Escherichia coli FabG 3-ketoacyl-ACP reductase proteins lacking the assigned catalytic triad residues are active enzymes

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The FabG 3-ketoacyl-acyl carrier protein (ACP) reductase of Escherichia coli has long been thought to be a classical member of the short-chain alcohol dehydrogenase/reductase (SDR) family. FabG catalyzes the essential 3-ketoacyl-ACP reduction step in the FAS II fatty acid synthesis pathway. Site-directed mutagenesis studies of several other SDR enzymes have identified three highly conserved amino acid residues, Ser, Tyr, and Lys, as the catalytic triad. Structural analyses of E. coli FabG suggested the triad S138-Y151-K155 to form a catalytically competent active site. To test this hypothesis, we constructed a series of E. coli FabG mutants and tested their 3-ketoacyl-ACP reductase activities both in vivo and in vitro. Our data show that plasmid-borne FabG mutants, including the double and triple mutants, restored growth of E. coli and Salmonella enterica fabG temperature-sensitive mutant strains under nonpermissive conditions. In vitro assays demonstrated that all of the purified FabG mutant proteins maintained fatty acid synthetic ability, although the activities of the single mutant proteins were 20% to 50% lower than that of wildtype FabG. The S138A, Y151F, and K155A residue substitutions were confirmed by tandem mass spectral sequencing of peptides that spanned all three residues. We conclude that FabG is not a classical short-chain alcohol dehydrogenase/reductase, suggesting that an alternative mode of 3-ketoacyl-ACP reduction awaits discovery.

The architecture of fatty acid synthase (FAS) has three forms (1–3). Mammalian fatty acid synthase (FAS I) consists of two copies of a single large multifunctional polypeptide derived from a single gene. This structure contains all the active sites and performs all the steps in the synthetic pathway, whereas in yeast and mycobacteria the FAS I enzymes are encoded as two different polypeptide proteins. Type II bacterial fatty acid synthases (FAS II) are composed of multiple separate enzymes, with each enzyme encoded as a separate open reading frame. Each enzyme catalyzes a discrete step in the pathway. These fundamental differences between the FAS types aids the development of antibacterial agents, although, as yet, none have been of wide clinical utility (1, 4–6).

The bacterial 3-ketoacyl-ACP reductase (FabG) catalyzes the essential keto reduction step in the elongation cycle of FAS II (1, 2) (Fig. 1A). This enzyme is the only known isozyme of this type in bacteria and is highly conserved and ubiquitously expressed in bacteria (2, 7, 8). Thus, FabG has become the focus of numerous attempts to develop antimicrobials (4, 6, 9, 10). Based on its sequence and structure FabG has long been considered a canonical member of a very large family of enzymes, the short-chain alcohol dehydrogenase/reductase (SDR) family, enzymes that perform a wide variety of reduction and dehydrogenation reactions adding or removing hydrogen in a NAD(H)- or NADP-(H)-dependent manner from specific substrates (1, 2, 8). The SDR proteins are generally proteins of 25 to 35 kDa that function as dimers or tetramers (11–13). Although only 15% to 30% sequence identity exists among different SDR proteins, the SDRs share select distinct sequence motifs such as the N-terminal coenzyme binding site Gly-Xaa3-Gly-Xaa-Gly and the centrally located catalytic site Tyr-Xaa3-Lys. Mutagenesis of other SDRs has identified Tyr, Lys, and Ser as the catalytic triad residues. The Tyr side chain functions as the catalytic base, the Ser side chain stabilizes the substrate, and the Lys side chain interacts with the nicotinamide ribose and lowers the pKa of the Tyr-OH (11, 12). Note that SDRs have been divided into five types: classical, extended, intermediate, divergent, and complex by Oppermann, Jornvall, and colleagues (11–13). The most recent review is 12 years old, and, given the rapid expansion of the databases, there may be additional types. Hence, SDRs are rather a hazy grouping. Several sequence signature motifs, most of which are parts of the Rossmann fold, can be identified (Fig. 1) (11–13).

Structural studies of Escherichia coli FabG suggested it to be a typical example of an SDR protein. This enzyme of 244
E. coli FabG reductase

A

\[
\text{NADPH} + \text{H}^+ + 3\text{-ketoacyl-ACP} \rightarrow \text{FabG} \rightarrow \text{NADP}^+ + 3\text{-hydroxyacyl-ACP}
\]

B

MNFEGKALV\_GASRGRGK\_DAETLAARGAVKITSGENQAISDYLNGKGLMLNVTDEG62+
+ PASIESLEKRAFEQEGVDLNYNAGITRDNLMRIMKDEENDITEIYNLSSFLVSKAVORMAM625+
+ MKXXHGRITGSGVVTGNGGGYHQAAAKGLIGFSGKLRVARSGTVNVRAPGDHEID9E89+
+ MTRALSQVRAGLQAQVPAGRLGQAQFANAVALFASDEAAYITGETLHVNGGMYMV244++

C

Figure 1. The 3-ketoacyl-ACP reductase reaction, the amino acid sequence of E. coli FabG, and the postulated catalytic mechanism of FabG. A, the NADPH-dependent reduction of 3-ketoacyl-ACP catalyzed by 3-ketoacyl-ACP reductase. B, the amino acid sequence of E. coli FabG. The cofactor binding sequence (Gly motif [GlyXXXGlyXGly]) is boxed. Asterisks highlight the putative catalytic triad of FabG (S138, Y151, and K155). C, The putative FabG catalytic mechanism (15). ACP, acyl carrier protein.

amino acid residues is tetrameric and has an α/β structure with the signature Rossmann fold motif (2, 8, 14–17). FabG also contains the highly conserved Y151-XXX-K155 motif, near the carboxy terminus of helix α4 (Fig. 1B). In addition, FabG has a conserved serine residue (S138) located near the tyrosine and lysine residues. Price and colleagues (8, 15) postulated that E. coli FabG has a catalytic mechanism similar to that of other SDR enzymes, although they observed that cofactor binding caused a significant conformational change in FabG that buried the signature lysine residue (K155). They proposed that, in E. coli FabG, protons are shuttled via a water network containing the highly conserved Y151-Xaa3-K155 motif, near the signature Rossmann fold motif (2, 8, 14). In E. coli FabG, protons are shuttled via a water network containing the highly conserved Y151-Xaa3-K155 motif, near the signature Rossmann fold motif (2, 8, 14–17). (Fig. S1). We turned to E. coli FabG because it is considered a classical SDR protein and is the subject of several crystal structure studies and genetic analyses. The R. solanacearum results suggested that the postulated E. coli FabG catalytic triad may not be active site residues. We report that substitution of the putative E. coli FabG residues S138, Y151, and K155 with A, F, or A resulted in proteins that retained FabG activity both in vivo and in vitro.

Results

Analysis of the FabG mutants in vivo

To test whether the S138-Y151-K155 motif of E. coli FabG is the catalytic triad of the enzyme, panels of E. coli fabG mutants were constructed on plasmids of high copy and low copy numbers. The plasmids were tested for complementation using two temperature-sensitive mutant strains that lack 3-ketoacyl-ACP reductase activity at 42 °C. We first used E. coli strain CL104 and subsequently switched to Salmonella enterica serovar Typhimurium LT2 strain CL95 (7). (The use of conditionally mutant strains is required since FabG is an essential enzyme in E. coli.) We first substituted FabG S138 with A or T, Y151 with F or H, and K155 with T or A and inserted these mutant fabG genes into the high-copy-number vector pBAD24M to produce six plasmids (pTWH22, 23, 24, 25, 29, and 30). Transcription of the genes proceeded from the arabinose-regulated araBAD promoter in these plasmids. The vector also supplied the ribosome-binding site necessary for translational initiation. Following transformation into strain CL104, all of the resulting transformants were found to grow at 42 °C in the presence or absence of arabinose (Fig. S2, A–C) as previously seen with the R. solanacearum FabG1 (Fig. S1). In addition, all transformants grew better in the absence than in the presence of arabinose suggesting that FabG overexpression is somewhat toxic (Fig. S2, A–C). These data argued strongly that these mutant fabG genes encode functional 3-ketoacyl-ACP reductases and that the S138-Y151-K155 motif is not the E. coli FabG catalytic center. To strengthen this argument, we constructed six additional FabG mutant plasmids: Y151S, Y151I, Y151R, K155R, K155I, and K155E. These mutant proteins all remained active in vivo (Fig. S2, D and E).

To exclude the possibility that combinations of two or three of these residues were essential for FabG activity, additional mutant plasmids were constructed including three double mutants (S138A/Y151F, S138A/K155A, Y151F/K155A) and the triple mutant (S138A/Y151F/K155A). Upon transformation of the resulting plasmids, pTWH38 (S138A/Y151F double mutant), pZH197 (S138A/K155A double mutant), pZH198 (Y151F/K155A double mutant), and pZH199 (S138A/Y151F/K155A triple mutant), into the E. coli strain CL104, all conferred the ability of the mutant strain to grow in the presence or absence of arabinose at 42 °C (Fig. S2F).

To investigate the possibility that complementation of E. coli strain CL104 resulted from elevated expression of the FabG mutant proteins, three single mutant fabG genes, three doubly mutant genes, and one triple mutant gene were
We next tested the possibility that growth was due to homologous recombination between the plasmid-borne mutant genes and the chromosomal gene. In this scenario the two mutant alleles, the temperature-sensitive or wildtype alleles encoded on the genome and the residue substitution allele mutant alleles, the temperature-sensitive or wildtype alleles genes and the chromosomal gene. In this scenario the two recombination is equivalent to that given by loss of RecA procedures and refolded on the puriﬁcation column. FabG refolded with high efﬁciency (probably owing to spacing of the proteins on the columns preventing aggregation); the refolded wildtype protein had full activity.

In later work we used the pQE2 vector, which allowed high-level expression in the S. enterica fabG(Ts) strain CL65 (7), and puriﬁed the proteins by denaturation–renaturation as given above. Use of this expression system had the advantage of precluding homologous recombination, and since each protein contains a lysine residue absent in the other protein (K72 of E. coli FabG, K43 of S. enterica FabG) this provided an assay to detect the presence of any S. enterica protein in our puriﬁed mutant protein preparations. This was done by trypsin digestion of the proteins followed by HPLC peptide separation and tandem mass spectral analyses of the recovered peptides. Analyses using the exponentially modiﬁed Protein Abundance Index, which denotes the ratio of observed to observable peptides, showed that the Y151F preparation contains less than 0.5% of the S. enterica FabG, whereas the S138A and K155A preparations contain only about 0.14% and 0.19% S. enterica FabG, respectively. The triple mutant E. coli FabG contained only 0.6% of the S. enterica FabG. In each case at least 93% of the possible peptides were observed experimentally.

Finally, fragmentation by tandem mass spectroscopy (MS/MS) was used to sequence tryptic peptides that span the three
E. coli FabG reductase

Figure 3. Fatty acid biosynthesis from [1-14C]acetate in the E. coli fabG(Ts) mutant strain CL104 carrying fabG mutant genes at the nonpermissive temperature. Phosphorimage of argentation thin-layer chromatographic analysis of [1-14C]acetate-labeled E. coli fabG(Ts) strain CL104 carrying the pZH201 (wildtype FabG gene, lane 1), pZH200 (pHSG575, lane 2), pZH208 (FabG S138A, Y151F, and K155A triple mutant, lane 3), pZH206 (FabG S138A and K155A double mutant, lane 4), pZH205 (FabG S138A and Y151F double mutant, lane 5), pZH207 (FabG Y151F and K155A double mutant, lane 6), pZH202 (FabG S138A, lane 7), and pZH203 (FabG Y151F, lane 8) plasmid. The migration positions of the methyl esters of the fatty acids are shown. SFA, saturated fatty acid ester; UFA, unsaturated fatty acid ester.

The putative active site residues of the triple mutant protein. Figure 4A shows MS/MS sequencing of a peptide that contained all three assigned active site residues. Fragments that thoroughly documented the Y151F and K155A substitutions were present in this spectrum, whereas cleavages to document the S138A substitution were sparse. However, a longer peptide (owing to lack of trypsin cleavage at R132 and K163) gave fragments that documented the S138A substitution (Fig. 4B).

Figure 4. Tandem MS sequencing of triple mutant FabG peptides. The spectra were chosen based on Mascot quality (141 in A, 73 in B) and coverage from the amino (b ions) and carboxyl (y ions) ends of the peptide. A, the I133 to K163 peptide. B, the H130 to R167 peptide due to partial trypsin digestion. Note that MS/MS does not degrade peptides sequentially from the ends but rather somewhat randomly such that b and y ions overlap. The fragments shown in red are those used in identification.

These data were confirmed by Sanger sequencing of the relevant portion of the triple mutant fabG gene (Fig. S4). These data demonstrated that all three expected residue substitutions had been made. Tandem MS sequencing of each of the singly mutant proteins also showed the expected residue substitutions (Fig. S5). Therefore, the putative active site residues had all been eliminated. These protein preparations were utilized in Figure 5 and for the kinetic analyses.

To determine the in vitro 3-ketoacyl-ACP reductase activities of the FabG proteins wildtype fabG, three fabG single mutants (S138A, Y151F and K155A), three double fabG mutants (S138A/Y151F, S138A/K155A, and Y151F/K155A), and one triple fabG mutant (S138A/Y151F/K155A) were inserted into vector pET28. The amino terminal hexahistidine-tagged wildtype and mutant FabG proteins were then expressed in E. coli strain BL21 (DE3) and purified by nickel chelation chromatography under denaturation conditions and refolded. SDS-gel electrophoresis showed that all of the N-terminal Histagged proteins were highly purified and had the expected molecular weights (Fig. S6A).

The enzymatic activity of the wildtype FabG and mutant FabG proteins was first tested in the initiation step of fatty acid synthesis. The reactions were constituted by sequentially adding initiation enzymes FabD, FabH, FabG or a mutant FabG (FabG S138A, FabG Y151F, and FabG K155A), FabA, and FabI, followed by analysis by conformationally sensitive gel electrophoresis (FabA and FabI were added to stabilize the short-chain products). In the absence of FabG, only the holo-ACP substrate was detected (Fig. 5A, lane 7). After the addition of wildtype FabG (Fig. 5A, lane 2), FabG S138A (lane 3), FabG Y151F (lane 4), FabG K155A (lane 5), or FabG S138A/Y151F/K155A (lane 6) to the reaction, butyryl-ACP was produced. However, the activities of FabG S138A, FabG Y151F, and FabG K155A were lower than that of the wildtype FabG protein. These data showed that the mutant FabG proteins S138A, Y151F, K155A and triple mutant, like the wildtype FabG, could complete the initial cycle of fatty acid synthesis to produce butyryl-ACP.
A more direct assay of the enzymatic activities of the mutant FabG proteins in reduction of a long-chain 3-ketoacyl-ACP was performed. We synthesized malonyl-ACP and octanoyl-ACP as described in “Experimental procedures.” As expected, the incubation of FabB with malonyl-ACP and octanoyl-ACP resulted in the formation of 3-ketodecanoyl-ACP (Fig. 5B, lane 7) (the longer-chain species are more stable to electrophoresis). Upon the addition of NADPH, FabG (or one of the mutant FabG proteins), and EcFabA to the reaction mixture (lanes 1–6 of Fig. 5B), all incubations yielded the expected enoyl-ACP species. FabG converted 3-ketoacyl-ACP to 3-hydroxacyl-ACP, whereas EcFabA dehydrated 3-hydroxacyl-ACP to enoyl-ACP. Therefore, these data showed that all of the mutant FabG proteins were active with long-chain 3-ketoacyl-ACP substrates.

We also tested the function of the double mutant FabG proteins in fatty acid biosynthesis reactions in vitro. The data (Fig. S6) showed that all these mutant FabG proteins were active in in vitro fatty acid biosynthesis, although the activity of the triple mutant protein was much lower than that of the wildtype FabG in the initial cycle of fatty acid synthesis (Fig. S6).

**Kinetic analysis of the wildtype and single mutant FabG proteins**

Acetoacetyl-CoA, an unnatural substrate, has often been used in place of acetoacetyl-ACP to assay the activity of FabG 3-ketoacyl-ACP reductase and to determine the kinetic constants of this enzyme (20, 21). However, we failed to detect any activity of the mutant FabG proteins using acetoacetyl-CoA as the substrate, although the wildtype FabG was active (data not shown). We therefore turned to an acyl-ACP-dependent spectroscopic assay to determine the kinetic constants of the wildtype and single mutant FabG proteins for NADPH binding. As shown in Table 1, the mutant FabG proteins Y151F (147.7 μM) and K155A (166.5 μM) had higher KM values than the wildtype FabG (99.2 μM) for NADPH binding, whereas the mutant FabG protein S138A had a KM value (101.5 μM) almost identical to that of the wildtype FabG. Our data also showed that the Kcat values of these three mutant enzymes were similar to that of the wildtype FabG, although all mutant FabG proteins exhibited a slightly lower activity than that of wildtype FabG. As a result, although these mutant FabG proteins had somewhat increased KM values, they retained high 3-ketoacyl-ACP reductase activity.

**Circular dichroism spectroscopy analyses of the wildtype FabG and mutant FabG proteins**

Circular dichroism (CD) spectroscopy analysis is an excellent tool for the rapid determination of the secondary structure and folding properties of proteins. To investigate the effect of the mutations on the FabG structure, we investigated the changes of the physicochemical characteristics of FabG caused...
by three single mutant FabG proteins (S138A, Y151F and K155A) through CD spectroscopy analyses. The CD spectra of the wildtype FabG and mutant FabG proteins exhibited a characteristic signature of a helix with minima at 208 and 222 nm, respectively (Fig. S7A). The helical contents of the S138A, Y151F and K155A mutants and the wildtype FabG were 32.2%, 42.6%, 47.1%, and 33.2%, respectively. The helical content of the mutants Y151F and K155A was much higher than that of wildtype FabG, whereas the helical content of the mutant S138A was almost identical to that of the wildtype FabG. We also assayed the helical contents of the three double mutant FabG proteins (S138A/Y151F, S138A/K155A and Y151F/K155A) and the triple mutant FabG protein (S138A/Y151F/K155A). The helical contents of these mutant proteins were much higher than that of the wildtype FabG (Fig. S7B). These results, taken together, showed that the introduced mutations at the sites of S138, Y151, and K155 caused conformational changes in *E. coli* FabG.

The presence of NADPH has been suggested to affect the conformation of FabG (22). Our results (Fig. S7B) showed that the molar ellipticity of wildtype FabG and all mutant FabG proteins increased when NADPH (200 μM) was added. This observation is in agreement with previous studies (22).

### NADPH binding to wildtype FabG and mutant FabG proteins

The spectral parameters of tryptophan fluorescence, position, shape, and intensity are dependent on the electronic and dynamic properties of the chromophore environment. Therefore, tryptophan fluorescence has been extensively applied to obtain information on the structural and dynamic properties of proteins. Tryptophan is fluorescent with an emission \( \lambda_{\text{max}} \) at 325 nm at pH 7.0. Buried tryptophan residues in a variety of folded proteins show a red or blue shift at the emission maximum. The *E. coli* FabG molecule contains a single tryptophan residue at position 103 and displays intrinsic fluorescence with an emission \( \lambda_{\text{max}} \) at 363 nm (Fig. 6A). To investigate the effect of the mutated S138, Y151, and K155 residues on the FabG structure, the fluorescence spectra of the wildtype FabG and mutant FabG proteins were studied by exciting the samples at 280 nm and recording the emission spectrum over a range of 300 to 500 nm. As shown in Figure 6A, the fluorescence spectra of the wildtype FabG and mutant FabG proteins exhibited the characteristic signature of an emission \( \lambda_{\text{max}} \) at 363 nm. Mutant K155A also displayed increased fluorescence intensity at 363 nm, whereas the mutant S138A and Y151F resulted in decreased fluorescence intensity.

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**Table 1**

| FabG       | \( K_M \) (μM) | \( K_{cat} \) (min\(^{-1}\)) | \( K_{cat}/K_M \) (μM\(^{-1}\) min\(^{-1}\)) | Relative activity % |
|------------|----------------|-----------------|----------------------------------|--------------------|
| Wildtype   | 99.22          | 2.74            | 27.66                            | 100                |
| S138A      | 101.47         | 2.31            | 22.84                            | 82                 |
| Y151F      | 147.72         | 2.45            | 16.57                            | 59                 |
| K155A      | 166.45         | 2.31            | 13.89                            | 50                 |

\( K_M \) denotes the Michaelis constant, \( K_{cat} \) is the turnover number, \( K_{cat}/K_M \) denotes the catalytic efficiency of the enzyme. Relative activity is the ratio of \( K_{cat}/K_M \) value of each mutant protein to that of wildtype FabG as 100%. These proteins were puriﬁed from BL21(DE3) and puriﬁed by Ni chelate chromatography under denaturing conditions and refolded.

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Price et al. (15) observed that *E. coli* FabG exerted a conformational change when binding its cofactor NADPH and suggested that NADPH binding conferred on FabG the ability to exhibit a slight negative homotropic cooperativity (with a Hill constant of \( n_H = 0.9 \)). We investigated the effect of the mutations on the NADPH binding of FabG by determining the affinity of FabG for NADPH by fluorescence spectroscopy. Owing to tryptophan fluorescence quenching at 363 nm, the fluorescence intensity of the wildtype FabG and mutant FabG proteins decreased as a function of the increasing concentrations of NADPH (Fig. 6B). Because of the enhancement of NADPH fluorescence at 450 nm, the fluorescence intensity of NADPH binding by wildtype FabG, S138A, or Y151F increased as a function of increasing concentrations of NADPH. The shape of the binding curve for S138A and Y151F saturated at a lower NADPH concentration, whereas the fluorescence intensity of the NADPH-binding K155A was not changed as the concentration of NADPH increased (Fig. 6B). Analysis of the binding curves showed that wildtype FabG and mutant proteins S138A and Y151F could bind NADPH with \( K_d \) values of 13.22, 13.89, and 15.75 μM, respectively, and that the fluorescence intensity of NADPH binding was maximized at 312.5, 56.17, and 126.58 μM, respectively. These data suggested that, although wildtype FabG and the mutant FabG S138A and Y151F had similar \( K_d \) values for NADPH binding, the mutant proteins had lower fluorescence intensity than wildtype FabG. Therefore, the mutant proteins S138A and Y151F had a decreased affinity for NADPH binding. For mutant protein K155A, no significant NADPH binding was detected at the concentrations that could be tested with this assay, suggesting that the K155A mutation may have resulted in loss of *E. coli* FabG cooperative behavior toward NADPH.

### Discussion

SDR NAD(P)(H)-dependent oxidoreductases are a very large protein family (perhaps 50,000 members). The Y151-Xaa3-K155 (numbering of *E. coli* FabG) segment is one of the distinct sequence motifs of SDR family enzymes (11, 13). S138 is also conserved in most SDR proteins (11). Thus, a triad of S138, Y151, and K155 was proposed to be the catalytic site of SDR enzymes (11). The first evidence supporting Y151 and K155 as SDR family catalytic site residues came from site-specific mutagenesis analyses of the *Drosophila* alcohol dehydrogenase (23, 24). When Y151 was substituted with F, H, or E and K155 was substituted with Ile, the resulting mutants lacked catalytic activity. To establish S138 as a member of a
catalytically important “triad” of residues also involving Y151 and K155, Oppermann (25) exchanged S138 with A or T in the 3β/17β-hydroxysteroid dehydrogenase from Comamonas testosteroni and found that a Ser-to-Ala exchange at position 138 resulted in an almost complete (>99.9%) loss of enzymatic activity that was not observed with an S-to-T replacement (25). Additional evidence supporting the S138, Y151, and K155 triad as the catalytic site of SDR enzymes was provided by site-specific mutagenesis analysis of the E. coli 7 α-hydroxysteroid dehydrogenase (26).
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However, there are examples of atypical SDRs that retain SDR structure but lack canonical active site triad residues. A recent example is a Vibrio vulnificus protein of unknown function that is tetrameric and has SDR topology [32]. The protein binds NADPH tightly and with extreme specificity but lacks the putative triad tyrosine and lysine residues found in FabG. The authors postulate another tyrosine-dependent site, but since the substrate of the reaction is unknown, testing of the postulated site was precluded. Another example of an atypical SDR is human peroxisomal enoyl CoA reductase where the active site Y is replaced by an F residue [27]. To our knowledge, although various alternative active site residues have been proposed for a few atypical SDR proteins, we can find no tests of these hypotheses. A problem is that many proteins annotated as atypical SDRs result from high-throughput crystallography consortia. The proteins are assigned only by their conserved structures and lack of canonical active site residues. The reactions catalyzed are unknown.

We also observed that the Pseudomonas aeruginosa fabV gene, encoding a new isoform of SDR enoyl-ACP reductase, remained able to complement an E. coli fabI temperature-sensitive mutant strain upon substitution of Y235 with F or K244 with M [28].

We considered mutating the three FabG Tyr residues other than Y155, but these residues are located well away from the NADPH cofactor and two of the three seem involved in interface interactions (FabG is a dimer of dimers, hence two different interfaces are present). The Ts mutants we isolated previously all mapped in interface regions showing the very strong interface dependence of this enzyme activity [17]. The third Tyr is on the surface and exposed to solvent. Attempts to deduce a mechanism are hindered by the lack of information on the location of the acyl chain of the 3-ketoacyl-ACP in the structure. Since FabG is required for elongation of C4 to C16 acyl chains, we would expect to see a long hydrophobic tube or cleft in the structure (as seen for other fatty acid elongation cycle enzymes) but the prior crystal structures give no hints of how acyl chains are bound. A known acyl chain binding site would focus the active site hunt. Untargeted mutagenesis of a plasmid-borne fabG seems likely to be unrewarding because the interfaces are significantly larger targets for mutation than the active site.

E. coli FabG seemed a typical example of an SDR protein and contains the highly conserved triad of S138-Y151-K155 [8] and the triad was assumed to be the catalytic site of FabG. Previously, Price and colleagues [8, 15] determined the structure of FabG and the mutant FabG Y151F and suggested that cofactor binding induced a conformational rearrangement of FabG, causing the triad of S138-Y151-K155 to establish a catalytically competent active site. In addition, the mutant FabG Y151F lost the ability to reduce acetoacetyl-CoA in vitro [8, 15]. In this regard, recall that none of our mutant proteins could reduce acetoacetyl-CoA, although they were active with the natural substrate, acetoacetyl-ACP. Therefore, based on the acetoacetyl-CoA result Price and coworkers reported that the S138-Y151-K155 triad comprised the FabG catalytic center (8, 15). However, no additional experimental evidence was provided to support this hypothesis.

In conclusion we have demonstrated that substitution of E. coli FabG residues S138, Y151, and K155 with a variety of other residues failed to inactivate the enzyme. We have thoroughly characterized the S138A, Y151F, and K155A substitutions as single mutant, double mutant, and the triple mutant. All mutant sequences were confirmed by DNA sequencing of plasmids extracted from the cell pellets of cultures grown for protein production. Moreover, the relevant tryptic peptides of the S138A, Y151F, and K155A single mutant proteins plus the triple mutant peptide were sequenced by tandem mass spectroscopy (MS/MS). The MS/MS results directly demonstrated that each of the designed residue substitutions had been made. We have ruled out contamination of our purified proteins with recombinant species resulting from genetic recombination or formation of tetramer species containing a mixture of plasmid and chromosomally encoded proteins. The fabG(Ts) strain CL65 of S. enterica serovar LT2 prevented genetic recombination and also provided a heterologous host with a distinguishable FabG sequence that could be used to assay for mixed tetramers by tandem mass spectroscopy. Urea denaturation and subsequent renaturation of the column-bound proteins also precluded assay of mixed tetramers. We believe that we have eliminated each of the possible pitfalls and therefore conclude that E. coli FabG does not catalyze the reduction of 3-ketoacyl-ACPs using the classical S-Y-K triad.

Experimental procedures

Bacterial strains and growth media

The E. coli strains and plasmids are listed in Table S1. Luria-Bertani (LB) medium was used as the rich medium for the E. coli strains. The phenotypes of the E. coli fab strains were assessed on rich broth medium [29]. Antibiotics were used at the following concentrations (in μg/mL): sodium ampicillin, 100; kanamycin sulfate, 30; chloramphenicol, 30; and gentamicin, 20. L-Arabinose was used at a final concentration of 0.02%. Isopropyl-β-D-thiogalactoside (IPTG) was used at a final concentration of 1 μM, and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was used at a final concentration of 20 μg/mL.

Recombinant DNA techniques and construction of plasmids

The E. coli fabG gene was amplified using the genomic DNA of E. coli MG1655 as the template. The PCR amplification was performed with Pfu DNA polymerase and two primers, EcfabF-Ndel and EcfabG-R-HindIII, as listed in Table S2. The PCR product was inserted into the T-vector plasmid pMD19 to produce plasmid pTWH1. All mutated fabG genes were prepared by overlapping PCR using pTWH1 as the template, and the mutagenic primers are listed in Table S2. The mutated fabG genes were inserted into the T-vector plasmid pMD19. All cloned fabG sequences were confirmed by sequencing performed by Shanghai Sangon, Inc. The fabG gene fragments digested from the T-vectors with Ndel and HindIII were gel purified and ligated into pBAD24M (28).
pET28(b) or pQE2, which was digested with the identical enzymes, to produce the plasmids listed in Table S1. To construct the pHSG575 series of fabG expression vectors, the fabG genes were digested from the pBAD24M series fabG expression vectors by BamH1 and HindIII and were inserted into the identical sites of pHSG575. To facilitate screening, a 750-bp gentamicin resistance cassette was obtained from plasmid p34s-Gm by HindIII digestion and inserted into the identical sites of the above intermediate plasmids.

**Expression and purification of hexahistidine-tagged proteins**

The pQE2 plasmids encoding fabG mutant proteins were transformed into *S. enterica* serovar LT2 mutant strain CL65 for expression of the mutant FabG proteins at a high level upon IPTG induction (protein production by the phage T5 pQE promoter rivals that of the pET system). The FabG wildtype and mutant proteins were purified as follows. In total, 1 l of LB cultures of FabG expression strains was collected by centrifugation (4000g, 4 °C, 20 min) after IPTG induction at 30 °C for 3 h. The cells were suspended in 10 ml of lysis buffer (100 mM NaH2PO4, 300 mM NaCl, pH 8.0, and 8 M urea) and were disrupted by two passages through a French press cell at maximum pressure. The soluble cell extract was obtained by centrifugation at 20,000g for 20 min. The supernatant was applied to a Ni-NTA agarose-containing column and mixed gently for 1 h at 4 °C. The bottom cap of the column was removed, and the flow-through was collected. After washing with buffer (100 mM NaH2PO4, 300 mM NaCl, pH 8.0) containing a step-gradient concentration of urea (8–0 M) at 4 °C, the lysate-resin was washed twice using an identical buffer plus 20 mM imidazole. The His-tagged proteins were eluted with the elution buffer (100 mM NaH2PO4, 300 mM NaCl, and 250 mM imidazole, pH 8.0) and then dialyzed overnight at 4 °C against 4 L of buffer (100 mM NaH2PO4, 300 mM NaCl, pH 8.0). The purified proteins were stored at −80 °C with addition of 20% glycerol at final concentration. The mutant proteins are also expressed and purified from BL21(DE3) using as described above. We also purified the *E. coli* fatty acid synthetic proteins (FabB, FabH, FabG, FabZ, FabB, FabI, and holo-ACP) and *Vibrio harveyi* acyl-ACP synthetase (AasS) as described (28, 30).

**Analysis of fatty acid compositions**

The ability of the FabG mutants to restore fatty acid synthesis was tested in the *E. coli* fabG(Ts) mutant strain CL104. Briefly, the IPTG-inducible vector pHSG575-carrying genes encoding FabG or FabG mutant proteins was transformed into CL104. The cultures were grown at a permissive temperature, induced with IPTG, and shifted to 42 °C. After the incubation, [1-14C]acetate was added as described (28). The labeled fatty acids were extracted, analyzed by argentation thin-layer chromatography (TLC), and quantified by phosphorimaging (31).

**ACP-dependent gel reconstitution assay of FabG activity**

Malonyl-ACP was synthesized from holo-ACP and malonyl-CoA using *E. coli* FabD. Octanoyl-ACP was synthesized from octanoic acid, ATP, and *E. coli* holo-ACP by use of *V. harveyi* acyl-ACP synthetase as described (32). The ability of FabG or FabG mutant proteins to function in fatty acid synthesis was assessed in reactions reconstituted by the sequential addition of purified *E. coli* fatty acid synthetic enzymes and cofactors in sodium phosphate (pH 7.0) as described (28, 30). The reaction products were separated by conformationally sensitive gel electrophoresis (33).

**Spectrophotometric assay of FabG activity**

The spectrophotometric assay for FabG activity was measured by the oxidation rate of NADPH at 340 nm. Two substrates (acetoacetyl-CoA and acetoacetyl-ACP) were used for the assay. When using acetoacetyl-CoA as a substrate, the reaction mixture contained 0.5 mM acetoacetyl-CoA, 0.2 mM NADPH, 10 μg of FabG (or a mutant FabG), and 0.1 M sodium phosphate buffer with a pH of 7.4 in a final volume of 300 μl. When using acetoacetyl-ACP as a substrate, the mixture contained 100 μM ACP, 1 mM β-mercaptoethanol, 500 μM malonyl-CoA, 500 μM acetyl-CoA, varying concentrations of NADPH, 0.5 μg of purified *E. coli* FabD, 0.5 μg of purified EcFabH in 0.1 m sodium phosphate buffer (pH 7.0), and 5 μg of FabG (or a mutant FabG) protein in a final volume of 100 μl. The kinetic constants were determined using GraphPad Prism software, version 4.

**Tandem mass spectroscopy of FabG peptides**

Protein samples were digested with trypsin (Promega sequencing grade modified) prior to LC-MS/MS analysis by the Protein Sciences Facility of the University of Illinois Carver Biotechnology Center. Trypsin cuts proteins at the carboxyl side of K or R residues. LC-MS/MS analysis was performed on a Thermo Scientific Fusion Orbitrap mass spectrometer coupled to a Dionex Ultimate 3000 RSLC nano UHPLC. The separation was performed on a Thermo Scientific Acclain PepMap RSLC (75 μm × 15 cm) C18, 2-μm, 100-Å column at 300 nl/min. The gradient (A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile) was run over 76 min with the following compositions: 0 to 6 min 1% to 4% B, 6 to 51 min 4% to 35% B, 51 to 56 min 35% to 50% B, 56 to 60 min 50% to 90% B, 60 to 64 min 90% B, 64 to 66 min 90% to 2% B, 66 to 76 min 2% B. In each case the volume was made up to 100% with A, the Fusion Trubrid settings were in the Peptide mode and the Universal method with resolution of 120,000, precursor mass range 300 to 1600, RFF lens 60%, AGC 2.0e5, Injection Time 100 ms, Intensity threshold 5.0 e3, Charge state 1 to 7, Exclude after 2 times in 30 s for 60 s, Mass tolerance ±10 ppm, Data Dependent MS2, collision induced dissociation, Isolation 1.6 m/z, CID activation, Collision energy 35%, IT detection in Rapid Scan mode, AGC 1.0e4, All parallelizable time, maximum Injection time 60 s, 3 s/cycle.

Raw data were processed into peak lists using Mascot Distiller 2.7.1.0 (Matrix Science) and searched using Mascot 2.7.0.1 against a custom database composed of two entries (mutant sequence and wildtype host sequence). Mascot search settings included three missed cleavages, no fixed
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modifications, oxidation (Met) variable modifications, precursor ion mass tolerance ±10 ppm, fragment ion mass tolerance ±0.3 Da, and the threshold for accepting individual spectra of 13. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (34) partner repository with the dataset identifier PXD023486 and 10.6019/PXD023486. The wildtype E. coli K-12 and S. enterica LT2 FabG sequences were taken from UniProtKB/Swiss-Prot entries P0AEK2 and P0A2C9, respectively.

Circular dichroism measurements

The CD spectra of FabG and mutant FabG proteins were obtained with a Chirascan (Applied Photophysics Limited) at 25 °C using a 1.0-nm bandwidth, 1-mm cell, 1.0-nm step, 0.5-s time-per-point, and 1.0-min time interval. The CD spectra were measured at an enzyme concentration of 4 μM in 50 mmol/L phosphate-buffered saline (PBS, pH 7.5), and when required, a final concentration of 200 μM NADPH was added to each sample. The results were expressed as molar ellipticity (θ) deg cm² dmol⁻¹. The values were normalized by subtracting the baseline recorded for the buffer under similar conditions.

Fluorescence titration of FabG-NADPH binding

Equilibrium binding of a ligand to FabG or its mutant proteins was measured by fluorescence titration at 20 °C using a Jobin-Yvon Horiba spectrofluorometer and band-passes of 3 and 5 nm as the excitation and emission monochromators, respectively. The fluorescence spectrum of FabG was studied by exciting the samples at 280 nm and recording the emission spectrum over the range of 300 to 500 nm. For the NADPH association constant calculation, the samples were excited at 363 nm. Aliquots of 3 μl of NADPH (from stock solutions of 100 μM) were added to 4 μM FabG in 50 mM potassium phosphate buffer at a pH of 7.5. The solution was mixed after the addition of each aliquot, and the fluorescence intensity in the 400- to 500-nm region was recorded as the average of three readings.

Data availability

All data are in this article or Supporting Information excepting the tandem mass spectroscopy data, which have been deposited into the PRIDE database (Project Name: 20-175-Cronan-ZheHu and 20-200-Cronan-ZHu; Project accession: PXD02348 and Project DOI: 10.6019/PXD023486). The URL for PRIDE is https://www.ebi.ac.uk/pride/.

Supporting information—This article contains supporting information.

Author contributions—Z. H., J. M., H. W., and J. E. C. designed the experiments and interpreted data. Z. H., J. M., Y. C., W. T., and L. Z. performed experiments. Z. H., H. W., and J. E. C. wrote the paper.

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Abbreviations—The abbreviations used are: ACP, acyl carrier protein; FAS, fatty acid synthase; SDR, short-chain alcohol dehydrogenase/reductase.

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