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Substrate Recognition by β-Ketoacyl-ACP Synthases

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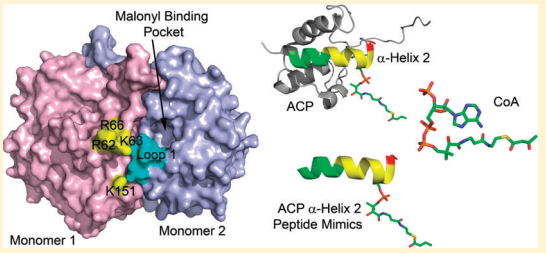
ABSTRACT: β-Ketoacyl-ACP synthase (KAS) enzymes catalyze Claisen condensation reactions in the fatty acid biosynthesis pathway. These reactions follow a ping-pong mechanism in which a donor substrate acylates the active site cysteine residue after which the acyl group is condensed with the malonyl-ACP acceptor substrate to form a β-ketoacyl-ACP. In the priming KASIII enzymes the donor substrate is an acyl-CoA while in the elongating KASI and KASII enzymes the donor is an acyl-ACP. Although the KASIII enzyme in *Escherichia coli* (ecFabH) is essential, the corresponding enzyme in *Mycobacterium tuberculosis* (mtFabH) is not, suggesting that the KASI or II enzyme in *M. tuberculosis* (KasA or KasB, respectively) must be able to accept a CoA donor substrate. Since KasA is essential, the substrate specificity of this KASI enzyme has been explored using substrates based on phosphopantetheine, CoA, ACP, and AcpM peptide mimics. This analysis has been extended to the KASI and KASII enzymes from *E. coli* (ecFabB and ecFabF) where we show that a 14-residue malonyl-phosphopantetheine peptide can efficiently replace malonyl-ecACP as the acceptor substrate in the ecFabF reaction. While ecFabF is able to catalyze the condensation reaction when CoA is the carrier for both substrates, the KASI enzymes ecFabB and KasA have an absolute requirement for an ACP substrate as the acyl donor. Provided that this requirement is met, variation in the acceptor carrier substrate has little impact on the k_{cat}/K_m for the KASI reaction. For the KASI enzymes we propose that the binding of ecACP (AcpM) results in a conformational change that leads to an open form of the enzyme to which the malonyl acceptor substrate binds. Finally, the substrate inhibition observed when palmitoyl-CoA is the donor substrate for the KasA reaction has implications for the importance of mtFabH in the mycobacterial FASII pathway.



Fatty acids are essential for the survival of microorganisms,[1,2] and consequently the bacterial fatty acid biosynthesis (FASII) pathway (Figure 1) has emerged as a promising target for antibacterial drug discovery. Genetic knockout and knockdown experiments combined with target-specific inhibitors support the importance of the FASII pathway for bacterial viability,[2,3] and it has also been shown that the front line tuberculosis drug isoniazid prevents mycolic acid synthesis by inhibiting the FASII enoyl-ACP reductase.[4−7] There is consequently interest in exploring the interaction of substrates and inhibitors with enzymes in the pathway.

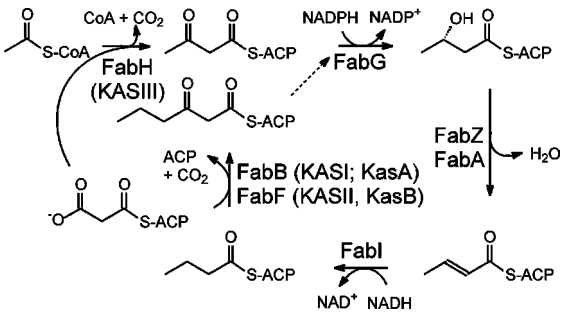


Figure 1. The bacterial fatty acid biosynthesis pathway in *E. coli*. FabH (KASII), the β-ketoacyl-ACP synthase is responsible for condensing acetyl-CoA with malonyl-ACP to yield β-ketoacyl-ACP. This product is subsequently reduced, dehydrated, and reduced by the successive action of a β-ketoacyl-ACP reductase, FabG, a dehydratase, FabZ or FabA, and a *trans*-2-enoyl-ACP reductase, FabI. A repetitive series of elongation reactions are then performed that utilize the same enzymes except that the condensation reaction is performed by the ACP specific β-ketoacyl synthases FabB (KASI) and FabF (KASII). In *M. tuberculosis*, FabH condenses malonyl-AcpM with long chain (C24+) acyl-CoAs that are provided by the mycobacterial FASI pathway. The homologues of the KASI, II, and III enzymes in *M. tuberculosis* are KasA, KasB, and mtFabH, respectively.

In addition to the enoyl-ACP reductases, inhibitor discovery efforts have also focused on the β-ketoacyl-ACP synthase (KAS) enzymes in the pathway which catalyze the Claisen condensation of an acyl donor and malonyl-ACP to form a β-ketoacyl-ACP.[8] At least three classes of natural product KAS inhibitors have been discovered, including thiolactomycin, cerulenin, and platensimycin/platensin, suggesting that these enzymes are sensitive targets for therapeutic intervention.[9−14] In *E. coli* there are three KAS enzymes, two involved in the elongation of the growing fatty acid, KASI and KASII, and a third priming KAS, KASIII, which catalyzes the initial condensation of acetyl-CoA with malonyl-ecACP. In *E. coli* the KASI, II, and III enzymes carry the designation ecFabB, ecFabF, and ecFabH, respectively, while in *Mycobacterium tuberculosis* they are KasA, KasB, and mtFabH.[15,16] The

Received: August 2, 2011
Revised: October 21, 2011
Published: October 21, 2011

principal difference between the enzymes from *M. tuberculosis* and *E. coli* is that in the former organism the FASII pathway synthesizes fatty acids up to C54−C56 in length, and so the priming step involves a C22−C24 acyl-CoA rather than acetyl-CoA since *de novo* fatty acid biosynthesis is catalyzed by a separate FASI pathway in *M. tuberculosis*. In addition, the specific roles of KasA and KasB in fatty acid elongation have been studied by Slayden et al., who demonstrated that KasA extended palmitoyl-CoA to monounsaturated fatty acids averaging 40 carbons units while KasB synthesized longer multi-unsaturated acyl chains averaging 54 carbons units.[17] ecFabF and ecFabB are also involved in the elongation of both saturated and unsaturated fatty acids with the highest activity observed with C6−C12 acyl-ACPs.[16] Specifically, ecFabB catalyzes the elongation of C10:1,[18] a critical step in unsaturated fatty acid biosynthesis, while ecFabF plays an essential role in thermal regulation of fatty acid composition.[19,20] In the absence of ecFabB, ecFabF can catalyze both saturated and unsaturated fatty acid biosynthesis.[21]

In order to avoid futile cycling, substrates in the fatty acid biosynthesis and oxidation pathways are transported by two fundamentally different carrier molecules: a phosphopantetheinylated acyl carrier protein (ACP) for fatty acid biosynthesis and the phosphopantetheine moiety of coenzyme A (CoA) for fatty acid oxidation. ACPs are small, highly conserved acidic proteins that not only transport fatty acids but also play essential role in the synthesis of a variety of bacterial macromolecules such as glycerol phospholipids and polyketides.[22−24] CoA is the most prominent acyl-group carrier coenzyme in living systems and serves as an intermediate in the metabolism of almost all kinds of biological compounds such as amino acids, carbohydrates, and fats. CoA differs from ACP by the replacement of the carrier molecule with ADP.

While many FASII enzymes will accept CoA-based substrates, generally the K_m values are larger than for the corresponding ACPs so that selectivity toward the natural substrate is maintained. FabH is unique since it is the only FASII enzyme that will accept a CoA substrate. Furthermore, since FabH catalyzes the first step in the FASII pathway, it directly regulates the rate of fatty acid synthesis.[25−28] While ecFabH is known to be essential, transposon mutagenesis has indicated that mtFabH is not essential for bacterial viability.[29,30] This suggests that KasA and/or KasB, which are essential,[15,31] must be able to accept an acyl-CoA and act as both the initiation and elongation condensing enzyme for FASII. To address this question, we have explored the substrate specificity of KasA, as well as ecFabB and ecFabF, using substrates based on phosphopantetheine (PPant), CoA, ACP, and AcpM peptide mimics, and propose a structural explanation for the specificity of KASI/KASII for ACP substrates. While short peptide mimics of ACP α-helix 2 efficiently replace *E. coli* ACP (ecACP) as the substrate for ecFabF, the KASI enzymes ecFabB and KasA have an absolute requirement for ACP as the donor substrate. For the KASI enzymes we propose that ACP binding causes a conformational change that leads to an open form of the enzyme to which the acceptor substrate can more readily bind. In addition, the severe substrate inhibition observed with KasA when palmitoyl-CoA is the donor substrate suggests that mtFabH plays an essential role for initiation of the FASII pathway in *M. tuberculosis*.

MATERIALS AND METHODS

Materials. Malonyl, palmitoyl, and lauroyl CoA substrates were from Sigma. All solvents used were either ACS or HPLC grade, and all reagents were commercially available unless otherwise noted.

Wild-Type KasA and C171Q KasA. N-terminally His-tagged KasA proteins were purified from *Mycobacterium smegmatis* strain mc^{2}155 essentially as described.[10] pFPCA1 vectors containing the coding regions for these proteins were transformed into *M. smegmatis* competent cells by electroporation and plated on 7H10 solid media containing 30 μg/mL kanamycin, 200 μg/mL ampicillin, and 15 μg/mL cyclohexamide. Colonies from these plates were then cultivated in 1 L of 7H9 liquid media supplemented with glycerol and grown at 37 °C to an optical density (OD_{600}) of 0.6−0.8, after which protein expression was induced with 0.2% acetamide. After incubating overnight at 25 °C, cells were harvested by centrifugation, resuspended in 40 mL of 20 mM Tris-HCl, 500 mM NaCl, 5 mM imidizole buffer, pH 7.9 (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 an 8 mL nickel affinity column (8 × 1 cm). The column was washed with 50 mL of buffer A, 50 mL of 20 mM Tris-HCl, 500 mM NaCl, and 60 mM imidizole buffer, pH 7.9 (buffer B), and eluted using a linear gradient of imidizole from 100 to 800 mM imidizole in buffer A. The fractions containing KasA were subsequently loaded onto a G-25 size exclusion chromatography column preequilibrated in 50 mM sodium phosphate buffer, pH 8.5, 0.3 M NaCl. KasA was eluted in the same buffer and analyzed by SDS-PAGE. The protein was stored at −20 or −80 °C with 50% glycerol.[10]

ecFabB, ecFabF, MabA, and PanK. The KASI (ecFabB) and KASII (ecFabF) enzymes from *E. coli* as well as the β-ketoacyl-ACP reductase (MabA) and pantothenate kinase (PanK) were expressed as N-terminally His-tagged constructs in *E. coli* pLysS cells and purified using standard nickel affinity chromatography as described previously.[10,11,32,33]

Mutagenesis. The R207G mutant of ecFabF together with the R62Q, K63Q, R66Q, and K151Q mutants of ecFabB were prepared by Quikchange mutagenesis using the following primers:

R207G ecFabF (for) 5′-GGTTTTGGCGCGGC-AGGCGCATTATCTACCCGC-3′,
(rev) 5′-GCGGGTAGATAATGCGCCTGCCGCGC-CAAAACC-3′;
R62Q ecFabB (for) 5′-ACTGGCCTCATTGACCA-GAAAGTTGTGCGCTTT-3′,
(rev) 5′-AAAGCGCACAACTTTCTGGTCAAT-GAGGCCAGT-3′;
K63Q ecFabB (for) 5′-GGCCTCATTGACCGC-CAGGTTGTGCGCTTTATG-3′,
(rev) 5′-CATAAAGCGCACAACCTGGCGGTCAAT-GAGGCC-3′;
R66Q ecFabB (for) 5′-GACCGCAAAGTTGTG-CAGTTTATGAGCGACGCA-3′,
(rev) 5′-TGCGTCGCTCATAAACTGCA-CAACTTTGCGGTC-3′;
K151Q ecFabB (for) 5′-ACTGGCCTCATTGACCAG-CAGGTTGTGCGCTTTATG-3′,
(rev) 5′-GTTAACGCCATGAATCT-GAAACGGGGTGGCGAG-3′.

After confirming the correct sequence of the modified plasmids by ABI sequencing, the mutant proteins were expressed and purified as described for the wild-type enzyme.

Holo-ACP Synthase (AcpS). AcpS was expressed and purified as described previously.[34,35] The pET-AcpS plasmid containing the coding region for AcpS was transformed into *E. coli* BL21-DE3 competent cells. After selection on solid LB media containing ampicillin (200 μg/mL), individual colonies were used to inoculate 1 L of YT media which contained 16 g of tryptone, 10 g of yeast extract, 5 g (85 mM) of NaCl, and 2.4 g (20 mM) of sodium phosphate. The culture was incubated at 37 °C until the OD_{600} reached 0.8−1.0, after which protein expression was induced with 0.1 mM IPTG. After incubating for a further 3 h at 30 °C, cells were harvested by centrifugation, resuspended in 40 mL of 50 mM Tris-HCl buffer, pH 8.0, and sonicated for 6 min using 30 s pulses at 4 °C. Cell debris was removed by centrifugation at 33 000 rpm, and the cell free extract was then gently mixed with 1 g of DE-52 resin to form a slurry which was kept at 4 °C for 15 min. After removing the resin by centrifugation and repeating the treatment with DE-52, the supernatant was adjusted to pH 6.5 with a saturated 4-morpholineethanesulfonic acid monohydrate (MES) solution and then loaded onto an 8 mL SP-sepharose column (8 × 1 cm) equilibrated with 50 mM Tris-HCl buffer, pH 6.5. AcpS was eluted with a linear gradient of NaCl, and protein purity was analyzed by SDS-PAGE.

Acyl-Carrier Proteins. The apo form of the *M. tuberculosis* acyl-carrier protein (apo-AcpM) was expressed and purified as described previously,[10] while the apo form of the *E. coli* acyl-carrier protein (apo-ecACP) was obtained using a method adapted from that described by Haas et al.[36] Briefly, the pET-ecACP plasmid containing the coding region for ecACP was transformed into *E. coli* BL21-DE3 competent cells. After selection on solid LB media containing kanamycin (30 μg/mL), individual colonies were used to inoculate 1 L of LB media. The culture was incubated at 37 °C until the OD_{600} reached 0.6−0.8, after which protein expression was induced with 0.1 mM IPTG, 2 g/L casamino acids, and 0.05 g/L pantothenic acid. After incubating for a further 3 h at 30 °C cells were harvested by centrifugation, resuspended in 50 mL of 100 mM Tris-HCl buffer, pH 8.0, containing 0.15 mg/mL of lysozyme, DNase, and RNase, and sonicated for 6 min using 30 s pulses at 4 °C. Cell debris was removed by centrifugation at 33 000 rpm, and the cell free extract was then diluted with one volume of 25 mM MES buffer, pH 6.1 (buffer A). The mixture was then loaded onto a Q-sepharose (Amersham Biosciences) column (50 × 150 mm) equilibrated in buffer A, and the column was washed with 400 mL of buffer A. The protein was eluted from the column in 10 mL fractions with an 800 mL linear gradient from 0.1 to 0.85 M NaCl in buffer A. The fractions containing ecACP were further purified by subsequently loading them onto a Hi-load 16/60 Superdex-75 column pre-equilibrated in buffer A, containing 100 mM NaCl. SDS-PAGE indicated that the ecACP was predominantly in the apo form, and ESI mass spectrometry (ESI-MS) revealed that the apo-ecACP sample was comprised of two forms in which the N-terminal Met was present (8637 Da) or had been cleaved (8506 Da).[37]

Substrates Based on ecACP and AcpM. Apo-AcpM or apo-ecACP (100 μM) was incubated in 1.5 mL of AcpS reaction buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTT, pH 8.5) with 200 μM of either malonyl-CoA (Mal-CoA), palmitoyl-CoA, or lauroyl-CoA and 6 μM AcpS for 1 h at 37 °C. Acyl ACPs were isolated from the reaction mixture by ion exchange chromatography using a Mono Q 5/50 GL column (GE Healthcare) pre-equilibrated with 20 mM Tris-HCl buffer, pH 8.0 (buffer A). Elution of the different ACP substrates was achieved using a shallow linear gradient with buffer A containing 1 M NaCl. Malonyl-AcpM/ecACP, palmitoyl-AcpM, and lauroyl-ecACP eluted at ∼330 mM NaCl. ESI-MS determined the molecular weights of all products as expected based off the primary amino acid sequence. ESI-MS: malonyl-AcpM, 13047 Da; malonyl-ecACP, 8932 Da (9036 Da with N-terminal Met); palmitoyl-AcpM, 13 200 Da; lauroyl-ecACP, 9027 Da.

Peptide Synthesis. A study performed by Walsh et al. identified a short peptide fragment (A1-GDSLDMLEWSL) that was efficiently post-translationally modified by AcpS.[38] This information was used in the design of the peptides used here since not only were we interested in exploring their interaction with KasA and the other KAS enzymes, but they also had to be substrates for AcpS. Consequently, our peptides were designed as a combination of residues from the A1 peptide (namely S_3, L_4, D_5, or M_6 which were shown to specially interact with the AcpS recognition core) and from α-helix 2 of AcpM. Peptides containing 8 (DSLDMLEI-NH_2), 14 (DSLSMLEIAVQTED-NH_2), and 16 (DPDSLSMLE-IAVQTED-NH_2) residues were synthesized on a 0.25 mmol scale using an Applied Biosystems 433A peptide synthesizer, with 9-fluornylmethoxycarbonyl (Fmoc) chemistry. Use of a 5-(4′-Fmocaminomethyl-3′,5-dimethoxyphenol)valeric acid (PAL-PEG) resin afforded peptides with amidated C-termini. Standard Fmoc reaction cycles were used, and peptides were cleaved from the resin using 90% trifluoroacetic acid (TFA), 3.33% anisole, 3.33% thioanisole, and 3.33% ethanedithiol.[39]

The crude peptides were redissolved in 0.5% NH_4OH and purified using reversed-phase HPLC. Chromatography was performed at a flow rate of 4 mL/min with a Vydac protein/peptide semipreparative column (10 × 250 mm) and by running a linear gradient of 100% buffer A (95% H_2O, 5% acetonitrile, 0.1% TFA) to 100% buffer B (95% acetonitrile, 5% H_2O, 0.1% TFA) over 30 min. The 8-mer, 14-mer, and 16-mer peptides eluted at around 19 min and were subsequently analyzed by ESI-MS: 8-mer ($[M + H]^+$) calcd $C_{39}H_{67}N_9O_{15}S$, 934.07 m/z found 934.0. Yield 25%, 14-mer ($[M + 2H]^+$) calcd $C_{64}H_{108}N_{16}O_{26}S$ 775.35 m/z found 775.0. Yield 10%, 16-mer ($[M + 2H]^+$) calcd $C_{73}H_{120}N_{18}O_{30}S$ 880.40 m/z found 880.0. Yield 50%.

Acyl-Phosphopantetheine Peptide Preparation. Peptides (200 μM) were incubated in 1 mL of AcpS reaction buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 6.5) with 400 μM malonyl-CoA and 6 μM AcpS for 30 min at 37 °C and purified using reversed-phase HPLC. Chromatography was performed at a flow rate of 1 mL/min with a Vydac protein/peptide analytical column (10 × 250 mm) and by running a linear gradient of 100% buffer A (95% H_2O, 5% acetonitrile, 0.1% TFA) to 100% buffer B (95% acetonitrile, 5% H_2O, 0.1% TFA) over 45 min. The malonyl-phosphopantetheine-8-mer (MalPPant-8-mer), malonyl-phosphopantetheine-14-mer (MalPPant-14-mer), and malonyl-phosphopantetheine-16-mer (MalPPant-16-mer) eluted around 21 min and were subsequently analyzed by ESI-MS: MalPPant-8-mer $[M + 2H]^+$ calcd $C_{53}H_{90}N_{11}O_{24}PS_2$ 680.25 m/z found 680.0. Yield 33%, MalPPant-14-mer $[M + 2H]^+$ calcd $C_{78}H_{131}N_{18}O_{35}PS_2$ 988.42 m/z found 988.0. Yield 20%, MalPPant-16-mer $[M + 2H]^+$ calcd $C_{87}H_{143}N_{20}O_{39}PS_2$ 1094.45 m/z found 1094.0. Yield 50%.

Synthesis of Malonyl-Phosphopantetheine (4). *Pantetheine (1).* Pantethine (271 μmol) and tris(2-carboxyethyl)-phosphine (1.2 mmol) were dissolved in 1 mL of deionized

water and stirred at RT overnight with subsequent purification using reversed-phase HPLC. Chromatography was performed at a flow rate of 4 mL/min with a Vydac C18 semipreparative column (10 mm × 250 mm) and by running a linear gradient of 95% buffer A (20 mM ammonium acetate, pH 6.7) to 95% buffer B (acetonitrile) over 20 min. Product **1** eluted at 8.5 min and was subsequently concentrated and lyophilized to afford a viscous clear oil. Yield 50%. ESI-MS: $[M - H]^-$ calcd $C_{11}H_{22}N_2O_2S$ 277.1, m/z found 277.1.

*Phosphopantetheine (**2**).* PanK (1 mg) was added slowly to a solution of ATP (180 μmol, pH 7) and **1** (72 μmol) dissolved in 2.6 mL of 20 mM Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$ buffer, pH 8 and stirred at RT for 3 h with subsequent purification using reversed-phase HPLC. Chromatography was performed at a flow rate of 4 mL/min with a Vydac C18 semipreparative column (10 mm × 250 mm) and by running a linear gradient of 95% buffer A (20 mM ammonium acetate, pH 6.7) to 95% buffer B (acetonitrile) over 20 min. Product **2** eluted at 11 min and was subsequently concentrated and lyophilized to afford a white solid. Yield 20%. ESI-MS: $[M - H]^-$ calcd. $C_{11}H_{22}N_2O_7PS^-$ 356.09, m/z found 356.0.

*Malonylthiophenol (**3**).* The synthesis of malonylthiophenol is based on the procedure of Wieland and Koppe.[40] Typically, 10 mmol of malonic acid was dissolved in 5 mL of anhydrous tetrahydrofuran (THF) at −20 °C followed by the dropwise addition of 5 mmol of ethyl chloroformate in 1 mL of THF. Precipitation of triethylamine hydrochloride was observed after several minutes. The mixture was incubated for 30 min at −20 °C to ensure completion of the reaction.

The mixed anhydride was treated with 5 mmol of thiophenol and incubated at RT for 24 h. The solvent was then removed under vacuum, and the residue was suspended in 2 mL of water. The oily product was extracted in diethyl ether and dried over sodium sulfate. Evaporation removed the ether layer to afford **3** as a viscous oily residue. Yield 85%. ESI-MS: $[M - H]^-$ calcd $C_9H_8O_3S$ 196.02, m/z found 196.0.

*Malonyl-Phosphopantetheine (**4**).* **3** (25 μmol) dissolved in 1 mL of THF was added to 5 μmol of **2** dissolved in 1 mL of 100 mM $NaHCO_3$ buffer, pH 8.2. The mixture was stirred vigorously for 3 h and acidified with Dowex 50 to pH 3. The Dowex was removed by centrifugation and washed once with 1.5 mL of water. The supernatant and the wash were combined, and the thiophenol was extracted with diethyl ether. The aqueous solution containing the malonyl-phosphopantetheine was then lyophilized and purified using reversed-phase HPLC. Chromatography was performed at a flow rate of 1 mL/min with a Varian analytical column (4.6 mm × 150 mm) and by running a linear gradient of 95% buffer A (20 mM ammonium acetate, pH 6.7) to 75% buffer B (acetonitrile) over 16 min. The final product **4** eluted at 12 min and was subsequently concentrated and lyophilized to afford a white solid. Yield 25%. ESI-MS: $[M - H]^-$ calcd $C_{14}H_{25}N_2O_{10}PS$ 443.10, m/z found 443.0.

Enzyme Kinetics. Kinetic parameters for the reaction catalyzed by KasA were determined in 50 mM Tris-HCl, 1 mM DTT buffer, pH 8.5, at 25 °C using a coupled assay with MabA, the NADPH-dependent FASII β-ketoacyl-ACP reductase.[10,15] Reactions were initiated by the addition of KasA (100 nM) to reaction mixtures containing 250 μM NADPH, 10 μM MabA, and varying concentrations of palmitoyl and malonyl substrates. The kinetic parameters for ecFabF and ecFabB were assayed in a similar fashion, except they were determined in a buffer containing 50 mM potassium phosphate buffer, 50 mM NaCl, 1 mM DTT, pH 6.5. Reactions were initiated with either 0.6−0.8 μM Wt ecFabB, 1 μM R62Q, K63Q, R66Q, K151Q ecFabB, or 3 μM ecFabF, and initial velocities were measured at several fixed concentrations of one substrate while keeping the second substrate constant. While no lag phase was observed when assaying KasA, a lag phase of 20−30 s was observed when ecFabF and ecFabB were assayed with CoA-based substrates. Preincubation experiments suggested that this lag phase was due to rate-limiting acylation of the enzymes (data not shown) rather than to the coupling enzyme. In agreement with this hypothesis calculations using the equations described by Storer and Cornish-Bowden and published kinetic parameters for MabA indicated that no lag phase was expected under the conditions of the assay (v_1/V_2 = 10^{-3}−10^{-4}).[33,41] To determine if the inhibition of KasA by palmitoyl-CoA was reversible, 50 μM palmitoyl-CoA and 1 μM KasA were preincubated for 10 min prior to a 10-fold dilution into the assay mixture. Kinetic parameters were obtained by fitting the initial velocity data to the Michaelis−Menten equation using GraFit 4.0 (Erithacus Software Ltd.) or to the equation for substrate inhibition (eq 2).

$$v = V_{max}[S]/(K_m + [S] + [S]^2/K_i) \quad (2)$$

RESULTS

Kinetic Characterization of KAS Proteins. ecFabF and ecFabB were expressed in *E. coli* while the expression of KasA was performed in *M. smegmatis*.[15] All peptides and ACPs were post-translationally modified to the respective malonyl, lauroyl or palmitoyl substrates by AcpS.[37,42] Kinetic parameters were determined by monitoring the NADPH-dependent reduction of the β-ketoacyl-ACP product in a coupled assay with MabA, the subsequent enzyme in the FASII pathway in *M. tuberculosis*.[15]

Substrate Specificity of ecFabF. ecFabF has previously been reported to catalyze substrate condensation with substrates that are thioesters of CoA.[10,13,43] The kinetic parameters determined here (k_{cat} 2.5 ± 0.1 min^{-1} and K_m of 510 ± 84 μM) closely match values previously reported for this enzyme (k_{cat} 2.5 min^{-1} and K_m 502 μM) when malonyl-CoA was varied at a fixed concentration of lauroyl-CoA (75 μM).[10] However, kinetic parameters have not been reported for the natural ecACP substrates with this enzyme, and when malonyl-ecACP was varied at a fixed concentration of lauroyl-ecACP (10 μM) k_{cat}/K_m increased 50-fold due mainly to a decrease in the K_m for the acceptor substrate from 510 to 8 μM (Table 1). Similar k_{cat} values were obtained for all three malonyl-peptides (1−1.4 min^{-1}), while the K_m values decreased from 24 μM for the malonyl-8-mer to 6 μM for the malonyl-14 and -16-mers, when the peptide substrates were varied at a fixed concentration of lauroyl-CoA (100 μM) (Table 1). Thus, the 14- and 16-mer peptides are able to efficiently replace ecACP as the substrate for ecFabF. To evaluate the possibly that R206 was preventing efficient binding of CoA to ecFabF, the R206G mutant was prepared and a 10-fold increase in k_{cat}/K_m was observed for this enzyme when malonyl-CoA was varied at a fixed concentration of lauroyl-CoA (100 μM) (Table 1). The increase in k_{cat}/K_m of R206G ecFabF compared to the wild-type enzyme results mainly from an effect on K_m (Table 1), suggesting that R206 impairs the binding of CoA.

Malonyl-Phosphopantetheine as KAS Substrate. To explore minimum requirements for catalysis, MalPPant was

Table 1. Kinetic Parameters for ecFabF

enzyme	donor	acceptor	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M/min^{-1}$)	% activity[e]
Wt	lauroyl-CoA	malonyl-CoA[a]	510 ± 84	2.5 ± 0.1	0.005 ± 0.001	2
R206G	lauroyl-CoA	malonyl-CoA[b]	28.3 ± 6.5	1.28 ± 0.06	0.05 ± 0.01	20
Wt	lauroyl-CoA	MalPPant[a]	590 ± 133	1.3 ± 0.2	0.0022 ± 0.0002	1
Wt	lauroyl-CoA	MalPPant-8-mer[a]	23.7 ± 5.2	1.1 ± 0.1	0.05 ± 0.01	22
Wt	lauroyl-CoA	MalPPant-14-mer[a]	6.1 ± 1.5	1.4 ± 0.1	0.25 ± 0.07	108
Wt	lauroyl-CoA	MalPPant-16-mer[a]	6.2 ± 1.4	1.34 ± 0.02	0.23 ± 0.06	100
Wt	lauroyl-ecACP	malonyl-ecACP[c]	8.2 ± 2.1	1.74 ± 0.05	0.23 ± 0.06	100
Wt	lauroyl-CoA[d]	malonyl-CoA	53.7 ± 15	2.8 ± 0.4	0.06 ± 0.02	25

[a]Calculated by varying the malonyl substrate with respect to 75 μM lauroyl-CoA. [b]Calculated by varying the malonyl substrate with respect to 100 μM lauroyl-CoA. [c]Calculated by varying the malonyl substrate with respect to 10 μM lauroyl-ecACP. [d]Calculated by varying the lauroyl substrate with respect to 500 μM malonyl-CoA. [e]% activity is in respect to k_{cat}/K_m of malonyl-ecACP varied at a fixed concentration of lauroyl-ecACP.

Table 2. Kinetic Parameters for ecFabB

enzyme	donor	acceptor	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M/min^{-1}$)	% activity[d]
Wt	lauroyl-ecACP	malonyl-CoA[a]	153 ± 19	7.1 ± 0.4	0.047 ± 0.008	8
Wt	lauroyl-ecACP	MalPPant-8-mer[a]	14.2 ± 2.5	1.1 ± 0.1	0.08 ± 0.02	14
Wt	lauroyl-ecACP	MalPPant-14-mer[a]	15.8 ± 6	2.4 ± 0.2	0.18 ± 0.07	27
Wt	lauroyl-ecACP	MalPPant-16-mer[a]	29.0 ± 5.5	6.5 ± 0.4	0.23 ± 0.03	37
Wt	lauroyl-ecACP	malonyl-ecACP[a]	11.5 ± 2.2	6.6 ± 0.5	0.60 ± 0.16	100
Wt	lauroyl-CoA[b]	malonyl-ecACP	58.6 ± 5.4	4.4 ± 0.1	0.076 ± 0.009	13
Wt	lauroyl-ecACP[b]	malonyl-ecACP	3.2 ± 0.5	3.4 ± 0.2	1.1 ± 0.2	100
Wt	lauroyl-ecACP[c]	malonyl-CoA	3.9 ± 0.7	2.0 ± 0.1	0.5 ± 0.06	100
R62Q	lauroyl-ecACP[c]	malonyl-CoA	50 ± 7.0	4.6 ± 0.4	0.09 ± 0.02	18
K63Q	lauroyl-ecACP[c]	malonyl-CoA	60 ± 12	5.0 ± 0.5	0.07 ± 0.05	14
R66Q	lauroyl-ecACP[c]	malonyl-CoA	56 ± 10	3.2 ± 0.2	0.05 ± 0.03	10
K151Q	lauroyl-ecACP[c]	malonyl-CoA	6.7 ± 1.7	1.5 ± 0.1	0.22 ± 0.07	50

[a]Calculated by varying the malonyl substrate with respect to 10 μM lauroyl-ecACP. [b]Calculated by varying the lauroyl substrate with respect to 10 μM malonyl-ecACP. [c]Calculated by varying lauroyl-substrate with respect to 200 μM malonyl-CoA. [d]% activity for top section is in respect to k_{cat}/K_m of malonyl-ecACP varied at a fixed concentration of lauroyl-ecACP; for the middle section is in respect to the k_{cat}/K_m of lauroyl-ecACP varied at a fixed concentration of malonyl-ecACP; for the bottom section is in respect to the k_{cat}/K_m of lauroyl-ecACP varied at a fixed concentration of malonyl-CoA.

synthesized and tested for activity against all three KAS enzymes. This substrate did not show activity with ecFabB when using a fixed concentration of lauroyl-CoA nor with KasA when using a fixed concentration of palmitoyl-AcpM. However, it did show activity with ecFabF giving k_{cat} and K_m values of 1.3 ± 0.2 min^{-1} and 590 ± 133 μM, respectively, when MalPPant was varied at a fixed concentration of lauroyl-CoA (75 μM, Table 1). The kinetic parameters were similar to those of malonyl-CoA, indicating no improvement on specificity.

Substrate Specificity of ecFabB. Kinetic parameters have so far not been reported for ecFabB. Varying malonyl-ecACP at a fixed concentration of lauroyl-ecACP (10 μM) gave k_{cat} and K_m values of 6.6 ± 0.5 min^{-1} and 11.5 ± 2.2 μM, respectively, while the k_{cat} and K_m values were 3.4 ± 0.2 min^{-1} and 3.2 ± 0.5 μM, respectively, when lauroyl-ecACP was varied at a fixed concentration of malonyl-ecACP (10 μM) (Table 2). Unlike ecFabF, no activity could be detected with ecFabB when both substrates were based on CoA. However, varying malonyl-CoA at a fixed concentration of lauroyl-ecACP (10 μM) gave k_{cat} and K_m values of 7.1 ± 0.4 min^{-1} and 153 ± 19 μM, respectively. In addition, when the concentration of lauroyl-CoA was varied at a fixed concentration of malonyl-ecACP (10 μM), the k_{cat} and K_m values were 4.4 ± 0.1 min^{-1} and 58.6 ± 5.4 μM, respectively (Table 2). ecFabB was also active with the malonyl-peptides when assayed at a fixed concentration of lauroyl-ecACP (10 μM, Table 2). Taken together, little variation in k_{cat}/K_m values were observed for the various acceptor substrates provided that ecACP is used as the donor substrate.

Substrate Specificity of KasA. The K_m value for malonyl-AcpM (5.8 ± 1.5 μM) determined at a fixed concentration of palmitoyl-AcpM (10 μM, Table 3) is similar to the K_m value determined previously (13.5 μM) while the k_{cat} value of 28 ± 1.5 min^{-1} is ~6-fold larger than that determined by Schaeffer et al., which we speculate could be due to the use of mycobacterial acyl carrier protein in our study compared to the ACP from *E. coli* (Table 3).[15] Although no activity could be detected when both donor and acceptor substrates were based on CoA, malonyl-CoA efficiently replaced malonyl-AcpM as the acceptor substrate when palmitoyl-AcpM (12 μM) was used as the donor (k_{cat} 21 ± 0.5 min^{-1} and K_m 9.0 ± 0.8 μM; Table 3). Similar results were obtained when malonyl-CoA was replaced with the malonyl-peptides (Table 3). In contrast, when palmitoyl-CoA was used as the donor molecule at a fixed concentration of malonyl-AcpM, substrate inhibition was

Table 3. Kinetics Parameters for KasA

donor	acceptor	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M/min^{-1}$)	% activity[d]
palmitoyl-AcpM	malonyl-CoA[a]	9.0 ± 0.8	21 ± 0.5	2.3 ± 0.3	48
palmitoyl-AcpM	MalPPant-8-mer[a]	4.7 ± 0.6	21 ± 0.6	4.5 ± 0.7	99
palmitoyl-AcpM	MalPPant-14-mer[a]	8.6 ± 1.9	23 ± 1.5	2.9 ± 0.7	66
palmitoyl-AcpM	MalPPant-16-mer[a]	5.1 ± 0.6	18 ± 0.5	3.6 ± 0.5	73
palmitoyl-AcpM	malonyl-AcpM[a]	5.8 ± 1.5	28 ± 1.5	5.2 ± 1.4	100
palmitoyl-CoA[b]	malonyl-AcpM	0.40 ± 0.06 36 ± 5 (K_i)	5.0 ± 0.2		
PalmPPant-16-mer[c]	malonyl-CoA	NA[d]	NA[d]	NA[d]	

[a]Calculated by varying the malonyl substrate with respect to 12 μM palmitoyl-AcpM. [b]Calculated by varying the palmitoyl substrate with respect to 10 μM malonyl-AcpM. Parameters were obtained by fitting the data to eq 2. [c]Calculated by varying the palmitoyl substrate with respect to 50 μM malonyl-CoA. [d]% activity is in respect to k_{cat}/K_m of malonyl-AcpM varied at a fixed concentration of palmitoyl-AcpM.

detected in which the measured enzyme activity decreased at palmitoyl-CoA concentrations above ~3 μM, a concentration that is well below the critical micelle concentration of palmitoyl-CoA determined in the same reaction buffer (200 μM[44]). To rule out the possibility that inhibition of KasA by palmitoyl-CoA was due to an irreversible detergent effect, palmitoyl-CoA (50 μM) and KasA (1 μM) were preincubated for 10 min prior to dilution (1:10) into the reaction mixture. The activity of KasA was found to be unaffected by the preincubation, demonstrating that the effect of palmitoyl-CoA was reversible. The observation that palmitoyl-CoA does not have a detergent effect on KasA is also in agreement with a previous study in which we analyzed the interaction of thiolactomycin (TLM) with the acyl-enzyme of KasA and with the C171Q KasA acyl-enzyme mimic.[10] In both cases we observed similar binding kinetics which is significant since the acyl-KasA was generated by including 50 μM palmitoyl-CoA in the assay buffer. Finally, no activity could be detected when the enzyme was assayed with either an acyl peptide and malonyl-CoA (25 μM), or a malonyl-peptide substrate and an acyl-CoA, indicating that the peptide mimics could not efficiently substitute for AcpM.

Effect of Surface Mutations on the Proposed FabB:ACP Complex. Structural analysis of the KAS enzymes identified several basic residues that are conserved between FabB, FabF, and KasA and that we propose are important for ACP recognition. In FabB, these residues are R62, K63, and R66 from α-helix 3 and K151 from α-helix 9. These residues were replaced with Gln which resulted in a 12−15-fold increase in K_m when lauroyl-ecACP was varied at a fixed concentration of malonyl-CoA (200 μM) (Table 2).

DISCUSSION

Bacterial fatty acid biosynthesis utilizes substrates that are thioesters of acyl-carrier protein, a small acidic protein that has MWs of ~8.5 and ~13 kDa in *E. coli* and *M. tuberculosis*, respectively. However, while the KAS enzyme(s) involved in fatty acid elongation (KASI and KASII) utilize ACP-based substrates, the priming KAS enzyme (KASIII) is initially acylated by an acyl-CoA substrate (acetyl-CoA in *E. coli*, C20−24-CoA in *M. tuberculosis*). In order to compare and contrast the specificity of KASI/II and KASIII for the substrate carrier, we have synthesized substrates based on PPant, CoA, ACP, and AcpM peptide mimics and explored their interactions with the KASI enzyme from *M. tuberculosis* (KasA) as well as with the KASI and KASII enzymes from *E. coli* (ecFabB and ecFabF).

For ecFabF, the K_m values with CoA-based substrates are ~50-fold larger than the values obtained when ecACP substrates are used. Thus, ecFabF behaves like many of the other FASII enzymes that have been characterized, such as FabI and FabG, where replacement of ACP with CoA results in significant (10−100-fold) changes in K_m without generally affecting k_{cat}.[6,37] Since the principal interaction between ACP and ACP-binding proteins involves ACP α-helix 2, we synthesized a series of peptide substrates derived from α-helix 2 (Figure 2) in order to explore the interaction of ecFabF and

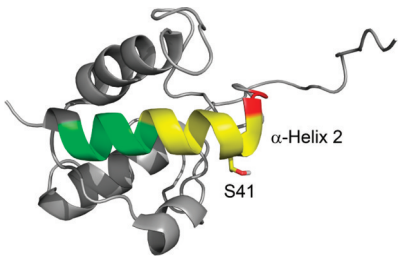


Figure 2. Structure of AcpM colored to show peptides derived from α-helix 2. Structure of AcpM with the conserved serine on α-helix 2 colored in yellow. The lengths of the three synthesized peptide mimics are indicated in colors where the 8-mer (DSLDMLEI-NH_2) is the yellow segment, the 14-mer (DSLSMLEIAVQTED-NH_2) is the yellow plus the green segment, and the 16-mer (DPDSLSML-EIAVQTED-NH_2) is the yellow, green, and red segments. The peptides were designed based on sequences of AcpM and ecACP together with information gleaned by Walsh et al., who identified short peptides that could be phosphopantetheinylated by AcpS and Sfp.[38] This figure was made using PyMol[52] and the PDB entry 1KLP (AcpM).

ACP in more detail. These experiments indicate that binding can be partially recovered with an 8-residue peptide derived from α-helix 2 and completely recovered if the peptide is extended to 14 or 16 residues. Thus, the ~8.5 kDa ACP can be efficiently replaced by a much smaller recognition element. These experiments were prompted by the efforts of Walsh and co-workers, who used phage display to identify a 12-residue peptide that is efficiently post-translationally modified by AcpS.[38]

In contrast to the KASI and KASII enzymes, the priming KASIII enzymes, such as ecFabH, efficiently bind both CoA and ACP substrates. Comparison of the structures of ecFabH and ecFabF reveals that R249 in ecFabH is replaced with P308 in ecFabF (Figure 3A). This substitution prevents ecFabF from

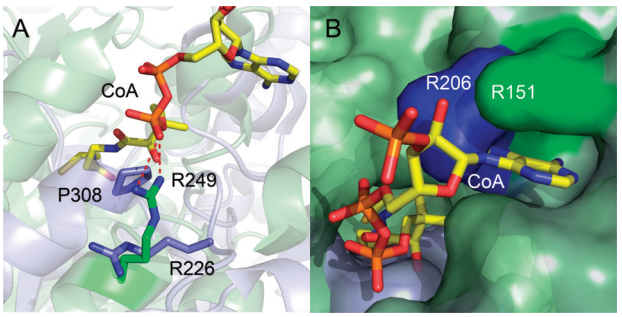


Figure 3. Structure of ecFabH-CoA Superimposed with ecFabF. The interaction of CoA with ecFabF was explored by superimposing the active sites and malonyl binding channels of each enzyme with the respective portions of ecFabH complexed with CoA.[47,52] (A) While R249 in ecFabH (green) forms an interaction to the pyrophosphate of the PPant chain, R226 in ecFabF (blue) is flipped 90° and replaced by P308, preventing bond formation to the pyrophophate of CoA. (B) R151 in ecFabH which binds to the adenine portion of CoA is replaced by R206 in ecFabF which apparently cannot form the same favorable interaction. The figure was made using PyMol[52] and the PDB entries 2eft (ecFabH) and 2gfw (ecFabF).

binding productively to the pyrophosphate group of CoA, thus providing insight into the specificity of ecFabF for ecACP substrates. A similar conclusion was drawn by Smith et al., who proposed that the presence of a tyrosine residue instead of an arginine at the homologous position in the ACP-specific decarboxylase CurF resulted in the preference of this enzyme for ACP compared to CoA substrates.[45]

Comparison of the ecFabH and ecFabF structures also implicates a second arginine, R151, in stabilizing the interaction of acyl-CoA with ecFabH. R151 interacts with the adenine portion of CoA in the structure of acyl-CoA bound to ecFabH.[46] Although an arginine (R206) is present at a similar location in ecFabF, modeling studies indicate that the side of chain of this residue forms unfavorable interactions with the CoA ribose, assuming that this residue is in a similar position when CoA is bound to ecFabF (Figure 3B). Since the corresponding residue in ecFabB is a glycine, and since ecFabB discriminates to a smaller extent against CoA than does ecFabF, we replaced R206 in ecFabF with a glycine. This resulted in 10-fold increase in k_{cat}/K_m for the reaction of ecFabF with malonyl-CoA, substantiating the belief that residues in this region play an important role in substrate selectivity. Consequently, in addition to lacking a homologue of R249, other residues in ecFabF are not appropriately positioned to stabilize the interaction of CoA with ecFabF. The observation that malonyl-PPant and malonyl-CoA have similar K_m values with ecFabF indicates that the poor K_m of the latter substrate for ecFabF is not a consequence of unfavorable interactions of the ADP portion of CoA with the enzyme.

For ecFabB and KasA, no activity can be detected with either enzyme if both donor and acceptor substrates are based on CoA. Similar to ecFabF, structural analysis of KasA and ecFabB reveals that the side chains of R226 and R234, respectively, are facing away from the putative location of the CoA pyrophosphate, preventing these residues from fulfilling the same function as R249 in ecFabH. Instead, as in ecFabF, a proline is present at this position in both KasA and ecFabB. However, the situation is more complex than in ecFabF since if ACP is used as a donor then variation in the acceptor carrier has only a small effect on k_{cat}/K_m. To rationalize this observation, we hypothesize that ACP binding to ecFabB or KasA results in a conformational change that facilitates the binding of the second substrate, regardless of the carrier molecule. This hypothesis is supported by Reynolds et al., who determined that ecFabH can adopt multiple conformations and that the distribution of these conformations is influenced by the carrier molecule.[47] Like other KAS enzymes, ecFabH is dimeric, and Reynolds et al. observed that the binding of acyl-CoA substrate to one ecFabH subunit induces a closed conformation in the second subunit that reduces its affinity for acyl-CoA. This negative cooperativity is only observed in the absence of ecACP, indicating that binding of ecACP to ecFabH leads to a more accessible open form of the active site that facilitates binding of the second substrate.

The operation of a similar mechanism in KasA is supported by X-ray structural data which indicate that M146 and M277 alter their relative positions when KasA is acylated, causing helices α5 and α6 to move which modulates access to the active site.[10] In the structural studies both subunits are either of the apoenzyme or the acyl-enzyme. However during catalysis we propose that binding of ACP to one subunit alters the conformation of the second (acylated) subunit to facilitate malonyl-ACP binding and subsequent departure of the long acyl chain following the condensation reaction. Interestingly, these key methionines are conserved in ecFabB (M137 and M269) but not in ecFabF (I138, M269).

In ACP binding proteins, acidic residues from ACP α-helix 2 interact with basic residues on the surface of the target protein.[37,48] The ACP recognition sites have been identified previously in ecFabH,[48] FabI,[37] and FabG,[49] and this information was used to identify a putative ACP binding site on the KASI and II enzymes comprised of several basic residues that are adjacent to the active site. For ecFabB these residues are R62, K63, R66, and K151 (Figure 4A). Although K151Q ecFabB has similar activity to wild-type, replacement of R62, K63, and R66 with Gln residues resulted in a significant increase in K_m (Table 2), confirming their importance for

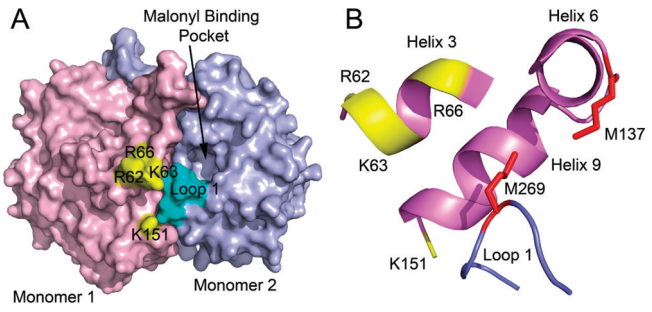


Figure 4. Proposed ecACP binding site on FabB. (A) The homodimeric structure of ecFabB (2VB9) in which monomer 1 is colored in purple and monomer 2 is colored in blue. The basic patch that forms the principal ACP binding site in monomer 1 is colored in yellow. R62, K63, and R66 are located on α-helix 3, while K151 is located on α-helix 9 all from monomer 1. Interaction of ACP with this basic patch would result in delivery of the substrate to monomer 2 and interaction of ACP with loop 1 (cyan) in monomer 2. (B) A detailed view of the locations of the conserved methionines that link the active site to helices α5 and α6 are shown in red. They are thought to alter their position upon ecACP binding and aid in the transition from free enzyme to the acyl-enzyme. M269 is located in the middle of loop 1 of monomer 2, and M137 is part of α-helix 6 of monomer 1. The figure was made using PyMOL.[52]

dx.doi.org/10.1021/bi201199x|*Biochemistry* 2011, 50, 10678–10686

ecACP binding. R62, K63, and R66 all lie on helix α3, and modeling studies suggest that the interaction of ecACP with this basic patch results in delivery of the substrate to the active site of the *adjacent* monomer. Thus, ecACP binding at this site provides a mechanism that allows ecACP to interact with both monomers at the same time. This basic patch is also adjacent to loop 1 on the adjacent monomer which includes M269 (Figure 4B). It is thus plausible that ecACP binding to ecFabB causes M269 to move closer to M137 which subsequently causes the helices to flex.

This model is consistent with the absolute requirement of KasA and ecFabB for ACP since the methionine residues that are responsible for the hinge movement are conserved in ecFabB and KasA but not in ecFabF. This suggests that ecFabF can freely adopt open and closed conformations irrespective of ecACP binding. Thus, for ecFabF the K_m values are simply a consequence of the interaction of substrates with an individual subunit, whereas in KasA and ecFabB binding to one subunit causes coupled motions in the adjacent monomer. For ecFabF we observe that a 14-residue peptide derived from ACP α-helix 2 can efficiently replace ecACP in the condensation reaction. However, remote interactions between ACP and the KASI enzymes must play a critical role in altering the conformation of the adjacent subunit since our peptide substrate mimics of ACP cannot replace the full length carrier protein in the reactions catalyzed by KasA or ecFabB. Finally, when palmitoyl-CoA is used as the donor for the KasA reaction, substrate inhibition is observed at concentrations above ~3 μM. This again stresses the importance of AcpM for the reaction catalyzed by this enzyme and also suggests that KasA would be unable to substitute for the loss of mtFabH, supporting the relevance of the latter enzyme in ongoing drug discovery efforts.[47,50,51]

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Funding

This work was funded by NIH Grant AI044639.

ABBREVIATIONS

KAS, β-ketoacyl-acyl carrier protein synthase; PPant, phosphopantetheine; ACP, acyl carrier protein; AcpM, *M. tuberculosis*; ecACP, *E. coli*; PanK, pantothenate kinase; AcpS, holo-ACP synthase; MalPPant, malonyl-phosphopantetheine; PalmPPant, palmitoyl-phosphopantetheine.

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