Roles of the Active Site Water, Histidine 303, and Phenylalanine 396 in the Catalytic Mechanism of the Elongation Condensing Enzyme of Streptococcus pneumoniae

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β-Ketoacyl-ACP synthases catalyze the condensation steps in fatty acid and polyketide synthesis and are targets for the development of novel antibiotics and anti-obesity and anti-cancer agents. The roles of the active site residues in Streptococcus pneumoniae FabF (β-ketoacyl-ACP synthase II; SpFabF) were investigated to clarify the mechanism for this enzyme superfamily. The nucleophilic cysteine of the active site triad was required for acyl-envelope formation and the overall condensation activity. The two active site histidines in the elongation condensing enzyme have different electronic states and functions. His³⁰³ is essential for condensation activity, and its protonated NE stabilizes the negative charge developed on the malonyl thioester carbonyl in the transition state. The NE of His³⁰³ accelerated catalysis by deprotonating a structured active site water for nucleophilic attack on the C3 of malonate, releasing bicarbonate. Lys³³² controls the electronic state of His³⁰³ and also plays a critical role in the positioning of His³³⁷. Phe³⁹⁶ functions as a gatekeeper that controls the order of substrate addition. These data assign specific roles for each active site residue and lead to a revised general mechanism for this important class of enzymes.

The condensing enzymes play a central role in fatty acid biosynthesis by elongating the growing acyl chain by two carbon atoms to initiate each elongation cycle (1, 2). Somewhat uniquely in biological synthesis, the enzymes create a carbon-carbon bond via a Claisen-like condensation reaction (3). Specifically, they catalyze the condensation of malonyl-acyl carrier protein (ACP) with an acyl-ACP intermediate via a two-step ping-pong kinetic mechanism. In the first step, an acyl chain from either acyl-CoA or acyl-ACP is transferred to an active site cysteine, and the cofactor is released. During the second step, malonyl-ACP bands, and a carbanion is generated on the C2 of malonate concomitant with the release of the C3 carboxyl group (4–6). The carbanion then attacks the acyl-enzyme intermediate to produce the β-ketoacyl-ACP product. In the dissociated, type II synthases, the condensation reaction is carried out by monofunctional enzymes (7), and most bacteria have only a single elongation-condensing enzyme that belongs to the FabF class. In mammals and yeast, the condensing enzyme component, KS, is fused into a multidomain complex referred to as the type I or associated FAS system (8). However, it is clear from primary sequence analysis that the active site of the FAS I condensation module is very similar to the FAS II elongation enzymes (Fig. 1A). The polyketide synthases also contain a condensing enzyme module in which the same signature active site residues can be identified (Fig. 1A).

The importance of the elongation condensing enzymes in regulating fatty acid formation (7, 9) and the unique chemistry of the reaction that they catalyze (3) have focused our interest on understanding the specific tasks of each active site residue in catalysis. In addition, these enzymes have emerged as attractive targets for the development of new broad-spectrum antibiotics (10–12) and anti-obesity/anti-cancer drugs (13–17), and there is growing interest in engineering the polyketide synthases to produce novel therapeutic agents (18). These efforts will be facilitated by a complete mechanistic understanding of the active site. At their catalytic cores, the elongation enzymes possess a Cys-His-His triad. These residues have been mutated and are thought to be critical to the overall forward condensation reaction (8, 19–22), although the functions of these residues in the partial reactions of the catalytic cycle are not precisely known. There are also conserved lysine, glutamate and phenylalanine residues in the vicinity of the active site, and the structural and/or catalytic roles for these residues are not understood (Fig. 1A). Our systematic analysis of a panel of mutants in a model elongation condensing enzyme and the correlation of this new information with new and previously determined structures led to a reevaluation of the roles for the active site residues in the catalytic cycle. We selected SpFabF as the basis for this work, since its x-ray structure is known at the highest resolution (1.3 Å) (21). A stereo view of the SpFabF active site illustrating the orientation of the key residues investigated in this study is shown in Fig. 1B.

**EXPERIMENTAL PROCEDURES**

**Materials**—Myristoyl-ACP was prepared using an established acyl-ACP synthetase method (23, 24). [2-¹⁴C]Malonyl-CoA and sodium [¹³C]bicarbonate were purchased from Amersham Biosciences. [1-¹⁴C]Lauroyl-CoA was purchased from American Radiolabeled Chemicals. Malonyl-ACP was prepared using AcpS (ACP synthase) with apo-ACP and malonyl-CoA as described (25). His-tagged AcpS, FabD, FabG, FabI, FabZ, FabB (β-ketoacyl-ACP synthase I), FabF, and FabH (β-ketoacyl-ACP synthase III) from Escherichia coli and FabF...
from *Streptococcus pneumoniae* were purified as described previously (21, 26, 27). Proteins were quantitated by the Bradford method (28).

**Construction of Mutants and Protein Purification**—A DNA fragment encoding the wild-type *S. pneumoniae* FabF was cloned into the pET11b vector between the NdeI and BamHI sites. Mutations at the active site residues were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All clones were checked by automated sequencing on an ABI Prism 3700 DNA Analyzer. His-tagged mutant proteins were expressed in Rosetta (DE3) cells by automated sequencing on an ABI Prism 3700 DNA Analyzer. The 40-μl reaction mix was spotted on a preadsorbent Silica Gel G plate (Analtech) developed in chloroform/methanol/acetic acid (24:1:1, v/v/v), dried, and exposed to a phosphor storage imager (Amersham Biosciences). The distribution of radioactivity was detected and quantitated as described above.

**Malonyl-ACP Bicarbonate Exchange Reaction**—The carbon exchange between malonyl-ACP and bicarbonate is the reverse reaction of the condensing enzyme (5). The reaction contained 50 μM malonyl-ACP, 5 μM myristoyl-ACP, 10 mM [14C]bicarbonate and 8 μg of wild-type *SpFabB* or mutants in 100 mM potassium phosphate, pH 6.8, in a final volume of 20 μl. Following incubation at 37 °C for 20 min, the reaction was stopped by placing the tubes in an ice slurry after being loaded onto a conformationally sensitive 0.5 μm urea, 13% acrylamide gel. Radioactivity was detected and quantitated as described above.

**Complementation of the fabB(Ts) Growth Phenotype**—Strain CY274 (*fabB15(Ts)*) was unable to grow at the nonpermissive temperature (42 °C) unless unsaturated fatty acids (oleate) were supplied or the *fabB* gene was expressed in trans. The ability of wild-type and mutant FabB to complement the *fabB(Ts)* phenotype was tested after transformation of strain CY274 with plasmids carrying wild-type or mutant *fabB* (32).

**SpFabF-ACP Interactions**—AlphaScreen technology is an experimental method to quantitate protein-protein interactions (33). Upon laser excitation, a chemical signal is generated on the donor beads (streptavidin-coated). When a specific interaction takes place, the acceptor beads (coated with a specific antibody or nickel chelater) emit a signal that is proportional to the proximity of the donor beads. A cascade of energy transfers take place, emitting highly amplified fluorescence (AlphaScreen signal) at a wavelength that is lower than that of the excitation. We measured ACP affinity using the competitive binding approach. Briefly, 1 μM biotinylated ACP was mixed with 1 μM His-tagged wild-type *SpFabF* or mutant in the presence of different concentrations of regular ACP (120 nM to 10 μM) in a 384-well ProxiPlate (PerkinElmer Life Sciences). After 30 min incubation, streptavidin donor beads and Ni2+ chelate acceptor beads (50 μg/ml final concentration for each bead; Alphascreen Histidine Detection Kit, PerkinElmer Life Sciences) were added to the above solutions. The reaction mixtures were then incubated for 1 h before being read by Fusion Universal Microplate Analyzer (PerkinElmer Life Sciences), with excitation at 680 nm and emission at 600 nm.

**X-ray Crystallography**—Crystals of the *SpFabF* [H103A] mutant were grown in 20% polyethylene glycol 3350, 0.2 M potassium acetate and were in space group P2_121 with cell dimensions *a* = 60.39 Å, *b* = 88.82 Å, and *c* = 61.05 Å. Rod-shaped crystals measuring ~0.25 mm on edge were harvested after 9 days and frozen in liquid nitrogen using 25% PEG 400 as a cryoprotectant. A 2.6 Å data set was collected at the SER-CAT ID beamline (Sector 22 at the Advanced Photon Source in Argonne National Laboratory), and processed using MOSFLM (34) and SCALA (35). The structure was solved by molecular replacement, using the program AMoRe (36) and the native *SpFabF* structure (Protein Data Bank code 1OX0) as the search model, and refined using a combination of REFMAC (37) and CNS (38). Data collection and refinement statistics are shown in Table 3. Model building was carried out using the O program (39).

**RESULTS**

**Condensing Enzyme Activity of FabF Mutants**—A subset of conserved residues within four protein segments in the elongation class of condensing enzymes of FAS I, FAS II, and the polyketide synthases was selected for analysis (Fig. 1). The active site cysteine (Cys164) and the two histidines (His303 and His307) that comprise the central Cys-His-His triad were obvious choices for site-directed
FIGURE 1. Conservation and orientation within the SpFabF active site of the amino acid residues participating in condensing enzyme catalysis. A, partial sequence alignment of the elongation condensing enzymes. This study focused on four conserved segments that are present in all members of the elongation condensing enzyme family. Sp, S. pneumoniae; Ec, E. coli; Mt, Mycobacterium tuberculosis. Also included in the sequence analysis are two condensing enzymes from human and yeast mitochondrial type II FAS (MitKS), the condensation domain from the human and yeast multifunctional type I FAS, and the condensation domain from the type I polyketide synthase from Streptomyces coelicolor A3(2) (ScA3PKS). For SpFabF, the residues investigated correspond to Cys164, His303, Lys332, and His337, Phe396, and Glu346, and for EcFabB the residue numbers are Cys163, His298, Lys328, His333, Phe392, and Glu342. B, stereo view of the SpFabF active site showing the orientation of the residues mutated in this study. The electronic states of the two histidines are controlled by the interaction of their NH nitrogens with either a backbone amide for His303 or a complex hydrogen bond network involving Lys332, Glu346, and a structured water (W2) for His337. Importantly, His303 forms a strong hydrogen bond with a structured water (W1), which also interacts with the backbone carbonyl of Phe392. Cys164 lies at the terminus of the N-terminal helix, and the Phe396 side chain is adjacent to the oxyanion hole formed by the backbone amides of Cys334 and Phe396. The pink ball indicates the focus of the oxyanion hole, and the pink dots show the interactions with the backbone amides. The red dots show hydrogen bond interactions.

Condensing Enzyme Mechanism

The overall condensation reaction was determined using [2-14C]malonyl-ACP and myristoyl-ACP as substrates. With the exception of SpFabF[H303A] and SpFabF[E346A], which retained 26 and 100% of the wild-type activity, respectively, all of the mutant condensing enzymes were deficient in their overall condensation activity (Fig. 2A, Table 1). The function of the mutants was also evaluated in an assay to reconstitute multiple rounds of fatty acid synthesis starting with acetyl-CoA as the primer (Fig. 2B). The SpFabF[H303A] and SpFabF[E396A] mutants retained activity, whereas the other mutants were inactive in this assay format, although neither active mutant produced as much long-chain acyl-ACPs as the wild type. In order to detect very low levels of activity, the assays were repeated at higher protein concentrations, and this revealed that SpFabF[K332A] exhibited a low, but significant, activity that was estimated at 0.2% of wild-type (Table 1). The activity of the other mutant proteins was not detected at the highest protein concentrations permitted by the assay format, setting the condensation activity of these constructs to <0.2% (Table 1).

The results with SpFabF[H303A] and SpFabF[K332A] were inconsistent with previous conclusions from work on EcFabB (40). This study concluded that the equivalent residues of His303 and Lys332 in EcFabB are both essential, whereas our analysis indicated that the two residues have important, but not absolutely essential, roles in promoting the overall condensation reaction. This apparent inconsistency might be attributable to subtle differences between the FabB and FabF subfamilies. We explored this point by constructing the same panel of mutants in EcFabB as reported previously (40); all of the FabB mutants appeared to be inactive using a protein concentration in the linear range for the wild-type enzyme (not shown). However, we were able to detect a trace of activity (~1% of wild type) in the EcFabB[H298A] and EcFabB[K328A] mutants when assayed at higher protein concentrations, illustrating that they retained partial condensation activity in vitro. We corroborated this conclusion using an in vivo complementation test to verify the activities of the FabB mutants (Table 2). The panel of fabB mutant alleles was introduced into E. coli strain CY274 (fabB15[Ts]), and the resulting strains were scored for correction of the temperature-sensitive growth phenotype (Table 2). The EcFabB[H298A] and EcFabB[K328A] mutants restored growth at the nonpermissive temperature, demonstrating that they retained condensing enzyme activity in vivo. In contrast, the inactive EcFabB[C163A] and EcFabB[H333A] mutants were unable to complement the growth phenotype. Importantly, since the condensation enzymes function as dimers, the lack of activity of the latter two mutants ruled out the possibility that complementation arose from the stabilization of the temperature-sensitive fabB allele by the expression of a properly folded protein. The results with the E. coli mutant panel (Table 2) were qualitatively the same as with the SpFabF panel (Table 1), although the level of enzyme activity in the EcFabB[H298A] was less than the residual activity in SpFabF[H303A]. Thus, the different condensing enzyme mutant sets differed in their absolute values of activity, but the rank order of activity in the mutant proteins was the same.

Active Site Residues Participating in Transacylation—The first step in the condensing enzyme reaction is the transfer of the acyl group to the active site cysteine, and this activity was assayed by monitoring the transfer of the isotopic labeled acyl group from [1-14C]lauroyl-CoA to ACP via the acyl-enzyme intermediate. As expected, mutants lacking this critical cysteine were completely inactive in the transacylation step (Fig. 2C). In contrast, SpFabF[H337A], SpFabF[H303A], SpFabF[E346A], SpFabF[E346A], and SpFabF[K332A] all retained transacylation activity (Fig. 2C, Table 1), mutagenesis. Also selected were Lys332 and Glu346 that have been implicated in catalysis, although Glu346 is not conserved in FAS I and polyketide synthases (Fig. 1). Finally, Phe396 was also included in the analysis, because it is located in a flexible region of the active site surrounded by conserved glycines. Each of these residues (Fig. 1) was mutated to alanine in SpFabF, and the mutants were analyzed for their ability to carry out the biochemical activities that comprise the full and partial reactions of the condensing enzyme catalytic cycle.

The function of the mutants was also evaluated in an assay to reconstitute multiple rounds of fatty acid synthesis starting with acetyl-CoA as the primer (Fig. 2B). The SpFabF[H303A] and SpFabF[E396A] mutants retained activity, whereas the other mutants were inactive in this assay format, although neither active mutant produced as much long-chain acyl-ACPs as the wild type. In order to detect very low levels of activity, the assays were repeated at higher protein concentrations, and this revealed that SpFabF[K332A] exhibited a low, but significant, activity that was estimated at 0.2% of wild-type (Table 1). The activity of the other mutant proteins was not detected at the highest protein concentrations permitted by the assay format, setting the condensation activity of these constructs to <0.2% (Table 1).
indicating that none of these residues were absolutely essential for trans-thioesterification. The transacylation assay can also detect accumulation of the [14C]acyl-enzyme intermediate, and this was only measurable for the SpFabF[K332A] mutant (not shown), indicating the removal of the conserved lysine either accelerated the rate of formation or reduced the rate of release of the acyl-enzyme intermediate. These results showed that the only active site residue required for transacylation was Cys164.

The finding that the SpFabF[H337A] mutant had 12% the transacylation activity of wild-type (4.15 ± 0.46 pmol/µg/min) suggests that it has little influence on the ionization of the active site cysteine.

FIGURE 2. Enzymatic activities of the panel of SpFabF mutants. A, the overall condensation reaction using myristoyl-ACP and [2-14C]malonyl-ACP as substrates. ●, wild type; ○, SpFabF[H303A]; ▲, SpFabF[H337A]; ▼, SpFabF[C164A]. B, the activity of the SpFabF mutant panel in a reconstituted fatty acid synthase assay. These reactions were initiated with FabH and acetyl-CoA, and the formation of intermediate- and long-chain acyl-ACPs was analyzed by conformationally sensitive gel electrophoresis. C, the transacylation half-reaction of the condensing enzyme mutant panel assayed by the transfer of [1-14C]lauroyl group from CoA to ACP. ●, wild type; ○, SpFabF[H303A]; ▲, SpFabF[K332A]; ▼, SpFabF[H337A]; ▼, SpFabF[C164A]. D, [2-14C]malonyl-ACP decarboxylase activity of the condensing enzyme mutants analyzed by conformationally sensitive gel electrophoresis. Mal-ACP, malonyl-ACP; Ac-ACP, acetyl-ACP. E, TAL formation by the condensing enzymes analyzed by thin layer chromatography.

TABLE 1
Summary of the activities of S. pneumoniae FabF active site mutants
The specific activities for the 100% reactions were as follows: condensation, 147 ± 7 pmol/µg/min; transacylation, 4.15 ± 0.46 pmol/µg/min; acetyl-ACP formation, 2.49 ± 0.04 pmol/µg/min; and TAL formation, 0.122 ± 0.03 pmol/µg/min.

| SpFabF     | Condensation (14C-β-ketopalmitoyl-ACP) | Transaclylation (14C-lauroyl-CoA) | Decarboxylation (HCO₃⁻ + 14C-Ac-ACP) | TAL formation (3 14C-TAL) |
|------------|--------------------------------------|----------------------------------|--------------------------------------|--------------------------|
|            | %                                    | %                                | %                                    | %                        |
| Wild-type  | 100                                  | 100                              | 100                                  | 100                      |
| C164A      | 25.6                                 | 12                               | 2.5                                  | 2.5                      |
| K332A      | 0.2                                  | 28.5                             | 3.5                                  | 3.5                      |
| H303A      |                                      | 60                               |                                      | 500                      |
| H337A      |                                      | 12                               |                                      |                          |
| C164A/K332A|                                      |                                  |                                      |                          |
| C164A/H303A|                                      |                                  |                                      |                          |
| C164A/H337A|                                      |                                  |                                      |                          |
| E396A      | 0.7                                  | 59.4                             | 4.3                                  | 39.9                     |
| E346A      | 100                                  |                                  | 100                                  | 100                      |

* <0.2% of control activity.
His$^{337}$ is within hydrogen-bonding distance of Cys$^{164}$, its electronic state is inconsistent with a role for this residue in deprotonating the active site sulfhydryl. The N$_6$ nitrogen of His$^{337}$ accepts a hydrogen bond from a backbone amide, meaning that the lone pair cannot be on the N$_8$ nitrogen adjacent to Cys$^{164}$ (Fig. 1B). Thus, the fixed electronic state of the histidine renders it unable to facilitate the abstraction of a proton from Cys$^{164}$. Perhaps one could postulate a conformational change, but none of the many available structures give any hint of this. The cerulenin and thiolaocmycin inhibitor structures (27, 41) show that the Ne nitrogen of His$^{337}$ donates a hydrogen bond to the carbonyl of the antibiotics, supporting the placement of the lone pair on N$_8$ rather than on the Ne adjacent to Cys$^{164}$. In the related FabH condensing enzymes, this histidine is replaced by an asparagine residue, and the O$_6$1 receives the same hydrogen bond from the backbone amide as does the N$_6$ nitrogen of His$^{337}$, again placing the protonated nitrogen adjacent to the active site cysteine. Although there may be mechanistic differences between the FabH and FabB/F classes of condensing enzymes, the requirement to generate the thiolate is a common feature and is attributed to the strong helix dipole conserved between the two proteins rather than the histidine, which is not conserved (26).

**Residues Participating in the Decarboxylation Step**—The formation of acetyl-ACP from malonyl-ACP measures the ability of the enzymes to create the carbonyl intermediate via the release of the C3 carboxyl group and occurs in the absence of an acyl-enzyme intermediate. Although this is a futile, nonproductive reaction, it independently measures the status of the decarboxylation half-reaction. This partial reaction requires that malonyl-ACP bind to the active site prior to the formation of the acyl-enzyme intermediate. This reversal of the normal order of substrate binding occurred at a low rate in the wild-type enzyme, indicating that the protein was able to limit this nonproductive reaction (Fig. 2D). However, the SpFabF[C164A] mutant exhibited robust malonyl-ACP decarboxylase activity that generated copious amounts of acetyl-ACP (Fig. 2D). A second derangement reaction that complicates the analyses of the decarboxylation activity of the SpFabF single mutants was the formation of the TAL by-product. TAL formation requires the presence of the active site cysteine (see below); thus, we eliminated TAL formation and maximized decarboxylation activity by constructing double mutants. SpFabF[C164A/H337A] did not exhibit decarboxylation activity, whereas SpFabF[C164A/H303A] retained activity, indicating that only His$^{337}$ played a critical role in the formation of the carbonyl (Fig. 2D). Somewhat surprisingly, SpFabF[C164A/H332A] was also defective in acetyl-ACP formation (Fig. 2D, Table 1), indicating that Lys$^{332}$ and His$^{337}$ were both involved in the formation of the carbonyl. This was a counterintuitive result, because the crystal structure of SpFabF reveals that the side chain of Lys$^{332}$ interacts via a water molecule to His$^{330}$ rather than to His$^{337}$ (Fig. 1B).

The Formation of TAL—TAL is an abortive product of the elongation condensing enzymes (31) arising from the reverse order of substrate binding. Malonyl-ACP binds to the free enzyme and is decarboxylated. The resulting acetyl moiety is then transferred to the active site cysteine, and ACP is released. A second molecule of malonyl-ACP then binds to the acetyl-enzyme intermediate and is condensed to form β-ketobutyryl-ACP, which is released. In the absence of the β-ketoacyl-ACP reductase to convert β-ketobutyryl-ACP to β-hydroxybutyryl-ACP (43–45), the ketoacyl group is again transferred to the cysteine and condensed with a third molecule of malonyl-ACP to release 3,5-diketohexanoyl-ACP. This molecule undergoes an internal, spontaneous cyclic rearrangement to produce TAL and ACP. It was important to measure this derangement condensation reaction in our analyses of the activities of the mutants to verify that any decrease in either condensation or acetyl-ACP formation was not due to an increase in TAL formation. TAL formation requires a different assay, because it is not attached to ACP and therefore is not detectable in the gel electrophoretic assays employed in Fig. 2, B and D. TAL formation by wild-type SpFabF was low (0.122 ± 0.03 pmol/μg/min) but detectable (Fig. 2E, Table 1). SpFabF[C164A], SpFabF[H337A], and the double mutants containing any one of these mutations did not produce TAL (Fig. 2E, Table 1). SpFabF[K332A] produced only 10% of the amount of TAL as wild type, consistent with its low condensation activity. However, SpFabF[H303A] displayed a robust TAL formation (Fig. 2E). This was a significant finding, which revealed that the apparent deficiency in the formation of acetyl-ACP by SpFabF[H303A] was not due to an inability to decarboxylate the substrate per se but rather due to efficient TAL production (Fig. 2E). Another important observation was that SpFabF[F396A] also produced more TAL than would be predicted based on its deficient condensation activity assayed with long-chain acyl-ACP (Table 1).

**Malonyl-ACP/Bicarbonate Exchange Reaction**—The diagram of the overall chemistry performed by the condensing enzymes typically shows that the C3 carboxyl group of malonate is released as CO$_2$. However, the first assay developed for ACP used the condensing enzymes to carry out the malonyl-CoA/[14C]bicarbonate exchange reaction (5), and this activity, which corresponded to the reverse reaction, pointed to bicarbonate as the product of the reaction. Indeed, bicarbonate was recently verified as the product of the mammalian FAS I condensation step (6). We tested the ability of our SpFabF enzymes to perform the reverse exchange reaction and were only able to detect activity in wild type and SpFabF[H303A] mutant (Fig. 3A). The data suggested that SpFabF[H303A] utilized the same mechanism for the reverse reaction as the wild-type enzyme, albeit at a slower rate (Fig. 3A). Thus, not only did SpFabF[H303A] retain significant condensation activity, it was also able to carry out the reverse reaction. Conversely, the inability of the other mutants to carry out the exchange reaction was consistent with their defects in acetyl-ACP formation, TAL formation, and the overall condensation reaction (Table 1). We then tested whether bicarbonate can act as a product inhibitor of malonyl-ACP decarboxylation (Fig. 3B). The activities of SpFabF[C164A] and SpFabF[C164A/H303A] were compared with elimination of the complication of TAL formation in the decarboxylation assay, and both enzymes were equally inhibited by bicarbonate in the assay. These experiments were consistent with bicarbonate being the product of the reaction in the presence and absence of His$^{303}$.

The crystal structure of SpFabF shows a water molecule (W1) in the active site forming a strong hydrogen bond with the side chain of His$^{303}$ (Fig. 1B). This water molecule has a B factor similar to those of the surrounding residues (21), and we hypothesize that it represents a catalytic water that is activated by the fixed electron state of His$^{303}$ to attack the carboxyl group of malonyl-ACP, leading to the release of bicarbonate. If His$^{303}$ functions in this capacity to accelerate catalysis, then SpFabF[H303A] would be more catalytically defective at
acidic than alkaline pH, where hydroxide ion is present at higher concentrations. Measurement of the pH rate profiles supported this hypothesis. For the wild-type protein, the pH rate profile for the overall condensation activity was rather flat between pH 6 and 8, with a 20% decrease at pH 6.0 compared with pH 7.0. In contrast, the SpFabF[H303A] mutant activity was reduced by 75% at pH 6.0 compared with pH 7.0 (Fig. 3C). The malonyl-ACP/bicarbonate exchange of SpFabF[H303A] exhibited the same pH dependence with the lowest activity at pH 6.0 (Fig. 3D).

Interaction between ACP and SpFabF Mutants—The defects in catalytic activity in the SpFabF mutants could possibly be due to structural changes that compromised their interaction with ACP substrates. Because many of the mutants were catalytically inactive in one or more of the reactions, the proteins could not be systematically compared by kinetic analysis. Examining the crystal structures of the mutants did not give any indication that the mutations caused changes in the protein surface. This conclusion was verified by measuring the ability of ACP to dissociate the interaction with each His-tagged SpFabF mutant protein and biotinylated ACP (Fig. 4). The base-line signal was obtained with 1 μM each SpFabF and biotinylated ACP. Although there were some small differences in the ACP displacement curves between the proteins, each of the mutant proteins bound ACP with essentially the same affinity, indicating that alteration in protein-protein interactions did not contribute to the catalytic deficiencies of the SpFabF mutants.

Structure of the SpFabF[H303A] Mutant—The removal of the His303 side chain from the active site had additional important effects on the structure of the FabF active site (Table 3; Fig. 5A). The absence of the His303 side chain resulted in the inability to detect structured water molecules in the active site tunnel. This observation is consistent with the idea that His303 is central to a hydrogen bond network that organizes the water molecules that fill the active site tunnel through a strong hydrogen bond interaction with W1. Also, the electron density of the Phe396 side chain was weak (Fig. 5A). Analysis of the SpFabF[H303A]
multiple conformations in the absence of the His303 side chain (Fig. 5A).

In the native structure (Fig. 1B), the fixed position of W1 allows only a single conformation for the Phe396 side chain (Table 3).

FIGURE 5. Structures of the SpFabF[H303A] and the FabF–cerulenin binary complex. A, the structure of the SpFabF[H303A] mutant active site. The His303 imidazole ring is missing, leading to the absence of structured waters within the active site and the free rotation of the Phe396 side chain. The structure of the native protein is shown in translucent green. B, structure of the FabF–cerulenin complex (accession number 1B3N) (41) compared with the free enzyme. Cerulenin forms a covalent derivative with the active site cysteine, and its hydrophobic tail extends into the acyl chain binding pocket of (41). In order for His303 to activate the water molecule, the lone pair of electrons must reside on the N imidazole ring. The 1.3-Å crystal structure of SpFabF revealed that the Nδ of His303 has significant additional electron density that we interpret as a hydrogen atom (21), and this places the lone pair on the N of the imidazole ring. The 1.3-Å crystal structure of SpFabF revealed that the Nδ of His303 has significant additional electron density that we interpret as a hydrogen atom (21), and this places the lone pair on the N of the imidazole ring. The 1.3-Å crystal structure of SpFabF revealed that the Nδ of His303 has significant additional electron density that we interpret as a hydrogen atom (21), and this places the lone pair on the N of the imidazole ring.

FIGURE 4. Interaction of wild-type SpFabF and mutant proteins with ACP. Concentrations (120 nM to 10 μM) of unlabeled ACP were added to the reaction mix, and the decrease in the AlphaScreen signal was recorded to measure the ability of the SpFabF mutant proteins to bind ACP. The lines represent the data fitted to one-phase exponential decay and showed no large differences in the binding of ACP to the wild-type (▲) and mutant SpFabF proteins (□, C164A; ○, H303A; ●, K332A; △, H337A; △, F396A).

mutant using mass spectrometry confirmed that the Phe396 residue was missing, leading to the absence of structured waters within the active site and the free rotation of the Phe396 side chain. The structure of the native protein is shown in translucent green. B, structure of the FabF–cerulenin complex (accession number 1B3N) (41) compared with the free enzyme. Cerulenin forms a covalent derivative with the active site cysteine, and its hydrophobic tail extends into the acyl chain binding pocket of the enzyme. Numbering of residues is according to the EcFabF sequence. The orientation of Phe396 (Phe396 in SpFabF) in the free enzyme is shown in translucent green to illustrate the movement of this side chain when the active site cysteine is acylated.

TABLE 3
Statistics of data collection and refinement

| Parameter (Unit) | SpFabF[H303A] |
|------------------|---------------|
| Space group      | P2₁₂₂         |
| Unit cell dimensions (Å) (a, b, c) | 60.4, 88.8, 61.0 |
| Resolution range (Å) | 61.1 to 2.6 |
| R merge (Å)      | 0.046 (0.472) |
| Complete (Å)     | 37.1 (3.5) |
| Completeness (%) | 99.5          |
| Redundancy*      | 4.7 (4.7)     |
| Reflections      | 254,362       |
| Unique reflections| 10,590        |
| Resolution range included in refinement (Å) | 30 to 2.6 |
| No. of reflections in working set | 10,506 |
| No. of reflections in test set (%) | 500 |
| No. of protein atoms in ASU | 3090 |
| No. of water molecules ASU | 40 |
| R work           | 0.194         |
| R free           | 0.256         |
| Root mean square deviation from ideal stereochemistry | 0.007 |
| Bond lengths (Å) | 1.315         |
| Mean B overall (Å²) | 34.75 |
| Ramachandran plot | 86.9  |
| Most favored region (%) | 11.9 |
| Additionally allowed region (%) | 0.6  |
| Generously allowed (%) | 0.6 |
| Disallowed (%)   | 0.6           |

* Values in parentheses refer to the highest resolution shell.

† R_work = Σ|I − I_model|/ΣI, where I is the intensity of the measured reflection, and I_model is the mean intensity of all symmetry-related reflections.

DISCUSSION

Roles of His303, His337, and W1—A primary conclusion from our biochemical and structural analyses is that the elongation condensing enzymes use His303 to activate a catalytic water molecule (Fig. 6). The active site of SpFabF (21) has a structured water molecule (W1) that is hydrogen-bonded to the Ne of His303 (Fig. 1B), and we propose that His303 activates W1 to promote a nucleophlic attack on the malonate carboxylate. Our examination of other elongation condensing enzyme structures (19, 21, 46) reveals that they contain water molecules in a very similar location to W1 that are hydrogen-bonded to the histidine residues equivalent to His303 in SpFabF. In order for His303 to activate the water molecule, the lone pair of electrons must reside on the Ne of the imidazole ring. The 1.3-Å crystal structure of SpFabF revealed that the Nδ of His303 has significant additional electron density that we interpret as a hydrogen atom (21), and this places the lone pair on the Ne. In contrast, the Ne of His337 is protonated, because the Nδ nitrogen receives a hydrogen bond from the backbone amide of Leu339 and therefore contains the lone pair of electrons (Fig. 1B). These structural insights mesh with the activities of the SpFabF mutant panel (Table 1). His303 is not absolutely essential for the condensation reaction in SpFabF but rather functions to accelerate the reaction rate, especially at lower pH. Wild-type SpFabF readily incorporates [14C]bicarbonate into malonyl-ACP in the reverse reaction, consistent with bicarbonate as the product of the reaction. SpFabF[H303A] also performs the malonyl-ACP exchange reaction with [14C]bicarbonate, albeit at a slower rate, and the presence of His303 is therefore not essential to carry out either the forward or the reverse reaction. A role for His303 as a base is supported by the biochemical and structural analysis of EcFabF[H298Q] and EcFabB[H298E] mutants in the decarboxylation half-reaction (47). However, the minor reduction of catalytic activity in the SpFabF[H303A] mutant argues against a major role for His303 in the SpFabF condensing enzyme. In contrast, the severe impairment of catalytic activity following mutation of the analogous His298 in EcFabB (40) indicates that His298 is more critical for catalysis in this elongation condensing enzyme. It is possible that rather than acting as a base, His303 is more critical for catalysis in this elongation condensing enzyme.
functions primarily to position the water molecule for attack on the malonate carboxyl group. The differences in the effects of removing this histidine on the enzymatic activity of different elongation condensing enzymes, the fact that we do not know the rate-controlling step in this complex ping-pong reaction, and the fact that we do not have the malonyl-ACP enzyme complex structure preclude a definitive conclusion on the role of His337 in the reaction mechanism.

Our proposed mechanism is not consistent with the electronic states of the active site histidines deduced from the structures of condensing enzyme binary complexes with cerulenin and thiolactomycin (27, 41). In both complexes, a carbonyl oxygen on the inhibitor receives hydrogen bonds from the Nε nitrogens of both His298 and His333 of EcFabB. The inhibitor carbonyl groups were initially interpreted as being equivalent to the thioester carbonyl group of malonate, but a close examination of the two binary complexes (27) reveals that the side chain of His298 moves ~2 Å to make a hydrogen bond with the inhibitor carbonyl group and the Nδ nitrogen is thereby shifted to within 3 Å of the Nε nitrogen of Lys328. We propose that this repositioning results in the movement of the lone pair from the Nε nitrogen of His298 to the Nδ nitrogen in the formation of the inhibitor complex and that the orientation and electronic states of the histidines in the drug complexes differ from those in the native protein.

**Activation of Cys164**—A key step in the transacylation reaction is the nucleophilic activation of the active site cysteine. One idea for how this occurs is that His337 and/or His303 facilitates the reaction by abstracting a proton from the sulphydryl group (19, 21). However, the electronic state of the Nε of His337 is protonated due to the strong hydrogen bond between its Nε with the backbone amide (Fig. 1), and none of the SpFabF active site mutants, except for SpFabF[C164A], are defective in the transfer of the primer acyl group from acyl-ACP to Cys164. Instead, the activation of the cysteine occurs through the dipole moment of helix Ne3, which is directed toward the sulfur atom and lowers the pKₐ of the cysteine sulfhydryl (21). The orientation of this helix dipole toward the active site sulphydryl within this protein superfamily was noted in the first thiolestate structure (48), and there is little doubt that it has a profound effect on the electrostatic environment of the condensing enzyme active site.

**Role of Lys332**—Lys332 is clearly a critical component of a hydrogen bond network that includes Glu346 and a structured water (W2) that interacts with His303 (Fig. 1B). This network maintains the correct resonance form of His303, which allows it to abstract a proton from the catalytic water, W1 (Fig. 6). If Lys332 controls the electronic state of His303, then one would predict that mutation of this lysine would result in a protein with catalytic properties similar to those observed with the SpFabF[H303A] mutant. Instead, the properties of SpFabF[K332A] more closely reflected the properties of SpFabF[H337A] (Table 1) (6, 40). This conundrum is resolved by the recently released crystal structure of the EcFabF[K328A] mutant (accession number 1OEO) (47). This structure shows that His333 (His337 in SpFabF) rotates into the space generated by the removal of Lys328 (Lys332 in SpFabF) (47) and has no effect on the position or orientation of His303 (His332 in SpFabF). Thus, the loss of Lys328 has a dramatic effect on the spatial positioning and hence the function of His337.

**Role of Phe396**—Our studies are the first to assign a key function for Phe396. Phe396 is flanked by conserved glycine residues and displays conformational variability in the available crystal structures. The structures of the FabB acyl-enzyme intermediate and the FabB–cerulenin complex (Fig. 5B) show that the aromatic ring rotates away from the active site and acts as a platform that directs the acyl chain into its binding pocket. The observation that TAL formation exceeds the forward condensation activity in the SpFabF[F396A] mutant suggests that Phe396 acts as the “gatekeeper” that controls the order of the reaction. In the free enzyme, the Phe396 side chain is closely packed into the active site, preventing the binding and decarboxylation of malonyl-ACP prior to the formation of the acyl-enzyme intermediate. After transacylation, the carbonyl of the thioester intermediate engages the oxyanion hole formed by the amide nitrogens of Cys164 and Phe396, causing the aromatic ring of Phe396 to rotate away from the active site, creating the necessary space to accommodate the incoming malonyl-ACP substrate. This movement is most clearly seen comparing the E. coli FabF structure with FabF with bound cerulenin (Fig. 5B). SpFabF[H303A] also exhibits enhanced TAL formation, and this is explained by the crystal structure, which reveals that the side chain of Phe396 is disordered and creates the necessary space for the second substrate, malonyl-ACP, to bind prior to the formation of the acyl-enzyme intermediate.

**A General Mechanism for Condensing Enzymes**—Our analysis is consistent with the model for the catalytic mechanism of the elongation condensing enzymes shown in Fig. 6. The first step involves the binding of acyl-ACP and the transfer of the acyl group to the active site cysteine.
The nucleophilicity of Cys$^{164}$ is enhanced by the strong Nα3-helix dipole effect, and the oxygen hole formed by the backbone amides of Cys$^{164}$ and Phe$^{396}$ (hole 1) promotes the reaction by accommodating the negative charge on the thioester carbonyl that develops in the tetrahedral transition state. ACP is released, and malonyl-ACP binds to the acyl-enzyme intermediate. His$^{303}$ activates a structured catalytic water dral transition state. ACP is released, and malonyl-ACP binds to the negative charge on the thioester carbonyl that develops in the tetrahe-longation condensing enzymes (Fig. 1).

The carbanion attacks the acyl-enzyme intermediate, and the tetrahe-longation transition state is again stabilized by the Cys$^{164}$/Phe$^{396}$ oxygen hole 1. The transition state resolves to release the β-ketoacyl-ACP prod-uct and bicarbonate. The condensation active sites of the FAS I multi-functional polypeptide and the polyketide synthases have the same Cys-His-His triad and the other key conserved residues as the FAS II functional polypeptide and the polyketide synthases have the same Cys-His-His triad and the other key conserved residues as the FAS II elongation condensing enzymes (Fig. 1A), suggesting that the catalytic mechanisms of these condensation modules are the same as for FpFabF. The role of a catalytic water in FAS I is established by the demonstration of bicarbonate as a reaction product (6).

Our model differs from that proposed by Olsen et al. (49), which suggested that the malonyl thioester engages the FabB active site with the carboxylic acid leaving group interacting with the Nε nitrogen of His$^{298}$ and two conserved threonines (300 and 302). This model is inconsistent with how the panetheine arm must enter the active site tunnel, and the C2 of malonate is also too far away from the acylated cysteine (about 5 Å) for the condensation reaction to occur. In our model, the malonate binds in the opposite orientation, which brings C2 into closer proximity to the acyl-enzyme intermediate and positions the distal peptide bond of the prosthetic group in a conformation to possibly interact with the two conserved threonines that line the active site tunnel.

The use of a catalytic water probably also extends to the initiation class of condensing enzymes that possess a Cys-His-Asn catalytic triad. We have verified that bicarbonate is incorporated into the malonyl-ACP in the FabH back reaction (not shown), suggesting that bicarbonate is also the product of this reaction. Close inspection of the FabH crystal structures (26, 50–53) reveals the presence of a catalytic water hydrogen-bonded to the active site histidine and in proximity to the cysteine sulfhydryl in an orientation very similar to that found in the elongation enzymes. This water molecule is interpreted by others as being responsible for the deprotonation of the active site sulfhydryl (53); however, our results suggest that the histidine and water may function in the same capacity as outlined in Fig. 6.

Comparison of Thiolase and Condensing Enzyme Mechanisms—The condensing enzymes belong to the thiolase superfamily, whose members catalyze the synthesis and degradation of carbon-carbon bonds. The synthetic thiolases deprotonate the α-carbon of acetyl-CoA, and the carbamation attacks the acetyl-enzyme intermediate to form the carbon-carbon bond. Although structurally similar, the condensing enzymes generate the same carbamion by decarboxylating a malonate thiouester. There are four differences between the active sites of the two enzyme clades that reflect these distinct mechanisms. First, thiolase contains two cysteines, one that accepts the acetyl intermediate and a second that performs the deprotonation step. The deprotonating cysteine spatially corresponds to the location of the histidine-water group in- ing in the condensing enzymes that drives the decarboxylation step. Second, the pair of histidines in the condensing enzymes is replaced by a histidine-asparagine pair in the thiolases. The asparagine is associated with a water molecule and participates with the adjacent histidine in the formation of an oxygen hole that stabilizes the carbamation. Third, it has been proposed that the Nε nitrogen of the thiolase histidine (equivalent to His$^{303}$ in FpFabF) activates the cysteine in the formation of the acetyl-enzyme intermediate (42). However, this is not possible in the condensing enzymes, because the lone pair of electrons is clearly located on the Nδ nitrogen (Fig. 1). We have also demonstrated that mutation of either conserved histidine does not significantly affect the transacylation step. Finally, structural studies of a number of thiolase complexes suggest that the histidine-water oxygen hole is involved in both the transacylation and deprotonation steps. Kursula et al. (42) propose that the acetyl carbonyl oxygen engages the oxygen hole during the transacylation step. The acetylated cysteine then rotates to engage a second oxygen hole, making the first oxygen hole available to participate in the condensation reaction. This is unlikely to occur in the condensing enzymes, because the long acyl chain is buried within its narrow binding pocket, preventing rotation of the acetyl-enzyme intermediate.

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