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Inhibition of InhA, the Enoyl Reductase from *Mycobacterium tuberculosis*, by Triclosan and Isoniazid[†]

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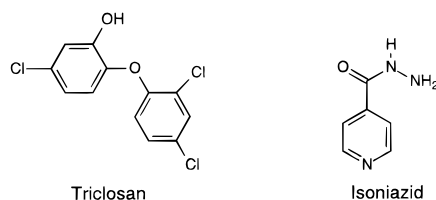
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ABSTRACT: Structural and genetic studies indicate that the antibacterial compound triclosan, an additive in many personal care products, is an inhibitor of EnvM, the enoyl reductase from *Escherichia coli*. Here we show that triclosan specifically inhibits InhA, the enoyl reductase from *Mycobacterium tuberculosis* and a target for the antitubercular drug isoniazid. Binding of triclosan to wild-type InhA is uncompetitive with respect to both NADH and *trans*-2-dodecenoyl-CoA, with K_i' values of 0.22 ± 0.02 and $0.21 \pm 0.01 \mu\text{M}$, respectively. Replacement of Y158, the catalytic tyrosine residue, with Phe, reduces the affinity of triclosan for the enzyme and results in noncompetitive inhibition, with K_i and K_i' values of 36 ± 5 and $47 \pm 5 \mu\text{M}$, respectively. Consequently, the Y158 hydroxyl group is important for triclosan binding, suggesting that triclosan binds in similar ways to both InhA and EnvM. In addition, the M161V and A124V InhA mutants, which result in resistance of *Mycobacterium smegmatis* to triclosan, show significantly reduced affinity for triclosan. Inhibition of M161V is noncompetitive with $K_i' = 4.3 \pm 0.5 \mu\text{M}$ and $K_i = 4.4 \pm 0.9 \mu\text{M}$, while inhibition of A124V is uncompetitive with $K_i' = 0.81 \pm 0.11 \mu\text{M}$. These data support the hypothesis that the mycobacterial enoyl reductases are targets for triclosan. The M161V and A124V enzymes are also much less sensitive to isoniazid compared to the wild-type enzyme, indicating that triclosan can stimulate the emergence of isoniazid-resistant enoyl reductases. In contrast, I47T and I21V, two InhA mutations that occur in isoniazid-resistant clinical isolates of *M. tuberculosis*, show unimpaired inhibition by triclosan, with uncompetitive inhibition constants (K_i') of 0.18 ± 0.01 and $0.12 \pm 0.01 \mu\text{M}$, respectively. The latter result indicates that InhA inhibitors targeted at the enoyl substrate binding site may be effective against existing isoniazid-resistant strains of *M. tuberculosis*.

Drug-resistant strains of *Mycobacterium tuberculosis* are a major factor in the worldwide spread of tuberculosis, a disease that kills 3 million people each year (1–3). Resistance to isoniazid (Scheme 1), a front-line antitubercular drug, results primarily from mutations in the mycobacterial KatG enzyme, a mixed function catalase/peroxidase enzyme responsible for activating isoniazid (4). In addition, isoniazid resistance also correlates with mutations in other mycobac-

Scheme 1



[†] This work was supported by NIH Grant AI44639.

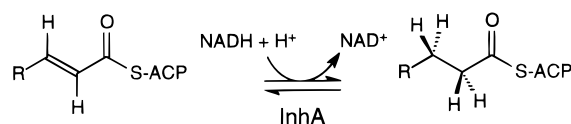
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terial enzymes including InhA,¹ an enoyl reductase (5, 6) and KasA,¹ a β -ketoacyl-ACP synthase (7). Both InhA and KasA are involved in mycolic acid biosynthesis and are putative in vivo targets for activated isoniazid. InhA, which catalyzes the NADH-dependent reduction of long-chain

Scheme 2



trans-2-enoyl-ACP fatty acids (Scheme 2) (5, 8), is inhibited by isoniazid via the formation of a covalent adduct between an activated isoniazid metabolite and the nicotinamide headgroup of NAD(H) (9, 10). InhA mutations identified in isoniazid-resistant strains of *M. tuberculosis* reduce the affinity of NADH for the enzyme, consistent with the hypothesis that NADH has to be bound for adduct formation to occur (6, 10). Consequently, enzyme inhibitors that are targeted at the enoyl substrate binding site and are active against both wild-type and isoniazid-resistant InhA enzymes have potential application as novel antituberculosis drugs.

Recent genetic, kinetic, and crystallographic studies have shown that the antibacterial compound triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) (Scheme 1), an additive in many personal care products such as soaps and toothpaste, inhibits EnvM (FabI), the enoyl reductase from *Escherichia coli* (11–18). Subsequent genetic studies have also revealed that mutations in the enoyl reductase from *Mycobacterium smegmatis* result in resistance to triclosan (19). The *M. tuberculosis* enoyl reductase (InhA) is 36% identical, 65% similar to EnvM and 87% identical, 97% similar to the *M. smegmatis* enoyl reductase (20), suggesting that triclosan might also be an inhibitor of the *M. tuberculosis* enzyme. Additionally, triclosan has identical MICs for *M. tuberculosis* (5 $\mu\text{g/mL}$) and *M. smegmatis* (5 $\mu\text{g/mL}$), and selection for triclosan resistance in *M. tuberculosis* results in a mutation in the InhA promoter, further supporting the hypothesis that triclosan is an InhA inhibitor (Slayden and Barry, personal communication). Consequently, we have studied the inhibition of InhA by triclosan and analyzed the mutations identified by Levy and co-workers.

Our studies show that triclosan is a submicromolar inhibitor of InhA. Importantly, the inhibition is uncompetitive, indicating that the inhibition cannot be alleviated by increasing the concentration of substrate. Analysis of the EnvM–triclosan structure reveals that the triclosan 2-hydroxy group interacts with the hydroxyl of Y156 (14). Since Y156 is a conserved residue, we explored whether the InhA homologue (Y158) might also interact with triclosan. Significantly, the Y158F mutant is much less sensitive to triclosan, suggesting that triclosan binds to EnvM and InhA in similar ways.

The genetic studies identified three enoyl reductase mutations, M161V, M103T, and A124V, that correlated with resistance of *M. smegmatis* to triclosan (19). Both the M161V and A124V InhA mutations result in reduced sensitivity to triclosan. In addition, the M161V and A124V enzymes are much less sensitive to isoniazid in an in vitro inactivation reaction compared to wild-type enzyme. This suggests that mutations resulting from exposure to triclosan may com-

promise the in vivo effectiveness of isoniazid as an antitubercular drug.

To further explore the relationship between isoniazid and triclosan resistance, we also studied the binding of triclosan to two InhA mutants (I47T and I21V) identified in isoniazid-resistant clinical isolates (6). Both of these InhA mutants were inhibited uncompetitively by triclosan with K_i' values similar to wild-type InhA. The latter studies indicate that inhibitors targeted at the substrate binding site may be useful for treating existing isoniazid-resistant tuberculosis.

EXPERIMENTAL PROCEDURES

Substrates and Enzymes. *trans*-2-Dodecenoyl-CoA (DDCoA)¹ and *trans*-2-hexadecenoyl-CoA (HXDCoA)¹ were synthesized from *trans*-2-dodecenoic acid and *trans*-2-hexadecenoic acid, respectively, using the mixed anhydride method as described previously (8, 21). Wild-type and mutant InhA proteins were constructed, expressed, and purified as described previously (21). Protein concentrations were determined using $\epsilon_{280} = 37.3 \text{ mM}^{-1} \text{ cm}^{-1}$ for the wild-type, M161V, A124V, M103T, S94A, I47T, and I21V proteins and $\epsilon_{280} = 35.7 \text{ mM}^{-1} \text{ cm}^{-1}$ for the Y158F mutant. Triclosan (Irgasan DP 300) was a gift of Ciba Specialty Chemicals Corp.

Preparation of Triclosan Solutions. Initial experiments were performed by diluting stock solutions of triclosan in either 100% CH_3CN or DMSO directly into aqueous buffer. However, subsequent analysis revealed a much lower triclosan absorbance at 282 nm than anticipated, suggesting that triclosan was not soluble under these conditions. Consequently, triclosan stock solutions (200–400 mM) were prepared at high pH (1 M NaOH or KOH), followed by dilution into lower pH buffers (22). After the solutions were filtered through a 0.45 μm filter, the triclosan concentration was calculated using $\epsilon_{282} = 4.0 \text{ mM}^{-1} \text{ cm}^{-1}$.

Steady-State Kinetics and Determination of Inhibition Constants. Kinetic parameters were determined at 25 °C in 30 mM Pipes and 150 mM NaCl at pH 6.8 as described (21). Assays were performed with *trans*-2-dodecenoyl-CoA (DDCoA) or *trans*-2-hexadecenoyl-CoA (HXDCoA). Inhibition constants (K_i and K_i') were calculated by determining k_{cat} and K_m at several fixed concentrations of triclosan. For wild-type InhA (35 nM), K_i' was calculated by determining the k_{cat} and K_m DDCoA at a fixed, saturating concentration of NADH (600 μM) and by varying the concentration of DDCoA (0–200 μM) and triclosan (0, 0.1, 0.4, and 1.4 μM). Alternatively, K_i' was calculated by determining k_{cat} and K_m NADH at a fixed, saturating concentration of DDCoA (175 μM) and by varying the concentration of NADH (0–600 μM) and triclosan (0, 0.05, 0.2, 0.5, and 1 μM).

For the A124V, S94A, I47T, and I21V variants, inhibition constants were calculated by determining k_{cat} and K_m NADH at a fixed, saturating concentration of DDCoA and by varying the concentration of NADH and triclosan. Enzyme, substrate, and inhibitor concentrations were as follows: A124V 26 nM, DDCoA 175 μM , NADH 0–800 μM , and triclosan 0, 0.3, 0.6, and 1.2 μM ; S94A 25 nM, DDCoA 175 μM , NADH 0–800 μM , and triclosan 0, 0.2, 0.35, and 1 μM ; I47T 44 nM, DDCoA 175 μM , NADH 0–800 μM , and triclosan 0, 0.15, 0.3, and 0.6 μM ; and I21V 49 nM, DDCoA 175 μM , NADH 0–800 μM , and triclosan 0, 0.15, 0.3, and 0.6 μM .

¹ Abbreviations: DDCoA, *trans*-2-dodecenoyl-CoA; HXDCoA, *trans*-2-hexadecenoyl-CoA; InhA, the enoyl reductase from *Mycobacterium tuberculosis*; EnvM, the enoyl reductase from *Escherichia coli*; KasA, the β -ketoacyl-ACP synthase from *M. tuberculosis*.

Each initial velocity was determined in triplicate, and at least five different substrate concentrations were examined. Initial velocity data obtained at various substrate concentrations in the presence of varying amounts of inhibitor were initially analyzed by plotting v versus $v/[S]$. Inspection of these plots enabled us to discriminate between noncompetitive and uncompetitive inhibition. The experimental data for wild-type, A124V, S94A, I47T, and I21V enzymes were analyzed using eq 1 for uncompetitive inhibition, where S is the concentration of varied substrate, I is the inhibitor concentration, V is the maximum velocity, K_m is the Michaelis–Menten constant, and K_i' is the inhibition constant for I binding to the ES complex.

$$v_i = \frac{VS/(1 + I/K_i')}{S + K_m/(1 + I/K_i')} \quad (1)$$

Inhibition constants for the Y158F mutant (345 nM) were calculated by determining k_{cat} and K_{mDDCoA} at a fixed, saturating concentration of NADH (180 μ M) and by varying the concentration of DDCoA (0–200 μ M) and triclosan (0, 12, 30, and 72 μ M). For the M161V mutant, K_i and K_i' were calculated by determining k_{cat} and K_{mNADH} at a fixed saturating concentration of HXDCoA (35 μ M) and by varying the concentration of NADH (0–1 mM) and triclosan (0, 3, 6, and 9 μ M). The experimental data from both the M161V and Y158F mutants were analyzed using eq 2 for noncompetitive inhibition, where K_i is the inhibition constant for the binding of the inhibitor to an enzyme form that precedes binding of the varied substrate.

$$v_i = \frac{V(S/K_m)}{1 + I/K_i + S/K_m + SI/(K_i' + K_m)} \quad (2)$$

Inactivation of Wild-Type and Mutant Enzymes by Isoniazid. Time-dependent inactivation of wild-type and mutant proteins by isoniazid was performed at 25 °C in 100 mM Na_2HPO_4 , pH 7.5 (23). Wild-type or mutant proteins (3 μ M) were incubated with NADH (10 μ M or 1.5 mM), isoniazid (200 μ M), and MnCl_2 (2 μ M) in a total volume of 1 mL. Aliquots were taken periodically, and specific activities were determined from the rate of decrease in absorbance at 340 nm using *trans*-2-dodecenoyl-CoA (80 μ M) and NADH (200 μ M) in 30 mM Pipes and 150 mM NaCl at pH 6.8. Observed activities were plotted as a function of time, and first-order rate constants for inactivation were obtained by fitting the data to a single exponential decay using Grafit 3.09b (Erithacus Software Ltd.).

RESULTS

Kinetic Analysis of Wild-Type and Mutant Proteins. Previously determined kinetic parameters for wild-type InhA and the Y158F mutant are given in Table 1. Replacement of Y158 with Phe results in a 24-fold reduction in k_{cat} , from $501 \pm 23 \text{ min}^{-1}$ to $21 \pm 7 \text{ min}^{-1}$, and a 30-fold reduction in the K_m for NADH (K_{mNADH}), from $66 \pm 7 \mu\text{M}$ to $2.0 \pm 0.1 \mu\text{M}$ (21). In contrast, the A124V, S94A, I47T, and I21V proteins all had k_{cat} and K_{mDDCoA} values similar to those of wild-type InhA (Table 1). However, the latter mutants exhibited a 2–5-fold increase in K_{mNADH} , consistent with previous studies (6). The M161V mutant had a K_{mDDCoA}

Table 1: Kinetic Parameters for Wild-Type and Mutant InhA Enzymes^a

enzyme	k_{cat} (min^{-1})	K_m (μM)		k_{cat}/K_m ($\mu\text{M}^{-1} \text{min}^{-1}$) (DDCoA)
		DDCoA	NADH	
wild type	501 ± 23	46 ± 5	66 ± 7	10.9 ± 1.7
Y158F	21 ± 7	70 ± 6	2.0 ± 0.1	0.3 ± 0.1
S94A	993 ± 63	104 ± 16	250 ± 30	8.9 ± 1.9
I47T	720 ± 36	36 ± 6	152 ± 22	20.0 ± 4.3
I21V	925 ± 31	70 ± 9	120 ± 11	13.2 ± 2.1
M161V ^b	700 ± 76	10 ± 2	332 ± 44	70 ± 21
A124V	861 ± 18	72 ± 10	142 ± 8	11.9 ± 1.9

^a All kinetic parameters were determined in 30 mM Pipes and 150 mM NaCl at pH 6.8 (25 °C); see details in Experimental Procedures.

^b *trans*-2-Hexadecenoyl-CoA was used.

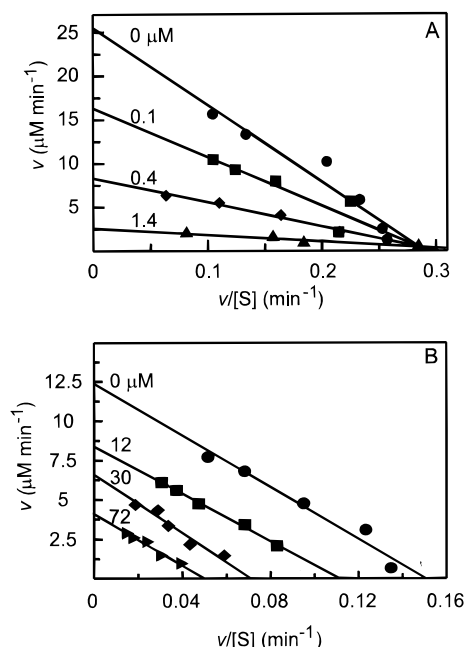


FIGURE 1: Eadie–Hofstee plot (v versus $v/[S]$) for the inhibition of wild-type InhA (A) and Y158F InhA (B) by triclosan. For wild-type InhA, triclosan inhibition was studied while both [NADH] and [DDCoA] were varied. Plot A only contains the data obtained while the [DDCoA] was varied. For Y158F, triclosan inhibition was studied while the [DDCoA] was varied only.

> 200 μM , and consequently, kinetic studies were performed using HXDCoA, giving $k_{cat} = 700 \pm 76 \text{ min}^{-1}$, $K_{mNADH} = 332 \pm 44 \mu\text{M}$, and $K_{mHXDCoA} = 10 \pm 2 \mu\text{M}$ (Table 1). The $K_{mHXDCoA}$ observed for M161V is 7-fold higher than the previously reported wild-type value (8). Finally, replacement of M103 with Thr resulted in an enzyme that was unable to bind NADH. In addition, no activity could be observed even at NADH concentrations up to 10 mM.

Inhibition of Wild-Type and Mutant InhAs by Triclosan. For wild-type InhA, plots of v versus $v/[S]$ for each triclosan concentration intersected on the x -axis, indicating uncompetitive inhibition with respect to both NADH and DDCoA (Figure 1). Subsequently, eq 1 was used to calculate K_i' values of 0.22 ± 0.02 and $0.21 \pm 0.01 \mu\text{M}$ when NADH and DDCoA were varied, respectively (Table 2).

Due to the much lower K_{mNADH} for the Y158F mutant compared to wild-type InhA, it was not possible to study the inhibition of Y158F by varying the concentration of NADH. Consequently, triclosan inhibition was only studied for this mutant by varying the concentration of DDCoA. Plots

Table 2: Inhibition Constants for Triclosan Binding to Wild-Type and Mutant Enzymes and Relative Rates of Inactivation of Wild-Type and Mutants Enzymes by Activated Isoniazid^a

enzyme	K_i' (triclosan) (μM)	K_i (triclosan) (μM)	inhibition type with triclosan	inactivation rate (10^{-3} min^{-1}) with isoniazid (% WT)	
				10 μM NADH	1.5 mM NADH
wild type	0.22 ± 0.02 0.21 ± 0.01^b		U	7.9 ± 0.7 (100)	8.2 ± 0.7 (100)
Y158F ^b	47 ± 5	36 ± 5	N	1.2 ± 0.1 (15)	1.1 ± 0.1 (15)
S94A	0.14 ± 0.01		U	5.3 ± 0.5 (66)	7.9 ± 0.5 (96)
I47T	0.18 ± 0.01		U	6.0 ± 0.5 (75)	7.6 ± 0.6 (93)
I21V	0.12 ± 0.01		U	6.5 ± 0.4 (79)	7.9 ± 0.6 (96)
M161V ^c	4.3 ± 0.5	4.4 ± 0.9	N	0.14 ± 0.02 (2)	0.10 ± 0.03 (1)
A124V	0.81 ± 0.11		U	0.9 ± 0.2 (11)	1.1 ± 0.2 (14)

^a All kinetic parameters were determined in 30 mM Pipes and 150 mM NaCl at pH 6.8 (25 °C); see details in Experimental Procedures. Inhibition constants were determined with NADH as the varied substrate unless otherwise stated. ^b *trans*-2-Dodecenoyl-CoA was used as the varied substrate.

^c *trans*-2-Hexadecenoyl-CoA was used. Time-dependent inactivation of wild-type and mutant proteins was carried out in 100 mM Na₂HPO₄ (pH 7.5) at 25 °C; see details in Experimental Procedures.

of v versus $v/[S]$ gave parallel lines (Figure 1), indicating noncompetitive inhibition of the Y158F mutant by triclosan. Equation 2 was used to calculate values of K_i and K_i' of 36 ± 5 and $47 \pm 5 \mu\text{M}$, respectively (Table 2).

Inhibition constants for M161V, A124V, and S94A mutants are reported in Table 2. For the M161V mutant, k_{cat} and $K_{\text{m NADH}}$ were determined at several concentrations of triclosan by varying the concentration of NADH at saturating concentration of *trans*-2-hexadecenoyl-CoA. A plot of v versus $v/[S]$ indicated noncompetitive inhibition of the M161V mutant by triclosan, and use of eq 2 yielded K_i and K_i' values of 4.4 ± 0.9 and $4.3 \pm 0.5 \mu\text{M}$, respectively. k_{cat} and $K_{\text{m NADH}}$ were determined at several concentrations of triclosan for the A124V and S94A mutants by varying the concentration of NADH at saturating concentration of DDCoA (175 μM). Plots of v versus $v/[S]$ indicated uncompetitive inhibition; consequently, data were analyzed using eq 1, giving K_i' values of 0.81 ± 0.11 and $0.14 \pm 0.01 \mu\text{M}$ for the A124V and S94A mutants, respectively.

Inhibition constants for triclosan with the S94A, I47T, and I21V mutants are reported in Table 2. Plots of v versus $v/[S]$ indicated uncompetitive inhibition for each enzyme, and eq 1 was used to calculate K_i' values of 0.14 ± 0.01 , 0.18 ± 0.01 , and $0.12 \pm 0.01 \mu\text{M}$ for the S94A, I47T, and I21V mutants, respectively.

Inactivation of Wild-Type and Mutant InhAs by Isoniazid. The relative rates of inactivation of wild-type and mutant enzymes by isoniazid are reported in Table 2. The inactivation of wild-type protein at 10 μM and 1.5 mM NADH (Figure 2) resulted in apparent first-order rate constants of $(7.9 \pm 0.7) \times 10^{-3} \text{ min}^{-1}$ and $(8.2 \pm 0.7) \times 10^{-3} \text{ min}^{-1}$, respectively.

Inactivation of the S94A, I47T, and I21V mutant enzymes at 10 μM NADH (Figure 2) resulted in apparent first-order rate constant values of $(5.3 \pm 0.5) \times 10^{-3} \text{ min}^{-1}$, $(6.0 \pm 0.5) \times 10^{-3} \text{ min}^{-1}$, and $(6.5 \pm 0.4) \times 10^{-3} \text{ min}^{-1}$, respectively. Thus the S94A, I47T, and I21V mutants inactivate 1.5–1.2-fold more slowly than wild-type InhA using 10 μM NADH, within the range of values previously reported (6, 9). Interestingly, when the NADH concentration was increased to 1.5 mM, the S94A, I47T, and I21V mutant enzymes inactivated at rates identical, within experimental error, to those of wild-type InhA (Table 2).

The A124V and M161V enzymes exhibited a 9- and 56-fold decrease in the rate of isoniazid inactivation at 10 μM

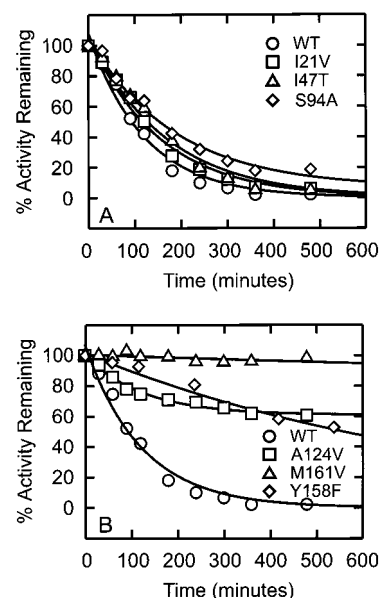


FIGURE 2: Inactivation of wild-type and mutant InhAs by isoniazid. In each case the specific activity is plotted as a function of time for incubations of enzymes with isoniazid, NADH, and MnCl₂. The data have been fit to a single exponential decay, giving first-order inactivation rate constants. Inhibition experiments were determined at both 10 μM and 1.5 mM NADH; however, only data obtained at 10 μM NADH are shown. Panels: (A) wild-type InhA and the S94A, I47T, and I21V InhA mutants; (B) wild-type InhA and the Y158F, M161V, and A124V InhA mutants.

NADH, respectively (Figure 2 and Table 2). In contrast to the S94A, I47T, and I21V enzymes, when the NADH concentration was increased to 1.5 mM, the rate of inactivation for the A124V and M161V mutants did not change (Figure 2 and Table 2). Finally, the rate of inactivation of the Y158F mutant (Figure 2 and Table 2) decreased 7-fold when compared to the wild-type protein in the presence of either 10 μM NADH [$(1.2 \pm 0.1) \times 10^{-3} \text{ min}^{-1}$] or 1.5 mM NADH [$(1.1 \pm 0.1) \times 10^{-3} \text{ min}^{-1}$].

DISCUSSION

Binding of Triclosan to Wild-Type InhA. The inhibition of wild-type InhA by triclosan is uncompetitive with respect to both NADH and 2-dodecenoyl-CoA, with K_i' values of 0.22 ± 0.02 and $0.21 \pm 0.01 \mu\text{M}$, respectively. Previous kinetic studies have revealed that substrates bind randomly to the enzyme (21). Thus, since uncompetitive inhibition

indicates that the inhibitor is binding after the varied substrate, triclosan must be binding to the substrate ternary complex (EAB), the product ternary complex (EPQ), or one of the product binary complexes (EP or EQ) (24).

Kinetic studies have demonstrated that triclosan binds tightly to EnvM in the presence of NAD⁺ (13, 15). Crystallographic studies indicate that the phenolic triclosan ring forms a stacking interaction with the NAD⁺ nicotinamide ring and that the phenolic hydroxyl group is hydrogen bonded to Y156 and the NAD⁺ ribose hydroxyl (13–15, 18). To further investigate the mode of triclosan binding to InhA, we studied the inhibition of the Y158F InhA mutant in which the catalytic tyrosine, homologous to Y156 in EnvM, is replaced by Phe. Previous kinetic studies have shown that k_{cat} for the Y158F enzyme is reduced 24-fold compared to the wild-type InhA (21). The inhibition studies revealed that removal of the tyrosine 158 hydroxyl group also significantly reduces the affinity of triclosan for the enzyme. Due to the low $K_{\text{m NADH}}$ for Y158F, inhibition kinetics were only determined by varying the enoyl substrate at a fixed saturating concentration of NADH. The inhibition of Y158F InhA by triclosan is noncompetitive, with K_i and K_i' values of 36 ± 5 and $47 \pm 5 \mu\text{M}$, respectively. Thus triclosan is binding to the Y158F mutant both before and after the varied substrate (DDCoA).

Combining the data on InhA with the structural and crystallographic studies on EnvM, we propose that triclosan can bind to both the E–NADH (EA) and E–NAD⁺ (EP) forms of the InhA enzyme. In the E–NAD⁺ complex, triclosan is hydrogen bonded to Y158, which has rotated from the position it occupies in the E–NADH complex (21, 25). Due to the interaction with Y158, triclosan binds more tightly to the E–NAD⁺ binary product complex than to the E–NADH binary substrate complex ($K_i' \ll K_i$) and so, for the wild-type enzyme, inhibition is uncompetitive. However, in the Y158F enzyme, in which the specific hydrogen bond between Y158 and triclosan has been removed, there is now little preference for the E–NAD⁺ complex over the E–NADH complex ($K_i' \approx K_i$), so noncompetitive inhibition is observed.

Characterization of the Triclosan-Resistant Mutants. Using genetic selection methods, Levy and co-workers identified three mutations in the *M. smegmatis* enoyl reductase, M161V, M103T, and A124V, that correlated with increased resistance to triclosan (19). In addition, Levy also demonstrated that the S94A mutation provided resistance to triclosan. We have studied the effect of these four mutations on the inhibition of InhA by triclosan.

The M161V and A124V mutants had k_{cat} values ca. 1.4–1.7-fold higher than for the wild-type enzyme (Table 1). In addition, $K_{\text{m NADH}}$ has increased for both mutants while $K_{\text{m HXDCOA}}$ is higher for M161V compared to wild type. Significantly, both mutants showed decreased affinity for triclosan. For A124V inhibition was uncompetitive with $K_i' = 0.81 \pm 0.11 \mu\text{M}$, while for M161V the inhibition was noncompetitive with $K_i' = 4.3 \pm 0.5 \mu\text{M}$ and $K_i = 4.4 \pm 0.9 \mu\text{M}$. The reduced affinities of M161V and A124V for triclosan are consistent with the genetic studies and support the contention that the antibacterial activity of triclosan results from the inhibition of the enoyl reductase enzymes.

In one of the triclosan-resistant EnvM mutants, M159, the InhA M161 homologue is mutated to a Thr (26). Analysis of the EnvM–triclosan structure reveals that replacement of

M159 with Thr will remove hydrophobic contacts between the bound triclosan and the methionine side chain (14). If triclosan binds similarly to InhA and EnvM, then mutation of the InhA M161 residue to a Thr could affect triclosan binding by a similar mechanism. In contrast to M161, the InhA A124 residue is on a helix behind the NADH binding pocket, ca. 13 Å from the substrate carbonyl in the ternary InhA–NAD⁺–substrate complex (25). Consequently, it is not clear how replacement of A124 with Val affects triclosan binding.

We also studied the InhA M103T mutant; however, this enzyme was completely inactive. Although CD spectroscopy provided no evidence for major changes in secondary structure compared to the wild-type enzyme (data not shown), no activity could be detected for M103T even at concentrations of NADH above 10 mM. Clearly, the experiments to select for triclosan resistance will not select an inactive InhA enzyme. However, it is possible that the M103T *M. smegmatis* enzyme is active in vivo. In the InhA structure (27), M103 is part of a one-turn helix that also contains T101, one of the residues that differs between the *M. tuberculosis* and *M. smegmatis* enzymes. In the *M. smegmatis* enoyl reductase, T101 is a serine, and it is possible that the subtle change of S to T alters the consequence of mutating M103 such that the M103T InhA enzyme is inactive. In addition, we also studied the S94A InhA mutant. This mutant bound triclosan similarly to the wild-type enzyme. Thus, the observations on the M103T and S94A InhA enzymes cannot account for the triclosan resistance observed in *M. smegmatis*. However, subtle differences between the *M. smegmatis* and *M. tuberculosis* enoyl reductases could explain this.

Isoniazid Inhibition of the Triclosan-Resistant Enzymes. Levy et al. also reported that *M. smegmatis* carrying the A124V or M161V enoyl reductase mutations had increased resistance to isoniazid (19). We compared the rates of inactivation of these mutants with isoniazid using Mn²⁺ to activate the isoniazid. Initial studies were performed using 10 μM NADH, similar to the in vivo concentration of the cofactor (6). Significantly, the A124V and M161V mutants inactivated 9- and 56-fold more slowly, respectively, than the rate of inactivation of wild-type InhA. These decreases are similar to or larger than for any of the InhA mutants observed clinically (6). Consequently, the mutations leading to triclosan resistance also significantly reduce the sensitivity of InhA to isoniazid. These observations reinforce the fact that triclosan resistance can result in resistance to isoniazid, a frontline antitubercular drug.

It has been postulated that the mechanism of isoniazid resistance in clinical strains carrying InhA mutations results from a decreased affinity of NADH for the enzyme (6). This is based on the hypothesis that the NADH cofactor has to be bound to InhA for the acylpyridine–NAD adduct to form (6, 10). To test this hypothesis, we generated and characterized the I47T and I21V InhA mutants previously studied by Blanchard and co-workers (6). At 10 μM NADH, the rate of isoniazid inactivation of the I47T and I21V enzymes was 1.3- and 1.2-fold slower, respectively, than the rate of inactivation of wild-type InhA, as previously observed (6). In addition, consistent with previous observations, the S94A InhA mutant inactivated 1.5-fold more slowly than the wild-type InhA (9). However, with a saturating concentration of NADH (1.5 mM), the I47T, I21V, and S94A InhA mutants

inactivated at rates indistinguishable from the rate of inactivation of wild-type InhA (Table 2). Thus, resistance to isoniazid in these enzymes can be accounted for by the decreased occupancy of NADH that occurs under physiological conditions, as proposed previously (6). Consequently, since the two triclosan-resistant mutants, M161V and A124V, have increased K_m 's for NADH, we studied their inhibition using 1.5 mM NADH. However, increasing the NADH concentration had no effect on the rate of inactivation of the M161V and A124V mutants by isoniazid (Table 2). Therefore, isoniazid resistance in the M161V and A124V enzymes cannot be accounted for simply by the reduced binding of NADH, and these mutations must also interfere with the binding of the isoniazid metabolite and/or the formation of isoniazid–NAD adduct directly. Interestingly, mutation of Y158 also results in a decrease in sensitivity to isoniazid. The Y158F mutant inactivates 6.7-fold more slowly than the wild-type InhA, and in addition, this rate of inactivation is unaffected by increasing the concentration of NADH. Indeed, the Y158F binds NADH 2.5-fold more tightly than wild type (21), so clearly isoniazid resistance in this mutant cannot result from impaired binding of NADH.

Inhibition of Isoniazid-Resistant Mutants by Triclosan. Since the clinically observed InhA mutants with increased resistance to isoniazid result from amino acid substitutions in or near the NADH binding site, there is a general expectation that inhibitors targeted at the enoyl binding site will be effective against these enzymes. On the basis of the comparison with the EnvM structural data, triclosan is hypothesized to bind in the InhA enoyl substrate binding site, and thus we studied the inhibition of the I47T and I21V InhA mutants by triclosan. For both enzymes the inhibition was uncompetitive, with K_i' values similar to those of wild type. Consequently, these data support the general expectation that inhibitors targeted at the enoyl binding site will be effective against clinically observed InhA mutants.

SUMMARY

Triclosan is an uncompetitive inhibitor of wild-type InhA and is proposed to bind preferentially to the E–NAD⁺ binary complex, in which the triclosan phenolic hydroxyl interacts with the hydroxyl group of Y158. Two of the mutations identified in *M. smegmatis* by Levy and co-workers, M161V and A124V, show reduced affinity for triclosan, consistent with the observation that these mutations correlate with an increase in resistance to triclosan. In addition, the M161V and A124V enzymes show increased resistance to isoniazid, raising the possibility that triclosan could stimulate the emergence of isoniazid-resistant InhA. However, triclosan is an effective inhibitor of two InhA mutants previously identified in isoniazid-resistant clinical isolates. Consequently, the design of inhibitors based on triclosan and targeted at the enoyl substrate binding site may prove to be an effective strategy for developing novel antitubercular therapeutics.

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