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Raschle, Thomas; Arigoni, Duilio; Brunisholz, René; Rechsteiner, Helene; Amrhein, Nikolaus; Fitzpatrick, Thérésa Bridget

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Reaction Mechanism of Pyridoxal 5′-Phosphate Synthase

*DETECTION OF AN ENZYME-BOUND CHROMOPHORIC INTERMEDIATE**

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Thomas Raschle[‡], Duilio Arigoni[§], René Brunisholz[¶], Helene Rechsteiner[¶], Nikolaus Amrhein[‡], and Teresa B. Fitzpatrick[‡1]

From the [‡]Institute of Plant Sciences, ETH Zurich, 8092 Zurich, the [§]Organic Chemistry Laboratory, ETH Zurich, 8093 Zurich, and the [¶]Functional Genomics Center Zurich, 8057 Zurich, Switzerland

Vitamin B6 is an essential metabolite in all organisms. *De novo* synthesis of the vitamin can occur through either of two mutually exclusive pathways referred to as deoxyxylulose 5-phosphate-dependent and deoxyxylulose 5-phosphate-independent. The latter pathway has only recently been discovered and is distinguished by the presence of two genes, *Pdx1* and *Pdx2*, encoding the synthase and glutaminase subunit of PLP synthase, respectively. In the presence of ammonia, the synthase alone displays an exceptional polymorphic synthetic ability in carrying out a complex set of reactions, including pentose and triose isomerization, imine formation, ammonia addition, aldol-type condensation, cyclization, and aromatization, that convert C3 and C5 precursors into the cofactor B6 vitamer, pyridoxal 5′-phosphate. Here, employing the *Bacillus subtilis* proteins, we demonstrate key features along the catalytic path. We show that ribose 5-phosphate is the preferred C5 substrate and provide unequivocal evidence that the pent(ul)ose phosphate imine occurs at lysine 81 rather than lysine 149 as previously postulated. While this study was under review, corroborative crystallographic evidence has been provided for imine formation with the corresponding lysine group in the enzyme from *Thermotoga maritima* (Zein, F., Zhang, Y., Kang, Y.-N., Burns, K., Begley, T. P., and Ealick, S. E. (2006) *Biochemistry* 45, 14609–14620). We have detected an unanticipated covalent reaction intermediate that occurs subsequent to imine formation and is dependent on the presence of Pdx2 and glutamine. This step most likely primes the enzyme for acceptance of the triose sugar, ultimately leading to formation of the pyridine ring. Two alternative structures are proposed for the chromophoric intermediate, both of which require substantial modifications of the proposed mechanism.

Pyridoxal 5′-phosphate (PLP)[2] is an essential cofactor of many enzymes in all living systems. It is involved in amino acid and carbohydrate metabolism and has recently been implicated as an antioxidant with a potent ability to quench singlet oxygen and the superoxide anion (2–4). Two distinct pathways for its *de novo* biosynthesis have been identified (5–13). One, referred to as the DXP-dependent pathway, is found in a relatively small number of eubacteria and has been extensively studied in *Escherichia coli*. In this pathway, pyridoxine 5′-phosphate is derived from DXP and 4-phosphohydroxy-L-threonine (9, 10). The second pathway, referred to as DXP-independent, has only been identified recently and appears to be far more prevalent, *i.e.* in archaea, fungi, plants, and the majority of bacteria (2, 13). It is characterized by the presence of two genes, *Pdx1* and *Pdx2*. The corresponding proteins function together as the glutamine amidotransferase, PLP synthase with Pdx2 as the glutaminase domain and Pdx1 as the acceptor domain. As a result of extensive labeling studies in yeast and biochemical analysis with the recombinant enzymes, the substrates of Pdx1 have recently been identified (11, 12, 14, 15). In the presence of Pdx1, Pdx2, and glutamine, a combination of either ribose 5-phosphate (R5P) or ribulose 5-phosphate (Ru5P) with either glyceraldehyde 3-phosphate (G3P) or dihydroxyacetone phosphate resulted in the reconstitution of PLP biosynthesis. As either of two pentose or triose sugar phosphates could be utilized, it was proposed that Pdx1 can catalyze their isomerization (11).

In analogy to the reactions catalyzed by imidazole glycerol-phosphate synthase (HisH/HisF) (16) and thiazole synthase (17), it has been hypothesized that a pentulose phosphate imine adduct occurs during the reaction sequence of Pdx1 and Pdx2 (11). The observation of the expected mass for such an adduct with the isolated recombinant protein corroborated this hypothesis. This adduct was proposed to involve lysine 149, as deduced from tandem mass spectrometric analysis (11). With the knowledge of substrates and product at hand, and furthermore, the observation that 2′-hydroxypyridoxol is converted into pyridoxamine in whole yeast cells (18), a mechanism of action was proposed (11). In this context, Pdx1 appears to be exceptional in that it can catalyze pentose and triose isomerizations, imine formation, ammonia addition, and aromatic ring formation, all in a single enzymatic system. Many of the mechanistic steps proposed are hypothetical, and additional information on the actual mechanism of such an intriguing enzyme is highly desirable given its fundamental interest and its potential as a drug target in certain parasitic organisms (19, 20).

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[1] To whom correspondence should be addressed. Tel.: 41-44-6323841; Fax: 41-44-6321044; E-mail: tfitzpatrick@ethz.ch.

[2] The abbreviations used are: PLP, pyridoxal 5′-phosphate; DXP, deoxyxylulose 5-phosphate; G3P, glyceraldehyde 3-phosphate; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; HPLC, high pressure liquid chromatography; RP-HPLC, reverse-phase HPLC; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; ESI, electrospray mass ionization.

Here we report on the identification of an unanticipated Pdx1 reaction intermediate. This intermediate is chromophoric; its formation is subsequent to that of the imine adduct and is concomitant with the release of phosphate from the pent(ul)ose substrate. Furthermore, it is dependent on glutamine hydrolysis by the Pdx2 subunit. In addition, we show that ribose 5-phosphate is the preferred C5 substrate of Pdx1. Lastly, although we can corroborate the participation of a pent(ul)ose phosphate-derived imine as a reaction intermediate, this adduct is now unequivocally shown to involve, in *Bacillus subtilis* Pdx1, lysine 81 rather than lysine 149, as suggested previously (11). Our results have implications for the annotation of the active site of Pdx1 and necessitate considerable revision of the previously proposed mechanism (11).

EXPERIMENTAL PROCEDURES

Materials—The constructs pETBsPdx1, pETBsPdx2, and pETBsPdx2-His$_6$, described in Ref. 12, and pETBsPdx1-His$_6$, described in Ref. 21, in addition to the mutants Pdx1-His$_6$ D24A, Pdx1-His$_6$ K81A, Pdx1-His$_6$ K149A, and Pdx2-His$_6$ H170N (21), were used in this study.

Site-directed Mutagenesis—Pdx1-His$_6$ K81R and Pdx1-His$_6$ K149R were generated using the QuikChange site-directed mutagenesis kit (Stratagene) employing the following oligonucleotides: K81R, forward 5′-CCGGTAATGGCACGAGCGCGTATCGG-3′, and reverse, 5′-CCGATACGCGCTCGTGCCATTACCGG-3′; K149R, forward 5′-CTATGCTTCGCACAAGAGGTGAGCCTGG-3′, and reverse, 5′-CCAGGCTCACCTCTTGTGCGAAGCATAG-3′ using pETBsPdx1-His$_6$ as the template. Expression and purification of all proteins were carried out as described in Ref. 12.

Generation of Free Pdx1—Isolated Pdx1-His$_6$ was incubated with 0.5 mM DL-G3P and 20 mM ammonium sulfate for 3 h at room temperature prior to dialysis against 20 mM Tris-Cl, pH 7.5, containing 20 mM sodium chloride. Dialyzed protein samples were frozen in liquid nitrogen and stored at −80 °C until use.

Synthesis of U-^{14}C- and U-^{13}C-labeled Ru5P and R5P—Uniform labeling of Ru5P with either ^{13}C or ^{14}C was carried out by incubation of the respective uniformly labeled glucose (0.5 mM) with enzymes of the pentose phosphate pathway (hexokinase (2 units), glucose 6-phosphate dehydrogenase (1 unit), 6-phosphogluconic dehydrogenase (1 unit)) in 50 mM Tris-Cl buffer, pH 8, containing 10 mM $MgCl_2$ and 0.6 mM each of ATP, NAD^+, and $NADP^+$ at 37 °C for the indicated time intervals. When labeled R5P was required, 1 unit of phosphoriboisomerase was included.

PLP Synthase Activity Assays—The activity of PLP synthase was monitored in 96-well plates (PowerWave™ microplate spectrophotometer, Bio-Tek) by following the absorbance at 414 nm, due to the formation of PLP (12). Reactions were carried out at 37 °C in 50 mM Tris-Cl, pH 8, containing 20 μM free Pdx1 and 20 μM Pdx2, 1 mM DL-G3P, and 20 mM glutamine while varying the concentration of either R5P or Ru5P in the range of 0.01–1.0 or 0.3–6 mM, respectively.

HPLC/Protein Methods of Pdx1—Pdx1 species were separated by RP-HPLC (Agilent 1100 series capillary LC System) employing a Zorbax SB C18 column (3.5 μm, 0.5 × 150 mm). Protein was eluted with a linear gradient of 0–45% acetonitrile (v/v). Peak fractions were collected and analyzed by either MALDI-TOF (Bruker Ultraflex II) or ESI mass spectrometry (Waters quadropole time-of-flight Ultima API). Pdx1 tryptic peptides were separated by RP-HPLC employing a Reprosil 100 C18 column (5 μm, 100 × 4.6 mm) equilibrated in 0.05% trifluoroacetic acid (v/v). The online addition of scintillation liquid (Flo-scint A, PerkinElmer Life Sciences) and radio detection (Radiomatic FLO-ONE-Beta, PerkinElmer Life Sciences) permitted identification of radiolabeled fractions. A gradient of 0–60% acetonitrile/0.5% trifluoroacetic acid (v/v) at 1 ml/min was established over 45 min, held for 5 min, raised to 80% acetonitrile/0.5% trifluoroacetic acid (v/v) over 5 min, and then returned to the starting conditions over 15 min. Fractions of interest were analyzed by MALDI-TOF/TOF mass spectrometry (Bruker Ultraflex II). Total protein and phosphospecific staining was carried out using SYPRO® Ruby and Pro-Q® Diamond phosphoprotein gel stain according to the manufacturer's (Invitrogen) instructions and Ref. 22, respectively.

Reconstitution of the Chromophoric Adduct—Free Pdx1 (20 μM) was incubated with R5P in the presence of either Pdx2 (20 μM) and 10 mM glutamine or 20 mM ammonium sulfate in 50 mM Tris-Cl buffer, pH 7.5.

Measurement of the Release of Inorganic Phosphate—The inorganic phosphate released was monitored spectrophotometrically employing a coupled enzyme assay (EnzChek® phosphate assay kit, Molecular Probes). Typically, equimolar concentrations of Pdx1 and Pdx2 (20 μM) were incubated with glutamine and the kit constituents in 50 mM Tris-Cl, pH 8.0, containing 20 mM $MgC1_2$. The reaction was started by the addition of R5P to a final volume of 300 μl and monitored by the increase in absorbance at 360 nm.

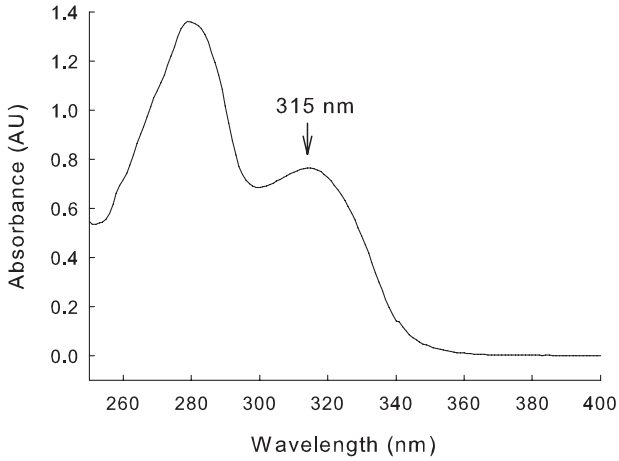


FIGURE 1. **UV-visible absorbance spectrum of freshly isolated Pdx1 (42 μM).** In addition to the typical protein maximum at 278 nm due to aromatic residues, an additional absorbance maximum at 315 nm (indicated by the *arrow*) is observed.

RESULTS

Detection of a Chromophoric Reaction Intermediate of PLP Biosynthesis—During the preliminary characterization of the PLP synthase subunit, Pdx1, we frequently observed an absorbance maximum at 315 nm with the freshly isolated protein, which gradually disappeared over a period of days, in addition to the typical maximum at 280 nm (Fig. 1). Although this spe-

cies could not be assigned to any known chromophore, denaturation experiments indicated that it is covalently attached to the protein (data not shown). Thus, to aid in its characterization, we attempted its reconstitution. The addition of the individual sugar substrates to Pdx1 alone did not affect the UV-visible absorbance spectrum. However, if the glutaminase subunit, Pdx2, and its substrate, glutamine, were included in the presence of the pent(ul)ose sugar, a time-dependent increase in the absorbance at 315 nm was observed (Fig. 2*A*). The increase was not observed in the presence of either Pdx2 or glutamine alone, suggesting that formation of the chromophore is dependent on glutamine hydrolysis. This was corroborated by employing the catalytically incompetent Pdx2 H170N mutant (21), which does not allow formation of the chromophore (Fig. 2*A*, *inset*). Pdx2 H170N can still bind glutamine when complexed to Pdx1 (21) but is unable to carry out its hydrolysis due to the replacement of the catalytic histidine (23). Interestingly, it was also possible to reconstitute the chromophore employing ammonium sulfate in place of Pdx2 and glutamine, albeit at a rate ~10-fold slower (data not shown).

The rate of chromophore formation is a function of the concentration of ribose 5-phosphate (Fig. 2*B*) from which an apparent K_m (126 ± 27 μM) could be estimated. The intriguing possibility that the species observed may be a reaction intermediate of PLP formation prompted us to test its catalytic competence. Indeed, the addition of a stoichiometric amount of G3P resulted in formation of the reaction product, PLP (absorbance maximum at 414 nm) with a clear isosbestic point at 348 nm (Fig. 2*C*). The involvement of the supposed active site residues, Asp-24 and Lys-149 (11, 24), in formation of the chromophore could be confirmed by the inability of the mutants, D24A and K149A, to support its formation (data not shown).

The Site of Covalent Adduct Formation Is Lysine 81 of Pdx1—It was recently postulated that one of the early steps in PLP biosynthesis is the formation of an imine adduct between Lys-149 of Pdx1 and its pent(ul)ose substrate (11). As we suspected that formation of the imine precedes formation of the chromophore, we sought to confirm its localization. In contrast to the chromophoric adduct, the imine can be fully reconstituted simply by preincubation of Pdx1 with the pent(ul)ose substrate (11). A tryptic digest of the fully reconstituted imine adduct was performed, and the resulting peptide mixture was analyzed by MALDI-TOF mass spectrometry, but peak suppression prevented identification of the modified peptide. It was, therefore, necessary to first partially reduce sample complexity by isolating the peptide from the complete protein digest. For this, we employed [U-^{14}C]R5P to reconstitute the imine followed by tryptic digestion and RP-HPLC as described above, permitting us to identify the fraction containing the modified peptide (Fig. 3*A*). Repetition of this experiment using either natural abundance material or [U-^{13}C]R5P followed by comparative MALDI-TOF mass spectrometry of the analogous fraction revealed peptides with masses of 2686.246 and 2691.261 Da, corresponding to the ^{12}C- and ^{13}C-labeled species, respectively (Fig. 3*B*). Unexpectedly, tandem mass spectrometry of these peptides yielded spectra that matched the predicted fragmentation pattern of the peptide, MADPTIVEEVMNAVSIPVMAKAR (Fig. 3*C*), corresponding to resi-

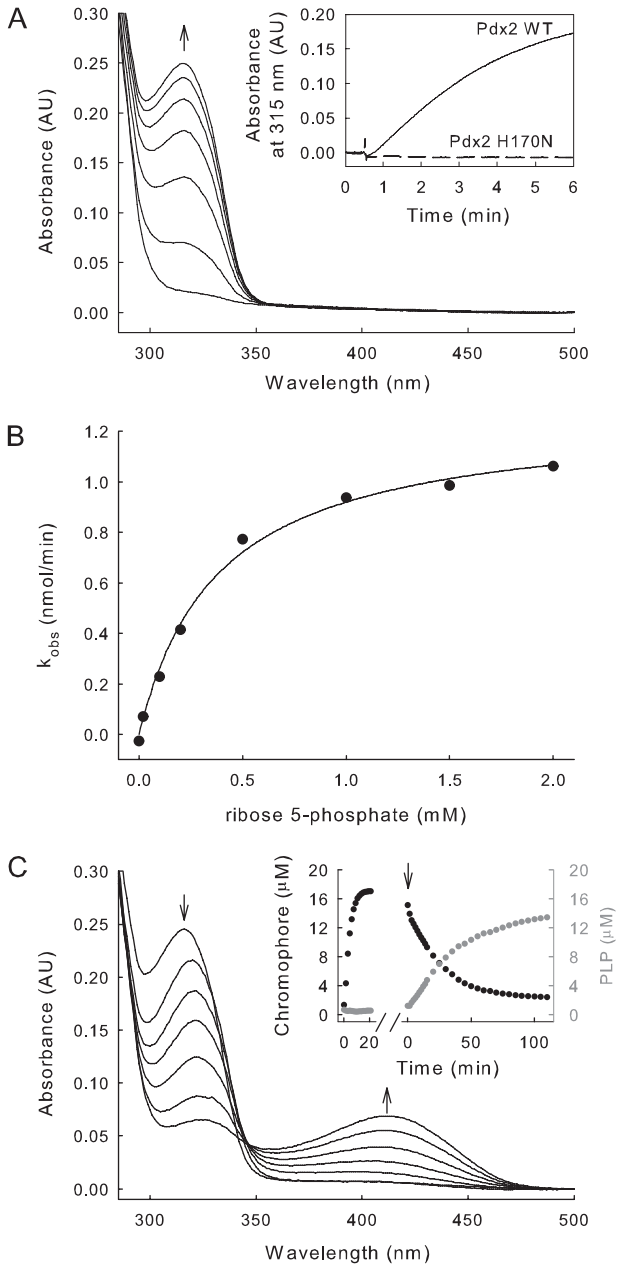


FIGURE 2. **UV-visible absorbance changes of Pdx1 in the presence of R5P.** *A*, reconstitution of the chromophoric adduct. Incubation of Pdx1 (20 μM) in the presence of R5P (0.2 mM), Pdx2 (20 μM), and glutamine (10 mM) results in an absorbance maximum at 315 nm. The spectra were recorded at 0-, 1.5-, 3-, 4.5-, 6-, 7.5-, and 9-min intervals. The *inset* shows the increase in absorbance at 315 nm observed when Pdx2 wild type (*WT*, *solid line*) was substituted for Pdx2 H170N (*dotted line*). *AU*, arbitrary units. *B*, the rate of chromophore formation is dependent on the concentration of R5P. The changes in absorbance at 315 nm were recorded as a function of the concentration of R5P (0–2 mM). Fitting of the data was according to $f(x) = a \times x/b + x$. *C*, demonstration of the catalytic competence of the chromophoric adduct. The chromophoric adduct was first reconstituted as described in *A* and represents time = 0. DL-G3P (0.4 mM) was then added, and the spectra were recorded at 3-, 9-, 15-, 25-, 40-, and 60-min intervals. A decrease in the absorbance at 315 nm concomitant with an increase in the absorbance at 414 nm due to the formation of PLP is evident. The *inset* demonstrates that chromophore formation (*black circles*) precedes PLP formation (*gray circles*). The molar concentrations of adduct (ϵ_{315} = 16,200 $M^{-1}cm^{-1}$) and PLP (ϵ_{415} = 5380 $M^{-1}cm^{-1}$) are plotted as a function of time. Note that following reconstitution of the adduct, dialysis was performed to remove excess R5P and glutamine, and DL-G3P (0.4 μM) was added (indicated by an *arrow*). The reaction was carried out in 50 mM Tris-Cl, pH 8.0.

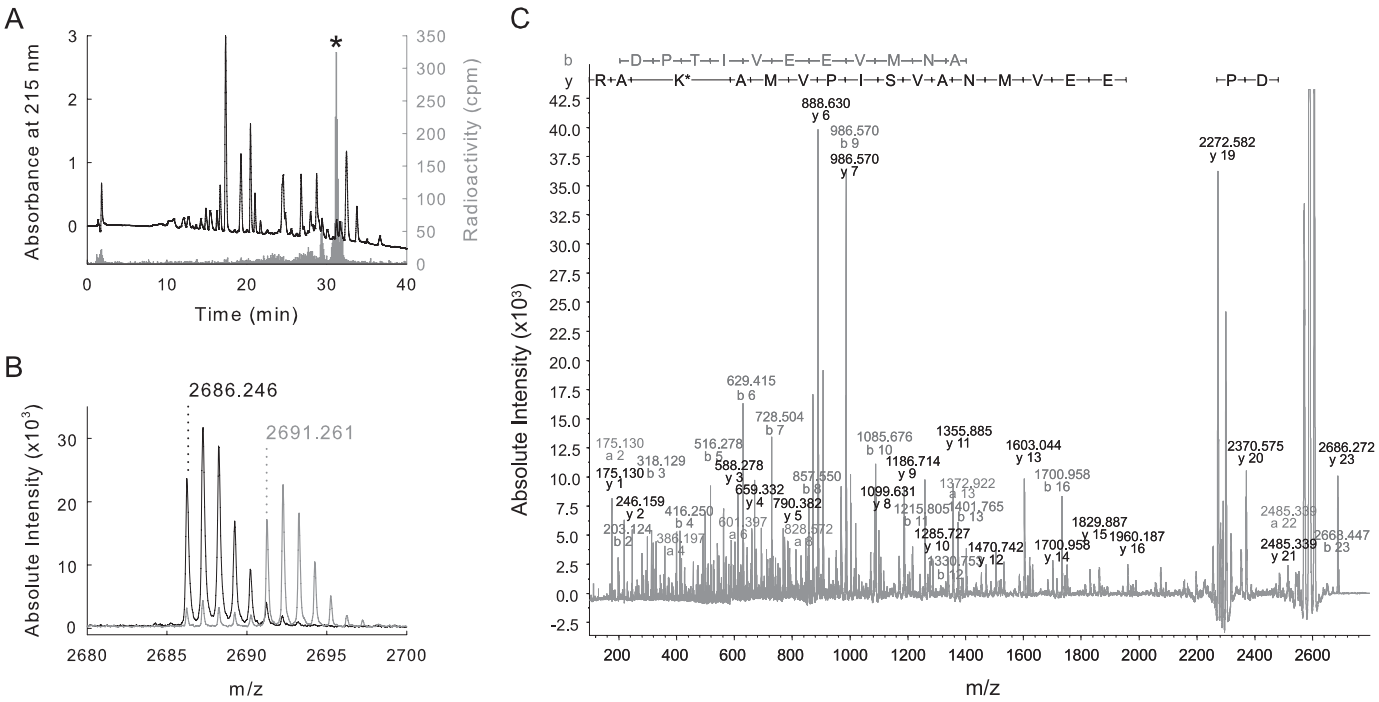


FIGURE 3. **RP-HPLC and mass spectrometry of the Pdx1 imine adduct.** *A*, RP-HPLC elution profile of tryptic peptides of Pdx1 preincubated with [U-^{14}C]R5P. The *black line* indicates the absorbance at 215 nm, and the *gray line* indicates the radioactivity detected. *B*, MALDI-TOF mass spectrometry of the fraction labeled with * (retention time = 31 min). The spectra shown are an overlay of the modified peptide identified by comparison of tryptic peptides of [U-^{12}C]R5P and [U-^{13}C]R5P preincubated with Pdx1 as described in *A*. The *m/z* are as indicated. *C*, MALDI-TOF/TOF analysis of the precursor ion, *m/z* = 2686.272 $[M^+H]^+$, yielded a fragmentation pattern matching the predicted profile for the peptide MADPTIVEEVMNAVSIPVMAK†AR (residues 61–83) with Lys-81 forming the imine adduct (†). Observed b (*gray*) and y (*black*) ions are as indicated.

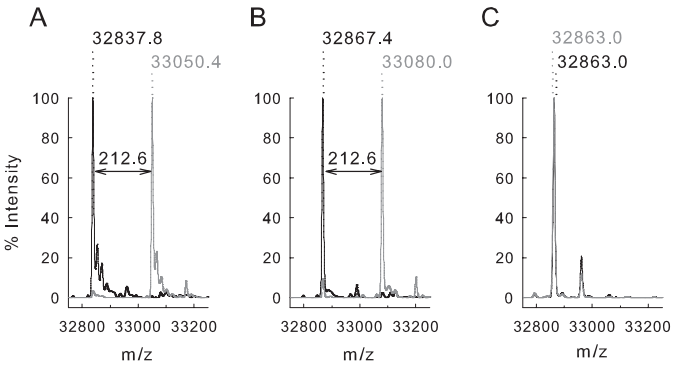


FIGURE 4. **Imine adduct formation by Pdx1 mutants.** Shown are ESI-mass spectra of Pdx1 wild type (*A*), Pdx1 K149R (*B*), and Pdx1 K81R (*C*) in the presence (*gray line*) and absence (*black line*) of R5P (preincubated for 3 h at 37 °C). The *m/z* of the different Pdx1 forms are as indicated.

dues 61–83, with the pentulose phosphate imine adduct residing at Lys-81 (^{12}C predicted mass, 2686.286). An overlay of the unlabeled and labeled spectra substantiates incorporation of the label at Lys-81 (data not shown). This implied that the imine adduct in Pdx1 occurs at Lys-81 rather than Lys-149 as reported previously (11).

To corroborate the finding further, we attempted reconstitution of the imine adduct with the K81R/K81A and K149R/K149A mutants of Pdx1. ESI-mass spectrometry analysis revealed that K149A could not form the imine adduct (as has been shown previously (11)), nor could K81A or K81R. However, K149R retains the ability to form the imine (Fig. 4). Furthermore, formation of the chromophoric adduct could be observed with K149R (albeit at a rate 12 times lower than that of the wild type, k_{cat} = 0.020 and 0.240 min^{-1} for K149R and wild type, respectively), whereas no activity could be detected for K81R. The difference in rate was mainly manifested in the k_{cat} as the K_m of K149R was close to that of the wild type (185 ± 26 and 126 ± 27 μM for K149R and wild type, respectively). However, we were unable to detect formation of PLP with K149R (data not shown).

R5P Is the Preferential C5 Substrate of PLP Synthase—During the imine adduct reconstitution experiments, we noted a preference for R5P as a substrate when compared with Ru5P (Fig. 5*A*). This was already reflected in previous analyses where initial rates of PLP formation were substantially higher with R5P as the C5 sugar (11, 12). However, in these analyses, a fraction of the isolated protein would have the C5 sugar incorporated, thus interfering with an accurate comparison of the efficiency of the C5 sugars in PLP synthesis. To provide a more precise assessment of PLP synthesis, we isolated free Pdx1 as described under "Experimental Procedures" and reassessed PLP formation. Under these conditions, k_{cat} values for R5P and Ru5P were very similar (0.040 and 0.042 min^{-1}), whereas there was a 60-fold difference in the K_m values (0.053 and 3.18 mM, respectively) (Fig. 5*B*).

The preferential binding of R5P was also reflected in studies in which a mixture of [U-^{14}C]Ru5P and [U-^{14}C]R5P was synthesized from [U-^{14}C]glucose as outlined under "Experimental Procedures." After incubation of Pdx1 with this mixture, the reaction was subjected to SDS-PAGE followed by autoradiography to detect co-migration of the label with Pdx1 (Fig. 5*C*). Indeed, a signal could be detected (Fig. 5*C*). However, if phosphoriboisomerase was omitted from the reaction mixture, thus

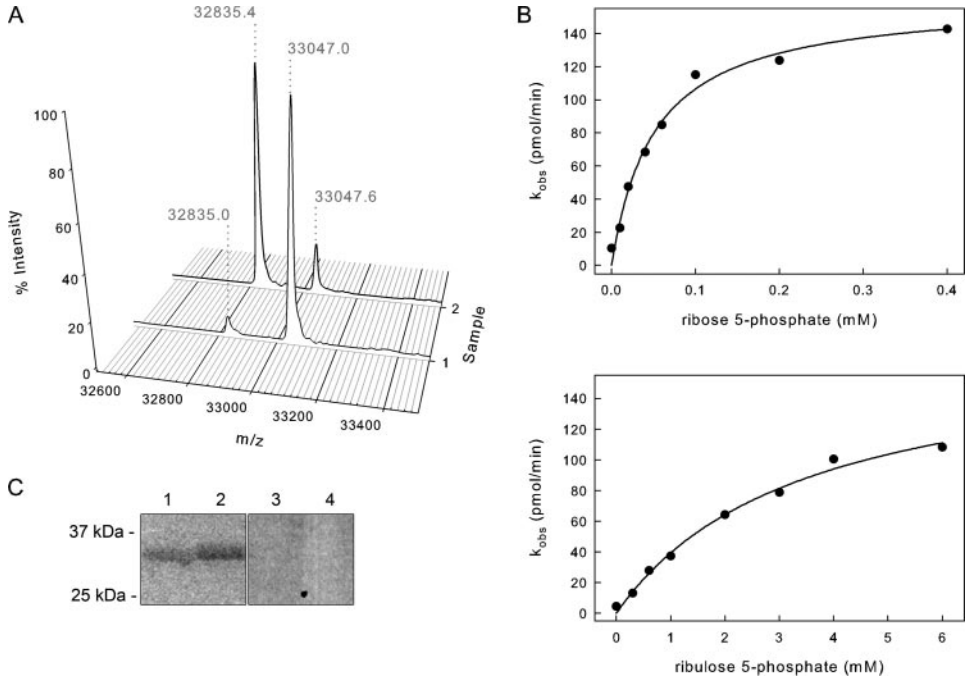


FIGURE 5. **R5P is the preferential C5 substrate of Pdx1.** *A*, ESI-mass spectra of Pdx1 preincubated for 1 h at 37 °C with either R5P (*sample 1*) or Ru5P (*sample 2*). The mass of 32835 and 33047 Da corresponds to that of the free protein and imine adduct, respectively. *B*, PLP biosynthesis as a function of the concentration of R5P or Ru5P. The rate of PLP synthesis was determined from the absorbance at 415 nm. Assays were carried out in 50 mM Tris-Cl, pH 8.0, and at 37 °C employing free Pdx1 (20 μM), Pdx2 (20 μM), glutamine (20 mM), and DL-G3P (1 mM) while varying the C5 substrate in the range of 0.0–0.4 mM (R5P) and 0–6 mM (Ru5P). Steady state kinetic parameters k_{cat} and K_m were estimated by fitting to the Michaelis-Menten equation. *C*, SDS-PAGE (15%) and autoradiography of Pdx1. *Lane 1* preincubated with a mix of [U-^{14}C]R5P and [U-^{14}C]Ru5P, *i.e.* phosphoriboisomerase was included in the synthesis (see "Experimental Procedures"); *lane 2*, as for *lane 1* but in the presence of Pdx2; *lane 3*, Pdx1 preincubated with [U-^{14}C]Ru5P, *i.e.* phosphoriboisomerase was omitted from the synthesis (see "Experimental Procedures"); *lane 4*, as for *lane 3* but in the presence of Pdx2.

resulting in formation of [U-^{14}C]Ru5P predominantly, no such signal could be detected (Fig. 5*C*). Furthermore, we observed that Pdx2 was not required for pentose binding to Pdx1, indicating that the sugar can bind to Pdx1 alone.

Characterization of the Chromophoric Adduct—We employed RP-HPLC to assess the feasibility of separating the imine and chromophoric adduct, respectively, from the unmodified Pdx1 protein to further aid in their characterization. In the first instance, we selected a sample of isolated Pdx1 in which the chromophore could no longer be observed. This sample yielded two clearly resolvable peaks, annotated *a* and *b* (Fig. 6*A*, *Sample 1*). ESI-mass spectrometry of these two peak fractions revealed masses of 33049.0 and 32835.0 Da, corresponding to the pent(ul)ose phosphate imine (predicted mass difference 212 Da) and free protein, respectively (Table 1). Confirmation of the imine adduct was substantiated by RP-HPLC analysis of the reconstituted imine for which an expected increase in species **a** and a concomitant decrease in species **b** was observed (Fig. 6*A*, *Sample 2*). Moreover, the presence of a phosphate group in species **a** could be corroborated employing a phosphospecific stain (Fig. 6*B*). The marker protein, egg ovalbumin (45 kDa, contains two phosphate groups) and alkaline phosphatase-treated Pdx1 acted as controls.

Upon treatment of reconstituted **a** with ammonium salt and subjection to RP-HPLC as described for the imine adduct, three resolvable peaks, **a**, **b**, and **c**, were obtained. Notably, only the newly observed species **c** had an absorbance corresponding to that of the chromophore (Fig. 6*A*, *Sample 3*). ESI-mass spectrometry confirmed that species **a** and **b** correspond to the pent(ul)ose phosphate imine and free protein, respectively. Species **c** (observed mass 32,930 Da) on the other hand has an additional mass of 95 Da when compared with the native protein. Tryptic digestion followed by separation of the resulting peptides in a fashion analogous to that described above for the imine adduct localized the chromophoric adduct to Lys-81 in Pdx1 (data not shown). In addition, mass spectrometric comparison of the relevant natural abundance and U-^{13}C-labeled peptide revealed that the adduct had retained all five carbon atoms from the pentose sugar. Furthermore, reconstitution of the chromophore employing ^{14}N- and ^{15}N-labeled ammonium sulfate in place of Pdx2 and glutamine indicated that nitrogen was not incorporated into the chromophore (data not shown). Additionally, we observed that species **c** did not stain positive for a phosphate group (Fig. 6*B*). In agreement with this, an enzyme assay confirmed that inorganic phosphate was released in an amount stoichiometric with the protein concentration (Fig. 6*C*) and that its formation is concomitant with the formation of the chromophore (Fig. 6*D*). The observed mass spectrometric difference of 95 Da between species **b** (the free protein) and species **c** requires that in addition to the phosphate group and one molecule of water, a proton is lost during formation of the chromophore. Furthermore, we observed by UV-visible spectrophotometry that chromophore formation (k_{cat} 0.212 min^{-1}) is approximately five times faster than PLP formation (k_{cat} 0.040 min^{-1}), indicating that it is not a rate-limiting step in the reaction (Fig. 2*C*, *inset*).

DISCUSSION

Vitamin B6 research has attracted renewed attention recently for two reasons: firstly, for the discovery of an alternate route of its *de novo* biosynthesis, which is different from that previously assumed to occur in most organisms (2, 3, 11, 12, 19, 20, 25–27), and secondly, because the vitamin appears to have previously unrecognized potent antioxidant capabilities equivalent to those of vitamins C and E and may thus play an important role in the defense against oxidative stress (2, 4, 28–31). Aside from the general interest in the biosynthesis of the vitamin, the potential of the pathway as a drug target has provided a strong impetus to define the mechanism of action of the proteins involved, Pdx1 and Pdx2 (19, 20). The participation of only two proteins and the remarkable ability of Pdx1 to carry out a

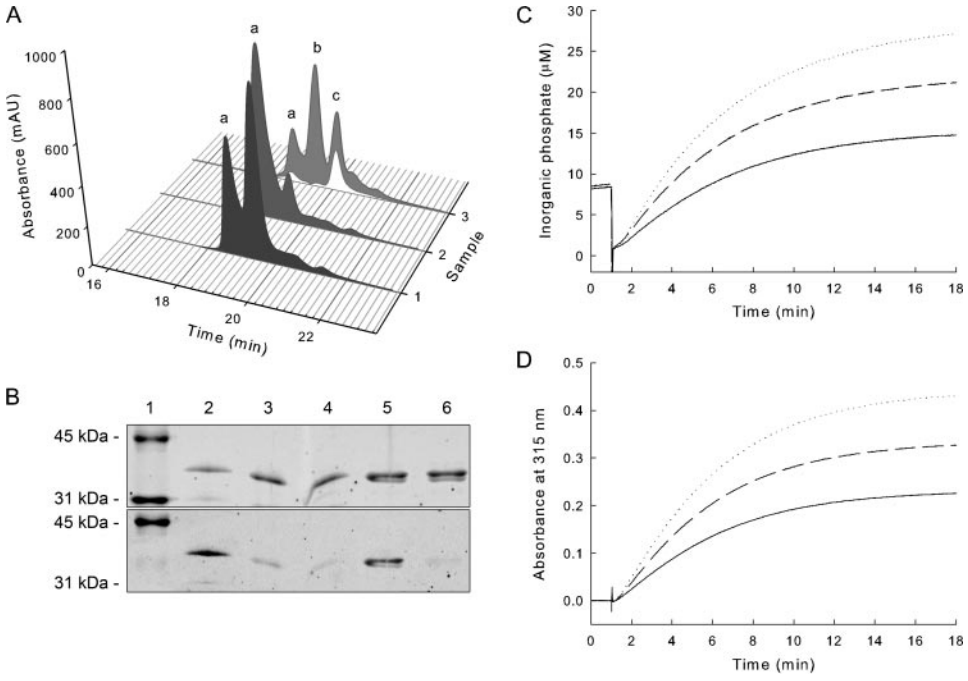


FIGURE 6. **Characterization of the Pdx1 chromophoric adduct.** *A*, RP-HPLC of isolated Pdx1 (*sample 1*), reconstituted imine adduct (*sample 2*), and reconstituted chromophoric adduct (*sample 3*). The absorbance was monitored at 280 nm, and in the case of sample 3, also at 325 nm (area shown in *white*). The species observed are labeled *a*, *b*, and *c*, corresponding to the imine adduct, free protein, and chromophoric adduct forms of Pdx1, respectively. *B*, the *upper panel* shows SYPRO® Ruby staining of the loaded protein; *lane 1*, molecular mass standards, *lanes 2–4*, species **a**, **b**, and **c**, respectively; *lanes* 5 and *6*, Pdx1 (10 pmol) before and after treatment with alkaline phosphatase, respectively. The *lower panel* shows phosphospecific staining of the same samples as in the *upper panel*. *C*, the release of inorganic phosphate was monitored employing a coupled enzyme assay; molar concentrations measured are plotted as a function of time. Inorganic phosphate release is concomitant with chromophore formation (*D*). In *C* and *D*, the *solid*, *dashed*, and *dotted lines* represent 10, 20, and 40 μM protein, respectively, demonstrating that both phosphate release and chromophore formation are stoichiometric with the protein concentration.

TABLE 1
Molecular mass of observed species Pdx1

Species	Predicted mass	Observed mass	Observed mass − observed mass of b[a]
	Da	*Da*	
a	33046.8	33047.0	212.1
b	32834.8	32834.9	
c	32929.8	32930.2	95.3

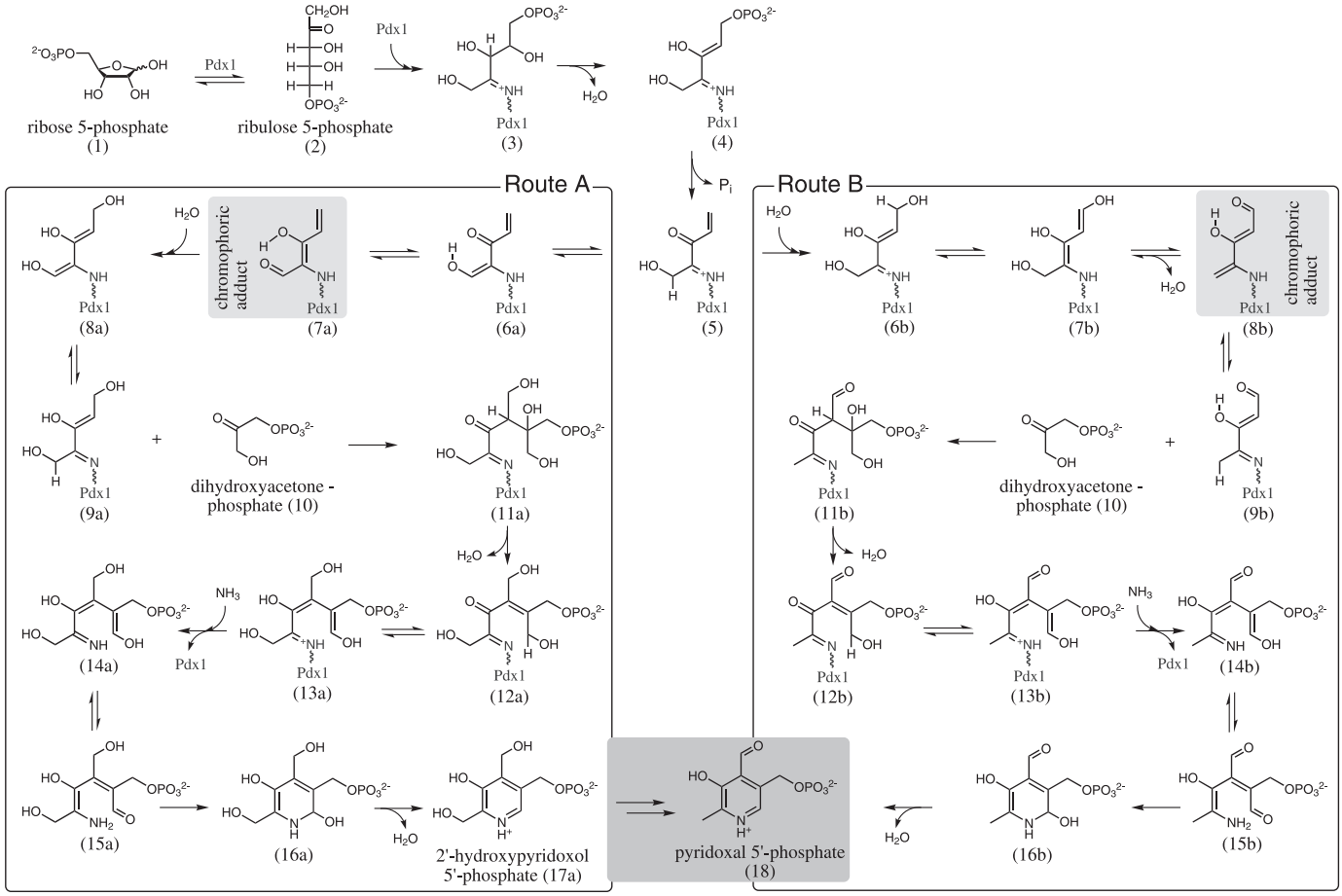
[a] Difference in mass observed when compared with that of the free-protein (species b).

variety of reaction steps directly yielding PLP from primary metabolites are expected to warrant complex chemistry. Here, the observation and characterization of an unanticipated chromophoric reaction intermediate covalently bound to Pdx1 allows us to formulate the early steps of catalysis, with the consequence that the previously postulated mechanism (11) must be revised. An alternative mechanism is provided (Scheme 1).

As formation of neither the imine nor the chromophore requires the presence of the triose sugar, it is assumed that PLP synthesis is initiated by binding of the pent(ul)ose sugar in the Pdx1 active site. Despite the fact that the enzyme can utilize both R5P (**1**) and Ru5P (**2**) (Scheme 1) as substrates, there is a clear preference for the pentose rather than the pentulose sugar phosphate. Although R5P in solution is normally present as an equilibrium of three species, *i.e.* the α and β furanoses and the open chain form, Ru5P exists only in the latter form (32). The ring forms of R5P are the predominant species in solution (64% for the β and 36% for the α-furanose when compared with 0.1% for the free aldehyde) (33). Thus, the preference of PLP synthase for R5P may be manifested in recognition of the ring form. Evidence has been provided that R5P can be isomerized to Ru5P by Pdx1 (11); as isomerization requires the open chain form of the sugar, we propose that PLP synthase first binds the furanose form and catalyzes its opening prior to isomerization to Ru5P (**2**).

The next stage in PLP biosynthesis involves formation of an imine adduct (**3**). It was previously proposed that Lys-149 is the active site residue involved at this stage (11). Indeed, the recently described structure of free Pdx1 from *Geobacillus stearothermophilus*, which folds as a $(\beta\alpha)_8$ barrel (24), embraced a model of such an imine adduct between Ru5P and Pdx1. This model was based on the co-crystallization of the cryoprotectant 2-methyl-2,4-pentanediol and a sulfate ion, which were believed to mimic the carbon backbone and phosphate, respectively, of the pent(ul)ose phosphate substrate. However, such a model necessitated a rather drastic reorientation of Lys-149 as it points away from the active site. It is now clear from the present study and the very recent one of Ref. 1 that the pent(ul)ose phosphate imine occurs at the better poised Lys-81 rather than Lys-149 in Pdx1. Furthermore, our studies clearly indicate that the pentose sugar remains covalently bound to Lys-81 at least until formation of the chromophoric adduct. This observation rules out the recently proposed transfer of the pentulose phosphate imine from Lys-81 to Lys-149 (1). However, we cannot exclude the possibility that Lys-149 is involved in imine formation at a later step, *i.e.* subsequent to formation of the chromophore and before the transimination with ammonia occurs. Indeed, Lys-149 is absolutely conserved in Pdx1 sequences, and the site-directed mutagenesis studies clearly indicate that it has a role in PLP formation. However, as the conservative K149R mutant described here retains the ability to form the chromophoric adduct, exemplified by a lowered k_{cat} but unaltered K_m, we suggest that it is likely that this residue could alternatively act as an acid/base catalyst.

In this study, a key finding is provided by the isolation and characterization of a novel chromophoric reaction intermediate. The accumulated experimental evidence demonstrates that the chromophoric group of this intermediate is appended to the ϵ-amino group of Lys-81 and that the new residue has the composition $C_5H_6O_2$, corresponding to the elimination of one equivalent of inorganic phosphate, one molecule of water, and



SCHEME 1. **Mechanistic proposal for formulation of PLP by the DXP-independent pathway.**

one additional proton from the original protonated imine adduct (Scheme 1, **3**). In combination with the observed extinction coefficient (ϵ_{315} = 16,200 $M^{-1}cm^{-1}$), which clearly points to a π-π^* transition, these stringent requirements can be accommodated by the two structural proposals **7a** and **8b** of Scheme 1. In both cases, the path involves dehydration of **3** to **4**, initiated by the removal of the acidic hydrogen at *C*3 of the substrate followed by a β-elimination of the phosphate group leading to **5**, in which the iminium group is destabilized by the adjacent keto group and suffers deprotonation to **6a** that can easily tautomerize to the desired chromophoric adduct (**7a**). This step is then followed by the addition of water to form **8a**, the tautomer of which, **9a**, is an appropriate partner for condensation with dihydroxyacetone phosphate (**10**), the second building block of vitamin B6, to give **11a**. From here on, a sequence of mechanistically plausible steps (**11a–17a**), involving dehydration (**11a–12a**), tautomerization, and a transimination step with ammonia to cleave the covalent bond of the substrate to the protein, can lead to the pyridine ring moiety of **17a**. Compound **17a** has been considered as a putative intermediate of PLP biosynthesis (11) on the basis of the demonstration by the Spenser group (18) that its dephosphorylated counterpart is transformed into pyridoxamine by whole cells of *Saccharomyces cerevisiae*; a hypothetical mechanistic scheme for this transformation has been outlined before (15, 18).

The second possible structure, **8b**, for the chromophoric intermediate is accessible from **5** via the addition of water to provide **6b** followed by acid-catalyzed tautomerization of the latter to **7b**, which can collapse by the elimination of water to the desired compound **8b**. Nucleophilic attack of its tautomeric form (**9b**) on dihydroxyacetone phosphate to give **11b** followed by the usual combination of tautomerizations, transimination, and dehydration steps provides an alternative pathway (**11b–18**) for the eventual formation of PLP, which in contrast to route A does not necessitate the postulated intermediacy of 2′-hydroxypyridoxol 5′-phosphate (**17a**). It is interesting to note in this context that preliminary experiments with the *B. subtilis* protein failed to disclose any observable activity upon incubation with this compound.

The DXP-dependent pathway of vitamin B6 biosynthesis requires the interplay of seven enzymes to form PLP, three of which are exclusive to the pathway. Vitamin B6 biosynthesis has been extensively studied in *E. coli* (5, 7, 10, 34–36), and it has recently been pointed out that much of the complexity arises through the formation of 3-amino-1-hydroxyacetone 1-phosphate (37); the action of a total of four enzymes is required to facilitate its biosynthesis. This complexity is substantially reduced in the DXP-independent pathway, where Pdx1 itself incorporates the nitrogen atom and directly yields the cofactor vitamer PLP. In this context, PLP synthase simply

provides a more economical solution to vitamin B6 biosynthesis when compared with its *E. coli* counterpart.

In summary, significant progress has been made toward the mechanistic understanding of the PLP synthase reaction. However, additional work is necessary for the unequivocal identification of the chromophoric intermediate described in this work and for a more complete description of the later steps of the mechanism. As PLP synthase is one of the most conserved enzymes found in nature, it is highly likely that the mechanism proposed pertains to all organisms that utilize the DXP-independent pathway for vitamin B6 synthesis.

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Addendum—In a publication that appeared after the original submission of this work, independent x-ray crystallographic evidence has been presented for imine formation at the equivalent lysine (lysine 82) in the enzyme from *Thermotoga maritima* (1). While this study was under review, the crystal structure of Pdx1 from *T. maritima* with ribulose 5-phosphate bound was published, where imine formation with the pentulose phosphate was observed at Lys-82 (equivalent to Lys-81 in *Bs*Pdx1) (1).

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