Crystal Structures of Salmonella typhimurium Biodegradative Threonine Deaminase and Its Complex with CMP Provide Structural Insights into Ligand-induced Oligomerization and Enzyme Activation*

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Two different pyridoxal 5′-phosphate-containing L-threonine deaminases (EC 4.3.1.19), biosynthetic and biodegradative, which catalyze the deamination of L-threonine to α-ketobutyrate, are present in Escherichia coli and Salmonella typhimurium. Biodegradative threonine deaminase (TdcB) catalyzes the first reaction in the anaerobic breakdown of L-threonine to propionate. TdcB, unlike the biosynthetic threonine deaminase, is insensitive to L-isoleucine and is activated by AMP. In the present study, TdcB from S. typhimurium was cloned and overexpressed in E. coli. In the presence of AMP or CMP, the recombinant enzyme was converted to the tetrameric form accompanied by significant enzyme activation. To provide insights into ligand-mediated oligomerization and enzyme activation, crystal structures of S. typhimurium TdcB and its complex with CMP were determined. In the native structure, TdcB is in a dimeric form, whereas in the TdcB-CMP complex, it exists in a tetrameric form with 222 symmetry and appears as a dimer of dimers. Tetrameric TdcB binds to four molecules of CMP, two at each of the dimer interfaces. Comparison of the dimer structure in the ligand (CMP)-free and -bound forms suggests that the changes induced by ligand binding at the dimer interface are essential for tetramerization. The differences observed in the tertiary and quaternary structures of TdcB in the absence and presence of CMP appear to account for enzyme activation and increased binding affinity for L-threonine. Comparison of TdcB with related pyridoxal 5′-phosphate-dependent enzymes points to structural and mechanistic similarities.

Enzymes that use pyridoxal 5′-phosphate (PLP)4 as a cofactor catalyze many important reactions involving amino acids such as transamination, decarboxylation, β- or γ-replacement/elimination, and racemization (1). On the basis of the carbon atom subjected to covalent changes, PLP-dependent enzymes are classified into at least three distinct families: α, β, and γ (2). The α- and γ-families might be distantly related but are clearly not homologous to the β-family. The β-family members whose structures are known include tryptophan synthase (3), biosynthetic threonine deaminase (4), O-acetylserine sulfhydralase (5), cystathionine β-synthase (6), serine dehydratase (7), threonine synthase (8), and 1-aminoacyclopropane-1-carboxylate deaminase (9). All these enzymes exhibit fold type II, which is characteristic of the β-family of PLP-dependent enzymes. In this family of enzymes, each subunit is formed by two distinct domains, both having typical open α/β architecture. The active sites of these enzymes are composed of residues from only one subunit. Their PLP-binding lysine residue is positioned in the N-terminal segment of the polypeptide chain.

Escherichia coli and Salmonella typhimurium are known to possess two distinct PLP-containing threonine deaminases (EC 4.3.1.19), one biosynthetic and the other biodegradative (10, 11). Both the enzymes catalyze the deamination of L-threonine to yield α-ketobutyrate and ammonia. Biosynthetic threonine deaminase (IlvA) encoded by the gene ilvA, is constitutively expressed under normal conditions and catalyzes the first reaction in the isoleucine biosynthesis pathway. L-Isoleucine and L-valine act as allosteric inhibitor and activator, respectively. IlvA provided one of the earliest examples of feedback inhibition (12).

Biodegradative (catabolic) threonine deaminase (TdcB), encoded by the gene tdcB in E. coli and S. typhimurium, is induced anaerobically and catalyzes the first reaction in the degradation of L-threonine to propionate. Unlike IlvA, this enzyme is insensitive to L-isoleucine and L-valine and is activated by AMP. The feedback-resistant TdcB is more suited for the industrial production of L-isoleucine when compared with the feedback-inhibited IlvA (13).

Studies on the TdcB from E. coli and S. typhimurium have shown that the enzymatic activity is enhanced in the presence

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4 The abbreviations used are: PLP, pyridoxal 5′-phosphate; TdcB, biodegradative threonine deaminase; IlvA, biosynthetic threonine deaminase; r.m.s.d., root mean square deviation.
of AMP due to a large decrease in $K_{p}$ for L-threonine and apparent increase in $V_{\text{max}}$ (14–16). Using various analogs of AMP and other natural nucleotides, the functional atoms or groups of AMP, which are involved in the ligand binding and activation of enzymatic activity have been identified (17, 18). Among other mononucleotide phosphates, CMP showed significant enzyme activation compared with GMP, UMP, and IMP. Further, no enzymatic activation was observed in the presence of ATP, whereas ADP showed slight activation.

In the absence of AMP, TdcB exists in monomer–dimer equilibrium at low concentration (19–21). This equilibrium shifts toward the tetrameric form as the concentration of TdcB is increased. Even at low concentrations of TdcB, the presence of AMP induces oligomerization from monomer to tetramer. A number of other biochemical properties of this enzyme, including the capacity and nature of its binding to PLP, kinetic studies and molecular behavior in the presence and absence of AMP, substrate specificity, spectral changes upon addition of L-threonine, inhibition by the reaction product $\alpha$-ketobutyrate and other $\alpha$-keto acids, inactivation by certain intermediaries metabolites of the tricarboxylic acid cycle, and reaction mechanism have been studied extensively (14–16, 20, 22–24). However, these investigations did not reveal the exact site of AMP binding and its role in the formation of oligomers or enzyme activation. It is believed that the regulation of enzymatic reaction by an effector involves conformational changes associated with its binding. In oligomeric proteins, these changes may involve relative rearrangement of subunits as well as subtle changes in the conformation of individual subunits. Three-dimensional structure of TdcB and local and global conformational changes associated with AMP binding are important to understand the mechanism by which the activity of TdcB is regulated by AMP.

Here, we report the crystal structures of *S. typhimurium* biodegradative threonine deaminase with PLP bound to the active site Lys$^{58}$ (as an internal aldimine) in two different crystal forms I and II. The crystal structure of PLP-bound TdcB in complex with CMP has also been determined in another crystal form (crystal form III). This is the first structural description of the biodegradative threonine deaminase. Apart from the overall structural features, the structures of TdcB reveal the mode of PLP binding and its relationship to the expected binding site of L-threonine. Comparison of TdcB structures with IlvA, serine dehydratase, and other members of the $\beta$-families of PLP-dependent enzymes has revealed the differences as well as similarities in the overall structure and reaction mechanism. Analysis of the tertiary and quaternary structural changes observed in the presence of CMP has provided a structural basis for understanding CMP-directed oligomerization and enzyme activation.

**EXPERIMENTAL PROCEDURES**

**Crystallization and Data Collection**—Cloning, expression, purification, crystallization, and collection of complete x-ray diffraction data sets for TdcB in two crystal forms I and II have been described previously (25). Briefly, TdcB from *S. typhimurium* was cloned in pSET C vector and overexpressed in *E. coli* BL21(DE3)pLysS along with an N-terminal hexa-histidine tag. TdcB was purified to homogeneity using nickel-nitrilotriacetic acid affinity column chromatography. Diffraction data for TdcB in two crystal forms were collected to resolutions of 2.2 and 1.7 Å, respectively. Both the crystal forms belonged to the space group P1. The volumes of the asymmetric units in these crystals forms were compatible with two and four subunits of TdcB, respectively. Matthews coefficient ($V_M$) and solvent content of both forms were 2.1 Å$^3$ Da$^{-1}$ and 41.7% (v/v), respectively (26). The unit cell volume of crystal form II was almost twice that of crystal form I.

Despite extensive efforts, diffraction quality crystals of TdcB in complex with various L-threonine analogs could not be obtained. Attempts to co-crystallize TdcB with AMP and CMP were also made. Crystals of TdcB obtained in the presence of AMP diffracted poorly, whereas crystals of TdcB obtained in the presence of CMP diffracted to a resolution of 3–3.5 Å at an in-house x-ray source. A complete data set to a resolution of 2.5 Å was collected on a TdcB crystal obtained in the presence of CMP and the substrate analog O-methylthreonine on beamline BL44XU at SPring-8, Hyogo, Japan. The data were processed and scaled using the programs DENTOZ and SCALEPACK of the HKL suite (27). TdcB-CMP complex (crystal form III) belonged to the space group P622. The asymmetric unit of the crystal was compatible with one subunit of TdcB with a $V_M$ of 3.6 Å$^3$ Da$^{-1}$ and a solvent content of 65.6% (26). The crystal parameters and the data collection statistics are summarized in Table 1.

**Structure Solution and Refinement**—Amino acid sequence alignment shows an identity of 34% between TdcB and the N-terminal domain of IlvA. The sequence corresponding to the C-terminal regulatory domain of IlvA is absent in TdcB. Therefore, a model consisting of the atomic coordinates of the N-terminal domain of the IlvA from *E. coli* (PDB code 1TDJ) (4) with non-identical residues converted to alanine was used as the search model for structure solution of TdcB by molecular replacement with the program AMoRe (28). Because the quality of the data was better for crystal form II, reflections in the resolution range 15.0–3.0 Å of this crystal form were used for the rotation and translation searches. The highest peak of the translation search had a correlation coefficient of 47.2% and an $R$-factor of 55.6%. Refinement was initiated with the program REFMAC5 (29). The $F_o - F_c$ map calculated after initial positional refinement showed good fit for the majority of the main chain. Map improvement by atom update and refinement was carried out using the program Arp/Warp (30). The quality of the electron density map obtained from Arp/Warp was sufficient to allow unambiguous assignment and building of most amino acid residues using the program COOT (31). In the final stages of refinement, inspection of the difference ($F_o - F_c$) map showed strong positive density for the expected ligand PLP. This was followed by identification of potential sites of solvent molecules by automatic water-picking algorithm of COOT (31). The positions of these automatically picked waters were manually checked, and a few more waters were manually identified on the basis of electron density contours at 1.0 $\sigma$ in the $F_o - F_c$ map and 3.0 $\sigma$ in the $F_o - F_c$ map. Electron density peaks, which were significantly higher than those of water molecules and were within the coordination distance from the surrounding atoms, were assigned as sodium ions, because the crystals were grown in presence of sodium ions (100 mM trisost-
Structure of Biodegradative Threonine Deaminase

**TABLE 1**

Crystal parameters and statistics on data collection, refinement, and model quality

Values in parentheses correspond to the highest resolution shell.

| Dataset | TdcB-crystal form I | TdcB-crystal form II | TdcB-CMP-crystal form III |
|---------|---------------------|----------------------|---------------------------|
| **Crystal parameters** | | | |
| Unit cell parameters | | | |
| a, b, and c (Å) | 46.32, 55.30, 67.24 | 56.68, 76.83, 78.50 | 161.15, 161.15, 68.72 |
| α, β, and γ (°) | 103.09, 94.70, 112.94 | 66.12, 89.16, 77.08 | 90.00, 90.00, 120.00 |
| Space group | P1 | P1 | P622 |
| Temperature (K) | 100 | 100 | 100 |
| Wavelength (Å) | 1.542 | 1.542 | 0.93 |
| **Data collection** | | | |
| Resolution range (Å) | 30.0-2.2 (2.28-2.20) | 30.0-1.7 (1.76-1.70) | 30.0-2.50 (2.59-2.50) |
| No. of monomers per asymmetric unit | 2 | 4 | 1 |
| Total no. of reflections | 360,350 | 2,012,989 | 733,136 |
| No. of unique reflections | 29,556 | 128,680 | 18,646 |
| Data redundancy | 12.19 | 15.64 | 39.32 |
| Vₚ/λ (Å³ Da⁻¹) | 2.1 | 2.1 | 3.6 |
| I/σ(I) | 22.18 (4.98) | 26.58 (2.76) | 53.41 (7.56) |
| Completeness (%) | 94.7 (84.5) | 93.1 (86.9) | 99.8 (100.0) |
| Rmerge (%) | 5.3 (22.2) | 4.1 (42.6) | 5.5 (38.3) |
| **Refinement** | | | |
| R (%) | 21.0 (22.9) | 19.0 (31.5) | 19.7 (23.8) |
| Rfree (%) | 25.3 (29.6) | 22.1 (36.0) | 22.6 (24.1) |
| No. of atoms | | | |
| Protein atoms | 4,765 | 9,665 | 2,417 |
| Ligand atoms | 31 | 77 | 37 |
| Solvent atoms | 274 | 782 | 62 |
| **Model quality** | | | |
| r.m.s.d. from ideal values | | | |
| Bond length (Å) | 0.008 | 0.007 | 0.007 |
| Bond angle (deg.) | 1.025 | 1.031 | 1.062 |
| Dihedral angles (deg.) | 5.470 | 5.106 | 5.360 |
| Wilson B factor (Å²) | 31.42 | 26.0 | 66.0 |
| Average B factors (Å²) | | | |
| Protein atoms | 35.8 | 24.8 | 66.1 |
| Ligand | 31.4 | 22.5 | 62.0 |
| Water | 37.4 | 32.5 | 66.1 |
| Residues in Ramachandran plot (%) | | | |
| Most favored region | 89.6 | 91.4 | 90.4 |
| Allowed region | 9.6 | 8.3 | 8.9 |
| Generously allowed region | 0.5 | 0.1 | 0.7 |
| Disallowed region | 0.2 | 0.2 | 0.0 |

* Rmerge = Σh_i |I_h| - |F̅_h|/Σh_i |F̅_h|, where I_h is the jth measurement of the intensity of reflection h, and |F̅_h| is the average intensity.

Dium citrate buffer and 50 mM NaCl in the crystallization condition). The four subunits of TdcB in the asymmetric unit were labeled A-D as shown in Fig. 2a, thus forming AB and CD dimers. During the final stages of model building, large stretches of amino acids in the D subunit showed probable dimers. Based on the difference (Fo - Fc) map, occupancies for the two conformations were manually fixed to 80 and 20%, respectively, which implied that one conformation was major (D’) and the other was minor (D”). Final rounds of refinement were performed using REFMAC5 (29), incorporating TLS restraints (29). The final model of native TdcB in crystal form II contains four subunits of TdcB, four PLP molecules (bound to Lys)²⁸, one sodium ion, and 274 water molecules. For TdcB co-crystallized with CMP and O-methylthreonine (crystal form III), the molecular replacement solution using the program MOLREP had a correlation coefficient of 58.4% and an R-factor of 43.9% in the resolution range 30-3.5 Å. The initial difference Fourier map indicated alternate positions for a few stretches of residues in the polypeptide chain and electron density for CMP, which were incorporated into the model. No electron density corresponding to O-methylthreonine was observed. The final model of TdcB in complex with CMP (hereafter referred to as “TdcB-CMP”) includes one subunit of TdcB, one molecule of PLP (bound to Lys), CMP, sodium ion, and 62 water molecules. The final refinement statistics are given in Table 1.

Structure Analysis—The geometry of the final models was checked using PROCHECK (33). Structural superpositions and the r.m.s.d. calculations between the subunits of TdcB were done using the program ALIGN (34). The rotation and translation relating individual subunits of TdcB dimers were also obtained from ALIGN. The r.m.s.d. values for TdcB and the other related proteins were determined using the DALI server (35). Average B-factors for protein atoms, water molecules, and to the one described for crystal form II. The final model of native TdcB in crystal form I contains two subunits of TdcB, two PLP molecules (bound to Lys), one sodium ion, and 274 water molecules. For TdcB co-crystallized with CMP and O-methylthreonine (crystal form III), the molecular replacement solution using the program MOLREP had a correlation coefficient of 58.4% and an R-factor of 43.9% in the resolution range 30-3.5 Å. The initial difference Fourier map indicated alternate positions for a few stretches of residues in the polypeptide chain and electron density for CMP, which were incorporated into the model. No electron density corresponding to O-methylthreonine was observed. The final model of TdcB in complex with CMP (hereafter referred to as “TdcB-CMP”) includes one subunit of TdcB, one molecule of PLP (bound to Lys), CMP, sodium ion, and 62 water molecules. The final refinement statistics are given in Table 1.
ligands were calculated using the BAVERAGE program of the CCP4 suite (36). Interactions were evaluated using the CONTACT module of the CCP4 suite. Interface residues were identified by recognizing the residues of one subunit, which were within a cut-off distance of 4.0 Å from the atoms of the other subunit. NACCESS5 was used for surface area calculations. The figures were prepared using the programs PyMOL,6 MOLSCRIPT (39), Raster3D (40), GRASP (41), and TOPDRAW (42).

Activity Assay and Kinetic Studies—Enzymatic activity of the TdcB was routinely examined by measuring directly the formation of α-ketobutyrate at 310 nm (20) on a Jasco UV-visible spectrophotometer model V-530 (Japan Spectroscopic Co.) at 25°C. The standard reaction mixture (0.2 ml) contained 100 μM potassium phosphate buffer, pH 8.0, 50 μM of L-threonine and rate-limiting concentration of TdcB. The reactions were carried out in the presence and absence of 5 mM CMP or AMP. α-Ketobutyrate produced was determined assuming molar absorbance of 25.6 M⁻¹ at 310 nm. For kinetic studies, the concentration of AMP/CMP was held in excess at 5 mM. Experiments were repeated at least three times from three independent purifications. Kinetic parameters were calculated by fitting the initial velocity versus substrate concentration to the Michaelis-Menten equation, v = (Vmax[S])/(Km + [S]), using the non-linear regression analysis option of the GraphPad Prism software.

Gel-filtration Chromatography and Glutaraldehyde Cross-linking—Gel-filtration analysis was carried out using Sephacryl-S 200 column (Amersham Biosciences) previously equilibrated with 100 mM phosphate buffer, pH 8.0, at 4°C for the native TdcB and with additional 5 mM AMP/CMP in case of TdcB-AMP or TdcB-CMP complex. The column was calibrated with molecular standards containing aldehyde dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29.0 kDa), and cytochrome c (12 kDa). Elution was monitored by measuring absorption at 280 nm as well as at 400 nm (peak corresponding to the internal aldime).

Purified TdcB in the native form and in the presence of AMP and CMP was incubated with 0.04% (v/v) glutaraldehyde in 50 mM phosphate buffer, pH 8.0, 50 mM NaCl and 2 mM dithiothreitol at 4°C in dark for various time intervals. The protein samples were then mixed with an equal volume of 2× SDS-PAGE loading buffer and immersed in a boiling water bath for 5 min. Cross-linked adducts were resolved using SDS-PAGE followed by Coomassie Blue R-250 staining.

Protein Data Bank Accession Numbers—The coordinates and structure factors for native TdcB in crystal forms I and II and TdcB-CMP (crystal form III) have been submitted to the Protein Data Bank and assigned accession codes 2GN0, 2GN1, and 2GN2, respectively.

RESULTS AND DISCUSSION

Biochemical Studies on TdcB—Enzymatic activity was measured by monitoring increases in absorbance at 310 nm at 25°C due to the product α-ketobutyrate, formed by deamination of L-threonine. Using the purified S. typhimurium TdcB, the Km value for L-threonine was estimated as 123 ± 7 mM. In the presence of 5 mM AMP and CMP, the Km values for L-threonine were 16 ± 2 mM and 32 ± 5 mM, respectively. Thus, AMP and CMP caused a decrease in Km for L-threonine by ~7.7- and 3.5-fold. The ratios of the observed Vmax values of the enzyme in the presence of AMP and CMP to that of native TdcB were ~9 and 3, respectively. Thus, interaction of TdcB with AMP/CMP results in the activation of the enzyme.

Gel-filtration experiments with TdcB in the absence and presence of CMP showed a shift in the apparent molecular mass of the enzyme from 45 kDa for native TdcB to 100 kDa in the presence of CMP and 120 kDa in the presence of AMP (data not shown). These results are indicative of rapid equilibrium between various forms of the enzyme in solution as observed previously in E. coli as well as in S. typhimurium (14, 19–21). Previous studies involving sucrose density ultracentrifugation have shown that the sedimentation velocity of the enzyme increases smoothly from 4.8 to 7.6 S with increasing enzyme concentration or even at low concentrations of the enzyme in the presence of AMP/CMP (43). The increase in the S value from 4.8 to 7.6 S corresponded to a shift of dimeric to tetrameric form of TdcB.

TdcB was cross-linked in the absence and presence of AMP and CMP with 0.04% (v/v) glutaraldehyde and was analyzed using SDS-PAGE. The cross-linked enzyme in all the three cases showed four polypeptide bands corresponding to one each for monomeric and tetrameric forms and two bands near the expected dimeric form of TdcB (data not shown). Previous studies involving cross-linking of the native TdcB and in the presence of AMP with dimethyl suberimidate followed by reduction and alkylation showed three polypeptides corresponding to the molecular weights of monomer, dimer, and tetramer (14). Thus, the results obtained by various other groups and the present gel-filtration and glutaraldehyde cross-linking experiments strongly suggest that, in the presence of AMP or CMP or at high concentration of the enzyme, TdcB is in a tetrameric form.

Model Quality—In all the three crystal forms, electron density is of good quality throughout the polypeptide chain except for a few surface residues. In crystal forms I and II, relatively poorer density is observed for the side chains of residues ranging from 108–121 and 125–135. Some of the residues in these regions as well as a few other surface residues have been truncated according to the extent of density observed for their side chains. In all the three structures, besides the N-terminal hexahistidine tag, a few residues at the N and C termini were not included in the model due to absence of well defined electron density. The most favored and additionally allowed regions of the Ramachandran plot (33, 44) contained 89–91% and 7–8%, respectively, of non-glycine and non-proline residues. In all the three structures, two or three residues were present in the boundary region between generously allowed and disallowed regions of the Ramachandran map. These residues are either in a region of poor electron density or near the CMP binding pocket. One of the common outliers in all the three structures is Ser121. This residue with weak electron density in crystal forms

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Structure of Biodegradative Threonine Deaminase

I and II is situated next to Tyr^{120}, which is hydrogen-bonded to the CMP molecule in TdcB-CMP complex.

Tertiary Structure of TdcB—As expected, TdcB exhibits fold type II, characteristic of the β-family of PLP-dependent enzymes (Fig. 1). Each subunit comprises nine β-strands (13.5%), sixteen α-helices (48.8%), and four 3_10-helices (3.1%). The tertiary structure and the topology diagram of the secondary structural elements of TdcB as defined by the program DSSP (45) are shown in Fig. 1. The structure consists of a small and a large domain. Residues from Ile^{39} to Tyr^{153} form the small domain, whereas the large domain is formed by two parts of the polypeptide chain, extending from the N terminus (Met^{1}) to Lys^{58} and from Asp^{154} to the last C-terminal residue (Asp^{328}). The small domain is an open twisted α/β structure with four parallel β-strands (β5, β2, β3, and β4) surrounded by five α-helices (α1, α2, α9, α10, and α12) on one side and by four α-helices (α3, α13, α14, and α15) on the other side. The C-terminal helix α16 protrudes away from this domain toward the solvent. The corresponding helix in IlvA connects the N-terminal catalytic domain with the C-terminal regulatory domain. Unlike most of the other members of the β-family of PLP-dependent enzymes, the first β-strand in the small domain in TdcB (β2) is preceded by an α-helix (α5). Two helices, α14 and α15, present in TdcB form a single long helix in most of the other members of the β-family of PLP-dependent enzymes. Between the two domains, there is a large internal gap that provides space for the active site. The PLP cofactor is covalently bound as a Schiff base to the ε-amino group of Lys^{58} present at the N-terminal end of the helix α4 yielding an internal aldimine.

Dimeric TdcB—The presence of one dimer (AB dimer) in the asymmetric unit of crystal form I and two dimers (AB and CD dimers) in the asymmetric unit of crystal form II of native TdcB (Fig. 2a) allows for a comparison of three independent models of the dimeric TdcB in different crystallographic environments and therefore provides a measure of structural variability of the protein. Careful examination of both crystal forms I and II indicated that differences occur mainly due to the presence of the D subunit in two different conformations (D' and D") in crystal form II. The electron density map associated with both the conformations is shown in Fig. 2b. Most of the crystal packing interactions of the two different conformations of D subunit are similar. However, there is enough space to accommodate D' and D" subunits with subtle differences at the unit cell interface. In crystal form II, the interactions between the AB and CD dimers are much weaker than those at the A and B or C and D interfaces (Fig. 2a). Structural superposition of D' and D" subunits indicated no significant structural differences between the two conformations. Careful examination of CD' and CD" dimers indicated that the two conformations of the D subunit result from slightly altered dimer interfaces formed between C and D' and C and D" subunits. The maximum deviation
Structure of Biodegradative Threonine Deaminase

observed between corresponding Cα atoms of the two conformations of the D subunit is 4.8 Å. In the TdcB crystal form II, transformation of AB dimer by a matrix that superposes A and C subunits transforms the B subunit such that its new position is related to the D′ subunit by a rotation of 9.9°. The dimer formed by the C and D′ subunits is similar to the AB dimer. In crystal form I, a sodium ion has been modeled between one of the subunits in the unit cell and the subunit in the next unit cell. Similarly, two sodium ions have been modeled in crystal form II, one at the interface of the two dimers in the asymmetric unit and another between dimers of neighboring unit cells.

**Inter-subunit Interactions in the Dimeric TdcB**—In the dimeric TdcB, inter-subunit interactions (≤4.0 Å) between residues in the AB and CD′ dimers of crystal form II have been analyzed. In each subunit, the large domain contributes to the dimer formation. Accessible surface area calculations show that a single subunit of TdcB with bound PLP has a surface area ranging from 12,368 to 12,671 Å². In the AB dimer, the total surface area buried on dimerization is 1,046 Å² (8.3%) per subunit, of which 63.2% (661.4 Å²) is non-polar and 36.8% (385.0 Å²) is polar. In the CD′ dimer, the total surface area buried on dimerization is 978 Å² (7.8%) per subunit, of which 62.4% (610.9 Å²) is non-polar and 37.6% (367.7 Å²) is polar. Atoms of 19–21 residues of one subunit make hydrophobic and polar interactions (≤4.0 Å) with the atoms of the other subunit. Residues that mainly contribute to the interface in both the dimers are 26–29, 31–38, 48–53, 274–280, and 313–324, which are on the helices α3, α13, and α16 and the loops preceding α3, α4, α14, and α16 helices. The interface between the two subunits is mainly formed by hydrophobic interactions and hydrogen bonds. There is a salt bridge between Glu38 and Arg53 at the interfaces of AB and CD′ dimers. There are 8 and 11 hydrogen bonds (cut-off value of 3.5 Å) across the dimer interfaces of AB and CD′ dimers, respectively. Hydrogen bonds, which are common to interfaces of both AB and CD′ dimers, are between Lys278–Gly113, Lys278–Ile280, and their dyad symmetry mates. Other hydrogen bonds that are unique to AB interface are between Asn13–Met17, Asn34–Arg53, and their dyad symmetry mates. Hydrogen bonds that are unique to CD′ interface are between Lys27–Asn34, Lys27–Arg32, Glu38–Tyr26, and Arg32–Tyr120. The contacts at the dimer interface of native TdcB are not as extensive as in other members of β-family of PLP-dependent enzymes. This may lead to energetically inexpensive movement of the subunits leading to slightly altered dimer interface as observed in the case of the CD′ dimer. In this dimer, the D′ subunit is stabilized by a salt bridge between Arg75 from the D′ subunit and Asp264 from the B subunit of the neighboring unit cell. Therefore, AB dimers of crystal forms I and II and CD′ dimer of crystal form II are likely to represent the physiological dimeric state.

**Tetrameric TdcB in Complex with CMP**—The tetrameric TdcB-CMP structure with 222 symmetry is shown in Fig. 3a. The four subunits of the tetramer are designated A–D. The arrangement of subunits in the tetrameric TdcB structure is similar to the tetrameric association observed in the crystal structure of IlvA from *E. coli* (4). Superposition of the A subunit of tetrameric TdcB with the A subunit of the dimeric TdcB structure using the program ALIGN (34) gave an r.m.s.d. of 0.39 Å between corresponding Cα atoms. This difference is mainly due to alternate tracing of main-chain atoms for residues 104–121, 126–146, 274–277, and residues at the N and C termini (Fig. 3b). Most of these changes are associated with either the residues of the small domain lining the entry to the active site...
Structure of Biodegradative Threonine Deaminase

![Diagram](image)

**FIGURE 3.** The quaternary structure of tetrameric TdcB-CMP complex and the tertiary structural differences between dimeric and tetrameric TdcB. 

a, ribbon representation of the tetrameric TdcB is shown in an orientation such that two of the three perpendicular 2-folds of the tetramer are along the plane of the paper. The tetramer with subunits A–D appears as a “dimer of dimers” in which each subunit interacts with two other subunits along different subunit interfaces. The CMP molecule at the dimer interface is shown in stick model representation. The black spheres represent sodium ions present at the dimer-dimer interface. b, stereo view of superposition of the A subunit from dimeric and tetrameric TdcB showing the tertiary structural changes due to the binding of CMP. The subunits are in different shades of gray in regions where tertiary structural changes are observed. PLP is shown to indicate the active site pocket.

Pocket or the residues involved in inter-subunit interactions. In comparison with the dimeric TdcB structure, residues Leu$^{326}$–Gly$^{146}$ of tetrameric TdcB-CMP (present in the helix α8 and the loop preceding it) lining the entrance to the active site pocket move away from the active site, thus broadening the area available for substrate entry to the active site pocket. Between the dimeric and tetrameric TdcB, the largest deviation (3.42 Å) is observed for the Cα atom of Phe$^{131}$.

**Inter-subunit Interactions in the Tetrameric TdcB** — In the tetrameric TdcB structure, each subunit interacts with only two other subunits. The contacts between A and B subunits are most extensive, whereas the presence of only sparse interactions between A and C and no contacts between A and D subunits gives a dimer of dimers appearance to the tetramer. Accessible surface area calculations show that a single subunit in the tetrameric TdcB has a surface area of 13,357.1 Å$^2$. The total surface area buried on dimerization is 1,124.3 Å$^2$ (8.4%) per subunit, which is 78 Å$^2$ more than that of the AB dimer of dimeric TdcB. Two molecules of CMP in the dimer interface interact with residues from both the subunits. The electrostatic surface representation of a dimer showing the CMP binding pocket in the tetrameric TdcB-CMP complex is shown in Fig. 4a. Besides CMP, atoms from 20 residues of one subunit make hydrophobic and polar interactions (≈4.0 Å) with the atoms of the other subunit. In the TdcB-CMP, residues that mainly contribute to the interface in both the dimers are 34–35, 50–53, 271–280, and 313–325, which are in helices α3, α13, and α16 and in the loops preceding α4, α14, and α16. There are 20 hydrogen bonds across the dimer interface of TdcB-CMP dimer formed by protein atoms and CMP. Each CMP molecule forms three hydrogen bonds with Asn$^{34}$, Gln$^{275}$, and Lys$^{278}$ of another subunit of the dimer. Hydrogen bonds between Lys$^{278}$–Gly$^{313}$, Lys$^{278}$–Ile$^{280}$, and their dyad symmetry mates are common to the interface of AB dimer of dimeric and tetrameric TdcB.

Hydrogen bonds unique to tetrameric TdcB-CMP dimer interface are between Asn$^{34}$–Arg$^{53}$, Lys$^{278}$–Asn$^{314}$, Ser$^{321}$–Thr$^{324}$, Ser$^{321}$–Gly$^{325}$, and their dyad symmetry mates.

There is a small region of contact between subunits at the dimer-dimer interface (between A and C or between B and D subunits), which involves hydrophobic as well as polar interactions. These contacts are formed mainly by residues in two stretches of the polypeptide chain between Leu$^{167}$–Tyr$^{174}$ at the C-terminal region of helix α9 and Ile$^{199}$–Thr$^{202}$ at the C-terminal region of helix α10. In IlvA, residues involved in dimer-dimer contacts are also at the C-terminal region of α-helices corresponding to α9 and α10 of TdcB (4). The solvent-accessible surface area buried at this interface is 877.2 Å$^2$ per dimer (corresponding to 438.6 Å$^2$ (3.3%) per subunit), out of which 69.3% (607.6 Å$^2$) is non-polar and 30.7% (269.6 Å$^2$) is polar.

The dimer-dimer interface contains four hydrogen bonds formed by Ile$^{199}$–Asn$^{200}$ and Ile$^{199}$–Thr$^{202}$ and their dyad symmetry mates. Other residues at this interface are involved in van der Waals contacts and hydrophobic interactions. A sodium ion bound to the main-chain oxygen atom of Glu$^{271}$ from both the subunits has been modeled at the 2-fold axis.

To investigate the possibility of tetramer formation by the native (dimeric) TdcB, the structures of dimers in dimeric and tetrameric forms of TdcB were compared. A tetrameric form of native TdcB was generated by superposing two copies of native AB dimer on the AB and CD dimers of tetrameric TdcB-CMP (Fig. 4b) by two separate transformations. In the first transformation, the A subunit of native TdcB was made to superpose on the A subunit of TdcB-CMP, whereas in the second transformation, the A subunit of the native dimer was superposed on the C subunit of TdcB-CMP. The residual rotation and transla-
between the transformed B subunit of native TdcB and the B subunit of native TdcB was used for structural superposition. The native TdcB tetramer so generated had a large number of short contacts (2.2 Å) at the interface of the transformed B subunits (Fig. 4b). Atoms from 17 residues (Met170, Leu173–Asn198, Lys197–Ile203, and Asn203–Lys205) from each B subunit are involved in the short contacts at this interface. From the above analysis, it can be concluded that the conformational changes accompanying the binding of CMP at the dimer interface are essential to facilitate the tetramerization of TdcB.

Active Site Pocket—The active sites of TdcB are situated in clefts between the small and large domains of each subunit. The coenzyme PLP is deeply buried in the active site and is accessible only via a narrow channel. The 2Fo – Fc electron density map corresponding to PLP bound to Lys58 and the associated hydrogen-bonding network are shown in Fig. 5. The pyridine ring is sandwiched between Phe57 on the side facing the protein interior and Gly237, Cys238, and Ala284 on the other side. The aromatic ring of Phe57 is almost perpendicular to the pyridine ring of PLP. The N1 atom of the pyridine ring of PLP forms a hydrogen bond to the Oγ of Ser311 (2.66 Å). The 3′-hydroxyl group of PLP is hydrogen-bonded to the N82 atom of Asn85 (2.83 Å) and to the N6 atom of Lys58 (2.54 Å). The side-chain amide group of Asn85 is coplanar with the pyridine ring. This will allow the expected pyridine ring tilt during transaldimination without any steric hindrance and loss of hydrogen bond. The semicircular tetraglycerine loop formed by Gly184, Gly185, Gly186, and Gly187 and the residues Leu188 and Ile189 form the binding site for the phosphate moiety of PLP. The phosphate group forms six hydrogen bonds with the main-chain amides of the semicircular loop and three hydrogen bonds with water molecules. These water molecules interact with Pro152, Gln162, and Ile189 present in the active site pocket. The positive side of the helix dipole of α10 present at the carboxyl end of the semi-circular loop is close to the phosphate group and compensates for its negative charge. All the amino acid residues that are hydrogen-bonded to PLP are well conserved in TdcB and IlvA.

TdcB and IlvA structures have not been determined in the presence of the substrate, l-threonine, or its analogs. To find the probable substrate binding site, we have carried out cavity calculations using the program VOIDOO (46) of the Uppsala program package. This revealed a small cavity in the active site pocket near PLP. The wall of this cavity is formed mainly by His86, Pro152, Tyr153, Val158, and Gln162. All these residues are also conserved in IlvA, providing further evidence of their role in substrate binding or catalysis. In IlvA, auxotrophic mutants of some of these residues have been shown to affect enzyme activity either by decreasing the substrate affinity or by destabilizing the catalytic intermediates.

**CMP Binding Site**—Previous studies carried out using various structural analogs of AMP, and other natural nucleotides had indicated that considerable alteration in the adenine base could be tolerated, mainly in the imidazole portion of the ring (17, 18). Substitution at N6 position of AMP appeared to decrease the binding affinity. These studies had also shown that the atoms at 2′- and 3′-hydroxyl groups of ribose moiety and 5′-phosphate group of AMP are of primary importance, both for activation and binding. Structural information obtained from the present work fully explains these observations. Two molecules of CMP in the dimer interface help in the formation of a tight dimer, which in turn interacts with another dimer forming a tetramer. The 2F0 – Fc electron density map associated with CMP and the hydrogen-bonding interactions with the two subunits at the dimer interface are shown in Fig. 6. CMP molecules interact with residues from both subunits. The ribose sugar is in the C2′-endo form. The cytosine base is in the anti conformation with respect to the ribose sugar. CMP forms 14 hydrogen bonds with the protein atoms and 4 hydrogen bonds with water molecules. The cytosine base is sandwiched between Ala116 of one subunit and Arg276 of the other subunit. The N4 atom of cytosine base is hydrogen-bonded to the side-chain oxygen atom of Asp119, and the O2 atom is hydrogen-bonded to the side-chain oxygen of Gln275. Superposition of AMP on the bound CMP indicates that N3 and N6 atoms of adenine are expected to correspond to N4 and O2.
atoms of cytosine and would form hydrogen bonds with Gln275 and Asp119, respectively. Because the base region of CMP faces the solvent, there is enough space for the purine ring of AMP to bind at this site. Presence of the bulkier adenine base is expected to further strengthen the interaction between the two subunits. In the sugar, O2* is hydrogen-bonded to the side-chain atom of Gln88, Asn314, and main-chain oxygen of Thr54, all from the same subunit. The O3* atom of the ribose forms a strong hydrogen bond with Asn314 from one subunit and with N6 atom of Lys278 from the other subunit. The O5* atom is hydrogen-bonded to the phenolic hydroxyl group of Tyr120.

The oxygen atoms of the phosphate group bind with the guanidium group of Arg53, the phenolic hydroxyl group of Tyr120, the main-chain oxygen atom of Thr54 from one subunit, and the side-chain nitrogen atom of Asn314 from the other subunit. Thus, all the three moieties, base, sugar, and phosphate, form one hydrogen bond each with the atoms from the second subunit of the dimer. Previous studies to identify the AMP binding site in E. coli TdcB by photolabeling using the photoreactive AMP analog, 8-azido-AMP, followed by tryptic digestion revealed one tryptic peptide Thr230–Arg242 with radioactivity (37). In this work, this region is not present near the CMP binding pocket, which indicates that 8-azido-AMP might have bound nonspecifically to the enzyme.

Role of CMP in Oligomerization and Enzyme Activation—Comparative analysis of the crystal structures of dimeric and tetrameric forms of TdcB provides direct structural insight on the ligand-induced oligomerization and the framework needed for understanding the significant increase in the affinity of substrate binding and $V_{max}$. In the absence of CMP, TdcB exists in a dimeric form, the structure of which is different from the dimer structure observed in the CMP-induced tetrameric TdcB-CMP. In the structure of the TdcB-CMP complex, the movement of the residues from the small domain away from the active site pocket lead to an increase in the size of the channel for the entry of substrate to the active site pocket, which in turn can increase the rate of the reaction. However, residues at the active site pocket do not show significant structural changes from those of the dimeric TdcB structure. The presence of CMP in the dimer interface, far from the active site pocket, supports the previous observation that the CMP binding does not have a direct role in the activation per se.

The present results can also be interpreted in terms of the allosteric model with a low activity T state and a high activity R state. The enzyme exists mainly in the T state in the absence of AMP. The T ↔ R equilibrium is shifted to the R state in the presence of AMP or at high concentrations of TdcB. Dimeric TdcB structure can represent the T state, whereas the dimer structure observed in the TdcB-CMP tetrameric form may correspond to the R state of the enzyme. However, the absence of sigmoidal kinetics or allosteric behavior suggests that TdcB has some atypical properties. In this respect, TdcB differs from Clostridium tetanomorphum ADP-activated threonine deaminase, which has similar subunit structure and undergoes a dimer-tetramer transition by either the activator ADP or high levels of threonine (38) and exhibits sigmoidal kinetics in the absence of ADP and Michaelis-Menten kinetics in the presence of ADP (38). Studies on AMP-activated TdcB in S. typhimurium and E. coli and ADP-activated threonine deaminase from C. tetanomorphum suggest that energy production by controlled catabolism of L-threonine by allosteric effectors may be a regulatory mechanism during anaerobic condition. It is likely that, during periods in which the level of energy in the cells is low, the higher concentration of AMP provides a signal...
Structure of Biodegradative Threonine Deaminase

for activation and conversion of L-threonine to propionate and ATP. In E. coli and S. typhimurium, propionate can be further catabolized into pyruvate and succinate by the 2-methylcitric acid cycle.

**Structural Comparison with Related Enzymes**—A comparison between TdcB and a representative subset of structures from the Protein Data Bank using the DALI server (35) revealed that the structures most similar to TdcB were within the fold type II or β-family of PLP-dependent enzymes. The first nine crystal structures with the highest Z scores are IlvA, serine racemase, O-acetylserine sulfhydrase, cystathionine β-synthase, β-subunit of tryptophan synthase, serine dehydratase, O-phosphoserine sulfhydrlyase, threonine synthase, and 1-aminocyclopropane-1-carboxylate deaminase. Most of these enzymes can catalyze the cleavage of the Cβ–O bond of serine or threonine. Structural alignment of TdcB with these structures resulted in r.m.s.d. values of 1.6–3.1 Å and Z scores ranging from 43.6 to 22.0 for 265–314 aligned Cα residues. Superposition of all these structures indicates that, besides overall structural similarity, structural equivalence extends to the PLP binding pocket and the regions related to reaction specificity. Structural conservation observed among these enzymes provides strong evidence of their phylogenetic relationship.

**Mechanistic Considerations**—The general catalytic mechanism of PLP-dependent enzymes has been well studied (1). In the TdcB structure, the N1 atom of the PLP is hydrogen-bonded to the Oy of Ser311. In TdcB, as seen in IlvA, serine group of partial reactions in which the last two steps occur in solution non-enzymatically. During catalysis, an external aldimine is formed between PLP and L-threonine by transaldimination. It was proposed in the case of serine dehydratase that the phosphate group of PLP abstracts a proton from the α-amino group of the substrate during the formation of external aldimine and then acts as a strong general acid by donating a proton to the Oy of serine. The hydrogen atom from the Cα carbon atom of serine is then abstracted by the active site lysine in a concerted fashion (7). A similar catalytic reaction in TdcB will result in β-elimination of water from the PLP-Thr leading to the formation of PLP-aminocrotonate. A second transaldimination reaction results in the release of aminocrotonate leading to the formation of a PLP-Lys Schiff base. Non-enzymatic tautomerization of aminocrotonate forms α-iminobutyrate, which is then spontaneously hydrolyzed into α-ketobutyrate and ammonia. Because of the similarities in the architecture of the active site, substrate structure, and chemistry performed, it might be appropriate to assume that threonine and serine deaminases have similar catalytic mechanisms.

**Conclusions**—We present here the first report on crystal structures of TdcB and its complex with the activator molecule, CMP. Structural comparison of native TdcB and its complex with CMP has revealed interesting differences. In the native structure, TdcB is in a dimeric form, whereas in complex with CMP, it forms a tetramer, which appears as a
Structure of Biodegradative Threonine Deaminase

binding are conserved in TdcB, IlvA, and serine dehydratase suggesting a similar catalytic mechanism for these enzymes. Mutational analysis of residues interacting with the CMP molecule at the dimer interface may further increase our understanding of the role of CMP in enzyme activation.

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Structure of Biodegradative Threonine Deaminase

39641