Identification and Analysis of the Acyl Carrier Protein (ACP) Docking Site on β-Ketoacyl-ACP Synthase III*

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The molecular details that govern the specific interactions between acyl carrier protein (ACP) and the enzymes of fatty acid biosynthesis are unknown. We investigated the mechanism of ACP-protein interactions using a computational analysis to dock the NMR structure of ACP with the crystal structure of β-ketoacyl-ACP synthase III (FabH) and experimentally tested the model by the biochemical analysis of FabH mutants. The activities of the mutants were assessed using both an ACP-dependent and an ACP-independent assay. The ACP interaction surface was defined by mutations that compromised FabH activity in the ACP-dependent assay but had no effect in the ACP-independent assay. ACP docked to a positively charged/hydrophobic patch adjacent to the active site tunnel on FabH, which included a conserved arginine (Arg-249) that was required for ACP docking. Kinetic analysis and direct binding studies between FabH and ACP confirmed the identification of Arg-249 as critical for FabH-ACP interaction. Our experiments reveal the significance of the positively charged/hydrophobic patch located adjacent to the active site cavities of the fatty acid biosynthesis enzymes and the high degree of sequence conservation in helix II of ACP across species.

The 4′-phosphopantetheine prosthetic group is a central and universal feature in the mechanism of fatty acid biosynthesis that provides two crucial functionalities to the process: a long and flexible arm that can reach into active sites and a terminal thioester group for the attachment of acyl groups through a thioester linkage. Two types of fatty acid synthase architectures exist in nature, and the 4′-phosphopantetheine moiety operates quite differently in each type. The type I, or associated structure, found in mitochoendria, is a subunit of NADH-ubiquinone oxidoreductase (16, 17).

In bacteria, the involvement of ACP in these various synthetic pathways means that it must interact with a plethora of functionally different enzymes. However, analysis of the primary structures of these enzymes does not reveal a common ACP-binding motif. The interaction must be specific to enable the prosthetic group to deliver its cargo precisely to the active site, but it must also be relatively weak to allow rapid on and off rates for the substrates. It is also anticipated that conformational changes facilitate the entry and exit of the extended prosthetic group from the active site. Biophysical (18, 19) and structural studies (20) point to an interaction between the acyl chain and the ACP protein. However, crystal structures reveal that the active sites of the fatty acid biosynthetic enzymes are generally located at the bottom of hydrophobic clefts or tunnels with varying dimensions (for two examples, see Refs. 21, 22).

Therefore, the fatty acid intermediate must dissociate from ACP and be injected into the active site cavity. The physico-chemical and structural basis of these interactions is not known.

β-Ketoacyl-acyl carrier protein synthase III (FabH) is a con-

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The atomic coordinates and structure factors (code 1G5a) 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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The abbreviations used are: ACP, acyl carrier protein; FabH, β-ketoacyl-ACP synthase I; FabF, β-ketoacyl-ACP synthase II; FabH, β-ketoacyl-ACP synthase III; PCR, polymerase chain reaction; bp, base pair(s).

This paper is available on line at http://www.jbc.org
denaturing enzyme that catalyzes the initial step in the elongation of fatty acids. Its two substrates, acetyl-CoA and malonyl-ACP, bind sequentially in a ping-pong enzyme mechanism to produce acetoacetyl-ACP (23–25). We and others have recently determined the crystal structure of FabH (22, 26) and have identified the active site and the specific binding site for CoA. The active site is at the base of a 20-Å-deep tunnel, and a bound CoA molecule in the crystal structure reveals that the interactions with the 4′-phosphopantetheine moiety are mainly hydrophobic in character. The weak interaction between FabH and ACP thioesters (ca. 5 μM) (27) and the flexibility of the ACP molecule (4) have made it difficult to crystallize the FabH-ACP complex. As an alternative strategy, we have investigated the interaction between the two proteins using a combination of computational, biochemical, and structural methods. Using this approach, we have identified specific surface features on both proteins that are critical to their interaction and propose a model by which ACP can deliver and extract substrates from FabH and other fatty acid biosynthetic enzymes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Amersham Pharmacia Biotech supplied the [1-14C]acetyl-CoA, Sigma supplied the acetyl-CoA, malonyl-CoA, and ACP, and Promega supplied the molecular biology reagents. NH$_2$-terminally His-tagged FabD, FabF, and FabH were expressed in *E. coli* strain BL21(DE3) (Novagen) and purified by nickel chelation affinity chromatography as described previously (27–29). Protein was stored in 50% glycerol at −20 °C. Protein concentration was determined using the Bradford method with γ-globulin as the standard (30). All other reagents were of the highest available purity.

**Modeling of the FabH-ACP Complex**—The program SurfDock, which is described in detail elsewhere (31, 32), was used to investigate the FabH-ACP complex. The coordinates of ACP and FabH were both retrieved from the Protein Data Bank (accession codes 1acp and 1ebl, respectively), and hydrogen atoms were added using the suite of programs from Molecular Simulations Inc. The partial charges were assigned according to the ff91 forcefield (33), and the solvent-excluded surfaces were created with the MSMS program (34) using probe radii of 1.5 and 1.56 Å for ACP and FabH, respectively. These probe radii are slightly larger than normal to avoid the possibility of creating artificial tunnels in the protein surfaces, because the spherical harmonic approximation in SurfDock requires a genus 0 surface (i.e. no holes). At this point, sets of spherical harmonic surfaces of various resolutions ranging from order 6 to 40 at approximate intervals of 5 were generated for ACP and FabH. The electrostatic potentials and hydrophobicities were then mapped to these generated surfaces. The former were calculated on the basis of a distance-dependent dielectric constant with no shielding may be dampened relative to the shielding in the solvent-accessible regions.

**In the docking procedure, FabH was considered as the fixed molecule, and no constraints were placed on the positioning of the mobile ACP molecule. During the docking calculations, ~60,000 docking pairs were evaluated by analyzing the interactions at the interfaceal surface between the two proteins. The interfaceal surface is defined as the locus of points equidistant between the interaction surfaces. SurfDock maps atomic properties onto the surfaces of the interacting molecules and uses a combination of geometric and chemical criteria to score putative complexes. In the current implementation, the total score is a linear combination of the following terms: contact area, geometric shape, chemical properties, steric overlap, and interface topology. The initial population size was set to 200, and at every generation during the competition algorithm 50 individuals were kept and 150 new individuals were generated. The process was repeated for 300 generations, at which point all the top scoring complexes were analyzed visually.

**Crystal Structure Analysis of FabH**—His-tagged FabB was expressed and purified as described previously (29). Crystals of FabH in space group P2$_1$2$_1$2$_1$ were grown by the hanging drop vapor diffusion method using M9 medium solution that contained 2.0 M ammonium sulfate, 20% polyethylene glycol 400 and 100 mM Tris, pH 6.5. Data from a single crystal were processed using HKL (38). The structure was solved by molecular replacement using the FabF structure (39) and the programs SegMod (40) and AMoRe (41). The final structure was obtained by alternating rounds of refinement using XPLOR (42) and visual inspection using O (43). Solvent flattening and NCS averaging using the DM program (44) were included in all maps. Omit maps were used extensively to avoid model bias. The stereochemistry was evaluated using PROCHECK (45). Data and refinement statistics are shown in Table I.

**Construction of FabH Mutants**—Mutations were introduced into the fabH gene in pET-fabh (27) using an overlap extension PCR method. All of the FabH mutants were prepared using the same two outside primers: HNnsFor (5′-CATTTGAGTTGCAAGCAGGT) and HNnsRev (5′-ACGCTTAAATGAGCTGTGCT). The internal primers for all the FabH mutants are listed in Table II. To construct each mutant, two PCR reactions with pET-fabH as the template and consisting of one outside primer and the respective inside primer were performed, and the products were then pooled and used as a template for a second PCR using both outside primers. The 677-bp PCR product was purified from 1% agarose gel using the QIAquick gel extraction kit (Qiagen, Inc.) and ligated into pCR2.1 (Invitrogen). Following transformation into E. coli InvaF, plasmid DNA was isolated and sequenced. A clone with the sequence containing the desired mutation was digested with NsiI and BamHI and ligated into pET-fabH, which had been digested with the same enzymes and dephosphorylated with calf intestinal alkaline phosphatase. The resultant plasmids were transformed into competent E. coli strain BL21(DE3) cells by electroporation. Expression and purification was as for the wild type protein.

**Circular Dichroism Spectroscopy**—The correct folding of the mutant proteins was verified by analysis of their CD spectrum between 200 and 280 nm using an AVIV 62A DS spectrometer (22). Protein concentration was determined by measuring the absorbance at 280 nm, immediately prior to collecting the spectrum. The extinction coefficient of His-tag FabH was taken to be 27,493 (m × cm)$^{-1}$ using the Biopolymer calculator (available on the Web), and the measured ellipticity was converted to molar values for direct comparison of the mutants.

**Coupled Assay of FabH Activity with Malonyl-ACP**—The coupled assay of FabH, as described previously (25, 27), contained 25 μM ACP, 1 mM β-mercaptoethanol, 65 μM malonyl-CoA, 45 μM [1-14C]acetyl-CoA (specific activity 80 μCi/μmol), 2.64 μg of purified FabH, 0.1 μM sodium phosphate buffer, pH 7.0, and 1 ng to 0.1 μg of FabH protein in a final volume of 40 μl. The ACP, β-mercaptoethanol, and buffer were preincubated at 37 °C for 30 min to ensure the complete reduction of ACP. The reaction was initiated by the addition of FabH. After incubation at 37 °C for 15 min, 35 μl of reaction mixture was removed and dispensed onto a paper filter disc (Whatman 3MM filter paper). The disc was washed successively with ice-cold 10%, 5%, and 1% trichloroacetic acid with 20 min for each wash. The filter discs were dried and counted for 14C-isotope in 3 ml of scintillation fluid.

**Spectrophotometric Assay of FabH Activity with Malonyl-CoA**—The reaction mixture contained 0.1 mM acetyl-CoA, 5 mM MgCl$_2$, 50 μg of FabH protein, 5 mM malonil-CoA, 0.1 mM Tris-HCl buffer, pH 7.0, in a final volume of 300 μl. The reaction was initiated by the addition of
malonyl-CoA. β-Ketobutyryl-CoA, the final product of this reaction, formed a complex with Mg²⁺, which absorbs at 305 nm (46). Increase in the absorbency at 305 nm was recorded for 2 min. The ability of ACP to inhibit either FabH or FabH[R249A] activity in the spectrophotometric assay was tested by incubating ACP with the protein at room temperature for 5 min before the addition of malonyl-CoA to initiate the reaction.

**Surface Plasmon Resonance**—Binding studies were performed on a Biacore 3000 surface plasmon resonance instrument. ACP was covalently attached to a carboxymethyl-dextran-coated gold surface (CM-5 Chip, Biacore). The carboxymethyl groups on the chip were activated with N-ethyl-N-(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide to activate the carboxymethyl-dextran. The ACP was attached at pH 4.5 to this activated surface by reaction of the carboxyl groups of the dextran with primary amines on the ACP to form an amide linkage. Any remaining reactive sites on the surface were blocked by reaction with ethanolamine. A reference cell was prepared similarly except that no ACP was added. Binding was measured by flowing FabH in 10 mM HEPES, 150 mM NaCl, pH 7.4, at a flow rate of 20 μl/min through the reference and ACP-containing flow cells in sequence. A blank was also run consisting of buffer only. Following the injection, release of the bound FabH was measured by flowing only buffer through the flow cells. Regeneration of the chip surface to remove bound FabH was achieved with ethanolamine. Following the incubation of FabH with ACP, the surface was regenerated by flushing with ethanolamine.

**RESULTS**

**Computational Docking of ACP and FabH**—The interaction between FabH and ACP was computationally investigated using the program SurfDock. This automated protein-protein docking algorithm has been fully described elsewhere (32, 47) and was used successfully to predict the structural complex of β-lactamase with its inhibitory protein RTEM (31) and the ternary TF-VIIaXa complex (32). SurfDock is a hierarchical docking program that performs a rigid-body six-dimensional search to dock two proteins of known three-dimensional structure. Briefly, surface properties such as hydrophobicity and electrostatic potential are abstracted onto the molecular surfaces of the interacting proteins and represented as expansions of spherical harmonics functions. By varying the number of terms used in the expansion function, the resolution of the surface and the property representation may be controlled (48).

During the docking procedure, we moved progressively from the dimer axis of FabH to inhibit the FabH active site. This is significant, because the ACP docking procedure was completely unconstrained, and the result was consistent with the proposed substrate binding site based on the surface electrostatic potential of the FabH crystal structure (22, 26). In the smaller subset, which contained the next lowest scoring group (~380 to ~250), ACP was predicted to dock in a cavity at the dimer axis of FabH. We describe in detail only the top scoring complex (score = ~524), which has a number of features that are consistent with the requirements of the interaction and with protein-protein interactions in general.

![Fig. 1](image1.png)

Using the NMR structure of ACP (5–7) and the x-ray structure of FabH (22, 26), we generated an optimal FabH-ACP complex using SurfDock as described under “Experimental Procedures.” The top scoring complexes from the docking computation could be grouped into two subsets. In the larger subset, which contained the lowest scores (~524 to ~400), the ACP preferred to dock at the entrance to the FabH active site. This is significant, because the ACP docking procedure was completely unconstrained, and the result was consistent with the proposed substrate binding site based on the surface electrostatic potential of the FabH crystal structure (22, 26). In the smaller subset, which contained the next lowest scoring group (~380 to ~250), ACP was predicted to dock in a cavity at the dimer axis of FabH. We describe in detail only the top scoring complex (score = ~524), which has a number of features that are consistent with the requirements of the interaction and with protein-protein interactions in general.

**Fig. 1** shows an overview of this complex, and it highlights the matching physicochemical surface properties of the two molecules that were detected by SurfDock. Table III lists the favorable amino acid contacts at the FabH-ACP interface, and Fig. 2 shows a stereo view of the interface that illustrates these interactions. There are several attractive features of this model. First, Ser-36 of ACP, the attachment site for the 4′-phosphopantetheine, is adjacent to the FabH active site entrance and oriented appropriately for insertion of the prosthetic group into the tunnel. Second, the interfacial amino acids from both proteins are highly conserved (Fig. 3). Third, the closest contact between the proteins is mediated by two α-helices (helix II from ACP and helix Co2 from FabH) that are oriented at a relative angle of ~60°, which is the optimal angle for this type of protein-protein interaction (49). Fourth, two alanine residues (Ala-45 from ACP and Ala-253 from FabH) permit the close approach of the two α-helices. Fifth, the contacting surfaces are oppositely charged, and there are many possible ionic interactions between them (Table III). We emphasize “possible” because SurfDock does not determine the orientations of the surface side chains in the complex and their conformations in the complex will certainly differ from their orientations observed in the free proteins. Sixth, the basic patch on FabH is quite extensive and actually comprises two nonoverlapping binding sites, one for ACP described here and another for CoA characterized earlier (22, 26). The fact that the two sites are adjacent but distinct supports our model of the FabH-ACP interaction. Finally, the conserved and unusually exposed Phe-213 of FabH forms a hydrophobic interaction with the conserved Met-44 of ACP.

**Effect of Surface Mutations on the Putative FabH-ACP Complex**—The model predicted by the SurfDock program was tested by introducing a number of mutations into FabH that should disrupt FabH-ACP interactions. Five residues were selected that are both integral to the interface (see Fig. 2) and also highly conserved (see Fig. 3), namely, lysines 214, 256, and 257, Arg-249, and Ala-253. We replaced the positively charged arginine and lysines with glutamates to introduce electrostatic

### Table II

| Mutant  | Forward primer | Reverse primer |
|---------|----------------|----------------|
| Ala-253 | 5'-ATTATCGATTACCGGGCAA | 5'-TCGCCGTGTTAACCTGATAATAC |
| Arg-249 | 5'-AACCTGGAAATTATCAGTGCAAC | 5'-GATAAATTCCAGGGTACCTGATAATG |
| Arg-249 | 5'-AACCTGGAAATTATCAGTGCAAC | 5'-GATAAATTCCAGGGTACCTGATAATG |
| Lys-214 | 5'-ATTTTCCTGCTTCTGGTTACCGGGAAAC | 5'-TCCCTCAGCCGAACCTCTGCTTGTTGTTGTTG |
| Lys-214 | 5'-ATTTTCCTGCTTCTGGTTACCGGGAAAC | 5'-TCCCTCAGCCGAACCTCTGCTTGTTGTTG |
| Lys-256 and Lys-257 | 5'-GCCAACCGGGAAAGAAAAGGACCTGGAATGGT | 5'-ACCAGAGTCTTCTGCGGGCTTGACCT |
| Lys-256 and Lys-257 | 5'-GCCAACCGGGAAAGAAAAGGACCTGGAATGGT | 5'-ACCAGAGTCTTCTGCGGGCTTGACCT |

*a* Bases changed to introduce mutations are underlined.
repulsion between FabH and ACP and to test whether ACP associates with FabH over the entire predicted surface. We also changed these same positively charged residues to alanines to determine which of the electrostatic interactions are the most important for promoting the binding to ACP. Finally, we replaced Ala-253 with a bulky tyrosine residue to test the importance of the hydrophobic depression on the FabH surface. None of these mutations introduced significant structural changes into the FabH molecule based on their CD spectra (data not shown).

Two assays were used to evaluate the effects of these mutations on the FabH-specific activity. The first was ACP-dependent, and used malonyl-ACP as the substrate in a standard biochemical procedure that was originally developed to evaluate FabH activity (24, 25, 27). The second assay was ACP-independent and was developed specifically for this analysis. In this case, malonyl-CoA was used as the substrate instead of malonyl-ACP, and the formation of the acetoacetyl-CoA-Mg\(^{2+}\) complex was monitored spectrophotometrically at 305 nm. One would predict that any mutation that interferes with the FabH:ACP interaction would adversely affect the ACP-dependent assay but not the ACP-independent assay. Table IV lists the FabH mutants that were produced and their performance in the two assays. It should be noted that the wild type FabH enzyme was about 40-fold less active with the malonyl-CoA substrate than with its normal substrate malonyl-ACP. This result was anticipated, and the reduced activity with the substrate analogue was compensated for by an increased protein concentration in the spectrophotometric assay.

With the exception of FabH[A253Y], the mutant proteins did not have impaired activity with the CoA substrate analogue when compared with the wild-type protein. This was expected, because none of the mutations were directed against the known binding site for CoA on FabH (22, 26). This result also confirmed that the mutations did not significantly alter the overall structure of the protein (Table IV). In contrast, all of the mutants exhibited reduced enzyme activity with malonyl-ACP compared with the wild-type enzyme (Table IV), thereby supporting the general location of the ACP interaction surface shown in Fig. 3. In the case of the FabH[A253Y] mutant, the activity with malonyl-CoA was 17% of wild type, which indicates a general structural effect of this mutation. However, this mutant retained only 0.1% of the wild type activity in the ACP-dependent assay (Table IV), indicating that the alanine to tyrosine substitution introduced a change into the protein that was specific for ACP over CoA substrates. If the overall structure was perturbed randomly, then the activity in both assays should be reduced equally.

A closer analysis of the specific activities of the mutant proteins in the ACP-dependent assay supports the more detailed features of the model. The substitution of Ala-253 with the bulky side chain of tyrosine was predicted to hinder the close approach of the protein

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**FIG. 1.** A surface rendition of the FabH:ACP complex predicted by SurfDock. **Left panel,** a surface rendition of the complex in which the FabH and ACP surfaces are colored according to their hydrophobic/hydrophilic properties. ACP is on the left differentiated from FabH by the net surface. **Red to blue** indicates the shift from hydrophobic to hydrophilic character. Most of the FabH surface is hydrophilic, with only a few hydrophobic patches evident. **Right panel,** a ribbon diagram of the modeled complex showing the interaction of the two molecules and the location of the active site entrance.

**TABLE III**

| Interacting amino acid residues | ACP | FabH |
|---------------------------------|-----|------|
| Arg-6                           | His-222 | |
| Glu-13                          | Lys-256 | |
| Ser-36                          | Arg-36 | |
| Glu-41                          | Arg-249 | |
| Met-44                          | Phe-213 | |
| Ala-45                          | Ala-253 | |
| Glu-47                          | Lys-214 | |
| Glu-48                          | Lys-214 | |
| Glu-49                          | Thr-218 | |
| Glu-49                          | Lys-257 | |

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**FIG. 2.** A stereo view showing the important interactions at the interface of the FabH:ACP complex predicted by SurfDock. **Left panel,** overview of the interacting helices on ACP helix II (chocolate brown) and FabH Co2 (blue). The location of Ser-36 of ACP, which is the attachment site for the 4'-phosphopantetheine prosthetic group, is indicated in relation to the active site entrance. **Right panel,** a magnified stereo view of the predicted interaction surface. The amino acid residue pairs (see also Table III) that interact are color-coded as follows (ACP/FabH): orange, Glu-13/Lys-256; blue, Glu-41/Arg-249; purple, Met-44/Phe-213; green, Ala-45/Ala-253; yellow, Glu-47 and Glu-48/Lys-214; cyan, Glu-49/Lys-257. The interacting α-helices at the interface, helix II from ACP and Cα2 from FabH, are highlighted in chocolate brown and blue, respectively. For clarity, some of the peripheral interactions listed in Table III have been omitted from the figure. Arg-249 from FabH and Glu-41 from ACP are located just above Ser-36 of ACP and are proposed to form a crucial ionic interaction adjacent to the active site entrance. The figure was produced using MOLSCRIPT (55) and rendered with Raster3D (56).
molecules, and the specific activity is indeed minimal. Also, the substitution of negatively charged glutamic acid for the positively charged arginines or lysines (FabH[R249E], FabH[R249E/K257E], and FabH[K214E]) uniformly dropped the activities of the mutant proteins, presumably by introducing repulsive ionic interactions into regions where attractive interactions were predicted to occur. In contrast, although the FabH[R249A] and FabH[K256A/K257A] mutants displayed reduced activities, the effects were less severe. The only mutation that produced an unexpected result was FabH[R249A]. Like the other alanine mutations, this was predicted to show a subtle change in activity, but it turned out to be very defective in the ACP-dependent assay (Table IV). The identical residues are in uppercase (minus leader sequences) were compared. The identical residues are in uppercase.

**Fig. 3. Conservation of residues on FabH and ACP.** A primary sequences of FabH enzymes from bacteria and plants are compared, and the region containing the residues proposed to interact with ACP is highlighted. The His in the active site is marked with an asterisk. Numbering is for **E. coli** FabH. The ACP proteins from representative prokaryotes and eukaryotes (minus leader sequences) were compared. The identical residues are in uppercase, whereas those identified as interacting with FabH by SurfDock are highlighted. The region from Leu-32 to Phe-50 is highly conserved and includes helix II and the conserved loop containing the prosthetic group attachment site (Ser-36; indicated with an asterisk).

**Table IV**

| ACP specific activity in FabH and ACP docking site mutants in ACP-dependent and ACP-independent assays |
|--------------------------------------------------|
| **FabH**                                 | Malonyl-ACP   | Malonyl-CoA   |
| Wild type                               | 4.156 ± 0.900 | 0.093 ± 0.010 |
| A253Y                                   | 0.004 ± 0.001 | 0.016 ± 0.001 |
| R249E                                   | 0.009 ± 0.002 | 0.110 ± 0.002 |
| K256E/K257E                             | 0.081 ± 0.011 | 0.115 ± 0.006 |
| K256A/K257A                             | 0.043 ± 0.020 | 0.117 ± 0.007 |
| K256E/K257E                             | 0.081 ± 0.012 | 0.070 ± 0.006 |
| K214E                                   | 0.294 ± 0.006 | 0.103 ± 0.002 |
| K214A                                   | 1.560 ± 0.050 | 0.111 ± 0.006 |

* Numbers in parentheses, %.

**Fig. 4. ACP inhibition of FabH and FabH[R249A].** The ability of ACP to function as an inhibitor of the condensing enzyme reaction was evaluated using a spectrophotometric assay utilizing malonyl-CoA as described under "Experimental Procedures" and the indicated concentration of ACP and either 50 μg of FabH (●) or 50 μg of FabH[R249A] (○).
FIG. 5. FabH-ACP binding studies using surface plasmon resonance. ACP was immobilized to the surface of a CM-5 chip, either FabH or FabH[R249A] was injected, and the change in the relative refractive index was measured using the Biacore 3000 as described under "Experimental Procedures." The refractive index change with FabH at 0.83 μM protein is indicative of binding to the immobilized ACP, whereas a binding signal was not detected with FabH[R249A] at 133 μM. An apparent binding constant of 2 ± 1 μM was calculated by determining the relative extent of FabH binding at seven different protein concentrations.

FIG. 6. The positively charged/hydrophobic region of FabH that is predicted to be the binding site for ACP. A, the region in the context of the whole molecule. Note that the surface of FabH is generally electronegative. The active site tunnel entrance is flanked on one side by the ACP-binding site and on the other side by the known coenzyme A binding site. The two sites are contiguous but distinct. B, a magnified view of the ACP binding surface. The locations of the residues mutated in this study are indicated. The extreme ranges of red (negative) and blue (positive) represent electrostatic potentials of <-9 to >+9 kBT, where kB is the Boltzmann constant and T is the temperature. The figure was calculated using the GRASP program (57).

Arg-249 as a critical residue in the interaction between FabH and ACP.

FIG. 7. Comparison of the electropotential surfaces of six ACP-binding enzymes. The figure was made using the known coordinates for FabA (21); FabB (our coordinates were deposited in the Protein Data Bank, accession code 1G5X); FabD (58); FabF (39); FabH (59); and LpxA (60). Each protein has a basic/hydrophobic patch (X) suitable for binding ACP that is adjacent to the active site (○). The distances between these zones are: FabH, 10.1 Å; FabA, 11.9 Å; FabB, 13.7 Å; FabD, 11.7 Å; FabF, 13.3 Å; FabI, 13.3 Å; and LpxA, 14.9 Å. The extreme ranges of red (negative) and blue (positive) represent electrostatic potentials of <-9 to >+9 kBT, where kB is the Boltzmann constant and T is the temperature. The figure was calculated using the GRASP program (57).

Support the idea that ionic interactions are important docking determinants and that ACP interacts with each of these residues.

To eliminate the ability of lysines 214, 256, and 257 to form ionic bonds with ACP, we mutated them to alanines and demonstrated only a moderate effect on FabH catalytic activity (Table IV). In contrast, when Arg-249 was changed to an alanine, the mutant FabH was severely impaired in the ACP-dependent assay (Table IV) and refractory to ACP inhibition (Fig. 4). To confirm the importance of Arg-249, we used surface plasmon resonance to directly monitor the interaction between FabH and ACP. Wild type FabH clearly bound to ACP, but FabH[R249A] showed no detectable interaction even at a relative 100-fold excess (Fig. 5). These data point to Arg-249 as the most important electropositive residue involved in FabH-ACP docking, and it is therefore significant that Arg-249 is the only residue on this surface that is completely invariant in FabH proteins (Fig. 3). Although Arg-249 appears to make a strong electrostatic interaction with Glu-41 of ACP, it is also likely that the guanidinium moiety and the proximal elements of the side chain form more extensive interactions with ACP.

A magnified view of the ACP-binding electrostatic surface of FabH (Fig. 6B) reveals the positive patches from Arg-249 and the other basic residues, but these are interspersed with additional hydrophobic regions that presumably contribute to the binding specificity. Ala-253 is a highly conserved residue located in a hydrophobic depression on the surface, and it is predicted to interact with a sister alanine of ACP. The importance of this depression was tested by introducing a bulky side chain in place of Ala-253. This modification effectively attenuates the interaction between ACP and FabH. Thus, we suggest that the small hydrophobic alanines in the FabH-ACP interface are critical for allowing the close approach of the two proteins.

Our model predicts that other ACP-binding proteins will also contain a conserved arginine (lysine) residue in a hydrophobic/electropositive patch adjacent to their active sites. The crystal structures of five other ACP-binding proteins are known: FabA, FabD, FabF, FabI, and LpxA. FabB is highly homologous to FabF, and we determined its crystal structure to 2.5 Å resolution using molecular replacement methods (see "Experimental Procedures"). While this analysis was underway, the FabB

**Features of ACP Binding Sites**—The surface of FabH surrounding the active site tunnel is generally electropositive, in contrast to the rest of FabH, which is highly electronegative (Fig. 6A). However, ACP is an acidic protein, and it is reasonable to suppose that ACP is attracted to this general locale on the FabH surface and away from the rest of the protein. Accordingly, the top scoring complexes from SurfDock all involve ACP bound to the electropositive FabH surface. The specificity determinants of the interaction cannot easily be determined from the models, because the precise orientations of the side chains at the interface are not known. However, the mutagenesis experiments that replaced the lysine and arginine residues in this region of FabH with oppositely charged glutamates
structure was reported elsewhere (50), and we refer the reader to this citation for a more detailed description of the FabB structure. Each of these six proteins has a region adjacent to its active site that incorporates the important features of the putative FabH ACP-binding site (Fig. 7). In all cases, the active site entrance is adjacent to a positively charged/hydrophobic patch that would be predicted to dock the incoming acyl-ACP substrate. The distances between the positively charged/hydrophobic patches and the active site entrances varied from 10.1 Å for FabH to 14.9 Å for LpxA (Fig. 7). Compelling support for the generality of the model comes from examination of the recent crystal structure of the AcpS-ACP complex (51). In this case, a salt bridge between Glu-41 of ACP and Arg-221 of AcpS is a critical interaction that determines the binding of ACP to AcpS.

Features of ACP that Support the Model—A comparison of the primary structures of ACPs from a variety of species (Fig. 3B) reveals that residues 32 to 50 are highly conserved, in contrast to the rest of the protein where the primary structure is quite variable. This pattern of sequence conservation supports our model, because the conserved region encompasses the entire interface with FabH. Specifically, it includes the whole of helix II, which is predicted to pack against helix C. The face region predicted by SurfDock in the highest scoring ACP complex is that the 4-helix bundle of ACP interacts with FabA. In addition, changes during the binding process. These NMR data are entirely consistent with the docking model generated by SurfDock. Specifically, the majority of the ACP residues that experience chemical shift changes are located on the face of helix II within the relatively rigid three-helix bundle. This is precisely the interface region predicted by SurfDock in the highest scoring FabH-ACP complex described here. Also, the apparent conformational movement in the ACP binding process would not disrupt our predicted FabH-ACP complex, and it would provide a reasonable mechanism whereby the prosthetic group attached to Ser-36 could be injected into the active site tunnel.

A Model for the Interaction of ACP with Its Target Proteins—A crucial requirement of any proposed FabH-ACP complex is that the 4-phosphopantetheine prosthetic group attached to Ser-36 must be able to deliver the acyl group on its terminal sulfhydryl to the active site. As already noted, the orientation of Ser-36 in our model is ideal, and one reason for this may be the proposed ion pair between Arg-249 and Glu-41 (Table III). This interaction between two completely conserved residues is directly adjacent to Ser-36 on helix II. It is tempting to speculate that this ion pair has a critical role in orienting the prosthetic group relative to the active site tunnel, and this may be a crucial interaction in all the complexes that ACP forms with the enzymes of fatty acid biosynthesis. Although the orientation of Ser-36 is ideal in the docking model, it is not close enough to allow the attached prosthetic group to deliver the substrate to the FabH active site. We estimate that a movement of some 10 Å would be required in the ACP molecule to bridge this gap. Because the NMR data show that ACP has conformational flexibility in this region, we propose that this is a required feature of the interaction. Biochemical evidence suggests that the lipid molecule attached to ACP interacts with and modifies the ACP structure (18, 54). Therefore, the required dissociation of the lipid during the binding process may help to promote this proposed conformational change in the ACP molecule.

Thus, we envisage three discrete steps in a dynamic ACP-binding process. The first step involves a weak but specific interaction between the target protein and the rigid three-helix bundle of ACP. This is the interaction found by SurfDock for FabH, and effectively aligns Ser-36 and the prosthetic group with respect to the active site entrance. In the second step, a conformational change in ACP injects the substrate attached to the prosthetic group into the active site. These movements include an unfolding of an extended flexible loop, and might be driven by the release of the acyl group from its interactions with ACP after the initial binding step. Finally, the modified substrate is removed from the active site by a reversal of the conformational changes, and the ACP structure is stabilized through interactions with the new lipid intermediate. This mechanism has two attractive features. First, it accommodates the various architectures of the target ACP-binding proteins in which the distance from Ser-36 to the active site is not constant. Second, it provides a way of easily introducing and extracting the prosthetic group from the active site openings without forming a tight complex between the two proteins. The FabH-ACP model forms the basis for strategies to stabilize the interaction with a view to growing diffraction quality crystals of this and other complexes.

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