The structural basis for substrate versatility of chloramphenicol acetyltransferase CATI

Tapan Biswas,1† Jacob L. Houghton,1,2† Sylvie Garneau-Tsodikova,1,2* and Oleg V. Tsodikov1*

1Department of Medicinal Chemistry, University of Michigan, Ann Arbor, Michigan 48109
2Life Sciences Institute, University of Michigan, Ann Arbor, Michigan 48109-2216

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Abstract: Novel antibiotics are needed to overcome the challenge of continually evolving bacterial resistance. This has led to a renewed interest in mechanistic studies of once popular antibiotics like chloramphenicol (CAM). Chloramphenicol acetyltransferases (CATs) are enzymes that covalently modify CAM, rendering it inactive against its target, the ribosome, and thereby causing resistance to CAM. Of the three major types of CAT (CATI-III), the CAM-specific CATIII has been studied extensively. Much less is known about another clinically important type, CATI. In addition to inactivating CAM and unlike CATIII, CATI confers resistance to a structurally distinct antibiotic, fusidic acid. The origin of the broader substrate specificity of CATI has not been fully elucidated. To understand the substrate binding features of CATI, its crystal structures in the unbound (apo) and CAM-bound forms were determined. The analysis of these and previously determined CATI-FA and CATIII-CAM structures revealed interactions responsible for CATI binding to its substrates and clarified the broader substrate preference of CATI compared to that of CATIII.

Keywords: antibacterial agent; antibiotic resistance; chloramphenicol acetyltransferase; fusidic acid; specificity; substrate recognition

Introduction
Chloramphenicol (CAM) [Fig. 1(A)] is a potent broad-spectrum antibacterial agent. Since its isolation from Streptomyces venezuelae in 1948,1 CAM was one of the primary agents used to treat many infections in the decades that followed. To date, despite its relatively high toxicity,2 CAM is used in many countries because of its affordability and its broad spectrum of activity. In the Western world, CAM is used in treatment of ophthalmic infections and as a last resort in cases of life-threatening brain infections, such as those caused by Neisseria meningitidis, which do not respond to other agents. CAM’s ability to cross the blood-brain barrier makes it a potent therapeutic against brain infections. Because of the emergence of pathogens resistant to multiple drugs, CAM is now being reconsidered as a wider-spectrum therapeutic.3

CAM inhibits protein biosynthesis by binding to the 50S subunit of the bacterial ribosome. Recent crystal structures of the 50S subunit of the Escherichia coli and Thermus thermophilus ribosome in complex with CAM revealed that CAM binds to the A-site of the 50S subunit and occupies the binding site for the amino-acyl moiety of the A-site tRNA.4,5 The 3-hydroxyl of CAM is buried in the interface
with the ribosome through direct hydrogen bonding, potassium ion-mediated electrostatic interactions, as well as through van der Waals interactions with the RNA phosphosugar backbone.\textsuperscript{4,5} The 1-hydroxyl of CAM forms hydrogen bonds with RNA bases. Therefore, any modification of the 1-hydroxyl or the 3-hydroxyl of CAM is predicted to be disruptive of CAM-ribosome binding.\textsuperscript{5} Bacterial resistance to CAM is caused by the chromosomally or plasmid-encoded enzyme chloramphenicol acetyltransferase (CAT) that catalyzes the transfer of an acetyl group from acetylcoenzyme A (AcCoA) to the 3-hydroxyl group of CAM [Fig. 1(B)].\textsuperscript{6} A subsequent slow, non-enzymatic transfer of this acetyl group to the neighboring 1-hydroxyl group allows for a second CAT-catalyzed acetyl transfer from AcCoA onto the 3-hydroxyl group of the same CAM molecule, resulting in a di-acetylated CAM.\textsuperscript{7,8} However, a single acetylation of CAM is sufficient to abolish its affinity for the ribosome\textsuperscript{9} as explained by the above-mentioned structural observations.\textsuperscript{4,5}

CAT proteins are historically divided into three types: CAT\textsubscript{I}, CAT\textsubscript{II}, and CAT\textsubscript{III}, with all three types capable of catalyzing the acetyl transfer to CAM to generate 3-O-acetyl-CAM. Genomic analysis of different CAT sequences indicates that the boundaries between these CAT types are not sharp. Members of the CAT\textsubscript{I} family are present in many important pathogens such as \textit{E. coli}, \textit{Shigella flexneri}, \textit{Serratia marcescens}, and \textit{Salmonella enterica}. CAT\textsubscript{I} family enzymes display high sequence conservation among themselves (e.g. \textit{S. flexneri} and \textit{S. marcescens} CAT\textsubscript{I} proteins are 98\% and 99\% identical to \textit{E. coli} CAT\textsubscript{I}, respectively); however, they display only a modest sequence identity to CAT\textsubscript{II} (\~{}46\%) and CAT\textsubscript{III} (32–47\%) (Fig. 2). The CAT\textsubscript{II} family is not easily distinguishable from CAT\textsubscript{III} and has been defined historically only through its extreme susceptibility to thiol-modifying agents compared with that of CAT\textsubscript{I} and CAT\textsubscript{III}.\textsuperscript{11} There are no obvious additional Cys residues or other sequence features in CAT\textsubscript{II} distinguishing it from the CAT\textsubscript{III} variants. A slight variation in the pK\textsubscript{a} of the Cys31 (in CAT\textsubscript{III} nomenclature), the only Cys in vicinity of the substrate or the cosubstrate binding sites, was suggested to be responsible for the difference in reactivity with thiol-modifying agents,\textsuperscript{12} although there is no evidence confirming this idea.

The sequence differences between CAT\textsubscript{I} and CAT\textsubscript{III} include several substitutions in the binding
site (Fig. 2), potentially resulting in positional differences of CAM bound to these two proteins. A major consequence of this divergence is reflected in different substrate selectivities of CATI and CATIII. In addition to binding and modifying CAM, unlike CATIII, binds a much bulkier antibiotic,
fusidic acid (FA).\textsuperscript{13} FA [Fig. 1(A)] is a steroidal antibacterial agent that is used topically or systemically, usually against infections caused by Gram-positive pathogens. CAT\textsubscript{I} does not modify FA; rather, it sequesters it through binding by its CAM binding site. This type of mechanism of resistance through sequestration is not uncommon and has been observed for other antibiotics such as bleomycin and thiocoraline.\textsuperscript{14–18} Kinetic studies have shown that FA competes with CAM for binding to CAT\textsubscript{I}, but not the other CAT types.\textsuperscript{13} Various bile salts and some triphenylmethane dyes also exhibit similar competitive binding to CAT\textsubscript{I}, but not to CAT\textsubscript{II/III}.\textsuperscript{13,19–21}

CAT\textsubscript{I} plays an important role in antibiotic resistance of many pathogenic bacteria. In addition, CAT\textsubscript{I} has been used as a biochemical and proteomic tool in a number of systems\textsuperscript{22–26} and as a common CAM-resistance marker encoded in laboratory plasmids. Despite its importance in drug resistance and biotechnology, CAT\textsubscript{I} has been much less investigated than CAT\textsubscript{III}.\textsuperscript{13,19–21} Structural and biochemical studies of CAT\textsubscript{III}\textsuperscript{28–34} have been mostly used to understand general features of CAT\textsubscript{I} proteins. Despite this progress, differences in the substrate selectivity between CAT\textsubscript{I} and CAT\textsubscript{III} remain unclear in absence of analysis of CAT\textsubscript{II/III}-CAM and CAT\textsubscript{I}-FA structures.

Herein we report crystal structures of CAT\textsubscript{I} alone (apo) and in complex with CAM, which explain how CAT\textsubscript{I} binds CAM despite differences in its binding site residues from those in CAT\textsubscript{III}. Analysis of these structures along with that of the structure of CAT\textsubscript{I} in complex with FA (deposited in the Protein Data Bank (PDB) by Roidis and Kokkinidis; accession code: 1Q23\textsuperscript{35}) provides an explanation of the differences in substrate preference among CAT types.

**Results**

**Overall structure of CAT\textsubscript{I}**

*E. coli* CAT\textsubscript{I} protein was initially co-crystallized with CAM in the P1 space group (Table I). Molecular replacement using either a monomer or trimer of apo-CAT\textsubscript{I} (from the structure of a serendipitous complex of the nitric oxide synthase oxygenase domain with CAT\textsubscript{I}; PDB code: 1NOC\textsuperscript{37}) as a search model did not yield a solution. This complication likely arose due to the presence of several copies of the protein molecules within a very large unit cell. Further crystallization trials yielded crystals of CAT\textsubscript{I} alone in the P2\textsubscript{1} space group with a smaller asymmetric unit. These crystals grew under conditions similar to those of the CAT\textsubscript{I}-CAM crystals. Molecular replacement with a CAT\textsubscript{I} trimer from the 1NOC entry as a search model, yielded an apo-CAT\textsubscript{I} structure with three CAT\textsubscript{I} trimers in the asymmetric unit (Table I). This three-trimer structure was then successfully used as a molecular replacement search model to determine the structure of the CAT\textsubscript{I}-CAM complex in the P1 crystal form. The asymmetric unit of the P1 crystal form contained six CAT\textsubscript{I}-CAM trimers. The crystal structure of the apo-CAT\textsubscript{I} and that of the CAT\textsubscript{I}-CAM complex were refined to 3.2 Å and 2.9 Å resolution, respectively (Table I).

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<th>Table I. X-ray Diffraction Data Collection and Refinement Statistics for apo-CAT\textsubscript{I} and CAT\textsubscript{I}-CAM Structures</th>
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<td><strong>Data collection</strong></td>
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| **Structure refinement statistics** |
| Resolution (Å) | 40.0–3.2 | 35.0–2.9 |
| R (%) | 23.8 | 24.0 |
| Rfree (%) | 30.1 | 30.9 |
| Bond length deviation (RMSD) from ideal (Å) | 0.009 | 0.006 |
| Bond angle deviation (RMSD) from ideal (°) | 1.08 | 0.907 |
| Ramachandran plot statistics\textsuperscript{b} | | |
| % of residues in most allowed regions | 84.9 | 88.7 |
| % of residues in additional allowed regions | 12.9 | 10.5 |
| % of residues in generously allowed regions | 2.3 | 0.8 |
| % of residues in disallowed regions | 0 (0 residues) | 0 (0 residues) |

\textsuperscript{a} Numbers in parentheses indicate the values in the highest-resolution shell.

\textsuperscript{b} Indicates Procheck statistics.\textsuperscript{36}
replacement (Cα RMSD = 0.7 Å) and to another previously deposited structure of apo-CAT1 (PDB code: 1PD5; Cα RMSD = 0.7 Å). Furthermore, the structures of apo-CAT1 are highly similar to the structure of CAT1 in complex with CAM (Cα RMSD ~ 0.4 Å), suggesting that no major protein conformational changes occur upon CAM binding. Analogously, no major conformational differences were observed for CATIII in the apo and the CAM-bound forms.28 The overall fold and the oligomeric organization of CAT1 [Fig. 3(A)] resemble those of the previously characterized CATIII28 variant. Three identical monomers of CAT1 form a trimer with a 3-fold rotational symmetry. The overall trimeric scaffold is formed by three 7-stranded β-sheets, each of which is formed by six strands (β6, β5, β7, β9, β10, and β2) from one monomer and one strand (β8) from another monomer [Fig. 3(B)]. In each monomer, this β-sheet is flanked on the outside by five α-helices and a small three-stranded β-sheet. In the trimeric core, the aliphatic parts of buried Asp157 side-chains (in strand β8) of the three monomers come together to form intimate hydrophobic contacts with each other, while their carboxyl groups are engaged in intricate, asymmetric network of hydrogen bonding interactions with the side-chains of Ser155 and Asn159. The hydrophobic interactions between the Asp157 residues are likely critical for complex stability as this residue is either an Asp or an Asn in most CAT1/CATIII proteins. Ser155 could however be substituted by a Gly (Fig. 2). The side-chains of Asp157 residues are distorted so that the carboxyl groups
form hydrogen bonds with their own backbone amide NH moieties justifying a weaker conservation of the Ser155. As the side-chains in these β-strands are generally buried away from the solvent, their identities are well conserved among CAT homologs.

**Chloramphenicol interactions in the active site**

Upon trimerization, the active site is formed at the interface of two β-sheets predominantly with residues from strands β6, β5, β7, β9, and β8 of one monomer (termed as the binding monomer) and strands β2 and β10 of the other (the catalytic monomer). Each trimer contains three identical substrate binding sites [Fig. 3(A)]. The nature of this conserved trimeric assembly strongly suggests that CAT1 monomers either require trimeric assembly for proper folding or, if folded, CAT1 would be catalytically active only in the context of a trimer. Indeed, monomeric mutants of the CATIII, whose overall fold is highly similar to that of CAT1, were shown to be catalytically inactive.38 Below, we discuss features of the active site of CAT1 and highlight its differences from that of CATIII that specify the distinct substrate recognition properties of these two proteins.

In the structure of CAT1-CAM complex, all three active site pockets of the CAT1 trimer are occupied with CAM molecules [Fig. 3(A)], whose positions are clearly defined in the electron density map [Fig. 3(C,D)]. One of the two monomers forming a binding site (called here the binding monomer) provides the majority of the residues (Cys91, Phe102, Ser104, Phe134, Phe144, Ser146, Leu158, and Val170) involved in binding of the CAM while the other one (called here the catalytic monomer) provides His193, which has been demonstrated to be one of the primary conserved catalytic residues7,39,40 [Fig. 3(B)]. A few other residues from the catalytic monomer (Phe25 and Cys31) also provide an important CAM-binding surface in the binding pocket. The disposition of the conserved catalytic residues [e.g. His193, Ser146, and Asp197; highlighted in red in Fig. 3(B)] in the CAT1-CAM structure is highly similar to that observed previously in CAT1-CAM complex.41 The position of His193, the likely general base, relative to the bound CAM is identical to that observed in CAT1-CAM structure. The side-chains of Leu158, Val160, and Phe166 that line the very bottom of the substrate binding pocket [Fig. 4(C)] are positioned through interactions of the trimeric assembly and show only minor alterations between CAT1 and CATIII. The dichloroacetonyl moiety of CAM closely interacts with Phe134 (Phe135 in CATIII), likely indicating a strong hydrophobic interaction. A major difference between the CAT1-CAM and CATIII-CAM structures is that the residue analogous to Tyr133 of CAT1 is nonpolar (Leu134) in CATIII. Tyr133 forms a strong hydrogen bond (2.9 Å) with the carbonyl group of CAM. Interestingly, this interaction occurs in place of the interaction of that between the hydroxyl of Tyr25 in CATIII (Phe25 in CAT1) and the carbonyl group of CAM, located at an O-O distance of 2.8 Å from each other.

**Fusidic acid interactions in the active site**

In the CAT1-FA complex [Fig. 4(A)], FA occupies the same binding site as CAM, which explains its observed behavior as a competitive inhibitor of CAM acetylation.13 The differences between the active site residues of CAT1 (as described above) and those of CATIII, while having little effect on CAM binding,41,44 create a unique surface suitable for binding to FA in CAT1. In particular, the placement of Ala24
and Ala29 of CAT$_1$ shapes the substrate binding cavity such that the ring D and the 2-methylhex-2-ene "tail" of FA can be accommodated. The hydrophobic steroid ring system of FA makes numerous hydrophobic contacts with active site residues, including Thr93, Phe102, Phe134, Phe144, Ser146, Phe156, Leu158, Val160, Phe166, and Val170 of the binding subunit, as well as Ala24, Phe25, Val28, and Ala29 of the catalytic subunit [Fig. 4(A,C)].

The hydroxyl moiety of ring A of FA closely aligns with the 3-hydroxyl of CAM and forms a very strong hydrogen bond with the N$_e$2 atom of His193 at a distance of 2.9 Å [Fig. 4(C)]. The hydroxyl group of Tyr133 points inward towards the binding pocket forming a hydrogen bond with the hydroxyl moiety of ring C, the atoms being separated by a distance of about 2.6 Å [Fig. 4(C)], similarly to the interaction between the 3-hydroxyl and Tyr133 in the CAT$_1$-CAM structure [Fig. 3(E)]. Residues from both the binding monomer (Phe134) and the catalytic monomer (Ala24 and Val28) form a hydrophobic zone near the entrance of the binding pocket in CAT$_1$ that cradle the “tail” section of FA and dictate its conformation [Fig. 4(A,C)].

Valuable insight can be gained by comparing this structure with the previously reported...
structures of CATIII in complex with CAM\textsuperscript{29} and a quadruple mutant of CATIII in complex with FA.\textsuperscript{43} In the quadruple mutant of CATIII, four catalytic pocket residues were mutated (Gln92Cys/Asn146Phe/Tyr168Phe/Ile172Val) to mimic those of CATI. This comparison [Fig. 4(B)] indicates a disruption of the FA tail-interacting hydrophobic zone in the quadruple mutant of CATIII, in particular due to the Ala24-Phe and Val28Arg substitutions. The carboxylic acid and acetoxy moieties of ring D are highly solvent exposed when bound to both CATI and the CATIII mutant. The acetoxy group of Phe forms hydrogen bond with FA, at a distance of 2.8 Å to the hydroxyl on the A-ring [Fig. 4(D)], and stabilizes the altered FA orientation. Phe168 and Val172 residues in the CATIII quadruple mutant make direct hydrophobic contacts with the FA molecule, which explains the contribution of these substitutions to the change in binding affinity to FA.\textsuperscript{43}

We observe no major differences in the backbone conformations near the active site of CATI in the structures of apo-CATI (PDB code: 3U9B), CATI bound to CAM (PDB code: 3U9F), and CATI bound to FA (PDB code: 1Q23\textsuperscript{36}). This strongly suggests that CATI has evolved to bind multiple ligands, even as large as an FA molecule, without any major protein conformational changes in its backbone.

Discussion

Chloramphenicol acetyltransferase (CAT) is found in many pathogenic bacteria and is often the cause of resistance against chloramphenicol (CAM), once a widely used antibiotic. Of many known CATs, the type-I appears to be the most prevalent. Recent studies have found CATI in many pathogenic bacteria. CATI has a preference for binding to a variety of substrates; not only does it inactivate CAM but it also binds and sequesters other antibiotics such as FA. A clear understanding of the mechanism of substrate binding by CATI is important to address the intriguing question of how CAT proteins from different classes with similar overall structures display different substrate selectivity profiles. In comparison to CATIII that has been studied almost exclusively, there are only few mechanistic studies that have been performed on CATI.

The general fold and the trimeric organization of CAT proteins have been observed in enzymes of primary metabolic pathways in bacteria and eukaryotes, such as pyruvate dehydrogenases\textsuperscript{45,46} and α-keto acid dehydrogenases.\textsuperscript{47,48} Therefore, CAT appears to be a product of an ancient gene duplication event, which underwent subsequent specialization through evolution to serve a protective role against toxic compounds such as CAM. The general catalytic mechanism proposed for CAT proteins is based on studies of many such proteins. The residue primarily responsible for catalysis of CATI appears to be His193 (His195 in CATIII).\textsuperscript{28} This role was proposed based on a previous study in which a mutant CATIII (His195Tyr) was shown to be devoid of catalytic activity.\textsuperscript{49} Another conserved residue, Ser146, likely stabilizes the oxyanion formed upon an attack on the AcCoA carbonyl carbon by the 3-hydroxy of CAM. Mutagenesis studies with CATIII confirmed that Ser148 (Ser146 in CATI) is crucial for efficient catalysis.\textsuperscript{42}

The CATI protein structure is similar in the apo form and in the CAM- and the FA-bound states, indicating that no major changes in the backbone conformations or in positions of the side-chains occur upon ligand binding. It is quite remarkable that such nearly rigid scaffold is evolutionarily conserved and yet CATI can bind chemically diverse substrates. Analysis of the aligned sequences shows that several residues of CATI are different than corresponding residues in CATIII. Our investigation of the CATI structures indicates that many of these differences are in residues lining the substrate binding pocket (Fig. 2, blue circles). The most striking differences are concentrated around a small patch of residues (Ala24-Cys31, contributed by the catalytic monomer) that enable the FA molecule to be accommodated only in the pocket of CATI. The bulkier residues of CATIII in this patch would push the FA towards the opposite side of the pocket and consequently disrupt the structure. Interestingly, the flexibility (apparently resulting in the reduced rigidity and increased disorder of the backbone) of the quadruple
mutant of CATIII helps it accommodate the pushed out FA in a different conformation. The “tail” of FA now finds a different hydrophobic pocket to rest in and in turn provides stability to the FA in this altered binding pocket. The mutant CATIII shows a 200-fold higher affinity to FA than the wild-type CATII. However, the quadruple mutant of CATIII binds FA with a much (4-fold) weaker affinity than CATI. In addition, the $K_m$ for CAM acetylation by the CATIII quadruple mutant was somewhat compromised (with respect to either CATI or CATIII) and the value of $k_{cat}$ was between those for CATIII and CATI. With the direct structural evidence, it is now clear how the tail of FA nests in a hydrophobic pocket and renders CATI more energetically favorable to bind to FA. In CATIII, a similarly positioned FA “tail” would be sterically blocked by Phe24 and Arg28, and it is not surprising that CATIII does not show affinity towards FA.

Our understanding of CAM’s mechanism of action as well as the mechanisms of resistance to it were largely based on biochemical and structural information available on CAM binding to CATIII and to the bacterial ribosome. The present structural study augments this knowledge by filling in the gap in our understanding of the recognition of both CAM and FA by CATI. CAM has been largely removed from the clinic in the Western world due to its safety concerns, even though cases of extreme toxicity are exceedingly rare. CAM has remained a popular drug in underdeveloped areas due to its low cost and effectiveness against a variety of pathogens. However, with other antibiotics, development of resistance against CAM is a major obstacle to its power to save lives. The detailed picture of the CATI structure is expected to aid in design of inhibitors of CAT enzymes that could re-sensitize CAM-resistant strains. In addition, structure-guided design of CAM analogs could lead to new antibiotics of this class that would be less toxic and more refractory to inactivation by CAT.

**Materials and Methods**

**Expression and purification of CATI**

CATI was expressed in BL21 (DE3)/RIL cells (Stratagene), which harbor a plasmid containing a constitutively expressing CAM resistance gene camR encoding untagged CATI protein. The cells were grown in LB medium (200 rpm, 37°C) containing CAM (25 μg/mL) until the culture reached an attenuation of 0.4 at 600 nm. The cells were harvested after an additional 3 h growth. Pelleted cells (centrifugation at 5,000 g, 10 min, 4°C) were resuspended in the lysis buffer (MES pH 6.5 (40 mM), NaCl (200 mM), glycerol (5%), β-mercaptoethanol (2 mM), and EDTA (0.1 mM)) and lysed by sonication. The lysate was clarified by centrifugation at 35,000 × g for 45 min at 4°C. We took advantage of the thermostability of CAT proteins in purifying CATI without an affinity tag. The clarified lysate was heated (75°C, 20 min) and subsequently centrifuged (35,000 × g, 45 min, 4°C) to remove unfolded precipitated proteins. The CATI in the soluble fraction was further purified by size-exclusion chromatography on an S-200 column (GE Healthcare) equilibrated with buffer (Tris pH 8.0 (40 mM) and NaCl (100 mM)). The fractions containing pure CATI, as determined by SDS-PAGE, were concentrated to 5 mg/mL using an Amicon Ultra centrifugal filter device (Millipore) and used for crystallization.

**Crystallization of CATI alone and in complex with CAM**

Crystals of CATI alone and a complex of CATI with CAM (CATI-CAM) were grown by vapor diffusion in hanging drops containing 1 μL of protein and 1 μL of the reservoir solution (HEPES (100 mM) pH 7.5 (pH of 1 M stock of HEPES acid was adjusted by adding NaOH), PEG 4000 (20% w/v), isopropanol (10% v/v)) or 1 μL of the reservoir solution containing CAM (1 mM), respectively. Irregularly shaped crystals, 40–60 μm in each of the three dimensions were formed in 7–10 days when incubated at 22°C against the respective reservoir solutions. The crystals were gradually transferred into the reservoir solution containing glycerol (15% v/v) and flash frozen in liquid nitrogen.

**Data collection and structure determination**

X-ray diffraction data were collected at 100 K at the 25 beamline of the National Synchrotron Light Source at the Brookhaven National Laboratory. The data were processed with HKL2000. The crystals of apo-CATI and CATI-CAM complex were in the P2₁ and P1 space groups, respectively. The structures of both apo-CATI and CATI-CAM complex were determined by molecular replacement with MOLREP as described in Results. The locations of the CAM molecules in the active sites of CATI were clearly identified and positioned in the omit Fo-Fc density and then refined. The structures were iteratively manually built and refined using programs Coot and REFMAC, respectively. The data collection and refinement statistics are given in Table I. The structures of apo-CATI and CATI-CAM complex were deposited in the Protein Data Bank with accession codes 3U9B and 3U9F, respectively.

**References**


