Structure−Activity Studies of the Inhibition of FabI, the Enoyl Reductase from *Escherichia coli*, by Triclosan: Kinetic Analysis of Mutant FabIs[†]

Sharada Sivaraman,[‡] Jacque Zwahlen,[‡] Alasdair F. Bell,[‡] Lizbeth Hedstrom,[§] and Peter J. Tonge*[,‡]

Department of Chemistry, State University of New York at Stony Brook, Stony Brook, New York 11794-3400, and Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02454

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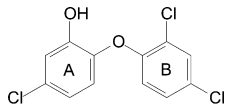
ABSTRACT: Triclosan, a common antibacterial additive used in consumer products, is an inhibitor of FabI, the enoyl reductase enzyme from type II bacterial fatty acid biosynthesis. In agreement with previous studies [Ward, W. H., Holdgate, G. A., Rowsell, S., McLean, E. G., Pauptit, R. A., Clayton, E., Nichols, W. W., Colls, J. G., Minshull, C. A., Jude, D. A., Mistry, A., Timms, D., Camble, R., Hales, N. J., Britton, C. J., and Taylor, I. W. (1999) *Biochemistry 38*, 12514−12525], we report here that triclosan is a slow, reversible, tight binding inhibitor of the FabI from *Escherichia coli*. Triclosan binds preferentially to the E·NAD$^+$ form of the wild-type enzyme with a K_1 value of 23 pM. In agreement with genetic selection experiments [McMurry, L. M., Oethinger, M., and Levy, S. B. (1998) *Nature 394*, 531−532], the affinity of triclosan for the FabI mutants G93V, M159T, and F203L is substantially reduced, binding preferentially to the E·NAD$^+$ forms of G93V, M159T, and F203L with K_1 values of 0.2 μM, 4 nM, and 0.9 nM, respectively. Triclosan binding to the E·NADH form of F203L can also be detected and is defined by a K_2 value of 51 nM. We have also characterized the Y156F and A197M mutants to compare and contrast the binding of triclosan to InhA, the homologous enoyl reductase from *Mycobacterium tuberculosis*. As observed for InhA, Y156F FabI has a decreased affinity for triclosan and the inhibitor binds to both E·NAD$^+$ and E·NADH forms of the enzyme with K_1 and K_2 values of 3 and 30 nM, respectively. The replacement of A197 with Met has no impact on triclosan affinity, indicating that differences in the sequence of the conserved active site loop cannot explain the 10000-fold difference in affinities of FabI and InhA for triclosan.

Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether) (Scheme 1) is an antibacterial additive in many consumer products such as toothpastes, mouthwashes, deodorant soaps, children's toys, and cutting boards. The rationale behind the prolific use of triclosan in the consumer market resulted from the presumption that triclosan has a nonspecific mode of action, therefore making it unlikely that bacteria could become resistant to this biocide (*1*). However, this theory has been challenged by evidence that triclosan is a specific FabI inhibitor. Genetic studies have shown that triclosan blocks lipid biosynthesis in *Escherichia coli* and that mutations in the *fabI* gene result in triclosan resistance (*2*).

Scheme 1

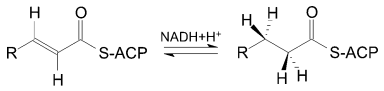


Triclosan

Subsequent kinetic and crystallographic studies have provided direct evidence that triclosan inhibits the FabIs from *E. coli* (*3*−*8*), *Staphylococcus aureus* (*9*), *Bacillus subtilis* (*10*), the malarial parasite, *Plasmodium falciparum* (*11*−*14*), and InhA, the ENR[1] from *Mycobacterium tuberculosis* (*15*, *16*).

The FabI ENR utilizes NADH to catalyze the stereospecific reduction of enoyl-ACPs (Scheme 2) and has been validated as an excellent target for antibacterial drug development (*17*). The fatty acid biosynthesis cycle (FAS) differs significantly in eukaryotes and prokaryotes (*18*).

Scheme 2



FabI Reaction

Most eukaryotes contain type I FAS, where all the catalytic domains reside on one polypeptide. Prokaryotes and plants contain the type II or the dissociated FAS II system, where each enzyme is found on a separate polypeptide. ENR catalyzes the last step in the bacterial FAS II cycle, the reduction of α,β-unsaturated fatty acids esterified to the acyl carrier protein (ACP). Rock and co-workers have demon-

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* To whom correspondence should be addressed. Telephone: (631) 632-7907. Fax: (631) 632-7960. E-mail: peter.tonge@sunysb.edu.

[‡] State University of New York at Stony Brook.

[§] Brandeis University.

[1] Abbreviations: ENR, enoyl reductase; InhA, enoyl reductase from *M. tuberculosis*; FabI, enoyl reductase from *E. coli*; CoA, coenzyme A (lithium salt); DDCoA, *trans*-2-dodecenoyl-CoA; K_{mNADH}, kinetically determined K_m for NADH; K_{iNAD}, K_i determined using NAD$^+$ in product inhibition studies; $K_{m'NAD}$, K_m for NAD$^+$ calculated from the NADH concentration, K_{mNADH}, and K_{NAD}, where $K_{NAD} = K_{iNAD}$.

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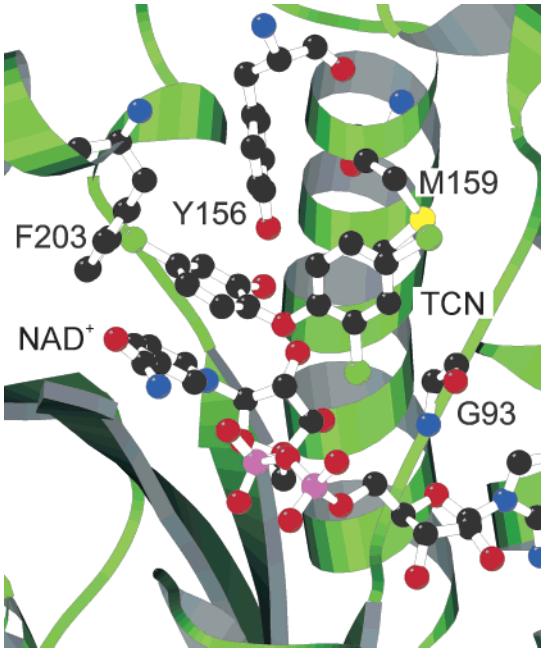


FIGURE 1: Active site region of the FabI−NAD$^+$−triclosan crystal structure showing the interaction between residues Y156, G93, F203, and M159 and triclosan (TCN) (*6*). This figure was generated using MOLSCRIPT (*42*).

strated that the ENR reaction is the rate-limiting step of fatty acid biosynthesis (*19*) and have identified three distinct classes of ENR enzymes that are the products of the *fabI*, *fabK*, and *fabL* genes (*10*, *20*). The diazaborines are a class of boron-containing antibacterial compounds that have been shown to inhibit the FabI from *E. coli* (*21*−*25*), while isoniazid, a frontline antitubercular drug, targets InhA, the ENR from *M. tuberculosis* (*26*−*29*). Interestingly, triclosan is a relatively weak inhibitor of InhA compared to FabI (*16*). InhA is 36% identical and 67% similar to the *E. coli* FabI. While triclosan also binds preferentially to the E·NAD$^+$ form of InhA, the affinity of triclosan for this enzyme is 10000-fold lower than for the *E. coli* FabI. In addition to these three compounds, two new classes of novel ENR inhibitors have been identified, and their interaction with the *E. coli* and *S. aureus* FabIs has been analyzed (*30*, *31*).

This paper presents investigations of the mechanism of triclosan inhibition and selectivity in *E. coli* FabI. We have characterized three mutations of FabI, G93V, M159T, and F203L, that have been found to correlate with triclosan resistance and in which the minimum inhibitory concentration (MIC) of triclosan has increased 95-, 12-, and 6-fold, respectively (*2*). Interestingly, the mutation of G93 to Ser leads to diazaborine resistance, while the mutation of the analogous residue in InhA (S94A) leads to isoniazid resistance (*28*). All three FabI residues are located close to the inhibitor and are involved in the formation of the cofactor-binding site (Figure 1) (*4*−*6*, *8*). In addition, we have replaced the conserved active site Tyr156 with Phe. Examination of the FabI−NAD$^+$−triclosan X-ray structure reveals a hydrogen bond (2.7 Å) between the Y156 hydroxyl group and the phenolic group of triclosan. Previously, we demonstrated that replacement of the analogous residue in InhA, Y158, with Phe decreased k_{cat} 24-fold and decreased the affinity of triclosan for this enzyme 200-fold (*16*). Furthermore, the preference of triclosan for the E·NAD$^+$ form of the InhA enzyme was lost. Last, to explore the difference in the affinity of triclosan between FabI and InhA, we have examined the role of a mobile active site loop in triclosan inhibition. This loop is disordered in the absence of the inhibitor but becomes ordered upon inhibitor binding (*6*, *8*). A sequence alignment of this loop region reveals that it is strongly conserved among all bacterial FabIs except that A197 is a methionine in mycobacterial InhAs (Table 1). We have examined the binding of triclosan to the A197M FabI mutant.

Table 1: Alignment of Enoyl Reductases[a]

Enoyl Reductase	Accession Number	
E. coli	P29132	RYMANAMGPEGVRVNAISAGPIRTLA**A**SGIKD
Salmonella typhimurium	P16657	RYMANAMGPEGVRVNAISAGPIRTLA**A**SGIKD
Haemophilia influenzae	P44432	RVMAADLGKEGIRVNAISAGPIRTLA**A**SGIKN
Pseudomonas aeruginosa	Q9ZFE4	RYLAGSLGAEGTRVNAVSAGPIRTLA**A**SGIKS
M. tuberculosis InhA	P46533	RFVAREAGKYGVRSNLVAAGPIRTLA**M**SAIVGGAL
M. avium InhA	O07400	RFVAREAGPHGVRSNLVAAGPIRTLA**M**AGIVGGVL
M. smegmatis InhA	P42829	RFVAREAGKVGVRSNLVAAGPIRTLA**M**SAIVGGAL

[a] Accession numbers are from the EMBL protein database. The conserved alanine in the FabIs and the methionine in the InhAs are bold.

Our results show that the G93V, M159T, and F203L mutations decrease the affinity of triclosan. The Y156F mutation of FabI also decreases the affinity and selectivity of triclosan; however, the A197M mutation has no effect on triclosan inhibition.

EXPERIMENTAL PROCEDURES

Preparation of Substrate. trans-2-Dodecenoylcoenzyme A (DDCoA) was synthesized from *trans*-2-dodecenoic acid using the mixed anhydride method as described previously (*32*).

Construction of Expression Plasmids for Wild-Type (WT) and Mutant FabIs. Mutations were introduced using the QuikChange mutagenesis kit (Stratagene). A list of the primers that were used for mutagenesis is given in Table 2. Wild-type and mutant plasmids were purified from XL1Blue cells (Stratagene) using a DNA purification and gel extraction kit from Qiagen Inc. After verification of the correct sequence, the plasmid was transformed into BL21(DE3) pLysS cells (Novagen) for protein expression.

Overexpression and Purification of Wild-Type and Mutant FabIs. Cultures of BL21(DE3) pLysS cells carrying the wild-type and mutant plasmids were grown in 500 mL of LB-ampicillin (300 μg/mL) medium at 37 °C to an OD_{600} of 1.7. The cells were harvested by centrifugation, resuspended in 500 mL of fresh LB-ampicillin medium containing isopropyl β-D-thiogalactoside (360 μg/mL), and allowed to induce overnight at 25 °C. The cells were harvested by centrifugation, resuspended in 30 mL of His-bind buffer, and lysed using a French press (five passes at 1500 psi). The cell debris was then removed by ultracentrifugation (33 000 rpm for 90 min) and the supernatant applied to a His-bind resin column (Novagen; 5 mL bed volume). The His-bind column was washed successively with His-bind buffer (25 mL) and His-wash buffer (20 mL), and the protein was eluted using a gradient of 30 mL 0 to 1 M imidazole in 20 mM Tris-HCl and 500 mM NaCl (pH 7.9). Fractions eluted from

Table 2: Primers Used for Mutagenesis[a]

mutant	primer[b]
Y156F (F)	5′-CGCGCTATCCCGAAC**TTC**AACGTTATGGG-3′
Y156F (R)	5′-CCCATAACGTT**GAA**GTTCGGGATAGCGCG-3′
G93V (F)	5′-CGTACACTCTATT**GTT**TTTGCACCTGGCG-3′
G93V (R)	5′-CGCCAGGTGCAAA**AAC**AATAGAGTGTACG-3′
M159T (F)	5′-CCGAACTACAACGTT**ACG**GGTCTGGCAAAAGCG-3′
M159T (R)	5′-CGCTTTTGCCAGACC**CGT**AACGTTGTAGTTCGG-3′
F203L (F)	5′-CCGGTATCAAAGAC**CTG**CGCAAAATGCTGGC-3′
F203L (R)	5′-GCCAGCATTTTGCG**CAG**GTCTTTGATACCGG-3′
A197M (F)	5′-CCGATCCGTACTCTGGCG**ATG**TCCGGTATCAAAGACTTCCGC-3′
A197M (R)	5′-GCGGAAGTCTTTGATACCGGA**CAT**CGCCAGAGTACGGATCGGACC-3′

[a] Forward (F) and reverse (R) primers are listed. [b] The mutation site is underlined in bold.

the His-bind buffer were assayed for activity as described below. Those fractions containing the His-tagged FabI were immediately pooled and applied to a 1.5 cm × 50 cm Sephadex G-25 column (Pharmacia-Amersham) equilibrated with 30 mM PIPES, 150 mM NaCl, and 1 mM EDTA (pH 8.0). Fractions containing the His-tagged protein from the G-25 column were pooled and concentrated. The concentration of the protein was calculated using an ϵ_{280} of 14 mM^{-1} cm^{-1} for the wild type and G93V, F203L, M159T, and A197M mutants and an ϵ_{280} of 12.6 mM^{-1} cm^{-1} for the Y156F mutant.

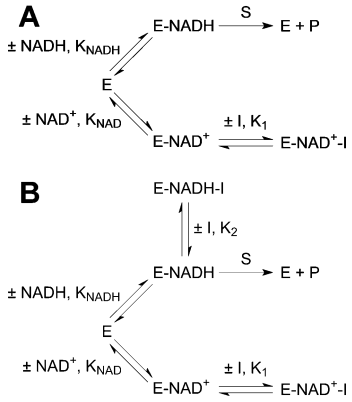
Preparation of Triclosan Solutions. Triclosan (Irgasan DP 300) was a gift from Ciba Specialty Chemicals Corp. (High Point, NC). Triclosan was added to the indicated final concentrations from serially diluted stock solutions in Me_2SO. The Me_2SO concentration in all the assays was maintained at 0.5%. Control experiments demonstrated that this concentration of Me_2SO did not affect FabI activity.

Steady State Kinetics and Determination of Inhibition Constants of Triclosan Binding to Wild-Type and Mutant FabIs. Triclosan is a slow, tight binding inhibitor of FabI and interacts specifically with the E·NAD^+ form of the enzyme (*7*). Consequently, since formation of the enzyme−inhibitor complex occurs slowly with respect to substrate reduction and since triclosan binds preferentially to an enzyme−product complex, typical progress curve analysis of slow, tight binding inhibition cannot be used to analyze inhibitor binding. Instead, the enzyme is preincubated with triclosan and NAD^+ in the presence of saturating NADH. Under these conditions, only a small fraction of the enzyme is in the E·NAD^+ form, thus reducing the affinity of triclosan to measurable levels.

All experiments were carried out on a Cary 100 Bio (Varian) spectrophotometer at 25 °C in 30 mM PIPES and 150 mM NaCl (pH 8.0). Kinetic parameters were determined spectrophotometrically by following the oxidation of NADH to NAD^+ at 340 nm (ϵ = 6.3 mM^{-1} cm^{-1}) or 370 nm (ϵ = 2.4 mM^{-1} cm^{-1}). Following the above protocol, wild-type FabI (15 nM) was preincubated in the presence of fixed concentrations of NAD^+ and NADH (250 μM) and triclosan concentrations (0, 0.07, 0.15, 0.22, 0.35, 0.5, and 1 μM) for 5 h at 4 °C. This experiment was repeated at varying concentrations of NAD^+ (7.4−200 μM). The mixture was warmed to room temperature, and the assay was initiated by the addition of DDCoA (80 μM). Equation 1 was used to estimate the apparent inhibition constant, K_i'

$$v = v_0/(1 + [I]/K_i') \quad (1)$$

Scheme 3: (A) Proposed Mechanism of Inhibition of WT, G93V, M159T, and A197M FabIs by Triclosan and (B) Proposed Mechanism of Inhibition of F203L and Y156F FabIs by Triclosan



where v_0 is the rate in the absence of inhibitor and [I] is the inhibitor concentration.

The dependence of K_i' on [NAD^+] was fit to the following equations (eqs 2−4) (*33*). K_1 and K_2 are defined in panels A and B of Scheme 3, respectively, and represent the inhibition constant for triclosan binding to the E·NAD^+ and E·NADH forms, respectively. Triclosan binds exclusively to the E·NAD^+ form

$$K_i' = K_1(1 + K_{m'NAD}/[NAD^+]) \quad (2)$$

Triclosan binds exclusively to the E·NADH form

$$K_i' = K_2(1 + [NAD^+]/K_{m'NAD}) \quad (3)$$

Triclosan binds to both E·NAD^+ and E·NADH forms

$$K_i' = K_2\{(1 + [NAD^+]/K_{m'NAD})/[1 + [NAD^+]/(K_{m'NAD}K_1/K_2)]\} \quad (4)$$

The K_m value for NAD^+ ($K_{m'NAD}$) was calculated from K_{NAD}, the constant for dissociation of NAD^+ from the enzyme, using eq 5:

$$K_{m'NAD} = K_{NAD}(1 + [NADH]/K_{mNADH}) \quad (5)$$

K_{NAD} was assumed to be equivalent to the inhibition constant for NAD^+ (K_{iNAD}) determined from product inhibition studies. K_{iNAD} was evaluated by measuring initial velocities at different NAD^+ (0−10 mM) and NADH (0−1 mM) concentrations while using a fixed concentration of DDCoA

(80 μM). The product inhibition data were fitted to eq 6 which describes linear competitive inhibition:

$$v = V[\mathrm{NADH}]/[K_{\mathrm{mNADH}}(1 + [\mathrm{NAD}^+]/K_{\mathrm{iNAD}}) + [\mathrm{NADH}]] \quad (6)$$

Enzyme concentrations for the inhibition studies were 15 (WT), 20 (A197M), 30 (F203L), 60 (Y156F), and 500 nM (M159T). A 0.5 cm path length cell was used to allow kinetic data to be obtained at high NAD^+ and NADH concentrations.

The extent of triclosan binding to the M159T, A197M, F203L, and Y156F FabI mutants as a function of NAD^+ concentration was monitored as described for the wild-type enzyme. Enzyme and cofactor concentrations were as follows: 593 nM M159T, 1 mM NADH, and 25−500 μM NAD^+; 19 nM A197M, 1 mM NADH, and 7.4−400 μM NAD^+; 27 nM F203L, 250 μM NADH, and 6.4−1000 μM NAD^+; and 60 nM Y156F, 250 μM NADH, and 10−800 μM NAD^+. Triclosan concentrations were varied up to 1 μM for A197M and Y156F, 1.5 μM for F203L, and 5 μM for M159T while maintaining an at least 5-fold excess of inhibitor over enzyme. For F203L and Y156F, the experiment was repeated keeping the NAD^+ concentration fixed at 50 μM and varying the NADH concentration from 7 to 500 μM for F203L and from 6.8 to 350 μM for Y156F. When the NADH concentration was varied at a constant NAD^+ concentration, equations analogous to those given above (eqs 2−4) were employed for data analysis in which K_1 and K_2 were transposed and K_{mNADH} replaced $K_{\mathrm{m'NAD}}$.

The affinity of triclosan for the G93V mutant was sufficiently reduced compared to that of the wild type that normal Michaelis−Menten kinetics could be used to quantitate inhibitor binding. Consequently, the inhibition constant K_{ii} was calculated for G93V (32 nM) by determining k_{cat} and K_{mDDCOA} at a fixed, saturating concentration of NADH (158 μM) and by varying the concentration of DDCoA (2.5−35 μM) and triclosan (0, 0.4, 1, and 5 μM). Alternatively, K_{ii} was calculated by determining k_{cat} and K_{mNADH} at a fixed, saturating concentration of DDCoA (50 μM) and by varying the concentration of NADH (0−60 μM) and triclosan (0, 0.4, 1, and 5 μM). Each initial velocity was determined in duplicate, and at least five different DDCoA concentrations were examined. Initial velocity data obtained at various substrate concentrations in the presence of various amounts of triclosan were initially analyzed by plotting v versus v/[S], which demonstrated that inhibition was uncompetitive for the G93V mutant. The experimental data were analyzed using eq 7 for uncompetitive inhibition

$$v_{\mathrm{i}} = V[\mathrm{S}]/[K_{\mathrm{m}} + [\mathrm{S}](1 + [\mathrm{I}]/K_{\mathrm{ii}})] \quad (7)$$

where [S] is the concentration of the varied substrate, [I] is the concentration of the inhibitor, V is the maximum velocity, K_{m} is the Michaelis−Menten constant, and K_{ii} is the inhibition constant.

RESULTS

Kinetic Analysis of Wild-Type and Mutant Proteins. Kinetic parameters for wild-type and mutant proteins are given in Table 3. The G93V, F203L, Y156F, and A197M mutations have relatively little effect on the catalytic parameters (less than 10-fold), with the exception of K_{mNADH} for A197M which is increased by 17-fold. In contrast, M159T exhibited a 27-fold decrease in k_{cat} and a 20-fold increase in K_{mNADH}. The K_{NAD} value for WT FabI was 1.1 ± 0.1 mM, similar to the value of 1.9 ± 0.3 mM determined by Ward et al. (*7*). In addition, the K_{NAD} values for F203L, Y156F, M159T, and A197M were 1.5 ± 0.2, 2.3 ± 0.5, 7 ± 1, and 5 ± 1 mM, respectively.

Table 3: Kinetic Parameters for Wild-Type and Mutant FabI Enzymes[a]

enzyme[b]	k_{cat} (min^{-1})	K_{m} (μM) DDCoA	K_{m} (μM) NADH	$k_{\mathrm{cat}}/K_{\mathrm{m}}$ ($\mu\mathrm{M}^{-1}\ \mathrm{min}^{-1}$) (DDCoA)	K_{NAD} (mM)
wild-type	712 ± 23	30 ± 6	20 ± 5	24 ± 6	1.1 ± 0.1
G93V	300 ± 16	3 ± 1	10 ± 2	100 ± 25	ND[c]
M159T	27 ± 1	79 ± 9	400 ± 40	0.33 ± 0.05	7 ± 1
F203L	634 ± 44	35 ± 6	37 ± 10	18.1 ± 4.4	1.5 ± 0.2
Y156F	105 ± 6	29 ± 4	51 ± 2	3.6 ± 0.7	2.3 ± 0.5
A197M	489 ± 23	9 ± 2	345 ± 37	53 ± 13	5 ± 1

[a] All kinetic parameters were determined in 30 mM PIPES and 150 mM NaCl at pH 8.0 and 25 °C. [b] All enzymes contain an N-terminal His tag. [c] Not determined. Unlike the case for the other enzymes, triclosan is not a slow, tight binding inhibitor of G93V, and consequently, K_{NAD} is not required for this enzyme.

Table 4: Inhibition Constants for Triclosan Binding to Wild-Type and Mutant FabIs

enzyme	K_1[b] (triclosan)	K_2 (triclosan) (nM)	binding preference[c]
wild-type	23 ± 1 pM		E·NAD^+
G93V[a]	0.25 ± 0.09 μM		E·NAD^+ ([DDCoA] varied)
	0.15 ± 0.01 μM		E·NAD^+ ([NADH] varied)
M159T[a]	4.0 ± 0.2 nM		E·NAD^+
F203L[a]	0.9 ± 0.1 nM	51 ± 1	E·NAD^+ and E·NADH ([NAD^+] varied)
	0.28 ± 0.02 nM	350 ± 90	E·NAD^+ and E·NADH ([NADH] varied)
Y156F	3 ± 1 nM	30 ± 1	E·NAD^+ and E·NADH ([NAD^+] varied)
		6.3 ± 0.1	E·NADH ([NADH] varied)
A197M	26 ± 2 pM		E·NAD^+

[a] Triclosan-resistant FabI mutants. [b] K_{ii} in the case of G93V. [c] Slow binding except for G93V.

Inhibition of Wild-Type and Mutant FabIs by Triclosan. Table 4 lists the inhibition constants obtained for triclosan binding to the wild-type and mutant enzymes. No inhibition is observed when triclosan is incubated in the absence of NAD^+ both for the wild-type and mutant FabIs, indicating that the inhibitor does not bind to the free enzyme. The apparent K_{i} for triclosan inhibition (K_{i}') will depend on the NAD^+ concentration under these conditions as described by eq 2. If the value of K_{m} for NAD^+ is constrained to 14.9 mM, the data are described well by eq 2 with a K_1 of 23 ± 1 pM, in agreement with previous studies (Figure 2 and Scheme 3A) (*7*). This strategy was also used to analyze the triclosan inhibition of M159T, F203L, Y156F, and A197M. Both M159T and A197M showed slow binding inhibition with a best fit to eq 2 yielding K_1 values of 4.0 ± 0.2 nM and 26 ± 2 pM, respectively (data not shown). Thus, like wild-type FabI, triclosan binds exclusively to the E·NAD^+ forms of M159T and A197M, albeit with a substantially reduced affinity for the M159T mutant (Scheme 3A).

For F203L, the K_{i}' values were consistent with eq 4, indicating that triclosan binds to both E·NAD^+ and E·NADH forms with K_1 and K_2 values of 0.9 ± 0.1 and 51 ± 1 nM,

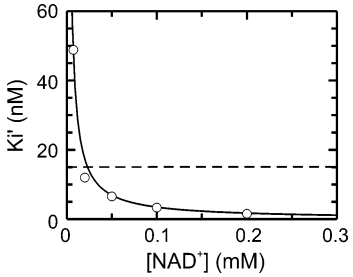


FIGURE 2: Effect of NAD^+ on the apparent inhibition constant for triclosan binding to wild-type FabI. Best fit lines are shown for eq 2 (—) and eq 4 (- - -). An acceptable fit is obtained for eq 2 using a $K_{m'NAD}$ of 14.9 mM, indicating the preference of triclosan for the E·NAD^+ form of the enzyme and giving a K_1 of 23 ± 1 pM.

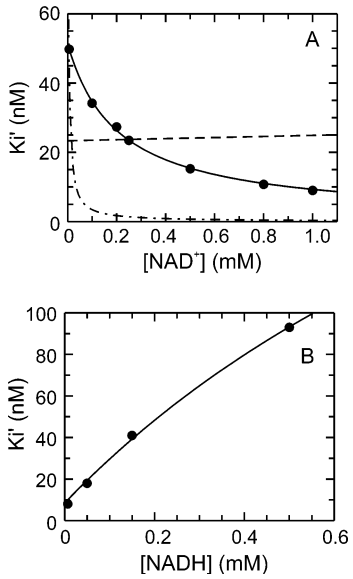


FIGURE 3: Triclosan inhibition of F203L. (A) Effect of NAD^+ on the apparent inhibition constant for triclosan binding to F203L. Best fit lines are shown for eq 4 (—), eq 2 (– - –), and eq 3 (– – –). An acceptable fit is obtained with eq 4 using a $K_{m'NAD}$ of 24.5 mM, indicating that triclosan binds to both E·NAD^+ and E·NADH forms of the enzyme and giving a K_1 of 0.9 ± 0.1 nM and a K_2 of 51 ± 1 nM. (B) Effect of NADH on the apparent inhibition constant for triclosan binding to F203L. An acceptable fit is obtained with eq 4 using a K_{mNADH} of 37 μM and giving a K_1 of 8.5 ± 0.6 and a K_2 of 350 ± 90 nM. Since the NAD^+ concentration was 50 μM, only a small fraction of the enzyme formed a complex with NAD^+ and the true value of K_1 was obtained by dividing the observed value by 1.5/0.05, giving a K_1 of 0.28 ± 0.02 nM.

respectively (Figure 3A and Scheme 3B). When the experiment was repeated with varying NADH concentrations, the data fit well to two models: binding exclusively to the E·NAD^+ form with a K_1 value of 0.21 ± 0.3 nM (eq 3, data not shown) and binding to both E·NAD^+ and E·NADH forms with a K_1 of 0.28 ± 0.02 nM and a K_2 of 350 ± 90 nM (eq 4, Figure 3B and Table 4). On the basis of the results of the experiment with the varied NAD^+ concentration, we prefer the model where triclosan binds to both E·NAD^+ and E·NADH forms of the enzyme. The K_1 values in the experiments with varied NAD^+ and varied NADH concentrations are comparable (0.9 and 0.28 nM, respectively); however, the values of K_2 differ by 7-fold (51 and 350 nM, respectively). The discrepancy in the K_2 values can be attributed to the strong preference of triclosan for the E·NAD^+ complex, which will make the contribution of the E·NADH complex difficult to measure.

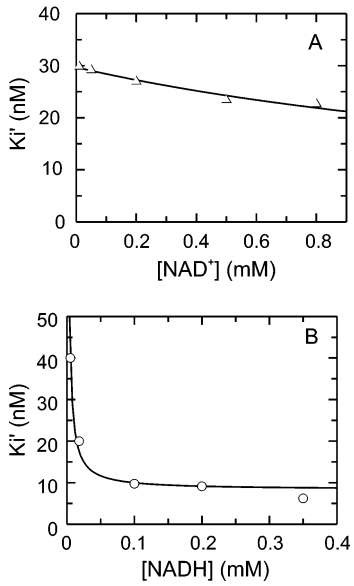


FIGURE 4: Triclosan inhibition of Y156F. (A) Effect of NAD^+ on the apparent inhibition constant for triclosan binding to Y156F. Equation 4 gave an acceptable fit with a $K_{m'NAD}$ of 22.1 mM, giving a K_1 of 3 ± 1 nM and a K_2 of 30 ± 1 nM. (B) Effect of NADH on the apparent inhibition constant for triclosan binding to Y156F analyzed using eq 2 with a K_{mNADH} of 51 μM, giving a K_2 of 6.3 ± 0.1 nM.

Like that of F203L, triclosan inhibition of the Y156F mutant monitored as a function of NAD^+ concentration also best fit eq 4, giving a K_1 of 3 ± 1 nM and a K_2 of 30 ± 1 nM (Figure 4A and Scheme 3B). Thus, triclosan has similar affinities for both the E·NAD^+ and E·NADH forms of Y156F. When the experiment was repeated by varying the NADH concentration, the apparent K_i' values decreased as the NADH concentration increased, indicating that triclosan bound preferentially to the E·NADH form of the enzyme under the experimental conditions that were used. Indeed, the data fit well to eq 2, which assumes that triclosan binds exclusively to the E·NADH form of Y156F, giving a K_2 of 6.3 ± 0.1 nM (Figure 4B and Table 4). We speculated that we were unable to detect binding to the E·NAD^+ form in the experiment with varied NADH concentrations given the small fraction of enzyme present in the E·NAD^+ complex. When the data were reanalyzed using eq 4, we obtained a K_2 of 6 ± 1 nM and a poorly defined K_1 of 150 ± 50 nM. Given the fact that the concentration of NAD^+ in the experiment with varied NADH concentrations is 50 μM and that K_{NAD} = 2.3 mM for this mutant, the true value of K_1 is 2.3/0.05-fold smaller than the observed value which gives a corrected K_1 of 3 ± 1 nM.

The triclosan inhibition of G93V did not exhibit slow binding, allowing the use of usual steady state kinetic methods for determining K_i. Triclosan is an uncompetitive inhibitor with respect to both DDCoA and NADH with K_{ii} values of 0.25 ± 0.09 and 0.15 ± 0.01 μM, respectively (Table 4, data not shown). These data indicate that triclosan binds to the E·NAD^+ form of G93V.

DISCUSSION

Characterization of the Inhibition of Triclosan-Resistant Mutants by Triclosan. In agreement with previous studies, our data show that triclosan is a slow, tight binding reversible

inhibitor of FabI, binding preferentially to the E·NAD^+ form of the enzyme with a K_1 of 23 pM (Scheme 3A) (*7*, *34*). Triclosan resistance is conferred by the point mutations G93V, M159T, and F203L (*2*). All three residues are located close to the inhibitor (Figure 1). Our data indicate that the G93V mutation reduces the affinity of the enzyme for triclosan by 9000-fold with little effect on catalysis (Table 4), consistent with the 95-fold increase in the MIC caused by this mutation (*2*). Significantly, the G93V mutant did not exhibit slow binding inhibition, although triclosan still bound preferentially to the E·NAD^+ complex ($K_1 = 0.2\ \mu$M). The G93V substitution could obstruct the triclosan binding site. Interestingly, the mutation of Gly93 to Ser leads to diazaborine resistance (*35*), and mutation of the homologous residue in InhA (S94A) results in decreased sensitivity of InhA to isoniazid (*27*, *36*). In addition, although the S94A InhA mutation also gives rise to triclosan resistance in *Mycobacterium smegmatis* (*15*), it does not appear to change the affinity of triclosan for InhA (*16*). Thus, this residue is a general determinant of bacterial resistance to FabI inhibitors, since the above data clearly indicate that while mutations at this position have little effect on the kinetic parameters for substrate reduction, inhibitor binding is severely compromised. Clearly then, novel FabI inhibitors must be designed to circumvent mutation at G93 (FabI) or S94 (InhA).

Unlike the G93V mutation, the M159T mutation significantly affects both the catalytic constants and triclosan affinity (Table 3). The value of k_{cat}/K_m for DDCoA is decreased by 80-fold; however, M159T must still be sufficiently active to support cell growth. Triclosan binds preferentially to the E·NAD^+ form, but the affinity is decreased by ca. 170-fold, consistent with the observed 12-fold increase in the MIC (*2*). Met159 makes several hydrophobic interactions with triclosan, in particular with ring B (*6*). These contacts will be lost with the Thr substitution, resulting in weaker binding. The homologous residue in InhA, M161, when mutated to a valine also confers triclosan resistance (*2*). Triclosan binds to both E·NAD^+ and E·NADH forms of the M161V InhA mutant, exhibiting noncompetitive inhibition, and the K_i is 20-fold larger than for wild-type InhA (*16*).

The third FabI mutation, F203L, leads to a 6-fold increase in the MIC (*2*), and examination of the crystal structure reveals that F203 forms the inner surface of the hydrophobic pocket into which ring A of triclosan is nestled. Ring A stacks nearly perpendicular to F203. This edge-on π interaction constitutes an important interaction in proteins (*37*−*39*), and substitution of F203 with an aliphatic leucine residue will likely reduce the affinity of the enzyme for triclosan. Indeed, while k_{cat} and K_m for F203L are comparable to those of the wild type (Table 3), inhibition by triclosan is reduced and triclosan binds to the E·NAD^+ form of the enzyme with a K_1 value of 0.9 nM. In addition, unlike the wild-type enzyme, triclosan also binds to the E·NADH form of this mutant FabI with a K_2 value of 51 nM (Scheme 3B).

Mechanism of Triclosan Binding to Y156F FabI and A197M FabI. InhA, the enoyl reductase from *M. tuberculosis*, and FabI, the ENR from *E. coli*, share a high degree of three-dimensional structural homology and are 36% identical in primary sequence (*40*). Triclosan inhibits both FabI and InhA, but it binds 10000-fold more tightly to FabI than it does to InhA. We are interested in exploring the molecular basis for the differential sensitivity of these two enzymes to triclosan, since this information will be important in designing novel antitubercular compounds that would be specific for the mycobacterial enzyme. FabI is a member of the short chain alcohol dehydrogenase (SCAD) family of enzymes which have a conserved Tyr and Lys residue organized in a characteristic YxxxK motif, and sequence homology modeling studies have suggested that these residues are important for catalysis (*41*). The conserved Tyr residue is Y156 in the *E. coli* FabI, and X-ray crystallographic data indicate that the phenolic hydroxyl group of triclosan is hydrogen bonded to Y156 (Figure 1), which supports the hypothesis that Y156 plays an important role in triclosan binding. To probe this interaction, Y156 was replaced with a Phe. Consistent with the structural data, the Y156F mutation caused the affinity for triclosan to decrease and the mechanism of inhibition to change. In contrast to the wild-type enzyme, triclosan binds to both E·NAD^+ and E·NADH forms of Y156F with K_1 and K_2 values of 3 and 30 nM, respectively. Thus, removal of the hydrogen bond between Y156 and the triclosan phenol hydroxyl not only substantially reduces the affinity of the inhibitor for the enzyme but also abolishes the preference of triclosan for the E·NAD^+ product complex. Similar results were observed when the analogous substitution was introduced in InhA where noncompetitive inhibition by triclosan was observed for Y158F InhA ($K_i' = 47\ \mu$M, $K_i = 36\ \mu$M) in contrast to uncompetitive inhibition for the wild-type enzyme ($K_i' = 0.22\ \mu$M) (*16*). For InhA, the analogous residue (Y158) rotates by 60° about the $C_\alpha - C_\beta$ bond from its position in the E·NADH complex upon addition of a substrate analogue (*40*). This movement of Y158 may be important for triclosan's discrimination between the E·NADH and E·NAD^+ forms of InhA. However, there is no evidence for a similar movement of Y156 in FabI. In addition, on the basis of homology with InhA, Y156 is expected to be an important catalytic residue in the FabI-catalyzed reaction. However, the Y156F mutation only reduced k_{cat} 7-fold compared to that of the wild type (Table 3). Thus, either Y156 does not play a major role in the FabI-catalyzed reaction, or this residue is involved in one or more catalytic steps that are not rate-limiting.

The origin of the slow binding step in the inhibition of FabI by triclosan can be attributed to a conformational change in the protein structure that occurs as a consequence of triclosan binding. Significantly, this feature is absent in the triclosan inhibition of InhA. Examination of crystal structure data for both enzymes reveals the presence of a substrate binding loop that forms a lid over the active site which is longer in the case of InhA than FabI (*6*, *40*). Interestingly, this loop is disordered in the FabI−NAD^+ structure but becomes ordered upon formation of the FabI−NAD^+−triclosan ternary complex (*6*). The loop forms van der Waals interactions with triclosan and hydrogen bonds to the bound NAD^+ cofactor. The opening and closing of the loop may constitute an important event in substrate or ligand binding and could also be responsible for the conformational change that is required to account for the slow binding inhibition of the enzyme by triclosan. Ala197 is conserved among bacterial FabIs, but is replaced with Met in the mycobacterial InhAs, suggesting that A197 might be the difference in triclosan inhibition. The A197M mutation has little effect on k_{cat}, but

increases K_{mNADH} by 20-fold and decreases K_{mDDCoA} by 6-fold. However, the mutation has no effect on triclosan inhibition. Consequently, the difference in binding of triclosan to the two enzymes cannot be attributed to this single change.

One important conclusion that results from these observations is that while triclosan binding requires the presence of NAD^+, mutagenesis can differentially affect the affinity of triclosan for the two enzyme forms that contain the oxidized and reduced cofactor. Thus, while triclosan is only observed to bind to the E·NAD^+ form of the wild-type, G93V, and M159T enzymes, for F203L and Y156F the affinities of triclosan for E·NADH and E·NAD^+ forms are sufficiently similar that binding to both the forms can be observed. For F203L, triclosan has a ca. 50-fold preference for E·NAD^+ over E·NADH, while this difference is reduced to ca. 10-fold for Y156F. Interestingly, similar results were seen for wild-type and mutant InhAs with mutations altering the relative affinity of triclosan for the E·NADH and E·NAD^+ forms of this enzyme, suggesting that this is a general determinant in ligand binding to enoyl reductases. While the structural basis for discrimination between E·NADH and E·NAD^+ forms remains to be elucidated, the X-ray structure of the wild-type FabI−triclosan−cofactor complex indicates that the triclosan A ring forms a stacking interaction with the nicotinamide ring. Modulation of this stacking interaction may be partly responsible for differential binding to E·NADH and E·NAD^+ forms in the wild-type and mutant enzymes, since the strength of the interaction will depend on both the oxidation state of the cofactor and enzyme−triclosan interactions that alter electron density in the inhibitor.

CONCLUSION

Triclosan is a slow, tight binding, reversible picomolar inhibitor of the wild-type *E. coli* FabI, binding preferentially to the E·NAD^+ complex. All three of the mutations identified by Levy and co-workers (G93V, M159T, and F203L) significantly reduce the affinity for triclosan, consistent with the observation that these mutations correlate with an increase in triclosan resistance. In the Y156F mutant, removal of the tyrosine hydroxyl causes a substantial decrease in triclosan affinity and abolishes the preference of triclosan for the E·NAD^+ form of the enzyme. The latter observation is consistent with the X-ray structure of the FabI−triclosan complex in which the inhibitor is shown to make critical interactions with the conserved tyrosine residue. Finally, the A197M FabI mutant has the same affinity for triclosan as the wild-type enzyme. Thus, while the active site loop likely plays a critical role in ligand binding, specific differences in the loop sequence between the *E. coli* FabI and the *M. tuberculosis* InhA cannot account for the 10000-fold difference in triclosan affinity for the two enzymes. Consequently, both the role of the loop and the involvement of other residues in triclosan binding need to be further investigated to improve our understanding of the specificity of triclosan for the two ENRs.

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