Inhibition of the *Staphylococcus aureus* NADPH-dependent Enoyl-Acyl Carrier Protein Reductase by Triclosan and Hexachlorophene*

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Richard J. Heath‡, Jing Li§, Gregory E. Roland¶, and Charles O. Rock‡‡

From the ¤Department of Biochemistry, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105, the ¶Department of Infectious Diseases, Parke-Davis Pharmaceutical Research, Ann Arbor, Michigan 48105, and the ‡Department of Biochemistry, University of Tennessee, Memphis, Tennessee 38163

Enoyl-acyl carrier protein reductase (FabI) plays a determinant role in completing cycles of elongation in type II fatty acid synthase systems and is an important target for antibacterial drugs. The FabI component of *Staphylococcus aureus* (saFabI) was identified, and its properties were compared with *Escherichia coli* FabI (ecFabI). ecFabI and saFabI had similar specific activities, and saFabI expression complemented the *E. coli fabI* (Ts) mutant, illustrating that the Gram-positive FabI was interchangeable with the Gram-negative FabI enzyme. However, ecFabI was specific for NADH, whereas saFabI exhibited specific and positive cooperative binding of NADPH. Triclosan and hexachlorophene inhibited both ecFabI and saFabI. The triclosan-resistant ecFabI(G93V) protein was also refractory to hexachlorophene inhibition, illustrating that both drugs bind at the FabI active site. Both the introduction of a plasmid expressing the *safabi* gene or a missense mutation in the chromosomal *safabi* gene led to triclosan resistance in *S. aureus*; however, these strains did not exhibit cross-resistance to hexachlorophene. The replacement of the ether linkage in triclosan by a carbon bridge in hexachlorophene prevented the formation of a stable FabI-NAD(P)H-drug ternary complex. Thus, the formation of this ternary complex is a key determinant of the antibacterial activity of FabI inhibitors.

Bacterial fatty acid biosynthesis is carried out by a universal series of reactions catalyzed by a collection of enzymes each encoded by a separate gene (1, 2). Fatty acids are assembled 2 carbon units at a time in a cyclical sequence of reactions. The enoyl-ACP reductase (FabI) catalyzes the last step in each cycle and plays a regulatory role in determining the rate of fatty acid synthesis (3, 4). The FabI enzymes are receiving increased attention not only because of their regulatory significance, but also because of the recent discovery that inhibitors of this step in the pathway are effective antibacterials. The diazaborines inactivate FabI through the formation of a covalent bond with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH. Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6). Isoniazid, an inhibitor of the mycobacterial FabI homolog (termed InhA), is activated within the cell and forms a covalent complex with NADH in the active site of the enzyme (5, 6).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF197058 (fabI gene from *S. aureus* strain RN4220).

† To whom correspondence should be addressed: Dept. of Biochemistry, St. Jude Children’s Research Hospital, 332 N. Lauderdale, Memphis, TN 38105-2794. Tel.: 901-495-3491; Fax: 901-525-8025; E-mail: charles.rock@stjude.org.

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¶ The abbreviations used are: ACP, acyl carrier protein; ecFabI, enoyl-ACP reductase from *E. coli*; saFabI, enoyl-ACP reductase from *S. aureus*; triclosan, 2,4,4′-trichloro-2′-hydroxydiphenylether; hexachlorophene, 2,2′-dihydroxy-3,3′,5,5′,6,6′-hexachlorodiphenylmethane; MIC, minimum inhibitory concentration; PCR, polymerase chain reaction; bp, base pair(s).
Chemicals, Inc. supplied the [2,8-3H-adenine]NAD$^2$,2-dihydroxy-4,4-triclosan while hexachlorophene, 2,2-nylether were the generous gifts from Rocco Gogglioti and John Domagala at Parke-Davis Pharmaceuticals. KIC (Aronomk, NJ) supplied the triclosan while hexachlorophene, 2,2-dihydroxydiphenylmethane, and 2,2-dihydroxy-4,4'-dichlorodiphenylmethane were purchased from TCI America. Sigma provided NADH and NADPH. American Radiolabeled Chemicals, Inc. supplied the [2,8-3H-adenine]NAD$^+$ (specific activity: 25 Ci/mmol). All other reagents were of the highest available grade.

**EXPERIMENTAL PROCEDURES**

**Materials**—His-tag FabI and FabI(G93V) from *E. coli* were purified as described previously (3, 12). The trans-2-oxenoyl-N-acetylstea-ine, trans-2-butenoyl-N-acetylstea-ine, and 2,2'-dihydroxydiphe-nylhydroxy were the generous gifts from Rocco Gogglioti and John Domagala at Parke-Davis Pharmaceuticals. KIC (Aronomk, NJ) supplied the triclosan while hexachlorophene, 2,2-dihydroxydiphenylmethane, and 2,2-dihydroxy-4,4'-dichlorodiphenylmethane were purchased from TCI America. Sigma provided NADH and NADPH. American Radiolabeled Chemicals, Inc. supplied the [2,8-3H-adenine]NAD$^+$ (specific activity: 25 Ci/mmol). All other reagents were of the highest available grade.

**Cloning of the safabI Gene**—The fabI gene from *S. aureus* was identified in the unfinished microbial genomic database by comparison with *ecFabI*. The fabI gene was cloned from the genome of *S. aureus* strain RN4220 using the primers RJH102 (bp 771–745) (5'-GGATCTTATTTAATTGCGTGGAATCCGCTATC) and ligated into the TA vector pCR2.1 (Invitrogen). The gene was sequenced and then excised with NdeI and BamHI and ligated into the expression vector pET15b digested with the same enzymes. This construct, pETsai, was transformed into *E. coli* strain BL21(DE3) for expression following isopropyl–thiogalactoside induction. The N-terminus of the His-tagged protein was purified by Ni$^{2+}$-chelation chromatography. Following purification, the protein was exchanged into 20 mM Tris, pH 7.6, 1 mM dithiothreitol, 100 mM EDTA by dialysis overnight. Omission of the EDTA led to precipitation of *saFabI* in the dialysis tubing. The protein was homogeneous as judged by SDS-gel electrophoresis. The yields and purity were similar to those reported previously for *ecFabI* (3, 4).

**Plasmid pETsai was digested with XbaI and EcoRV, and the 0.8-kilobase pair fragment was ligated into similarly digested pBluescript KSII(+) to create pAN2 to express saFabI in *E. coli*. pAN2 was transformed into strain RJH13 (fabI(Ts)) and plated at 30 °C. Individual colonies were then tested for growth at 42 °C by spotting onto LB agar plates and were incubated for 20 min at room temperature prior to addition to the assay, and the change in absorbance was followed with time.

**Spectrophotometric Assay of FabI**—Reduction of trans-2-oxenoyl-N-acetylstea-ine substrates was measured spectrophotometrically as described previously (10). Briefly, a standard reaction contained 0.1 mM sodium phosphate, pH 7.5, 100 μM trans-2-oxenoyl-N-acetylstea-ine, 200 μM NADH or NADPH, and 6 μg of FabI in a final volume of 300 μl. Decrease in absorbance at 340 nm was measured at 25 °C for the linear period of the assay (usually the first 1–2 min). Modifications to this assay are noted in the text and figure legends where applicable. The rate of substrate-independent hydrolysis of NAD(P)H was monitored and subtracted from each point from at least two independent experiments. Kinetic parameters were obtained by varying the concentration of cofactor or substrate as indicated. Drugs were added as serial dilutions from a stock in dimethyl sulfoxide, and the final volume of carrier was kept constant (1.7%) throughout the entire assay. In one series of experiments, the enzyme and drug were preincubated for 20 min at room temperature prior to addition to the assay, and the change in absorbance was followed with time.

**1H/NAD$^+$ Binding Assay**—Assays were performed as described (12) in 0.1 mM sodium phosphate, pH 7.5, containing 4 μM NAD$^+$ (specific activity, 4 Ci/mmol), 1 μM triclosan, and 8 μg of protein in a total volume of 50 μl and were incubated for 20 min at room temperature, then placed into an Ultrafree-ProB bind centrifugal filtration unit containing a 0.2-cm$^2$, 0.45-μm polyvinylidene difluoride membrane (Millipore Corp.). These units were then centrifuged, and the filtrate was washed with 0.1 ml sodium phosphate buffer and then counted in 5 ml of ScintSafe scintillation fluid.

**MIC Determinations**—The MIC values were determined by the microbroth dilution assay according to the National Committee for Clinical Laboratory Standards methods for antimicrobial susceptibility tests for aerobically growing bacteria (Approved Standard M7-A2, NCCLS, Villanova, PA, 1990). All of the determinations were performed twice with comparable results.

**Isolation of Triclosan-resistant *S. aureus***—Approximately 6.5 × 10$^8$ cells of strain RN4220 (25) were plated on to brain-heart infusion agar medium (Becton-Dickinson) containing 0.5 μg/ml triclosan. The plates were incubated at 37 °C with periodic monitoring. After 48 h, resistant clones were picked and their phenotype confirmed by regrowth on the original selective concentration of triclosan. The *S. aureus fabI* gene from triclosan-resistant mutants was characterized by double-strand nucleotide sequencing of PCR products amplified from chromosomal DNA (26). All PCR reactions utilized PCR SuperMix (Life Technologies, Rockville, MD). The reactions contained 100 μM of the primer GER68 (5'-ATGGTTAATCTGAAACAAAC; complementary to bp 1–23) and primer GER71 (5'-TTATTTAATGTCGGAAATCCGC; complementary to bp 771–749). Sequencing was performed on an Applied Biosystems model 377 DNA sequencing system using a PRISM Sequenase$^*$ dye terminator sequencing kit using GER68, GER71, and two additional internal fabI primers (5'-GCCGGACGCTTTTTGAAACTTCAG and 5'-GCCCACTCTAATATGGTGCAACATTGC) to ensure complete coverage.

**Isolation of saFabI Clones**—A chromosomal DNA plasmid library derived from strain RN4220 was constructed by ligating sucrose gradient-purified *Sau3A1* fragments (3–7 kilobase pairs) into BamHI-restricted plasmid pSK265 (27). After electroporation into strain RN4220, approximately 2,000 clones were pooled and frozen at –70 °C. A 1:10 or 1:100 dilution of an overnight culture of this library incubated in tryptic soy broth plus chloramphenicol (7 μg/ml) at 37 °C was plated onto brain-heart infusion medium containing 5 μg/ml chloramphenicol and 0.5 μg/ml triclosan. After 48 h of incubation at 37 °C, resistant clones were confirmed by regrowth on the same media. To confirm the presence of *saFabI* on the plasmid of all the resistant clones, two PCR reactions were performed per isolate. Each reaction contained saFabI-specific primer GER71 and either primer GER23 (5'-GTTTTATGCTTAAAACCTACAG) or GER24 (5'-GAATTGAATATATTTTGGCT-C), which flank the multiple cloning site of plasmid pSK265. In all cases, the isolated plasmids harbored the *saFabI* gene.

**RESULTS**

**Cloning of safabI**—The gene for the trans-2-oxenoyl-ACP reductase from *S. aureus* was identified by a BLAST search of the preliminary sequence data of unfinished microbial genomes obtained from the Institute for Genomic Research web site. The predicted protein sequence was 48% identical to *ecFabI* (Fig. 2) and included the Y6K sequence motif containing the key tyrosine and lysine active site residues.2 The gene was amplified by PCR from the chromosome of *S. aureus* strain RN4220 and cloned into the expression vector pET15b. Homogeneous protein was obtained by Ni$^{2+}$ affinity chromatography as de-

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2 R. J. Heath and C. O. Rock, unpublished observations.
Activity of saFabI—We utilized the spectrophotometric assay described under “Experimental Procedures” to analyze the nucleotide dependence of ecFabI and saFabI (Fig. 3). The ecFabI exhibited a marked preference for NADH over NADPH (Fig. 3A). The apparent $K_m$ of ecFabI for NADH was $25 \pm 4 \mu M$ (Fig. 3A, inset). We were not able to get a reliable estimate for the NADPH $K_m$ for ecFabI due to the low reaction rates, even at high enzyme concentrations. However, it was clear that the apparent $K_m$ was $>2 \text{mM}$. Whereas saFabI utilized NADH as a cofactor, the specific activity was very low; however, when NADPH was employed, a robust rate was observed (Fig. 3B).

The saFabI displayed non-linear kinetics with NADPH, and a distinct upward curvature of the double-reciprocal plot indicated positive cooperative NADPH binding (Fig. 3B, inset). The data fitted almost perfectly to a second order polynomial equation ($r^2 = 0.9997$), and analysis by a Hill plot revealed that NADPH binding to saFabI was highly cooperative with a Hill coefficient, $n_H$, of 2.2 (Fig. 3C). The cofactor concentration that yielded 50% of the maximal rate was 65 $\mu M$. The very low rate of reaction with NADH precluded a detailed analysis of NADH binding to saFabI, but the apparent $K_m$ was $>1 \text{mM}$. Analysis of the ecFabI and saFabI with crotonyl-N-acetylcysteamine and NADH or NADPH, respectively, revealed that both enzymes also had similar affinities for this substrate analog (ecFabI, $K_m = 5 \pm 0.3 \text{mM}$ and $V_{max} = 2 \pm 0.5 \text{nmol/min/\mu g}$; saFabI, $K_m = 8 \pm 0.3 \text{mM}$ and $V_{max} = 0.3 \pm 0.1 \text{nmol/min/\mu g}$).

The gene for saFabI was expressed in E. coli using plasmid pAN2 as described under “Experimental Procedures.” The introduction of plasmid pAN2 into strain RJH13 (fabI(Ts)) (3) complemented the temperature-sensitive growth defect of this strain at 42 °C. Analysis of the fatty acid composition of the transformed strains by gas-liquid chromatography show a normal E. coli profile that was the same as the parent strain (data not shown). Additionally, saFabI was able to substitute for ecFabI in the type II fatty acid synthase system reconstituted in vitro with purified enzymes of E. coli (data not shown). These experiments illustrated that saFabI was functionally equivalent to ecFabI and was capable of catalyzing all of the reactions required for the synthesis of straight chain saturated and unsaturated fatty acids.

Inhibition of ecFabI and saFabI by Triclosan and Hexachlorophene—The efficacy of hexachlorophene as a FabI inhibitor was compared with triclosan in a series of kinetic experiments where the reactions were initiated by the addition of enzyme and initial rates were measured over the first 90 s of the reaction (Fig. 4). ecFabI was inhibited by triclosan with an IC$_{50}$ of 2 $\mu M$ in this assay (Fig. 4A), which was essentially identical to the result obtained previously (12). The saFabI was also inhibited by triclosan with an IC$_{50}$ of 3 $\mu M$ (Fig. 4A), demonstrating that saFabI was also a target for triclosan. As expected, the ecFabI(G93V) active site mutant was more resistant to triclosan inhibition (Fig. 4A and Ref. 12). Hexachlorophene also inhibited ecFabI and saFabI in this in vitro assay (Fig. 4B). Both enzymes exhibited an IC$_{50}$ of approximately 4 $\mu M$, making hexachlorophene almost as potent a FabI inhibitor as triclosan in this kinetic assay as triclosan. The ecFabI(G93V) protein was resistant to hexachlorophene inhibition (Fig. 4B), indicating that hexachlorophene, like triclosan, functioned by interacting with the FabI active site.

Triclosan is a particularly effective FabI inhibitor due to the slow formation of a stable, ternary FabI-NAD$^+$-triclosan ternary complex, and this property of triclosan is responsible for its antibacterial activity (12). Two methods were used to determine if hexachlorophene also formed a similar ternary complex with FabI. First, a binding assay with $^3$H]NAD$^+$ and ecFabI was performed (Fig. 5). ecFabI forms a stable ternary complex with NAD$^+$ and triclosan, resulting in the isolation of radiolabeled ecFabI in the presence of triclosan. In contrast, hexachlorophene did not trigger an increase in $^3$H]NAD$^+$ binding, indicating that a ternary ecFabI-NAD$^+$-hexachlorophene complex did not form. ecFabI did not bind detectable amounts of $^3$H]NAD$^+$ in the absence of triclosan. We were unable to demonstrate ternary complex formation with saFabI using this assay. However, since saFabI has a low affinity for NADH (Fig. 3), this negative result was likely due to the poor interaction of the radiolabeled ligand with the enzyme. Labeled NAD$^+$ was not available, precluding similar experiments with this cofactor.

The second method used to detect ternary complex formation was to examine the effect of preincubation of the enzymes with
drug on the kinetics of the reaction (Fig. 6). Slow-binding inhibitors form time-dependent, stable complexes during the preincubation period, resulting in little to no reaction rate following initiation of the reaction by the addition of substrate. In contrast, inhibitors that do not form tight binding complexes exhibit kinetic behavior in reactions initiated with substrate that is indistinguishable from initiating the reaction with the addition of enzyme (28). Triclosan exhibits the hallmarks of a slow-binding inhibitor. The FabI-NAD\(^+\)-triclosan ternary complex takes several minutes to form and eventually results in complete inhibition (12). This experiment clearly showed that triclosan was a slow-binding, dead end inhibitor with either ecFabI (Fig. 6A) or saFabI (Fig. 6B). Furthermore, triclosan inactivated saFabI with essentially the same time course as we observed previously with ecFabI (data not shown) (12). In contrast, hexachlorophene did not irreversibly inhibit either of the FabI enzymes (Fig. 6), but rather the inhibition pattern was similar to that observed when enzyme was used to initiate the reaction (Fig. 5). Thus, triclosan formed a stable inhibitory complex with ecFabI and saFabI; however, hexachlorophene did not form a slowly dissociating ternary complex with either FabI enzyme.

Structural Features That Determine Inhibitory Activity—Triclosan and hexachlorophene have two structural differences that could account for the different behavior of the compounds as FabI inhibitors. A carbon bond in hexachlorophene replaces the ether bond in triclosan, and the oxygen bridge may be critical to the formation of a ternary complex. Alternately, hexachlorophene has two hydroxyl groups and three additional bulky chlorine atoms that may interfere with the proper positioning of the molecule within the FabI active site. Molecular modeling of the energy-minimized hexachlorophene structure overlaid on the FabI-NAD\(^+\)-triclosan structure indicated that neither the extra hydroxyl group nor the chlorine substituents would interfere with drug binding. We further investigated the structural basis for the differences in activity by testing three additional compounds as FabI inhibitors. Dihydroxydiphenylether was a potent ecFabI inhibitor; however, dihydroxydichlorodiphenylmethane was not (Fig. 7). The only difference in these two compounds is the substitution of a carbon for the oxygen bridging the two phenyl rings. We also examined a dihydroxydichlorodiphenylmethane (Fig. 7). This compound was not a very potent ecFabI inhibitor, although it was more effective than the compound without the chlorine substituents. The
chlorine on the 4-position of triclosan promotes tight binding of the drug to the FabI active site by forming a hydrogen bond interaction with the backbone amide of Ala-95 (12). Increased opportunities for hydrogen bond formation between the chlorines and the protein is one possible explanation for understanding the potency of hexachlorophene as a FabI inhibitor compared with analogs that lack chlorine substitutions. These data indicated that the ether linkage was an essential feature standing the potency of hexachlorophene as a FabI inhibitor compared with analogs that lack chlorine substitutions. These manipulations affected the sensitivity of the cells to hexachlorophene. Therefore, we then sequenced the safabI gene to determine if it was altered. A single missense mutation in the safabI gene that converted saFabI to saFabI(G23S) increased the resistance of S. aureus to triclosan by an order of magnitude (Table I). Second, we introduced the safabI gene on plasmid pTri-1 into S. aureus and observed increased resistance to triclosan. However, neither of these manipulations affected the sensitivity of the cells to hexachlorophene. These data are consistent with triclosan targeting the FabI in S. aureus, and suggest that FabI inhibition is not a component of the biocidal activity of hexachlorophene against this organism.

**TABLE I**

| Bacterial strains | MIC* | Hexachlorophene |
|-------------------|------|-----------------|
| S. aureus RN4220 (wild-type) | 0.25 | 1 |
| S. aureus RN4220/pTri-1<sup>1</sup> | 2 | 1 |
| E. coli GR666 (fabIG23S) | 2 | 1 |
| E. coli W3110 (wild-type)<sup>2</sup> | 0.5 | >64 |
| E. coli W3110/pFabI | 2 | >64 |
| E. coli RJH108 (fabIG93V) | 32 | >64 |

* MICs of drugs were determined according to the current National Committee for Clinical Laboratory Standards as described under “Experimental Procedures.”

<sup>1</sup> Strain genotypes and derivations are described in Ref. 10.

**DISCUSSION**

The Gram-negative (ecFabI) and Gram-positive (saFabI) enoyl-ACP reductases have many properties in common, but are distinguished by their cofactor selectivities. The cofactor specificity of ecFabI has been the subject of some debate. The initial report of enoyl-ACP reductase activity in cell extracts suggested that two enzymes were present in E. coli, one requiring NADH and the other utilizing NADPH (29). The pH optima of the two enzymes were slightly different, with the NADH activity being maximal at pH 7.5 and the NADPH activity preferring pH 6.5. Subsequent genetic and biochemical experiments lead to the conclusion that there is only a single NADH-
dependent enoyl-ACP reductase gene in *E. coli* (3, 30). However, Bergler et al. (31) recently reported that ecFabI has both NADH- and NADPH-dependent activities. The NADPH-dependent activity was only revealed when ecFabI was purified at acidic pH, and this activity was irreversibly lost when the pH was raised to 7.0 or above (31). We have attempted to exactly repeat these experiments without success, and conclude from our present study that ecFabI is highly selective for NADH. In contrast, saFabI is clearly a NADP-dependent reductase that exhibits a high degree of positive cooperativity. This property cannot be considered characteristic of Gram-positive FabI enzymes because the enoyl-ACP reductase from *Bacillus subtilis* is a NADH-dependent enzyme.2 Otherwise, saFabI has specific activities that are very similar to ecFabI for synthetic substrates and is able to complement *E. coli* strains that are defective in ecFabI. *S. aureus* produces branched-chain fatty acids (32), and its ability to substitute for ecFabI in an organism that produces straight-chain saturated and unsaturated fatty acids illustrates the broad substrate specificity of enoyl-ACP reductases.

The structural similarities between triclosan and hexachlorophene suggest that they have a common target, and both drugs inhibit enoyl-ACP reductases in vitro. The resistance of FabI(93V) to both triclosan and hexachlorophene support the concept that both drugs interact with FabI in the vicinity of the substrate binding site. However, the resistance of *E. coli* to hexachlorophene and the genetic analysis of *S. aureus* argues that FabI is not a biologically relevant target for the cellular action of hexachlorophene. The ability of FabI inhibitors to form stable ternary complexes with the enzyme is the critical feature required for antibacterial activity (Fig. 8). This class of agents are called slow-binding inhibitors because inhibitor complexes with the enzyme take a long time (seconds to minutes) to form relative to the catalytic rate (28). This is due to a slow conformational isomerization of the enzyme-drug complex from a state where the enzyme and drug are in rapid equilibrium to a state where the enzyme-drug complex undergoes very slow dissociation (Fig. 8). Triclosan exhibits all the hallmarks of a classic slow-binding inhibitor. The inhibition of FabI by triclosan becomes progressively stronger with time and is essentially irreversible after several minutes (12). This irreversible inhibition correlates with the formation of a stable FabI-NADH-triclosan ternary complex (12, 13) that is accompanied by a conformational change in a flexible loop in FabI including residues 194 to 210 (14). The diazaborines are another class of potent FabI inhibitors that act via the formation of a tight binding bisubstrate complex (6). The formation of the FabI-NADH-diazaborine inhibitory complex is also accompanied by an analogous conformational change in this flexible loop (5). In contrast, hexachlorophene neither exhibits the kinetic properties of a slow-binding inhibitor nor forms a ternary complex.

Our results point to the ether linkage in triclosan as a structural feature that is essential to the formation of the inhibitory ternary complex. This conclusion is drawn from the fact that dihydroxydiphenylether is a FabI inhibitor, whereas dihydroxydiphenylethane is not. Triclosan is sandwiched between the protein and the cofactor and a hydrogen bond network and stacking interactions form the bridge that connects the drug, protein and NAD+ (12–14). Clearly, the substitution of a carbon bridge in hexachlorophene for the ether oxygen in triclosan results in a compound that cannot orient itself to optimally participate in this network. This is most likely attributed to the different angle of the carbon bridge compared with the ether bridge, preventing hexachlorophene from forming hydrogen bond connections and the stacking interactions with NAD+.

Stewart et al. (14) propose that the ether oxygen of triclosan is part of a hydrogen bond network that also includes the hydroxyl groups of Tyr-156, triclosan, and the NAD+ ribose (14). However, the distance between these atoms is about 4 Å in the various published structures (12–14), and it seems unlikely that this weak hydrogen bond acceptor will significantly contribute to the binding affinity at that distance. The substitution of a thioether for the ether in hydroxydiphenyl compounds also significantly reduces antibacterial activity (10), indicating that the bulkier sulfur atom also prevents the proper alignment of the aromatic rings and hydroxyl group in the FabI active site. Thus, the presence of the ether oxygen is clearly important in promoting tight drug binding and the ensuing conformational change that leads to essentially irreversible FabI inhibition and potent antibacterial activity.

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Inhibition of the *Staphylococcus aureus* NADPH-dependent Enoyl-Acyl Carrier Protein Reductase by Triclosan and Hexachlorophene

Richard J. Heath, Jing Li, Gregory E. Roland and Charles O. Rock

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