of any of the seven partial activities needed for the *de novo* synthesis (13). The cohesive properties of this complex justifiably led to the assumption that in the yeast system the seven enzyme components were held together by sufficiently strong protein–protein interactions to allow the isolation of a single physical entity. The precedents for the existence of multienzyme aggregates were the pyruvate dehydrogenase complexes of *E. coli* and muscle (14,15). While more recent genetic evidence requires a redefinition of the term multienzyme complex as applied to fatty acid synthetases, the distinction between aggregated, or type I, and nonaggregated, or type II, synthetases remains operationally valid.

The natural distribution of the two synthetase prototypes is shown in Table II. No phylogenetic pattern or trend is discernible except perhaps for the beginning and the end of the evolutionary tree. The structurally primitive eubacteria and a representative blue-green alga (*Phormidium lunidum*) (16) contain type II synthetases, while in animal species, invertebrates (17) and vertebrates (nonmammalian and mammalian), the synthetases are without exception multienzyme complexes. It may be assumed that multienzyme complexes are more efficient and therefore more advanced. However, since type II synthetases occur in plants and multienzyme complexes occur in certain bacteria and since bacterial phylogeny is uncertain, the notion that the function of these enzymes improved in the course of evolution remains to be verified.

In nonaggregated (more primitive?) type II systems, which lack a higher level of organization, each intermediate is released into solution so that the further progress of the reaction sequence depends on collision between substrate and enzyme at each step. Conceivably, the component enzymes of type II systems are not entirely disorganized but are loosely associated in the membrane space. However, no such evidence exists except for the suggestive observation that the bulk of *E. coli* ACP as seen by radioautography is localized in the bacterial membrane (18).

By contrast, all intermediates generated by a multienzyme complex remain enzyme bound throughout the synthetic process. Because it remains covalently linked, the *enzyme-bound* product of

TABLE II

Occurrence of Mutienzyme Complex (Type I) and Nonaggregated (Type II) Fatty Acid Synthetases

Only relatively well-characterized fatty acid synthetases known to be type I or type II systems have been included. The synthetases from vertebrate sources are thoroughly reviewed by Volpe and Vagelos (3). The recently isolated synthetase from goose uropygial gland (24a) forms branched acids from acetyl-CoA and methylmalonyl-CoA. The type II *B. subtilis* system (31) produces long-chain iso and ante-iso acids.

		Type I	Type II
A.	Animal tissues (3)		A. Procaryotic
	1. Mammalian	Rat liver (19)	*Escherichia coli* (10, 11, 29)
		Adipose tissue	*Clostridium butyricum* (11)
		Mouse brain (24)	*Pseudomonas aeruginosa* (30)
		Mammary gland (22, 23)	*Bacillus subtilis* (31)
	2. Avian	Chicken (21) and pigeon (20)	*Mycobacterium smegmatis*[a] (32)
		Liver	*Phormidium lunidum* (16) (blue-green alga)
		Goose uropygial bland (24a)	
	3. Invertebrate	*Ceratitis capitata* (17) (insect)	B. Eucaryotic
			Euglena gracilis (33, 34) (photoauxotrophic)
B.	Microbial		*Chlamydomonas reinhardii* (35)
	1. Eucaryotic	*Saccharomyces cerevisiae* (13)	Avocado mesocarp (36)
		Neurospora crassa (25)	Lettuce chloroplasts (37)
		Euglena gracilis (26)	Spinach chloroplasts (38)
	2. Procaryotic	*Corynebacterium diphtheriae* (27)	
		Mycobacterium smegmatis (28)	

[a] Palmitoyl-CoA elongating system.

the first partial reaction is directly transferred to the second catalytic site, which necessarily must be in close spatial proximity to the donor site and in turn adjacent to the center that catalyzes the following step. Such successive transfers of covalently bound intermediates between neighboring sites are possible only if the topological ordering of catalytic centers corresponds to the sequence of chemical events. Kinetically, the important property of the multienzyme complex is the absence of free intermediates. The rate of the overall reaction is determined only by the rate of transfer between sites, not by the K_m of the individual intermediates.

Evolutionary considerations prompted the decision in this laboratory to investigate fatty acid biosynthesis in a species of mycobacteria, the subject under review. It was thought that multienzyme complexes might have evolved in stages from nonaggregated systems as a result of modifications in protein structure, amino acid substitutions in individual enzymes producing mutual affinities until protein–protein interactions become sufficiently strong to stabilize the fatty acid synthetase system in the form of a single multifunctional complex. The hypothesis predicted the possible existence of transitional fatty acid synthetases that lacked the tight organizational features of the known type I systems but showed some tendency to form loose aggregates. It was further felt that the isolation of aggregates of limited stability might yield valuable information on developmental aspects of FAS multienzyme complexes and, more generally, on protein–protein interactions between functionally related enzymes. For this purpose *Mycobacterium smegmatis* was selected as a source (28) because mycobacteria are relatively advanced procaryotes. The initial results obtained with the mycobacterial synthetase seemed to support the working hypothesis. First of all, the ACP-independence of the system established it as a type I synthetase, the first demonstration that FAS multienzyme complexes are not restricted to eucaryotic cells. Also, the enzyme system was much more labile under normal assay conditions than known type I synthetases, but could be stabilized in an environment of high ionic strength. Later, it became increasingly doubtful, however, that the *M. smegmatis* synthetase represented a loose

complex or aggregate of individual enzymes. The instability in solutions of low ionic strength was not due to weak interactions between monofunctional entities, but was caused by the facile dissociation of the complex into subunits of molecular weight 200–250,000 (39). Presumably, these subunits are polyfunctional polypeptide chains. It now appears that all type I fatty acid synthetases are susceptible to dissociation into subunits and that they are always of similar size. However, the ease of the ionic-strength-dependent dissociation differs from case to case. At low ionic strength the *M. smegmatis* synthetase is exceptionally labile, the animal tissue synthetases are moderately stable (40), and the yeast synthetase retains its native structure even in distilled water. To effect the dissociation of the yeast complex requires 1–2 *M* salt solutions (41). The stabilizing noncovalent interactions in the various multienzyme complexes must therefore differ not only in strength, but also in kind.

Up to the time of this writing the original hypothesis postulating the existence of labile, transitional fatty acid synthetase complexes has remained without experimental support. In fact, the protein structure and organization of the procaryotic *M. smegmatis* synthetase does not appear to differ in principle from others in this category. However, various properties of this enzyme system proved to be sufficiently unique to warrant further investigation. Most prominent among these properties are (*1*) a bimodal product pattern that includes fatty acyl chains of unusual length, (*2*) the extraordinarily high K_m for acetyl-CoA, (*3*) stimulation by certain mycobacterial polysaccharides, and (*4*) catalysis of palmitoyl-CoA elongation, as well as of *de novo* synthesis.

In addition to the multienzyme FAS complex, mycobacterial extracts contain a second, ACP-dependent, type II synthetase that extends but does not start long carbon chains. Systems of this type have not been found elsewhere.

A. IDENTITY OF ENZYME SOURCE

For the investigations described in this chapter the bacterial strain listed as ATCC 356, *Mycobacterium phlei,* was used throughout in this laboratory. From information now available it is clear that ATCC 356 is in fact a strain of *M. smegmatis* and not of *M.*

phlei. This culture had earlier been mislabled accidentally (42). The revised designation of ATCC 356 as *M. smegmatis* is based, *inter alia,* on the observation that this strain produces mycobactin S rather than mycobactin P, and on several other diagnostic tests (42).

II. *M. smegmatis* Acetyl-CoA Carboxylase

Acetyl-CoA carboxylases, though separate enzymes, function in concert with fatty acid synthetase systems. They catalyze the formation of malonyl-CoA, the first specific event in lipogenesis. In most instances this is also the committed step because malonyl-CoA is not used elsewhere in metabolism.

In analogy with the existence of two FAS prototypes, acetyl-CoA carboxylases also occur as aggregated and nonaggregated enzyme systems (3). From sources such as animal tissues and yeast the carboxylases can be isolated as single physical entities of high molecular weight, whereas in *E. coli* three separable proteins, biotin-carrier protein (BCP), biotin carboxylase, and biotin transcarboxylase, catalyze the overall conversion of acetyl-CoA to malonyl-CoA (43). One of the plant carboxylases (from wheat germ) seems to be intermediate in molecular properties between the animal–yeast and bacterial systems (44), whereas the spinach chloroplast enzyme system can be resolved into three active components analogous to those obtained from *E. coli* (44). Since fatty acid synthesis in *M. smegmatis* conforms with the "animal–yeast" pattern rather than the "bacterial" pattern* a characterization and classification of the mycobacterial acetyl-CoA carboxylase seemed of interest. This enzyme appears to be of the aggregated type (45). However, the possibility that *M. smegmatis* contains two distinct carboxylating systems has not been ruled out.

Conventional fractionation yielded a 35- to 60-fold enriched fraction that catalyzed the overall conversion of acetyl-CoA to malonyl-CoA. Under conditions that allow separation of the *E. coli* carboxylase system into three components, the *M. smegmatis* enzyme remained an intact entity.

* This statement applies only to *de novo* fatty acid synthesis. The second enzyme, the nonaggregated, palmitoyl-CoA elongating system of *M. smegmatis* requires external ACP and on this basis is to be classified as "bacterial."

The most notable property of the *M. smegmatis* carboxylase is that it catalyzes the carboxylation of both propionyl-CoA and acetyl-CoA. Both reactions are affected by inorganic sulfate. The respective kinetic parameters are, for acetyl-CoA, V_{max} = 0.79 m*M* in the absence of SO_4^{2-} and 1.35 m*M* in the presence of SO_4^{2-}; and K_m = 0.30 in the presence and 0.22 in the absence of SO_4^{2-}; for propionyl CoA, SO_4^{2-} changes V_{max} from 1.28 to 1.48, but leaves the K_m unchanged (at 0.1 m*M*). Thus the enzyme carboxylates propionyl-CoA somewhat more effectively than acetyl-CoA. All the data indicate that these two activities reside in a single catalytic entity. However, since sulfate, regardless of the nature of the countercation, raises the activity for acetyl-CoA carboxylation substantially and of propionyl-CoA carboxylation only slightly, it is clear that the relative specificity of the enzyme for the two substrates can be modulated. The sulfate effect does not relate to ionic strength; it is not duplicated by Cl^- or HPO_4^{2-}.

Differential effects on the two activities are also shown by palmitoyl-CoA. Even at the highest concentrations tested (250 μM), palmitoyl-CoA inhibited propionyl-CoA carboxylation by less than 50%, while acetyl-CoA carboxylase activity was reduced to 50% at 30–40 μM palmitoyl-CoA. Interestingly, 0.75 *M* $(NH_4)_2SO_4$ completely relieved palmitoyl-CoA inhibition of both substrates. Since acetyl-CoA carboxylation is far more sensitive to palmitoyl-CoA than the carboxylation of propionyl-CoA, this response may be related to end-product inhibition of straight-chain fatty acid synthesis. It should be noted, however, that the effective palmitoyl-CoA concentrations are very much higher than those needed to inhibit yeast or liver acetyl-CoA carboxylase (43). For the latter enzymes the regulatory role of palmitoyl-CoA is well established.

If the dual substrate specificity should persist on further purification of the *M. smegmatis* carboxylase, then the reactions that the enzyme catalyzes would not be the "committed steps" as usually defined for pathways that utilize malonyl-CoA and methylmalonyl-CoA. Methylmalonyl-CoA is not a substrate for chain elongation by the *M. smegmatis* fatty acid synthetase (45) in accord with the absence of branched-chain acids (mycocerosic or phthienoic acids) in this particular mycobacterial strain (46). Methylmalonyl-CoA mutase has been detected in *M. smegmatis*