Crystal Structure of Escherichia coli Diaminopropionate Ammonia-lyase Reveals Mechanism of Enzyme Activation and Catalysis

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Pyridoxal 5′-phosphate (PLP)-dependent enzymes utilize the unique chemistry of a pyridine ring to carry out diverse reactions involving amino acids. Diaminopropionate (DAP) ammonia-lyase (DAPAL) is a prokaryotic PLP-dependent enzyme that catalyzes the degradation of D- and L-isoforms of DAP to pyruvate and ammonia. Here, we report the first crystal structure of DAPAL from Escherichia coli (EcDAPAL) in tetragonal and monoclinic forms at 2.0 and 2.2 Å resolutions, respectively. Structures of EcDAPAL soaked with substrates were also determined. EcDAPAL has a typical fold type II PLP-dependent enzyme topology consisting of a large and a small domain with the active site at the interface of the two domains. The enzyme is a homodimer with a unique biological interface not observed earlier. Structure of the enzyme in the tetragonal form had PLP bound at the active site, whereas the monoclinic structure was in the apo-form. Analysis of the apo and holo structures revealed that the region around the active site undergoes transition from a disordered to ordered state and assumes a conformation suitable for catalysis only upon PLP binding. A novel disulfide was found to occur near a channel that is likely to regulate entry of ligands to the active site. EcDAPAL soaked with D,L-DAP revealed density at the active site appropriate for the reaction intermediate aminoacrylate, which is consistent with the observation that EcDAPAL has low activity under crystallization conditions. Based on the analysis of the structure and results of site-directed mutagenesis, a two-base mechanism of catalysis involving Asp120 and Lys77 is suggested.

Background: DAPAL is a novel PLP-dependent enzyme involved in the degradation of DAP, a nonstandard amino acid.

Results: Striking structural differences were observed between apo- and holo-EcDAPAL, and structure with a PLP-aminoacrylate intermediate bound at the active site was obtained.

Conclusion: Enzyme undergoes apo to holo structural transitions and follows a two-base mechanism of catalysis.

Significance: The results provide insights into the catalytic mechanism of DAPAL.

Diaminopropionate (DAP)3 is a nonstandard amino acid found in both prokaryotes and higher organisms (1–3). In prokaryotes, DAP is an important intermediate in the synthesis of nonribosomal peptides exhibiting antibiotic properties such as viomycin (3), capreomycin (4), and zwittermicin (5). It has been demonstrated that consumption of large quantities of a drought-resistant legume Lathyrus sativus leads to neurolathyrism, a degenerative disease caused by the neurotoxin β-N-oxalyl-L-α,β-diaminopropionate (6, 7). L-DAP is the immediate precursor in the synthesis of the neurotoxin (8). High levels of D-DAP have been found in the gut of Bombyx larvae (2), which reduce drastically in the pupal stage (9).

Pyridoxal 5′-phosphate (PLP)-dependent enzymes are crucial for the metabolism of amino acids and have been extensively investigated in terms of their structure and function. These enzymes carry out a variety of reactions, such as transamination, racemization, deamination, decarboxylation, elimination, and replacement, using PLP as an electron sink for stabilization of reaction intermediates. They have been classified into four major folds (I–IV) based on their structure and sequence similarities (10, 11). Diaminopropionate ammonia-lyase (DAPAL) (EC 4.3.1.15), a prokaryotic enzyme, has been classified as a fold type II PLP-dependent enzyme, although it shares low sequence identity (18–23%) with other well studied fold type II enzymes. DAPAL catalyzes the conversion of both D- and L-isoforms of DAP to pyruvate and ammonia (Fig. 1) (12). The only other substrate it can degrade, although poorly, is serine. In eukaryotes, which appear to lack DAPAL, cystathionase has been shown to carry out degradation of DAP, albeit at a lower rate compared with that of its cognate substrate cystathionine (13). Biochemical studies on the catalytic properties of DAPAL from Pseudomonas sp. (12), Salmonella typhimurium (14, 15), and Escherichia coli (14, 16) have been reported in the literature. However, in the absence of its crystal structure, these

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3 The abbreviations used are: DAP, diaminopropionate; PLP, pyridoxal 5′-phosphate; DAPAL, DAP ammonia lyase; EcDAPAL, E. coli/DAPAL; SeMet, selenomethionine; DSD, D-serine deaminase; LSD, L-serine dehydratase; SR, serine racemase; r.m.s.d., root mean square deviation; PDB, Protein Data Bank.
investigations did not reveal the structural basis of its substrate and reaction specificity and mechanism of catalysis.

Three-dimensional structures of DAPAL from *E. coli* (*Ec*DAPAL) in holo- and apo-forms and structures of the complexes of the enzyme with substrates are described for the first time in this paper. The structures and observations on ligand binding and catalysis provide insights into the mode of activation of enzyme upon PLP binding and mechanism of efficient degradation of both D and L stereo forms of the substrate.

**EXPERIMENTAL PROCEDURES**

Cloning, Overexpression, and Purification—Cloning, expression, and purification of *Ec*DAPAL have been described previously (14). *Ec*DAPAL polypeptide consists of 398 residues with a molecular mass of 43.3 kDa. Selenomethionine (SeMet)-incorporated *Ec*DAPAL was prepared by the metabolic inhibition method (17). Details are described in supplemental Methods.

Crystallization and Data Collection—Crystals of *Ec*DAPAL suitable for structural studies were obtained as reported previously (18). Two crystal forms were obtained under similar conditions containing 20% PEG 3350, 70 mM MgCl₂, 50 mM Li₂SO₄, 6 mM Na⁺ “C₂H₅COO⁻”, 40 mM Tris-HCl, pH 8.3, and varying concentrations of β-mercaptoethanol (0–5 mM). Plate-like crystals belonged to the tetragonal (P4₃2₁2) space group, although the rod-shaped crystals were of the monoclinic (P₂₁) space group. Only tetragonal crystals appeared when excess PLP (50–100 μM) was added to the crystallization drops. SeMet *Ec*DAPAL also crystallized in the same two forms under similar conditions. Crystals were briefly soaked in a cryo-protectant solution (crystallization condition with additional PEG 3350 up to 30%), mounted on a cryo-loop and frozen in liquid nitrogen for x-ray diffraction data collection.

Diffraction data were collected for native and SeMet *Ec*DAPAL in the tetragonal form, native monomeric crystals, tetragonal crystals soaked in crystallization solution containing 10 mM D-Ser, L-Ser, or DL-DAP, and tetragonal crystals soaked with DL-DAP at a lower pH (6.5). Details are described in supplemental Methods. All datasets were processed and scaled using the programs DENZO and SCALEPACK of the HKL suite (19). Both tetragonal and monomeric crystals showed large batch to batch variations in the unit cell parameters (*b* in tetragonal and both *b* and *c* in monoclinic), due to which initial attempts to determine structure by isomorphous replacement method were not successful. These variations were found to result from weak packing of molecules along the variable cell edges as discussed in the supplemental Results and supplemental Fig. S1.

Structure Determination and Refinement—The structure was determined at 2.5 Å using the data collected from a SeMet *Ec*DAPAL crystal by single wavelength anomalous dispersion method using the Autosol program of Phenix suite (20). The program HySS (21) located 36 tentative Se positions corresponding to a dimer in the asymmetric unit, out of which 26 were accepted for phase calculation in Phaser (22). The figure of merit based on these sites was 0.54. The map was improved by density modification and NCS averaging using Resolve of Phenix suite. The improved map was used for chain tracing. Three noncontiguous segments consisting of a total of 758 residues (698 with side chains) were identified by Resolve. The model and the map had a correlation coefficient of 0.81 and *R*ₘₐₓ and *R*ₜₒₓ of 25.0 and 28.0%, respectively. The model was improved by several rounds of refinement using REFMACS (23) and manual model building using COOT (24) of CCP4 suite (25). Solvent molecules were identified by the automatic water-picking algorithm of COOT. The positions of these automatically picked waters were manually checked, and a few more waters were identified on the basis of electron density contoured at 1.0σ in the 2Fo–Fc and 3.0σ in the Fo–Fc map. In the final stages of refinement, inspection of the difference (Fo–Fc) map showed strong positive density into which a model of the co-factor PLP could be built. The final structure of SeMet *Ec*DAPAL modeled in tetragonal crystal form contains two polypeptide chains, two PLP molecules (bound to Lys⁷⁷), two Tris ions, and 242 water molecules.

Other structures were determined using the SeMet *Ec*DAPAL as the phasing model. The refinement statistics for all structures are given in Table 1. The first (Met¹) and the last (Pro⁹⁹) residues of the polypeptide were disordered in most of the structures. Crystals soaked with DL-DAP, pH 6.5, and L-Ser did not have any extra density corresponding to bound substrate or reaction intermediate. These structures were similar to *Ec*DAPAL structure obtained without soaking with substrates and therefore have not been deposited in the Protein Data Bank. Residue Cys⁴⁸ of all protomers in monoclinic form and SeMet derivative structure in the tetragonal form has been modified by β-mercaptoethanol to hydroxyethylthiocysteine. This cysteine is a surface residue, and its chemical modification is unlikely to have any implication on the activity of the protein.

Similar modification of surface-exposed residues by β-mercaptoethanol has been observed in 165 other protein structures deposited in the PDB.

Structure Analysis—The geometries of the final models were checked using PROCHECK (26). Structural superposition and root mean square deviation (r.m.s.d.) calculations between the subunits of *Ec*DAPAL were carried out using the program ALIGN (27) and SSM superpose (28) feature of COOT. DALI server was used for the identification of structural homologs (29) in the PDB. Average *B*-factors for protein atoms, water molecules, and ligands were calculated using the BAVÉRAGE program of the CCP4 suite. PISA was used for analysis of interfaces and identification of interface residues (30). Figures were prepared using the programs PyMOL (31) and ChemDraw.

Site-directed Mutagenesis—Single site mutants of *Ec*DAPAL targeting residues Lys⁷⁷, Asp¹²⁰, and Asp¹⁸⁹ were constructed by site-directed mutagenesis (32). *Ec*DAPAL cloned in pRSET C vector was used as the template for generation of mutants. The primers used for generating mutants are listed in supplemental Methods. All the mutants were confirmed by sequencing.
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| Dataset name | SeMet | Native-P4 | Native-P2 | DL-DAP | D-Šer |
|--------------|-------|-----------|-----------|--------|-------|
| Wavelength   | 0.98  | 1.0       | 0.93      | 1.54   | 1.54  |
| Cell parameters |       |           |           |        |       |
| a | 85.64 Å | 85.53 Å | 85.78 Å | 85.51 Å | 85.61 Å |
| b | 85.64 Å | 85.53 Å | 94.15 Å | 85.21 Å | 85.61 Å |
| c | 207.27 Å | 207.27 Å | 94.69 Å | 205.08 Å | 207.65 Å |
| α, β, γ (degrees) | 90, 90, 90° | 90, 90, 90° | 90, 110.01, 90° | 90, 90, 90° |
| Space group | P4_2_2_2 | P4_2_2_2 | P2_1 | P4_2_2 | P4_2_2 |
| Resolution range | 50.0–2.45 Å | 20.0–2.00 Å | (2.28–2.20 Å, 2.54–2.50 Å) | 50.0–2.50 Å |
| Rmerge<sup>a</sup> | 7.5% (19.9%) | 6.6% (37.5%) | 7.8% (35.7%) | 8.1% (41.8%) |
| Unique reflections | 53,936 | 52,570 | 71,324 | 26,585 |
| | 49,3 (14.0) | 22.2 (2.2) | 18.9 (2.5) | 29.2 (3.9) |
| Completeness | 99.7% (93.4%) | 99.2% (93.6%) | 98.3% (97.5%) | 97.4% (98.1%) |
| Redundancy | 15.4 (13.1) | 5.0 (3.0) | 3.6 (3.4) | 6.1 (6.1) |
| Rwork<sup>b</sup> | 19.3% | 19.2% | 21.3% | 21.8% |
| Rfree<sup>c</sup> | 24.1% | 23.3% | 26.1% | 26.7% |
| r.m.s.d. bond length | 0.008 Å | 0.009 Å | 0.005 Å | 0.006 Å |
| r.m.s.d. bond angle | 1.16° | 1.22° | 1.02° | 0.99° |
| Ramachandran plot |       |           |           |        |       |
| Favored | 92.3% | 91.1% | 91.1% | 90.8% |
| Additionally allowed | 7.3% | 8.4% | 8.1% | 8.7% |
| Generously allowed | 0.1% | 0.1% | 0.4% | 0.2% |
| Outliers | 0.3% | 0.3% | 0.4% | 0.3% |
| TLS groups per chain | 9 | 4 | 8 | 4 |
| Average B-factor |       |           |           |        |       |
| Protein atoms | 27.43 Å<sup>2</sup> | 37.62 Å<sup>2</sup> | 44.10 Å<sup>2</sup> | 44.28 Å<sup>2</sup> |
| Water atoms | 11.44 Å<sup>2</sup> | 32.10 Å<sup>2</sup> | 33.79 Å<sup>2</sup> | 25.28 Å<sup>2</sup> |
| Other hetero-atoms | 17.34 Å<sup>2</sup> | 29.21 Å<sup>2</sup> | 57.89 Å<sup>2</sup> | 34.55 Å<sup>2</sup> |

<sup>a</sup> Rmerge = Σ|Fo(hkl) – Fc(hkl)|/ΣFo(hkl), where Fo(hkl) is the intensity of the Fo measurement of reflection (hkl) and Fc(hkl) is its mean intensity.
<sup>b</sup> Rwork = Σ|Fo(hkl) – Fc(hkl)|/ΣFo(hkl), where Fo(hkl) and Fc(hkl) are the observed and calculated structure factors.
<sup>c</sup> Rfree = Σ|Fo(hkl) – Fc(hkl)|/ΣFo(hkl), where Fo(hkl) and Fc(hkl) are the observed and calculated structure factors but from a randomly selected subset of the data (5%), which were excluded from the refinement process.

Activity Assay and Spectral Studies—Activity measurements of EcDAPAL and its mutants were carried out by a coupled enzyme spectrophotometric method by monitoring the decrease in absorption at 340 nm due to consumption of NADH (14), as described in supplemental Methods. All assays were carried out in 50 mM potassium phosphate buffer containing 10 μM PLP at pH 7.5. The visible absorbance spectrum of EcDAPAL (1 mg ml<sup>−1</sup>) and mutants in the range of 300–550 nm in potassium phosphate buffer or crystallization solution was recorded using a Jasco UV-Visible V-630 spectrophotometer. Spectra were also recorded as a function of time upon addition of substrates (10 mM).

RESULTS AND DISCUSSION

Subunit Structure from the Tetragonal Crystal Form—The structure of EcDAPAL has been determined to reasonable R_work and R_free values (Table 1). The structures of the two protomers of tetragonal crystal form are nearly identical as revealed by the low r.m.s.d. of 0.24 Å obtained after superposition of corresponding Ca atoms of the two chains. As in other fold type II PLP-dependent enzymes, each protomer consists of two domains as follows: a large and a small domain, with PLP bound in a cleft between the two domains (Fig. 2A). The large domain is made up of segments from both the N (residues 2–69) and C (residues 197–398) termini of the polypeptide, whereas the small domain is constituted by residues 78–187. Two loops consisting of residues 70–77 and 188–196, respectively, connect the two domains and are shown in red in Fig. 2A. Each domain has a β-sheet core surrounded by helices. The large domain is made up of a seven-stranded (S1–S3 and S8–S11) mixed and twisted β-sheet surrounded by 11 helices (H1–H3 and H10–H17). The central five strands (S3 and S8–S11) are parallel, whereas the strands at the edges (S1 and S2) are antiparallel and are at an angle of 70° to each other due to twisting of the sheet. The small domain is made up of a four-stranded parallel β-sheet (S4–S7, in the order 6547) and six helices (H4–H9). Helix H10, which is a part of the large domain, has extensive interactions with the small domain. Residues 93–113, helices H5 and H6, and the adjoining loops are in slightly different conformations in the two protomers. This region is involved in crystal packing and its similar conformation in both the protomers would lead to steric clashes. Residues 279–284 of chain A and 279–283 of chain B were not modeled due to absence of well defined electron density. These residues are part of the solvent-exposed loop connecting helix H12 and H13.

Structural comparisons of the protomer with other structures in the PDB using DALI server (29) revealed that EcDAPAL is similar to fold type II PLP-dependent enzymes. The structures most similar are d-serine deaminase (DSD; PDB code 3R0X) (33), serine racemase (SR; PDB code 3L6B) (34), l-serine dehydratase (LSD; PDB code 1PW) (35), biodegradative threonine deaminase (PDB code 2GNI) (36), and biosynthetic threonine deaminase (PDB code 1TDI) (37), with r.m.s.d. in ranges of 2.1–3.0 Å. Structural comparisons indicated that except for strand S1 of the large domain and the two helices H5 and H6 of the small domain, other secondary structural elements are conserved in these homologs. Apart from EcDAPAL, similar insertions are observed in S. typhimurium DSD (PDB code 3R0X).
Interfaces and Biological Unit—EcDAPAL has been shown to be a stable dimer in solution (14, 16). The solvent-accessible surface area of an isolated protomer is about 14,973 Å². Analysis of all the intermolecular interfaces from the tetragonal crystal structure revealed that the largest interface is formed by the two protomers of the asymmetric unit and corresponds to a buried surface area of 1,055 Å² per protomer. This represents 7.7% of the accessible surface of a protomer. This interface is likely to correspond to the physiological dimer as other interfaces found in the crystal structure have buried areas lower than 695 Å² per monomer. Thus, the two protomers as shown in Fig. 2B represent a biological dimer. The interface is formed by the large domains of two protomers related by noncrystallographic 2-fold symmetry about an axis inclined to the crystal b by ~10°. It involves interactions of residues from four helices and three loops of each subunit (Fig. 2C). Residues that are involved in intersubunit interactions belong to the N-terminal loop (Ser² and Phe⁴), H2 (Leu⁵³, Leu⁶⁶, and Phe⁵⁷), H14 (Leu³¹⁹, Arg³²², Val³²³, and Gly³²⁵), the loop between helix H14 and H15 (Asn³²⁶, Pro³²⁷, Tyr³²⁸, Arg³³³, and Ile³⁴⁵), H15 (Tyr³⁵²), H17 (Tyr³⁸⁵, Arg³⁸⁶, Glu³⁸⁷, and Val³⁸⁹), and the C-terminal loop (Trp³⁹⁰, Glu³⁹¹, Gly³⁹², Ala³⁹⁵, and Val³⁹⁶). The interface is stabilized by both polar and hydrophobic interactions. Out of the 48 residues of the interface, 20 (10 from each subunit) are polar. 20 pairs of hydrogen-bonded residues were found in the interface (Ser⁴–Glu³⁸⁷, Ser⁷–Glu³⁹¹, Arg³²²–Trp³⁹⁰, Asn³²⁶–Tyr³⁸⁵, Arg³³³–Asn³²⁶, Arg³³³–Pro³²⁷, Arg³⁸⁶–Asn³²⁶, Arg³⁸⁶–Pro³²⁷, Tyr³⁹⁰–Asn³²⁶, Val³⁹⁶–Glu³⁹¹, and their dyad symmetry mates).

Even though the EcDAPAL protomer shares overall structural similarity with other fold type II PLP-dependent enzymes, the quaternary arrangement of the two subunits of the dimer is different. Two types of quaternary arrangements have been commonly observed in fold type II enzymes. In interfaces that resemble O-acetylserine sulfhydrylase (38), both small and large domains are involved in interface formation, and the buried surface areas range from 1,500–4,000 Å² in different enzymes. In serine racemase-like interfaces (34), only large domains are involved in the interface, and the buried surface areas are in the range of 800 – 1,200 Å². The dimeric interface of EcDAPAL is also formed by interaction of only the large domains, although the relative arrangement of the two protomers is different from that of SR. Analysis using the PISA server did not lead to the identification of any other enzyme with a similar interface. Hence, EcDAPAL is a dimeric protein with a biological interface that is novel and distinct from interfaces observed earlier in fold type II PLP-dependent enzymes.

Active Site—The active site of holo-EcDAPAL is constituted by residues from a single polypeptide chain and occurs in a cleft between the two domains. As established earlier (14), Lys⁷⁷ anchors the co-factor PLP as a Schiff base through its ε-amino group. Lys⁷⁷ is at the N-terminal end of a helix (helix 4, residues 78–93) and is also the last residue of the loop (residues 70–77) that connects the two domains. Apart from this interaction, PLP is stabilized by several other noncovalent interactions. Oxygen atoms of the phosphate moiety of PLP are hydrogen bonded to two water molecules, a Tris ion and to the main chain nitrogen atoms of five residues (Gly²³², Val²³³, Gly²³⁴, Ala²³⁵, and Met²³⁶) occurring in a loop (Fig. 3A). A similar glycine-rich loop anchors the phosphate group of PLP in other PLP-dependent enzymes (39). O3 of PLP is at hydrogen bonding distance from the side chain nitrogen of Asn¹²², and N1 is hydrogen bonded to the hydroxyl group of Thr¹⁷⁶ (Fig. 3B). The pyridine ring of PLP is nearly orthogonal to the phenyl ring of Phe⁷⁶.
leading to a CH-π interaction. In most of the fold type II enzymes, pyridine N1 of PLP is hydrogen bonded to a Ser or Thr. The residue hydrogen bonded to N1 is an Asp or Glu in fold type I, which enhances the electron withdrawing capacity of PLP. In contrast, an Arg is hydrogen bonded to N1 in fold type III enzymes leading to lower electron withdrawing potential of PLP. These interactions are significant for the functional differences between PLP-dependent enzymes of different folds. Thus, in EcDAPAL, instead of a carbanion intermediate, a neutral aminoacrylate is likely to be stable.

EcDAPAL substrate-binding site is lined by PLP and residues from loops 119–123, 188–190, 232–236, 287–291, and 166–169. Lys77, Asp120, Asp189, and Tyr168 are close to the substrate-binding site and could be important for the catalytic function of the enzyme. Electron density suitable for a Tris ion was observed at a site close to the phosphate group of PLP in the holo-EcDAPAL structure. The Tris ion fitted to this density could be refined with full occupancy and reasonable B-factors. It has extensive interactions with the surrounding groups (Fig. 3C). The amino group of Tris is hydrogen bonded with nearly tetragonal geometry to the phosphate group of PLP and the main chain oxygen atoms of Gly288 and Ala290. Hydroxyl groups of Tris have hydrogen bonding interactions with carboxyl groups of Asp120 and Asp189, hydroxyl group of Tyr168, main chain nitrogen of Ala290, and three water molecules. EcDAPAL is less active in Tris buffer compared with phosphate buffer (data not shown). Similar inhibition in Tris buffer has been reported for DSD (40). NH4+/H11001 and K+/H11001 prevented inhibition of DSD by Tris in a concentration-dependent manner. Na+/H11001 ions also prevented the inhibition, although less effectively. EC-DAPAL structure suggests that the observed inhibition is due to the binding of Tris at the active site and might represent a property shared by several other PLP-dependent enzymes.

A novel disulfide bond linking Cys265 and Cys291 was observed close to the substrate access channel in holo-EcDAPAL (Fig. 4A). The homologous enzymes DSD (33), SR (34), and LSD (35) bind a metal ion (Na+, K+, Mg2+, or Mn2+) close to this site with the residue corresponding to Cys265 involved in metal coordination. In holo-EcDAPAL, residual density at a position corresponding to the metal-binding site was observed.
in one of the two subunits of the crystallographic asymmetric unit. In the SeMet EcDAPAL, both the subunits had density at this site. The density was interpreted as a water molecule based on the distance of this site to the nearest polar groups (Fig. 4A). This suggests that unlike homologous enzymes, there is no metal ion bound in the vicinity of the EcDAPAL active site. Instead, a novel disulfide linkage is observed. The metal ion in these enzymes is likely to stabilize structure of the loop corresponding to residues 261–295 of EcDAPAL. In EcDAPAL, the disulfide linkage may be important for maintaining the conformation of the substrate access channel. Residues 279–283, which occur in the solvent-exposed region of the active site access channel (Fig. 4B), are disordered indicating that the loop is highly flexible. These residues may regulate the access of substrates to the active site. Disulfide bond might limit the disorder in the 261–295 segment. However, further mutational analysis is required to unravel the role of this unique disulfide in the function of EcDAPAL.

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(helix H9) residues. Well defined electron density was absent for some of these regions in the monoclinic form, which include residues 109 and 263–293 in chain A; 102–110 and 262–293 in chain B; 111 and 262–293 in chain C; and 262–291 in chain D. Also, electron density for an intact PLP was not observed in any of the four protomers. Thus, the tetragonal and monoclinic forms correspond to the holo- and apo-forms of EcDAPAL, respectively. However, both the forms resemble the closed form of the fold type II PLP-dependent enzymes. This is in contrast to DSD (33) and SR (34) in which the closed form is observed only upon substrate binding.

The electron density observed at the active site of protomers in the apo-form could be fitted with a phosphate ion and two water molecules. These are bound close to the positions corresponding to the PLP phosphate and substrate carboxyl-binding sites, respectively, of the holo-form. Unlike in the holo-form, density corresponding to a Tris ion is absent. As mentioned above, a stretch of 32 residues (262–293 residues, which include the disulfide-bonded cysteines) is disordered in the apo-form, leaving the active site partially unstructured and exposed to the solvent. In the holo-form, this region is mostly ordered and occurs as a helix (H12, residues 265–272) followed by a loop, and only five residues remain disordered (Fig. 4 and C). These results indicate that the structure undergoes disorder to order transition upon binding PLP. The corresponding loop in LSD has also been shown to assume different conformations in the holo- and apo-forms (35). Residues 31–33 and Asp120 are near the active site of the enzyme and are in different conformations in apo- and holo-forms. Residues 31–33 are part of the loop that interacts with the PLP phosphate in the holo structure, and the side chain of Asp120 is hydrogen bonded to the main chain N of Gly288, which is disordered in the apo structure. Binding of PLP leads to considerable structural changes leading to shielding of the active site from the bulk solvent and leaving an access channel for the exchange of substrates and products (Fig. 4B).

Side chains of some other residues away from the active site are also in different orientations in the apo and holo structures. These include Trp299, Arg203, Phe15, and Lys14 (Fig. 4D). These changes appear to be triggered by the order-disorder transition between the holo- and apo-forms. Trp299 of the apo structure occupies the same position as that of Ile206, which is ordered only in the holo-form. The changes are largest where the segment 262–293 is ordered upon binding of PLP and become less pronounced toward the surface involved in crystal contacts near Lys14 (Fig. 4D). Interestingly, the electron density for these residues is good in the apo-form, and the associated B-factors are also comparable with the average B-factor for all atoms. In the holo-form, these residues have relatively larger B-factors, and Lys14 is partially disordered in one of the two protomers of the asymmetric unit.

It was found that the holo-dimer is incompatible with the monoclinic crystal packing due to steric clashes involving residues 267–278 (in chains C and D) and Lys14 (in chains A, C, and D). Only the structure with disordered segments resulting from loss of PLP appears to be compatible with the packing arrangement of the monoclinic form. Also, the appearance of only tetragonal crystals in the presence of excess PLP during crystallization provides further evidence that structural changes brought about by loss of PLP favor monoclinic packing arrangement.

**Structures Obtained after Soaking with dL-DAP**—Structure determined from crystals soaked with dL-DAP at pH 8.3 was similar to that of the holo-EcDAPAL except at the active site. Superposition of the corresponding Ca atoms of the two structures resulted in an r.m.s.d. of 0.74 Å. The electron density observed at the active site did not correspond to DAP but to PLP-linked aminoacrylate, a reaction intermediate expected to be formed during the catalytic cycle (Fig. 5A). Orientation of the pyridine ring of PLP in this structure is related to that of the internal aldimine form by a rotation of 32° (Fig. 5B). In spite of this rotation, the CH-π interaction with Phe76 is maintained. Lys77 side chain is free in this structure, and its ε-amino group is hydrogen bonded to the hydroxyl group of Tyr206, side chain carboxyl of Asp139, and phosphate group of PLP. There is no density for Tyr168 side chain in the A subunit. Apart from these, all other active site residues have conformations similar to those of the holo-EcDAPAL structure. The carboxyl group of aminoacrylate makes hydrogen bonding interactions with the side chain hydroxyl of Thr119 and the main chain N of Asp120 and His123. Even though the overall structure of the complex is similar to that of holo-EcDAPAL, well defined density was not observed for the region consisting of residues 103–112 and 104–110, in chains A and B, respectively. This region corresponds to helix H6 (residues 102–107) and the subsequent loop.

EcDAPAL is the first example of a single substrate fold type II PLP-dependent enzyme in which an aminoacrylate has been observed at the active site. PLP-aminoacrylate complex has been observed in five other PLP-dependent enzyme structures determined earlier (41–46). Occurrence of aminoacrylate at the active site of EcDAPAL could be due to the reduced rate of the catalysis in the crystallization condition (see under “Activity Assay and Spectral Studies”). In fold type II enzymes with bound aminoacrylate, the residues corresponding to Lys77 and Thr119 of EcDAPAL are conserved. Except for these similarities, the active site of EcDAPAL is considerably different from those of other enzymes with bound aminoacrylate.

 Structures obtained for crystals soaked with dL-DAP at pH 6.5 did not reveal additional density at the active site corresponding to DAP or aminoacrylate, unlike the crystals soaked with dL-DAP at pH 8.3. The structure is similar to that of holo-EcDAPAL. PLP is linked to the enzyme in the internal aldimine form, and density corresponding to Tris ion is present. Biochemical studies have indicated that the enzyme degrades DAP much more slowly at pH 6.5 than at pH 8.3. Absence of density corresponding to the ligand at the active site indicates that lowering of pH not only affects catalysis but also reduces the affinity of the enzyme for the substrate. Good electron density corresponding to Tris ion in both the structures (for crystals soaked with dL-DAP at pH 8.3 and 6.5) indicates that binding of Tris is not affected by pH.

**Structures Obtained after Soaking with d-Ser and l-Ser**—Structures of EcDAPAL were also obtained after soaking the crystals in the presence of d- and l-Ser. Both are substrates for EcDAPAL, although the reaction rates are much lower when compared to that with DAP. In these structures, PLP is bound to the enzyme in the internal aldimine form as in the holo-
FIGURE 5. Stereo diagrams showing active site of EcDAPAL-ligand complexes. A, modeled aminoacrylate with the corresponding electron density ($2F_{o} - F_{c}$, contoured at 1σ) at the active site in EcDAPAL crystals soaked with DAP. PLP-aminoacrylate complex is labeled as PLP-AA. Hydrogen bonds are shown as dashed lines. B, superposition of the active sites of EcDAPAL (dark gray) and EcDAPAL aminoacrylate complex (light gray). Distances of active site residues from the Ca of aminoacrylate are shown (light gray dashes). C, D-Ser with corresponding electron density ($2F_{o} - F_{c}$, contoured at 1σ) at the active site of crystals soaked with D-Ser. Tris ion bound in the EcDAPAL structure (not soaked with ligands) is shown in a line representation, to illustrate the overlap between the Tris and the substrate-binding site.
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TABLE 2

Kinetic parameters of EcDAPAL and its mutants with D- and L-DAP

|           | D-DAP                      | L-DAP                      |
|-----------|----------------------------|----------------------------|
|           | $K_m$ (μM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (min$^{-1}$ μM$^{-1}$) | $K_m$ (μM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (min$^{-1}$ μM$^{-1}$) |
| EcDAPAL   | 0.05 ± 0.002 | 2.675 ± 15 | 5.35 × 10$^4$ | 0.03 ± 0.002 | 1.476 ± 76 | 4.92 × 10$^4$ |
| D120N     | ND*                  | ND                      | ND                   | 15.57 ± 0.05 | 183 ± 5    | 3.21 × 10$^4$ |
| D189N     | 12.9 ± 2.3          | 21 ± 4                | 1.6                  | 11.5 ± 4.4 | 11 ± 2     | 0.96            |

*ND means not determined. Activity of D120N EcDAPAL was not detectable with D-DAP as substrate.

EcDAPAL structure. r.m.s.d.s of corresponding Cα atoms upon superposition of D- and L-Ser-soaked structures with the native EcDAPAL structure were 0.35 and 0.32 Å, respectively.

Structure from crystals soaked with D-Ser had additional electron density at the substrate-binding site. A molecule of D-Ser could be fitted into the density with its side chain partially occupying the Tris-binding site (Fig. 5C). Density corresponding to a Tris ion was absent in this structure. The substrate carboxyl interacts with the residues of the conserved carboxyl-binding loop. In the external aldimine form, the amino group of D-Ser is expected to form a Schiff base with the PLP. The amino nitrogen of D-Ser is at the distance of ~3.5 Å from the C4 ‘atom of PLP and is hydrogen bonded to the side chain carboxyl of Asp189 and phosphate group of PLP. Asp189 or phosphate group of PLP might be important for activation (deprotonation) of the substrate (DAP) had a reaction intermediate bound at the active site. Consistent with this observation, biochemical assays have shown that the $K_m$ value for L-Ser is much higher than that for D-Ser (12).

Spectral Observations and Activity Measurements—The structure of EcDAPAL obtained from crystals soaked with the substrate (DAP) had a reaction intermediate bound at the active site. Consistent with this observation, the $k_{cat}$ value of the enzyme was 360 times lower for D-L-DAP in the crystallization solution ($k_{cat} = ~5.6$ min$^{-1}$) as compared with that in phosphate buffer ($k_{cat} = ~2.038$ min$^{-1}$). The enzyme showed no activity with D- or L-Ser in the crystallization solution in conformity with the observations on the structures of D-Ser and L-Ser EcDAPAL complexes.

Spectral properties of EcDAPAL have earlier been characterized in phosphate buffer (14). Similar experiments were carried out in the crystallization solution. In phosphate buffer at pH 7.5, enzyme showed a characteristic absorbance maximum at 412 nm, corresponding to the holo-form with PLP bound as an internal aldimine (supplemental Fig. S2A). Upon addition of 10 mM DL-DAP, a red shift to 422 nm and a peak at 317 nm were observed within 15 s. The absorbance maximum reverted to 412 nm and the absorbance at 317 nm stabilized within 10 min (supplemental Fig. S2A). In contrast, upon addition of 10 mM DAP in the crystallization condition, no change in the absorbance at 412 nm was observed, and the peak at 317 nm corresponding to pyruvate increased with time at a much lower rate (supplemental Fig. S2B). This suggests that under the crystallization condition, the reaction rate is greatly reduced.

Residues that are in close proximity to Cα of the bound substrate were mutated by site-directed mutagenesis. The residues Asp120 and Asp189 were individually mutagenized to Asn, and Lys77 was mutated to both His and Arg. The mutant enzymes were overexpressed and purified by procedures similar to that used for the wild type enzyme. The purified proteins were checked for the presence of PLP by estimating the absorbance at ~412 nm. No detectable absorbance at 412 nm was observed for the Lys mutants, indicating that these did not bind PLP. This illustrates the critical role of Lys77 in anchoring PLP at the active site. In the structure of the L-form of the substrate. D189N mutant showed only marginal activity toward both the substrates, as expected by their inability to bind PLP. Kinetic parameters obtained for mutants D120N and D189N are compared with those of wild type EcDAPAL in Table 2. Although D120N EcDAPAL had measurable activity with L-DAP, the catalytic efficiency ($k_{cat}/K_m$) was reduced by 150-fold as compared with that of native holo-EcDAPAL. No activity could be detected with D-DAP as substrate. In contrast, for the wild type enzyme, the catalytic efficiency was nearly the same toward both the stereo isomers (Table 2). Thus, the mutation D120N resulted in a stereospecific enzyme that is active (although lower) only toward the L-form of the substrate. D189N mutant showed only marginal activity with both the isomers. The catalytic efficiency was 4 orders of magnitude lower with both D- and L-DAP as substrates.

To further probe into the effect of mutations on the activity of the enzyme, the visible absorbance spectra of the wild type and mutant enzymes were recorded as a function of time upon addition of substrates. Fig. 6, A and B, corresponds to the spectra of holo-EcDAPAL incubated with D- and L-DAP, respectively. As observed earlier, there was a red shift in the absorbance maximum from 412 to 422 nm immediately after the addition of both D- and L-DAP (Fig. 6, A and B, red line). With increase in time, this peak reverted back to 412 nm with concomitant increase in the absorbance at 317 nm (because of the product pyruvate). Addition of D-DAP to D120N also resulted in a shift of absorbance maximum from 412 to 422 nm, indicating the formation of ketoenamine form of PLP external aldimine (47). However, no further changes in the spectrum were observed with change in time (Fig. 6C). The small peak
observed at 340 nm may correspond to enolimine tautomer of PLP (47). The peak at 422 nm was higher than the peak at 412 nm in contrast to that of the wild type enzyme spectrum. Addition of L-DAP to the D120N also resulted in a similar red shift and increase in absorbance at 422 nm, which reverted to lower wavelengths very slowly (Fig. 6). As with the wild type enzyme, increase in absorbance at 317 nm due to the formation of pyruvate could be observed. The spectral changes observed when D- or L-DAP was added to the D189N mutant were similar (Fig. 6, E and F). There was a red shift of the peak to 422 nm. The shifted peak had lower intensity and the peak height reduced marginally with time. The slight increase in absorbance in the 317–340-nm region could be a combined effect of product formation and enolimine tautomer of PLP. Taken together, these results suggest that Asp120 might be involved in a step beyond the formation of external aldimine, probably in the stereo-specific proton abstraction from C\(^\text{H}9\) of D-DAP.

Substrate Binding and Plausible Catalytic Mechanism—It was shown earlier that EcDAPAL has a narrow reaction and substrate specificity (14–16). However, the enzyme can act on both the D- and L-forms of the substrate. In PLP-dependent deaminases, after the formation of external aldimine, the reaction is initiated by abstraction of C\(\alpha\) proton by a general base catalytic group. The ability to recognize both stereo forms of the substrate has been accounted for by either a single base or a dual base mechanism in other enzymes (48). The single base mechanism requires the two isoforms of the substrate to bind in different orientations at the active site such that the same group on the protein can carry out deprotonation. In the dual base mechanism, the two forms of the substrate are held at the active site by the same set of interactions, but the enzyme must have two suitably positioned bases for proton abstraction. Examination of the substrate binding pocket of EcDAPAL (Fig. 3) and the mode of binding of aminoacrylate (Fig. 5, A and B) and D-Ser (Fig. 5C) suggests that both D- and L-forms of the substrate are likely to be held by same set of interactions by the carboxyl-binding loop of the enzyme. In the external aldimine, the \(\alpha\)-amino group of the substrate is covalently bonded to the C4' atom of PLP. Therefore, the proton to be abstracted will point in nearly opposite directions for the two enantiomeric forms of the substrate. The side chains of four residues (Asp\(^{120}\), Asp\(^{189}\), Tyr\(^{168}\), and Lys\(^{77}\)) are close to the bound substrate and could function as bases for proton abstraction. Although Lys\(^{77}\) is covalently linked to PLP in the internal aldimine form, it is free
to abstract Cα proton from the PLP substrate external aldimine. Side chain functional groups of Asp120, Asp189, Lys77, and Tyr168 are at distances of 3.4, 4.1, 4.4, and 5.4 Å, respectively, from the Cα of aminoacrylate. The distance and orientation of Tyr168 is not appropriate for the abstraction of the proton. Furthermore, Tyr168 is not conserved in SR and LSD, indicating that it is not crucial for catalysis. Asp120 corresponds to a threo- 

Conclusions—The work presented here has revealed key differences between EcDAPAL and other fold type II PLP-dependent enzymes. EcDAPAL is a dimeric protein with an interface that has not been observed earlier in other fold type II PLP-dependent enzymes. EcDAPAL has a novel disulfide bond close to the site at which a metal ion binds in other fold type II PLP-dependent enzymes. The disulfide bond or the metal-binding site could be important for stabilizing the loops lining the substrate channel. Structures of the PLP-bound and -unbound form of the enzyme highlighted important structural differences near the active site and substrate channel, which might represent the transition that the enzyme undergoes upon binding of PLP. Unlike other homologs (which undergo transition from open to closed form on substrate binding), all structures of EcDAPAL resemble a closed form. Examination of catalytic properties of EcDAPAL and active site geometry of the holo-form suggests that the Tris ion is likely to be an inhibitor of PLP-dependent enzymes. Soaking EcDAPAL with D-Ser revealed a molecule of D-Ser bound at the active site without forming a Schiff base linkage to PLP. This represents the initial mode of binding of substrate to the active site before being covalently attached to PLP. Structure determined from EcDAPAL crystals soaked with D,L-DAP provides direct evidence for the formation of aminoacrylate intermediate in the reaction. The work has highlighted the importance of Lys77, Asp120, and Asp189 in the function of the enzyme. Asp120 and Lys77 are likely to be the residues responsible for proton abstraction from Cα of D- and L-DAP,
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respectively. Further structural studies on mutant enzymes and their complexes will provide deeper insights into this unique PLP-dependent enzyme.

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