Crystal Structures Capture Three States in the Catalytic Cycle of a Pyridoxal Phosphate (PLP) Synthase**

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Background: Pyridoxal phosphate (PLP) synthase generates PLP from three common metabolites in a reaction with three covalent intermediates.
Results: Crystal structures capture the synthase active site at three steps of catalysis.
Conclusion: Protein dynamics are correlated with formation of covalent intermediates.
Significance: New information is provided about the complex catalysis in the synthase active site and the roles of conserved amino acids.

Pyridoxal 5′-phosphate (PLP),2 a biologically active form of vitamin B6, includes a pyridine ring with hydroxyl, methyl, formyl, and phosphate substituents. The carboxylation at the 4′-position makes this B6 vitamin the most versatile co-factor in nature through formation of Schiff–base intermediates with both enzymes and substrates. PLP-dependent enzymes are predominantly involved in amino acid metabolism, lipid metabolism, and gluconeogenesis where they catalyze transamination, decarboxylation, racemization, elimination, or replacement of an electrophilic group and phosphorolysis reactions (1, 2). In addition to being an efficient co-factor, PLP has antioxidant properties, as the core aromatic pyridine ring of PLP is an efficient singlet oxygen scavenger (3–5).

Two PLP biosynthetic pathways are known. The deoxyxylulose 5-phosphate-dependent pathway synthesizes PLP through seven enzymatic steps and is present in a subset of gamma-proteobacteria (6). The more common deoxynxylulose 5-phosphate-independent, ribose 5-phosphate (R5P)-dependent pathway comprises two gene products that together form pyridoxal 5′-phosphate synthase (PLPS) (7, 8). The gene pair is widely distributed in archaea, fungi, plants, protists, and bacteria (6). PLPS has been characterized from several biological sources, including Bacillus subtilis (Pdx1/Pdx2 or PdxS/PdxT) (7–9), Thermotoga maritima (YaaD/YaaE) (10), Saccharomyces cerevisiae (Snz1/Sno1) (11), Plasmodium falciparum (Pdx1/Pdx2) (12), and Geobacillus stearothermophilus (PdxS/PdxT, the subunit nomenclature used here) (13). Lack of PLP synthesis in humans suggests that the single-enzyme pathway may be a potential drug target. There is also great interest in understanding the mechanism by which this remarkable single-enzyme pathway synthesizes PLP from the following three common metabolites: glutamine, R5P, and glyceraldehyde 3-phosphate (G3P).

PdxS and PdxT have distinct and spatially separated catalytic functions (7, 8). PdxT, a glutamine amidotransferase (GAT) of the Triad (class I) family (7, 8, 14), uses a Cys-His-Glu catalytic

PLP synthase (PLPS) is a remarkable single-enzyme biosynthetic pathway that produces pyridoxal 5′-phosphate (PLP) from glutamine, ribose 5-phosphate, and glyceraldehyde 3-phosphate. The intact enzyme includes 12 synthase and 12 glutaminase subunits. PLP synthesis occurs in the synthase active site by a complicated mechanism involving at least two covalent intermediates at a catalytic lysine. The first intermediate forms with ribose 5-phosphate. The glutaminase subunit is a glutamine amidotransferase that hydrolyzes glutamine and channels ammonia to the synthase active site. Ammonia attack on the first covalent intermediate forms the second intermediate. Glyceraldehyde 3-phosphate reacts with the second intermediate to form PLP. To investigate the mechanism of the synthase subunit, crystal structures were obtained for three intermediate states of the Geobacillus stearothermophilus intact PLPS or its synthase subunit. The structures capture the synthase active site at three distinct steps in its complicated catalytic cycle, provide insights into the elusive mechanism, and illustrate the coordinated motions within the synthase subunit that separate the catalytic states. In the intact PLPS with a Michaelis-like intermediate in the glutaminase active site, the first covalent intermediate of the synthase is fully sequestered within the enzyme by the ordering of a generally disordered 20-residue C-terminal tail. Following addition of ammonia, the synthase active site opens and admits the Lys-149 side chain, which participates in formation of the second intermediate and PLP. Roles are identified for conserved Asp-24 in the formation of the first intermediate and for conserved Arg-147 in the conversion of the first to the second intermediate.

2 The abbreviations used are: PLP, pyridoxal 5′-phosphate; PLPS, PLP synthase; R5P, ribose 5-phosphate; G3P, glyceraldehyde 3-phosphate; GAT, glutamine amidotransferase.

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triad to produce ammonia by hydrolysis of glutamine (Fig. 1A). PdxS is a remarkable synthase that produces PLP using readily available metabolites from the pentose phosphate pathway (R5P), from PdxT (NH₃), and from glycolysis (G3P) (7, 8). The activity of PdxT is coupled to its PdxS synthase in a different manner than for other GATs (15). Typically, GATs have abrogated glutamine hydrolysis in the absence of their synthase substrates. PdxT is unique in that its glutaminase activity depends only on formation of the intact PLPS (PdxS₁₂PdxT₁₂) and is independent of the PdxS substrates (8). As is typical of glutamine-dependent synthases, PLPS or the PdxS subunit can form PLP using exogenous ammonia, although glutamine is a more efficient nitrogen source (8, 16).

The complex chemical reactions that occur in the PdxS active site have been the subject of intense investigation, and at least two covalent intermediates have been identified (Fig. 1B) (7, 17). A PdxS catalytic lysine initially forms a Schiff base imine adduct with the R5P C1 atom in a manner that is independent of PdxT, glutamine, or G3P (7). The imine undergoes spontaneous isomerization to a stable amino ketone intermediate, here named I₁ (18, 19). Ammonia, either exogenous or from glutamine hydrolysis, reacts with I₁ to release phosphate and water, forming a chromophore intermediate, here named I₂, with an absorption maximum at 320 nm (16, 17). Nitrogen addition from ammonia was shown to occur at the C2 atom, and a structure was proposed for I₂, in which the covalent bond to the catalytic lysine is shifted from C1 to C5 in I₂ (16). Additionally, a second phosphate-binding site outside the synthase active site has been identified for PLP and is proposed to play a role in catalysis (11, 19).

The overall architecture of PLPS (10, 14, 21) and the structures of PdxS (11, 13, 21) and PdxT (12, 22) are well understood. The 24-subunit PLPS comprises 12 PdxS subunits and 12 PdxT subunits (14). PdxS forms a D6-symmetric cylindrical decamer of two opposing hexameric rings (13). The inner surface of the dodecameric cylinder is lined with the active sites of the 12 PdxS monomers, which possess the (H₉₋₂₅/H₂₋₅)₈ barrel fold (13). The 12 PdxT subunits dock at the outside of the PdxS cylinder with each PdxT active site facing the exterior end of a PdxS (H₉₋₂₅/H₂₋₅)₈ barrel. The hydrophobic interior of the PdxS (H₂₋₅/H₉₋₂₅)₈ barrel is thus inferred to be an ammonia channel, in an identical manner to imidazole glycerol phosphate synthase, another GAT (23). All crystal structures that include the PdxS synthase subunit lack 14–28 amino acids at the C terminus (10, 11, 13, 14, 21). Despite their disorder, the last 21 amino acids are essential to PLP synthesis and become progressively ordered upon binding of PdxT and substrates (24).

To investigate the intriguing aspects of the PdxS mechanism, we characterized PLPS from G. stearothermophilus and obtained crystal structures for substrate complexes of PdxS alone (PdxS-Glu) and of the intact enzyme (PLPS-Glu). The structures provide snapshots of PdxS at distinct steps in its complicated catalytic cycle and provide insights into the elusive mechanism and the structural elements that drive the conversion of I₁ to I₂. PdxS alternates between open and closed conformations dependent on PdxT and substrates. Interaction of PLPS with glutamine and R5P induced
Structure of PLP Synthase

partial ordering of the C-terminal tail, which forms a lid over the R5P site in the closed state of the synthase subunit. Synthase closure is stabilized by several charged residues and appears to be essential for the formation of the I$_2$ chromophore. The structure of PdxS-I$_1$/I$_2$ provides snapshots of PdxS from the I$_1$ to the I$_2$ state.

EXPERIMENTAL PROCEDURES

Cloning of G. stearothermophilus pdxS and pdxT—The plasmids pET0881 (13) for PdxS and pET0669 for PdxT were used for all mutageneses and subclonings. pET0669 was generated by subcloning the PdxT coding region into the pETTEV281 vector (13). The PdxT coding sequence was kindly provided by Dr. Boris Belitsky. PdxT H169N (here designated H169N$_r$) and some substitutions in PdxS (R53K, R53E, R53A, R53Q, E151S, E151A, E151Q, R288K, R288E, R288Q, R288A, D24N, D24A, D102A, R147K, R147A, and R147Q) were generated by site-directed mutagenesis followed by subcloning and insertion into an appropriate expression vector. The T7 promoter, lac operator, and ribosome-binding site sequences. These sequences were replaced by insertion of a synthetic DNA carrying the trp/lac fusion promoter trc (25), the translational enhancer sequence for the E. coli atp genes (26), and the ribosome-binding site (AGGAGG) spaced 8 bp from the ATG start sequence (27). All mutagenesis was verified by sequencing.

Expression and Purification of PdxS and PdxT—PdxS and PdxT were purified separately. Cells of Escherichia coli strain BL21(DE3) (or strain BL21 for pPdxSMBP_K149R and pPdxSMBP_K149Q) were transformed with the appropriate expression plasmid, grown at 37 °C in either Luria-Bertani (LB) medium until expression plasmid, grown at 37 °C in either Luria-Bertani (LB) or Terrific Broth (TB) medium until expression was induced with either 200 μM isopropyl 1-thio-D-galactopyranoside (IPTG) or 20 mM glutamine, 2 mM DTT), dialyzed (20 mM Tris-HCl, pH 7.9, 10 mM glutamine), and stored at −80 °C.

PLPS Assays—Published assays were used with slight modifications (8, 17). Glutaminase activity was assayed in a coupled reaction with glutamate dehydrogenase. Reactions were carried out at 37 °C in a total reaction volume of 250 μl containing 50 μM PdxS, 50 μM PdxT, 100 mM Tris-HCl, pH 8.5, 20 mM glutamine, 50 mM KCl, 0.375 mM 3-acetylpyridine adenine dinucleotide, and 1.8 μM glutamate dehydrogenase (Sigma). Glutamate formation was monitored by glutamate dehydrogenase reduction of 3-acetylpyridine adenine dinucleotide to the 3-acetylpyridine adenine dinucleotide reduced form detected at 363 nm using a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices). Formation of PdxS-I$_2$ was assayed at 37 °C in a total reaction volume of 50 μl containing 50 μM PdxS, 50 μM PdxT, 50 mM Tris-HCl, pH 7.9, 20 mM glutamine, and 10 mM R5P. I$_2$ was detected by absorbance at 315 nm in a SpectraMax M5. PLP synthesis was assayed at 37 °C in a total reaction volume of 50 μl containing 50 μM PdxS, 50 μM PdxT, 50 mM Tris-HCl, pH 7.9, 20 mM glutamine, and 10 mM R5P. After a 7-min incubation to form I$_2$, PLP synthesis was initiated by addition of G3P (20 mM). PLP was detected by absorbance at 415 nm in a SpectraMax M5. I$_2$ formation and PLP synthesis for PdxS mutants at Lys-149 were assayed in a sub-micro quartz cuvette (Starna Cells, Inc.) with a total reaction volume of 100 μl using an Ultrospec 3100 pro spectrophotometer (Amersham Biosciences). For determination of kinetic constants, 1 μM PLPS was used, and the reactions were carried out as described above.

Intact Protein Mass Spectrometry—For detection of PdxS covalent intermediates, reaction mixtures with a total volume 100 μl were incubated for 15 min at 37 °C, quenched with 10% formic acid, and analyzed by LC-MS. Samples were eluted at a flow rate of 0.5 ml/min, with a linear gradient of 5–95% acetonitrile, over 15 min employing an Aeris widepore C4 column (3.6 μm, 50 × 2.10 mm) and directly analyzed by ESI mass spectrometry (Agilent 6520 Accurate Mass Q-TOF). Data were analyzed using the maximum entropy deconvolution algorithm within the Agilent Mass Hunter Qualitative Analysis software.

Assays of intermediate formation at Lys-81 in PdxS D24N, D24A, R147K, R147Q, and R147A were carried out with 150 μM PdxS and 200 μM PdxT, 20 mM Tris-HCl, pH 7.9, 10 mM R5P, 20 mM glutamine. Assays of adduct formation in wild type PLPS were carried out with 31.25 μM PdxS, 60 μM PdxT, 20 mM Tris-HCl, pH 7.9, 10 mM R5P, 0–20 mM glutamine. Assays of adduct formation in wild type PdxS were carried out with 31.25 μM PdxS, 10 mM R5P, 0–50 mM (NH$_4$)$_2$SO$_4$, 20 mM Tris-HCl, pH 7.0; excess R5P was removed by gel filtration (PD-10 Desalting column, GE Healthcare); and the eluate was diluted to 1 mg/ml total protein prior to addition of (NH$_4$)$_2$SO$_4$ and formic acid treatment. Assays of a covalent adduct of product PLP with PdxS were done under single turnover conditions according to a published protocol (18) in which the I$_2$ chromophore was formed by addition of R5P and ammonia to PdxS; excess substrates were removed during buffer exchange to phosphate, pH
7.4; G3P was added, and the protein was subject to MS analysis as described above.

Crystallization—PdxS/R5P (PdxS/I1) and PdxS/R5P/NH3 (PdxS/I1/I2) were crystallized at 20 °C via sitting drop vapor diffusion using 4 μl of protein solution (5 mg/ml PdxS, 20 mM Tris-HCl, pH 7.9, 0.060 mM MgCl2) and 4 μl of reservoir solution (6% PEG 8K, 100 mM sodium cacodylate, pH 7.0, 200 mM lithium citrate for PdxS/R5P, and 200 mM ammonium citrate for PdxS/R5P/NH3). Crystals appeared within 3 days, grew over 7 days, and were cryo-protected with 15% ethylene glycol in reservoir solution, and flash-cooled in liquid nitrogen. PLPS/H169N/I1/R5P/Gln was crystallized at 20 °C by hanging drop vapor diffusion using 1 μl of protein solution (7 mg/ml PLPS/H169N, 20 mM Tris-HCl, pH 7.9, 5 mM R5P, 10 mM Gln) and 1 μl of reservoir solution (14% PEG 3350, 200 mM sodium malonate, pH 7.0). Crystals appeared within 2 days, grew over 7 days, and were dehydrated by serial transfer of the coverslip to wells containing the original mother liquor with the PEG 3350 concentration increased by 5% every 12 h to a final concentration of 39% (28). Crystals were directly flash-cooled in liquid nitrogen. Crystal dehydration resulted in a 14% decrease in the unit cell volume (7,315,128 to 6,312,125 Å3) and an improvement in the diffraction limit from 3.9 to 2.69 Å.

X-ray Data and Structure Determination—Data were collected at the Advanced Photon Source on the GM/CA beam line 23ID-D and were processed and scaled with the HKL2000 suite (Table 1) (29). Initial phases for each structure were determined by molecular replacement. The structure of PLPS/H169N/I1/R5P/Gln was solved using the program BALBES (30) and the structures of PdxS (13) (1ZNN) and PdxT (14) (2NV2). PdxS from the PLPS/H169N/I1/R5P/Gln structure was used to solve the PdxS/R5P (PdxS/I1) structure using the program Phaser (31). The PdxS/R5P (PdxS/I1) structure was then used to solve the PdxS/R5P/NH3 (PdxS/I1/I2) structure also using Phaser. Iterative model building was done in COOT (32), and refinement was carried out with REFMAC (33–35) in the CCP4 suite (36) and Buster (37). TLS groups for all three structures were determined using the webserver TLS MD (38, 39). Link records for the covalent adducts PdxS Lys-81-I1, Lys-81-I2, Lys-81-I1/I2, Lys-81-I1/I2/I2, and the synthase subunit (PdxS) (8, 14, 16). The initial velocity formation of intact PLPS from the glutaminase (PdxT) and synthase (PdxS) subunit (8, 14, 16). The initial velocity was six times faster for glutamine hydrolysis than for PLP synthesis when the synthase substrates are present. In contrast, the PLPS glutaminase was neither dependent on nor accelerated by R5P or G3P (data not shown), but it was dependent on formation of intact PLPS from the glutaminase (PdxT) and the synthase (PdxS) subunit (8, 14, 16). The initial velocity was six times faster for glutamine hydrolysis than for PLP formation. This indicates weak coupling of the active sites under saturating conditions, which may not pertain in vivo. Although the synthase activity could be reconstituted using ammonia as a nitrogen source, the efficiency was 2-fold lower than with glutamine (Fig. 2).

We attempted to capture distinct biochemical states of the synthase by crystallization of intact PLPS or the synthase subunit, PdxS, alone. Crystal structures were obtained at 2.7 Å for L1 in PdxS (PdxS/I1), at 2.3 Å for a mixture of I1 and I2 in PdxS (PdxS/I1/I2), and at 2.7 Å for intact PLPS with L1 in PdxS and the glutamate thioester intermediate in PdxT (PLPS/I1/Glu) (Table 1). These structures and additional probes by site-directed mutagenesis are presented below.

First Covalent Intermediate (I1)—We visualized the synthase active site with its first covalent intermediate (I1) by crystallographic asymmetric unit (Fig. 3). Covalent modification of PdxS in solution was confirmed by whole-protein mass spectrometry (MS) (Fig. 4A). The overall dodecameric structure is virtually identical to the structure of the PdxS free enzyme (13), although the crystal forms differ. Electron density corresponding to an R5P adduct at Lys-81 was present in the active sites of all six subunits in the crystallographic asymmetric unit (Fig. 3). The density was most consistent with formation of a Lys-81 adduct at the R5P C1 atom, based on the appearance of (R5P Kₘ = 10 ± 2 μM, G3P Kₘ = 1.06 ± 0.4 μM, and kₐₙ = 0.010 ± 0.005 min⁻¹), and they yielded values roughly similar to the range reported for B. subtilis PLPS (8, 19). PLPS displayed Michaelis-Menten behavior with no detectable cooperativity in any of its catalytic activities. The glutaminase activity of most GATs depends on or is accelerated by the synthase subunits (15) in a manner that tightly couples the glutaminase and synthase activities, efficiently generating ammonia only when the synthase substrates are present. In contrast, the PLPS glutaminase was neither dependent on nor accelerated by R5P or G3P (data not shown), but it was dependent on formation of intact PLPS from the glutaminase (PdxT) and the synthase (PdxS) subunit (8, 14, 16). The initial velocity was six times faster for glutamine hydrolysis than for PLP formation. This indicates weak coupling of the active sites under saturating conditions, which may not pertain in vivo. Although the synthase activity could be reconstituted using ammonia as a nitrogen source, the efficiency was 2-fold lower than with glutamine (Fig. 2).

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unbiased density and the distance between the lysine nitrogen atom and the Iₑ phosphate. This differs from the C₂ Schiff base adduct reported for *T. maritima* PLPS (10) but is consistent with NMR data (18). Refinement tests of both the 1-imino, 2-hydroxyl isomer and the 1-amino, 2-keto isomer resulted in a better fit to density for the amino-keto isomer, as expected (18). Refinement tests of both the 1-imino, 2-hydroxyl isomer and the 1-amino, 2-keto isomer resulted in a better fit to density for the amino-keto isomer, as expected (18).

When an R₅P covalent intermediate was discovered (7), Lys-149 was identified as the site of the R₅P adduct, but the site was reassigned to Lys-81 in light of a structure of PLPS co-crystallized with R₅P (10). The Lys-149 adduct is unlikely to be on the reaction pathway given its long formation time and given that the Arg variant at this position (K149R) was competent for PLPS synthesis, although at a reduced rate (Table 2). Lys-149 is at the top of strand β₆ in the PdxS β₆/α₆ barrel with the side chain and R₅P adduct directed outside the barrel away from the active site. Interestingly, in this position the adduct phosphate occupies an identical site as the phosphate of product PLP in a crystal structure of the yeast enzyme (Fig. 3) (11), and the site is occupied by free phosphate or sulfate ions in several crystal structures. The phosphate has ionic contacts with Arg-137 and Arg-138, which are part of an extensive salt bridge network (13). Other contacts of PdxS with Iₑ include hydrogen bonds to phosphate oxygens from the amide nitrogens of Gly-153, Gly-214, and Gly-235. The conserved side chain of Asp-24 at the top of strand α₈ extends ~15 Å across the barrel with the phosphate near the N terminus of helix α₈. Despite formation of Iₑ, the active site is open, perhaps due to the disorder of residues 49–56, which were also disordered in the free enzyme structure (13).

**TABLE 1**

| Crystallographic summary | Synthase₁ᵢ | Synthase₁ᵢ/I₂ | PLPS₁ᵢ,Glu |
|--------------------------|-----------|--------------|------------|
| **Diffraction data**     |           |              |            |
| X-ray source             | APS 23ID-D| APS 23ID-D   | APS 23ID-D |
| Wavelength (Å)           | 1.033     | 1.033        | 1.033      |
| Space group              | P₃₁₂₁     | P₂₁         | C22₁       |
| Unit cell lengths (Å)    | 179.79, 179.79, 104.57 | 94.68, 107.20, 178.22 | 141.02, 249.14, 179.66 |
| α, β, γ (°)              | 90, 90, 120 | 90, 92.24, 90 | 90, 90, 90 |
| Asymmetric unit          | 6 PdxS    | 12 PdxS      | 6 PdxS, 6 PdxT/H169N |
| dₑmin (Å)               | 2.70 (2.77–2.70) | 2.29 (2.35–2.29) | 2.69 (2.76–2.69) |
| Unique reflections       | 53,746    | 158,924      | 87,355     |
| Average F/σ              | 23.4 (3.8) | 15.5 (2.0)   | 15.5 (2.1) |
| Average multiplicity     | 11.4 (11.4) | 3.8 (3.8)    | 3.3 (5.1)  |
| Completeness (%)         | 100 (100) | 100 (100)    | 98.9 (98.9) |
| Rₑₐₚ (%)                 | 0.129 (0.838) | 0.093 (0.74) | 0.101 (0.695) |
| R free (%)               | 43.44–2.70 | 47.35–2.29   | 50–2.69    |
| Root mean square deviations | 21.8/26.2 | 19.1/22.3    | 20.9/25.7  |
| Bond lengths (Å)         | 0.0112    | 0.0062       | 0.0114     |
| Bond angles (°)          | 1.4084    | 1.0930       | 1.4022     |
| Average B factors (Å²)   | 63.1      | 49.6         | 59.5       |
| Protein                  | 46.2      | 49.6         | 40.5       |
| Water                    | 77.3      | 69.0         | 62.7       |
| Ligands                  | 97.4      | 97.2         | 96.3       |
| Ramachandran             | 10.012    | 2.5          | 3.2        |
| Favored (%)              | 97.4      | 97.2         | 96.3       |
| Allowed (%)              | 2.5       | 2.5          | 3.2        |
| Outlier (%)              | 0.1       | 0.3          | 0.5        |
| No. of atoms             | 193       | 1236         | 263        |
| Protein                  | 264       | 176          | 222        |
| Water                    | 193       | 1236         | 263        |
| Ligands                  | 264       | 176          | 222        |
| Rₑₐₚ (%)                 | 0.129 (0.838) | 0.093 (0.74) | 0.101 (0.695) |

* Values in parentheses refer to the highest resolution shell.
encounters $I_1$ (16, 17). This can be accomplished by addition of glutamine to PLPS-$I_1$ or by addition of ammonia to PdxS-$I_1$ or PLPS-$I_1$. PdxS-$I_2$ has a mass consistent with addition of ammonia to the R5P adduct and loss of phosphate and water (16, 17). We used intact-protein MS to identify conditions for maximal accumulation of $I_2$ in either PdxS or PLPS, but all conditions yielded mixtures of $I_2$, $I_1$, and the free enzyme. In no condition was $I_2$ the predominant species (Figs. 4C and 6), consistent with previous results (16).

We were unable to obtain a structure of PdxS-$I_2$ in the context of intact PLPS, but replacement of lithium citrate with ammonium citrate in the conditions for crystallization of PdxS-$I_1$ produced crystals in a different form (Table 1 and Fig. 7) in which the 12 PdxS subunits in the crystallographic asymmetric unit included three states of the synthase active site: $I_1$, $I_2$, and no intermediate (Fig. 7). Thus, the 2.3-Å structure is a snapshot of PdxS in transition between $I_1$ and $I_2$. $I_1$ is the predominant state in seven of the 12 subunits; two subunits contain
density for I₂ with additional density for a phosphate ion disconnected from I₂; two subunits have density for a phosphate ion; and the 12th subunit has density consistent with five carbon atoms not covalently attached to Lys-81 as well as disconnected density for a phosphate ion. Density interpreted as I₂ is consistent with a five-carbon planar species, and the intermediate was modeled according to the proposed structure (16) shown in Fig. 1. In all subunits, the density for the intermediates was poorer than for the surrounding protein, probably due to a mixture of states in each subunit.

FIGURE 4. Formation of I₁ and I₂ in PdxS. A, deconvoluted electrospray injection (ESI) mass spectrum showing complete conversion of PdxS to PdxS-I₁ upon a 15-min incubation with R5P. The calculated mass of the PdxS free enzyme is 31,849.6 Da. B, accumulation of a second adduct (+212 Da) upon a 48-h incubation with an excess of RSP. Calculated masses are shown on the spectra. C, deconvoluted electrospray injection mass spectrum showing accumulation of PdxS-I₂ upon a 15-min incubation with RSP and (NH₄)₂SO₄. A mixture of PdxS-I₂, PdxS-I₁, PdxS-I₂/Lys-149-R5P, PdxS-I₁/Lys-149-R5P, and free enzyme was detected.
FIGURE 5. Amino acids critical to accumulation of PdxS-I₁ and PdxS-I₂. A, deconvoluted ESI mass spectrum for PdxS/D24N after a 15-min incubation with R5P. B, deconvoluted ESI mass spectrum for PdxS/D24A after a 15-min incubation with R5P. No mass corresponding to I₁ (212 Da) accumulates for either variant at amino acid 24. C, deconvoluted ESI mass spectrum for PdxS/R147Q-I₁ after a 15-min incubation with R5P and (NH₄)₂SO₄. D, deconvoluted ESI mass spectrum for PdxS/R147A after a 15-min incubation with R5P and (NH₄)₂SO₄. Accumulation of I₁ is slightly reduced in PdxS/R147A, as a small amount of the free enzyme remained. No mass corresponding to PdxS-I₂ (+ 212 Da) was detected for either variant at amino acid 147.
The most significant change to the protein structure is the position of the Lys-149 side chain, which is directed into the active site where it may hydrogen bond with the O2 and/or O4 atoms of I1 or the O3 atom of I2. Lys-149 does not interact with the phosphate nor with other polar side chains in the active site. Its position is supported by electron density in all 12 subunits, and rotations of the backbone add a hydrogen bond to the β5-β6 connection (Thr-148 carbonyl and Ala-212 amide). This is a major change from the outward-pointing position of Lys-149 in all other structures of the PLPS synthase, whether from G. stearothermophilus or other sources. Crystallization conditions were similar (same buffer, same pH, and similar crystallization agent) for the two PdxS structures reported here and for our previous free enzyme structure (13). The free enzyme was crystallized with ammonia and no R5P, PdxS1, with R5P and no ammonia, and PdxS1/I1 with both R5P and ammonia, leading us to conclude that the inward conformation of Lys-149 is associated with formation of the chromophoric I2.

Apart from contacts with Lys-149, I1 interacts with the enzyme identically in PdxS1/I1 and in PdxS1, primarily through hydrogen bonds with the phosphate. In both structures, conserved residues Asp-102 (β4) and Arg-147 (β6) form a salt bridge beneath Lys-81-I1 but do not contact the intermediate. Substitution of Asp-102 with alanine had a negligible effect on I2 or PLP formation (Table 2). Conversely, mutagenesis of Arg-147 reduced I2 formation and PLP synthesis more than 10-fold. Intact-protein MS revealed that Arg-147 substitutions prevented conversion of I1 to I2 (Fig. 5, C and D).

PLPS with PdxS/I1 and PdxT-Glutamyl Thioester Intermediates—We obtained a crystal structure of intact PLPS (PdxS12PdxT12) with two covalent intermediates, the glutamate thioester intermediate at Cys-78 in the glutaminase subunit (PdxT) and I1 in the synthase subunit (PdxS). The crystal structure of the double covalent intermediate trapped the fully activated synthase active site and is informative about conformational changes that occur during the catalytic cycle (Figs. 8 and 9).

![FIGURE 6. I1 accumulation. A, effect of pH on distribution of PdxS species after incubation with R5P and (NH4)2SO4. Peak areas are from deconvoluted ESI mass spectra. B, effect of (NH4)2SO4 concentration on distribution of PdxS species after incubation with R5P and (NH4)2SO4. I1 accumulation is compared with the total modified PdxS, based on peak areas from deconvoluted ESI mass spectra. No conditions were identified in which I1 was the predominant form of PdxS.](image-url)
ate (14). Interaction of PdxT with PdxS sequesters the glutaminase active site from bulk solvent, in a manner that would slow the loss of labile ammonia generated in the glutaminase reaction. The ammonia generation site faces the entrance to the PdxS $B_\beta/\alpha_\alpha$ barrel at the opposite end from the synthase active site. The hydrophobic barrel interior is well suited to diffusion of uncharged ammonia to the synthase active site.

In contrast to the glutaminase active site, the synthase active site of the PLPS double covalent intermediate reflects a remarkable set of conformational changes relative to the other states of *G. stearothermophilus* PdxS. These changes sequester the site from bulk solvent and provide more specific contacts with I$_1$ (Fig. 8). Three elements of the synthase structure are changed. First and most dramatically, the C-terminal tail is partially ordered from residues 272 to 290, leaving only residues 291–294 without electron density. Second, an internal peptide (residues 49–56) becomes ordered and forms a short helix ($\alpha_2a$) and the KGEPG loop (Table 2). The initial velocity of I$_2$ formation decreased significantly in comparison with the initial velocity of PLP synthesis. However, glutaminase activity was not affected (data not shown).

The C-terminal tail serves as a lid that covers helices $\alpha_2a$ and $\alpha_8'$ and the KGEPG loop, but it does not directly contact the covalent intermediate. The tail is not fully ordered, but combination of densities from the six independent subunits in the crystallographic asymmetric unit permitted all but the last four amino acids to be built into electron density (Fig. 8C). Tail residues Pro-285, Glu-286, and His-287 contact Arg-53 and the KGEPG loop.

The C-terminal tail interacts with the PdxS core in a zippered network of hydrogen bonds alternating between side chain and backbone atoms. For example, the His-287 side chain (tail) hydrogen bonds with the Gly-150 carbonyl (core, KGEPG). The Gln-290 side chain (tail) hydrogen bonds to the Glu-112 backbone carbonyl (core), and the His-115 side chain (core) hydrogen bonds to the Gln-290 backbone amide (tail). Additionally, the C-terminal tail hydrogen bonds to core residues residing on the adjacent subunit. Hydrogen bonds are formed between the carbonyl of Met-275 (tail) and the side chain of Arg-60 (helix $\alpha_2a$ in the adjacent subunit), the carbonyl of Arg-276 (tail) and the amide nitrogen of Val-58 (neighboring helix $\alpha_2a$), and the carbonyl of Gly-155 (KGEPG loop) and the side chain of Arg-60 (neighboring helix $\alpha_2a$). Consistent with the lack of cooperativity, the orientation of each subunit relative to its neighbors is unchanged by active site closure. In all six monomers, density for the tail ends at Gln-290 (Fig. 8C). The Gln-290 backbone carbonyl is hydrogen-bonded to Arg-137 (core) at precisely the secondary phosphate site (phosphate of R5P in the adduct at Lys-149 shown in Fig. 3, sulfate ion in the PdxS free enzyme (13), phosphate ion in the *Plasmodium* PLPS structure (21), and PLP phosphate in the yeast synthase structure (11)). Thus, the ordered C-terminal tail is incompatible with phosphate-containing ligands in this site. Interestingly, the Lys-149 side chain is outside the active site and pointed toward the secondary phosphate site, unlike its position in the PdxS$I_1$/$I_2$ structure (Fig. 9B).

The full length of the C-terminal tail is essential to I$_2$ formation. We made a series of C-terminal truncations (PdxS$\Delta$271–294, PdxS$\Delta$279–294, PdxS$\Delta$287–294, PdxS$\Delta$291–294, and PdxS$\Delta$294) to evaluate the importance of the tail. All truncated variants had decreased I$_2$ formation and PLP synthesis, with a greater effect on I$_2$ formation in our initial velocity-based assay.
The reduction in activity was correlated with the size of the truncation, but removal of even one amino acid (Δ294) reduced I₁ formation 5-fold and PLP synthesis 2-fold. The four C-terminal residues not observed in the PLPS₁-Glu structure were Glu-291–Arg-292–Gly-293–Trp-294, of which only Trp-294 and Arg-292 are conserved. Substitutions at Arg-292 had a greater effect on both chromophore and PLP formation compared with substitutions at the nonconserved Glu-291. Retaining the positive charge at position 292 (R292K) was least deleterious. As expected, all truncations had wild type glutaminase levels (data not shown).

**DISCUSSION**

Our studies present new insights into the complex mechanism of the synthase subunit of PLPS and new information about the roles of three conserved synthase amino acids. Together with the structure of the *G. stearothermophilus* PLPS synthase subunit (13), the structures reported here provide...
The disorder of amino acids 48–56 in PdxS glutaminase Michaelis complex (Fig. 8); and I1/I2 (Fig. 7). As the KGEPG loop also shifts with movement of Lys-149 into the active site. A FEBRUARY 27, 2015 •

The KGEPG loop also shifts with movement of Lys-149 into the active site of each subunit, in a position well supported by electron density (Fig. 7). Here, a comparison of the G. stearothermophilus synthase structures is important because under very similar crystallization conditions Lys-149 pointed into the active site in the presence of both RSP and ammonia, but it was directed outward in the presence of either substrate alone (Fig. 3) (13). This is notable because Lys-149 points outside the active site in all other synthase structures (10, 13, 14, 21, 44). Thus, addition of ammonia to I1 is the trigger for transfer of Lys-149 to the active site, where it is known to be critical for I2 formation (Table 2) (17). We infer that after ammonia addition the synthase active site re-opens permitting entry of Lys-149.

The structure of intact PLPS with on-pathway intermediates in both the glutaminase and synthase active sites (PLPS1-Glu) captures the enzyme as it is poised for ammonia attack on the synthase I1 intermediate. This state has a closed synthase active site where the C-terminal tail and helices α2a and α8’ together bury the I1 intermediate. The closed synthase active site is an ideal environment to sequester labile ammonia en route to I2. Ordering of helix α2a is a key part of active site closure, as it is disordered in the other G. stearothermophilus synthase structures. The ordering of helix α2a (residues 49–56) and the C-terminal tail (270–294) may be coupled, as part of the tail (275–276 backbone) contacts residues 58 and 60 in an adjacent subunit. This is consistent with the observation that the B. subtilis synthase C terminus is protected from proteolysis by addition of the glutaminase subunit and substrates (24). Although the final four amino acids (291–294) remained disordered in PLPS1-Glu, these residues, particularly conserved Arg-292, are critical for the formation of I2 and PLP (Table 2). Strong electron density for the C-terminal tail ends precisely at the secondary phosphate site outside the (β/α)8 barrel (Fig. 3, inset). A role has been proposed for phosphate binding to this site during the catalytic cycle (19). Alternatively, the site may be a transient anchor for the synthase terminal carboxylate, perhaps to assist in shifting Lys-149 into the active site. Ordering of the C-terminal tail and closure of the synthase active site appear to have required both a Michaelis-like complex in the glutaminase and I1 in the synthase, based on structures with the glutaminase intermediate in B. subtilis PLPS (14) and with I1 in the P. horikoshii PLPS (44), both of which had an open synthase active site and a disordered C-terminal tail.
**Structure of PLP Synthase**

Several key questions remain concerning the synthase mechanism. Formation of the I₂ chromophore remains mysterious. The chemical steps to its formation have been proposed (17, 20) but not demonstrated. A proposed structure of I₂ is indirectly supported by the structure of an acid breakdown product (18) and by the electron density in PdxS₁₁₁₁ (Fig. 7), but neither is definitive. The mode of interaction of substrate G3P with the synthase is also unknown. We did not capture a G3P complex with either the free enzyme or the I₁ intermediate, and it is likely that G3P interacts only with the I₂ intermediate state. To trap a G3P complex, we searched for a residue that is required for PLP synthesis but not for formation of I₁ or I₂. No conserved amino acid in the vicinity of the active site had this property. Given the instability and hence reactivity of I₁, G3P binding near the intermediate will likely result in PLP formation with no catalytic assistance from the enzyme. We considered two possibilities for a G3P-binding site. The first, on the outside of the (β/α)₆ barrel, makes use of the secondary phosphate-binding site. G3P in this site would require an assist, perhaps through covalent attachment to Lys-149, to move into the active site upon I₂ formation. The observed shift of Lys-149 into the active site in PdxS₁₁₁₁ makes this an attractive hypothesis. However, substitution of Arg for Lys-149 did not prevent PLP synthesis, arguing against this possibility. A more probable scenario is for the G3P phosphate to replace the free phosphate that forms upon ammonia attack in the I₁ to create I₂, thereby binding in an optimal position for reaction with I₂. This would also explain (and others) inability to trap a meaningful PLPS-G3P complex.

This study also leaves open the classic question for all glutamine amidotransferases about how the synthase and glutaminase active sites communicate to couple their activities. For PLPS, the specific question is how engagement with a Michaelis complex of the glutaminase subunit leads to closure of the synthase active site in the I₁ state. The now four crystal structures of *G. stearothermophilus* PdxS provide no clues. Crystal structures of other PLPS enzymes (10, 14, 21) or synthase dodecamers (11, 44) are equally opaque on this point. PLPS is the only characterized GAT in which the glutaminase activity does not depend on, nor is it accelerated by, the synthase substrates but depends only on the formation of the intact PLPS (8). We note that the PLPS substrates (glutamine, R5P, and G3P), unlike those of other GATs, are readily available metabolites and that glutamine may be the limiting substrate in vivo.

Up to 1.5% of prokaryotic genes encode for PLP-dependent enzymes; therefore, the demand for PLP as a cofactor is immense (45). The remarkable single-enzyme PLP biosynthetic pathway is a marvel of enzyme catalysis. Our studies provide additional glimpses into the mechanism, but there remains much to learn.

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