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Lysine relay mechanism coordinates intermediate transfer in vitamin B6 biosynthesis

Matthew J Rodrigues^{#1,2}, Volker Windeisen^{#1,3}, Yang Zhang⁴, Gabriela Guédez³, Stefan Weber³, Marco Strohmeier³, Jeremiah W Hanes^{4,5}, Antoine Royant^{6,7}, Gwyndaf Evans², Irmgard Sinning³, Steven E Ealick⁴, Tadhg P Begley⁸, and Ivo Tews^{1,3,*}

¹Biological Sciences, University of Southampton, Southampton, UK.

²Diamond Light Source, Harwell Science and Innovation Campus, Didcot, UK.

³Heidelberg University Biochemistry Center (BZH), Heidelberg, Germany.

⁴Department of Chemistry and Chemical Biology, Cornell University, Ithaca, New York, USA.

⁵Pacific Biosciences, Menlo Park, California, USA.

⁶Institut de Biologie Structurale, Université Grenoble Alpes, CNRS, CEA, Grenoble, France.

⁷European Synchrotron Radiation Facility, Grenoble, France.

⁸Department of Chemistry, Texas A&M University, College Station, Texas, USA.

[#] These authors contributed equally to this work.

Abstract

Substrate channeling has emerged as a common mechanism for enzymatic intermediate transfer. A conspicuous gap in knowledge concerns the use of covalent lysine imines in the transfer of carbonyl-group-containing intermediates, despite their wide use in enzymatic catalysis. Here we show how imine chemistry operates in the transfer of covalent intermediates in pyridoxal 5'-phosphate biosynthesis by the *Arabidopsis thaliana* enzyme Pdx1. An initial ribose 5-phosphate lysine imine is converted to the chromophoric I₃₂₀ intermediate, simultaneously bound to two lysine residues and partially vacating the active site, which creates space for glyceraldehyde 3-

* ivo.tewsrt@soton.ac.uk.

Author contributions

M.J.R., V.W., G.G., S.W., M.S. and J.W.H. performed protein expression, purification and enzymatic essays. M.J.R., V.W., G.G., S.W., M.S. and I.T. performed crystallization and X-ray diffraction experiments. M.J.R., V.W., A.R. and I.T. performed online spectroscopy experiments. M.J.R., V.W., Y.Z., I.S., S.E.E., T.P.B. and I.T. performed crystallographic analysis and data deposition. M.J.R., A.R., G.E., T.P.B. and I.T. performed spectroscopic data analysis. M.J.R., G.E., I.S., S.E.E., T.P.B. and I.T. wrote the paper. M.J.R. and V.W. contributed equally to this work.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Any supplementary information, chemical compound information and source data are available in the online version of the paper. Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>. Correspondence and requests for materials should be addressed to I.T.

Accession codes. Coordinates and structure factors have been deposited in the Protein Data Bank under the accession codes 5LNS (Pdx1-R5P), 5LNT (Pdx1 K166R-pre-I₃₂₀), 5LNU (Pdx1-I₃₂₀-G3P), 5LNV (Pdx1-I₃₂₀ multi-crystal), 5LNW (Pdx1-I₃₂₀-G3P), 5LNR (Pdx1-PLP).

phosphate to bind. Crystal structures show how substrate binding, catalysis and shuttling are coupled to conformational changes around strand $\beta 6$ of the Pdx1 ($\beta \alpha$)₈-barrel. The dual-specificity active site and imine relay mechanism for migration of carbonyl intermediates provide elegant solutions to the challenge of coordinating a complex sequence of reactions that follow a path of over 20 Å between substrate- and product-binding sites.

Pyridoxal 5'-phosphate (PLP) is an active form of vitamin B6, which functions as an enzyme cofactor and antioxidant^{1,2}. More than 140 different PLP-dependent enzymes catalyze reactions in prokaryotic amino acid and carbohydrate metabolism, and an estimated 1.5% of the genes in microbial genomes encode PLP-dependent enzymes³. The PLP synthase complex, which consists of the enzymes Pdx1 and Pdx2, is conserved in all domains of life^{4,5}. In striking contrast, the related *Escherichia coli* pathway, used only by a small number of bacteria, requires six enzymes to achieve the same biosynthesis^{4,6}.

The Pdx1 enzyme requires three substrates to catalyze the formation of PLP^{7–9}. Ribose 5-phosphate (R5P) reacts with ammonia, which is produced by a separate Pdx2 glutaminase domain, to produce the chromophoric I₃₂₀ intermediate with an absorbance maximum at 320 nm (ref. 10). I₃₂₀ formation is followed by addition of the third substrate, glyceraldehyde 3-phosphate (G3P) (**Fig. 1a**)⁹. Pdx1 has a ($\beta \alpha$)₈-barrel fold in a dodecameric assembly; each subunit contains two phosphate-binding sites, named P1 and P2, that define the active site architecture^{11,12} (**Fig. 1b**). P1 and P2 are separated by 21 Å (phosphorus to phosphorus) and are known to bind the phosphate groups of R5P, but the mechanism by which Pdx1 transfers intermediates between the sites is unknown.

The crystallographic studies described here reveal five Pdx1 intermediate structures, and they have been used to propose a mechanism of intermediate transfer chemistry that is not evident from previous characterization of the I₃₂₀ structure by MS analysis, phosphine trapping, and NMR analysis^{9,13}. The I₃₂₀ intermediate is simultaneously bound to two lysine residues, which allows the transfer between catalytic centers, and we describe structural changes to support intermediate formation and transfer.

RESULTS

The I₃₂₀ intermediate is central to PLP biosynthesis

Various bacterial and eukaryotic Pdx1 proteins were screened to identify the *Arabidopsis* Pdx1 (ref. 14) crystal system that provided the Pdx1-intermediate complexes reported here (**Fig. 2; Supplementary Results, Supplementary Fig. 1 and Supplementary Table 1**). Although other crystal systems such as that in *Thermus thermophilus* yielded higher resolution diffraction (1.6 Å), they were not robust enough to survive crystal soaking experiments.

PLP formation is initiated with formation of the Pdx1-R5P complex, observed here at 1.9-Å resolution (**Fig. 2a**), and previously observed for *Plasmodium berghei* and *Geobacillus stearothermophilus* Pdx1 (refs. 15,16). A covalent imine forms between the ϵ -NH₂ of Lys98 and the R5P C1, with the R5P phosphate bound in P1 (refs. 10,15–17) (**Fig. 2a**). The proximity of the ϵ -NH₂ group of Lys98 to the side chains of Aspl19 and Ser121

(**Supplementary Fig. 2a,b**) of the totally conserved DESE sequence motif in PLP synthases suggests that Asp119 or Ser121 may play a role in Schiff base formation, but as of yet we are unable to clearly assign the base catalyzing this step.

Transient association with Pdx1 activates Pdx2 to catalyze glutamine hydrolysis, releasing ammonia as a nitrogen source for PLP biosynthesis^{8,18,19}. The ammonia produced by Pdx2 passes through a hydrophobic tunnel in the center of the Pdx1 ($\beta\alpha$)₈-barrel to the Pdx1 P1 active site; channeling of intermediates prevents their diffusion into the bulk solvent and is common in glutamine amidotransferase enzymes such as PLP synthase^{11,15,20}. Ammonia reacts with Pdx1-R5P to form the chromophoric I₃₂₀ intermediate^{10,21}. Since the product, PLP, binds in the P2 site²², we hypothesized that formation of the I₃₂₀ intermediate is central to the migration of reaction intermediates from Lys98 (P1) to Lys166 (P2).

Soaking Pdx1-R5P crystals with ammonium chloride generated Pdx1-I₃₂₀ (**Fig. 2c**). While the addition of ammonium salts decouples Pdx1-I₃₂₀ formation from Pdx2-dependent glutamine hydrolysis¹⁰, the change in osmotic pressure caused many crystals to become disordered. More than 1,000 crystals were tested to optimize the protocol used to provide both high-resolution diffraction and homogeneous accumulation of Pdx1 in the I₃₂₀ state. Online UV-Vis (visible) microspectrophotometry²³ was performed to ensure that crystals used in diffraction experiments contained Pdx1 predominantly in the I₃₂₀ state (**Fig. 3a**).

Pdx1-I₃₂₀ crystals typically diffracted to 2.3–2.0-Å resolution and showed continuous electron density between Lys98 and Lys166 and loss of the phosphate group (**Figs. 2c and 3b**). Interpretation of the highest-resolution data set at 1.7-Å resolution was aided by the earlier MS and NMR data showing that I₃₂₀ retains all five carbons of the R5P substrate¹⁰, with Cl covalently bound to a nitrogen atom¹³, observed here to be the ϵ -NH₂ of Lys98 (**Fig. 3b**).

Comparison of the ¹³C-NMR spectra of I₃₂₀ reconstituted *in vitro* using ¹⁴N- and ¹⁵N-labeled ammonium chloride showed that the nitrogen incorporation occurred at the C2 atom¹³, which allowed assignment of nitrogen and oxygen atoms bound to C2 and C3 of the intermediate, respectively (**Figs. 2c and 3b**). The C3-bound oxygen atom of I₃₂₀ is the sole oxygen that remains from the R5P substrate¹³. The C5 attachment of I₃₂₀ to a nitrogen atom was previously interpreted as being caused by I₃₂₀ decomposition under the denaturing and acidic conditions in the NMR experiment¹³; however, the crystallographic analysis now resolves the nature of the C5-N interaction, observed here to be a covalent bond between C5 and the ϵ -NH₂ of Lys166. Thus, the intermediate is not released from Lys98 before I₃₂₀ formation as previously thought¹³, but is simultaneously bound to two lysine residues.

A network of polar interactions between side chains of Asp119 and Glu122 of the DESE sequence motif and Arg164 (all residues completely conserved in PLP synthases) is in close proximity to the intermediates in the P1 site. Previous studies demonstrated that replacing Arg 164 in *G. stearothermophilus* Pdx1 with alanine causes a 95% reduction in the rate of I₃₂₀ formation¹⁶, and Arg164 is suitably positioned to activate the ϵ -NH₂ group of Lys166 for Schiff base formation in the Pdx1-I₃₂₀ structure (**Supplementary Fig. 2c**). This conserved

network of polar interactions is reminiscent of acetoacetate decarboxylase, a well-studied example of the electrostatic perturbation model for enzyme activation²⁴.

Several examples of crystallographic structures with imine-bound cofactors are known to suffer from site-specific radiation damage^{25,26}. Radiation damage may lead to artifacts in electron density maps that are not representative of the protein structure before X-ray exposure²⁷. UV-Vis spectra of PdxI-I₃₂₀ *in crystallo* during X-ray irradiation were collected. The spectra of the crystals changed in response to irradiation, with 20% of the changes occurring in the first 245 kGy. While their occupancy is below 20%, the species generated by X-ray irradiation are not expected to have a substantial effect on the interpretation of electron density maps²⁸. We constructed a 2.2-Å resolution multi-crystal data set using a protocol similar to that first described for investigation of site-specific radiation damage in horseradish peroxidase²⁹. The X-ray dose absorbed during data collection was calculated using RADDOS-3D³⁰; BLEND was used to merge the diffraction data collected from each crystal below the dose threshold of 245 kGy into a single data set³¹. The resulting structure confirmed the nature of the bridging structure of I₃₂₀ (**Supplementary Fig. 3**).

Conformational changes supporting intermediate transfer

Formation of PdxI-I₃₂₀ requires conformational changes in strand β6 of the PdxI (βα)₈-barrel, which results in reorientation of Lys166 toward P1; this residue points toward the P2 site in the PdxI-R5P structure (**Fig. 3b**; **Supplementary Fig. 4a**). We investigated the PdxI K166R variant^{10,16} to better understand whether the structural transition is linked to ammonia incorporation. Sequential exposure of PdxI K166R to R5P and ammonium chloride results in a complex termed PdxI-pre-I₃₂₀ (**Fig. 2b**). Like Lys166 in the PdxI-I₃₂₀ complex, Arg166 reorients toward P1, and a peptide flip between Arg 166 and Gly 167 causes reorientation of the Thr165 and Arg166 side chains as PdxI transitions from the PdxI-R5P state to the PdxI-pre-I₃₂₀ and PdxI-I₃₂₀ complexes (**Fig. 3b**; **Supplementary Fig. 4b,c**).

An earlier analysis predicted such changes for the wild-type enzyme by showing that the β6 strand lacks hydrogen bond stabilization from adjacent β-strands, allowing for additional flexibility in this region¹². The recent structural analysis of *G. stearothermophilus* PdxI also revealed that Lys166 adopts these two conformations¹⁶.

These conformational changes are likely linked to the passage of ammonia through the transient hydrophobic tunnel in the (βα)₈-barrel^{11,15}. Notably, the absolutely conserved methionine residue in this tunnel, Met 162, is located on strand β6 together with the catalytic residue Lys 166 (ref. 15). In the previous study, the equivalent residue to Met 162 in *Plasmodium berghei*, Met 148, was exchanged for leucine, causing an increase in the catalytic rate of Pdx2-dependent PLP biosynthesis with no effect on the rate of NH₄Cl-dependent catalysis, suggesting a coupling of PdxI synthase and Pdx2 glutaminase activities involving Met 148. We propose that the disruption of Met 162 on the β6 strand by the passage of ammonia couples glutamine hydrolysis to the conformational changes required for I₃₂₀ formation (**Supplementary Fig. 4d**).

Elimination of the R5P phosphate group bound in the P1 site immediately precedes the formation of the I₃₂₀ intermediate⁹. This phosphate-binding site is located in the typical

position for the ($\beta\alpha$)₈-fold at the C-terminal face of the β -barrel, and makes additional contacts with the $\beta 6$ - $\alpha 6$ loop that carries Lys 166 (refs. 11,12,32). The Pdxl-pre-I₃₂₀ structure highlights the role of this phosphate group as a rigid anchor that, together with the Lys98 imine, ensures correct positioning of the intermediate for ammonia incorporation and covalent attachment to Lys166 in the wild-type protein (**Fig. 2b**). Although incorporation of the ammonia nitrogen atom occurs in the Pdxl K166R variant, the different functionality of the Arg166 guanidino group prevents the catalysis of phosphate elimination^{10,16}. The retention of the phosphate group explains the different UV-Vis absorption spectrum of the pre-I₃₂₀ complex, which has a lower absorbance compared to Pdxl-I₃₂₀ (ref. 10).

Dual-specificity binding site for pentose and triose

The structural rearrangements that are associated with I₃₂₀ formation result in the displacement of carbon atoms C3, C4 and C5 of the intermediate away from the P1 phosphate-binding site (**Fig. 3b**), and the elimination reaction allows the R5P phosphate group to diffuse out of the P1 site. These actions create space for the binding of G3P in the P1 site. In the Pdxl-I₃₂₀-G3P complex, G3P is covalently bound to the C2 nitrogen atom of I₃₂₀, with the G3P phosphate in the P1 binding site (**Fig. 2d** and **3c**). Surprisingly, the intermediate is covalently attached to both Lys98 and Lys166, forming a horseshoe-like structure.

In solution, addition of G3P to Pdxl -I₃₂₀ allows the reaction to proceed to the PLP product state; however, this did not occur *in crystallo*. The total loss of diffraction upon prolonged G3P exposure provides strong evidence for extensive structural changes that accompany the final steps of PLP formation. The C terminus of Pdxl is disordered in crystal structures and is known to participate in the final stages of PLP biosynthesis^{32–34}. The packing of Pdxl in the crystalline state may prevent the C terminus from adopting the conformation required for Pdxl to complete catalysis of PLP biosynthesis.

The Pdxl-PLP state can be observed by directly soaking Pdxl crystals with PLP. The 1.6-Å resolution electron density reveals an imine attachment of PLP to Lys166 by the carbon atom that was initially the C5 of R5P (**Figs. 2e** and **3d**). A noncovalent yeast Pdxl-PLP complex was previously reported²²; however, examination of the deposited structure (PDB ID 3o05) shows conclusively that the PLP is covalently bound. The covalent nature of this complex is supported by UV-Vis spectroscopy of a Pdxl-PLP crystal that shows the characteristic absorption maximum at 408 nm as is typical for covalently bound PLP rather than 388 nm as is typical for free PLP (**Supplementary Fig. 5**)¹³.

The structural data presented here have been combined with the published biochemical biophysical data from Pdxl into a mechanistic proposal for catalysis and intermediate transfer (**Fig. 4**; **Supplementary Fig. 6**). The schematic shows the central role of the I₃₂₀ intermediate, linking between side chains Lys98 and Lys166, for transfer of the reaction between substrate- and product-binding sites.

DISCUSSION

PdxI is the first enzyme found to use a dual transamine intermediate to transfer a reaction across an extended active site within a $(\beta\alpha)_8$ -barrel enzyme³⁵, in addition to coupling delivery of ammonia from Pdx2 to PdxI to I₃₂₀ formation. PdxI enzymes that have been studied thus far span bacterial species^{11,12,16,17}, unicellular eukaryotes^{15,19,22,36,37} and plants¹⁴. The mechanism of I₃₂₀ formation and intermediate transfer is assumed to be conserved given the high overall conservation of the PdxI enzyme⁵, the consistency of findings across all kingdoms of life and, in particular, the conservation of Lys98 and Lys166. Central to the function of PdxI is the bridging structure of the I₃₂₀ intermediate between these conserved lysine residues (**Figs. 2c** and **3b**).

The structures reveal a mechanism for how PdxI uses covalent tethers to prevent loss of intermediates to surrounding solvent, and explain how the enzyme maintains a high local concentration of substrate and protects the reactive I₃₂₀ species. The use of covalent tethers is reminiscent of the protein-bound phospho-pantothenoyl thioesters that are used to transfer intermediates between active sites in the assembly line enzymology of fatty acid synthases³⁸, polyketide synthases³⁹ and non-ribosomal polypeptide synthases⁴⁰. PdxI uses a similar transfer strategy to those found in the glycine cleavage system⁴¹ and the classical pyruvate dehydrogenase complex⁴², where lipoic acid is used to chaperone intermediates between active sites. Similarly to PdxI, these enzymes use covalent tethers to prevent the loss of substrates or intermediates to surrounding solvent and maintain the local concentration of substrate. In contrast to the previous examples, PdxI transfers covalent intermediates within a single catalytic domain. The intricate relay mechanism displayed by the PdxI subunit of PLP synthase allows the enzyme to maintain precise control of the complex reaction performed across multiple active sites.

A conspicuous absence in Nature's repertoire is the use of lysine imines to channel carbonyl-group-containing intermediates; this is surprising because lysine imines are used extensively in enzymatic catalysis, and transamination reactions are facile. In this study we described PLP synthase as the first example of an enzyme that uses two lysine residues to shuttle intermediates between two active sites. Lys98 anchors intermediates in the P1 site for the first phase of PLP assembly. Lys166 then adds and facilitates intermediate transfer to the P2 site, where completion of PLP formation occurs. This transfer strategy enables a single enzyme to catalyze the complex assembly process required for the formation of the pyridine ring of PLP and stands in contrast to the use of six enzymes in *E. coli* to achieve the same biosynthetic goal.

Note: Another X-ray structure of the I₃₂₀ complex⁴³ appeared while this paper was under revision.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

ONLINE METHODS

Molecular biology and protein production.

Pdx1.1 and Pdx1.3, the two alleles of active Pdx1 from *Arabidopsis thaliana*, were cloned as C-terminal his-tagged proteins in *E. coli* BL21 (DE3) cells as previously described, using the NdeI/XhoI restriction sites of the pET21a expression plasmid (Novagen)¹⁴. The expressed proteins (including tag) have molecular weights of 33,532 Da (Pdx1.1) and 33,886 Da (Pdx1.3). Cells were grown in 1 L cultures at 37 °C to an optical density (OD) of 0.6. Protein expression was induced using 60 ml of 25% (w/v) lactose and then grown for a further 16 h at 30 °C.

Protein purification.

Cells were lysed by sonication, and centrifuged for 1 h at 140,000 × g. Proteins were purified from the supernatant using 1 ml Immobilized Metal Affinity Chromatography HiTrap columns (GE Healthcare Life Sciences) loaded with nickel and equilibrated with lysis buffer (50 mM Tris-Cl, pH 7.5, 500 mM sodium chloride, 10 mM imidazole, 2% glycerol). The column was washed with wash buffer (50 mM Tris-Cl, pH 7.5, 500 mM sodium chloride, 50 mM imidazole, 2% glycerol) and the protein was eluted in elution buffer (50 mM Tris-Cl pH 7.5, 500 mM sodium chloride, 500 mM imidazole, 5% glycerol). For subsequent size-exclusion chromatography a Superdex 26/60 column (GE Healthcare Life Sciences) was equilibrated with gel filtration buffer (20 mM Tris-Cl pH 8.0, 200 mM KCl).

Complex preparation.

Pdx1-R5P was prepared using crystals of Pdx1.3 grown in 0.5 M sodium citrate buffered with 0.1 M HEPES, pH 7.5. These crystals were soaked in 2.5 mM R5P. K166R-pre-I₃₂₀ was prepared using pre-formed crystals of K166R Pdx1.1 grown in 100 mM sodium cacodylate pH 6.5 and 15% (w/v) PEG 8000. 0.5 μl of 1:1 100 mM R5P and mother liquor were then added to 1 μl drops containing the crystals, leading to a final concentration of about 15 mM R5P in the drop. The well was closed for 5 min, allowing for equilibration of the sample. 0.5 μl of 1:1 1 M NH₄Cl and mother liquor were added to the same drop resulting in a final drop size of 1.5 μl and final NH₄Cl concentration of 125 mM. The well was sealed and allowed to equilibrate for 2 h. Pdx1-I₃₂₀ was prepared using pre-formed crystals of Pdx1.3 grown in 100 mM Tris-Cl pH 8.5 and 12.25% PEG 4000 (w/v). 0.5 μl of 50 mM R5P in mother liquor was added to 2 μl drops containing Pdx1.3 crystals resulting in a final concentration of 10 mM R5P in the drop. The well was sealed and equilibrated for 30 min. 0.5 μl of 500 mM NH₄Cl in mother liquor were then added to the same well with Pdx1.3 crystals, resulting in a final NH₄Cl concentration of 100 mM in the drop. The well was sealed and equilibrated for 4 d. Pdx1-I₃₂₀-G3P was prepared starting with crystals of Pdx1-I₃₂₀ described above. After 4 d, the Pdx1-I₃₂₀ crystals were transferred to a solution containing mother liquor containing 10 mM G3P and allowed to equilibrate for 150 s. Cryoprotectant buffer containing 10 mM G3P was then added to stabilize the crystals before flash cooling in liquid nitrogen. The Pdx1-PLP complex was prepared by adding 10 mM PLP to preformed Pdx1.3 crystals grown in 0.1 M Tris-Cl, pH 8.25, containing 8% PEG 8000.

In all cases except for the I₃₂₀-G3P complex, cryobuffer containing the well solution and an additional 20% glycerol was added, and crystals were either flash cooled in liquid nitrogen (R5P, I₃₂₀, PLP complexes) or directly transferred into the cryostream (pre-I₃₂₀ complex).

Data collection and structure determination.

Offline and online UV-Vis absorption spectra were recorded at the European Synchrotron Radiation Facility (ESRF), France, on beamline ID14-4 (ref. 44) and at the ID29S-Cryobench laboratory^{23,45}. Diffraction data for the Pdxl-R5P data set were collected on beamline ID14-1 (ref. 46) of the ESRF at a wavelength of 0.9760 Å. Diffraction data for the Pdxl K166R-pre-I₃₂₀ data set were collected on beamline ID29 (ref. 47) of the ESRF, at a wavelength of 0.933 Å. Diffraction data for the Pdxl-I₃₂₀ crystal single crystal data set were collected on beamline I04-1 of the Diamond Light Source, UK, at a wavelength of 0.9173 Å. Diffraction data for the Pdxl-I₃₂₀ multi-crystal data set were collected on beamline ID23-1 (ref. 48) of the ESRF, at a wavelength of 0.9763 Å. Diffraction data for the Pdxl-I₃₂₀-G3P crystal were collected on beamline ID23-1 of the ESRF at a wavelength of 0.9763 Å. Diffraction data for the Pdxl-PLP data set were collected on beamline ID14-1 of the ESRF, at a wavelength of 0.9334 Å. All data sets were collected at a temperature of 100 K. Data integration and scaling was carried out with XDS, DIALS and AIMLESS⁴⁹⁻⁵¹. Structure determination by molecular replacement used bacterial Pdxl from *Bacillus subtilis*⁴ as the search model (PDB ID 2NV1) and MOLREP⁵². Iterative model building and refinement were carried out using COOT⁵³, REFMAC5 (ref. 54) and PHENIX⁵⁵. Pdxl crystallized in a R3 unit cell with four monomers in the asymmetric unit. All monomers were first refined individually, i.e., without non-crystallographic symmetry (NCS) constraints, and inspected. The crystals selected in this study represent structures that were uniform with respect to accumulation of intermediates over all four monomers. For I₃₂₀ multi-crystal and I₃₂₀-G3P, the final round of refinement used the Cartesian protocol in PHENIX with Chain A as reference and NCS constraints over Lys98, Lys166 and the intermediate. Dictionary files defining ligand geometry restraints for refinement were created using JLigand⁵⁶. All further data manipulations were carried out by the CCP4 suite of programs⁵⁷. The refined crystal structures include Ramachandran favored residues (outliers) as Pdxl-R5P 98.8% (0.0%), Pdxl K166R-pre-I₃₂₀ 98.7% (0.0%), single crystal Pdxl-I₃₂₀ 98.8% (0.0%), multi-crystal Pdxl-I₃₂₀ 97.6% (0.0%), Pdxl-I₃₂₀-G3P 98.7% (0.0%), Pdxl-PLP 98.3% (0.0%). The illustrations in the main text were prepared using PyMOL (Schrödinger, LLC). Data collection and refinement statistics are shown in **Supplementary Table 1**. Coordinates and structure factors have been deposited in the Protein Data Bank under the accession codes 5LNS (Pdxl-R5P), 5LNT (Pdxl K166R-pre-I₃₂₀), 5LNU (Pdxl-I₃₂₀-G3P), 5LNV (Pdxl-I₃₂₀-G3P), 5LNR (Pdxl-PLP).

Multi-crystal analysis to assess beam damage.

About 50 complete data sets for I₃₂₀ complexes were collected at ID23-1, ESRF. Data collected before the 245 kGy threshold were integrated using XDS⁴⁹; all X-ray doses were calculated using RADDOSE-3D⁵⁸. BLEND was run in analysis mode to group data sets with similar unit cell dimensions³¹. Grouped wedges of data were input to synthesis mode to identify groups that merged well. A core group of five wedges with good merging statistics (R_{MEAS} 16.5, R_{PIM} 11.0, 62.3% complete, 2.0-Å resolution) was identified. BLEND was

then run in combination mode to add wedges to the core group individually and assess whether any improvements in completeness could be made without significantly worsening merging statistics. This resulted in a 19-wedge data set from nine crystals (R_{MEAS} 20.1, 99.5% complete, 2.2-Å resolution) leading to a low-dose structure of the I₃₂₀ complex. Coordinates and structure factors of the multi-crystal PdxI-I₃₂₀ complex have been deposited in the Protein Data Bank under the accession code 5LNV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1|. The reaction catalyzed by the Pdx1 and Pdx2 subunits of the PLP synthase complex and the structure of Pdx1.

(a) Pyridoxal 5-phosphate synthase catalyzes the complex condensation reaction between ribose 5-phosphate, glyceraldehyde 3-phosphate and ammonia. (b) The overall structure of the Pdx1 core. Pdx1 forms a dodecamer from two interlocked hexameric rings. This core complex is shown in two orientations, with subunit boundaries and the two phosphate-binding sites for each protein monomer indicated. The phosphate-binding sites P1 and P2 are required for substrate and product binding, respectively, and are separated by 21 Å (phosphorus to phosphorus). A cartoon representation for one monomer illustrates the $(\beta\alpha)_8$ -barrel fold of Pdx1.

Figure 2|. Crystallographic structures of five covalent intermediates in PLP biosynthesis. Pdx1 is shown in cartoon representation, and catalytic lysine side chains and intermediate atoms are shown in stick representation with carbon atoms in green (for Lys98 and Lys166) and orange (for intermediates), nitrogen atoms in blue, oxygen atoms in red, and phosphorus atoms in purple. $2F_o - F_c$ electron density maps are shown at 1σ for the complexes. **(a)** Binding of R5P (first substrate) uses P1 and occurs by covalent attachment through Schiff base formation with Lys98. **(b,c)** Addition of ammonia (second substrate) leads to the formation of an intermediate in P1 in the K166R mutant **(b)**, which converts to the I_{320} species in wild-type enzyme through formation of a second Schiff base with Lys166 **(c)**. **(d)** Incorporation of G3P (third substrate) leads to a covalent complex with I_{320} , with the G3P phosphate bound in the P1 site. **(e)** The PLP complex shows PLP covalently bound to the enzyme through Schiff base formation with Lys166, with its phosphate bound in the P2 site.

Figure 3]. The dual-specificity binding site P1 and the product-binding site P2.

(a) The UV-Vis spectrum of a Pdx1-I₃₂₀ crystal shows an absorption maximum at 280 nm, corresponding to protein, and near 320 nm for the I₃₂₀ intermediate. (b) The intermediate I₃₂₀ is covalently bound to both Lys98 and Lys166, shown in two views rotated by 90°. The overlay with the R5P complex (faded) shows the different positioning of Lys166 and adjacent residues Thr165 and Gly167. Selected amino acid atoms and the I₃₂₀ atoms are shown in stick representation, with carbon atoms in green (for amino acids) and orange (for intermediates), nitrogen atoms in blue, oxygen atoms in red, and phosphorus atoms in purple. Arrows indicate the different conformations of the Lys166 side chains and the Lys166-Gly167 peptide between Pdx1-I₃₂₀ and Pdx1-R5P complexes. Pep-flip, peptide flip. (c) The I₃₂₀-G3P complex horseshoe-like intermediate. The intermediate is covalently attached to both Lys98 and Lys166. Formation of the G3P binding site requires prior formation of the I₃₂₀ adduct to dissociate the C3-C5 atoms from the binding site, which allows the G3P phosphate to bind in the P1 site. The overlay with the R5P complex (faded) shows where the ribose and triose portions match. Atoms colored as described for **Figure 3b**. (d) The PLP adduct is covalently bound by a Schiff base to Lys166. 2F_o - F_c electron density map of the refined complex shown at 1 σ. Atoms colored as described for **Figure 3b**.

Figure 4|. The central role of I₃₂₀ intermediate transfer in vitamin B6 biosynthesis.

The proposed Pdx1 reaction mechanism is shown as a schematic. Lys98 points toward P1, and Lys166 points toward P2 in the free enzyme. Structural transitions support reorientation of the side chain of Lys166 toward the P1 site at the time of ammonia incorporation, leading to formation of I₃₂₀, creation of the G3P binding site, and the subsequent formation of the covalent I₃₂₀-G3P complex. The reversion of the structural transitions around Lys166 lead to release of the I₃₂₀-G3P intermediate from covalent attachment to Lys98 and observation of covalently bound PLP in the P2 site.