Domain Swapping Between *Enterococcus faecalis* FabN and FabZ Proteins Localizes the Structural Determinants for Isomerase Activity*

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Running Title: Structural determinants of isomerase activity

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Anaerobic unsaturated fatty acid synthesis in bacteria occurs through the introduction of a double bond into the growing acyl chain. In the *Escherichia coli* model system, FabA catalyzes both the dehydration of β-hydroxyldecanoyl-ACP and the isomerization of *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP as the essential step. A second dehydratase, FabZ, functions in acyl chain elongation, but cannot carry out the isomerization reaction. *Enterococcus faecalis* has two highly-related FabZ homologs. One of these termed *Ef*FabN, carries out the isomerization reaction in vivo, whereas the other, *Ef*FabZ, does not (Wang and Cronan, *J. Biol. Chem.* 279:34489, 2004). We carried out a series of domain swapping and mutagenesis experiment coupled with in vitro biochemical analyses to define the structural feature(s) that specify the catalytic properties of these two enzymes. Substitution of the β3 and β4 strands of *Ef*FabZ with the corresponding strands from *Ef*FabN was necessary and sufficient to convert *Ef*FabZ into an isomerase. These data are consistent with the hypothesis that the isomerase potential of β-hydroxylacyl-ACP dehydratases is determined by the properties of the β-sheets that dictate the orientation of the central α-helix and thus the shape of the substrate binding tunnel rather than the catalytic machinery at the active site.

Anaerobic unsaturated fatty acid (UFA) biosynthesis occurs through the insertion of a double bond into the growing acyl chain (Fig. 1), and the proportion of UFA produced is a critical determinant of the biophysical properties of biological membranes. In bacteria, fatty acids are synthesized using the dissociated, type II fatty acid synthase system in which each of the steps is catalyzed by distinct enzymes that are each encoded by separate genes (1,2). The key players in UFA synthesis in *Escherichia coli* were first defined by the isolation and characterization of UFA auxotrophs (3). The double bond is introduced at the 10-carbon intermediate by β-hydroxydecanoyl-ACP dehydratase, FabA (4), which is capable of both the removal of water to generate *trans*-2-decenoyl-ACP and the isomerization of this intermediate to the *cis*-3-decenoyl-ACP (1,5) (Fig. 1). A second unsaturated fatty acid auxotroph was isolated that corresponds to the *fabB* gene, which encodes β-ketoacyl-ACP synthase I (6). In *fabA* and *fabB* mutants, saturated fatty acid synthesis persists due to the presence of another dehydratase, FabZ (7), and another elongation condensing enzyme, FabF (8,9). The FabZ isozyme (β-hydroxylacyl-ACP dehydratase) is ubiquitously expressed in type II systems, cannot carry out the isomerization reaction, and is the only type of dehydratase that exists in most bacteria. While FabA and FabZ have many primary sequence characteristics in common, bioinformatic analysis clearly divide the two subtypes. Specifically, there are distinct...
differences in the active site residues, an Asp in FabA and a Glu in FabZ, and FabA is a dimer (10) whereas FabZ is a hexamer (11). FabZ does play a role in UFA synthesis by functioning in the elongation of both saturated and unsaturated long-chain acyl-ACP, whereas FabA most efficiently processes saturated chain lengths 10 carbons and shorter (12). Likewise, the FabF condensing readily elongates 16:1 to 18:1 UFA (9); however, the inability to support UFA synthesis in fabB mutants leads to the conclusion that FabF cannot elongate a key intermediate in UFA biosynthesis in vivo, most likely cis-3-decenoyl-ACP (1,2).

The availability of numerous bacterial genome sequences allows the reconstruction of type II fatty acid synthesis in these organisms using bioinformatics analysis tools. It is notable that fabA and fabB genes occur together in gram-negative bacteria that produce UFA (13). However, many anaerobes that synthesize UFA do not have a recognizable fabA homolog in their genomes, and also have a FabF rather than a FabB subtype of elongation condensing enzyme. In these organisms UFA are synthesized by a different mechanism. *Streptococcus pneumoniae* produces straight-chain saturated and monounsaturated fatty acids predominately of 16 and 18 carbon chain lengths (14). This organism does not utilize a FabA-like mechanism for introducing a double bond into the growing acyl chain, but rather accomplishes this task using FabM, a trans-2, cis-3-decenoyl-ACP isomerase (15). *Enterococcus faecalis* also has a fatty acid composition similar to *E. coli*, but also lacks a FabA, FabB and FabM. However, *E. faecalis* has two FabZ homologs and Wang and Cronan (16) show that one of these genes, now called FabN, functions as a dehydratase/isomerase analogous to FabA, whereas the other possessed only dehydratase activity (Fig. 1). Thus, one cannot predict the biochemical activity of this class of proteins based on bioinformatics.

The comparison of the X-ray structures of FabA (10) and FabZ (11), coupled with the analysis of site-directed mutants shows that the differences in the catalytic activities of the enzymes are not due to the distinct catalytic residues in the active site or to the side-chains of the residues that compose the substrate binding pocket (11). Both proteins adopt a “hot dog” fold with a long central α-helix (the hot dog) surrounded by several β-sheets (the bun). A detailed comparison of these closely related structures led to the hypothesis that the different biochemistry of these enzymes is due to differences in the β-sheet structures that alter the orientation of the central α-helix and thus the shape of the active site tunnel preventing the substrate from adopting a cis conformation in FabZ. The existence of two even more closely related enzymes, *Ef*FabN and *Ef*FabZ, with different catalytic properties but identical active site residues, allows us the opportunity to test the working hypothesis using domain swapping experiments to determine if the isomerase activity is due to the structure of the β-strands that control the shape of the active site tunnel.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sources of supplies were: Amersham Pharmacia Biotech, [2-\(^{14}\)C]malonyl-CoA (specific activity, 52 mCi/mmole); Sigma, antibiotics, acyl-CoA, *E. coli* ACP; Promega, molecular reagents and restriction enzymes, T4 ligase; Novagen, pET vectors and expression strains; Qiagen, Ni\(^{2+}\)-agarose resin. Protein was quantitated by the Bradford method (17). The *Mycobacterium tuberculosis* MtFabH, *E. coli* EcFabD, EcFabG, EcFabA, *S. pneumoniae* SpFabF proteins were purified as described previously (12,18-21). All other chemicals were reagent grade or better.

**Cloning and Construction of Chimeric Enzymes**—The *EffabN* and *EffabZ* genes were amplified from genomic DNA of *E. faecalis* by primer sets *Ef*FabNstart/*Ef*FabNend and *Ef*FabZstart/*Ef*FabZend. The PCR products were ligated into plasmid pCR2.1 and sequenced. The plasmids were isolated and digested with NdeI and BamHI, and the gene fragments were isolated and ligated into plasmid pET-15b digested with the same enzymes to generate the *Ef*FabN and *Ef*FabZ expression vectors pYL3 and pYL4. Chimeras were generated by overlapping PCR method. Sequences of internal primers are listed in Table I. T7 promoter primer with reverse
primers and T7 terminator primer with forward primers were used to generate PCR fragments corresponding to the specific region of the two enzymes. After purification from agarose gels, these fragments were mixed with right combination to serve as template, and T7 promoter and T7 terminator primers were used to amplify the recombinant molecule. PCR products from this step were ligated to PCR 2.1 vector and sequenced to ensure the chimerical constructs were correct. The chimerical genes were cut out with NdeI and BamHI, and transferred into pET15b to generate the expression vectors.

**Protein Purification**—The pET15b plasmids were used to transform *E. coli* Rosetta competent cells for protein expression. The selected transformants were cultured in LB medium with antibiotic (50 μg/ml carbenicillin and 34 μg/ml chloramphenicol) at 37°C until A\textsubscript{600} reached 0.6. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and incubation continued for overnight at 20°C. Cells were collected by centrifugation (6000 rpm, 4°C, 15 mins) and cell pellets were lysed using a French Press. Soluble proteins were applied to a Ni\textsuperscript{2+}-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). His-tagged proteins were eluted with 500 mM imidazole in the same buffer. Proteins were quantitated by the Bradford method (17). The purified proteins were stored at –20°C.

Purified *Efa* or *EfZ* proteins were applied to a Superdex\textsuperscript{TM} 200 HR 16/60 column (Amersham Biosciences) and eluted with 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 1 mM DTT). The molecular weights of *Efa* and *EfZ* were estimated using globular protein standards and a calibration curve.

**Enzymatic Assays**—The ability of the individual 6×His-tag *Efa* or *EfZ* to carry out the dehydratase and/or isomerase activities was measured using a reconstituted system essentially as described previously (12,15,22). The reaction mixtures contained 100 μM ACP, 1 mM β-mercaptoproethanol, 0.1 M sodium phosphate buffer, pH 7.0, 100 μM NADPH, 50 μM octanoyl-CoA, 100 μM [2-\textsuperscript{14}C] malonyl-CoA (specific activity, 52 mCi/mmol), MtFabH (1.0 μg), EcFabD (1.0 μg), EcFabG (1.0 μg), SpFabF (3 μg) with *EfN* (1.0 μg) or *EfZ* (1.0 μg) or chimeric proteins (1.0 μg) in a final volume of 40 μl. The assay mixtures were incubated at 37°C for 30 min, and analyzed by conformationally sensitive gel electrophoresis in 13% polyacrylamide gels containing 2.5 M urea. Electrophoresis was performed at 25°C and 32 mA/gel. The gels were dried, and the bands were quantitated using a PhosphoImager screen. In the experiments determining *EfN* isomerase activity, EcFabZ was added to reaction mixture and the reaction was incubated at 37°C for 30 min before *EfN* addition. Specific activities were calculated from the slopes of the plot of product formation versus protein concentration in the assay.

**RESULTS AND DISCUSSION**

**Characteristics of *E. faecalis* fab Genes**—An analysis of the type II fatty acid biosynthetic genes in *E. faecalis* shows that they are located in two clusters in the genome (Fig. 2A). *S. pneumoniae*, a closely related bacteria which also synthesizes unsaturated fatty acids, has only one fab gene cluster in its genome corresponding to the large gene cluster in *E. faecalis*. A comparison of the predicted protein sequences of *E. faecalis* fab genes to that of *S. pneumoniae* showed that the 12-gene cluster contains all the gene homologs of the *S. pneumoniae* fab gene cluster except fabM, which is an essential isomerase to make unsaturated fatty acids in *S. pneumoniae* (15,23). A separate 3-gene cluster contains homologs of *E. coli* fabI and fabF1, in addition to *FabN*, a fabZ homolog that possess the ability to introduce double bonds into growing acyl chains in vivo (16). FabT, the predicted transcriptional regulator in several Gram-positive bacterial fatty acid biosynthetic pathways (24), is located at the beginning of 12-gene cluster. FabT belongs to the MarR superfamily, which are typically dimers that utilize a winged-helix motif to bind a DNA palindrome (25,26). Often bacterial transcription factors are autoregulated and their DNA binding motifs are located within their own promoter regions. One DNA
palindrome was found in the promoter region of FabT (Fig. 2A). Significantly, this same palindrome is found in the promoters of the fabl, fabF1 and fabK genes (Fig. 2A). Unraveling the transcriptional regulation in this large cluster is beyond the scope of this study. The significance of the bioinformatics analysis is that it ties the 3- and 12-gene clusters for fatty acid biosynthesis and suggests that the fabl, fabF1-fabN and fabK genes may be coordinately regulated.

Purification of EfFabN and EfFabZ—EfFabN and EfFabZ were cloned into pET15b vector and purified as described under "Experimental Procedures." Purified His-tagged EfFabZ and EfFabN had monomer molecular weights of ≈18 kDa based on SDS gel electrophoresis, consistent with their primary sequence (Fig. 3). Recently, the crystal structure of Pseudomonas aeruginosa FabZ was solved, and it forms a classic "trimer of dimers" structure (11). Accordingly, PaFabZ behaves as a hexamer exhibiting a Stoke’s radius on gel filtration chromatography corresponding to a 112 kDa protein (11), while EcFabA is a dimer both in its X-ray structure (10) and in solution. Both EfFabN and EfFabZ are hexamers in solution as determined by gel filtration chromatography (Fig. 3), which illustrates that they have similar structures to PaFabZ.

Enzyme Activity of EfFabN and EfFabZ—We used a complex, multi-component reconstituted fatty acid biosynthetic system described under "Experimental Procedures" to detect the dehydratase and isomerase activities of purified EfFabN and EfFabZ in vitro (Fig. 4). This assay used enzymes from different sources to synthesize substrate for the dehydratases and followed the same principals as previously used in the characterization of the FabA and FabM isomerases (12,15). The identity of the band indicated as β-hydroxy-cis-5-dodecenoyl-ACP was established by mass spectrometry (15). The assay employed the MtbFabH enzyme (from Mycobacterium tuberculosis) to generate β-keto-[14C]decanoyl-ACP from octanoyl-CoA and [2-14C]malonyl-ACP (via EcFadD). The NADPH-dependent EcFabG reduced the intermediate to the substrate for the assays, β-hydroxy[14C]decanoyl-ACP (Fig. 4, lane 1). Addition of SpFabF, the elongation condensing enzyme of S. pneumoniae, to the reaction did not, and should not, yield any new products (Fig. 4, lane 2). The addition of EcFabA (Fig. 4, lane 3) resulted in the conversion of the β-hydroxy intermediate to a mixture of trans-2- and cis-3-dodecenoyl-ACPs. The enoyl-ACP cannot be elongated by the SpFabF condensing enzyme, but the cis-3 intermediate is a substrate. Accordingly, in the presence of SpFabF the cis-3-dodecenoyl-ACP is condensed with malonyl-ACP, and following reduction by EcFabG, a new band appeared on the gel corresponding to β-hydroxy-cis-5-dodecenoyl-ACP (Fig. 4, lane 4). Since this reaction mixture did not contain an enoyl-ACP reductase, additional rounds of elongation did not occur unless this product is isomerized again by FabA. FabA is selective for 10-carbon substrates and characteristically less active with unsaturated β-hydroxy intermediates (12), therefore there is only a trace conversion to β-hydroxy-14:2(Δ5,7)-ACP (the faint band below the β-hydroxy-12:1(Δ5)-ACP). The addition of EcFabZ converted the β-hydroxydecenoyl-ACP to the enoyl-ACP (Fig. 4, lane 5); however, the addition of SpFabF to this reaction did not lead to the appearance of the elongated 12-carbon unsaturated intermediate (Fig. 4, lane 6). The addition of EfFabN to the base reaction yielded enoyl-ACP (Fig. 4, lane 7) and β-hydroxy-cis-5-dodecenoyl-ACP appeared when SpFabF was added (Fig. 4, lane 8). Thus, EfFabN is capable of not only dehydrating β-hydroxydecanoyl-ACP but also isomerizing trans-2-decenoyl-ACP to cis-3-decenoyl-ACP in vitro. On the other hand, EfFabZ dehydrated β-hydroxydecenoyl-ACP (Fig. 4, lane 9) but lacked isomerase activity. These data establish that purified EfFabN is capable of isomerizing the trans-2-enoyl-ACP to cis-3-enoyl-ACP in vitro. This in vitro gel assay system was used to evaluate the relative activities of these two enzymes. The EcFabD/MtbFabH/EcFabG system was used to present EfFabN and EfFabZ with β-hydroxydecanoyl-ACP as a dehydratase substrate. The formation of enoyl-ACP was measured as a function of EfFabN or EfFabZ concentration and a
These data are consistent with the inability of condensing enzymes in the experiment. The formation of an elongation product with excess concentration of spFabF arising from EfFabN isomerase activity as a function of EfFabN in the assay was used to determine the isomerase specific activity of EfFabN (Fig. 5B). The dehydratase activity of EfFabN, 0.07 ± 0.002 pmol/min/μg, was much less than that of EfFabZ which was 2.2 ± 0.07 pmol/min/μg, whereas the dehydratase activities of the two controls, EcFabA and EcFabZ, were 0.34 ± 0.03 pmol/min/μg and 0.36 ± 0.003 pmol/min/μg respectively. Thus, EfFabN had the lowest dehydratase activity of the 4 enzymes in the coupled assay. The isomerase activity of EfFabN for the 10-carbon substrate was 0.6 ± 0.03 pmol/min/μg, while the specific activity of EcFabA under these same assay conditions was 52 ± 1 pmol/min/μg (Fig. 5B). Thus, FabN was 86-fold less efficient than EcFabA in the formation of cis-double bonds in the in vitro fatty acid synthase assay reconstituted with the indicated constellation of E. coli enzymes and ACP cofactor. Also, FabN was about 2-fold less active than the FabM isomerase required for unsaturated fatty acid synthesis in Strepotoccus pneumoniae (15). Although this assay is capable of clearly distinguishing dehydratases from isomerases, the activity of the FabM and FabN isomerases are low in comparison to FabA. The reasons for the low activities of the Gram-positive isomerases in this reconstituted assay are attributed in part to the use of heterologous ACP and elongation condensing enzymes in the experiment. These data are consistent with the inability of fabN expression to complement the growth defect in fabA(Ts) mutants (16).

Chimera Construction and Enzyme Activity—The working hypothesis developed from our structural analysis of EcFabA and PaFabZ (11) was that the isomerase activity depends on the shape of the substrate binding tunnel which is controlled by the positioning of the β-strands (the bun) surrounding the long central helix (the hot dog). The existence of these two closely related FabZ enzymes with different catalytic properties provided an opportunity to test this hypothesis concerning the basis for their distinct catalytic properties. The goal was to determine the minimal structural features required to convert a dehydratase (EfFabZ) into a dehydratase/isomerase (EfFabN). Structural models of EfFabN and EfFabZ were generated using the PaFabZ X-ray structure (11) as the template. There are several amino acid differences between EfFabN and EfFabZ that were predicted to reside in the substrate binding tunnel and which might be responsible for the isomerase activity of EfFabN. These include Asn16; Ile20; Thr57; Ile88; Asn92. We introduced each of these point mutations into EcFabZ and measured their activities. All of the mutants retained dehydratase activity and none acquired the isomerase function (data not shown). The next approach was to construct a series of chimeric EfFabZ/N proteins to determine the minimum structural elements required to transform EfFabZ into an isomerase. The transition points for chimera construction were selected by based upon the structure of PaFabZ (11) and are mapped onto the primary sequences in Fig. 2B. EfFabN and EfFabZ both have high similarity to each other and PaFabZ (EfFabN has 55% similarity and 41.6% identity to PaFabZ; EfFabZ has 56.5% similarity and 43.5% identity to PaFabZ), their structural folding patterns are clearly predicted to be similar to those determined for PaFabZ. The divisions between chimeras were made at the boundary of loops between the β-sheet structures with at least one identical residue in both proteins occurring after the joint (Fig. 2B). Fig. 2C schematically depicts the chimeras made from introducing different EfFabN domains into EfFabZ. All chimeras were hexamers as judged by gel filtration chromatography and retained catalytic activity. First, EfFabN and EfFabZ were divided to two parts, and a swap of the corresponding regions of EfFabN and EfFabZ was made to generate Chimera1 and Chimera2. The analysis of these two proteins showed that both of them retained the ability to dehydrate β-hydroxydecanoyl-ACP to enoyl ACP (Fig. 6, lanes 7 and 8). On the other hand, only Chimera2 containing the C-
terminal part of \( \text{EfFabN} \) possessed 43% the isomerase activity of \( \text{EfFabN} \) as indicated by the formation of the \( \beta \)-hydroxy-cis-5-dodecenoyl-ACP (Fig. 6, lane 8). We then divided the C-terminal part of \( \text{EfFabN} \) into 3 pieces to narrow down the region. Chimera3 and Chimera4 had only dehydratase activity (Fig. 6, lanes 9 and 10), leading to the conclusion that \( \beta_3 \) and the following loop region of \( \text{EfFabN} \) was an essential requirement for isomerase activity. However, the fact that Chimera5 lacked isomerase activity (Fig. 6, lane 11) illustrated that \( \beta_3 \) and following loop was not sufficient to support isomerase activity. The combination of \( \beta_3 \) and \( \beta_4 \) (Chimera6) possessed isomerase activity at 38% of the \( \text{EfFabN} \) level (Fig. 6, lane 12) while the combination of \( \beta_3 \) with \( \beta_5 \) (Chimera7) did not (Fig. 6, lane 13). These data show that the \( \beta_3\beta_4 \) region of \( \text{EfFabN} \) was necessary and sufficient to transform \( \text{EfFabZ} \) from a dehydratase to a dehydratase/isomerase.

The isomerase activity of Chimera6 showed the important role of the combination of the \( \beta_3\beta_4 \) strands in specifying isomerase activity. Two residues, Phe86 from \( \beta_3 \) and Val108 from \( \beta_4 \) in \( \text{EfFabZ} \), were candidates for mediating this effect based on the modeling of \( \text{EfFabN} \) and \( \text{EfFabZ} \) using the structure of \( \text{PaFabZ} \) as a template. The corresponding residues in \( \text{EfFabN} \) are Ile88 and Phe110. In both structures, these two residues sit face to face on the outside of \( \alpha_3 \) helix in a position to modulate the orientation of the helix, therefore, we prepared single and double mutants to replace these residues and assessed the isomerase activity of the constructs. \( \text{EfFabZ[F86I]} \), \( \text{EfFabZ[V108F]} \), and \( \text{EfFabZ[F86I,V108F]} \) were all found to retain dehydratase activity, but all three mutants lacked the ability to isomerize the substrate (not shown). These data suggest that the conversion of \( \text{EfFabZ} \) to \( \text{EfFabN} \) requires the sum of the differences between the two \( \beta_3\beta_4 \) strands, rather than a simple single amino acid substitution.

Conclusions—These data are consistent with the hypothesis that the shape of the substrate binding tunnel is the major determinant of the isomerase activity of the \( \text{FabZ} \) class of \( \beta \)-hydroxyacyl-ACP dehydratases. The replacement of the \( \beta_3\beta_4 \) strands in \( \text{EfFabZ} \) with the corresponding strands from \( \text{EfFabN} \) were the minimal requirements to convert \( \text{EfFabZ} \) from a dehydratase to a dehydratase/isomerase. Molecular modeling of the \( \text{EfFabZ} \) and \( \text{EfFabN} \) clearly indicate that their structures are both very similar to the \( \text{PaFabZ} \) crystal structure. The \( \beta_3\beta_4 \) strands that control the catalytic activity of the enzymes interact with both ends of the central helix \( \alpha_3 \) and the conformation of these strands along this interaction interface determines the orientation of helix \( \alpha_3 \), and hence the shape of the active site tunnel (Fig. 7). Single and double mutants within these strands failed to transform \( \text{EfFabZ} \) into an isomerase, only when the \( \beta_3\beta_4 \) strands were inserted as a unit was isomerase activity reconstituted. The structural analysis of \( \text{FabA} \) and \( \text{FabZ} \) (11) led to the hypothesis that differences in the shapes of the active site tunnels do to the structures of these \( \beta \) strands, rather than active site chemistry, explain why the two enzymes differ in their ability to carry out the isomerization reaction. \( \text{EcFabA} \) (10) and \( \text{PaFabZ} \) (11) possess long, narrow, hydrophobic tunnels which span both monomers. The substrate tunnels both begin at the surface with narrow openings that can be completely occluded by a tyrosine, snake under the N-terminus of the long, central \( \alpha_3 \) helix of the first monomer and extend along the side of the corresponding helix \( \alpha_3 \) from the second monomer on its way back to the surface (Fig. 7). The tunnels are roughly 20 Å long and the catalytic residues are located about half-way down their length. Both proteins have an active site histidine that acts as a general base with the N\( \delta \) atom hydrogen-bonded to a backbone carbonyl oxygen. Both proteins also have catalytic water molecules that are held in place by hydrogen bonds to a backbone amide at the N-terminal end of the central helix and to an acidic residue, Asp84 in \( \text{EcFabA} \) and Glu68 in \( \text{EcFabZ} \). Although the acidic residues are different, they hold the water molecules in essentially the same spatial position within the tunnels. Site-directed mutagenesis swapping the aspartate in \( \text{FabA} \) for the glutamate in \( \text{FabZ} \), and vice versa, do not change the catalytic properties of the proteins.
making it clear that the catalytic machinery is equivalent and capable of carrying out both the dehydratase and isomerase activities (11). Rather, the ability of FabA, but not FabZ, to place the trans-2 substrate in an appropriate conformation to allow isomerization was proposed to arise from the differences in the protein backbones in the distal part of the substrate binding tunnels, encompassing helix α3 and strands β3/β4. Differences in the orientation of helix α3 is the primary structural difference leading to differently shaped active site tunnels (Fig. 7) that in turn position on the substrate in a conformation that in FabA/N, but not FabZ, is appropriate for the isomerization reaction. Strand β4 pushes the α3 helix upward and strand β3 moves in the same direction to allow this movement and form one side of the active site tunnel (Fig. 7). Thus, the catalytic properties of the FabA/Z class of dehydratases is specified by the β3/β4 strands, not the catalytic residues, which alter the shapes of the active site tunnels.

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FOOTNOTES

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1Abbreviations used are: ACP, acyl carrier protein; FabZ, β-hydroxy-acyl-ACP dehydratase; FabA, β-hydroxydecanoyl-ACP dehydratase/isomerase; FabN, the FabZ-like protein of E. faecalis with β-hydroxydecanoyl-ACP dehydratase/isomerase activity. Species-specific proteins are designated with the genus-species abbreviation in italics in front of the protein name such as: EcFabZ for E. coli FabZ and EfFabN for E. faecalis FabN, etc.
TABLE I. Primers and Plasmids used in this study

All primers are listed in the 5′-3′ direction. Words in upper case indicate complementary to the sequence of EfFabN and those in lower case indicate complementary to the sequence of EfFabZ. NdeI and BamHI sites are underlined.

| Primer       | Sequence                              |
|--------------|---------------------------------------|
| EfFabNstart  | CGCCATATGAAAAAAGTAATGACTGCAAC         |
| EfFabNend    | CGCGGATCCCTTCTTATCTGCCCACAAT          |
| EfFabZstart  | CGCCATATGAAATTAACAATTACAGAAAT         |
| EfFabZend    | CGCGGATCCTTTCACCTATCCAATCA            |
| Chr1rev      | ccaccaaaagtatgccgtTTCACCTTCAAATTTGATCC|
| Chr1for      | GGATCAATTTGAAGGTGAaagccatactttgtgg    |
| Chr2rev      | CGGCAATATAGGCGTAtttcccttgAACCagg     |
| Chr2for      | cctgaattcaaaagggaaACAGCCTATATTGGCGG   |
| Chr3rev      | atgtaatttcaagacatgcGCTTGGTATCCCTTTTG |
| Chr3for      | CAAAAGGTAACACCGGCGatgtcttgaaattacat  |
| Chr4rev      | gtcgcatttgccgatgcAGCAAGACGCGGAGCT     |
| Chr4for      | AAGTCCGCCTCTTCTGCTgcatgggaaagcgac     |
| Chr5rev      | CTAAATTAATGTCacccggagccaccttttt      |
| Chr5for      | caaaaaagttgtgtccctgtgGACACATTTATTAG   |
| Chr6rev      | CACACCTTTACCCATTCCgacaaagtcagtaatt   |
| Chr6for      | attacgtgactttgtgcGGAATGGGTAAAGGTG     |
| Chr7rev      | gtcgcatttgccgatgcAGCAAGACGCGGACTT     |
| Chr7for      | AAGTCCGCCTCTTCTGCTgcatgggaaagcgac     |

| Plasmid   | Relevant characteristics            |
|-----------|-------------------------------------|
| pYL3      | pET15b carrying EfFabN             |
| pYL4      | pET15b carrying EfFabZ             |
| pYL5      | pET15b carrying chimera1           |
| pYL6      | pET15b carrying chimera2           |
| pYL7      | pET15b carrying chimera3           |
| pYL8      | pET15b carrying chimera4           |
| pYL9      | pET15b carrying chimera5           |
| pYL10     | pET15b carrying chimera6           |
| pYL11     | pET15b carrying chimera7           |
FIGURE LEGENDS

Fig. 1. The branch point for unsaturated fatty acid synthesis in E. coli and E. faecalis. The key intermediate in anaerobic olefin formation is β-hydroxydecanoyl-ACP. This molecule is dehydrated to trans-2-decenoyl-ACP and then utilized by enoyl-ACP reductase (FabI) and the elongation condensing enzymes to form saturated fatty acids. All of the dehydratases in E. coli and E. faecalis carry out this reaction and all the elongation condensing enzymes function in the production of saturated fatty acids. Unsaturated fatty acids are formed by the isomerization reaction of the EcFabA and EfFabN dehydratases. These specialized enzymes exhibit both dehydratase and isomerase activity against β-hydroxydecanoyl-ACP and form cis-3-decenoyl-ACP, which is elongated by the special activity of either the EcFabB or EfFabF1 elongation condensing enzymes.

Fig. 2. The E. faecalis fatty acid biosynthetic gene clusters and construction of the EfFabZ/EfFabN chimeras. Panel A, The genes required for type II fatty acid biosynthesis are located in two clusters in E. faecalis genome. The thick arrows indicate the relative sizes of the genes. The numbers above the arrows indicate the gene designations in the E. faecalis V583 data base, and the gene names below the arrows indicate the known type II genes that correspond to the open reading frames in the E. faecalis gene clusters (fabl, enoyl-ACP reductase; fabF1, β-ketoacyl-ACP synthase II; fabN, FabZ like β-hydroxydecanoyl-ACP dehydrase/trans-2-decenoyl-ACP isomerase; fabT, a helix-turn-helix DNA binding protein predicted to regulate the gene expression; fabH, β-ketoacyl-ACP synthase III; acpP, ACP; fabK, enoyl-ACP reductase II; fabD, malonyl-CoA:ACP transacylase; fabG, β-ketoacyl-ACP reductase; fabF2, β-ketoacyl-ACP synthase II; accB, acetyl-CoA carboxylase subunit; fabZ, β-hydroxyacyl-ACP dehydratase; accC, accD and accA, subunits of acetyl-CoA carboxylase. The asterisks (*) indicate the location of the palindromes identified in the promoter regions of the fabl, fabF1, fabT, and fabK genes. The alignment of these DNA sequences is shown under the gene organization chart and a consensus sequence is indicated. Panel B, alignment of two E. faecalis FabZ-like proteins. EcFabZ, E. coli FabZ; PaFabZ, Pseudomonas aeruginosa FabZ; EcFabA, E. coli FabA. The identical residues are indicated by black. The secondary structure diagramed on the top is drawn according to PaFabZ structure (11). Panel C, schematic diagram of EfFabZ/EfFabN chimeras. Chimeras were constructed based on the secondary structure predictions illustrated in Panel B and the locations of the junctions are indicated by arrows.

Fig. 3. Purification and apparent molecular weight of EfFabN and EfFabZ. His-tagged EfFabN and EfFabZ were purified as described under “Experiment Procedures.” Analysis of the purified EfFabN and EfFabZ proteins by SDS electrophoresis using a 10% bis-Tris gel (NuPAGE™) is shown in the lower inset. EfFabN and EfFabZ have monomer sizes of ≈18 kDa by SDS gel electrophoresis (lower inset). EfFabN and EfFabZ were applied to a Superdex™ 200 HR 16/60 column (Amersham Biosciences) equilibrated with buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 1 mM DTT) separately. The apparent molecular weights of EfFabN and EfFabZ were estimated to be 102 kDa and 100 kDa respectively by graphic analysis of a standard curve based on the elution volumes of protein molecular markers (Amersham Biosciences) (upper inset). Ferritin (440 kDa); catalase (232 kDa); aldolase (158 kDa), BSA, bovine serum albumin (66 kDa); ovalbumin (43 kDa); chymotrypsinogen (25 kDa); RnaseA (13.7 kDa).

Fig. 4. EfFabN catalyzes the formation of cis unsaturated acyl-ACP intermediates in vitro. The ability of the 6x-His-tag EfFabN and EfFabZ proteins to carry out the dehydratase and isomerase activities was measured using a reconstituted gel assay system. The enzymes used in each lane were indicated on the top. The assays contained the indicated Fab enzymes and were initiated with octanoyl-CoA and [2-14C]malonyl-CoA. The appearance of β-hydroxy-C12:1(Δ5c)-ACP indicated the ability of the dehydratase to isomerize C10:1(Δ2t)-ACP to C10:1(Δ3c)-ACP, which was elongated by SpFabF.
**Fig. 5. Specific activities of EfFabN and EfFabZ.** The specific dehydratase activities (Panel A) assay were performed using β-hydroxy-C10-ACP as substrate, and the isomerase activities (Panel B) were analyzed using trans-2-C10:1-ACP as substrate. The coupled assay using a series of Fab enzymes to generate the substrates was performed in the presence of different concentrations of either EfFabN or EfFabZ as described under “Experimental Procedures.” Radiolabeled product formation was quantitated using a phosphorimager. A standard curve of [14C]malonyl-CoA was used to calibrate the instrument.

**Fig. 6. Enzyme activities of the FabZ/FabN chimeric proteins.** The enzymes used in the assay were indicated on the top, and the formation of β-OH-C12:1(Δ5c) indicated the isomerase activity of the chimera. Assays were performed and analyzed as described under “Experimental Procedures.”

**Fig. 7. Differences in the position of helix α3 in FabZ and FabA.** The crystal structure of FabZ (pdb code 1U1Z) from *P. aeruginosa* shows the location of helix α3 (hot dog) relative to β-strands β3 and β4 within the curved β-sheet (the bun) which is characteristic of isomerase-negative dehydratases. Helix α3 is shown in orange, the β-sheets are yellow, and β3 and β4 sheets are magenta. The active site residues that span the interface with the associated monomer (blue/green) are indicated. Helix α1 would obscure α3 in this orientation has been removed for clarity. Superimposed on the FabZ structure are the helix α3 from the *E. coli* FabA structure (transparent) and the bound inhibitor (grey) that was captured in the complex (pdb code 1MKA). FabZ and FabA were structurally aligned based on their β-sheets, and this alignment reveals the subtle but significant differences in the placement of helices α3 in their respective buns. The acyl chain of the inhibitor indicates the location of the active site tunnel whose shape is modulated by the position of helix α3. The figure was produced using MOLSCRIPT (27) and rendered with RASTER3D (28).
Figure 1

Unsaturated Fatty Acids

Saturated Fatty Acids

EffFabN
EffFabZ
EcFabA
EcFabZ
EffFabN
EffFabF1
EcFabB
EffFabF1
EcFabF
EffFabF2
EcFabB
EcFabF
EffFabF1
EcFabB
EffFabF1
EcFabB
EffFabF
EffFabF2
EcFabB
EcFabF
EffFabF1
EcFabB
EffFabF
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EcFabB
EcFabF
EffFabF1
EcFabB
EffFabF
EffFabF2
EcFabB
EcFabF
EffFabF1
EcFabB
EffFabF
EffFabF2
EcFabB
EcFabF
Figure 2
Figure 3
| Protein   | Ac | Cc | Ac | Cc | Ac | Cc | Ac | Cc | Ac | Cc |
|-----------|----|----|----|----|----|----|----|----|----|----|
| EcFabD    | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| MlfabH    | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| EcFabG    | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| SpFabF    | -  | +  | -  | +  | -  | +  | -  | +  | -  | +  |
| EcFabA    | -  | -  | +  | +  | -  | -  | -  | -  | -  | -  |
| EcFabZ    | -  | -  | -  | -  | +  | +  | -  | -  | -  | -  |
| EfFabN    | -  | -  | -  | -  | -  | -  | +  | +  | -  | -  |
| EfFabZ    | -  | -  | -  | -  | -  | -  | +  | +  | -  | +  |

**Figure 4**
Figure 5

A

Protein (ng)

decenoyl-ACP (pmole/min)

B

Protein (μg)

β-OH dodecanoyl-ACP (pmol/min)

EffFabN
EffFabZ

EffFabN
EffFabZ
| Acyl-ACPs | EcFabD | MfFabH | EcFabG | SpFabF | EfFabN | EfFabZ | Chimera1 | Chimera2 | Chimera3 | Chimera4 | Chimera5 | Chimera6 | Chimera7 |
|----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| β-OH C10:0 | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     |
| β-OH C12:1(Δ5c) | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     |
| C10:1(Δ2t/3c) | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     |
| 1 2 3 4 5 6 7 8 9 10 11 12 13 |

**Figure 6**
Domain swapping between enterococcus faecalis FabN and FabZ proteins localizes the structural determinants for isomerase activity
Ying-Jie Lu, Stephen W. White and Charles O. Rock

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