Enoyl-Acyl Carrier Protein Reductase (fabI) Plays a Determinant Role in Completing Cycles of Fatty Acid Elongation in Escherichia coli*

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The role of enoyl-acyl carrier protein (ACP) reductase (E.C. 1.3.1.9), the product of the fabI gene, was investigated in the type II, dissociated, fatty acid synthase system of Escherichia coli. All of the proteins required to catalyze one cycle of fatty acid synthesis from acetyl-CoA plus malonyl-CoA to butyryl-ACP in vitro were purified. These proteins were malonyl-CoA:ACP transacylase (fabD), β-ketoacyl-ACP synthase III (fabH), β-ketoacyl-ACP reductase (fabG), β-hydroxydecanoyl-ACP dehydrase (fabA), and enoyl-ACP reductase (fabI). Unlike the other enzymes in the cycle, FabA did not efficiently convert its substrate to catalyze one cycle of fatty acid synthesis from acetyl-CoA to initiate fatty acid synthesis or the growing acyl chain to continue cycles of elongation. The first cycle of elongation is initiated by the condensation of acetyl-CoA with malonyl-ACP catalyzed by β-ketoacyl-ACP synthase III (FabH), the product of the fabH gene. Subsequent cycles of elongation are catalyzed by condensing enzyme I (FabB) or II (FabF). Recently, the existence of a fourth condensing enzyme was proposed (2). However, the map position and sequence of this putative new enzyme are the same as that of the fabF gene (3); therefore there is little concrete evidence for the presence of a fourth condensing enzyme. The β-ketoacyl-ACP is reduced by a NADPH-dependent β-ketoacyl-ACP reductase (FabG). Only a single enzyme is known to be responsible for this step, although the existence of other isozymes cannot be ruled out. There are two β-hydroxyacyl-ACP reductases (FabA and FabB2) capable of forming trans-2-enoyl-ACP. The product of the fabB gene is specifically involved in the introduction of a cis-double bond into the growing acyl chain at the β-hydroxydecanoyl-ACP step. The spectrum of reactions catalyzed by the FabB dehydrase is unknown, but the fact that mutations in the fabB2 gene were isolated based on their ability to suppress the temperature-sensitive growth phenotype in lpxA2(Ts) (β-hydroxymyristoyl-ACP:UDP-N-acetylglucosamine acyltransferase) mutants indicates that it plays an important role in long-chain saturated fatty acid formation (4). The last reaction in each elongation cycle is catalyzed by enoyl-ACP reductase (FabI). E. coli is thought to possess two enoyl-ACP reductases, one NADH-dependent and the other NADPH-dependent (5), suggesting that more than one isozyme catalyzes this step of fatty acid biosynthesis.

The gene encoding the NADH-dependent enoyl-ACP reductase (fabI) was discovered as an outgrowth of research on the mechanism of action of diazabornines, a class of heterocyclic antibiotics. Biosynthesis of fatty acids and phospholipids is inhibited when E. coli or Salmonella typhimurium are treated with diazabornines, and resistance to these antibiotics is associated with an allelic form of the envM gene product (6). The envM gene was first identified as a temperature-sensitive mutation with an osmotically-repairable membrane defect (7). Because the envM protein, purified from an overproducing strain, possesses NADH-dependent enoyl-ACP reductase activity and binds radiolabeled diazabornine, the gene was renamed fabI (8). The fabI (envM) gene has been sequenced, and the diazabornine resistance is associated with a point mutation in this locus (9). Similarly, the product of the fabI analog in Mycobacterium tuberculosis, the inhA gene product, is the target for the antibiotics isoniazid and ethionamide, and a single point mutation

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1 The abbreviations used are: ACP, acyl carrier protein; acyl-ACP, acyl-acyl carrier protein.
Enoyl-ACP Reductase of E. coli

Fig. 1. Cycles of fatty acid elongation are pulled by enoyl-ACP reductase (FabI). There are four reactions in each cycle of fatty acid elongation. The first step is catalyzed by β-ketoacyl-ACP synthase, which condenses malonyl-ACP with either acetyl-CoA (synthase III, FabH) or acyl-ACP (synthases I and II, FabB, and FabF, respectively). The malonyl-ACP used in the condensing enzyme step is formed by malonyl-CoA:ACP transacylase (FabD). Reduction of the ketoester is catalyzed by the NADPH-dependent β-ketoacyl-ACP reductase (FabG). The third step is catalyzed by β-hydroxyacyl-ACP dehydrase (either FabA or FabZ). The final step in each cycle is catalyzed by the NADH-dependent enoyl-ACP reductase (FabI), which converts trans-2-enoyl-ACP to acyl-ACP. The inner arrows indicate the overall direction of the cycle in fatty acid biosynthesis. The outer thick arrows diagrammatically indicate the equilibrium positions for the enzymatic reactions determined in this study.

in the inhA gene confers resistance to both antibiotics in M. tuberculosis (10). InhA has recently been purified, crystallized, and shown to be an NADH-dependent enoyl-ACP reductase (11). The focus of this study is to determine the number of enoyl-ACP reductase enzymes in E. coli and to define the role of enoyl-ACP reductase in regulating cycles of fatty acid elongation.

EXPERIMENTAL PROCEDURES

Materials—β-[3-3H]Alanine (specific activity, 56 Ci/mmol) and EnHance were purchased from DuPont NEN. [2-14C]Malonyl-CoA (specific activity, 57 mCi/mmol) was purchased from Moravek Biochemicals Inc. Cerulenin, tetradecanoinic acid, and ACP were purchased from Sigma. The cis-7-tetradecenoic acid was the generous gift of Dr. John E. Cronan, Jr. Fatty acids were enzymatically coupled to ACP (12, 13) and then concentrated, and the salt was removed by centrifugal filtration in a Centricon-3 concentrator (Amicon). Protein amounts were assayed by the Bradford method (14), and purity was estimated by electrophoresis in a 2.5 M urea, 15% acrylamide gel (15). Acetyl-CoA and malonyl-CoA were from Pharmacia Biotech Inc. All other chemicals were of reagent grade or better.

In Vivo Labeling of the ACP Pool—Strain R J H 13 (panD fabI) (16) was grown at 30°C to a density of 5 × 10⁸ cells/ml in M 9 medium (17) supplemented with 0.4% glucose and 0.5 μmol β-[3-3H]Alanine (specific activity, 56 Ci/mmol), at which time an aliquot of the culture was removed and placed into a flask prewarmed to 42°C. Samples (0.5 ml) were taken after 10 min of incubation, and cerulenin (1 mg/ml) was added to the remaining portions and incubated for a further 10 min. Samples were immediately pelleted by centrifugation in a microfuge (15,000 rpm, 4°C, 3 min) and then lysed by a procedure using successive treatments with sucrose, lyszyme, EDTA, and Triton X-100 (18).

Products were analyzed by conformationally sensitive electrophoresis in 13% polyacrylamide gels containing 0.5 μmol urea (15, 19), and the bands were visualized by fluorography. Strain Sj 16 (panD) (20) was grown at 37°C to a density of 5 × 10⁸ cells/ml, and the culture was split; one half was treated with 1 mg/ml cerulenin for 10 min, and the other half was harvested as a control.

Preparation of Cell-free Extracts—The crude fatty acid synthase preparation and the fabI (Ts) extract were obtained as described (21). Briefly, a 500-ml culture of either E. coli strain U81005 or RJ H 13 (fabI Ts) was grown to late log phase in LB medium, and the cells were harvested by centrifugation. Cells were resuspended in 5 ml of lysis buffer (0.1 M sodium phosphate, pH 7.0, 5 mM β-mercaptoethanol, 1 mM EDTA) and disrupted by passage through a French pressure cell at 18,000 psi. The lysate was centrifuged in a J A-20 rotor at 20,000 rpm at 4°C for 1 h to remove cell debris, and protein in the 45–80% ammonium sulfate cut of the supernatant was collected. This pellet was resuspended in 2 ml of lysis buffer and dialyzed overnight at 4°C against 1 liter of lysis buffer. Protein was determined by the Bradford method (14).

RESULTS AND DISCUSSION

Protein Purification and Substrate Synthesis—We purified the five enzymes required to carry out a single cycle of fatty acid synthesis to investigate the role of each enzyme in completing the cycle. Each gene encoding a component of the cycle was cloned into the pET-15b vector, and the proteins were purified by affinity chromatography as described under “Experimental Procedures.” In all cases, active enzymes were obtained in a highly purified state (Fig. 2). Acyl-ACPs (14:0 and 14:1Δ7) were prepared using the acyl-ACP synthetase method.

Protein purification...
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formation of [14C]malonyl-ACP. The addition of 
onyl-CoA:ACP transacylase plus [2-14C]malonyl-CoA led to the 
completing rounds of elongation (Fig. 3). The addition of mal-
entstopositively identify the intermediates formed by each of 
structed in vitro — structural sensitive gel electrophoresis (not shown).

Reconstruction of Fatty Acid Biosynthesis with Purified Components — A single round of fatty acid synthesis was recon-
structed in vitro by the sequential addition of purified compo-
ents to positively identify the intermediates formed by each of 
the enzymes in the cycle and to verify the role of FabI in 
completing rounds of elongation (Fig. 3). The addition of mal-
yl-CoA:ACP transacylase plus [2-14C]malonyl-CoA led to the 
formation of [14C]malonyl-ACP. The addition of β-ketoacyl-
ACP synthase III (FabH) resulted in the conversion of malonyl-
ACP to β-ketobutyryl-ACP. The gel electrophoresis technique 
underestimated the actual amount of β-ketobutyryl-ACP formed because the high pH, temperature, and urea used to 
achieve the separation led to degradation and loss of recovery of the unstable β-ketoesters. The addition of β-ketoacyl-ACP re-
ductase (FabG) to the mixture (which also contained NADPH) 
resulted in the complete conversion of the malonyl-ACP to 
β-hydroxybutyryl-ACP. Significantly, the addition of β-
hydroxyacyl-CoA dehydrase (FabA) to the mixture resulted in 
only an 8% conversion of β-hydroxybutyryl-ACP to crotonyl-
ACP. Increasing the amount of FabA enzyme in the assay 
mixture did not lead to increased formation of crotonyl-ACP (Fig. 3). The ratio of β-hydroxybutyryl-ACP and crotonyl-ACP 
products was determined in this and several other experiments 
by densitometric analysis of the fluorograph. Crotonyl-ACP 
accounted for 8–11% of the total density in the β-hydroxybuty-
ryl-ACP plus crotonyl-ACP bands and was independent of 
the amount of FabA enzyme added to the assay or the length of 
the incubation. These data indicated that the FabA reaction 
rapidly reached an equilibrium that favored β-hydroxybutyryl-
ACP. The addition of FabI to the reaction mixture yielded 
butyryl-ACP, and the extent of butyryl-ACP formation in-
creased as the amount of FabI in the assay increased. These 
data show that the activity of FabI is responsible for pulling 
cycles of fatty acid elongation.

Correction of the Fatty Acid Synthesis Defect in Exacts of 
fabl(Ts) Mutants with Purified FabI — Exacts isolated from 
wild-type and fabl(Ts) defective strains were used to examine 
in vitro the role of the FabI protein in fatty acid elongation (Fig. 4). As previously reported (20), extracts from the wild-type 
strain incorporated [14C]malonyl-CoA into butyryl-ACP and 
long-chain acyl-ACP. The addition of cerulenin blocked the 
activity of FabB and FabF but not FabH, leading to the accumu-
lation of butyryl-ACP. Extracts from strain RJH13 
(fabl(Ts)) were unable to form either butyryl-ACP or long-chain acyl-ACP either in the presence or the absence of cerulenin.

Two intermediates accumulated in these reactions. The major 
intermediate comigrated with β-hydroxybutyryl-ACP, and the 
minor species comigrated with crotonyl-ACP. Densitometric 
analysis of the fluorograph showed that the crotonyl-ACP band 
comprised 10% of the total density of the β-hydroxybutyryl-
ACP plus crotonyl-ACP bands. The ratio of these two products 
formed in the crude cell extract was virtually identical to the 
ratio of products observed in the purified system in the absence 
of FabI (Fig. 3). The addition of purified FabI protein to the 
extract from the fabl(Ts) strain restored the ability of the 
extract to synthesize both long-chain acyl-ACP and butyryl-
ACP in the presence of cerulenin. These data show that FabI 
is essential for the first cycle of fatty acid elongation and is 
the only component missing from the extracts of strain 
RJH13 (fabl(Ts)).

The data suggested that FabI participated in subsequent 
elongation cycles, but the possibility remained that another 
acyl-ACP reductase (the NADPH-dependent enzyme pre-
dicted by Weeks and Wakil (5)) was present in the extracts and 
contributed to the elongation of saturated or unsaturated fatty 
acids to produce long-chain acyl-ACP (Fig. 4). To verify that
FabI was capable of participating in the elongation of these long-chain species and to rule out the presence of another enzyme, we examined the elongation of 14:0-ACP and 14:1-ACP (Fig. 5). Extracts from strain R.1 H13 (FabI(Ts)) were unable to convert either 14:0-ACP or 14:1-ACP to 16:0-ACP or 16:1-9-ACP, respectively. These extracts contained FabB (or FabF) that catalyzed the condensation of [14C]malonyl-CoA with the acyl-ACP, FabG, and FabZ (or FabA) that carried out the NADPH-dependent reduction of the β-ketoacyl-ACPs and the dehydration of β-hydroxyacyl-ACP to the trans-2-enoyl-ACP intermediate. Two intermediates accumulated in each experiment that corresponded in electrophoretic mobility to the β-hydroxyacyl and trans-2-enoyl intermediates in the elongation cycles. Like the electrophoretic relationship between β-hydroxybutyryl-ACP and crotonyl-ACP observed in Fig. 3, the long-chain β-hydroxyacyl intermediates migrated more slowly than the corresponding acyl-ACP, and the long-chain trans-2-enoyl intermediates migrated slightly more quickly than the corresponding acyl-ACP. These relative mobilities were the same as reported previously for long-chain species and to rule out the presence of another enzyme, we examined this pool more closely to determine if our in vitro results with extracts from fabI(Ts) mutants and purified components correlated with the in vivo results. Strain R.1 H13 (panD fabI(Ts)) was labeled with β-[^3H]alanine to uniformly label the ACP pool, and the composition of this pool was analyzed by conformationally sensitive gel electrophoresis at both the permissive and nonpermissive temperatures using the wild-type strain Sj 16 for comparison (Fig. 6). To facilitate the analysis, a portion of each culture was incubated with cerulenin, an irreversible inhibitor of the FabB and FabF condensing enzymes. At the permissive temperature (30°C), the major ACP species in strain R.1 H13 co-migrated with acetyl-ACP with significant amounts of malonyl-ACP also detected. This pattern of ACP intermediates was essentially the same as in the control strain Sj 16. The addition of cerulenin to strain R.1 H13 grown at 30°C led to the accumulation of butyryl-ACP, which is the same major change in the pool composition observed in the wild-type control strain Sj 16. In contrast, treatment of strain R.1 H13 with cerulenin after a temperature shift to 42°C did not lead to the accumulation of butyryl-ACP indicating that the first cycle of fatty acid biosynthesis was blocked in the fabI(Ts) mutants. Shifting strain R.1 H13 to the nonpermissive temperature (42°C) lead to a significant change in the ACP pool composition. Acetyl-ACP essentially disappeared, malonyl-ACP accumulated, and a new species migrating slightly more quickly than ACP appeared. We also observed the appearance of a new minor species that migrated just ahead of butyryl-ACP. The migration positions of these two ACP intermediates in vivo corresponded to the migration positions of β-hydroxybutyryl-ACP and crotonyl-ACP (Figs. 3 and 4). One important conclusion from these experiments was that the ACP pool composition of fabI(Ts) mutants at the nonpermissive temperature (Fig. 6) was essentially identical to the distribution of products observed in the in vitro system with either purified components (Fig. 3) or extracts from fabI(Ts) mutants (Fig. 4). Densitometric analysis of the fluorograph showed that the band with the migration position of crotonyl-ACP was 13% of the total density in the β-hydroxybutyryl-ACP plus crotonyl-ACP bands. Thus, our in vitro reconstitution experiments accurately reflect the properties of the fatty acid synthase in vivo.

Conclusions—Our results point to enoyl-ACP reductase (the FabI gene product) as a determinant factor in completing rounds of fatty acid elongation (Fig. 1). The reactions catalyzed by FabH, FabG, and FabI all result in the extensive conversion to the next intermediate in the cycle as depicted by the thick-
ness of the arrows in Fig. 1. The amount of butyryl-ACP formed is dependent on the amount of FabI protein added due to the dehydratation reaction reaching a rapid equilibrium that favors the accumulation of \( \beta \)-hydroxybutyryl-ACP over crotonyl-ACP by a ratio of 9:1. These data indicate that increasing the rate of initiation of a cycle of fatty acid elongation by a factor of 10 would only double the amount of enoyl-ACP product produced by FabA. Although it is not clear whether FabA, FabZ, or both contribute to the dehydratation of \( \beta \)-hydroxybutyryl-ACP in vivo, the fact that our in vitro reconstitution experiments reflect the properties of the fatty acid synthase in vivo provides compelling support for the conclusion that FabI activity plays a determinant role in completing rounds of fatty acid elongation.

Our experiments show that enoyl-ACP reductase (the fabl gene product) is the only reductase required to complete both saturated and unsaturated fatty acid synthesis in the type II, dissociated fatty acid synthase system of E. coli. Extracts derived from fabl(Ts) strains were unable to synthesize butyryl-ACP or complete the elongation of long-chain saturated or unsaturated fatty acids without supplementation with exogenous FabI. We found no evidence for the NADPH-dependent isomerase postulated by Weeks and Wakil (5). The Mycobacterium tuberculosis FabI analog (inhA) is also specific for NADH (23) and participates in ACP-dependent fatty acid synthesis in a type II system similar to that in M. smegmatis (24). We do not have a complete picture of the substrate specificity of InhA enoyl-ACP reductase, but the enzyme effectively reduces trans-2-octenoyl-ACP as well as long-chain intermediates (24) indicating a broad chain length specificity.

Hypothesis—The fact that a single enoyl-ACP reductase plays a determinant role in completing cycles of chain elongation suggests that FabI may be a key regulatory component in dissociated fatty acid synthase systems. Two aspects of fatty acid synthesis may be influenced by modulation of FabI activity. First, FabI may participate in establishing the basal saturated:unsaturated fatty acid ratio. FabA is the critical branch point because it is the only dehydratase capable of forming both cis-3-decenoyl-ACP and trans-2-decenoyl-ACP (1). FabB is essential for unsaturated fatty acid synthesis presumably because it is the only condensing enzyme capable of utilizing cis-3-decenoyl-ACP. In contrast, FabI reduces trans-2-decanoyl-ACP to decanoyl-ACP to initiate saturated fatty acid biosynthesis. Genetic manipulation of the intracellular levels of FabB and FabA establish that altering the relative ratios of these two proteins has a dramatic impact on the saturated:unsaturated fatty acid ratio (25). One testable hypothesis is that increased levels of FabI protein would increase saturation of fatty acids by increasing the utilization of trans-2-decenoyl-ACP. Second, FabI may be a target for regulation of the total rate of chain elongation. Acyl-ACP thioesters of bacterial (26, 27) or plant (28–30) origin leads to the acceleration of fatty acid biosynthesis and the copious secretion of fatty acids into the medium. Furthermore, down-regulation of fatty acid synthesis following the cessation of phospholipid production at the glycerol-phosphate acyltransferase step correlates with the elevation of intracellular acyl-ACP (16, 31). The intracellular targets for acyl-ACP regulation are unknown. One tenable hypothesis is that acyl-ACPs attenuate FabI activity by product inhibition, and this mechanism may contribute to the regulation of pathway activity by acyl-ACP.

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