Roles of the FabA and FabZ β-Hydroxyacyl-Acyl Carrier Protein Dehydratases in Escherichia coli Fatty Acid Biosynthesis*  

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Richard J. Heath‡ and Charles O. Rock‡‡  

From the ‡Department of Biochemistry, St. Jude Children’s Research Hospital, Memphis, Tennessee 38101 and the ¶Department of Biochemistry, University of Tennessee, Memphis, Tennessee 38163

There are two genes, fabA and fabZ, encoding β-hydroxyacyl-acyl carrier protein (ACP) dehydratases that function in the dissociated, type II fatty acid synthesis system of Escherichia coli. We have investigated their roles in fatty acid synthesis by purifying the two proteins and reconstituting cycles of fatty acid synthesis in vitro using five other purified proteins. FabA and FabZ exhibited broad, overlapping chain length specificities. The FabZ dehydratase efficiently catalyzed the dehydration of short chain β-hydroxyacyl-ACPs and long chain saturated and unsaturated β-hydroxyacyl-ACPs. FabA was most active on intermediate chain length β-hydroxyacyl-ACPs and also possessed significant activity toward both short and long chain saturated β-hydroxyacyl-ACPs. Significantly, FabA was virtually inactive in the dehydration of long chain unsaturated β-hydroxyacyl-ACP. The introduction of the double bond at the 10-carbon stage of fatty acid synthesis by FabA was only detected in the presence of β-ketoacyl-ACP synthase I (FabB). A yeast two-hybrid analysis failed to detect an interaction between FabA and FabB, therefore the channeling of intermediates toward unsaturated fatty acid synthesis by FabB was attributed to the affinity of the condensing enzyme for cis-decenoyl-ACP. The broad substrate specificity of FabZ coupled with the inactivity of FabA toward a long chain unsaturated β-hydroxyacyl-ACP provides a biochemical explanation for the phenotypes of cells with genetically altered levels of the two dehydratases.

Escherichia coli is the paradigm for the dissociated (or type II) fatty acid synthase systems. The type II systems are found in most bacteria and plants, and the individual steps are catalyzed by a series of discrete proteins that are encoded by unique genes (for reviews, see Refs. 1 and 2). There are four chemical reactions required to complete each round of fatty acid elongation (Fig. 1). In some cases, multiple enzymes are available to catalyze a given step suggesting that the two proteins have different substrate specificities and/or physiological functions. Each of the three condensing enzymes, FabB, FabF, and FabH, has a distinct role in the pathway (1, 2). FabB is required to catalyze a unique step in unsaturated fatty acid biosynthesis, and fabB mutants are unable to produce unsaturated fatty acids. FabF is responsible for temperature-dependent alterations in fatty acid composition, and fabF mutants are unable to produce cis-vaccenate. FabH catalyzes the initial condensation reaction in the pathway. There are also two isozymes that catalyze the dehydration of β-hydroxyacyl-ACP to trans-2-acyl-ACP, the third step in each cycle of fatty acid elongation (Fig. 1). The FabA reaction mechanism has been characterized extensively and is best known for its unique ability to isomerize trans-2-decenoyl-ACP to cis-3-decenoyl-ACP, thus initiating unsaturated fatty acid biosynthesis (3–12). The sequence of the fabA gene is known, and the active site histidine (His-70) has been identified (13). FabA is thought to be highly selective for β-hydroxyacyl-ACP substrates between 9 and 11 carbons based on experiments with β-hydroxyacyl-N-acetylcysteamine substrate analogs and allenic inhibitors (5, 14, 15). In light of the data on the substrate specificity of FabA, we were somewhat surprised that FabA functioned efficiently in the first cycle of fatty acid elongation, generating crotonyl-ACP from β-hydroxybutyryl-ACP (16, 17), suggesting a broader role for FabA. One difference between our work and the earlier studies on FabA substrate specificity is that we used native ACP thiosesters rather than thioester analogs as substrates. The fabA mutants are unable to synthesize unsaturated fatty acids, but they produce saturated fatty acids normally, indicating the existence of at least one other dehydratase capable of elongating saturated fatty acids (18–21). A second dehydratase, called FabZ, was recently identified based on the ability of defective fabZ alleles to suppress temperature-sensitive (lpzA2) mutants in lipid A biosynthesis (22). The FabZ isozyme activity was characterized with native β-hydroxymyristoyl-ACP substrate (22); however, other substrates were not examined, and it is not clear whether FabZ is capable of playing a wider role in fatty acid biosynthesis. The goal of the present study was to determine the substrate preferences for the FabA and FabZ isozymes to elucidate their role(s) in fatty acid synthesis and provide a biochemical basis for interpreting the phenotypes of cells with altered levels of the two dehydratases.

EXPERIMENTAL PROCEDURES

Materials—Sources of supplies were: Moravek Biochemicals Inc., [2-14C]malonyl-CoA (specific activity, 57 mCi/mmol) and [1-14C]acetyl-CoA (specific activity, 54 mCi/mmol); DuPont NEN, En’Hance; Sigma, fatty acids, ACP, cerulenin, and acyl-CoAs; Promega, molecular biology reagents; Novagen, pET vectors and expression strains; Qiagen, Nt’-agarose column; and Pharmacia Biotech Inc., acetyl-CoA and malonyl-CoA. Acyl-ACPs were synthesized using the acyl-ACP synthetase purified from an overproducing strain (23) as described previously (24). The acyl-ACPs were concentrated and the buffer exchanged by centrifugal filtration in a Centricon-3 concentrator (Amicon). Yields were judged by

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† To whom correspondence should be addressed; Dept. of Biochemistry, St. Jude Children’s Research Hospital, 332 N. Lauderdale, Memphis, TN 38101. Tel.: 901-495-3491; Fax: 901-525-8025; E-mail: charles.rock@stjude.org.
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Fig. 1. Enzymes of fatty acid elongation in E. coli. There are four reactions in each cycle of fatty acid elongation. The first cycle is initiated by β-ketoacyl-ACP synthase III (FabH), which condenses malonyl-ACP with acetyl-CoA; subsequent cycles begin with the condensation of malonyl-ACP with acyl-ACP catalyzed by β-ketoacyl-ACP synthases I and II (FabB and FabF, respectively). Reduction of the resulting β-ketoester is accomplished by a single NADPH-dependent β-ketoester reductase (FabG). The focus of this paper is the third step in the cycle catalyzed by either the FabA or FabZ β-hydroxyacyl-ACP dehydratase. FabA also isomerizes trans-2-decenoyl-ACP to cis-3-decenoyl-ACP, which bypasses FabI and is used by FabB to initiate the first cycle of elongation in unsaturated fatty acid synthesis (reaction not shown). The final step in each cycle is catalyzed by a single NADH-dependent enoyl-ACP reductase (FabI) that converts trans-2-enoyl-ACP to acyl-ACP. The malonyl-ACP used by the condensing enzymes arises from the transacylation of malonyl-CoA to ACP catalyzed by malonyl-CoA:ACP transacylase (FabD). Malonyl-CoA is synthesized by acetyl-CoA carboxylase (not shown).

Bradford protein determination (25) and conformationally sensitive gel electrophoresis in 2.5 M urea, 13% acrylamide gels (26). All other chemicals were of reagent grade or better.

Construction of Expression Vectors and Purification of the His-tag Proteins—The individual enzymes were cloned, expressed, and purified essentially as described previously (17, 27). The genes were amplified from genomic DNA obtained from strain UB1005. Primers created novel restriction sites for NdeI at the N-terminal methionine and BamHI downstream of the stop codon. Polymerase chain reaction was performed with Taq DNA polymerase, and the fragments were ligated into the TA cloning vector pCRII (Invitrogen) and transformed into E. coli OneShot cells. Following overnight growth, plasmid was isolated and digested with NdeI and BamHI, and the appropriate fragments were isolated and ligated into NdeI- and BamHI-digested pET-15b. These mixtures were used to transform strain BL21(DE3) to ampicillin and digested with

One such clone was chosen for each gene and grown in 100 ml of M9 medium (28) supplemented with 1% casein amino acids, 0.4% glucose, and 1 mM MgCl2 to a density of approximately 5 × 108 cells/ml. Isopropyl-1-thio-β-galactosidase was then added to a final concentration of 1 mM, and incubation continued for a further 3 h at 37°C. Cells were collected by centrifugation (8,000 rpm, 4°C, 10 min) and stored at −20°C overnight. Cell lysis was performed as described with the addition of lysozyme to 0.1 mg/ml after resuspension (29). Soluble protein was applied to a Ni2+-agarose column and washed with 40 mM imidazole-containing metal chelation affinity chromatography buffer (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM β-mercaptoethanol). His-tagged proteins were eluted with 200 mM imidazole in the same buffer and the purified proteins stored at −20°C.

Dehydration of β-Hydroxybutyryl-ACP and β-Hydroxyhexanoyl-ACP—The ability of FabA and FabZ to function in the first cycle of fatty acid synthesis was assessed in reactions containing 0.1 mM sodium phosphate, pH 7.0; with 0.1 μg each of FabD, FabG, and FabH; 50 μM NADH; 50 μM NADPH; 1 mM β-mercaptoethanol; 100 μM acetyl-CoA; 30 μM [2-14C]malonyl-CoA (specific activity 57 mCi/mM); and 50 μM ACP in a final volume of 40 μl. FabA (0.2 μg) or FabZ (0.2 μg) was added to the appropriate reactions, and FabI (0.1 μg) was added as indicated in the figure legends. Reactions were incubated for 20 min at 37°C before placing in an ice slurry, adding gel loading buffer, and loading onto a conformationally sensitive 13% polyacrylamide gel containing 0.5 M urea. Following electrophoresis, gels were fixed, soaked in EnHance, and subjected to fluorography.

The specific activities of the two dehydratases toward these substrates was determined in assays as described above except that FabI was present at 0.1 μg in each reaction, and the amount of FabA or FabZ was varied from 0.006 to 0.2 μg/assay by the addition of the enzymes from a serially diluted stock. Bands were quantitated by exposure of the dried gel to a PhosphorImager. Specific activities were calculated from the slopes of the plot of product formation versus FabA or FabZ protein concentration in the assay. The rates of β-hydroxyhexanoyl-ACP dehydration by FabA and FabZ were determined in assays where acetyl-CoA was replaced with butyryl-CoA.

Ability of FabA and FabZ to Participate in Dehydration of Long Chain Saturated and Unsaturated Fatty Acids—The dehydration of β-OH-16:0-ACP and β-OH-16:1-9-ACP by FabA or FabZ was assessed in incubations primed with a 100 μM concentration of either 14:0-ACP or 14:1-17-ACP. Reactions contained 0.1 mM sodium phosphate, pH 7.0; 0.1 μg each of FabD, FabG, and FabB; 50 μM NADH; 50 μM NADPH; 1 mM β-mercaptoethanol; 30 μM [14C]malonyl-CoA (specific activity 57 mCi/mM); and 50 μM ACP in a final volume of 40 μl. FabA (0.2 μg) or FabZ (0.2 μg) was added to the appropriate reactions, and FabI (0.1 μg) was added as indicated in the figure legends. Reactions were incubated for 20 min at 37°C before being placed in an ice slurry, gel loading buffer added, and loading to a conformationally sensitive 13% polyacrylamide gel containing 0.5 M urea. Following electrophoresis, gels were fixed, soaked in EnHance, and subjected to fluorography. To determine the rate of each reaction, experiments were set up as above, with FabI present in each, and the concentration of FabA or FabZ varied between 3 and 48 ng. Product formation was quantitated using the PhosphorImager.

Dehydration of Long Chain Substrates—The dehydration of β-hydroxy substrates with chain lengths between 8 and 16 carbons was investigated by using the transacylation and condensation reactions of FabB to produce the β-hydroxyacyl-ACP substrate starting with a CoA thioester two carbons shorter. Reactions contained a 1 mM concentration of CoA thioester, FabD, FabB, and FabG (all at 1 μg); 100 μM NADH; 100 μM NADPH; 100 μM ACP; 0.1 mM sodium phosphate, pH 7.0; and 100 μM [2-14C]malonyl-CoA (specific activity 14.25 mCi/mM). After a 1-h incubation at 37°C, FabB was inactivated by addition of cerulien to a concentration of 100 μg/ml, FabI (1 μg) was added, and the mixture was aliquoted into individual reaction tubes. The yield of β-hydroxyacyl-ACP varied somewhat according to the chain length of acyl-CoA used as the primer and fell between 30 and 60 μM in a typical experiment. FabA or FabZ was then added from a serially diluted stock, and the mixtures were incubated for 20 min at 37°C in a final volume of 40 μl. Products were resolved on conformationally sensitive polyacrylamide gels containing an optimal concentration of urea to effect the separation. Bands were quantitated using the PhosphorImager.

Isomerase Assay—The dehydration and isomerization of β-hydroxy-decanoyl-ACP were assayed by preparing the substrate in incubations containing octanoyl-CoA (1 mM); 100 μM ACP; 100 μM [2-14C]malonyl-
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Fig. 2. Purity of FabA, FabZ, and the five other enzymes required to reconstitute fatty acid biosynthesis in vitro. The coding sequences for each of these proteins were cloned into the pET-15b His-tag expression vector, expressed to high levels following isopropyl-
1-thio-β-D-galactopyranoside induction, and purified by chromatography on Ni²⁺-agarose as described under "Experimental Procedures." The purity of the proteins was determined by sodium dodecyl sulfate-gel electrophoresis on 12% polyacrylamide gels.

CoA (specific activity, 14.25 mCi/mmol); 1 mm β-mercaptoethanol; 0.1 mM sodium phosphate, pH 7.0; 100 μM NADH, 100 μM NADPH; FabD, FabG, and FabB (each at 1.0 μg) were incubated at 37 °C for 1 h. The reaction was then aliquoted into a series of tubes, and, to indicated reactions, cerulenin was added to 100 μM and incubated at room temperature for 5 min to inactivate the FabB. FabA, FabZ, and FabI (0.1 μg) were then added as indicated, and incubation continued at 37 °C for 20 min. The final volume of each incubation was 40 μl. Reaction products were separated by electrophoresis on conformationally sensitive 15% polyacrylamide gels.

Yeast Two-hybrid Analysis—The yeast two-hybrid system (30) was employed to test for a direct interaction between the FabA and FabB proteins in vivo. The fabA and fabB genes were excised from the corresponding pET-15b vectors as Ndel-BamHI fragments and ligated directly into the Ndel and BamHI sites of the pAS1 vector (31). The resulting constructs expressed a fusion protein of FabA (pAS1-FabA) or FabB (pAS1-FabB) at the COOH-terminal end of the GALI DNA binding domain. Vectors with an activation domain fusion were constructed by ligating the faba and fabb genes (as Ndel-BamHI fragments) into the NcoI and BamHI sites of pACTII using NcoI-Ndel linkers (5'-CATGTTCCCCAC-3' and 5'-TATGTTGGCC-3'). Plasmids were then transformed into the Saccharomyces cerevisiae strain Y153 (MATa leu2-3, 112; ura3-52, trpl-901, his3-D200, ade2-101, gal4 AGL890 ΔURA3::GAL-lacZ, LYS2::GAL-HIS3) (31) using the lithium acetate protocol. Cells were plated onto complete minimal dropout plates lacking Leu and Trp and grown at 30 °C. Individual colonies were purified and then tested for the level of expression of HIS3 by growth on plates containing concentrations of 3-aminotriazole between 0 and 50 mM and lacking His, Leu, and Trp. Pairs of plasmids tested were: pACTI and pCOI (positive control); pAS1-FabA and pACTII-FabB; pAS1-FabB and pACTII-FabB; pAS1-FabA and pACTII-FabB; pAS1-FabB and pACTII-FabA; pAS1 and pACTII (negative control).

RESULTS

Assays for FabA and FabZ—The individual β-hydroxyacyl-ACP substrates for the FabA and FabZ dehydratase assays were prepared, and cycles of fatty acid elongation were reconstructed in vitro by purifying seven His-tag derivatives of enzymes in fatty acid biosynthesis (Fig. 2). FabD was used to generate malonyl-ACP from malonyl-CoA. FabH was used as the condensing enzyme for the first cycle of elongation and the formation of β-ketoheptanoyl-ACP, and FabG was used as the condensing enzyme to produce the β-ketoacyl-ACPs for all other cycles. FabG was used in all cases to generate the β-hydroxyacyl-ACPs from the β-ketoacyl-ACPs formed by the condensing enzymes. Likewise, FabI was used to convert trans-2-enoyl-ACPs to acyl-ACPs for all chain lengths.

Our first experiments were designed to determine if there are any absolute substrate preferences for FabA and FabB by examining the ability of the two dehydratases to function in the initiation of fatty acid synthesis and in the elongation of long chain saturated and unsaturated fatty acyl-ACPs (Fig. 3). Both dehydratases participated efficiently in the initiation of fatty acid synthesis since there was essentially no difference in the formation of butyryl-ACP in these experiments (Fig. 3A). Also, both FabA and FabB participated in the formation of 16:0-ACP, although the extent of product formation with FabA was not as great as in incubations containing FabB (Fig. 3B). In contrast, FabA functioned poorly in the formation of 16:1-ACP, whereas FabB participated efficiently in this cycle for the formation of long chain unsaturated acyl-ACP (Fig. 3C). These data suggested that the two dehydratases possess broad overlapping substrate specificities.

Substrate Specificities for FabA and FabZ—The subtle differences in the substrate specificities of FabA and FabZ were examined in coupled enzyme assays where the β-hydroxyacyl-ACPs were first generated in a preincubation with FabD, FabG (or FabH), FabG, an acyl-CoA primer, and [14C]malonyl-CoA. These preincubations typically generated concentrations of β-hydroxyacyl-ACP between 30 and 60 μM. Although we did not generate and purify enough labeled substrate to determine a kinetic Kₘ, these concentrations of β-hydroxyacyl-ACP are significantly higher than found in vivo. These intermediates have not been detected in vivo suggesting that they comprise only a few percent of the total ACP pool, which is approxi-
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**Fig. 4.** FabA and FabZ specific activity in the initial cycle of fatty acid synthesis. The ability of FabA and FabZ to function in the initial cycle of fatty acid synthesis was determined in assays containing a combination of the FabD, FabH, and FabG enzymes, ACP, NADH, and NADPH as cofactors, and acetyl-CoA plus [2,3-14C]malonyl-CoA as substrates to produce β-hydroxy[14C]butyryl-ACP as described under “Experimental Procedures.” The specific activities were determined the addition of serially diluted amounts of either FabA (panel A) or FabZ (panel B) in the presence of an excess of enoyl-ACP reductase (FabI) to convert all of the crotonyl-ACP formed by the dehydratases to butyryl-ACP. The products of the reactions were analyzed by conformationally sensitive gel electrophoresis using 13% polyacrylamide gels containing 0.5 M urea followed by autoradiography. The pmol of [14C]butyryl-ACP formed was determined using a PhosphorImager calibrated with a [14C]malonyl-CoA curve. The results were plotted as a function of dehydratase protein concentration, and the specific activities of FabA and FabZ were calculated from the slopes of the lines (panel C).

Similarly 100 μM under optimal growth conditions (17, 32, 33). Next, a series of FabA or FabZ protein concentrations was added along with a saturating amount of FabI to completely convert the trans-2-acyl-ACP products to the corresponding acyl-ACPs. Product formation was detected by conformationally sensitive gel electrophoresis followed by quantitation using a PhosphorImager (Fig. 4). Specific activities were determined by plotting product formation versus protein concentration. Fig. 4 is an example that illustrates how the data were collected and analyzed. FabZ was determined to have a specific activity 4.4-fold higher than FabA toward β-hydroxybutyryl-ACP. Thus, both FabA and FabZ enzymes participate in the initial cycle of fatty acid elongation, although FabZ is the most efficient enzyme.

This work was expanded to examine all of the saturated chain lengths up to 16:0, and the specific activities of FabA or FabZ toward each of these substrates are shown in Fig. 5. In these experiments, the corresponding β-hydroxy[14C]acyl-ACP was generated in incubations containing an acyl-CoA as a primer to take advantage of the fact that FabB will utilize acyl-CoAs as acceptors (34). Both dehydratases were active on all substrates tested. FabZ was most active on short chain substrates, and its overall activity decreased as the chain length increased. In contrast, FabA was more active on medium chain acyl-ACPs with lengths centered on 10 carbons. Both dehydratases possessed lower activity toward 14- and 16-carbon substrates.

We focused on the elongation of cis-7-tetradeccenoyl-ACP to cis-9-hexadecenoyl-ACP to evaluate the role of FabA and FabZ in the elongation of unsaturated fatty acids (Fig. 6). The specific activity of FabZ for β-hydroxy-cis-9-hexadecenoyl-ACP was 348 pmol/min/μg compared with barely detectable 13 pmol/min/μg for FabA. Thus, FabA was essentially inactive in the elongation of unsaturated fatty acids compared with FabZ and its higher activity toward the saturated homolog (101 pmol/min/μg) (Fig. 5). FabZ activity was the same toward β-hydroxy-16:0-ACP (298 pmol/min/μg) (Fig. 5) as it was on β-hydroxy-16:1(Δ7c)-ACP (Fig. 6). These data indicated that FabA does not participate in the cycles of elongation in the unsaturated fatty acid arm of the pathway.

**Isomerization Reaction of FabA**—The clear prediction was that FabZ would not have isomerase activity based on the phenotype of the fabA mutants and previous work on this enzyme, and this idea was confirmed with an in vitro assay (Fig. 7). The new insight from these experiments was that active FabB was required to detect significant formation of cis-acyl-ACPs in incubations containing FabA. The absence of FabI and NADH in the incubations shown in Fig. 7 (lanes 2, 4, 6, and 7) prevented the cycle of elongation from being completed leading to the conversion of β-hydroxy-10:0-ACP to trans-2-10:1-ACP. The inclusion of FabI and NADH in incubations where FabB was inactivated by cerulenin resulted in the formation of 10:0-ACP with either FabA or FabZ (Fig. 7, lanes 3 and 5). The conformationally sensitive gel electrophoresis system separates cis-un saturated acyl-ACPs from their saturated homologs (26), and the inability to detect a significant cis-3-decenoyl-ACP band indicated that it was a minor product. Guerra and Browse (35) report that equal amounts of β-hydroxy, trans-2, and cis-3 products were formed by FabA, al-
ACP produced in lane 6 containing 2.0 M urea, and the bands were visualized by autoradiography. cis-lanes was formed with either dehydratase (continued FabB activity) of FabI. Cerulenin was added to the four indicated incubations to block additional 20 min with either FabA or FabZ in the absence or presence of 1 mM NADH, and NADPH as cofactors, [2-14C]malonyl-CoA, and the indicated concentrations of FabA or FabZ dehydratases. Product formation as a function of dehydratase protein concentration was quantitated using a PhosphorImager following separation of the products by conformationally sensitive gel electrophoresis on 15% polyacrylamide gels as described under "Experimental Procedures." Figure 6. FabA is defective in the elongation of unsaturated acyl-ACP. The specific activity of FabA and FabZ in the elongation of cis-14:1(Δ7c)-ACP to cis-16:1(Δ8)-ACP was determined in assays containing cis-14:1(Δ7c)-ACP, FabD, FabB, FabI, and FabG enzymes, ACP, NADH, and NADPH as cofactors, [2-14C]malonyl-CoA, and the indicated concentrations of FabA or FabZ dehydratases. Product formation as a function of dehydratase protein concentration was quantitated using a PhosphorImager following separation of the products by conformationally sensitive gel electrophoresis on 15% polyacrylamide gels containing 2.5 M urea as described under "Experimental Procedures." Though these data were not consistent with our previous experience (16, 17). The most careful recent measurements that have been made with substrate analogs showed that the ratio of β-hydroxy to trans-2 to cis-3 products was 75:22:3 when the FabA reaction was at equilibrium (36). Thus, we are not surprised that our gel electrophoresis analysis did not show a significant difference between the product distribution in incubations containing either FabA (Fig. 7, lanes 2 and 3) or FabZ (Fig. 7, lanes 4 and 5), indicating that significant quantities of the cis-3-decenoyl-ACP were not formed in incubations containing FabA and inactive FabB. However, in assays that contained active FabB, virtually all of the of β-hydroxy-10:0-ACP was eluted to a mixture of β-hydroxy-12:1(Δ5c)-ACP and 12:2(Δ5c,Δ2c)-ACP (Fig. 7, lane 6). Since this type of assay lacked FabI and NADH, the further rounds of elongation were prevented. Incubations containing FabZ and active FabB (Fig. 7, lane 7) yielded the same product distribution as incubations performed in the absence of active FabB (Fig. 7, lane 4). These data illustrate that FabB functions to pull fatty acid synthesis in the direction of unsaturated fatty acid formation.

Interactions between FabA and FabB—There are two interpretations for the data in Fig. 7. One is that FabA produces an equilibrium mixture of three products, and the high activity of FabB toward the cis-3-decenoyl-ACP intermediate results in the formation of cis-unsaturated products by the irreversible conversion of cis-3-decenoyl-ACP to β-keto-cis-5-dodecenoyl-ACP. A second possibility is that there is a direct interaction between FabA and FabB which facilitates the channeling of intermediates. Since type I fatty acid synthases are multifunctional enzymes, there has been considerable speculation about whether the individual enzymes of the type II system interact to form functional complexes in vivo. We tested an aspect of this hypothesis using the yeast two-hybrid system to determine if there were interactions between FabA and FabB in vivo. Fusions of both genes to both the GAL4 DNA binding domain in plasmid pAS1 or the GAL4 transcriptional activation domain in plasmid pACTII were constructed as described under "Experimental Procedures." Yeast strain Y153 was transformed with the plasmids alone or in combination, and the transformants were selected on plates containing His and Trp. Potential interactions were assessed by growth of individual transformants on minimal plates lacking His and containing 3-aminotriazole concentrations from 0 to 50 mM. The four types of transformants containing each of the individual plasmids (pACTII-FabA, pACTII-FabB, pAS1-FabA, and pAS1-FabB) grew very weakly on 15 mM 3-aminotriazole and not at all on plates containing 25–50 mM 3-aminotriazole. This pattern of growth could not be distinguished from control cells that did not contain plasmids or from strains transformed with empty vectors, illustrating that the FabA and FabB fusions alone did not lead to transcriptional activation. Yeast transformants containing the combinations of either pAS1-FabA plus pACTII-FabB or pAS1-FabB plus pACTII-FabB exhibited robust growth on 50 mM 3-aminotriazole indicating strong interactions between the products of these plasmids. Since both FabA (13) and FabB (37) are homodimers, this pattern of growth was anticipated. These control experiments confirmed that the yeast strains expressed all four fusions and that the fusions did not interfere with dimerization. In contrast, strains containing combinations of either pAS1-FabA plus pACTII-FabB or pAS1-FabB plus pACTII-FabB did not grow on minimal plates containing 25–50 mM 3-aminotriazole, and we were unable to distinguish the growth of these strains from control strains containing any of the four plasmids alone or the parental strain. Based on the yeast two-hybrid analysis, FabA and FabB did not interact in vivo, thus the results in Fig. 7 can only be explained by the high affinity of FabB for the cis-3-decenoyl-ACP intermediate.

DISCUSSION

Role of FabG—Our results indicate that FabG, the NADPH-dependent β-ketoacyl-ACP reductase, is the only reductase required for fatty acid biosynthesis in E. coli. The fabG gene is located within the fab cluster at 24.5 min between the fabD and acpP genes (38) and encodes a protein with acetoacetyl-ACP reductase activity (16, 17). The protein has high similarities (>50% identical residues) to several acetoacetyl-CoA reduc-
tases and the plant \(\beta\)-ketoacyl-ACP reductases. Although we did not perform a detailed comparison of substrates, FabG efficiently reduced every \(\beta\)-ketoacyl-ACP substrate generated in this paper, illustrating that it has activity toward both short and long chain saturated and unsaturated \(\beta\)-ketoacyl-ACPs. Thus, FabG appears similar to the NADH-dependent enoyl-ACP reductase (FabI) in that it also functions in every cycle of the pathway (16), consistent with the initial characterization of the \(\beta\)-ketoacyl-ACP reductase by Toomey and Wakil (39). Sequence analysis of the \emph{Haemophilus influenzae} genome reveals that this free living organism has only a single \(fabG\) and a single \(fabI\) homolog (40), supporting the idea that the two reductase reactions in the cycle are carried out by single enzymes with broad substrate specificity. However, the conclusion that FabI is the only enoyl-ACP reductase in \emph{E. coli} fatty acid biosynthesis is corroborated by the biochemical and physiological defects of \(fabI\) temperature-sensitive mutants (16), and it will be important to perform a similar genetic analysis of the \(fabG\) gene to verify that it is the only \(\beta\)-ketoreductase in the pathway.

\textbf{Role of FabB—}FabB activity was first recognized as essential for unsaturated fatty acid synthesis based on the isolation of \(fabB\) mutants that require supplementation with exogenous unsaturated fatty acids for growth (41) which are specifically deficient in \(\beta\)-ketoacyl-ACP synthase I activity (42). Analysis of the fatty acid composition of strains that overexpress FabA, FabB, or both leads to the conclusion that FabB activity is the primary factor in determining the level of unsaturated fatty acids produced by the pathway (43). Since FabB is not directly involved in the introduction of the double bond into the growing acyl chain, it must be required for one or more of the elongation reactions in unsaturated fatty acid biosynthesis, most likely the elongation of \(\text{cis-3\text{-decanoyl-ACP}}\) (for review, see Refs. 1 and 2). In the absence of active FabB or FabI, FabB generates a mixture of \(\beta\)-hydroxydecanoyl-ACP and \(\text{trans-2\text{-decanoyl-ACP}}\). We did not detect a \(\text{cis-3\text{-product}}\), which is consistent with the minor contribution of \(\text{cis-3\text{-products}}\) formed in FabA reactions (36). However, in the presence of active FabB (and the absence of FabI), all of the products detected were two carbons longer and were derived from the \(\text{cis-3\text{-decanoyl-ACP}}\) (Fig. 7). The idea that FabA and FabB form a complex that channels substrates toward the unsaturated branch of the pathway is untenable in light of the lack of significant interaction between these two proteins in the yeast two-hybrid system. In addition, the recent crystal structure of FabA shows that the active site His resides halfway down a 6 \(\times\) 20 \(\text{Å}\) tunnel (44). Thus, the 4'-phosphopantetheine arm of ACP must be inserted into this tunnel and is constrained from "swinging" to another active site without first completely extracting itself from the substrate binding tunnel. These data support the hypothesis that the unique ability of FabB to elongate the low amounts of \(\text{cis-3\text{-decanoyl-ACP}}\) formed by FabA pulls the pathway in the direction of unsaturated fatty acid synthesis and explains the requirement for FabB in unsaturated fatty acid production. Thus, the hypothesis that competition between FabI and FabB is an important factor in regulating the ratio of saturated to unsaturated fatty acids will be important to test.

\textbf{Physiological Roles for FabA and FabZ—}The substrate specificities of FabA and FabZ dehydratases lead to a model (Fig. 8) that provides a biochemical explanation for the phenotypes of strains with genetically altered levels of the dehydratases. Although the equilibrium of the dehydratase reactions lies in the direction of \(\beta\)-hydroxyacyl-ACP, their activity is critical to supplying \(\text{trans-2\text{-acyl-ACP}}\) to FabI which pulls each cycle of elongation to completion (16, 17). Defects in FabA and/or reduced \(fabA\) gene expression lead to a deficit in unsaturated fatty acid production (18–21, 45); however, overproduction of FabA protein also leads to a decrease in the unsaturated fatty acid content of membrane phospholipids (43). The former effect is understood by the essential requirement for FabA isomerase activity in unsaturated fatty acid synthesis. The second effect is more clearly understood in light of our finding that FabA is essentially inactive in cycles of elongation involved in unsaturated fatty acid biosynthesis. The inability of FabA to catalyze the dehydration of \(\text{cis-unsaturated \(\beta\)-hydroxyacyl-ACPs}\) is consistent with the structure of the FabA active site (44). The bent chain of \(\text{cis unsaturated fatty acids}\) will not fit into the 6 \(\text{Å}\) diameter wormhole that gives access to the active site His. Thus, increasing FabA activity would contribute to increased flux through the saturated arm of the pathway rather than the unsaturated branch. Similarly, mutants defective in FabZ activity (i.e. \(fabZ8\), \(fabZ9\), and \(fabZ10\)) are proposed to suppress temperature-sensitive \(\text{lpxA2 mutations}\) by increasing the concentration of \(\beta\)-hydroxymyristoyl-ACP available to the LpxA acyltransferase (22). FabZ is the primary dehydratase involved in the unsaturated branch of the pathway and is more active than FabA on long chain saturated acyl-ACP. These two properties support the idea that reduced activity of FabZ selectively increases the levels of intermediates in the saturated branch of the pathway. It will be important to determine the actual levels of FabA and FabZ in \textit{vivo} and to measure the levels of pathway intermediates in wild type and mutant strains to substantiate these ideas.

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