

Mechanism and Inhibition of the FabV Enoyl-ACP Reductase from *Burkholderia mallei*[†]

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ABSTRACT: Enoyl-ACP reductases catalyze the final step in the elongation cycle of the bacterial fatty acid biosynthesis (FAS-II) pathway. At present, four distinct enoyl-ACP reductases have been identified, which are the products of the *fabI*, *fabL*, *fabK*, and *fabV* genes. The FabV enoyl-ACP reductase is the most recent member of this enzyme class and was originally identified in *Vibrio cholerae* by Cronan and co-workers [Massengo-Tiasse, R. P., and Cronan, J. E. (2008) *Vibrio cholerae* FabV defines a new class of enoyl-acyl carrier protein reductase. *J. Biol. Chem.* 283, 1308–1316]. In this work, a detailed kinetic analysis of the mechanism of the FabV enzyme from *Burkholderia mallei* (bmFabV) has been undertaken, which reveals that bmFabV catalyzes a sequential bi-bi mechanism with NADH binding first and NAD⁺ dissociating last. The enzyme is a member of the short chain dehydrogenase/reductase superfamily in which the catalytic tyrosine (Y235) and lysine (K244) residues are organized in the consensus Tyr-(Xaa)₈-Lys motif. The role of these active site residues has been investigated using site-directed mutagenesis which has shown that both Y235 and K244 are involved in acid–base chemistry during substrate reduction. Sequence alignment and site-directed mutagenesis also identify a second lysine in the active site (K245) that has an important role in binding of the enoyl substrate. Because of interests in developing inhibitors of bmFabV, a detailed analysis of the inhibition of the enzyme by triclosan has been conducted showing that triclosan is a competitive inhibitor with respect to NADH and an uncompetitive inhibitor with respect to the substrate 2-dodecenoyl-CoA ($K_i = 0.4 \mu\text{M}$). In combination with fluorescence binding experiments, we conclude that triclosan binds to the enzyme–NAD⁺ product complex which is in rapid and reversible equilibrium with other intermediates on the reaction pathway.

The bacterial fatty acid biosynthesis (FAS-II) pathway is a validated yet relatively unexploited target for antimicrobial development (1, 2). Fatty acids are important components of the cell membrane and are essential for bacterial viability (3, 4). While eukaryotes synthesize fatty acids using a multidomain enzyme complex (FAS-I), the FAS-II pathway found in prokaryotes consists of individual enzymes that catalyze each step of fatty chain elongation (5–7). Consequently, the fundamental structural differences that exist between the FAS-I and FAS-II pathways support the proposal that compounds can be developed that selectively target bacterial fatty acid biosynthesis.

The majority of the FAS-II enzymes are essential for bacterial viability (8), and detailed kinetic studies coupled with high-resolution crystal structures have provided a solid foundation for the development of compounds that target this pathway (2, 9). In particular, the enoyl-ACP reductase, which catalyzes the last reaction in each elongation circle, has been the most heavily targeted component of the pathway based on the discovery that antibacterial compounds such as triclosan and isoniazid target this enzyme (10–13). The FabI enoyl-ACP reductase, exemplified by the enzyme from *Escherichia coli*, was initially considered to be the only reductase in bacteria (14), and extensive effort has been spent to develop inhibitors of this enzyme from organisms such as *Mycobacterium tuberculosis* and *Staphylococcus aureus* (15, 16).

However, interests in targeting FabI for the development of an agent with activity against both *S. aureus* and *Streptococcus pneumoniae* were reduced by the discovery of FabK, an alternative flavin-dependent enoyl-ACP reductase from *St. pneumoniae* which was insensitive to the lead FabI inhibitor triclosan (17). At around the same time, Rock and co-workers also discovered a third enoyl-ACP reductase in *Bacillus subtilis* (FabL) which was homologous to FabI (18).

Recently, a fourth isoenzyme (FabV) was identified by Cronan's lab from the Gram-negative bacterium *Vibrio cholerae* (vcFabV) (19). vcFabV, like FabI and FabL, is a member of the short chain dehydrogenase/reductase (SDR) superfamily, although it is significantly larger than other enzymes in this family. However, there is no convincing sequence homology between FabV and the FabI and FabL enzymes. Subsequent sequence similarity studies revealed that FabV is well conserved among a variety of organisms, including several clinically important pathogens, such as *Yersinia pestis*, *Pseudomonas aeruginosa*, and *Burkholderia* species (19).

To date, no detailed kinetic study of any of the FabV enzymes has been conducted. Here we report the cloning and expression of bmFabV from *Burkholderia mallei*, the category B pathogen that causes glanders (20), and demonstrate that this enzyme is a NADH-dependent enoyl-ACP reductase. The impact of site-directed mutagenesis on catalytic activity is consistent with the knowledge that bmFabV is a member of the short chain dehydrogenase reductase superfamily. Finally, since Cronan and co-workers have reported that the antibacterial compound triclosan

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Table 1: Primers Used for Cloning and Mutagenesis

name	sequence ^a
bmFabV forward	5'GGAATTCCATATGATCATCAAACCGCGGTACGC3'
bmFabV reverse	5'CGCGGATCCTCATTCGATCAGATTTCGGAATCCG3'
Y235A forward	5'GTCACGCACGACATCGCTGGAACGGCTCGAT3'
Y235A reverse	5'ATCGAGCCGTTCAGGCGATGTCGTGCGTGAC3'
Y235S forward	5'ACGCACGACATCTCCTGGAACGGCT3'
Y235S reverse	5'AGCCGTTCAGGAGATGTCGTGCGT3'
K244A forward	5'ATCGGCGAAGCGGCGAAAGATCTCGACCG3'
K244A reverse	5'GGTCGAGATCTTTCGCCGCTTCGCCGAT3'
K244R forward	5'GATCGGCGAAGCGAGGAAAGATCTCGACCG3'
K244R reverse	5'GGTCGAGATCTTCTCGCTTCGCCGATC3'
K245M forward	5'CGAAGCGAAGATGGATCTCGACCGC3'
K245M reverse	5'GCGGTCGAGATCCATCTTCGCTTCG3'
K244A/K245A forward	5'ATCGGCGAAGCGGCGGCGAGATCTCGACCG3'
K244A/K245A reverse	5'CGGTCGAGATCTGCCGCCGCTTCGCCGAT3'
ftuACP forward	5'GGAATTCCATATGAGTACACATAACGAAGATTCTAAA3'
ftuACP reverse	5'CCGCTCGAGACCTACATCTTTAGATTTCGATATA3'

^aRestriction sites and mutated sites are underlined.

is only a weak inhibitor of vcFabV (19), we have studied in detail the mechanism of bmFabV inhibition by this diphenyl ether.

MATERIALS AND METHODS

Materials. His-bind Ni²⁺-NTA resin was purchased from Invitrogen, while a QuikChange site-directed mutagenesis kit was obtained from Stratagene. Biotinylated thrombin and streptavidin agarose were from Novagen. Triclosan was a gift from Ciba. *trans*-2-Dodecenoic acid was purchased from TCI. All other chemical reagents were obtained from Sigma-Aldrich.

Synthesis of *trans*-2-Dodecenoyl-CoA (DD-CoA) and Lauryl-CoA. DD-CoA and lauryl-CoA were prepared from *trans*-2-dodecenoic acid and lauric acid, respectively, using the mixed anhydride method (21). Product formation was confirmed by ESI mass spectrometry.

Cloning, Expression, and Purification of bmFabV. The entire putative *fabV* gene from *B. mallei* ATCC 23344 (NCBI Reference Sequence YP_102617.1) was amplified using the primers listed in Table 1 and inserted into the Novagen pET15b vector using the 5' NdeI and 3' BamHI restriction sites (underlined) so that a His tag was encoded at the N-terminus of the coding sequence. After purification from XL1Blue cells (Stratagene) using a DNA purification and gel extraction kit (Qiagen Inc.), the correct sequence of the insert was confirmed using ABI DNA sequencing.

Protein expression was performed using *E. coli* BL21(DE3)pLysS cells. After transformation, a single colony was used to inoculate 10 mL of Luria broth (LB) medium containing 0.2 mg/mL ampicillin in a 50 mL falcon tube, which was then incubated overnight at 37 °C in a floor shaker. The overnight culture was then used to inoculate 1 L of LB medium containing ampicillin (0.2 mg/mL) which was incubated on an orbital shaker at 37 °C until the optical density at 600 nm (OD₆₀₀) increased to ~1.0. Protein expression was induced via addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), and the culture was then shaken at 25 °C for 16 h. Cells were harvested by centrifugation at 5000 rpm for 25 min at 4 °C. The cell paste was then resuspended in 30 mL of His binding buffer [5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9)] and lysed by sonication. Cell debris was removed by centrifugation at 33000 rpm for 60 min at 4 °C. The resulting supernatant was loaded onto a His-bind

column (1.5 cm × 15 cm) containing 4 mL of His-bind resin (Novagen) that had been charged with 9 mL of charge buffer (Ni²⁺). The column was washed with 60 mL of His binding buffer and 30 mL of wash buffer [60 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9)]. Subsequently, the protein was eluted using a gradient of 20 mL of binding buffer and 40 mL of elute buffer [1 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9)]. Fractions containing bmFabV were collected, and imidazole was removed using a Sephadex G-25 column (1.5 cm × 55 cm long) using PIPES buffer [30 mM PIPES, 150 mM NaCl, and 1.0 mM EDTA (pH 8.0)] as the eluent. The purity of the protein was shown to be >95% by 12% SDS-PAGE, which gave an apparent molecular mass of ~45 kDa. The concentration of the protein was determined by measuring the A₂₈₀ and using an extinction coefficient (ε₂₈₀) of 42650 M⁻¹ cm⁻¹ calculated from the primary sequence. The enzyme was stored at -80 °C after being flash-frozen with liquid N₂.

To remove the N-terminal His tag, 1 μL of biotinylated thrombin was added to 1 mL of bmFabV (20 μM) in PIPES buffer, and the reaction mixture was incubated at room temperature for 16 h. Streptavidin agarose (20 μL) was then added, and the solution was incubated for an additional 1 h, after which the agarose was removed by centrifugation at 13000 rpm for 10 min. The supernatant was further purified by chromatography on a Sephadex G-25 column (0.5 cm × 15 cm long) using PIPES buffer as the eluent, and fractions containing bmFabV lacking the His tag were pooled and analyzed via 12% SDS-PAGE.

Site-Directed Mutagenesis, Expression, and Purification of bmFabV Mutants. The bmFabV mutants Y235A, Y235S, K244A, K244R, K245M, and K244A/K245A were prepared using the QuikChange mutagenesis kit from Stratagene with the primers listed in Table 1. The sequence of each mutant plasmid was confirmed by ABI DNA sequencing, and the expression and purification of all the mutants followed the same procedure described above for the wild-type enzyme.

Cloning, Expression, and Purification of ftuACP. The open reading frame (NCBI Reference Sequence YP_170325) which encodes the putative acyl carrier protein (ACP) in *Francisella tularensis* SCHU S4 was amplified using the primers listed in Table 1. The protein product of this ORF is 68% identical and 79% similar to the ACP from *B. mallei* ATCC 23344. The purified PCR product was inserted into the Novagen pET23b

vector using the 5' BamHI and 3' XhoI restriction sites (underlined), so that a His tag was encoded at the C-terminus of the protein. The sequence of the construct was confirmed by ABI DNA sequencing.

Expression and purification of ftuACP followed a protocol similar to that described above for bmFabV except that 0.1 M potassium phosphate buffer (pH 8.0) was used for G-25 chromatography. The purified protein was analyzed by 15% SDS-PAGE and MALDI-TOF mass spectrometry. The concentration of the protein was determined by measuring the absorption at 280 nm and by using an extinction coefficient of $2560 \text{ M}^{-1} \text{ cm}^{-1}$ calculated from the primary sequence. The protein was stored at 4 °C for >3 months without losing any activity.

Preparation of Crotonyl-ACP (Cr-ACP). Purified ftuACP was concentrated to 900 μM in the reaction buffer [0.1 M potassium phosphate solution (pH 8.0), 3 mL], and an equimolar amount of dithiothreitol was added to the solution. The reaction mixture was then stirred under nitrogen at 0 °C for 2 h to ensure complete reduction of the ACP thiol group, after which a 1.5-fold molar excess of crotonic anhydride was added to the reaction mixture. After the mixture had been stirred for 15 min at 0 °C, small molecules were removed from the Cr-ACP by chromatography on a Sephadex G-25 column (0.5 cm \times 15 cm) using PIPES buffer as the eluent.

Steady-State Kinetic Analysis. Steady-state kinetic parameters were determined at 25 °C in 30 mM PIPES buffer (pH 7.9) containing 1.0 mM EDTA and 0.62 mM NaCl. The optimum value for ionic strength was determined by varying the concentration of NaCl from 0 to 700 mM, while the pH optimum was obtained by varying the pH of the reaction mixture from 6.6 to 8.5. Initial velocities were determined using a Cary 300 Bio (Varian) spectrophotometer to monitor the oxidation of NADH to NAD^+ at 340 nm ($\epsilon = 6300 \text{ M}^{-1} \text{ cm}^{-1}$). Initial characterization of the enzyme mechanism was performed in reaction mixtures containing 5 nM bmFabV and measuring initial velocities at several fixed concentrations of NADH (33, 110, and 250 μM) and by varying the concentration of DD-CoA (1.5–35 μM), or at several fixed concentrations of DD-CoA (6 μM , 12 μM and 18 μM) and by varying the concentration of NADH (25–250 μM). Double-reciprocal plots were then used to differentiate between ping-pong and ternary complex mechanisms.

To further investigate the binding order of substrates, product inhibition studies were performed in which each substrate concentration was varied in the presence of several fixed concentrations of one of the products, NAD^+ (0, 50, and 110 μM) or lauryl-CoA (0, 50, and 100 μM). The type of inhibition in each case was subsequently determined using a Lineweaver–Burk plot.

Finally, the kinetic data in the absence of products were globally fit to the equation for the steady-state sequential bi-bi mechanism (eq 1) to determine the K_m values for DD-CoA and NADH.

$$v = V_{\max} [A][B] / (K_{iA} K_B + K_B [A] + K_A [B] + [A][B]) \quad (1)$$

where v is the initial velocity, V_{\max} is the maximum velocity, $[A]$ and $[B]$ are the concentrations of the two substrates, K_A and K_B are the Michaelis constants for A and B, respectively, and K_{iA} is the dissociation constant for A. Data analysis was performed using GraFit version 4.0 (Erithacus).

K_m values of wild-type bmFabV toward DD-CoA and NADH were also determined by varying the concentration of one

substrate at a fixed, saturating, concentration ($>10K_m$) of the second substrate. Data sets were then fit to the Michaelis–Menten equation (eq 2) using GraFit (Erithacus).

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

K_m values determined using the method described above were found to be very close to those obtained from global fitting. Consequently, all subsequent measurements of K_m utilized initial velocities determined at fixed saturating concentrations of one substrate while the concentration of the other was varied.

Circular Dichroism Spectroscopy. The far-UV CD spectra of the wild-type bmFabV protein and its mutants were recorded in 50 mM Tris buffer (pH 7.9) at 25 °C at a concentration of 10 μM using an AVIV 62 DS spectrometer equipped with a Peltier temperature control unit. Data analysis was performed using Microsoft Excel.

Fluorescence Titration of Triclosan Binding to Wild-Type bmFabV. Equilibrium fluorescence titrations were conducted using a Spex FL3-21 Fluorolog-3 spectrofluorimeter. Aliquots (1 μL) of triclosan (20.0 mM stock in DMSO) were added to a 1 mL solution of enzyme (2 μM) in the same buffer that was used in the steady-state kinetic experiments. The excitation wavelength was 295 nm (5 nm slit width), and the emission wavelength was fixed at 335 nm (1 nm slit width). Dilution of the protein concentration was controlled to minimum (<1%), and the change in fluorescence as a function of triclosan concentration was fit to a quadratic equation (eq 3).

$$\frac{F_e - F_1}{F_e(\max) - F_1(\max)} = \frac{K_d + [E]_0 + [L] - \sqrt{(K_d + [E]_0 + [L])^2 - 4K_d[E]_0}}{2[E]_0} \quad (3)$$

where F_e and F_1 are the fluorescence intensities in the presence and absence of enzyme, respectively, $F_e(\max)$ and $F_1(\max)$ are the maximum fluorescence intensities in the presence and absence of enzyme, respectively, K_d is the dissociation constant, $[E]_0$ is the total enzyme concentration, and $[L]$ is the amount of triclosan added to the reaction buffer. Data fitting was performed using GraFit.

Progress Curve Analysis. Progress curve analysis was used to determine if triclosan was a slow onset inhibitor of bmFabV. FabI from *F. tularensis* (ftuFabI) was used as a model system since it has been shown that triclosan is a slow onset inhibitor of ftuFabI. In the assay, a very low concentration of enzyme (2 nM) and a high concentration of substrate (200 μM DD-CoA and 250 μM NADH) were used so that initial velocities were linear over a period of 1 h. Since triclosan and other diphenyl ether inhibitors bind to enoyl-ACP reductases in the presence of the oxidized cofactor, 100 μM NAD^+ was added to the reaction so that $[\text{NAD}^+]$ was effectively constant during progress curve data collection. The concentration of triclosan was 60 μM , and data were collected for 1 h to ensure that the system had reached the steady state. GraFit was used for the data fitting.

Inhibition of bmFabV by Triclosan. Steady-state kinetics revealed that triclosan is an uncompetitive inhibitor of bmFabV with respect to DD-CoA and a competitive inhibitor with respect to NADH. Initial velocities were measured at a fixed concentration of NADH (250 μM) or DD-CoA (35 μM) and at various concentrations of the second substrate and inhibitor. The equilibrium constant for the uncompetitive inhibition of bmFabV by

Table 2: Kinetic Parameters for Wild-Type and Mutant bmFabV Enzymes

enzyme	k_{cat} (min^{-1})	K_m (μM)		k_{cat}/K_m ($\mu\text{M}^{-1} \text{min}^{-1}$)
		DD-CoA	NADH	
wild type without His tag	1728 ± 10	4.4 ± 0.3	not determined	392 ± 27
wild type with His tag	1242 ± 30	2.5 ± 0.4	23 ± 3	497 ± 79
Y235A	5.0 ± 0.1	2.7 ± 0.2	23 ± 2	1.8 ± 0.1
Y235S	6.1 ± 0.3	2.2 ± 0.1	6 ± 1	2.8 ± 0.1
K244A	11 ± 1	7.2 ± 0.6	66 ± 2	1.5 ± 0.1
K244R	1.3 ± 0.1	7.6 ± 0.7	69 ± 1	0.17 ± 0.01
K245M	18 ± 1	28 ± 2	28 ± 2	0.64 ± 0.03
K244A/K245A	not determined	not determined	65 ± 4	0.023 ± 0.002

triclosan (K_{ii}) was calculated using eq 4 for the data collected at a fixed concentration of NADH

$$v = [V_{\text{max}}[S]/(1 + [I]/K_{\text{ii}})]/[K_m/(1 + [I]/K_{\text{ii}}) + [S]] \quad (4)$$

where $[S]$ is the concentration of DD-CoA, K_m is the Michaelis–Menten constant for DD-CoA, V_{max} is the maximum velocity, $[I]$ is the concentration of inhibitor added, and K_{ii} is the inhibition constant.

Similarly, the equilibrium constant for the competitive inhibition of bmFabV by triclosan (K_{is}) was calculated using eq 5 for the data collected at a fixed concentration of DD-CoA

$$v = V_{\text{max}}[S]/[K_m(1 + [I]/K_{\text{is}}) + [S]] \quad (5)$$

where $[S]$ is the concentration of NADH, K_m is the Michaelis–Menten constant for NADH, V_{max} is the maximum velocity, $[I]$ is the concentration of inhibitor added, and K_{is} is the inhibition constant.

RESULTS AND DISCUSSION

Bioinformatic Analysis. To identify putative enoyl-ACP reductase homologues in *B. mallei*, the sequences of FabI from *E. coli* (ecFabI), FabL from *Ba. subtilis*, FabK from *St. pneumoniae*, and FabV from *V. cholerae* (vcFabV) were used as templates for a BLAST analysis of the *B. mallei* genome (ATCC 23344). Two open reading frames were identified as ecFabI homologues, one in chromosome 1 (63% identical to ecFabI) and the second in chromosome 2 (42% identical to ecFabI), while a homologue of vcFabV (bmFabV) was also located on chromosome 1 (56% identical to vcFabV). Subsequently, the vcFabV homologue was cloned, expressed, and purified, giving a protein with a molecular mass of ~45 kDa on SDS–PAGE, consistent with the expected molecular mass of 44965 Da for His-tagged bmFabV. The enzyme was shown to catalyze the NADH-dependent reduction of DD-CoA, demonstrating that bmFabV has in vitro enzymatic activity characteristic of an enoyl-ACP reductase.

Kinetic Mechanism. The optimal ionic strength (0.62 mM) and pH (7.9) values for the enzyme-catalyzed reaction were determined by measuring initial velocities at saturating levels ($> 10K_m$) of both DD-CoA and NADH. The Michaelis–Menten constants for DD-CoA and NADH were then determined by varying the concentration of one substrate at a saturating concentration of the second substrate. The values of k_{cat} , $K_{\text{m,NADH}}$, and $K_{\text{m,DD-CoA}}$ obtained using this method were $930 \pm 6 \text{ min}^{-1}$, $28.7 \pm 1.2 \mu\text{M}$, and $2.4 \pm 0.1 \mu\text{M}$, respectively, which are close to the values obtained from global fitting (see below). Because of reports that vcFabV had a much higher catalytic activity without the C-terminal His tag (19), we also determined the kinetic parameters of bmFabV after thrombin was used to remove the N-terminal His tag. Subsequently, we found that

bmFabV lacking the N-terminal His tag had k_{cat} and $K_{\text{m,DD-CoA}}$ values of $1728 \pm 10 \text{ min}^{-1}$ and $4.4 \pm 0.3 \mu\text{M}$, respectively (Table 2), indicating that the His tag did not have a dramatic effect on activity. Consequently, all subsequent kinetic experiments were performed using enzymes bearing an N-terminal His tag.

To demonstrate that the recombinant enzyme is also capable of catalyzing the reduction of ACP substrates, the ACP from *F. tularensis* SCHU S4 (ftuACP), which is 68% identical to the putative ACP from *B. mallei*, was expressed and purified. ESI mass spectrometry revealed that the purified ftuACP was entirely in the holo form, and a chemical coupling reaction with crotonic anhydride was subsequently used to synthesize Cr-ACP. The values of k_{cat} , $K_{\text{m,NADH}}$, and $K_{\text{m,Cr-ACP}}$ obtained were $1299 \pm 29 \text{ min}^{-1}$, $31 \pm 4 \mu\text{M}$, and $25.1 \pm 2 \mu\text{M}$, respectively.

After optimization of the ionic strength and pH, a matrix of initial velocities was measured at different concentrations of DD-CoA and NADH, and the kinetic data were then analyzed using double-reciprocal plots. Similar to those of other enoyl-ACP reductases, these studies confirmed that bmFabV catalyzes substrate reduction through a ternary complex mechanism, since all the lines in the double-reciprocal plot intersect with each other at a single point (Figure 1) (22–25). To further investigate the order of substrate binding, product inhibition studies using NAD^+ and lauryl-CoA were performed. Analysis of the results from double-reciprocal plots indicated that NAD^+ was a competitive inhibitor with respect to NADH but was a mixed inhibitor with respect to DD-CoA. However, when NADH or DD-CoA was varied in the presence of lauryl-CoA, mixed patterns of inhibition were observed for both substrates (Figure 2). This combination of product inhibition patterns is indicative of a sequential bi-bi mechanism with NADH binding to the enzyme first. The ternary complex steady-state kinetic parameters were subsequently obtained by globally fitting the initial velocity data to eq 1 for an ordered bi-bi mechanism, giving a $K_{\text{i,NADH}}$ of $13.0 \pm 4.1 \mu\text{M}$, a $K_{\text{m,NADH}}$ of $23.2 \pm 3.4 \mu\text{M}$, a $K_{\text{m,DD-CoA}}$ of $2.5 \pm 0.4 \mu\text{M}$, and a k_{cat} of $1242 \pm 30 \text{ min}^{-1}$ (Table 2). Due to the similarity in kinetic parameters obtained from global fitting and when only one of the substrates was varied at a saturating level of the second substrate, we subsequently analyzed the impact of site-directed mutagenesis by determining kinetic parameters at a single fixed concentration of one substrate while varying the second substrate.

Taken together, the steady-state kinetic data reveal that bmFabV functions as an enoyl-ACP reductase, possessing even higher catalytic activity than other enoyl-ACP reductases characterized previously (21, 23, 24, 26). bmFabV catalyzes substrate reduction through a sequential bi-bi mechanism with NADH binding first. This suggests that NADH forms part of the enoyl substrate binding site as seen in several other FabI enoyl-ACP

reductases (22, 25). In addition, we found that the N-terminal His tag in bmFabv did not have a dramatic effect on catalytic activity as reported for the C-terminal His tag in vcFabV.

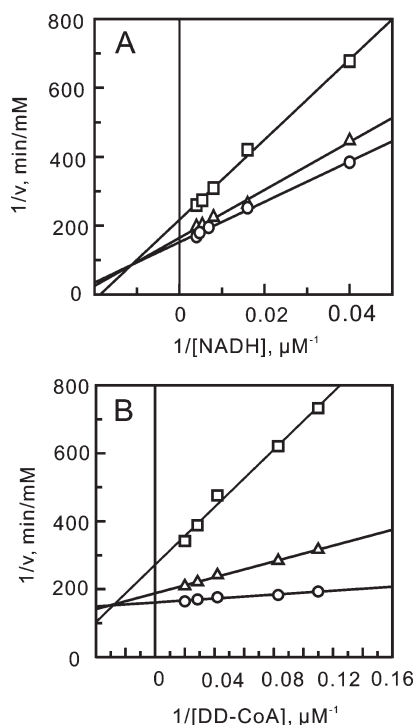


FIGURE 1: Two-substrate steady-state kinetics. Initial velocity patterns: (A) $1/v$ vs $1/[NADH]$ double-reciprocal plots in which the DD-CoA concentration was fixed at 6 (□), 12 (Δ), and 18 μM (○) and (B) $1/v$ vs $1/[DD-CoA]$ double-reciprocal plots in which the NADH concentration was fixed at 33 (□), 110 (Δ), and 250 μM (○).

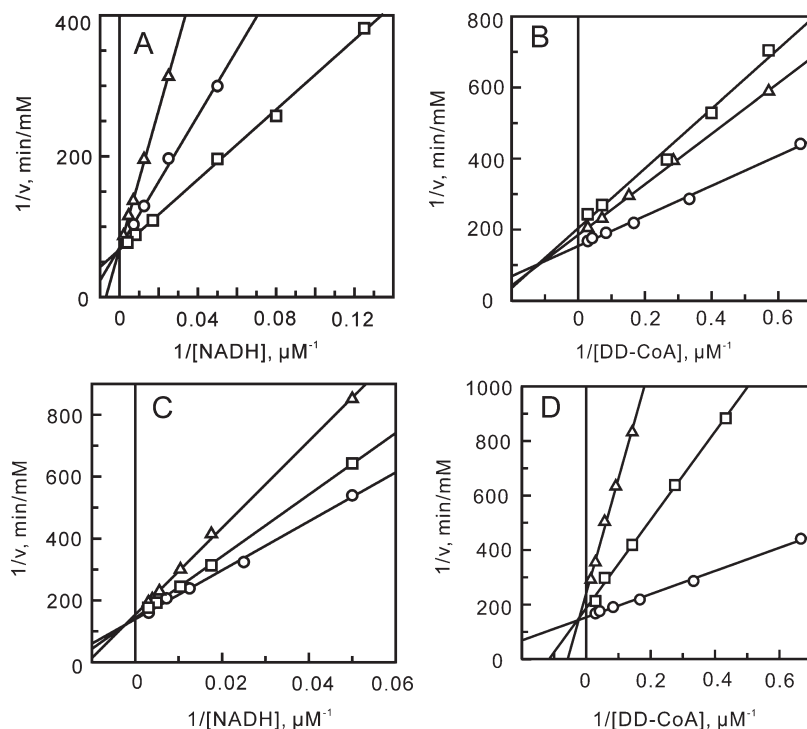


FIGURE 2: Product inhibition studies to determine the substrate binding order. Assays were performed by varying the concentration of one substrate at a fixed concentration of the second substrate and in the presence of one of the products of the reaction. (A) NADH concentration varied in the presence of NAD⁺ [0 (□), 55 (○), and 110 μM (Δ)] with the DD-CoA concentration fixed at 35 μM. (B) DD-CoA concentration varied in the presence of NAD⁺ [0 (○), 55 (Δ), and 110 μM (□)] with the NADH concentration fixed at 250 μM. (C) NADH concentration varied in the presence of lauryl-CoA [0 (○), 50 (□), and 100 μM (Δ)] with the DD-CoA concentration fixed at 35 μM. (D) DD-CoA concentration varied in the presence of lauryl-CoA [0 (○), 50 (□), and 100 μM (Δ)] with the NADH concentration fixed at 250 μM.

Active Site Residues and Catalytic Mechanism. The FabI and FabV enoyl-ACP reductases are members of the short chain dehydrogenase/reductase (SDR) superfamily (27–30). In this family, a catalytic tyrosine and lysine are found in a Tyr-(Xaa)_n-Lys motif, where *n* generally equals 3 for the dehydrogenases and 6 for enoyl-ACP reductases. The variation in *n* is thought to relate to the difference in chemistry between reduction of a carbonyl group and a carbon–carbon double bond conjugated to a thioester. Analysis of the bmFabV sequence suggests the presence of an even more extended active site motif, Tyr-(Xaa)₈-Lys, involving Y235 and K244. In addition, unlike that of other SDR reductases, K244 is followed by a second lysine (K245), which is conserved in the FabV homologues from a variety of organisms, including *Burkholderia* sp., *Yersinia* sp., and *Clostridium acetobutylicum* (Figure 3). To investigate the roles of these residues in catalysis, we expressed and purified the following mutant

		*		*		**
<i>ecFabI</i>	142	LTLSYLGAERAIPNYN	-	VMGLAKASLEANVR	171	
<i>bsFabL</i>	137	VSISLGLSIRYLENT	-	TVGVSKAALEALTR	166	
<i>bmFabV</i>	221	TAFTYLGEQVTHDIYWNGS	IGEA	KKDLDRTVL	252	
<i>ypFabV</i>	221	TAFTYLGEKITHDIYWNGS	IGAA	KKDLQDKVL	252	
<i>caFabV</i>	221	IAYSIGSPRTYKIYREGT	IGIA	KKDLEDKAK	252	
<i>paFabV</i>	221	TAFTYLGEKITHDIYWNGS	IGAA	KKDLQDKVL	252	
<i>vpFabV</i>	221	TAFTYLGERITHDIYWNGS	IGAA	KKDLQDKVL	252	
<i>mfFabV</i>	220	TAFTYLGEKITHDIYWNGT	IGAA	KKDLQDRV	251	
<i>etFabV</i>	221	TAFTYLGEKITHDIYWNGS	IGEA	KKDLDKRVL	252	
<i>vcFabV</i>	227	IAFSYMGPDVTHPIYLDGTL	GRAKID	LDLHQTSH	258	
		*		*		**

FIGURE 3: Sequence alignment of active site residues of the enoyl-ACP reductases from *E. coli*, *Ba. subtilis*, *B. mallei*, *Y. pestis*, *C. acetobutylicum*, *P. aeruginosa*, *Variovorax paradoxus*, *Methylobacillus flagellatus*, *Erwinia tasmaniensis*, and *V. cholerae*. Conserved residues are labeled with an asterisk. The sequence alignment was performed using Clustal W (48), and the figure was made using Jalview (49).

enzymes: Y235A, Y235S, K244A, K244R, K245M, and K244A/K245A. CD spectra of the wild-type and mutant proteins were superimposable (data not shown), suggesting that the alteration in catalytic activity resulting from mutagenesis was not a result of major structural changes in the protein. Subsequently, the kinetic parameters of the mutant enzymes were determined (Table 2).

Y235 in bmFabV is homologous to the conserved tyrosine that is found in all members of the SDR superfamily (21, 31). Replacement of this conserved tyrosine with phenylalanine in the dehydrogenases, such as β /17 β -hydroxysteroid dehydrogenase, results in an enzyme with virtually no activity (31), consistent with the notion that this residue plays a critical role in substrate oxidation and reduction. In contrast, equivalent mutations in the FabI enzymes from *E. coli* (ecFabI, Y156F) and *M. tuberculosis* (InhA, Y158F) result in much more modest decreases in k_{cat} values of 7- and 21-fold, respectively, without substantially affecting the K_{m} value for the substrate (21, 32). These data have raised questions concerning the importance of the conserved tyrosine in the enoyl-ACP reductases (33), and indeed, Anderson and co-workers have suggested that the enolate formed during reduction of the enoyl thioester does not need to be stabilized to a great extent by the enzyme (34). Consistent with this idea, the Y158S InhA enzyme has wild-type activity (21), although it now appears that a second tyrosine in ecFabI (Y146) may be involved in transition-state stabilization (33). This latter residue, which constitutes the third residue in the SDR “triad”, is a phenylalanine in mycobacterial FabI enzymes and is a serine in the dehydrogenases (21, 31).

With regard to bmFabV, replacement of Y235 with alanine gave a $k_{\text{cat}}/K_{\text{m}}$ value of $1.8 \pm 0.1 \mu\text{M}^{-1} \text{min}^{-1}$, which is 280-fold lower than that of the wild-type enzyme. This decrease in $k_{\text{cat}}/K_{\text{m}}$ primarily results from a reduction in k_{cat} , suggesting that Y235 plays a crucial role in substrate reduction. To further investigate the catalytic role of Y235, this residue was replaced with a serine. However, unlike InhA, this mutation could not restore the activity of bmFabV and instead resulted in an enzyme with activity similar to that of the Y235A mutant. Although the pK_{a} values of the Y235 hydroxyl group and of the S235 mutant in the enzyme are unknown, the pK_{a} values of these side chains differ by ~ 4.5 log in solution, suggesting that the acidity of Y235 is important for the function of this residue in catalysis.

In the SDR enzymes, the primary function of the active site lysine is to bind the cofactor through hydrogen bonds formed with the nicotinamide ribose. In addition, this lysine is also thought to interact directly with the conserved tyrosine in the dehydrogenases, facilitating protonation of the substrate carbonyl by the tyrosine (31). In bmFabV, the K244A mutant had k_{cat} , $K_{\text{m,DD-CoA}}$, and $K_{\text{m,NADH}}$ values of $11 \pm 1 \text{ min}^{-1}$, $7.2 \pm 0.6 \mu\text{M}$, and $66 \pm 2 \mu\text{M}$, respectively. This mutation thus causes small (3-fold) increases in the K_{m} values for both substrates, together with a 110-fold decrease in the k_{cat} value for substrate reduction. The corresponding mutation in InhA (K165A) resulted in an enzyme that was unable to bind cofactor. However, in the case of InhA, replacement of K165 with arginine completely restored activity. In contrast, the K244R bmFabV enzyme had a k_{cat} value 950-fold smaller than that of the wild-type enzyme and was thus even less active than the K244A mutant. The modest changes in K_{m} values coupled with the 10^2 – 10^3 -fold reduction in k_{cat} values caused by mutation of K244 (Table 2) thus support differing roles for the conserved lysine in FabI and FabV. In FabV, it appears that K244 plays a much more direct role in facilitating substrate reduction than does the corresponding residue in FabI where it

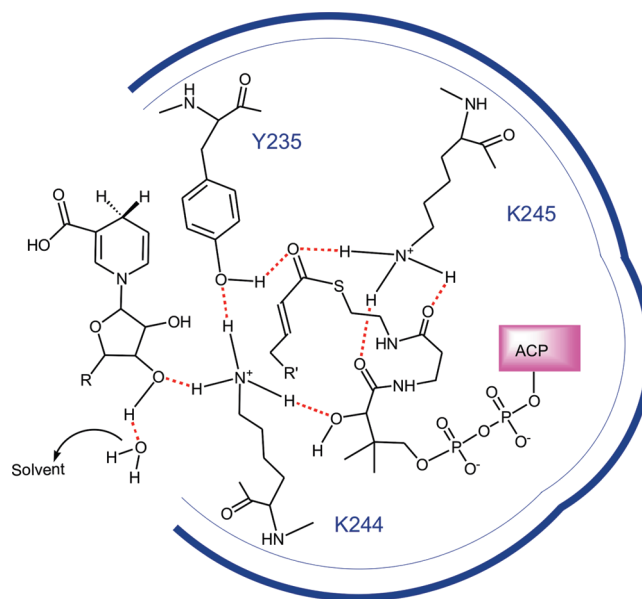


FIGURE 4: Proposed hydrogen bonding network among the three active site residues (Y235, K244, and K245), the cofactor, and the enoyl-ACP substrate. Y235 stabilizes the transition state of the enoyl-ACP substrate through a hydrogen bond with the carbonyl group, while K244 interacts directly with Y235 as proposed in the SDR dehydrogenases. K244 is also shown interacting with the NADH ribose hydroxyl and the secondary hydroxyl group in the ACP pantetheine, to account for the role of this residue in binding cofactor and substrate. K245 is shown interacting with the thioester carbonyl group, to account for the effect of mutating this residue on k_{cat} , and also with the ACP pantetheine.

functions primarily in cofactor binding. Indeed, the function of both Y235 and K244 in bmFabV is more akin to the role played by the conserved tyrosine and lysine residues in the dehydrogenases than in the FabI enzymes.

In bmFabV, a second lysine (K245) is found adjacent to K244. This lysine–lysine sequence is not observed in other enoyl reductases but is conserved in FabV from a variety of organisms (Figure 3). To probe the role of K245, the K245M and K244A/K245A mutants were generated. While the K_{m} value for NADH was unchanged in the K245M mutant, the K_{m} value for DD-CoA was increased 10-fold compared to the wild-type value. This mutation also caused a 70-fold reduction in the k_{cat} value for substrate reduction. Although the K244A mutation resulted in an only small 3-fold increase in $K_{\text{m,DDCoA}}$, combination of K244A with K245A resulted in an enzyme for which the K_{m} value for DD-CoA has increased to a value that cannot be measured. In contrast, the K_{m} values for NADH in the single and double mutants are very similar, suggesting that K245 plays an important role in binding the enoyl substrate.

In summary, we have identified three important active site residues in bmFabV using site-directed mutagenesis. The kinetic data support a model in which Y235, K244, and K245 form a hydrogen bond network that is important for substrate reduction. By analogy to the reaction mechanism in the SDR dehydrogenases, we suggest that Y235 is hydrogen bonded to K244 and/or K245, an interaction that would lower the pK_{a} of Y235 and facilitate the ability of this residue to stabilize and/or protonate the enolate intermediate formed during substrate reduction. In addition, K244 is thought to interact with both cofactor and acyl substrate, while K245 interacts solely with the acyl substrate. A schematic diagram showing these proposed interactions is given in Figure 4. This figure has been adapted from the proposed catalytic

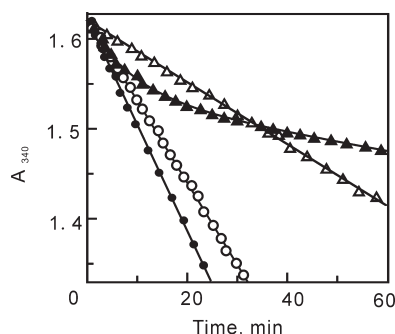


FIGURE 5: Progress curve analysis of ftuFabI and bmFabV. Progress curves for ftuFabI (2 nM) in the presence of DD-CoA (200 μ M), NADH (250 μ M), and either 0 (●) or 60 nM (▲) triclosan. Progress curves for bmFabV (2 nM) in the presence of DD-CoA (200 μ M), NADH (250 μ M), and either 0 (○) or 2 μ M (△) triclosan.

mechanism of 3 β - and 17 β -hydroxysteroid dehydrogenase in which the conserved tyrosine and lysine residues function together to protonate and deprotonate the substrate, respectively (35). The interactions that we propose for FabV differ somewhat from those in other FabI enoyl reductases where the conserved lysine is thought to be involved in only cofactor binding.

Inhibition of bmFabV by Triclosan. The diphenyl ether triclosan is a potent inhibitor of the FabI enzyme from a variety of organisms. Studies with the FabI enzymes from *E. coli* and *F. tularensis* have revealed that triclosan is a slow-onset inhibitor of these organisms, and it is thought that the slow step in formation of the final E-I* complex involves ordering of a substrate recognition loop that closes over the active site after triclosan binding (15, 26, 36–39). The high affinity of triclosan for ecFabI and ftuFabI, as well as related FabIs such as the enzyme from *S. aureus*, is generally a hallmark of the FabI enoyl-ACP reductases, with the outlier being the InhA enzyme from *M. tuberculosis*. Triclosan inhibits InhA with a K_i value of only 0.2 μ M (40) compared to the K_i values of 7 pM, 50 pM, and 5 nM for ecFabI, ftuFabI, and saFabI, respectively (15, 24, 32, 41). In addition, triclosan is not a slow-onset inhibitor of InhA in contrast to the other enzymes. However, a structure-based approach has successfully resulted in diphenyl ether-based inhibitors of InhA with improved affinity (42) that are slow-onset inhibitors of the enzyme (43). An important distinction between the FabI and FabK enoyl-ACP reductases is the insensitivity of the latter to triclosan (17), and Cronan and co-workers have shown that vcFabV is only weakly inhibited by triclosan (50% inhibition at 12 μ M) (19). Because of our success in developing high-affinity inhibitors of InhA, we were thus very curious to study the inhibition of bmFabV by triclosan.

Previous research in our lab has shown that triclosan is a slow-onset inhibitor of ftuFabI (41). In a standard progress curve analysis assay, a linear decrease in NADH concentration is observed in the absence of triclosan while a curved line is seen in the presence of inhibitor (Figure 5). This is a characteristic of slow-onset inhibition, since rapid reversible inhibitors are expected to also yield a linear decrease in substrate concentration under similar conditions. In the case of ftuFabI, the initial velocity decreases exponentially with time until a final steady-state velocity is achieved (44). However, in the studies with bmFabV, the NADH concentration decreased linearly with time, which strongly suggests that triclosan is a rapid, reversible inhibitor of bmFabV (Figure 5).

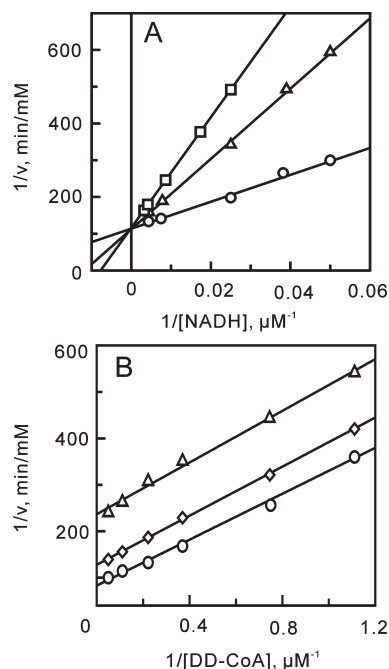


FIGURE 6: Mechanism of bmFabV inhibition by triclosan. Assays were performed by varying the concentration of one substrate at a fixed concentration of the second substrate constant and at various concentrations of triclosan. (A) NADH concentration varied in the presence of triclosan [0 (○), 0.8 (△), and 1.6 μ M (□)] with the DD-CoA concentration fixed at 35 μ M. (B) DD-CoA concentration varied in the presence of triclosan [0 (○), 0.8 (◇), and 1.6 μ M (△)] with the NADH concentration fixed at 250 μ M.

Initial velocities were subsequently obtained over a range of substrate and inhibitor concentrations, and data analysis revealed that triclosan is an uncompetitive inhibitor with respect to DD-CoA ($K_{ii} = 0.42 \pm 0.02$) and a competitive inhibitor with respect to NADH ($K_{is} = 0.48 \pm 0.04$ μ M) (Figure 6). Uncompetitive inhibition with respect to DD-CoA indicates that triclosan binds after DD-CoA. Since we have already shown that bmFabV catalyzes the reaction through a sequential mechanism with NADH binding first and NAD⁺ dissociating last, triclosan then can only bind to the substrate ternary complex (E–NADH–DD-CoA), the product ternary complex (E–NAD⁺–lauryl-CoA), or the binary product complex (E–NAD⁺). In addition, since triclosan is a competitive inhibitor with respect to NADH, triclosan must bind to either the NADH binding site in the free enzyme or the E–NAD⁺ complex. Taken together, we can conclude that triclosan binds to the E–NAD⁺ product complex as observed for the FabI class of enoyl-ACP reductases (15). In support of this hypothesis, fluorescence titration experiments demonstrated that the K_d value for the interaction of triclosan with the free enzyme is greater than 100 μ M, which indicates that triclosan binds only weakly to the free enzyme. Finally, the effect of NAD⁺ on triclosan binding was investigated via addition of NAD⁺ to the reaction mixture. When the inhibition of bmFabV by triclosan was measured by varying the concentration of NADH in the presence of 100 μ M NAD⁺, a 4-fold decrease in the K_{is} value (0.11 μ M) was observed. This means that NAD⁺ can potentiate the binding of triclosan to the enzyme by forming an E–NAD⁺–triclosan ternary complex. However, the formation and dissociation of this ternary complex must be rapid and reversible, occurring without ordering of the substrate binding loop. The mechanism of inhibition of bmFabV by triclosan thus closely follows that characterized previously for InhA (40).

The observation that triclosan is a rapid, reversible inhibitor of bmFabV with a K_i value of only $0.4 \mu\text{M}$ is consistent with the suggestion, based on studies with vcFabV, that the FabV enzyme class is relatively insensitive to this antibacterial diphenyl ether (19). This proposal has received additional support from the recent observation that deletion of FabV in *P. aeruginosa* (paFabV) increased the sensitivity of this organism to triclosan by more than 2000-fold, suggesting that resistance of *P. aeruginosa* to triclosan is due to the relative insensitivity of paFabV to triclosan rather than to efflux (45). This latter observation is of high interest since, like *B. mallei*, *P. aeruginosa* contains both FabI and FabV homologues. However, given our success at developing potent diphenyl ether inhibitors of InhA (42, 43), a FabI enzyme that is also relatively insensitive to triclosan [$K_i = 0.2 \mu\text{M}$ (40)], we are confident that the studies reported in this work will provide a firm foundation for the development of antibacterials that target pathogens with FabV enoyl-ACP reductases such as *B. mallei*, *Y. pestis*, and *P. aeruginosa*, as well as the closely related category B pathogen *B. pseudomallei*, which contains a FabV homologue that is 99% identical to bmFabV (46, 47).

CONCLUSION

In summary, studies on the mechanism of the reaction catalyzed by bmFabV support a sequential bi-bi mechanism in which NADH binds first to the free enzyme. Site-directed mutagenesis suggests that the two conserved active site residues in the Tyr-(Xaa)₈-Lys motif play a more significant role in the chemical steps of substrate reduction than in the FabI class of enoyl-ACP reductases, and that their function is more in line with that of the homologous residues in the members of the SDR dehydrogenase family. In addition, a second lysine in the active site has been identified and shown to play a key role in acyl substrate binding. Finally, detailed inhibition studies have revealed that triclosan binds to the bmFabV–NAD⁺ product complex through a rapid, reversible mechanism of inhibition that closely resembles the inhibition of InhA by triclosan, with a K_i value of $0.4 \mu\text{M}$.

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