Crystal Structure of *Escherichia coli* PdxA, an Enzyme Involved in the Pyridoxal Phosphate Biosynthesis Pathway*

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Pyridoxal 5'-phosphate is an essential cofactor for many enzymes responsible for the metabolic conversions of amino acids. Two pathways for its *de novo* synthesis are known. The pathway utilized by *Escherichia coli* consists of six enzymatic steps catalyzed by six different enzymes. The fourth step is catalyzed by 4-hydroxythreonine-4-phosphate dehydrogenase (PdxA, E.C. 1.1.1.262), which converts 4-hydroxy-L-threonine phosphate (HTP) to 3-amino-2-oxopropyl phosphate. This divalent metal ion-dependent enzyme has a strict requirement for the phosphate ester form of the substrate HTP, but can utilize either NADP+ or NAD+ as redox cofactor. We report the crystal structure of *E. coli* PdxA and its complex with HTP and Zn2+.

The protein forms tightly bound dimers. Each monomer has an α/βαα'-fold and can be divided into two subdomains. The active site is located at the dimer interface, within a cleft between the two subdomains and involves residues from both monomers. A Zn2+ ion is bound within each active site, coordinated by three conserved histidine residues from both monomers. In addition two conserved amino acids, Asp242 and Asp267, play a role in maintaining integrity of the active site. The substrate is anchored to the enzyme by the interactions of its phosphogroup and by coordination of the amino and hydroxyl groups by the Zn2+ ion. PdxA is structurally similar to, but limited in sequence similarity with isocitrate dehydrogenase and isopropylmalate dehydrogenase. These structural similarities and the comparison with a NADP+-bound isocitrate dehydrogenase suggest that these coenzyme-binding modes of PdxA are very similar to that of the other two enzymes and that PdxA catalyzes a stepwise oxidative decarboxylation of the substrate HTP.

Pyridoxal 5'-phosphate, the catalytically active form of vitamin B<sub>6</sub>, is an important cofactor for many enzymes responsible for the metabolic conversions of amino acids. Vitamin B<sub>6</sub> (pyridoxine) and its derivatives are also efficient singlet oxygen quenchers and potent fungal antioxidants (1). Two different pathways for *de novo* synthesis of pyridoxine are now recognized. One of these, found in all Archaea, eukaryotes, and in some bacteria, uses the singlet oxygen resistance (SOR1(Pdx1)) gene product, a highly conserved enzyme (2). A number of eubacteria, including *Escherichia coli*, utilize a specific pathway for pyridoxal phosphate synthesis that is distinct and has been characterized for some time (3). Analysis of sequences from several genomes has revealed that organisms may encode either the SOR1 or *E. coli*-like pyridoxine biosynthesis genes, but not both (4).

The pyridoxal 5'-phosphate biosynthesis pathway has been well characterized in *E. coli*, and consists of six enzymatic steps, beginning with erythrose 4-phosphate and deoxyxylulose 5-phosphate (5–7). All of the enzymes in this pathway have been identified. Of the six enzymes, PdxA<sup>3</sup>, *erythro*-4-phosphate dehydrogenase (PdxB), and pyridoxine-5'-phosphate oxidase are unique to this pathway (5), whereas SerC and GapA also function in other biosynthetic pathways (5).

The fourth step in this pathway is catalyzed by PdxA (E.C. 1.1.1.262), which converts 4-hydroxy-L-threonine 4-phosphate (HTP) to 3-amino-1-hydroxyacetone 1-phosphate, an oxidative decarboxylation that may involve 2-amino-3-keto-4-hydroxybutyric acid 4-phosphate as an intermediate (8) (Scheme 1). Either NADP<sup>+</sup> or NAD<sup>+</sup> function as cofactors, whereas the free alcohol 4-hydroxy-L-threonine is not a substrate for the reaction (8). The product of the PdxA reaction is used along with deoxxyxulose 5-phosphate by pyridoxine-5'-phosphate synthase (PdxJ) to generate pyridoxine 5'-phosphate along with inorganic phosphate. This key enzyme functions in closure of the aromatic pyridoxine ring (6, 9, 10).

Treatment of purified PdxA with 1 mM EDTA abolishes oxidation of HTP, suggesting the presence of a tightly bound divalent metal ion. Addition of 1 mM Mn<sup>2+</sup>, Mg<sup>2+</sup>, or Ca<sup>2+</sup> restores full activity (9), whereas 1 mM Ni<sup>2+</sup> or 2 mM Zn<sup>2+</sup> restored half of the original PdxA activity. Although the product of the reaction, 3-amino-1-hydroxyacetone 1-phosphate, undergoes facile dimerization in the absence of PdxJ and the co-substrate deoxyxylulose phosphate, we have directly detected 3-amino-1-hydroxyacetone 1-phosphate by electrospray ionization mass spectrometry of PdxA incubation mixtures.<sup>2</sup> None of the putative intermediate, 2-amino-3-oxo-4-hydroxybutyric acid 4-phosphate, could be detected in the reaction mixture, even as early as 30 s after initiation of the reaction.

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<sup>1</sup>The abbreviations used are: PdxA, 4-hydroxythreonine-4-phosphate dehydrogenase; HTP, 4-hydroxy-L-threonine phosphate; PdxJ, pyridoxine-5'-phosphate synthase; r.m.s., root mean square.
<sup>2</sup>J. Banks and D. E. Cane, unpublished observations.
suggestions that if this compound is formed, it never leaves the PdxA active site.

In principle, PdxA might catalyze either a stepwise or a concerted oxidative decarboxylation of HTP. For example, threonine dehydrogenase catalyzes the biochemically similar, reversible NAD-dependent oxidation of L-threonine to L-2-amino-3-ketobutyrate (Scheme 2) (11). The product, L-2-amino-3-ketobutyrate, which is normally converted to glycine and acetyl-CoA by 2-amino-3-ketobutyrate CoA ligase, can undergo pH-dependent decarboxylation, with a half-life ranging from 8.6 min at pH 5.9 to 140 min at pH 11.1 (12). PdxA and threonine dehydrogenase show no significant amino acid sequence similarity. By contrast, use of the PSI-BLAST sequence comparison algorithm (13) reveals ~30–35% sequence identity over a short ~25 residue segment between E. coli PdxA and both isocitrate dehydrogenase and 3-isopropylmalate dehydrogenases (9). Interestingly, each of the latter two enzymes catalyzes a nicotinamide- and divalent metal ion-dependent oxidative decarboxylation of a β-hydroxy acid substrate (Scheme 2). Significantly, the isocitrate dehydrogenase reaction has been shown to proceed by a stepwise mechanism that involves the corresponding 3-keto acid, oxaloacetic intermediate, based on multiple isotope effect (2H, 13C) studies, as well as the ability of isocitrate dehydrogenase to catalyze both the decarboxylation and the reduction of oxaloacetate, with a ~10-fold preference for decarboxylation (14).

Structural characterization of the enzymes of the pyridoxine biosynthetic pathway has so far been limited to E. coli PdxJ (15–17) and pyridoxine-5'-phosphate oxidase from Saccharomyces cerevisiae (Protein Data Bank number 1C1O), the enzyme that oxidizes pyridoxine 5'-phosphate and pyridoxamine 5'-phosphate to pyridoxal 5'-phosphate. The x-ray structure of PdxA bound to Zn$^{2+}$ as well as the HTP presented here reveals a protein that can be divided into two subdomains, and which shares structural similarities with both isocitrate dehydrogenase and isopropylmalate dehydrogenase. These structural similarities and, in addition, sequence conservation between PdxA and the two dehydrogenases of residues involved in the cofactor binding argue for a similar manner of nicotinamide cofactor binding and similar, stepwise, biochemical mechanisms for oxidative decarboxylation. Furthermore, based on the complex with HTP in the presence of Zn$^{2+}$, sequence conservation analysis among PdxA enzymes from different sources, and structural comparison with the isocitrate dehydrogenase family, the location of the PdxA active site is stipulated to be in a cleft between the two subdomains and at the interface between the two molecules of the dimer, involving a cluster of strictly conserved amino acids.

MATERIALS AND METHODS

Cloning, Expression, and Purification—The pdxA gene was cloned into a derivative of the pET-15b vector (Amersham Biosciences) containing a thrombin cleavage site to obtain an in-frame N-terminal fusion with a His$_6$ purification tag. Plasmid DNA was transformed into the E. coli methionine auxotroph DL41 for selenomethionine protein production (18). The transformed bacteria were grown at 37 °C to an A$_{600}$ of ~0.8 in Lb/LeMaster medium supplemented with 25 mg/liter of L-methionine. A 1-liter culture was induced with 100 μM isopropyl-β-D-thiogalactoside and the culture was continued at room temperature for an additional 15 h.

Cells were harvested by centrifugation (4000 x g, 4 °C, 25 min) and were re-suspended in 40 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 0.4 M NaCl, 5% (w/v) glycerol, 20 mg/ml imidazole, 10 mg/β-mercaptoethanol) containing one dissolved tablet of Complete™ protease inhibitor mixture (Roche Diagnostics). Cells were lysed by sonication on ice for a total of five 30 s cycles with 45 s between each cycle for cooling. The lysate was then cleared by centrifugation (100,000 x g, 4 °C, 30 min). The protein supernatant was first loaded on a 5-ml DEAE-Sepharose (Pharmacia) column equilibrated in lysis buffer and the flow-through fraction was collected. This protein was then applied to a 5-ml nickel-nitrilotriacetic acid (Qiagen) column, pre-equilibrated with lysis buffer. The column was washed extensively with buffer (50 mM Tris, pH 7.5, 50 mM imidazole, 0.4 M NaCl) and bound proteins were eluted with the same buffer containing 150 mM imidazole. Purified PdxA ran as a single band on both SDS-PAGE and native PAGE gels. Dynamic light scattering measurements were performed using a DynaPro801 molecular sizing instrument (Protein Solutions) at a protein concentration of 8.1 mg/ml$^{-1}$ and were carried out at 22 °C.

Crystalization and Data Collection—Two crystal forms of PdxA were obtained. Form I crystals were obtained with the intact His-tagged, selenomethionine-labeled protein by hanging drop vapor diffusion. A volume of 2 μl of protein solution (8.1 mg ml$^{-1}$) in buffer (20 mM Tris-HCl, pH 8, 0.2 M NaCl, 10 mM dithiothreitol, 5% (w/v) glycerol) was mixed with 4 μl of reservoir solution (7.5% (w/v) PEG 8000, 0.1 M NaOAc buffer, pH 5.5, 10 mM MgCl$_2$, 10 mM Na$_2$PO$_4$, pH 5.9) and suspended over the reservoir solution. Crystals grew to a size of 0.05 x 0.1 x 0.3 mm$^3$ in 1 day. These crystals are orthorhombic, space group P2$_1$2$_1$2$_1$, with cell dimensions a = 75.5, b = 79.3, c = 114.2 Å, and two molecules in the asymmetric unit. These crystals diffracted to ~2 Å resolution.

Form II crystals were obtained from the same starting protein solution using 20% (w/v) PEG 8000, 100 mM Hepes, pH 7.5, 75 mM citrate, and 100 mM MgCl$_2$ as a reservoir condition. These crystals belong to the monoclinic system, space group P2$_1$, with cell dimensions a = 92.1, b = 76.7, c = 95.4 Å, β = 109.7°, and four molecules in the asymmetric unit. These crystals diffracted to 2.45-Å resolution.

Crystals were briefly soaked in a cryoprotectant solution consisting of mother liquor supplemented with 20% (w/v) glycerol, picked up in a nylon loop, and flash cooled at 100 K in a N$_2$ gas cold stream (Oxford Cryosystems, Oxford, United Kingdom). Data were collected at beamline X8C, NSLS, Brookhaven National Laboratory using a Quantum 4-CCD detector (ADSC). All data sets, including Se multiwavelength anomalous diffraction data (Table I) were processed using the program HKL2000 (19).

Structure Solution and Refinement—Crystals of form I were used for structure determination. For phase determination, the resolution range from 12 to 2.4 Å was chosen. Twelve of the expected 16 Se sites within the asymmetric unit were found using the program SOLVE (20). The resulting phases gave an overall figure of merit of 0.58. Further improvement of the phases was achieved by using the program RESOLVE.
difference Fourier map, and was modeled using the program O. Refine-

Electron density corresponding to the HTP product was observed in the

tein Data Bank codes 1PTM, 1PS7, and 1PS6, respectively.

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and the complex of form I with HTP have been deposited with the

Å

for each monomer, one Zn2+

complex and crystal form II (P

early stages of refinement, but were removed as the resolution was

starting model built automatically using RESOLVE. Remaining parts

starting electron density map was of good quality with 69% of the

(21), resulting in an increase of the overall figure of merit to 0.77. The

Mean figure of merit for phasing = 0.58.

| Data set | Inflection point | Peak | Hard remote | HTP* complex | Crystal form II  
|----------|-----------------|------|-------------|--------------|----------------|
| Resolution range (Å) | 99.1-91.96 | 99.1-91.96 | 50.1-89.26 | 50.2-2.25 | 50.2-2.45 |
| Wavelength (Å) | 0.979322 | 0.979289 | 0.964092 | 1.5418 | 0.9950 |
| Observed hkl | 348,964 | 523,377 | 322,938 | 157,588 | 232,767 |
| Unique hkl | 95,128 | 95,296 | 99,161 | 29,976 | 44,115 |
| Completeness (%) | 99.5 | 99.6 | 98.7 | 90.3 | 94.6 |
| Overall R(work) | 11.1 | 12.7 | 11.0 | 16.2 | 9.4 |
| Rwork (%f) | 10.0 | 9.5 | 9.3 | 12.1 | 9.2 |
| Rwork resolution (Å) | 45.1-96.1 | 50.2-2.25 | 45-2.45 |
| Rfactor, (No. reflections) | 26.7 (2846) | 25.8 (2087) | 27.2 (2204) |
| Rfactor, (No. reflections) | 21.7 (85,302) | 20.6 (27,869) | 21.3 (41,903) |
| R.m.s. deviation bond lengths (Å) | 0.008 | 0.007 | 0.008 |
| R.m.s. deviation bond angles (°) | 1.6 | 1.4 | 1.5 |
| Average B-factors (Å²) (No. atoms) | 20.9 (2823) | 32.6 (2624) | 19.5 (5248) |
| Main chain atoms | 21.8 (2236) | 32.4 (2190) | 19.3 (4329) |
| Side chain atoms | 21.3 (4864) | 32.5 (4814) | 19.4 (9577) |
| Overall protein | 26.2 (263) | 35.6 (188) | 25.7 (454) |
| Solvent (No. waters) | 22.9 (2, Zn) | 42.6 (13, HTP) | 31.0 (4, Zn) |
| Mean coordinate error from | 23.5 (1, PO4) | 38.5 (2, Zn) |
| cross-validated Luzatti plot (Å) | 0.30 | 0.35 | 0.39 |

* PdxA crystal soak with 7 mM HTP using the P212121 form of native crystals.

† P21 crystal form of the selenomethionine PdxA. Complete multianal wavelength anomalous diffraction data set collected on the P212121 crystal form.

‡ Rwork = |Fobs| − |Fcalc| / |Fcalc|, Rfree = |Fobs| − |Fcalc| / |Fcalc| when Fcalc is omitted as for the first few and last few reflections.

§ Rfree = Rwork, but random sets of 3, 7, and 5% of the unique reflections not included in the refinement for P212121 form of native crystals.

### RESULTS

**Structure of the PdxA Monomer**—The structure of selenomethionine-labeled PdxA from *E. coli* was solved by the multianal wavelength anomalous diffraction and refined to a

final R-factor of 0.217 (Rfree = 0.267) at 1.96-Å resolution for the orthorhombic crystal form of native PdxA, R-factor of 0.213 (Rfree = 0.272) for the monoclinic crystal form of native PdxA at 2.45-Å resolution, and an R-factor of 0.206 (Rfree = 0.258) for the complex of PdxA with HTP at 2.25-Å resolution. These models have been refined with good stereochemical parameters (Table I). Statistics for the Ramachandran plot from an analysis using PROCHECK (25) for the model gave over 90% of non-glycine residues in the most favored region.

**E. coli** PdxA has an α/β/β architecture with a central 12-stranded mixed β-sheet flanked on both sides by α-helices. The β-strand order within this sheet is 3 1 -4 -1 -2 -1 -5 -12 -11 -6 -7 -10 -8 -9 (Fig. 1) with the first six strands being parallel, followed by two antiparallel strands and four parallel strands running in the opposite direction to the first strands. This extended sheet is twisted along its longitudinal axis (perpendicular to the strands) by −180°, so that the first few and last few strands while antiparallel, run in the same direction (Fig. 2). There are nine α-helices longer than one turn, aligned nearly parallel to the β-strands. Five helices line
one side of the sheet, whereas three helices lie along the other side of the sheet in its N-terminal part. Although there is no clear distinction of domains, the molecule could be divided into two subdomains, with residues Ala²-Lys¹⁴⁷ and Leu²⁸⁸-Ala³²⁹ forming subdomain 1 and Lys¹⁴⁸-Gly²⁸⁷ forming subdomain 2. The N and C termini are within subdomain 1 and are in close proximity to each other, separated by only 9.5 Å. The substrate-binding site is located near the middle of the β-sheet at the interface between the two subdomains, on the side with fewer α-helices. The overall fold of PdxA is clearly related to that found in the isocitrate/isopropylmalate dehydrogenases (26, 27) as classified in the Structural Classification of Proteins data base (28).

Structure of the PdxA Dimer—Dynamic light scattering measurements indicate that the PdxA molecules form homodimers in solution. This is in contrast to an earlier study where PdxA was characterized as being monomeric based on gel filtration data (8). The presence of homodimers is consistent with the dimeric arrangement observed in the crystal structure, with each dimer having approximate dimensions of 88 × 40 × 32 Å. The two monomers of the dimer are related by a 2-fold non-crystallographic symmetry axis parallel to the crystallographic a axis. The structures of the two independent monomers in the asymmetric unit are very similar, showing root mean square (r.m.s.) deviation of 1.2 Å for all Cα atoms, when refined without non-crystallographic symmetry restraints. Independent superposition of subdomains 1 or 2 gives an r.m.s. deviation of ~0.6 Å indicating that there is a small difference in the arrangement of the two subdomains relative to one another in the independent molecules.

The dimer is formed through the interactions of subdomains 2 of the PdxA monomers. These interactions are mediated by the α8 helices (residues 269–275) from each monomer and additional contacts from residues in the loops following parallel β-strands β7, β8, β9, and β10 (Fig. 3). There are a total of 16 hydrogen bonds (<3.2 Å), including 6 salt bridges, and a network of hydrophobic interactions. Analysis of the multiple sequence alignment for enzymes showing sequence similarity to PdxA (PFAM accession number PF04166) reveals that the residues found at the dimer interface are well conserved, suggesting that the observed mode of dimerization is common to all members of this family. A pair of Zn²⁺ ions is observed at the dimer interface, with residues from both monomers contributing to the coordination environment for each Zn²⁺ site (Fig. 3).
The surface area buried upon dimer formation calculated using a 1.4-Å probe radius is 1240 Å² or ~12% of the total surface area of each monomer.

Sequence and Structural Similarity—PdxA from E. coli and its orthologs form a conserved family of bacterial enzymes, and contain in particular the γ-subdivision of proteobacteria (7). Sequence analysis using PSI-BLAST (13) finds homologous sequences in 73 bacterial species and one similar sequence in the archaeon Ferroplasma acidarmanus. The sequence identity between E. coli PdxA and the bacterial orthologs varies from 93% for Salmonella to 27% for Burkholderia fungorum. There is 31% sequence identity between the E. coli PdxA and that from archaeal F. acidarmanus. The conserved residues cluster in the substrate- and cofactor-binding areas as well as at the dimerization interface (Fig. 3).

Structural comparison of E. coli PdxA with other protein structures was performed using the program DALI (29). Clearly the most structurally similar proteins were found to be isocitrate dehydrogenase (Protein Data Bank code 1ISO, Z = 16.5, r.m.s. of 4.0 Å for 244 C atoms) and isopropylmalate dehydrogenase (Protein Data Bank code 1CNZ, Z = 16.3, 5.1 Å for 249 C atoms). The limited amino acid sequence similarity between PdxA and the other two proteins evident from PSI-BLAST comparisons, as summarized above, has been previously noted (9). Notably, all three proteins catalyze analogous decarboxylative dehydrogenations of 3-hydroxy acids.
perposition of PdxA with the two above mentioned dehydrogenases shows that nine β-strands and seven α-helices are structurally and topologically equivalent in these proteins. With more stringent conditions 122 Cα atoms of PdxA, mostly from the β-strands, can be superimposed on IISO with an r.m.s. deviation of 1.65 Å (sPDBv (30)). Furthermore, the dimerization interface of PdxA and the other two dehydrogenases involves the same regions of the proteins and is similar overall. Structural alignment shows 16 identical residues of 329 for PdxA common to all three sequences (Fig. 4).

**Zn²⁺ Binding Sites**—In the electron density map of the native protein there are two strong peaks corresponding to metal ions. Based on the coordination and the type of liganding side chains we have interpreted these peaks as two Zn²⁺ ions. As no Zn²⁺ ions were explicitly part of the crystallization solution, these metal ions must have been acquired during expression in *E. coli* cells and remained associated with the enzyme throughout the purification and crystallization process. The presence of a tightly bound divalent metal cation has been previously reported by Cane *et al.* (9) as summarized earlier. The observed Zn²⁺ ions are at the dimer interface and are coordinated by His¹⁶⁶ and His²⁶⁶ from one monomer and His¹¹¹ from the second monomeric unit. The almost perfect octahedral coordination sphere of each Zn²⁺ atom is completed by three water molecules (Fig. 5). The distances between the Zn²⁺ and the liganding nitrogen or oxygens range from 2.1 to 2.3 Å. These three histidines are strictly conserved in all 74 homologous amino acid sequences indicating that all enzymes from this family are most likely Zn²⁺-dependent enzymes (PFAM family PF04166).³ The coordination of each Zn²⁺ ion by absolutely conserved histidine residues coming from both monomers of the dimer provides strong indication that dimerization is essential for enzymatic activity.

**Substrate Binding Site**—We have soaked the native crystals in a solution containing 7 mM substrate, HTP. The difference electron density map clearly showed a substrate molecule bound to one of the two molecules of the PdxA dimer (Fig. 6a). The HTP binds in a deep depression in the surface of the molecule. One side of this depression is lined by residues from the second monomer of the dimer, which converts it into a deep and rather narrow cleft (Fig. 6b). Superposition of the two independent molecules of the dimer shows that they differ somewhat in the disposition of the two subdomains. The molecule of HTP is bound to the monomer that has a less open conformation. In the native crystals of PdxA the same monomer subunit binds an inorganic phosphate ion in exactly the same place as the phosphate group of HTP. The somewhat larger opening of this cleft in the second subunit causes small displacements of atoms that would otherwise provide hydrogen bonds to the phosphate group, abolishing phosphate binding.

The incoming HTP molecule displaces the phosphate anion as well as the three water molecules that complete the Zn²⁺ coordination sphere. The phosphate group of the HTP replaces the inorganic phosphate ion, whereas the N and OG (hydroxyl oxygen) atoms replace the water molecules, resulting in a penta-coordinated central Zn²⁺ ion (Fig. 6c). In this HTP-Zn²⁺ complex the N, C-2, C-3, and OG atoms are essentially constrained to a common plane, leading to the eclipsed conformation of the scissile C1–C2 and C3–H bonds. This arrangement also establishes the potential for a hydrogen bond between the N atom of the HTP and Ne-2 of His¹⁶⁶ and between OG of HTP and the Ne-2 atoms of both His¹⁶⁶ and His¹¹¹, the latter protruding from the other monomeric subunit of the dimer. In addition, the carboxylate group of HTP is hydrogen bonded to the conserved residue Lys²⁷⁴ and the phosphate group is hydrogen bonded to Arg²⁹⁵ (conserved in ~90% of sequences) as well as to the backbone NH of Thr¹³⁷, found within the highly conserved sequence (Gly-His-Thr-Glu) (Fig. 6c).

**Putative NAD(P) Binding Site**—The PdxA enzyme utilizes NAD⁺ or NADP⁺ as the cofactor for oxidation of HTP. Although we grew crystals of PdxA in the presence of up to 10 mM of either of these cofactors, with and without HTP, in no case could electron density corresponding to the nicotinamide cofactor be found in the map. Efforts to soak the cofactors into the crystals were also unsuccessful. This may indicate that HTP normally binds first, although this has yet to be demonstrated experimentally. Although we did not directly locate the position of the NAD(P)⁺ cofactor in PdxA, its approximate position can be inferred by a comparison with the structure of the *E. coli* isocitrate dehydrogenase S113E mutant complexed with NADP⁺, isopropylmalate, and magnesium (Protein Data Bank code 1HJ6 (31). In this structure the isopropylmalate moiety of the NADP⁺ cofactor straddles the extended segment Thr³³⁸-Lys³⁴⁴, part of the loop following a β-strand, and the adenine and

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³ www.sanger.ac.uk/cgi-bin/Pfam/getacc?.
nicotinamide rings dip into depressions on either side of the loop. The adenine interacts with His339 on one side and Tyr345 on the other, whereas the nicotinamide packs between Gly340 and the substrate. The 2'-phosphate of the adenosine fragment is on the solvent exposed edge of the NADP. The corresponding part of the structure of isocitrate dehydrogenase, 1HJ6, PdxA and 1HJ6 were superimposed as shown in Fig. 4a, c, the hydrogen bonding network involving HTP and the coordination of Zn\(^{2+}\) ion. d, a model of the active site of PdxA with bound NADP\(^{+}\). The cofactor was modeled as described in b. Dashed lines show the contacts between zinc and liganding atoms and the close contact between C-4 of NADP and C-3 of HTP.

**DISCUSSION**

**Substrate Binding**—The present structure shows that binding of the HTP substrate requires a precise degree of opening between the subdomains and that the flexibility of the PdxA molecule is essential for substrate binding and product release. Moreover, the structure of the PdxA-HTP complex shows that the Zn\(^{2+}\) ion, coordinated by three conserved histidines, plays an essential role in substrate binding and that the phosphate group of HTP contributes substantially to this binding. The observed role of the phosphate group in HTP binding provides a structural explanation for the previous observation that the free alcohol 4-hydroxy-L-threonine is not a substrate for PdxA (8).

**Cofactor Binding and Catalytic Mechanism**—As illustrated in Fig. 6b, the 2'-phosphate of the adenosine fragment is on the solvent exposed edge of the NADP\(^{+}\) and would make few contacts with the protein, therefore explaining the dual specificity of PdxA for either cofactor, NAD\(^{+}\) or NADP\(^{+}\). The nicotinamide ring of the cofactor, if positioned as in isocitrate dehydrogenase, would be in a reasonable proximity to the bound substrate HTP molecule, with the proR face of its C-4 atom positioned ~4 Å from the H-3 proton of HTP that is to be removed during
oxidation (Fig. 6d). The calculated C4...H–C3 angle of $\sim 150^\circ$ in the crude model is consistent with this hypothesis. By comparison, a C4...H–C bond distance of 2.70 Å has been calculated for the reactive ternary nicotinamide-isocitrate complex of isocitrate dehydrogenase (32). Complexation with Zn$^{2+}$ should decrease the pK$_a$ of the C-3 hydroxyl (OG), similar to that in other metal ion-dependent dehydrogenases. Furthermore, because OG of HTP is within hydrogen bonding distance to the Ne-2 of His$^{166}$ and His$^{266}$, which are likely neutral at the pH of crystallization, the proton from this hydroxyl could be taken up by one of the histidines and transferred to the solvent, as these histidines are on the surface of the molecule. His$^{266}$ in addition makes a hydrogen bond to the conserved Asp$^{267}$, which likely assures its proper orientation for coordination of the Zn$^{2+}$ ion. Conservation of Asp$^{247}$ may be explained by its interaction with Lys$^{274}$ and, through a bridging water molecule, with His$^{266}$ and Asp$^{267}$ of the second molecule, therefore contributing to the proper orientation of side chains coordinating the substrate, and at the same time to dimerization.

Comparison with Isocitrate and Isopropylmalate Dehydrogenases—PdxA, isocitrate dehydrogenase, and isopropylmalate dehydrogenase all catalyze nicotinamide- and divalent metal ion-dependent oxidative decarboxylations of 3-hydroxy acids. Both isocitrate dehydrogenase (NAD$^+$ and NADP$^+$-dependent forms) and isopropylmalate dehydrogenase have all been shown to utilize the proR face of the nicotinamide cofactor, corresponding to the same stereospecificity predicted herein for PdxA (14). In contrast to PdxA, both isocitrate dehydrogenase and isopropylmalate dehydrogenase utilize Mg$^{2+}$ instead of Zn$^{2+}$. The comparison of the structures shows that the location of the cation is not the same in these enzymes. Indeed, the three histidines that coordinate Zn$^{2+}$ in PdxA, and are conserved in all PdxA enzymes, are not present in the other two dehydrogenases. In fact, previous kinetic studies of PdxA have shown that substitution of Mg$^{2+}$ for Zn$^{2+}$ results in a 2-fold increase in $k_{cat}$, without establishing which metal is present in the native protein (9). More significantly, the divalent metal in both isocitrate dehydrogenase and isopropylmalate dehydrogenase is complexed between the oxygen atoms of the hydroxyl and α-carboxylate of the substrate. Isocitrate and isopropylmalate are held in nearly identical conformations, with a $\sim 165^\circ$ torsion angle between the scissile C2-H and C3-carboxylate bonds (33), in contrast to the 0$^\circ$ torsion angle observed for the corresponding bonds in HTP. Both the eclipsed and anti-
geometries, however, are consistent with a mechanism in which the scissile C-carboxylate bond in the initially generated 3-keto carboxylate intermediate is essentially orthogonal to the plane of the newly formed carboxyl group, as required for the subsequent decarboxylation step. It is therefore particularly significant that isocitrate dehydrogenase has been shown to catalyze a stepwise oxidative decarboxylation involving the intermediacy of oxalosuccinate (14). Finally, the substrate binding sites of isocitrate dehydrogenase and isopropylmalate dehydrogenase are highly conserved, with the labile carboxylate being bound by interactions with active site Lys and Arg residues and hydrogen bonded to the hydroxyl group of a tyrosine (33), similar to the observed interactions of the carboxylate of HTP with Lys274 and Asn283 as well as a bound molecule of water.

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