A New Arrangement of \((\beta/\alpha)_8\) Barrels in the Synthase Subunit of PLP Synthase*

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Pyridoxal 5′-phosphate (PLP, vitamin B\(_6\)), a cofactor in many enzymatic reactions, has two distinct biosynthetic routes, which do not coexist in any organism. Two proteins, known as PdxS and PdxT, together form a PLP synthase in plants, fungi, archaea, and some eubacteria. PLP synthase is a heteromeric glutamine amidotransferase in which PdxT produces ammonia from glutamine and PdxS combines ammonia with five- and three-carbon phosphosugars to form PLP. In the 2.2-Å crystal structure, PdxS is a cylindrical dodecamer of subunits having the classic \((\beta/\alpha)_8\) barrel fold. PdxS subunits form two hexameric rings with the active sites positioned on the inside. The hexamer and dodecamer forms coexist in solution. A novel phosphate-binding site is suggested by bound sulfate. The sulfate and another bound molecule, methyl pentanediol, were used to model the substrate ribulose 5-phosphate, and to propose catalytic roles for residues in the active site. The distribution of conserved surfaces in the PdxS dodecamer was used to predict a docking site for the glutaminase partner, PdxT.

In the second B\(_6\) biosynthetic pathway (Fig. 1), which exists in some eubacteria and in archaea, fungi, plants, plasmodia, and some metazoa, the carbon skeleton of PLP is synthesized from an intact five-carbon pent(ul)ose unit and an intact three-carbon triose unit (6, 7). Very recently these substrates were shown to be ribulose 5-phosphate (or ribose 5-phosphate) and glyceraldelyde 3-phosphate (or dihydroxyacetone phosphate) (8). The PLP nitrogen is derived from the amide group of glutamine (9). Only two genes have been implicated in this pathway, which we refer to as the PdxS/PdxT pathway for the names of the corresponding gene products in \(B. subtilis\) (10). Homologs of \(pdxS\) are known as \(yaaD\), \(pdx1\), \(SNZ\), \(SOR1\), \(PYROA\), and \(HEVER\), and \(pdxT\) homologs are known as \(yaaE\), \(pdx2\), \(PDX2\), \(SNO\), and \(SNZB\) (11–18). The \(pdxS\)- and \(pdxT\)-like genes are proximal or adjacent to one another in most organisms where the PdxS/PdxT pathway exists.

PdxS-like proteins have among the most highly conserved sequences to emerge from proteome comparisons (19, 20). Our analysis of PdxS-like sequences shows that, among 91 sequences from all kingdoms of life (archaea, eubacteria and eukarya), all pairs of sequences are at least 60% identical, and ~22% of the residue positions are strictly conserved. Sequence searches with PdxS and its homologs do not identify proteins of known structure, although PdxS homology to some members of the \(\beta/\alpha\) barrel superfamly was predicted (19, 21).

PdxT is a homolog of the glutaminase domains (or subunits) of several glutamine amidotransferases (19), in accord with glutamine being the nitrogen source for the PdxS/PdxT pathway. PdxT is a member of the “Triad” family of glutaminase domains, characterized by a conserved Cys-His-Glu triad in the active site (22), and is most similar to the glutaminase domain of imidazole glycerol phosphate synthase (IGPS), an enzyme from the histidine biosynthetic pathway. The expected Triad structure was observed in crystal structures of PdxT from \(B. subtilis\) (21) and from its thermophilic cousin \(Geobacillus stearothermophilus\) (PDB code 1Q7R).

Considerable experimental evidence demonstrates that PdxS/PdxT is a heteromeric glutamine amidotransferase. PdxS and PdxT from \(B. subtilis\) form a physical complex (10), as do their homologs from yeast (20, 23). Glutaminase activity was detected for \(B. subtilis\) PdxT (10) and yeast SNO1 (23), and this activity required the presence of the second subunit, \(B. subtilis\) PdxS or yeast SNZ1. The recent observation of PLP synthase activity in vitro with glutamine as a nitrogen substrate shows that the PdxS/PdxT complex, now known as PLP synthase, is indeed a glutamine amidotransferase having glutaminase (PdxT) and synthase (PdxS) subunits (8). Glutamine amidotransferases are a group of about 20 enzymes that remove the amide nitrogen from glutamine and add it to another substrate (22). The active sites for glutamine hydrolysis and ammonia addition are chemically distinct and are carried on different
enzyme domains or subunits (24–29). Ammonia generated by the glutaminase domain of the enzyme is sequestered by the protein and transferred through an internal tunnel between the active sites. The working model for PLP synthase catalysis is that ribulose-5-phosphate or ribose-5-phosphate and glycer-aldehyde-3-phosphate or dihydroxyacetone phosphate bind in the active site of PdxS where they combine with ammonia generated by PdxT to form PLP.

Here we report the 2.2-Å crystal structure of PdxS from *G. stearothermophilus*. The PdxS dodecamer observed in the crystal exists in solution in equilibrium with a hexamer. For-tuitous binding of a crystal cryoprotectant molecule provides clues about the mode of ribulose-5-phosphate binding to PdxS and assignment of catalytic roles for some residues. A model of the PdxS/PdxT complex is proposed.

**EXPERIMENTAL PROCEDURES**

Cloning of *G. stearothermophilus* pdxS—*Escherichia coli* strains JM107 (30), XL1-Blue (Stratagene), and BL21(DE3) were used for plasmid construction and gene expression.

For crystallization experiments, plasmids expressing two versions of the His 

enzyme—tagged PdxS were constructed. pBB1188 encodes PdxS with a C-terminal His 

-tag. The modified pdxS gene was synthesized by PCR, using the chromosomal DNA of *G. stearothermophilus* strain 10 (www. genome.ou.edu/bstearo.html) as a template, and oligonucleotides oBB126 (5'-TTATTGCTTGGGATGCGGATATGCTGGGACGACCGGGGCC-TC, as 5'- and 3'-primers, respectively (the SphI and HindIII sites are underlined, and the histidine codons are in bold). The PCR fragment was cloned between the SphI and HindIII sites of the expression vector pBAD18 containing the inducible *E. coli araBAD* promoter (31).

pET0881 encodes PdxS with an N-terminal tag, including His 

and 26 other amino acids that provide thrombin and tobacco etch virus (TEV) protease cleavage sites (residue numbering of PdxS is relative to Met1 of the native protein). To create this plasmid, the modified pdxS gene was synthesized using pBB1186 as a template and oligonucleotides (5'-AACATATGCTGACGGGTATGCTGGGACGACCGGGCCGTC) and (5'-TTTTATATGCTGACGGGTATGCTGGGACGACCGGGCCGTC) as forward and reverse primers, containing the Nhel and HindIII sites, respectively (underlined). The PCR product was cloned between the Nhel and HindIII sites of pETTEV281.

Expression plasmid pETTEV281, a derivative of pET28a (Novagen), encodes a recognition site for TEV protease following the N-terminal His-Tag and the thrombin cleavage site. pETTEV281 was constructed as follows. First, the Nhel site upstream of the T7-Tag was replaced by a KpnI site by site-directed mutagenesis (QuickChange by Stratagene) using the primer 5'-CGCCGGCGCGGTATACGCTAGCATGACGTGGGAC-3' and its complement. A DNA sequence was designed to encode the TEV protease recognition site (32) and to insert between the newly created KpnI site and the BamHI site in the multiple cloning region. A new Nhel site upstream and adjacent to the BamHI site was created for convenient transfer of DNA frag-

ments between vectors. The annealed product of the designed oligonucleotides, had sticky ends (bold) for insertion between KpnI and BamHI sites (Nhel site is underlined, and the TEV protease cleavage site is indicated by ↓). The oligonucleotides were phosphorylated in a 120-ml reaction mixture (600 pmol of oligomer, 1 mm ATP, 40 units of T4 polynucleotide kinase (New England Biolabs), 70 mm Tris-HCl, pH 7.6, 10 mm MgCl2, 5 mm dithiothreitol) and incubated at 37 °C for 30 min. The reaction was quenched by addition of 25 mm EDTA (final concentration) and the kinase removed by phenol extraction. The phosphorylated oligonucleotides were annealed at 95 °C and slowly cooled to room temperature. The annealed duplex was ligated into modified vector pET28a (no Nhel site) that had been digested by KpnI and BamHI under standard conditions. The ligation product was transformed into competent XL1-Blue cells (Stratagene). Plasmid DNA from colonies was screened by restriction digestion for the new Nhel site, originating from the insert. DNA with the expected restriction pattern was sequenced at the Purdue Genomics Center to verify the construct and the plasmid was named pETTEV281.

We were able to produce crystals using either C-terminal or N-

terminal His 

-tagged versions of PdxS. Below we describe the procedures used for purification and crystallization of the N-terminal His 

form of PdxS.

Purification of PdxS—Cells of *E. coli* BL21(DE3) were transformed with plasmid pET0881 and grown in Luria-Bertani medium containing 50 μg/ml kanamycin. Expression of pdxS was induced by addition of IPTG (final concentration 400 μM) when the A600 reached 0.8 and the culture was incubated at 37 °C for 6 h. All subsequent steps were carried out at 4 °C unless otherwise noted. Cells were harvested by
from a 1:1 mixture of protein (5 mg/ml PdxS, 20 mM Tris-Cl, pH 8.1, and concentrated to 10 mg/ml.

SeMet-PdxS was produced by the same protocol, except cells were grown in the SeMet minimal medium (33) plus 50 mg of L-SeMet per liter. No reducing agent was used during purification. Purified SeMet-PdxS was extremely sensitive to pH and precipitated in solutions below pH 8.

centrifugation, resuspended in 25 ml of extraction buffer (20 mM imidazole, 20 mM Tris-Cl, pH 7.9, 500 mM NaCl), and lysed by two passes through a French press at 10,000 p.s.i. The lysate was centrifuged at 20,000 $\times g$ for 30 min. The clarified cell extract was loaded onto a Ni$^{2+}$-charged immobilized metal affinity chromatography column (HiTrap™ Chelating HP, Amersham Biosciences) pre-equilibrated with 20 mM Tris-Cl, pH 7.9, 500 mM NaCl) was applied to the column. The N-terminal His-tagged PdxS eluted at $\approx$250 mM imidazole. Purified PdxS was dialyzed at room temperature against 20 mM Tris-Cl, pH 8.3, and concentrated to 20 mg/ml. The purified PdxS was extremely sensitive to 5% of the data.

Structure of the Synthase Subunit of PLP Synthase

| Crystallographic summary | Native | Peak | Inflection | Remote | Hg derivative, peak |
|--------------------------|-------|------|------------|--------|-------------------|
| Data collection          |       |      |            |        |                   |
| Beamline                 | APS 19ID | APS 19BM | APS 19BM | APS 19BM | APS 14ID          |
| Wavelength ($\lambda$)   | 0.97951 | 0.98002 | 0.98027 | 0.96501 | 1.00750           |
| Space group              | C222   | C222  | C222      | C222   | C222              |
| Unit cell ($a$ (Å))      | 162.5  | 163.3 | 163.7     | 163.8  | 162.6             |
| $b$                      | 163.9  | 164.5 | 164.8     | 164.9  | 164.6             |
| $c$                      | 186.8  | 187.1 | 187.3     | 187.4  | 186.9             |
| $d_{min}$ ($\lambda$)    | 2.2    | 2.8   | 3.2       | 3.6    | 3.0               |
| Completeness (%)         | 99.9 (99.9) | 99.8 (99.8) | 99.5 (99.3) | 99.6 (99.5) | 100.0 (100.0)    |
| Redundancy               | 7.3 (6.6) | 9.5 (8.7) | 9.2 (9.0) | 8.3 (8.4) | 14.6 (14.6)       |
| $R_{merge}$ (%)          | 0.076 (0.55) | 0.09 (0.4) | 0.09 (0.45) | 0.11 (0.39) | 0.13 (0.45)       |
| $I/\sigma$ (last shell)  | 22.5 (3.7) | 20.9 (3.0) | 19.9 (3.1) | 15.3 (3.5) | 13.8 (3.6)        |

Structure Determination—We first attempted to solve the PdxS structure by SeMet MAD. As the PdxS polypeptide contains 15 Met residues, 90 selenium sites were expected in the asymmetric unit. However, we were unable to solve the selenium substructure using several programs (SOLVE (37), SHELX (38), ACORN (39), SAPI (40), and BnP (41)), presumably due to the poor quality of the data. We next turned to the mercury SAD method and was indexed and integrated using HKL2000 (34). A three-wavelength SeMet MAD data set was collected from one crystal at APS beamline 19-BM using the inverse beam method and was indexed and integrated using HKL2000. A 3.0 Å mercury SAD data set was collected from one crystal at APS beamline 19-ID. The wavelength of peak absorption at the mercury LIII edge (1.0075 Å) was used to collect 360° of data as 0.5° oscillation images, which were indexed and integrated using MOSFLM (35) in the CCP4 suite (36). Integrated intensities for all data sets were scaled using SCALa (36). Data processing statistics are shown in Table I.

Structure Determination—We first attempted to solve the PdxS structure by SeMet MAD. As the PdxS polypeptide contains 15 Met residues, 90 selenium sites were expected in the asymmetric unit. However, we were unable to solve the selenium substructure using several programs (SOLVE (37), SHELX (38), ACORN (39), SAPI (40), and BnP (41)), presumably due to the poor quality of the data. We next turned to the mercury SAD data set and solved the mercury substructure with both SHELX and BnP. Six pairs of mercury sites were found in the asymmetric unit. The PdxS polypeptide has two Cys residues in the sequence Cys-Gly-Cys. Each cysteine residue bound one mercury atom. The six pairs of sites were consistent with the self-rotation...
function, which revealed 6-fold noncrystallographic symmetry perpendicular to the crystallographic c axis. A 3 Å unaveraged map generated by the PHASES routine in BnP was interpretable. A Fourier map using Bijvoet differences from the SeMet-PdxS peak wavelength and mercury-SAD phases revealed most of the selenium sites, confirming the correctness of the phasing and also aiding chain tracing. The 6-fold redundancy was exploited to extend the experimental 3.0 Å SAD phases to 2.2 Å in the native data using RESOLVE (37). An 80%-complete model was auto-traced by ARP/wARP (42) in the phase-refined 2.2 Å map. All manual model building was done in program O (43). The model was refined with REFMAC5 (44). Water sites were identified automatically by ARP_WATERS in the CCP4 suite, refined, and verified manually. Density corresponding to MPD was initially modeled as unknown atomic sites, which were refined without non-bonded contact restraints using the dummy-atom feature of REFMAC5. Models for both enantiomers of MPD were fit to the resulting five dummy-atomic sites. Refinement statistics are shown in Table I. The final model is available in the Protein Data Bank with accession code 1ZNN. Structure superpositions were performed with program O, sequence alignment was with CLUSTALX (45), and molecular figures were prepared with PyMOL (46).

Analytical Ultracentrifugation—Sedimentation velocity experiments were performed in an Optima XL-I ultracentrifuge (Beckman-Coulter, Fullerton, CA). A double-sector charcoal-filled Epon centerpiece with sapphire windows and 1.2-mm pathlength was used. The reference sector was filled with 420 l of dialysis buffer (20 mM Tris-Cl, pH 8.1, without or with sulfate or phosphate) and the sample sector with an equivalent volume of sample containing ~1 mg/ml protein. Solvent densities and viscosities were calculated with SEDNTERP (47). Following thermal equilibration in the centrifuge at 20 °C for at least 1 h at 0 rpm, the velocity experiment was initiated by accelerating the rotor to 40,000 rpm. Boundary positions were determined using both the Rayleigh interference, and the absorbance (280 nm) optics with scans taken.
Structure of the Synthase Subunit of PLP Synthase

RESULTS

PdxS Fold—PdxS has a classic \((\beta/\alpha)_8\) barrel fold, consisting of eight parallel \(\beta\)-strands \((\beta_1-\beta_8)\) alternating with eight \(\alpha\) helices \((\alpha_1-\alpha_8)\) along the polypeptide chain (Fig. 2\(a\)). The barrel architecture is decorated by two major insertions, which participate in subunit contacts (see below). The first insertion is a large helical protrusion from the side and bottom of the barrel, formed by helix \(\alpha_6'\) (residues 179–189), helix \(\alpha_6''\) (residues 193–202), and the C terminus (residues 166–176) of helix \(\alpha_6\). The second insertion is a C-terminal helix \((\alpha_8';\text{residues 261–268})\), which lies on the side of the barrel such that the polypeptide C terminus is unconventionally at the top of the barrel. Two smaller deviations from the prototypical barrel include a small \(3_{10}\) helix, which we name \(\alpha_8\) (residues 237–240), on the top of the barrel, and a short \(3_{10}\) helix (residues 117–121), which we name \(\alpha_4\) by convention, in place of barrel helix \(\alpha_4\). Based on the sequence alignment of PdxS and 90 homologs, all of these deviations from the canonical barrel architecture are conserved. Several residues are disordered and missing from the structure, including 33 from the top of the barrel (residues 46–55 in the \(\beta_2-\alpha_2\) loop; residues 272–294 at the C terminus) and 17 from the bottom (residues 1–17 preceding \(\beta_1\)).

PdxS Quaternary Structure—PdxS crystallized as an unusual dodecamer in which the subunits form a cylinder 90 Å tall and 110 Å in diameter (Fig. 2\(b\)). The cylinder walls are 35 Å thick, creating an internal space 40 Å in diameter. The dodecamer is constructed of two opposing helical rings. The solid waist of the cylinder is formed by interdigitation of the helical protrusions from the two hexamers. Each end of the cylinder is a ring of six \((\beta/\alpha)_8\) barrels. The top of each \((\beta/\alpha)_8\) barrel points inward and the bottom toward the outside of the cylinder. The barrel axis of each subunit does not point directly inward but is tipped 25° toward the nearest end of the cylinder.

Subunits in the hexameric ring have extensive hydrophobic contacts that exclude water and bury 17% of the subunit surface. The helical insertion \(\alpha_8''\) is part of this interface. A strictly conserved salt bridge from Asp\(^{220}\) to Arg\(^{63}\) and His\(^{36}\) of a neighboring subunit is buried in the otherwise hydrophobic interface. By criteria of interface size, hydrophobicity, packing, and conservation, the hexameric ring appears to be a feature of PdxS and its 90 closest relatives. In contrast to contacts within the hexamer, subunit contacts between hexameric rings are more polar, less conserved, and include buried solvent. Twelve sulfate ions, which may mimic more physiologically relevant phosphate ions, are bound at an interface of hexamers. Each sulfate ion forms salt bridges with three strictly conserved residues from a subunit in one hexamer (His\(^{115}\), Arg\(^{137}\), and Arg\(^{138}\)) and conserved Lys\(^{187}\) from a subunit of the opposing hexamer. Conservation of a dodecamer quaternary structure among PdxS homologs is less clear than is conservation of the hexamer. However, residues in the helical protrusion \((\alpha_6', \alpha_6'',\text{and } \alpha_6'')\) and those contacting the buried sulfate ions are well conserved, and the hexamer-hexamer interface has nearly perfect shape complementarity. In addition, the yeast homolog of PdxS was observed to be considerably larger than a monomer (20).

PdxS Quaternary Structure in Solution—The quaternary structure of PdxS was determined by analytical ultracentrifugation (Fig. 3). PdxS formed two aggregated species with a ratio of the low mass to high mass species of 9 to 1, in buffer lacking sulfate or phosphate (Fig. 3, blue curve). Sedimentation values for a hexamer and dodecamer of PdxS, calculated by the program SEDPHAT, fit the two observed species well, consistent with the crystal structure. Addition of sulfate in concentration equal to that used for crystallization (200 mM) increased the proportion of the larger species (Fig. 3, red curve). Phosphate at 20 mM had the same effect (data not shown). Phosphate at 20 mM had a similar, although less pronounced, effect on the degree of dodecamer formation (Fig. 3, black curve). The effects of both sulfate and phosphate were concentration dependent, with higher concentrations of sulfate or phosphate increasing the proportion of the dodecamer.

Active Site—The \((\beta/\alpha)_8\) barrel is the most abundant enzyme fold in nature, and, without exception, the active sites are located among the loops at the top of the barrel. In the PdxS dodecamer, this places the active sites on the inside surface near the ends of
the cylinder. Consistent with the PdxS fold, genetic screens for mutations leading to B6 auxotrophy identified amino acid substitutions at six positions, all but one at the top of the barrel (11, 50) (Fig. 2a).

In the crystal structure, two molecules were bound in the presumed active site of PdxS (Fig. 4a). A sulfate ion from the crystallization buffer was bound at loops /H9252/6-H9251/6 and /H9252/7-H9251/7. This sulfate ion is in addition to the sulfates found between the hexamers and described above. Presumably the sulfate ion mimics the phosphate group of a substrate molecule. Within loop /H9252/6-H9251/6, the strictly conserved sequence Gly 153-Thr154-Gly155 has an unusual conformation, seemingly designed for phosphate binding (Fig. 4b). The sulfate ion is hydrogen-bonded to the glycine residues in the strictly conserved Gly153-Thr154-Gly155 sequence in loop /b6-a6. Special features of the loop include hydrogen bonds of the Thr154 side chain to backbone atoms (C=O of Glu151 and NH of Asn156) and conformations for Gly153 (φ = +84°, ψ = −4°) and Gly155 (φ = +58°, ψ = +20°) that are not generally accessible to amino acids other than glycine. Atoms in this stereo diagram are colored the same as described for Fig. 2a.

FIG. 4. PdxS active site. a, active site ligands. Bound sulfate and MPD molecules are shown in the presumed active site. Electron density, contoured at 1.0 σ, is from a |Fobs| − |Fcalc| map with the ligands and bridging water molecule omitted from the phasing model. Hydrogen bonds are drawn as dashed lines. Atoms are colored as in Fig. 2a, excepting the carbon atoms of MPD, which are green. b, phosphate-binding loop. Bound sulfate is hydrogen bonded to the glycine residues in the strictly conserved Gly153-Thr154-Gly155 sequence in loop /b6-a6. Special features of the loop include hydrogen bonds of the Thr154 side chain to backbone atoms (C=O of Glu151 and NH of Asn156) and conformations for Gly153 (φ = +84°, ψ = −4°) and Gly155 (φ = +58°, ψ = +20°) that are not generally accessible to amino acids other than glycine. Atoms in this stereo diagram are colored the same as described for Fig. 2a.
Fig. 5. Binding of phosphorylated substrates by PdxS and other proteins. a, a model of the PdxS/ribulose 5-phosphate (R5P) imine at Lys^{149}. The phosphate group is bound via two hydrogen bonds to loop β6-α6 and one to loop β7-α7. b, triose phosphate isomerase (PDB code 6TIM (57)) with bound glycerol 3-phosphate (G3P). The phosphate group is bound via two hydrogen bonds to helix α8', one to loop β6-α6 and one to loop β7-α7. c, pyridoxine 5'-phosphate synthase (PdxJ; PDB code 1IXN (52)) with substrates 1-deoxy-d-xylulose 5-phosphate (DXP) and glycerol 3-phosphate (G3P). d, thiamin phosphate synthase (PDB code 2TPS (53)) with product thiamin phosphate (TPS). Ligands and key secondary structures are labeled. Atoms in these stereo diagrams are colored the same as described for Fig. 2a.
DISCUSSION

Model of Substrate Binding—The discovery of a $\beta_6\alpha_6$ fold for PdxS establishes with near certainty the location of the active site on the internal surface of the dodecamer cylinder, and therefore the specific binding in this site of sulfate and MPD is potentially significant. Phosphorylated substrates were modeled into the PdxS active site based on the specific binding of R-MPD and its proximity to the bound sulfate ion, which serves as a likely mimic of a substrate phosphate group. Among the PdxS substrate molecules, ribulose 5-phosphate fit best. With the phosphate group bound in the sulfate-ion site, the hydroxyl groups at positions 1 and 3 of ribulose 5-phosphate can hydrogen bond with the unprotonated form of invariant Asp$^{24}$, as do the hydroxyls of MPD (Figs. 4a and 5a). Ribose
5-phosphate, the aldo form, could bind in the same manner to a protonated Asp<sup>24</sup>. Thus, Asp<sup>24</sup> is the prime candidate for a proton-shuffling catalytic residue of the observed ribose 5-phosphate keto isomerase activity of PdxS (8). The invariant residue Lys<sup>149</sup> forms an imine with ribulose 5-phosphate (8). In the crystal structure, the Lys<sup>149</sup> side chain points away from the active site. Re-orientation of the peptide bonds adjacent to Lys<sup>149</sup>, which are not hydrogen bonded, places the side chain in position to form the observed imine at position 2 of ribulose 5-phosphate. A model of the covalent intermediate is shown in Fig. 5a. A third residue that also may have a catalytic role is invariant Lys<sup>81</sup>. The positions of invariant residues Asp<sup>24</sup>, Lys<sup>81</sup>, and Lys<sup>149</sup> likely preclude binding of a shorter triose 3-phosphate to this site. A binding site for glyceraldehyde 3-phosphate or dihydroxyacetone phosphate is not obvious from the PdxS crystal structure. However, among invariant residues near the proposed ribulose 5-phosphate-binding site at the top of the barrel, three hydrogen-bonded side chains (Asp<sup>102</sup>, Glu<sup>105</sup>, and Arg<sup>147</sup>) and the adjacent Ser<sup>104</sup> provide a cluster of functional groups that could be used for triose 3-phosphate binding or for catalysis.

Comparison with Other Structures—Although PdxS obviously has a structure similar to all enzymes with the (βα)₈ barrel fold, automated searches of fold space were conducted to find its closest structural relatives. These were <i>E. coli</i> PdxJ involved in the first pathway of PLP biosynthesis (52), <i>B. subtilis</i> thiamin phosphate synthase (53), and archaeal TIM (54), using either of two search algorithms (55, 56). The biochemical similarities of these enzymes to PdxS are striking: PdxS and PdxJ have vitamin B₆ products; PdxS and TIM catalyze a triose phosphate isomerase reaction; all of the enzymes have sugar phosphate substrates. However, comparison of active sites did not reveal similarities in residues used for substrate binding or catalysis that would suggest specific roles for conserved residues in PdxS.

PdxS and the three other enzymes bind their phosphorylated substrates somewhat differently (Fig. 5). In PdxS the sulfate/phosphate is bound principally to the invariant Gly<sup>153</sup>-Thr<sup>154</sup>-Gly<sup>155</sup> in loop 6-8, whereas in the three other enzymes, as in many (βα)₈ barrel algorithms, phosphate is bound principally to a short helix, α<sup>8</sup>. In PdxS, helix α<sup>8</sup> interacts with the bound sulfate ion only indirectly through water molecules. Thus, the Gly<sup>153</sup>-Thr<sup>154</sup>-Gly<sup>155</sup> in loop 6-8 may be a primary phosphate-binding site in PdxS. We considered whether helix α<sup>8</sup> can bind the triose phosphate substrate at the same time as ribulose 5-phosphate is bound at loop 6-8, but this would result in a clash of the phosphate groups. However, conformational changes to loops at the top of the barrel, induced by substrate binding or by PdxT association, may allow binding of the second substrate to helix α<sup>8</sup> or may involve helix α<sup>8</sup> in binding the ribulose 5-phosphate.

The substrate sugar groups are bound by different loops in each of the three enzymes, with TIM most similar to PdxS. The TIM substrate is bound by loops β<sub>1</sub>-α<sub>1</sub>, β<sub>4</sub>-α<sub>4</sub>, and β<sub>6</sub>-α<sub>6</sub> (Fig. 5b), as proposed for ribulose 5-phosphate binding to PdxS (Fig. 5a). In PdxJ (Fig. 5c), the substrate is hydrogen-bonded to an asparagine side chain in an analogous position to Asp<sup>24</sup> of PdxS. In thiamin phosphate synthase, loops β<sub>6</sub>-α<sub>6</sub>, β<sub>7</sub>-α<sub>7</sub>, and β<sub>8</sub>-α<sub>8</sub> bind substrate (Fig. 5d). None of these enzymes is thought to use an imine intermediate, as has been detected for PdxS (8).

Two structure predictions for the PdxS family (19, 21) match the observed (βα)₈ fold. However, both predictions include a phosphate-binding site at loops β<sub>7</sub>-α<sub>7</sub> and β<sub>8</sub>-α<sub>8</sub>, similar to the sites in several other enzymes but different from the observed sulfate-binding site in PdxS.

**Formation of PLP Synthase from PdxS and PdxT—**PdxS and PdxT form a heteromeric glutamine amidotransferase (8, 10, 23) in which PdxT produces ammonia by glutamine hydrolysis and channels it through the protein to PdxS. Ammonia is labile and must be quenched by the enzyme (22). Precedent for binding of a glutaminase domain to an (βα)₈ barrel acceptor domain is found in the histidine biosynthetic enzyme imidazole-glycerol-phosphate synthase (IGPS). The glutaminase domain of IGPS is docked at the bottom of the (βα)₈ barrel acceptor domain in such a way that the barrel interior is the ammonia channel (29). This mode of interaction was predicted for PdxS and PdxT (21). However, residue packing within the PdxS barrel and the distribution of conserved residues argue against this hypothesis. The interior of the PdxS (βα)₈ barrel is well packed with side chains, unlike the open central cavity of the IGPS barrel. Strictly conserved residues are distributed disproportionately to the top of the PdxS barrel (Fig. 6, a and b), unlike conserved residues in IGPS, which are equally distributed to the active site at the top (Fig. 6c) and to the glutaminase docking site at the bottom of the barrel (Fig. 6f).

Regardless of how PdxT docks to PdxS, PLP synthase is expected to have several biochemical features common to glutamine amidotransferases. Glutaminase (PdxT) and synthase (PdxS) activities should be tightly coupled. In most amidotransferases, catalytic coupling is accomplished by a glutaminase that is conformationally impaired in the resting state of the enzyme and activated by binding of the synthase substrate.
PLP synthase appears exceptional in this regard because substantial glutaminase activity has been reported in absence of synthase substrates (10, 23). In a seeming reversal of the usual situation for glutamine amidotransferases, ribose 5-phosphate and ribulose 5-phosphate inhibit the glutaminase activity of yeast PLP synthase (23). This suggests that the imine adduct of ribulose 5-phosphate may be the "resting" state of PLP synthase in vivo, in which glutaminase is impaired and that conformational changes induced by binding of glyceraldehyde-3-phosphate (or dihydroxyacetone phosphate) may activate the glutaminase. Indeed the imine adduct is the predominant form of freshly isolated PdxS (8). Alternatively, binding of either synthase substrate alone may inhibit the glutaminase. Conformational changes associated with binding of substrates to PdxS could involve any of the loops at the top of the (β3αβ3) barrel. Prime candidates are loops β6-α6, which carries both the Gly153-Thr154-Gly155 phosphate-binding site and the Lys149 site of imine formation, and loop β2-α2, which is disordered in the crystal structure of PdxS and includes three invariant residues. Another property common to most glutamine amidotransferases is catalysis of both glutamine- and ammonia-dependent product formation (22). PLP synthase appears to share this property because the B6 auxotrophy of a product formation (22). PLP synthase appears to share this property because the B6 auxotrophy of a product formation (22). PLP synthase appears to share this property because the B6 auxotrophy of a product formation (22). PLP synthase appears to share this property because the B6 auxotrophy of a product formation (22). PLP synthase appears to share this property because the B6 auxotrophy of a product formation (22).

The overall catalytic conversion by PLP synthase (Fig. 1) is more complex than reactions catalyzed by other glutamine amidotransferases. In addition to ammonia transfer, the enzyme catalyzes condensation of two phosphosugars, closure of the pyridine ring, as well as isomerase reactions for its phosphosugar substrates (8). The complexity of the enzyme correlates well with the strong conservation of the PdxS sequence. Cross-talk between glutaminase and synthase active sites also appears more complex. Binding of one synthase substrate inhibits the glutaminase activity below basal levels, and full stimulation likely requires binding both phosphosugar substrates. A full understanding of this interesting enzyme will require further kinetic characterization as well as a structure of the intact enzyme.

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REFERENCES
1. Tanaka, T., Tateno, Y., and Gojobori, T. (2005) Mol. Biol. Evol. 22, 243–250
2. Drewek, C., and Leistner, E. (2001) Vitam. Horm. 61, 121–155
3. Hill, R. E., and Spenser, I. D. (1996) in Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (Neidhardt, F. C., Curtiss, R. L., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E., eds) pp. 695–703, American Society for Microbiology, Washington, D. C.
4. Tanaka, K., Tanzya, K., Yamada, K., and Kumaoka, H. (2000) J. Nutr. Sci. Vitam. (Tokyo) 46, 55–57
5. Cane, D. E. (2003) Chimia 57, 75–76
6. Zeidler, J., Gupta, R. N., Sayer, B. G., and Spenser, I. D. (2003) J. Org. Chem. 68, 3486–3493
7. Gupta, R. N., Hemscheidt, T., Sayer, B. G., and Spenser, I. D. (2001) J. Am. Chem. Soc. 123, 11353–11359
8. Burns, K. E., Xiang, Y., Kinsland, C. L., McLafferty, F. W., and Begley, T. P. (2001) J. Am. Chem. Soc. 123, 3682–3683
9. Tanzya, K., Adachi, Y., Masuda, K., Yamada, K., and Kumaoka, H. (1995) Biochim. Biophys. Acta 1244, 113–116
10. Belitzky, B. R. (2004) J. Bacteriol. 186, 1191–1196
11. Bean, L. E., Dvoracek, W. H., Jr., Braun, E. L., Errett, A., Saenz, G. S., Giles, M. W., Werner-Washburne, M., Nelson, M. A., and Natvig, D. O. (2001) Genetics 157, 1067–1075
12. Ehrenhau, M., Bilski, P., Li, M. Y., Chignell, C. F., and Daub, M. E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9374–9378
13. Ehrenhau, M., and Daub, M. E. (2001) J. Bacteriol. 183, 3383–3390
14. Osmani, A. H., May, G. S., and Osmani, S. A. (1999) J. Biol. Chem. 274, 21565–21569
15. Rodriguez-Novarro, S., Llorente, B., Rodriguez-Manzaneque, T. M., Ramne, A. U., Marquez, D., Dujon, B., Herrero, E., Sunnerhagen, P., and Perez-Oritz, J. E. (2002) Yeast 19, 1281–1276
16. Seack, J., Peverie, S., Gamulin, V., Schroder, H. C., Beuteimann, P., Muller, W. H. M., and Muller, W. E. (1998) Biochemistry 37, 5174–5176
17. Wengen, C., Eschbach, M. L., Muller, I. B., Warnecke, D., and Walter, R. D. (2005) J. Biol. Chem. 280, 5242–5248
18. Sakai, K., Kita, M., Tatsukawa, T., Osawa, N., and Tani, Y. (2002) J. Bacteriol. 184, 1043–1052
19. Osmani, A. H., May, G. S., and Osmani, S. A. (1999) J. Biol. Chem. 274, 21565–21569
20. Drewke, C., and Leistner, E. (2001) Vitam. Horm. 61, 121–155
21. Hill, R. E., and Spenser, I. D. (1996) in Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (Neidhardt, F. C., Curtiss, R. L., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E., eds) pp. 695–703, American Society for Microbiology, Washington, D. C.
22. Hill, R. E., and Spenser, I. D. (1996) in Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (Neidhardt, F. C., Curtiss, R. L., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E., eds) pp. 695–703, American Society for Microbiology, Washington, D. C.
23. Hill, R. E., and Spenser, I. D. (1996) in Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (Neidhardt, F. C., Curtiss, R. L., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E., eds) pp. 695–703, American Society for Microbiology, Washington, D. C.