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FOR THE RECORD

Molecular basis for triclosan activity involves a flipping loop in the active site

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Abstract: The crystal structure of the *Escherichia coli* enoyl reductase-NAD^+-triclosan complex has been determined at 2.5 Å resolution. The Ile192–Ser198 loop is either disordered or in an open conformation in the previously reported structures of the enzyme. This loop adopts a closed conformation in our structure, forming van der Waals interactions with the inhibitor and hydrogen bonds with the bound NAD^+ cofactor. The opening and closing of this flipping loop is likely an important factor in substrate or ligand recognition. The closed conformation of the loop appears to be a critical feature for the enhanced binding potency of triclosan, and a key component in future structure-based inhibitor design.

Keywords: antibiotics; crystal structure; enoyl reductase; fatty acid biosynthesis; NAD^+; triclosan

Triclosan, 5-chloro-2-(2,4-dichlorophenoxy)phenol, is a broad-spectrum antibacterial and antifungal agent that it is widely used in consumer products such as toothpastes, soaps, cosmetics, plastics, and textiles (Bhargava & Leonard, 1996). It inhibits specifically the *fab*I gene product of *Escherichia coli* (McMurry et al., 1998; Heath et al., 1999) and the inhA gene product of *Mycobacterium smegmatis* (McMurry et al., 1999), the enoyl reductases (ENR). ENR, catalyzing the reduction of enoyl-ACP (enoyl-acyl carrier protein), are essential enzymes for the biosynthesis of fatty acids (Heath & Rock, 1995). Besides being targeted by triclosan, *E. coli* enoyl reductase is also known to be inhibited by the antibacterial diazaborines (Baldock et al., 1996). The enoyl reductase of *Mycobacterium* is a target of the antitubercular drug isoniazid (Dessen et al., 1995; Rozwarski et al., 1998), although a β-ketoacyl ACP synthase has been proposed to be the primary target of the drug in *Mycobacterium tuberculosis* (Mdluli et al., 1998). These data suggest that bacterial ENR are important antimicrobial tar-gets, and the structural basis of triclosan inhibition may provide valuable insights into the development of novel antibiotics.

The crystal structure of the *E. coli* enoyl reductase-NAD^+-triclosan complex has been reported very recently (Heath et al., 1999; Levy et al., 1999). Unlike diazaborines and isoniazid that bind to the cofactor NAD^+ covalently, triclosan interacts with both the enzyme and the cofactor in a noncovalent fashion. The potent noncovalent interactions (IC_{50} of 120 nm as reported by Levy et al., 1999) have been mainly attributed to the face-to-face stacking of its phenol ring to the nicotinamide ring, and the hydrogen bonds between the phenolic hydroxyl and the hydroxyls of Tyr156 and 2′ nicotinamide ribose. We have also determined the crystal structure of the same complex independently and observed additional interactions with a flipping active site loop (Ile192–Ser198) that is crucial for defining the molecular basis for triclosan's potent inhibitory activity.

The *E. coli* enoyl reductase was overexpressed in *E. coli* and purified using published protocols (Rafferty et al., 1998). The ENR-NAD^+-triclosan complex was crystallized using the vapor diffusion sitting drop method by mixing 1:1 protein (10 mg mL^{-1}) and well (0.1 M HEPES pH 7.5, 2 M $(NH_4)_2SO_4$, 5% PEG400) solutions. The crystal was flash-frozen in 80% of the well solution plus 20% ethylene glycol. It belongs to the hexagonal space group $P6_122$ with $a = b = 79.0$ Å and $c = 328.8$ Å. The benzodiazaborine complex structure (Baldock et al., 1996) minus the inhibitor and the residues 193–205 was used as the starting structure. Rigid body refinement, iterations of model building, and refinement using X-PLOR (Brünger, 1987) led to the final structure. Statistics for the diffraction data set and final structural refinement are listed in Table 1. There is a dimer of the ternary complex in the asymmetric unit of the crystal, but the RMS difference between the two monomers is only 0.2 Å in α-carbon positions, and the cofactor and inhibitor binding modes are nearly identical in the two monomers. The atomic coordinates of the structure have been deposited at the Protein Data Bank (PDB) (accession 1C14).

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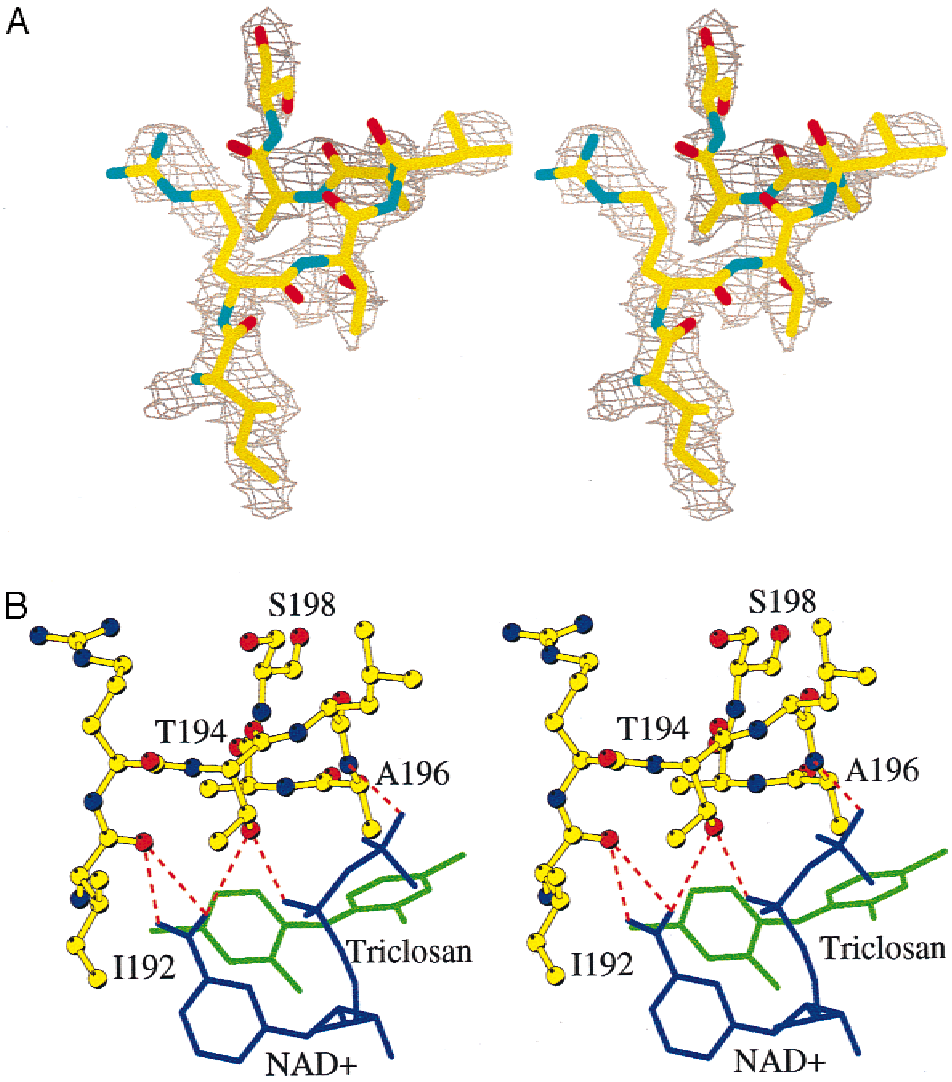

Fig. 1. The flipping loop. **A:** Final ($2F_o - F_c$) electron density for the Ile192–Ser198 loop. The electron density map is contoured at 1σ. Carbon, nitrogen, and oxygen atoms are drawn in yellow, blue, and red, respectively. **B:** Interactions of the flipping loop with triclosan and NAD^+. The flipping loop is shown in ball-and-stick models, with some of the amino acids labeled. The loop atom colors are as defined above. The triclosan inhibitor is shown in green bonds. The adenine part of the cofactor is omitted for clarity, while the rest of the cofactor is shown in blue bonds. Hydrogen bonding interactions are shown in dashed red lines.

Triclosan and the NAD^+ cofactor molecules are clearly defined in the electron density maps. The density for the protein, including that of the flexible Ile192–Ser198 loop (Fig. 1A), is also of good quality. Triclosan is a simple molecule made up of two aromatic rings. The phenol ring of the inhibitor is nestled into a hydrophobic pocket and may mimic the binding mode of the enolate anion intermediate (Levy et al., 1999). It stacks almost parallel to the nicotinamide ring of NAD^+, nearly perpendicular to Tyr146 and Phe203, and at an angle of about 60° with Tyr156. The phenol ring also makes hydrophobic contacts with residues Ala196–Ala197 and Ile200, while its 5-chloro group projects into a hydrophobic pocket composed of Pro191 and Met206. Moreover, the phenolic hydroxyl of the inhibitor makes a hydrogen bond (2.4 Å) with the Tyr156 hydroxyl and is 2.9 Å away from the 2′ hydroxyl of the nicotinamide ribose and 4.4 Å away from Lys163 NZ. The Tyr156 hydroxyl is the putative catalytic base for donating a proton (Rafferty et al., 1995), the ribose hydroxyl is the one forming the covalent attachment to the boron atom in diazaborines (Baldock et al., 1996), and the Lys163 NZ is believed to stabilize a negative charge in the transition-state (Rafferty et al., 1995). Given the likely high reactivity of these functional groups, there is a possibility of rather potent binding at this position. The phenoxy ring of triclosan is mostly involved in van der Waals contacts with the bound NAD^+, the main chains of Gly93–Ala95, the hydrophobic side chains of Leu100, Tyr156, Met159, Ala196 and Ile200, and a couple of water molecules. The ether oxygen linker, being 3.2 Å away from the 2′ hydroxyl of the nicotinamide ribose, contributes to the binding contacts as well.

In *E. coli* enoyl reductase, Ile192–Ser198 is a flexible loop above the active site. This loop is disordered in the ENR-NAD^+ binary complex (Baldock et al., 1998) and adopts an open conformation (average B of 70 $Å^2$) in the diazaborine ternary complexes

Table 1. *Data acquisition and refinement statistics*

Maximum resolution	2.5 Å
No. of observations	112,233
No. of unique reflections	21,505
Redundancy	5.2
Crystal mosaicity	0.3°
Completeness (last shell)	96.7% (93%)
R_{merge} (last shell)	0.063 (0.265)
Refinement resolution	7.0–2.5 Å
No. of reflections in refinement [$I > 2\sigma(I)$]	17,765
No. of nonhydrogen atoms	4,164
No. of solvent atoms	238
R-factor	0.199
R_{free}	0.263
RMS deviations	
Bonds	0.010 Å
Angles	2.0°
Dihedrals	23.2°
Impropers	1.6°
Ramachandran plot	
Favored	89%
Disallowed	0
Average *B* factors	
Protein	20 Å^2
Ile192–Ser198	34 Å^2
NAD$^+$ and triclosan	20 Å^2

(Baldock et al., 1996). In the open conformation, the loop is on the surface of the protein, does not form any secondary structure, and does not contribute to the inhibitor or cofactor binding. In the published triclosan complex structures, this loop was either not mentioned (Levy et al., 1999) or not observed (Heath et al., 1999). However, we have clearly observed this loop in a closed conformation (Fig. 1A). The Ile195–Ser198 part of the loop actually forms a one-turn helix that helps to define the active site cavity (Fig. 1B). We call this loop the flipping loop because it opens or closes in the presence or absence of triclosan, and possibly the enoyl substrate. Aside from numerous van der Waals interactions between Ala196–197 and triclosan, the loop also interacts with the bound NAD$^+$ cofactor (Fig. 1B). The dipole of the one-turn helix is positioned to bind the pyrophosphate (Ala196 N to O1PA, 3.2 Å), a feature that is often seen in nucleotide binding proteins (Hol et al., 1978). Thr194 makes hydrogen bonds with the pyrophosphate (OG1 to O1PN, 2.6 Å) and nicotinamide (OG1 to N7N, 3.1 Å). The backbone oxygen of Ile192 also hydrogen bonds with the nicotinamide (3.1 Å to N7N and O7N). The clever design of these specific interactions, as well as the amino acid sequence conservation in this loop region (Rafferty et al., 1995), propose an important role for the flipping loop in ligand binding.

Triclosan (Heath et al., 1999; Levy et al., 1999) and benzodiazaborine (Baldock et al., 1996) make similar interactions with amino acid residues lining the active site cavity (Fig. 2). The phenol ring of triclosan matches the portion of the diazaborine bicyclic ring that makes face-to-face stacking interaction with the nicotinamide ring. The phenol hydroxyl of triclosan and the boron oxygen in diazaborines make a similar hydrogen bond with Tyr156 and 2′ nicotinamide ribose. The phenoxy ring of triclosan and the tosyl group of benzodiazaborine, though oriented differently, interact with a similar set of protein atoms as well. Based only on these interactions, it is not clear why triclosan is such a potent inhibitor while the boron-less analogue of diazaborine, unable to bind covalently, is inactive (Grassberger et al., 1984). With the flipping loop observed in our structure, it is likely that the boron-less diazaborine analogue, having a bulky bicyclic ring, forces the active site loop to adopt an unfavorable, high energy conformation. Triclosan, however, has a small ether linker that permits the formation and proper positioning of the one-turn helix in the active site and allows the flipping loop to adopt a favorable, low energy conformation. Therefore, being able to stabilize the flipping loop is probably a crucial factor for the triclosan's enhanced potency.

The crystal structure of the triclosan complex is likely to make a more significant impact on future drug design than those of the

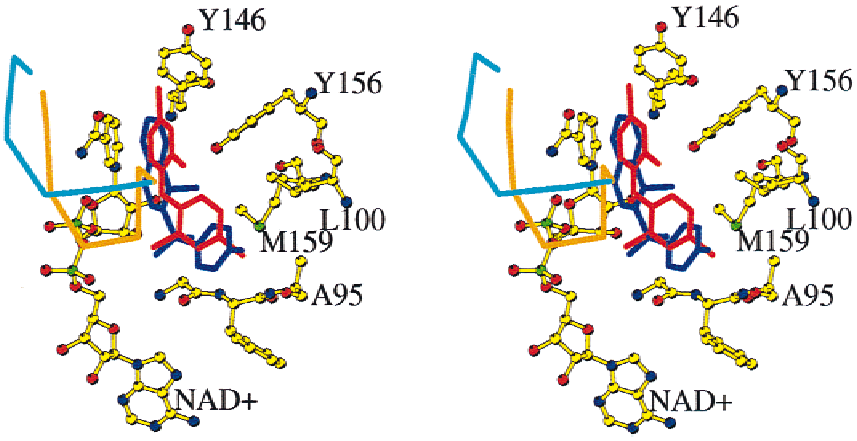

Fig. 2. Stereoviews comparing the interactions of triclosan to those of benzodiazaborine. Our triclosan complex structure is in yellow, with the C, O, N, and S (or P) atoms shown in yellow, red, blue, and green, respectively. Triclosan is shown in red bonds. Benzodiazaborine is shown in blue bonds. Most parts of the benzodiazaborine complex (including the cofactor) are nearly identical to those of the triclosan complex and are hence omitted. Some of the active site residues are removed for clarity. The flipping loop (Ile192 on the left, Ser198 on the right) is in an open conformation in the structure of the diazaborine complex (cyan), but in a closed conformation in that of the triclosan complex (orange).

isoniazid and diazaborine complexes. Isoniazid is a prodrug that needs to be activated by the *Mycobacterium* catalase-peroxidase KatG (Rozwarski et al., 1998). The activated molecule binds covalently to the nicotinamide ring of the NADH cofactor. Because it is small and acts as a suicide substrate analog, its structure with the enzyme is not a suitable template for the design of noncovalent inhibitors. The diazaborines, relying on a covalent interaction between a boron atom and the NAD^+ cofactor, are toxic compounds and also not ideal templates for drug design (Baldock et al., 1996). The structure of the triclosan complex has revealed an effective and completely noncovalent strategy for the inhibition of bacterial enoyl reductases. The structure offers valuable insights for the structure-based design of novel noncovalent inhibitors against this important antibacterial target, and the flipping loop observed in our structure will be a key component in such an effort.

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