Acyl carrier proteins play a central role in metabolism by transporting substrates in a wide variety of pathways including the biosynthesis of fatty acids and polyketides. However, despite their importance, there is a paucity of direct structural information concerning the interaction of ACPs with enzymes in these pathways. Here we report the structure of an acyl-ACP substrate bound to the Escherichia coli fatty acid biosynthesis enoyl reductase enzyme (FabI), based on a combination of x-ray crystallography and molecular dynamics simulation. The structural data are in agreement with kinetic studies on wild-type and mutant FabIs, and reveal that the complex is primarily stabilized by interactions between acidic residues in the FabI helix α2 and a patch of basic residues adjacent to the FabI substrate-binding loop. Unexpectedly, the acyl-pantetheine thioester carbonyl is not hydrogen-bonded to Tyr156, a conserved component of the short chain alcohol dehydrogenase/reductase superfamily active site triad. FabI is a proven target for drug discovery and the present structure provides insight into the molecular determinants that regulate the interaction of ACPs with target proteins.

Acyl carrier proteins (ACPs) play an essential role in a diverse array of metabolic pathways including the biosynthesis of fatty acids (1, 2), polyketides (3), membrane-derived oligosaccharides (4), lipopolysaccharides (5, 6), and phospholipids (7). In each case the growing substrate is attached via a thioester to the ACP phosphopantetheine group. ACPs must therefore be able to recognize and interact, in an acyl group-dependent manner, with a wide variety of enzymes. In eukaryotic type I fatty acid synthesis (FASI) and in polyketide biosynthesis, the ACP occurs as part of a larger polypeptide that is also associated with other catalytic activities. In contrast, in bacterial type II fatty acid biosynthesis (FASII), each of the enzyme activities as well as the ACP are encoded by separate polypeptide chains (2). ACPs that function in FASII-mediated biosynthesis are small, highly soluble, acidic proteins that vary in molecular mass from 7.5 kDa (Escherichia coli) to 13 kDa (Mycobacterium tuberculosis) (1, 8–11).

Despite the central role that ACPs play in metabolism, structural details of their interaction with target proteins are sparse. Whereas the structures of ACPs from a variety of different species have been determined by x-ray crystallography (12) and NMR spectroscopy (see for example, Refs. 13 and 14), only one structure has been determined of ACP in complex with another protein, the holo-ACP synthase (AcpS) (15), and no structural information is available for the interaction between ACP and enzymes of the fatty acid biosynthesis pathway. AcpS attaches the phosphopantetheine to the ACP serine and thus, although valuable, the complex of AcpS and ACP differs fundamentally from other ACP-protein complexes and does not provide insight into the delivery of substrate by ACP.

The NMR studies reveal that ACPs are highly flexible, a structural feature that is thought to be important for transporting the growing acyl chain and for protein recognition. In addition, based on x-ray crystallographic analysis of butyryl-ACP, it is thought that ACP adopts two major conformations, optimized either for delivering the acyl chain to the target enzyme or for transporting the growing acyl chain between enzymes (12). The flexibility of ACP, coupled with the relatively low affinity of ACP for target proteins, has hindered crystallographic efforts to obtain direct structural data on the interaction of ACP with any of the target proteins (16, 17).

Using docking and site-directed mutagenesis, Rock and co-workers (16, 17) have rigorously analyzed the interaction of ACP with two FASII enzymes. In agreement with the x-ray crystallography and molecular dynamics simulation. The structural data are in agreement with kinetic studies on wild-type and mutant FabIs, and reveal that the complex is primarily stabilized by interactions between acidic residues in the FabI helix α2 and a patch of basic residues adjacent to the FabI substrate-binding loop. Unexpectedly, the acyl-pantetheine thioester carbonyl is not hydrogen-bonded to Tyr156, a conserved component of the short chain alcohol dehydrogenase/reductase superfamily active site triad. FabI is a proven target for drug discovery and the present structure provides insight into the molecular determinants that regulate the interaction of ACPs with target proteins.

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The atomic coordinates and structure factors (code 2FHS) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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7. The abbreviations used are: ACP, acyl carrier protein; FabI, the enoyl reductase from Escherichia coli; InhA, the enoyl reductase from Mycobacterium tuberculosis; AcpS, holo-ACP synthase; DD-CoA, trans-2-dodecenoyl-CoA; DD-ACP, trans-2-dodecenoyl-ACP; C16-NAC, hexadecenoyl-N-acetylcycteamine; MES, 4-morpholineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; BisTris, 2-(bis[hydroxymethyl]amino)-2-(hydroxy-methyl)propane-1,3-diol; r.m.s., root mean square; MD, molecular dynamics.


Structure of ACP Bound to FabI

![Diagram of the type 2 fatty acid biosynthesis pathway and FabI inhibitors.](image)

**SCHEME 1.** The type 2 fatty acid biosynthesis pathway and FabI inhibitors.

The structure of ACP bound to AcpS (15), these studies confirmed the importance of the ACP recognition helix (helix α2) in FASII protein binding and identified a patch of basic residues on the E. coli β-ketoacyl-ACP synthase (FabH) and β-ketoacyl-ACP reductase (FabG) enzymes responsible for interaction with the ACP helix α2 (16, 17).

The efforts in our laboratory are focused on the FASII enoyl reductase enzyme FabI, a target for antibacterial diazaborine compounds (18) and triclosan (19–23), whereas the anti-TB drug isoniazid inhibits InhA, the FabI homolog from M. tuberculosis (24–26) (Scheme 1). Modeling studies, based on the information provided by the interaction of ACP with FabH and FabG (16, 17), suggest that ACP should interact with a cluster of basic residues adjacent to the FabI substrate binding loop. This loop is disordered in binary FabI-cofactor complexes, but becomes ordered in the ternary FabI-NAD̄-[triclosan complex. The ordered loop provides two entries into the active site, termed the major and minor portals. Sacchettini and co-workers (27) have determined the structure of a C16 substrate bound to InhA and have discussed the role of the major and minor portals in substrate recognition by the FabI enzymes. The orientation of the C16 substrate in InhA suggests that substrates enter the FabI active site through the major portal.

In the current study we have determined the structure of ACP bound to the E. coli FabI enzyme. X-ray crystallographic data obtained from a complex between FabI and dodecenoyl-ACP revealed most of the main chain electron density for both FabI and ACP. However, the observed relative orientation of ACP and FabI leaves the ACP Ser^36 residue too far from the active site to deliver the substrate through the major portal.

Because some aspects of the structure were not resolved by the crystallographic data, we employed computational methods to model the missing details and importantly to ascertain whether the ACP could deliver substrate into the FabI active site in the observed complex. Through molecular dynamics simulations, we generated a model for a productive complex between ACP and FabI. The details of the interaction between FabI and ACP in the resulting model are supported by mutagenesis studies, and provide the first detailed description of ACP recognition by a FASII enzyme. Intriguingly, the structural data indicate that the substrate enters the FabI active site through the minor portal and furthermore, suggest that the substrate thioester carbonyl group does not form a hydrogen bond with Tyr^156, a conserved active site residue.

**MATERIALS AND METHODS**

**Preparation of Substrates and Enzymes**—Plasmids for wild-type FabI and the Y156F mutant were available from a previous study (23). The Y146F, K201A, K201E, R204A, R204E, K205A, and K205E FabI mutations were introduced using the QuikChange mutagenesis kit (Stratagene). Wild-type and mutant FabI proteins were overexpressed and purified as described previously (23). Trans-2-dodecenoyl-CoA (DD-CoA) was synthesized from trans-2-dodecenonic acid using the mixed anhydride method (28). Apo-ACP was a gift from Dr. J. Shanklin and Dr. S. Booker. Apo-ACP was also expressed and purified from plasmid pET23b as previously described (29, 30) with the following modifications (Dr. S. Booker). After growing cultures at 37°C until an A_600 of 0.6 was reached, casamino acids were added to a final concentration of 2 g/liter and expression was induced by the addition of 500 μM isopropyl-1-thio-β-D-galactopyranoside. Following an additional 4 h of growth at 30°C, centrifugation yielded 8 g/liter of wet cell paste that was subsequently frozen. Frozen cell pellets (50 g) were then resuspended in 50 ml of 25 mM MES, pH 6.1, containing 200 mM NaCl (buffer A) and sonicated for 6 min using 30-s pulses at 4°C. Cellular debris was removed by centrifugation at 33,000 rpm for 1 h at 4°C and the supernatant was loaded onto a Q-Sepharose column (8 ml) equilibrated with 25 ml of buffer A. The column was washed with 50 ml of buffer A and ACP was eluted using a linear gradient (50 ml) of NaCl from 200 to 850 mM NaCl in buffer A. Fractions were analyzed by 18% SDS-PAGE, pooled, concentrated using an Amicon membrane (YM3, NMWL 3,000) and stored at −80°C.

Conformationally sensitive SDS-PAGE indicated that the ACP was predominately in the apo form and ESI mass spectrometry revealed that the apo-ACP sample was comprised of two forms in which the N-terminal Met was present or had been cleaved. The apo-ACP was used without further purification for the synthesis of trans-2-dodecenoyl-ACP (DD-ACP).

DD-ACP was a gift from Dr. M. Schaeffer and was also synthesized from DD-CoA and apo-ACP using E. coli AcpS (31), which was overexpressed and purified from E. coli as described previously (32). Briefly, 0.9 mg of apo-ACP was incubated with 50 μM DD-CoA (1.4-fold excess) and 50 μg of AcpS in 1.4 ml of 50 mM Tris-HCl, 25 mM MgCl₂, 1 mM dithiothreitol, pH 7.5, buffer for 1 h at 30°C, followed by quenching the reaction by placing it into a dry ice/ethanol bath for 5 min. Subsequently, an
equal volume of isopropyl alcohol was added and the reaction mixture was incubated for 2 h at 10 °C. AcpS was removed by centrifugation at 6000 rpm for 15 min and the supernatant was applied to a 1-ml Q-Sepharose column, equilibrated with 20 mM BisTris, 1 mM dithiothreitol, pH 6.5 (Buffer B), containing 50% isopropyl alcohol. The column was washed three times with Buffer B containing 50% isopropyl alcohol, and then five times with Buffer B alone. DD-ACP was eluted with 5 column volumes of Buffer B containing 600 mM NaCl. The fractions containing DD-ACP were identified by SDS-PAGE, pooled, concentrated, diazoyzed into 20 mM Tris-HCl, pH 7.0, and stored at −80 °C. The concentration of protein was determined by a BCA assay.

Kinetic Assays—All kinetic assays were performed on a Cary 100 Bio (Varian) spectrophotometer at 25 °C in 30 mM PIPES, 150 mM NaCl, pH 8.0. Kinetic parameters were determined by following the oxidation of NADH to NAD⁺ at 340 nm (ε = 6.3 mM⁻¹ cm⁻¹). kcat and kcat/Km for DD-CoA and DD-ACP were determined at a fixed, saturating concentration of NADH (250 μM) and by varying the concentration of DD-CoA (5–85 μM) or DD-ACP (1–200 μM), respectively. Kinetic parameters were obtained by fitting the initial velocity data to the Michaelis-Menten equation using Grafit 3.09b (Erithacus Software Ltd.).

Crystallization, Data Collection, and Refinement—The FabI-NAD⁺-DD-ACP complex was crystallized by the hanging drop vapor diffusion method. Crystals were grown at 22 °C from a protein solution containing FabI, NAD⁺, and DD-ACP in a 1:10:1 ratio mixed with a precipitant solution containing 18–22% PEG 4000 and 100 mM HEPES, pH 7.0. Crystals were flash-frozen in liquid nitrogen with 30% (w/v) glycerol as cryoprotectant and data were collected at beam line X26C at the National Synchrotron Light Source at Brookhaven National Laboratory. Data were indexed, integrated, and scaled using the HKL software package (33). Crystals belonged to the hexagonal space group P6₁,22, with a = b = 127.7 Å, and c = 206.7 Å and contained one FabI dimer and one ACP molecule in the asymmetric unit. The structure was solved by molecular replacement using the program MOLREP (34) and the FabI dimer as a search model (Protein Data Bank entry 1C14 (35)). Two unambiguous solutions with R-factors of 0.418 and 0.426 and correlation coefficients 0.569 and 0.552, respectively, were obtained. Structure refinement was performed with REFMAC (36). To locate the ACP molecule, several cycles of rigid-body refinement followed by solvent flattening and NCS averaging were carried out using DM (37). Additional electron density was observed after DM, which to a large extent was a good fit for the main chain of butyryl-ACP (12). All residues of the ACP were changed to Ala, because no density for the side chains was observed and the loop region 195–199 in molecule B of FabI was disordered as well. The model was improved with restrained refinement using REFMAC (36) and then manually improved using the program O (38). The structure was refined to an Rcryst of 0.263 and R of 0.226. An omit map calculated with CNS (39) for the final model showed no additional electron density. The stereochemistry of the model was good, with r.m.s. deviations of 0.010 Å and 1.235° in bond lengths and bond angles, respectively. The structure was analyzed with SFCHECK (40) and PROCHECK (41) and 87.1% of all residues were in the core region, 11.7% allowed, 1.2% generously allowed, and 0% in the disallowed regions of the Ramachandran diagram. Crystallographic statistics are given in Table 1. The coordinates and structure factors have been submitted to the RCSB (PDB code 2FHS).

Molecular Dynamics Simulations—Computational modeling and molecular dynamics simulations were performed with the Amber suite of programs (42). The missing ACP atoms were added using the butyryl-ACP crystal structure (PDB code 1L0I) (12) by overlapping the two structures, and replacing the partial coordinate set with the coordinates from 1L0I. Similarly, the structure of triclosan bound to FabI in the presence of NAD⁺ (PDB code 1QSG) (21) was used to place the NADH cofactor. Missing FabI side chains and all hydrogens were added using Xleap. The coordinates for the phosphopantetheine moiety were taken from the crystal structure of the holo-acyl carrier protein-synthase in complex with holo-acyl carrier protein (PDB code 1F80) (15). Maestro Molecular Modeling software was used to build the acyl chain for the phosphopantetheine moiety. Force field parameters were the ff99 set for proteins (43) and published parameters (44, 45) for NADH. The Amber antechamber module and GAFF force field (46) with am1bcc charges (47) were used to generate the parameters for phosphopantetheine and the attached acyl chain. To present the correct face of the crotonyl double bond to the NADH, the substrate must be bound in an s-trans conformation such that the si face of Cβ is oriented toward the NADH pro4(S) proton so that hydride transfer will generate the expected 3(S)-enoyl product (48). Because spontaneous isomerization of this double bond is unlikely to occur during relatively short molecular dynamics (MD) simulations, we modeled the crotonyl in the s-trans conformation.

**TABLE 1**

| Data collection and refinement statistics | Data collection |
|----------------------------------------|-----------------|
| Space group                            | P6₁,22          |
| Cell dimensions a, b, c (Å)             | 127.7, 127.7, 206.7 |
| Resolution (Å)                         | 50.0–2.7        |
| Rmerge (%)                             | 8.1 (71.2)      |
| Mean (I/σ(I))                          | 25.4 (2.7)      |
| Completeness (%)                       | 97.1 (98.4)     |
| Multiplicity                           | 10.1 (7.3)      |
| Refinement                             |                 |
| Resolution range (Å)                   | 30.0–2.7        |
| Unique reflections                     | 25,906          |
| Ramachandran statistics                | 87.1/11.7/1.2/0.0 |
| Rcryst                                | 0.226           |
| Rfree                                 | 0.263           |
| Number of nonhydrogen atoms (protein/solvent) | 3947/79       |
| r.m.s. deviations from ideal values    |                 |
| Bond length (Å)                        | 0.010           |
| Bond angle (°)                         | 1.235           |

a Rmerge = Σ ||Ii − 〈Ii〉||/Σ ||Ii||, where Ii is the ith measurement and 〈Ii〉 is the weighted mean of all measurements of Ii. Rfree = Σ ||Fo| − ||Fc||/Σ ||Fo||, where Fo and Fc are the observed and calculated structure factor amplitudes. Rcryst same as Rmerge, for 5% of the data randomly omitted from the refinement. Ramachandran statistics indicate the fraction of residues in the most favored, additionally allowed, generously allowed, and disallowed regions of the Ramachandran diagram, as defined by the program PROCHECK (41).
Structure of ACP Bound to FabI

Initial minimization of the FabI-NADH-ACP ternary complex was performed in a stepwise fashion by restraining the backbone Cα atoms and allowing the side chains to move, with each step consisting of 1000 cycles. The restraints were gradually removed in each step (force constants from 10, 7, 4, 1, and 0 kcal mol$^{-1}$ Å$^{-2}$). Equilibration dynamics was performed on the minimized structure, with a constant temperature of 300 K maintained by coupling to a thermostat using the Langevin algorithm with the collision frequency set to 1 ps$^{-1}$. This reduced viscosity has been shown to facilitate rapid structural rearrangement (49). During dynamics, restraints on the backbone atoms were gradually released in a stepwise fashion in 25-ps increments (force constants 10, 7, 4 to 1 kcal/mol/Å$^2$). Further equilibration with no restraints was performed for 500 ps at 300 K. The time step was 1 fs. During all simulations, all possible nonbonded interactions were evaluated at each time step (i.e. no cutoff was employed). Solvation effects were incorporated using the Generalized Born model as implemented in Amber (50–52). r.m.s. deviation values were calculated using the initial model structure (prior to equilibration) as a reference.

The production phase consisted of 1-ns simulations at 300 K with parameters as described above. Simulations were fully unrestrained with the exception of a distance restraint between the C-3 atom of the substrate and C-4 of the NADH nicotinamide ring, with a force constant of 10 kcal mol$^{-1}$ Å$^{-2}$. The initial separation was $\sim$18 Å, and the final distance was 4.6 Å. We modified Amber to apply the restraint force only to the substrate C-3 to avoid pulling NADH out of the binding pocket. After the substrate was drawn into the active site, a 100-ps simulation was performed to equilibrate the system with the substrate in the active site. Finally, this restraint was released and an additional 100 ps of fully unrestrained simulation was performed.

The sensitivity of the results to the force field parameters was studied by repeating the procedure using the ff99SB protein force field (53) and a newer variant of the GB solvation model (54, 55). We recently showed that this combination of force field and solvent model was able to accurately reproduce experimental data for large conformational changes in human immunodeficiency virus type 1 protease that occur upon addition or removal of an inhibitor (56, 57). After the substrate was drawn into the FabI active site, the system was simulated for an additional 2500 ps.

RESULTS

X-ray Crystallography—FabI forms a 110.9-kDa tetramer with 222 symmetry in which each monomer is formed by a central $\beta$-sheet that contains seven $\beta$-strands sandwiched by eight helices. Two FabI monomers (A and C) within the tetramer form a complex with ACP, resulting in a stoichiometry of 2:1 for the FabI-ACP interaction (Fig. 1). The ACP-bound FabI monomers and the uncomplexed FabI monomers are oriented in such a way that they form a nearly continuous $\beta$-sheet across the dimer interface.

Superposition of the ACP-bound FabI monomers with FabI from the triclosan-bound structure (PDB code 1QSG) (21) results in an r.m.s. deviation of 0.58 Å for 252 Cα atoms for residues 2–194 and 200–259. Thus, the overall structure of FabI does not change upon binding to ACP. However, the substrate binding loop (residues 191–200) undergoes a major conformational change upon complexation with ACP, whereas FabI helix a8 (residues 201–213) is shifted toward helix α2 of ACP (21) (Fig. 2). Although the density is missing for most of the side chains in ACP, the distance between the main chain atoms of these two helices indicates that helix a8 of FabI interacts with helix α2 of ACP with a main chain separation of about 8.5 Å, over a helix-helix interface length of roughly 11 Å. The substrate binding loop in the triclosan-bound structure, which is connected to helix a8, forms a lid on top of the triclosan moiety and the nicotinamide ring, thereby shielding them from the solvent. In contrast, this loop adopts an open lid conformation in the FabI-ACP structure, likely due to interaction with ACP,
Although the details of this interaction cannot be elucidated from the crystallographic data due to missing side chain density. Finally, the observation that only two ACP molecules are observed in the complex with the FabI tetramer could be due to crystal packing.

We attempted to dock ACP molecules to the corresponding positions on the other monomers and obtained steric conflicts between the existing ACP molecules, suggesting that the lack of 1:1 stoichiometry is biologically relevant.

**Model Building and Molecular Dynamics**—Because the side chains for both molecules in the region of the FabI-ACP interface were not resolved by the crystallographic data, we began our computational study by building a model that included these coordinates (see “Materials and Methods” for details), followed by a series of energy minimization and MD simulations to relax the resulting structure of the complex. The proteins were stable, with the r.m.s. deviation of FabI and ACP individually remaining below 3 Å. The relatively small contact interface between the proteins permitted a somewhat larger degree of relative motion of the two proteins, with the overall r.m.s. deviation of the system reaching a plateau of ~3.5 Å.

Structural analysis of the resulting model indicates that the complex is predominantly stabilized through hydrogen bonding interactions between basic residues of FabI in helix α8 and acidic residues of ACP in helix α2, consistent with the structural and modeling studies on the interaction of ACP with AcpS, FabH, and FabG (15–17). However, whereas the x-ray crystallographic data and computational modeling studies provide information on the nature of the interaction between FabI and ACP, the absence of electron density for the ACP pantetheine hindered our ability to predict how the ACP delivers the substrate into the FabI active site. Consequently, we set out to build a model of a productive FabI-ACP complex using the computationally refined crystallographic model as a starting point and attaching a crotonyl-phosphopantetheine group to the side chain of Ser236 in ACP. Importantly, these simulations also help determine whether the relative positions of FabI and ACP observed in the crystal structure permit delivery of substrate to the active site.

During MD simulations of this structure, the substrate remained outside the FabI active site. This is expected because diffusion of the substrate into the protein is likely an inaccessible event during standard MD simulations on the nanosecond time scale. We thus obtained a model for the productive complex by placing a distance restraint between the crotonyl C-3 carbon and the NADH C-4 carbon, reducing the target value during a 1-ns MD simulation to draw the substrate into the active site. No other restraints were employed to enforce any particular binding mode. Subsequently, the restraint between the substrate and FabI active site was released and a further completely unrestrained simulation was performed for 100 ps.

Similar to the behavior without substrate, the individual proteins remained relatively stable during these simulations, with backbone r.m.s. deviation less than or equal to 3.0 Å as compared with the initial model. We observed a greater movement of the ACP molecule with respect to FabI, which allowed Ser236 to be oriented toward the active site cavity. To gain more specific insight into the structural changes that occurred during substrate entry, we performed a superposition of the initial and final structures of the entire protein and then separately calculated the r.m.s. deviation values for each residue without refitting (Fig. 3). These show that the r.m.s. deviation for the major-
crotonyl C-3 and the NADH pro4(S) proton is 3 Å. Analysis of
the structure also reveals that the crotonyl thioester carbonyl is
located 4 Å from the Tyr146 hydroxyl group, suggesting that
Tyr146 may form a hydrogen bond to the thioester during sub-
strate reduction (Fig. 5).

To investigate the influence of the computational protocol
on the results, we repeated the process using a different protein
force field and solvent model (see “Materials and Methods”).
We recently employed this combination to successfully simu-
late ligand-induced conformational changes in human immu-
nodeficiency virus type 1 protease (56, 57). Importantly, we also
demonstrated that this particular implicit solvent model accu-
rately reproduces the stability of salt bridges between protein
side chains as compared with simulations in explicit solvent
(58). Simulations with these parameters gave very similar
results to those described above. The crotonyl thioester car-
bonyl is located even closer (2.7 Å) to the Tyr146 hydroxyl
group, providing further evidence that Tyr146 may form a
hydrogen bond to the thioester during substrate reduction.

Kinetic Analysis of Wild-type and Mutant FabI Enzymes—
The structural studies described above identify several key
interactions in the FabI-ACP complex that have been evaluated
by replacing the basic FabI residues with acidic groups and Ala.
The data in Table 2 demonstrates that replacement of Lys201,
Arg204, and Lys205 with Ala has little or no effect on the kinetic
parameters for reduction of DD-CoA, whereas \( k_{\text{cat}} / K_m \) for
reduction of DD-ACP is reduced 5 (Lys201 and Lys205) to 50
(Arg204)-fold. In addition, replacement of Arg204 and Lys205
with Glu causes a further reduction in \( k_{\text{cat}} / K_m \) for reduction of
DD-ACP without affecting \( k_{\text{cat}} / K_m \) for the DD-CoA substrate.
Similar to the Ala mutants, substitution of Glu for Arg204
has a larger impact on substrate reduction (250-fold) compared
with Lys205 (14-fold). Finally, replacement of Lys201 with Glu
resulted in an enzyme with little activity toward either substrate
and we were unable to determine accurate kinetic parameters
for this mutant.

We have also examined the importance of Tyr156 and Tyr146,
the two active site Tyr residues, in substrate reduction. Replace-
ment of Tyr156 with Phe has no effect on substrate reduction in
agreement with previous studies on both FabI (23) and InhA
(22, 28), which questioned the importance of Tyr156 (Tyr158 in
InhA) in catalysis (59). In contrast, mutagenesis of Tyr146 has a
larger impact on catalysis, with \( k_{\text{cat}} / K_m \) for DD-ACP
decreasing by around 50-fold compared with wild-type FabI. A
similar decrease in kinetic parameters for Y146F was also
observed for the DD-CoA substrate.

DISCUSSION

ACPs are small, acidic proteins that fulfill an essential role in
metabolism through their interactions with a diverse array of
target enzymes. However, despite their central role, detailed structural information on the acyl group–specific recognition of ACPS by their target proteins has remained elusive, presumably partly as a result of the conformational flexibility of the ACP molecule. Here we report the first structural data for the direct interaction of an acyl-ACP substrate with a target enzyme, the FASII FabI enzyme, based on a combination of x-ray crystallography and computational modeling. FabI is the enoyl reductase in the bacterial FASII pathway and a target for antibacterial drug discovery. Thus, not only does the structure of the FabI-ACP complex provide general insight into how target proteins recognize and bind to acyl-ACPs, the present structure also provides a foundation for the development of novel FabI inhibitors that target this interaction of FabI with its natural substrate.

In agreement with previous predictions, several acidic residues in and close to the ACP helix α2 (Asp$^{35}$, Asp$^{38}$, and Glu$^{45}$) form stable electrostatic interactions with three basic amino acids (Lys$^{201}$, Arg$^{204}$, and Lys$^{205}$) located adjacent to the ACP substrate binding loop. Importantly, replacement of Lys$^{201}$, Arg$^{204}$, or Lys$^{205}$ by Ala or Glu results in significant decreases in $k_{cat}/K_m$ for reduction of DD-ACP, caused both by a decrease in $k_{cat}$ and an increase in $K_m$, without affecting the kinetic parameters for reduction of the corresponding CoA substrate. The effect on DD-ACP reduction was larger for the Glu substituents, with Arg$^{204}$ demonstrating the most sensitivity to mutation. Thus, these residues are involved in specific interactions with the protein portion of DD-ACP, as was shown in similar experiments with FabH and FabG (16, 17). The FabI-ACP interaction positions Ser$^{36}$, the ACP residue that carries the phosphopantetheine, above an opening into the active site formed by the substrate binding loop, helix α8 comprised of residues 192–206 and a mobile loop comprised of residues 152–156. These two loops move apart to allow the phosphopantetheine to deliver the substrate to the active site through the minor portal (27). Whereas the C-3 carbon of the enoyl substrate is at a distance of ∼3 Å from the pro4(S) NADH proton, the position of the substrate into the active site is unexpected given previous structural data on inhibitors bound to FabI and the hexadecenoyl-N-acetylcysteamine (C16-NAC) substrate (27) bound to InhA. Below we discuss the orientation of the substrate with respect to the catalytic triad in the FabI active site.

FabI is a member of the short chain alcohol dehydrogenase/reductase family. This superfamily is characterized by a conserved triad of active site residues. In FabI the triad is comprised of Tyr$^{146}$, Tyr$^{156}$, and Lys$^{163}$, whereas in InhA, the M. tuberculosis enoyl reductase, the triad is Phe$^{149}$, Tyr$^{158}$, and Lys$^{165}$. Mechanistic information on the role of these residues in catalysis has been provided by site-directed mutagenesis coupled with structural data primarily arising from enzyme-inhibitor complexes rather than enzyme-substrate complexes. Inhibitors that have been structurally characterized in complex with these enzymes include compounds such as the diazaborines (18) and isoniazid (26), which modify the NAD(H) cofactor, and those, such as triclosan, that bind noncovalently to the enzyme-cofactor complex (20, 21).

Triclosan, which binds with picomolar affinity to the E. coli FabI (23, 60, 61), has been proposed to bind to the enzyme as a substrate analog (62). This hypothesis has gained support from the structure of C16-NAC bound in a stable ternary complex to InhA in the presence of NAD$^+$ (27). Tyr$^{156}$ of FabI (Tyr$^{158}$ in InhA) forms a hydrogen bond to the phenol of triclosan and the carbonyl oxygen of the C16 substrate. Mutagenesis clearly supports the importance of Tyr$^{156}$ (Tyr$^{158}$) in triclosan binding, and replacement of this residue by Phe increases the $K_c$ for triclosan inhibition by 400 (160)-fold (22, 23). However, the impact of mutating Tyr$^{156}$ (Tyr$^{158}$) with respect to its role in catalysis is much less pronounced. In FabI, the kinetic parameters are unaffected by replacement of Tyr$^{156}$ with Phe, whereas the corresponding mutation in InhA (Y158F) has a slightly stronger effect on catalysis, reducing $k_{cat}$ 24-fold compared with wild-type. Intriguingly, however, the Y158S InhA mutant displays wild-type activity (28). Thus, Tyr$^{156}$ does not play a significant role in substrate reduction and, in agreement with these data, the substrate carbonyl group in the present FabI-ACP structure is pointing away from Tyr$^{156}$ (Fig. 5).

The function of the second aromatic residue in the enoyl reductase triad (Tyr$^{146}$/Phe$^{149}$) is also not clear, and Rozwarski et al. (27) have argued that Phe$^{149}$ in InhA is involved in directing the NADH to deliver a hydride to the correct position on the substrate and/or in modulating the interaction of the bound substrate with a channel of water molecules that leads away from the active site. In the simulation model, the FabI Tyr$^{146}$ hydroxyl is located within hydrogen bonding distance from the substrate carbonyl, suggesting that Tyr$^{146}$ rather than Tyr$^{156}$ provides electrophilic assistance during substrate reduction. To probe the role of this residue in catalysis, the Y146F mutant was characterized and shown to catalyze substrate reduction with a $k_{cat}/K_m$ value 14 (DD-CoA) to 50 (DD-ACP)-fold lower than wild-type FabI. These data suggest that the Tyr$^{146}$
hydroxyl is directly involved in catalysis, consistent with the hydrogen bonding interaction revealed by the MD simulations. We note, however, that the homologue of Tyr146 in InhA is a Phe (Phe149), and so is unable to provide a hydrogen bond to the substrate. Indeed, Raman studies on deuterated NADD cofactor bound to InhA suggest that Phe149 most likely is involved in correctly positioning the cofactor for hydride transfer.8 Thus, one possibility is that Tyr146 (FabI) and Phe149 (InhA) play different roles in catalysis.

Returning to previous structural studies, Sacchettini and co-workers (27) have discussed two entry points for substrates into the active sites of enoyl reductases termed the major and minor portals. Based on the structures of inhibitors bound to FabI and InhA and that of the InhA-C16-NAC complex, the fatty acid substrate would have been expected to enter the FabI active site through the major portal. Instead, the present structure indicates that binding of ACP to FabI delivers the acyl-pantetheine through the major portal. Based on the structures of inhibitors bound to FabI and the active sites of enoyl reductases termed the major and minor portals, they provide the consistent view that ACP is able to successfully deliver the substrate into the FabI active site through the minor portal and from the position observed in the crystal structure of the complex. Knowledge of the structural determinants that control the interaction of ACP with target enzymes is of critical importance for the design of inhibitors against these enzymes and for fully understanding the multiple roles of ACP in metabolic pathways.

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