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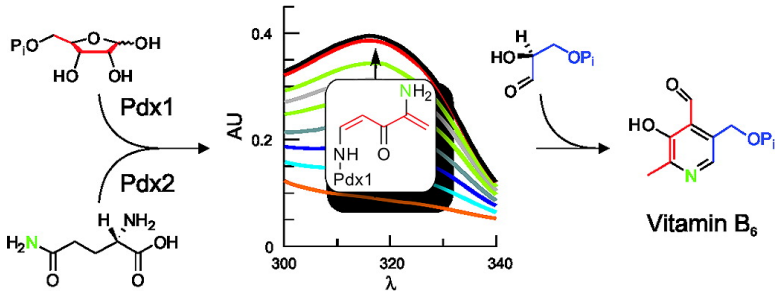

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Mechanistic Studies on Pyridoxal Phosphate Synthase: The Reaction Pathway Leading to a Chromophoric Intermediate

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Abstract: Two routes for the *de novo* biosynthesis of pyridoxal-5′-phosphate (PLP) have been discovered and reconstituted *in vitro*. The most common pathway that organisms use is dependent upon the activity of just two enzymes, known as Pdx1 (YaaD) and Pdx2 (YaaE) in bacteria. Pdx2 has been shown to have glutaminase activity and most likely channels ammonia to the active site of the PLP synthase subunit, Pdx1, where ribose-5-phosphate (R5P), glyceraldehyde-3-phosphate (G3P), and ammonia are condensed in a complex series of reactions. In this report we investigated the early steps in the mechanism of PLP formation. Under pre-steady-state conditions, a chromophoric intermediate (I_{320}) is observed that accumulates upon addition of only two of the substrates, R5P and glutamine. The intermediate is covalently bound to the protein. We synthesized C5 monodeuterio (pro-*R*, pro-*S*) and dideuterio R5P and showed that there is a primary kinetic isotope effect on the formation of this intermediate using the pro-*R* but not the pro-*S* labeled isomer. Furthermore, it was shown that the phosphate unit of R5P is eliminated rather than hydrolyzed in route to intermediate formation and also that there is an observed C5-deuterium kinetic isotope effect on this elimination step. Interestingly, it was observed that the formation of the intermediate could be triggered in the absence of Pdx2 by the addition of high concentrations of NH_4Cl to a preincubated solution of Pdx1 and R5P. The formation of I_{320} was also monitored using high-resolution electrospray ionization Fourier transform mass spectrometry and revealed a species of mass 34 304 Da (Pdx1 + 95 Da). These results allow us to narrow the mechanistic possibilities for the complex series of reactions involved in PLP formation.

Introduction

Pyridoxal-5′-phosphate (PLP; **1**) is the active cofactor form of Vitamin B_6 and is essential for primary metabolism in all known organisms. The cofactor participates in amino acid and carbohydrate metabolism and has recently been implicated in singlet oxygen resistance.[1] More than 140 different PLP-dependent enzymes have been identified, and it has been estimated that 1.5% of the genes in a typical bacterium encode PLP-dependent enzymes.[2] Two *de novo* pathways for the biosynthesis of PLP are currently known to exist. The system employed in *E. coli* is the most thoroughly characterized, requires six enzymes, and uses 1-deoxy-D-xylulose-5-phosphate and 4-hydroxy-L-threonine as direct precursors (DXP pathway).[3−9] This pathway is found in a relatively small number of bacteria. Another more prevalent route for PLP biosynthesis (R5P pathway) is present in prokaryotes, eukaryotes, and archaea and involves only two gene products collectively termed Pdx1/Pdx2, SNZ/SNO, or YaaD/YaaE, depending on the organism of origin.[10−12] The R5P PLP biosynthesis pathway from *Bacillus subtilis* has recently been reconstituted *in vitro* and shown to use ribose-5-phosphate (R5P; **2**), glyceraldehyde-3-phosphate (G3P; **3**) and glutamine as the substrates (Scheme 1).[13,14]

The PLP synthase holoenzyme has been structurally characterized and consists of two subunits: a glutaminase (Pdx2 or YaaE) and a PLP synthase (Pdx1 or YaaD). A highly chromophoric intermediate, with a λ_{max} of approximately 315 nm, accumulates in the active site of Pdx1 upon addition of glutamine and R5P. This intermediate is converted to PLP in

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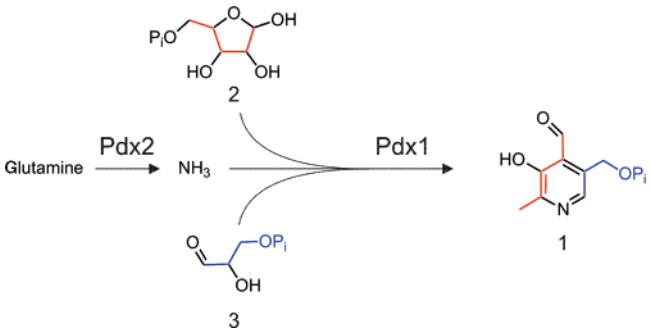
Scheme 1. Substrates Used by Pdx1 and Pdx2 in the Formation of PLP



the presence of G3P[15] and is covalently attached to the protein via Lys 81.[16] MS analysis of the labeled peptide indicated a mass increase of 95 Da which is consistent with loss of water and phosphate from a Ru5P−Pdx1 adduct. Here we provide additional information regarding this intermediate that enables us to further narrow down the mechanistic possibilities for PLP formation. Specifically, we are able to monitor the production of the intermediate under mild conditions using ESI-FTMS, observe a primary deuterium kinetic isotope effect at C5 of R5P on the formation of the intermediate, and determine that this isotope effect is due to removal of the pro-*R* proton. We also demonstrate that ammonia is covalently incorporated into the intermediate and that G3P is a much better substrate than dihydroxyacetone-phosphate (DHAP). Additionally, we show that phosphate loss occurs by an elimination reaction rather than by a hydrolysis reaction and that this occurs after C5 deprotonation and ammonia addition.

Experimental Section

Protein Overexpression and Purification. Overexpression and purification were performed as previously published[13] except for the following changes. A 1 mL HisTrap HP column (GE Healthcare Bio-Sciences Corp. Piscataway, NJ) was used according to the product literature. Subsequent to elution of Pdx1 and Pdx2 from the column the protein was buffer exchanged into the following buffer system: 50 mM Tris (Trizma base) pH 8.0 @ RT, 100 mM NaCl, 2 mM TCEP, and 10% glycerol using an Econo-Pac 10DG desalting column (Bio-Rad Laboratories, Hercules, CA). A portion of Pdx1 and Pdx2 were combined at this time and used for experiments where both were necessary. The two proteins were mixed at a 1:1.5 molar ratio (Pdx1:Pdx2) according to the Coomassie Plus Assay Reagent (Pierce Biotechnology, Rockford, IL). To free the enzyme from the bound Ru5P adduct that copurifies with Pdx1, 10 mM glutamine and 1 mM G3P were added and allowed to incubate overnight at 4 °C. For samples containing only Pdx1, 50 mM NH_4Cl was added in place of the glutamine. After the overnight incubation, the samples were again purified using the HisTrap procedure as detailed above, and subsequent to elution the proteins were buffer exchanged into 50 mM HEPES pH 7.5 @ RT, 100 mM NaCl, 2 mM TCEP, and 20% glycerol. 30 μL aliquots of protein were flash frozen using liquid nitrogen and stored at −80 °C. Control reactions demonstrated that this method of enzyme storage did not result in any activity loss.

Synthesis of (5*S*)-[5-$^2H_1$]- and (5*R*)-[5-$^2H_1$]-Ribose. The ribose diastereomers **4** and **5** were prepared from the known alcohol **9** using a modified procedure.[17,18] All solvents were reagent grade and distilled over CaH_2 when noted as dry. Proton and carbon NMR were recorded on a Bruker ARX-300.

Preparation of Deuteroribose Derivative 9 (Scheme 2). (−)-Isoborneol-1-^2H (1.6 g, 10.5 mmol)[19] was dissolved in dry ether (4 mL) and added to a previously prepared solution of *n*-butylmagnesium bromide [*n*-butyl bromide (1.2 mL, 11.4 mmol) in dry ether (4 mL) was added slowly to Mg in ether (4 mL) followed by 1 h of reflux]. After 30 min, dry benzene (13 mL) was added and the ether was distilled through a vigreux column under argon. Aldehyde **8**[16] (195 mg, 0.70 mmol) was dissolved in dry benzene (2.5 mL) and added to the prepared reagent. After refluxing for 3 h, the solution was cooled and treated with 0.1 N HCl (∼30 mL) and dichloromethane (30 mL). The layers were separated, and the organic phase was washed with saturated $NaHCO_3$ and brine. After drying over $MgSO_4$, evaporation of solvent was followed by column chromatography with 25% EtOAc in hexanes to provide the alcohol **9** (109 mg, 55% yield) as an oil; ^1H NMR (300 MHz, $CDCl_3$) δ 7.40−7.26 (m, 5H), 5.72 (d, J = 3.7 Hz, 1H), 4.76 (d, J = 11.9 Hz, 1H), 4.58 (d, J = 11.9 Hz, 1H), 4.59−4.55 (m, 1H), 4.10 (dd, J = 3.1, 9.1 Hz, 1H), 3.84 (dd, J = 4.3, 9.1 Hz, 1H), 3.60 (dd, J = 2.5, 8.4 Hz, 1H), 1.83 (d, J = 8.6 Hz, 1H), 1.59 (s, 3H), 1.36 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.5, 128.4, 128.0, 127.9, 113.0, 104.0, 78.6, 77.4, 76.5, 72.3, 60.2 (t, J = 21.5 Hz), 26.8, 26.4.

Conversion of Alcohol 9 into (5*S*)-[5-$^2H_1$]-Ribose 4. The alcohol **9** (32 mg, 0.11 mmol) was dissolved in AcOH (2 mL) and treated with 10% Pd/C (10 mg). Stirring was maintained under a balloon of H_2 for 3 h, and the reaction mixture was then filtered through Celite. Evaporation of the solvent followed by column chromatography on silica gel (3% MeOH in $CHCl_3$) afforded the diol **10** (21.5 mg, 99%) as an oil; ^1H NMR (300 MHz, $CDCl_3$) δ 5.80 (d, J = 3.8 Hz, 1H), 4.57 (t, J = 4.7 Hz, 1H), 4.05−3.95 (m, 1H), 3.82 (dd, J = 3.8, 8.9 Hz, 1H), 3.75−3.68 (m, 1H), 2.60 (d, J = 10.4 Hz, 1H), 2.24 (br s, 1H), 1.56 (s, 3H), 1.36 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 112.7, 103.9, 80.5, 78.7, 70.8, 60.4 (t, J = 24.2 Hz), 26.48, 26.45.

The diol **10** (21 mg, 0.11 mmol) was taken up in H_2O (3 mL) and treated with Dowex-50W resin (25 mg previously washed with 10% HCl, water, EtOH, and diethyl ether) at 50 °C. After 24 h, the resin was filtered off, NH_4OH was added, and the filtrate was lyophilized to provide (5*S*)-[5-$^2H_1$]-ribose **4** (16 mg, 96%) as an oily solid; ^1H NMR (300 MHz, D_2O) (chemical shifts for α- and β-pyranose and furanose) δ 5.38−5.20 and 4.90−4.80 (m, 1H), 4.20−4.07 (m, 1H), 3.89−3.78 (m, 2H), 3.70−3.47 (m, 1H); ^{13}C NMR (75 MHz, D_2O) (chemical shifts for α- and β-pyranose and furanose) δ 101.2, 96.0, 94.0, 93.8, 83.5, 83.2, 75.3, 71.3, 70.7, 70.3, 69.5, 69.3, 67.6, 67.4, 63.3 (t, J = 23.4 Hz).

Inversion of Configuration at the 5-Position of 9 (Scheme 3). Alcohol **9** (47 mg, 0.17 mmol) was dissolved in THF (5 mL) followed by addition of PPh_3 (175 mg, 0.67 mmol) and *p*-nitrobenzoic acid (112 mg, 0.67 mmol). After cooling to 0 °C, DEAD (116 mg, 0.67 mmol) was added dropwise and the solution was slowly warmed to room temperature. After 4 h, the solution was evaporated to an oil and chromatographed on silica with 10% EtOAc in hexanes to provide the *p*-nitrobenzoate ester **11** (72 mg).

The ester (72 mg, 0.17 mmol) was then dissolved in MeOH (3 mL) and treated with K_2CO_3 at room temperature. Evaporation of solvent followed by chromatography on silica gel (30% EtOAc in hexanes) provided the inverted alcohol **12** (46 mg, 99% over two steps); ^1H NMR (300 MHz, $CDCl_3$) δ 7.40−7.31 (m, 5H), 5.71 (d, J = 3.6 Hz, 1H), 4.75 (d, J = 11.9 Hz, 1H), 4.58 (d, J = 11.9 Hz, 1H), 4.56−4.52 (m, 1H), 4.10 (dd, J = 2.5, 9.1 Hz, 1H), 3.90−3.88 (m, 1H), 3.83 (dd, J = 4.3, 9.1 Hz, 1H), 1.90 (br s, 1H), 1.59 (s, 3H), 1.36 (s, 3H); ^{13}C NMR

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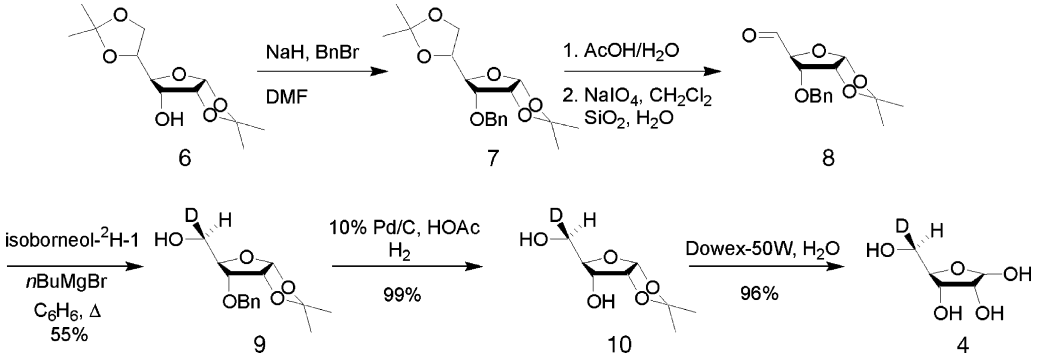
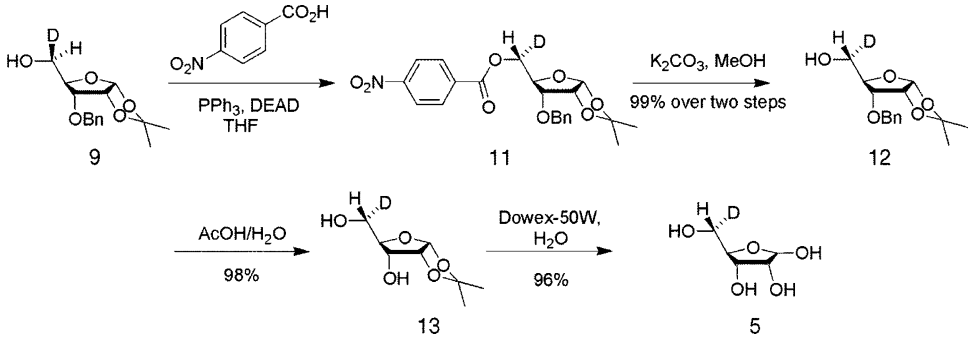
Scheme 2. Synthesis of (5*S*)-[5-$^2H_1$]-Ribose Diastereomer **4**



Scheme 3. Synthesis of (5*R*)-[5-$^2H_1$]-Ribose Diastereomer **5**



(75 MHz, $CDCl_3$) δ 137.5, 128.4, 128.0, 127.9, 113.0, 104.0, 78.6, 77.5, 76.5, 72.3, 60.1 (t, J = 21.2 Hz), 26.7, 26.4.

Conversion of 5*R*-Alcohol 12 into (5*R*)-[5-$^2H_1$]-Ribose 5. The alcohol **12** (46 mg, 0.16 mmol) was converted, as described for **9**, providing the diol **13** (30.5 mg, 98%) as an oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.80 (d, J = 3.8 Hz, 1H), 4.58 (t, J = 4.4 Hz, 1H), 4.09−3.95 (m, 1H), 3.92 (br s, 1H), 3.82 (dd, J = 2.7, 8.9 Hz, 1H), 2.55 (d, J = 10.4 Hz, 1H), 2.14 (br s, 1H), 1.56 (s, 3H), 1.37 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 112.8, 104.0, 80.5, 78.7, 70.8, 60.4 (t, J = 21.4 Hz), 26.5, 26.46.

The diol **13** (25 mg, 0.13 mmol) was treated, as described for **10**, to give 19 mg (96%) of (5*R*)-[5-$^2H_1$]-ribose **5** as an oily solid; ^{1}H NMR (300 MHz, D_2O) (chemical shifts for α- and β-pyranose and furanose) δ 5.41−5.20 and 5.00−4.80 (m, 1H), 4.24−4.09 (m, 1H), 4.00−3.75 (m, 2H), 3.73−3.45 (m, 1H); ^{13}C NMR (75 MHz, D_2O) (chemical shifts for α- and β-pyranose and furanose) δ 101.2, 94.1, 93.8, 83.5, 83.2, 75.3, 71.3, 70.7, 70.3, 69.6, 69.4, 67.6, 67.4, 63.3 (t, J = 23.4 Hz).

Enzymatic Synthesis and Purification of C5-Deuterium Labeled R5P. The *E. coli* ribokinase overexpression vector[20,21] was a gift from the S.L. Mowbray Laboratory. The enzyme was purified on a 1 mL HisTrap HP column using the following protocol. The overexpression strain was *E. coli* BL21, and overexpression was performed on 2 L of culture grown in LB media to an OD_{600} of 0.6 at which time the culture was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside. After growth overnight at 15 °C, the cells were pelleted by centrifugation at 4 °C and then resuspended in the following buffer for lysis by sonication: 50 mM Na^+-phosphate pH 8.0, 300 mM NaCl, 2 mM TCEP, and 10 mM imidazole. The cell debris was removed by centrifugation at 39 800 × g for 45 min at 4 °C. The lysate was passed through the HisTrap HP column and washed with 50 mL of 50 mM Na^+-phosphate buffer pH 8.0 containing 300 mM NaCl, 2 mM TCEP, and 20 mM imidazole and eluted with the same buffer but with 300 mM imidazole. Following elution the protein was buffer exchanged into 50 mM Tris pH 8.0 @ RT, 100 mM NaCl, 2 mM TCEP, and 20% glycerol using an Econo-Pac 10DG desalting column and flash frozen by submersion in liquid nitrogen in 80 μL aliquots. To carry out the phosphorylation reaction of the various C5-deuterium labeled ribose preparations, a total reaction volume of 5 mL was used according to the following conditions: 4 mg of *E. coli* ribokinase, 35 mM ribose, 45 mM ATP, 50 mM Tris, 100 mM KCl, and 2 mM TCEP. Prior to the addition of the kinase, the reaction mixture was adjusted to a pH of 7.2 using NaOH. The reaction was followed by TLC using polyethyleneimine-cellulose (PEI-cellulose-F) plates (EM Science, Gibbstown, NJ) run with 0.2 M KH_2PO_4 and visualized by *p*-anisaldehyde staining. The reaction was complete after 1 h at which time the protein was removed using an Amicon Ultra-15, 10 kDa cutoff centrifugal concentrator (Millipore, Billerica, MA). The flow through was diluted to 50 mL using ddH_2O and loaded onto a 20 mL HiPrep 16/10 Q XL anion exchange column (GE Healthcare, Uppsala Sweden) equilibrated with ammonium acetate pH 7. A gradient from 20 mM to 1 M ammonium acetate was run over 10 column volumes at a rate of 4 mL/min. The fractions containing R5P were pooled and lyophilized to constant weight and resuspended in ddH_2O giving a stock concentration of 50 mM.

Intermediate Formation. Intermediate formation was measured under the following conditions: 4 μM Pdx1/6 μM Pdx2, 2 mM R5P (or deuterium labeled R5P), 2 mM TCEP, 50 mM HEPES pH 7.6 @ RT, and 100 mM NaCl. R5P was preincubated with the enzyme for 15 min at room temperature prior to the initiation of the reaction upon addition of a final concentration of 15 mM glutamine. The absorbance was scanned or simply monitored at 320 nm using a Hitachi (Berkshire, UK) U-2010 UV/visible spectrophotometer. The concentrations of both glutamine and R5P were sufficiently high that the observed rate was essentially saturated. The reactions were performed in triplicate and averaged for analysis. Approximately 14 s of the reactions were missed during mixing and sample manipulation, therefore the data were shifted accordingly along the x-axis. The data were analyzed by nonlinear regression using the following double exponential equation:

$$\Delta Abs_{320} = A_1 e^{-\lambda_1 t} + A_2 e^{-\lambda_2 t} + C \qquad \text{(eq 1)}$$

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The parameters A_1 and A_2, λ_1 and λ_2 correspond to the amplitudes and observed rates of the fast and slow phases of the reaction, respectively. The term C is the offset. Data analysis and plotting were performed in the computer program GraFit 5 (Erithacus Software, Surrey, UK).

^{31}P NMR Analysis of I_{320} Formation. Purified Pdx1 (1.5 mM) was incubated with R5P (1.5 mM) in a total volume of 1 mL for 2 h at which time the protein was buffer exchanged into 50 mM HEPES pH 7.5, 200 mM NaCl, 1 mM TCEP, and 2 mM $MgCl_2$. This solution was then diluted 2× with $H_2{}^{18}O$ containing 1.2 M NH_4Cl to initiate I_{320} formation. The reaction was allowed to incubate overnight at room temperature at which time D_2O was added to a final concentration of 10% for NMR analysis. In order to catalyze the incorporation of ^{18}O into phosphate to generate a reference sample, a final concentration of 10 nM (by Bradford assay) of yeast inorganic pyrophosphatase (Sigma-Aldrich) was added and the reaction mixture was incubated for an additional 48 h at room temperature. ^{31}P spectra were recorded on a Varian INOVA 600 spectrometer operating at 242.785 MHz for ^{31}P observation using a 10 mm broadband direct observe probe head. Spectra were acquired observing a ^{31}P chemical shift range from 50 to −100 ppm with an acquisition time of 1.6 s and relaxation delay of 0.2 s using 90° pulses and broadband ^{1}H decoupling. Approximately 3500 scans were averaged for a total acquisition time of approximately 1.75 h. Spectra were zero filled to 256K complex points, and an unshifted Gaussian window was applied prior to Fourier transformation. ^{31}P chemical shifts were referenced to 85% phosphoric acid as an external standard.

Kinetics of Phosphate Elimination and Release. The kinetics of phosphate release were monitored by two different methods. First was by using the malachite green assay in a similar fashion to that reported elsewhere.[22] This assay is based on the formation, under acidic conditions (∼1 M HCl), of a malachite green/molybdate/phosphate complex that absorbs at 660 nm. The reaction mixture consisted of 20 μM Pdx1, 30 μM Pdx2, 2 mM R5P in a buffer composed of 50 mM HEPES pH 7.6 @ RT, 100 mM NaCl, and 2 mM TCEP. The enzyme was preincubated with R5P for 15 min prior to the addition of glutamine to a final concentration of 15 mM to start the reaction. The second method for measuring phosphate release was carried out using the EnzChek Phosphate Assay Kit from Invitrogen (Carlsbad, California). This assay is based on the formation of the chromophoric 2-amino-6-mercapto-7-methylpurine (λ_{max} = 360 nm) from 2-amino-6-mercapto-7-methylpurine riboside (λ_{max} = 330 nm) by the purine nucleoside phosphorylase catalyzed substitution with phosphate. The conditions for this assay were as follows: 8 μM Pdx1, 12 μM Pdx2, 2 mM R5P in addition to the assay components according to the manufacturer's instructions. This mixture was preincubated for 15 min at room temperature, and the reaction was initiated by the addition of a final concentration of 20 mM glutamine.

Formation of the Chromophoric Intermediate Using NH_4Cl. Reactions to form the chromophoric intermediate were carried out in the absence of the glutaminase subunit (Pdx2). Absorbance scans were taken under the following conditions: 20 μM Pdx1, 2 mM R5P, 2 mM TCEP in 50 mM HEPES pH 7.6 @ RT, with 100 mM NaCl. The reaction was initiated by the addition of solid NH_4Cl to a final concentration of 1 M, and absorbance scans were taken intermittently. To characterize the concentration dependence of the ammonia induced production of the enzyme bound intermediate, absorbance measurements were taken in a 96-well plate format (Greiner plate model 655801) purchased from Omega Scientific (Tarzana, CA) in a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT).

ESI-FTMS Analysis. The chromophoric intermediate was generated in the following manner. 25 μL of 538 μM Pdx1 stock solution (in 50 mM HEPES buffer pH 7.6 containing 100 mM NaCl, 2 mM TCEP and 20% glycerol) was diluted with 270 μL of water, followed by 400 μL of 250 mM HEPES buffer (pH 7.6, containing 500 mM NaCl). 27 μL of 50 mM R5P were then added, and the reaction mixture was incubated for 15 min at room temperature. 215 μL of 1 M NH_4Cl were then added to initiate chromophore formation. Two parallel control reactions were set up where the NH_4Cl or both the NH_4Cl and the R5P were substituted with an equivalent amount of water and were treated identically.

After allowing the reaction mixtures to incubate at room temperature for 80 min, 100 μL aliquots from each of them were quenched with 100 μL of 20% formic acid. Protein from the quenched reaction mixtures was desalted into 10 μL of 78% acetonitrile containing 0.1% TFA using C18 Zip Tip following the manufacturer's protocol. This was diluted to 50 μL with 50% methanol containing 0.1% formic acid, and the resulting solution was used for ESI-FTMS analysis (Thermo-Finnigan LTQ-FT). The resulting data were processed with the computer program Extract.

PLP Synthase Selectivity for G3P over DHAP. A steady state assay was performed under the following conditions using Pdx1 and Pdx2 which were isolated using the improved purification procedure as detailed in the "protein overexpression and purification section": 4 μM Pdx1, 6 μM Pdx2, 5 mM R5P, 10 mM glutamine in a buffer composed of 50 mM HEPES pH 7.6 @ RT, 100 mM NaCl, and 2 mM TCEP. The reactions were started by the addition of 5 mM DHAP or 1 mM G3P, and the UV−visible absorbance spectrum was monitored over time. The absorbance was converted to the concentration of PLP using an extinction coefficient of 6300 M^{-1} cm^{-1} at a wavelength of 390 nm.

Synthesis and Assay of 2′-Hydroxypyridoxol-5′-phosphate. 2′-Hydroxypyridoxol was synthesized as previously described.[23−25] 2′-Hydroxypyridoxol-5′-phosphate was synthesized with PdxK and purified by HPLC (LC-18-T column, 15 cm × 4.6 mm, 3 μm, isocratic elution with 25 mM NH_4OAc, pH 7). To test 2′-hydroxypyridoxol-5′-phosphate as a substrate for Pdx1, Pdx1 (50 μM) and Pdx2 (75 μM) were mixed with 330 μM 2′-hydroxypyridoxol-5′-phosphate in 25 mM Tris, pH 8, 60 μL total. After 16 h at 37 °C, the protein was removed with a Microcon and the sample was analyzed by HPLC (same column as above with the following solvent system: A, water; B, 100 mM KH_2PO_4 pH 6.6; C, methanol, 0−6 min, 100% B; 7 min, 10% C; 20 min, 30% C, 30% A; 22 min, 100% B).

Results and Discussion

Observation of a Chromophoric Species in the Active Site of Pdx1. When glutamine is added to a preincubated solution containing Pdx1 (4 μM), Pdx2 (6 μM), and R5P, a chromophoric species (I_{320}) accumulates in the active site (Figure 1). While this work was in progress, the formation and initial characterization of this intermediate was reported.[16] The intermediate is covalently bound to Lys 81 and has an extinction coefficient of approximately 16 200 M^{-1} cm^{-1} at 315−320 nm.[16] MS analysis of an I_{320}-containing tryptic peptide suggested that it is formed by loss of water and phosphate from a protein bound Ru5P adduct and that the glutamine derived ammonia is not covalently incorporated. Addition of G3P results in its conversion to PLP.

Isotope Effect on I_{320} Formation. The formation of I_{320} requires multiple elimination reactions to form a conjugated molecule from R5P. This means that there are also multiple deprotonation events leading up to I_{320} and suggests the possibility of a mechanistically informative deuterium isotope

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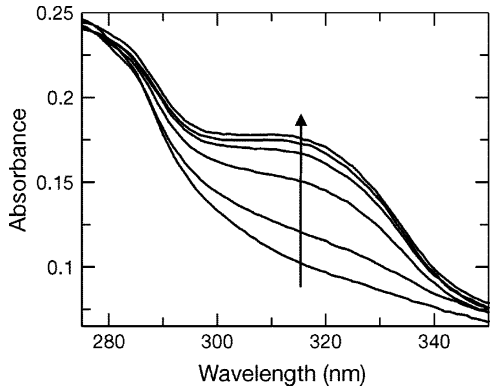

Figure 1. Formation of I_{320} in the active site of Pdx1. Pdx1 was preincubated with Pdx2 and a saturating concentration of R5P. Glutamine was added to initiate the reaction, and UV-absorbance scans were taken over a period of 200 s. No G3P was present in solution.

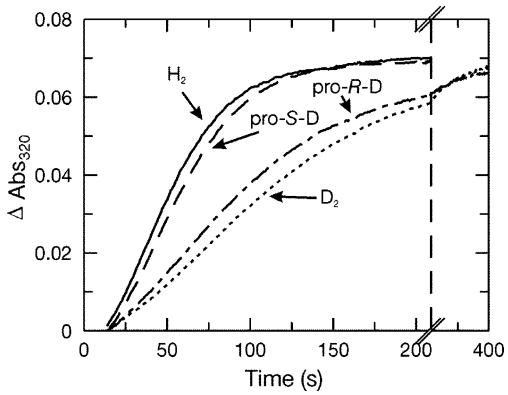

Figure 2. C5-deuterium kinetic isotope effect on I_{320} formation. The time dependence of I_{320} formation was monitored using the natural pentose (R5P, denoted "H_2") substrate, the C5-dideuterium labeled R5P analogue (denoted "D_2"), the specifically pro-*S*-D (denoted "pro-*S*-D") labeled R5P analogue, or the specifically pro-*R*-D (denoted "pro-*R*-D") labeled R5P analogue.

effect on the kinetics of its formation. Previous structural and biochemical data showed that Pdx1 has pentose-5-phosphate isomerase activity and that ribulose-5-phosphate (Ru5P) forms an adduct with an active site lysine.[13,26] Therefore, given the information that I_{320} seemed to be triggered by ammonia addition to the ribulose-5-phosphate adduct, the isotope effects on I_{320} formation were measured starting with Pdx1 already loaded with Ru5P to circumvent any possible rate-limiting steps involved in isomerization and imine formation that would attenuate the isotope effect. However, before an accurate deuterium kinetic isotope effect could be measured it was necessary to free the active site of the unlabeled Ru5P that is present following the overexpression and purification. This was accomplished by first purifying Pdx1 and Pdx2, combining them in a ratio of 1:1.5, and carrying out a single turnover reaction by adding glutamine and G3P. This converted the bound Ru5P to PLP. The resulting protein was subjected to an additional round of purification to give enzyme with no detectable bound Ru5P.

To obtain substrate to measure the KIE at C5, commercially available C5 dideuterium labeled ribose ([5-$^2H_2$]-ribose) was enzymatically phosphorylated using recombinant *E. coli* ribokinase and ATP[20,21] and the product was purified using anion exchange chromatography. The rate of formation of I_{320} from the Pdx1−[5-$^2H_2$]−Ru5P adduct was then compared to the rate of formation of I_{320} from the Pdx1−Ru5P adduct under pre-steady-state conditions. For this measurement, Pdx1 and Pdx2 were present at a molar ratio of 1:1.5, in which Pdx1 was at a concentration of 4 μM. The natural (C5-H_2) and labeled (C5-$^2H_2$) Ru5P substrates were loaded into the active site by preincubating the corresponding R5P with the enzyme for 15 min prior to the addition of the glutamine. The reactions were initiated with a final concentration of 15 mM glutamine, and the results are shown in Figure 2 as an increase in absorbance at 320 nm plotted over time. The data, which were collected in triplicate, exhibited biphasic behavior and were therefore analyzed by nonlinear regression using a double exponential equation (eq 1) to extract the observed rates for the two phases. The fast and slow phases using the natural pentose (labeled "H_2" on the plot) occurred at 0.050 ± 0.001 s^{-1} and 0.0354 ± 0.0002 s^{-1}, respectively. Simply a visual inspection of the plot reveals that there is a surprisingly large KIE for I_{320} formation when using the C5-$^2H_2$ labeled R5P (labeled "D_2") as a substrate and the data were again biphasic with fast and slow phase rates of 0.0204 ± 0.0003 s^{-1} and 0.0147 ± 0.0001 s^{-1}. The isotope effect was apparent on both phases of the reaction and was nearly identical in magnitude (2.45 and 2.41). The observation of a primary deuterium isotope effect of greater than 2.4 for deprotonation at C5 is strong evidence that the removal of one of the protons on C5 is partially rate limiting in the formation of I_{320} from the Pdx1−Ru5P adduct. The fact that the data show a clear lag phase is indicative that there are at least two reactions occurring in sequence at comparable rates prior to I_{320} production. It is not clear, at this time, what the identity of the other event is that limits I_{320} formation, but it is important to report that the lag phase cannot be altered by increasing the concentration of glutamine or R5P and is therefore likely to be a first-order reaction following substrate binding. Similar experiments were carried out using [3-$^2H_1$]-ribose and [4-$^2H_1$]-ribose. No isotope effects were observed for these isotopomers.

Stereochemistry of the C5 Deprotonation. The commercially available C5 dideuterium labeled ribose did not allow us to assign a specific stereochemistry to the deprotonation event. To obtain this information, syntheses of the two singly labeled R5P isomers were carried out.

Synthesis of (5*S*)-[5-$^2H_1$]- and (5*R*)-[5-$^2H_1$]-Ribose. The syntheses of (5*S*)-[5-$^2H_1$]- and (5*R*)-[5-$^2H_1$]-ribose utilize the known deuterium-labeled ribose derivative **9** (Scheme 2).[18] The absolute stereochemistry of this structure has been previously established by conversion to the relevant thymidine derivatives followed by 2D NMR analysis.[18,27]

The 5*S*-diastereotopically enriched alcohol **9** was prepared in several steps from the commercially available allofuranose **6**.[17] Straightforward hydrogenolysis followed by acetonide removal resulted in ribose **4**. Based on ^{1}H NMR data, the configuration at C5 was retained during the final two steps. Access to the 5*R*-stereochemistry was realized by inversion of **9** using Mitsunobu conditions followed by ester hydrolysis to afford the alcohol **11** in quantitative yield (Scheme 3). The same deprotection sequence resulted in the (5*R*)-[5-$^2H_1$]-ribose **5** in excellent yield.

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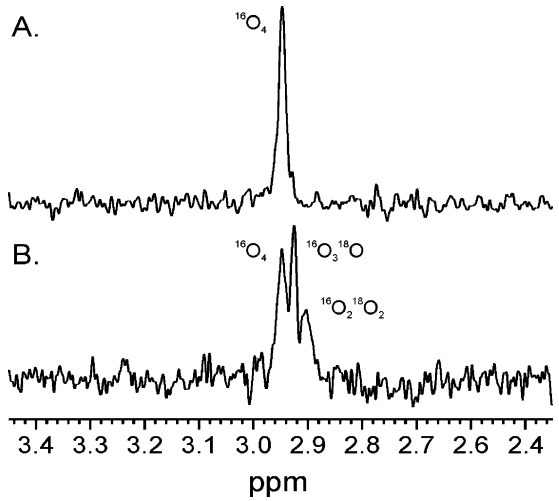

Figure 3. ^{31}P NMR analysis of phosphate release. (A) ^{31}P spectrum of a reaction mixture in which I_{320} was prepared stoichiometrically with Pdx1 in buffer containing 50% $H_2{}^{18}O$. The signal denoted "$^{16}O_4$" refers to inorganic phosphate with natural abundance oxygen bound in all four positions. (B) To generate a reference sample of ^{18}O-phosphate yeast inorganic pyrophosphatase was added to the reaction mixture described in (A) in order to catalyze the incorporation of ^{18}O into the released phosphate. The new peaks are denoted with the stoichiometric ratio of ^{16}O to ^{18}O bonded to the phosphorus atom of P_i.

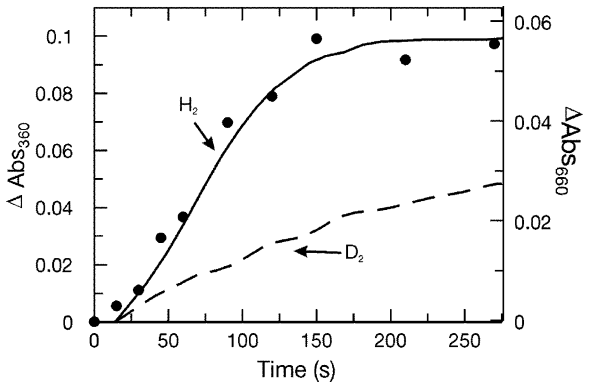

Figure 4. Time dependence of P_i elimination. Phosphate elimination during I_{320} formation was monitored over time by the malachite green method (●). The solid and dashed lines were data obtained using a continuous assay based upon an increase in absorbance at 360 nm as described in the main text. The solid line represents a reaction in which the natural substrate, R5P, was used (denoted "H_2"), and the dashed line represents a reaction in which the R5P was dideuterium labeled at position 5 (denoted "D_2").

The stereochemistry of the C5 deprotonation was determined using the deuterium KIE seen on the rate of formation of I_{320}. Shown in Figure 2 are the experiments performed with the stereospecifically labeled R5P derivatives (labeled "pro-*R*-D" and "pro-*S*-D" on the plot). In the experiment in which the pro-*R* isomer was present, there was a clear KIE, whereas there was not when using the pro-*S* isomer. Therefore, it can be unambiguously stated that the pro-*R* hydrogen is abstracted during the formation of I_{320} and that this deprotonation is partially rate limiting under these reaction conditions.

Phosphate is removed by elimination rather than hydrolysis during I_{320} formation. We know from previous studies that the P_i of R5P is lost during the course of I_{320} formation;[16] however, it is not known whether it is removed by elimination or hydrolysis. To differentiate between these two possibilities the intermediate was formed in the presence of 50% $H_2{}^{18}O$ and ^{31}P NMR analysis of the P_i released was performed. If the P_i group from R5P was removed by hydrolysis then 50% of the P_i would have an upfield isotopic shift of approximately 0.02 ppm[28] compared to that of the all ^{16}O containing P_i. As shown in Figure 3A, the ^{31}P spectrum of the reaction mixture is consistent with a single species. To illustrate that the resolution was adequate to clearly distinguish $^{16}O_4$ phosphate from $^{16}O_3{}^{18}O$ phosphate under the conditions used in this experiment, inorganic pyrophosphatase was added to the sample and ^{31}P NMR analysis was performed again (Figure 3B). Two new signals emerged upon addition of pyrophosphatase whose chemical shifts are consistent with P_i containing one and two ^{18}O atoms. These data demonstrate that P_i is eliminated in route to I_{320} formation.

C5 deprotonation occurs prior to phosphate elimination. To determine the relative order of phosphate elimination and C5 deprotonation, we measured the deuterium isotope effect on phosphate elimination. If phosphate elimination occurred before C5 deprotonation, there should be no large (or primary) isotope effect on the rate of phosphate formation while elimination after deprotonation should exhibit a kinetic isotope effect. Two separate approaches were taken to determine the kinetics of phosphate elimination. One was to perform a pre-steady-state reaction under similar conditions to those described above for measuring the kinetics of I_{320} formation. However, instead of monitoring the absorbance at 320 nm, the reaction was quenched at specific times and analyzed for phosphate using the malachite green assay[22] to determine the time course for phosphate elimination. Because this assay involves quenching the reaction mixture with a solution containing a final concentration of approximately 1 M HCl, the data represent the time course for the C−O bond cleavage reaction. As shown in Figure 4 (filled circles) the elimination of phosphate essentially mirrors the accumulation of I_{320} suggesting that loss of phosphate and chromophore formation are limited by the same microscopic rate constant or constants. Because of the difficulty in obtaining accurate measurements for free P_i in reactions performed under single turnover conditions using the malachite green assay, an alternative assay was developed. This alternative assay is based on the phosphorolysis of a purine nucleoside analog by purine nucleoside phosphorylase. The measurement of an increase in absorbance at 360 nm is an indicator of the liberation of inorganic phosphate from Pdx1 as the reaction occurs. The data denoted "H_2" were obtained under similar conditions as those detailed for the malachite green assay except that P_i liberation was measured in real time in the spectrophotometer (solid line in Figure 4). The absorbance increase mirrors the data obtained using the malachite green assay.

One important new observation can be made from this data. Because the malachite green assay is measuring the observed rate of C−O bond cleavage and the optical assay includes the additional step of P_i dissociation from the enzyme active site, then it can be concluded that P_i dissociation must be relatively rapid following the elimination step. Since the release of phosphate occurs in concert with the formation of I_{320}, it is likely that P_i elimination follows C5 deprotonation. However, a more rigorous test of this hypothesis would be to determine whether there is a C5 deuterium KIE on P_i elimination. Therefore, this experiment was repeated using (5-$^2H_2$)-R5P, and the results are

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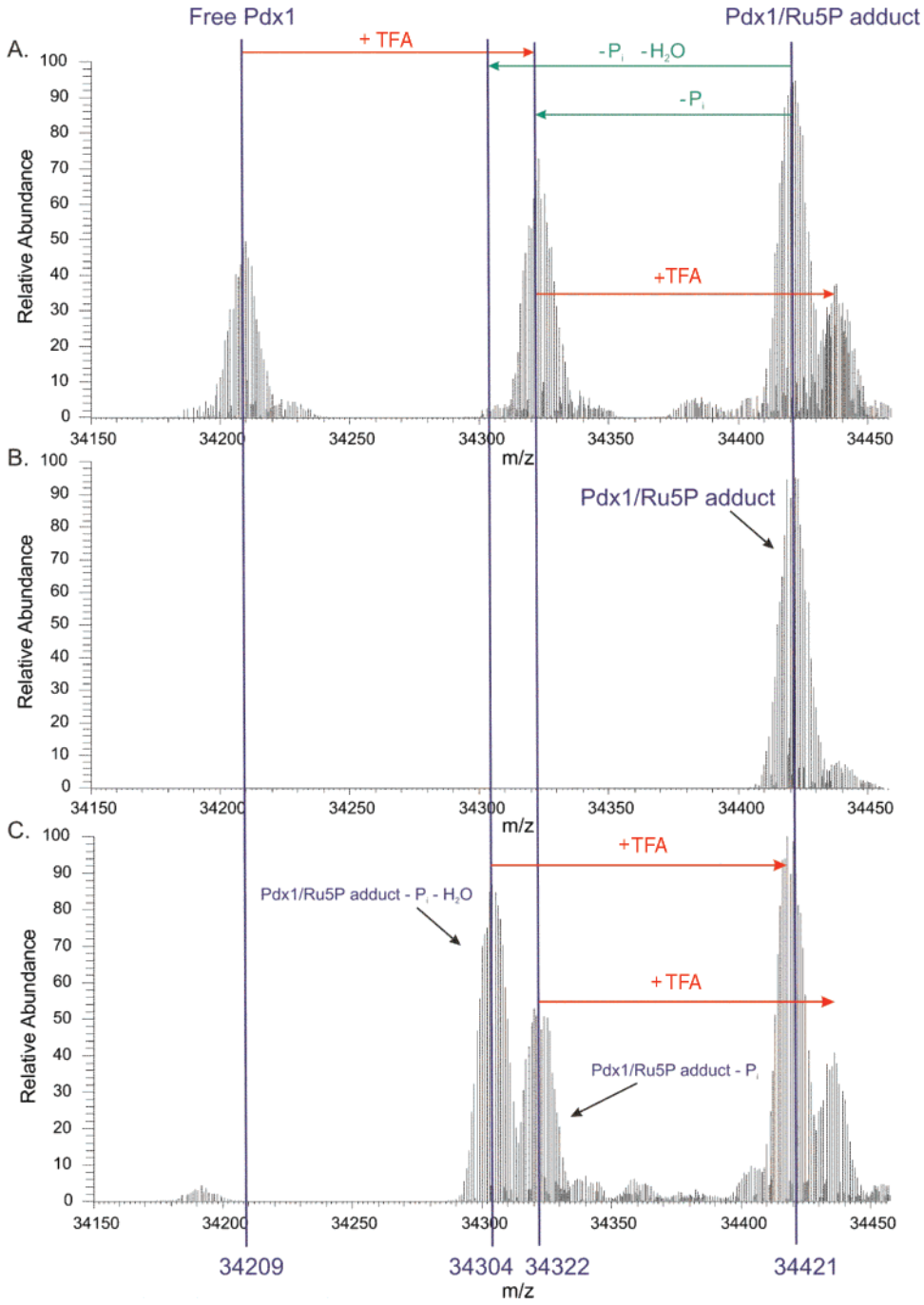


Figure 5. ESI-FTMS analysis of Pdx1. (A) Native Pdx1, not treated with R5P or NH_4Cl, (B) Pdx1 treated with R5P, and (C) Pdx1 treated with both R5P and NH_4Cl. Masses corresponding to free Pdx1 (34 209 Da), Pdx1 to which Ru5P is bound (34421 Da; $\Delta m = +212$), and adducts generated from Ru5P bound Pdx1 after ammonia incorporation via (i) phosphate loss only (34322 Da; $\Delta m = +113$) and (ii) both phosphate and water loss (34304 Da; $\Delta m = +95$) are marked with blue lines. The protocol used in desalting the sample preparation introduced TFA resulting in the observation of TFA adducts of all the peaks (+114 Da). These adducts are indicated with red arrows. In the deconvoluted spectrum "A", both the mono (34 323 Da) and the bis (34 437 Da) TFA adducts of Pdx1 are observed. These adducts are not observed with the complete conversion of free Pdx1 to the Ru5P bound Pdx1, as shown in spectrum "B". Addition of NH_4Cl results in the appearance of the two new peaks at 34 304 Da ($\Delta m = +95$) and 34 322 Da ($\Delta m = +113$) respectively, as shown in spectrum "C". The species with a mass of 34 322 Da overlaps with the mono TFA adduct of free Pdx1. However, the absence of free Pdx1 in spectrum "C" rules out the emergence of this species due to the mono TFA adduct of free Pdx1. The peak observed at the position overlapping with Pdx1 to which Ru5P is bound in "C" (observed mass 34 418 Da, slightly shifted from the line depicting the mass 34 421 Da) corresponds to the mono TFA adduct of the species with a mass of 34 304 Da rather than Pdx1 to which Ru5P is bound.

shown in Figure 4 along with the data for the natural substrate. There is clearly a KIE effect on phosphate elimination. This provides strong evidence that P_i elimination follows C5 deprotonation in route to the chromophoric intermediate.

High-resolution ESI-FTMS analysis of I_{320} formation. ESI-FTMS was performed to gain additional information regarding the identity of I_{320}. Previous data demonstrated that a tryptic peptide with an extra 95 Da could be isolated from the Pdx1−I_{320} complex.[16] This suggested that I_{320} was formed by loss of water and phosphate from the Pdx1−Ru5P adduct. However, since this experiment involved extensive sample manipulation, the possibility existed that the species detected

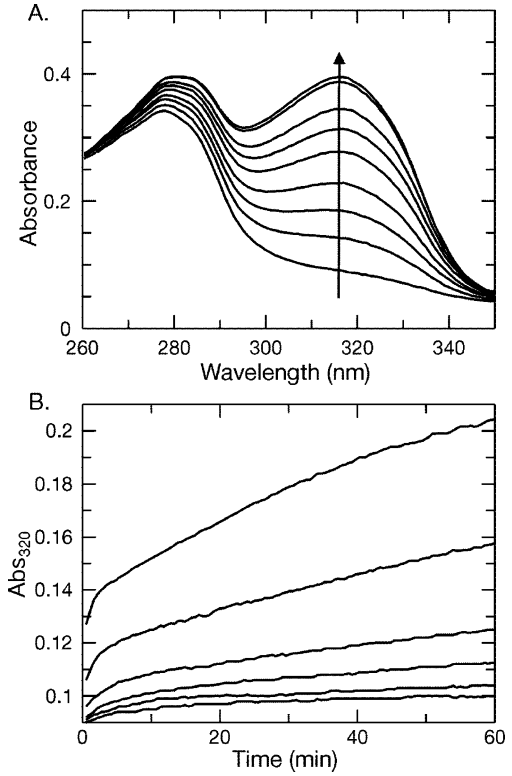


Figure 6. Addition of ammonia triggers I_{320} formation. (A) A sample of Pdx1 was preincubated with R5P and then NH_4Cl was added as a solid to a final concentration of 1 M. UV-absorbance scans were taken intermittently over a period of approximately 10 min. (B) The concentration dependence of I_{320} formation was characterized under similar conditions as in (A), except that varying concentrations of NH_4Cl were added to start the reaction (added as a solution). Starting with the reaction represented by the curve with the highest final amplitude to the one with the lowest final amplitude, the concentrations of NH_4Cl present were as follows: 320, 160, 80, 40, and 20 mM.

by MS was a decomposition product of I_{320}. We therefore carried out ESI-FTMS analysis on the intact Pdx1−I_{320} complex, prepared by reacting Pdx1 with R5P and NH_4Cl (see next section for details). This strategy required only mild desalting of the complex prior to MS analysis. The results are shown in Figure 5.

Two informative species are seen in the Pdx1 sample. These correspond to free Pdx1 (34209 ± 1 Da) and Pdx1 bound to an adduct of Ru5P (34421 ± 1 Da) in a ratio of approximately 2:3, respectively (Figure 5A). The additional species with mass values of 34 322 and 34 436 Da correspond to the mono- and bistrifluoroacetic acid (+114 Da) adducts of free Pdx1. When Pdx1 is treated with R5P, the Pdx1−Ru5P adduct (34 421 Da) is the major species observed (Figure 5B). Treatment of the Pdx1−Ru5P adduct with NH_4Cl results in the emergence of two new species with masses of 34 304 ± 1 Da and 34 322 ± 1 Da, respectively, as well as their monotrifluoroacetic acid adducts (Figure 5C). The species with a mass of 34 304 Da is consistent with an intermediate generated from the Pdx1−Ru5P adduct by the elimination of phosphate (−98 Da) and water (−18 Da). This species is likely to correspond to I_{320}. These data do not tell us if ammonia has been incorporated into this species because the precision of the MS analysis (±1 Da) does not differentiate between −OH and $-NH_2$, which differ by 1 Da. The species with a mass of 34 322 Da corresponds to one generated by phosphate elimination. This species is consistent

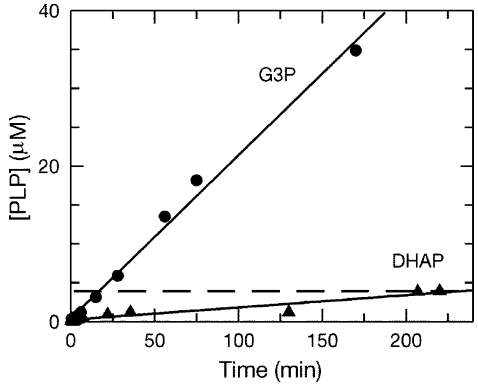


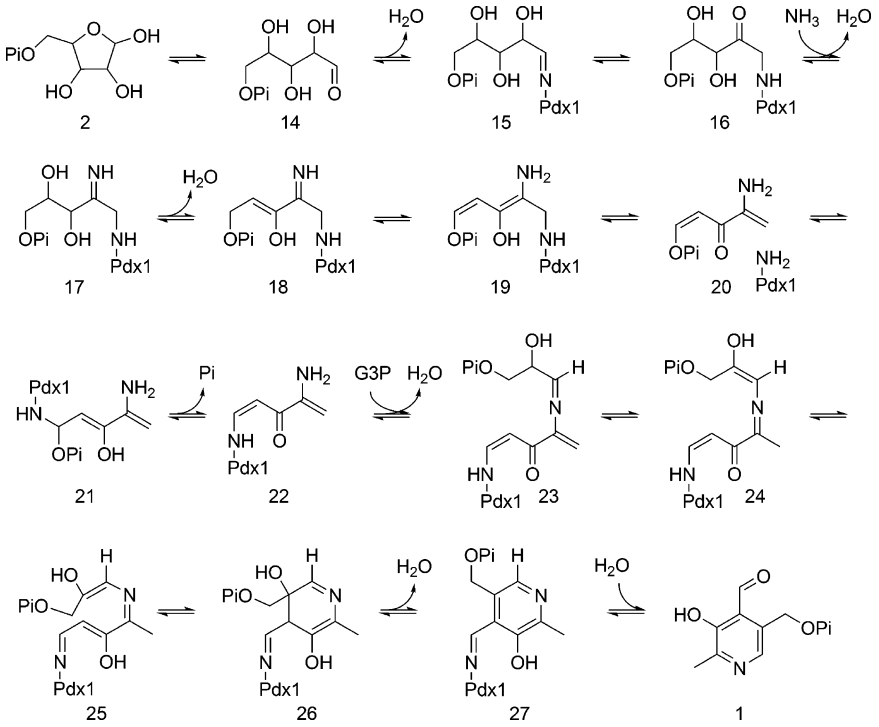
Figure 7. G3P is greatly preferred over DHAP. Shown in the plot is the steady-state accumulation of PLP using Pdx1 and Pdx2 at a ratio of 1:1.5. The substrates G3P and DHAP were present at saturating concentrations. The horizontal dotted line on the plot is to illustrate the concentration of Pdx1 used in the assay and is therefore at a point which multiple turnovers would be observed. When using DHAP as a substrate multiple turnovers are not observed in the time scale examined.

with a hydration product of I_{320}. Again, these data do not tell us if ammonia has been incorporated.

Is the nitrogen atom of ammonia covalently incorporated into I_{320}? I_{320} can be formed by treating glutamine and R5P with Pdx1/Pdx2 or by treating R5P and ammonia with Pdx1.[15,16] This suggests that the nitrogen atom of ammonia might be covalently attached to I_{320}. However, MS characterization of the I_{320} labeled tryptic peptide did not contain ammonia,[16] and the ESI-FTMS analysis described above did not differentiate between −OH and $-NH_2$, which differ by 1 Da.

When Pdx2 and glutamine are used as the ammonia source, the intermediate can be produced stoichiometrically with the protein in the absence of G3P. The enzyme bound intermediate can then be purified by gel filtration to remove all small molecules, and subsequent addition of only G3P results in PLP formation. At least two possible conclusions can be drawn from this observation: either ammonia is incorporated into the intermediate covalently or ammonia is tightly bound to Pdx2. To differentiate between these possibilities, R5P was preincubated with Pdx1 and NH_4Cl was then added to start the reaction. The results are shown in Figure 6A as UV−absorbance scans taken over time. From the large increase in absorbance at 320 nm, it is clear that the addition of NH_4Cl alone triggered I_{320} formation. Shown in Figure 6B is the concentration dependence of this reaction showing that good reconstitution of I_{320} requires exceedingly high concentrations of NH_4Cl, illustrating that the enzyme's affinity for ammonia from solution is weak (not yet saturated at concentrations up to 1 M NH_4Cl). However, once reconstituted this intermediate could not be separated from the protein and could be converted to PLP by the addition of only G3P following purification of the protein/I_{320} complex by gel filtration into a solution lacking ammonia. It is highly improbable that NH_4Cl induces the formation of I_{320} without covalent incorporation because the ammonia remains bound to Pdx1 during the gel filtration. This should not occur in the absence of a covalent bond because our analysis of the ammonia concentration dependence of I_{320} formation demonstrates that ammonia binds very weakly to Pdx1. Therefore, we conclude that I_{320} contains covalently bound ammonia and that the extensive sample preparation used in the reported MS analysis generated a hydrolysis product.[16]

Scheme 4. Mechanistic Proposal for PLP Synthase



G3P is the preferred substrate. Pdx1 can use either G3P or DHAP as the three carbon sugar for the synthesis of PLP.[13,14] This implied that Pdx1 has triose phosphate isomerase activity. Whether this activity is intrinsic to PLP synthase or due to a trace contaminating triose phosphate isomerase activity was initially unclear. During the course of this study, it became apparent that the enzyme prepared by a more thorough purification procedure had a much reduced ability to make PLP using DHAP. In order to quantify this observation, an experiment was performed under saturating conditions using either G3P or DHAP as the triose phosphate, and the results are shown in Figure 7.

Clearly DHAP was not utilized as efficiently as G3P, and its consumption could not account for even a single catalytic turnover (the enzyme concentration used is denoted by the horizontal dashed line on the plot). This suggests that the previously observed triose phosphate isomerase activity was due to a low concentration of the *E. coli* isomerase present in the preparation. This is not surprising considering that *E. coli* triose phosphate isomerase operates with a k_{cat} of nearly 10 000 s^{-1} ($k_{cat}/K_m \approx 1\ \mu M^{-1}\ s^{-1}$), whereas Pdx1 catalyzes the formation of PLP with a k_{cat} of approximately 0.000 87 s^{-1}, a difference of over ten million-fold.

2′-Hydroxypyridoxol-5′-phosphate is not converted to PLP by Pdx1. To test a previous suggestion that 2′-hydroxypyridoxol-5′-phosphate was an intermediate in the formation of PLP in *Saccharomyces cerevisiae*,[29] this compound was synthesized and incubated with Pdx1 and Pdx2 (close orthologues to SNZ and SNO in *S. cerevisiae*) or with Pdx1 alone. HPLC analysis of the reaction mixtures revealed no conversion to PLP.

Proposed Mechanism of PLP Formation. In this paper we have described additional properties of the pyridoxal phosphate synthase catalyzed reaction. Specifically, we can follow the production of an advanced intermediate under mild conditions using ESI-FTMS. We observe a primary deuterium kinetic isotope effect at C5 of R5P on the formation of the intermediate and determine that this isotope effect is due to removal of the pro-*R* proton. We also demonstrate that ammonia is likely to be covalently attached to the intermediate and that G3P is a much better substrate than dihydroxyacetone-phosphate (DHAP). Additionally, we show that phosphate loss occurs by an elimination reaction rather than by a hydrolysis reaction and that this occurs after C5 deprotonation and ammonia addition. A mechanistic proposal for PLP synthase that is consistent with these new observations is outlined in Scheme 4.

In this mechanism, ring opening of ribose-5-phosphate **2** gives **14**. Imine formation with Lys 81 results in **15**. Isomerization to **16** sets up imine formation using ammonia generated by the Pdx2-catalyzed glutamine hydrolysis to give **17**. Elimination of water yields **18**, which tautomerizes to give **19**. Elimination of K81 from C1 results in **20**, which then reacts at C5 with the same residue to give **21**. Elimination of phosphate provides **22** which is our proposed structure for I_{320}. Imine formation between **22** and G3P results in **23**. Two tautomerization reactions followed by an electrocyclic ring closure and aromatization give **27**. Imine hydrolysis gives PLP **1** in the final step of the reaction.

This mechanistic proposal now has a substantial level of experimental support. Treatment of the enzyme with R5P followed by proteolysis with trypsin gave a peptide with mass and sequence consistent with imine **15** attached to Lys 81.[16] However, such an imine is likely to be hydrolyzed during sample preparation. It is therefore more likely that the isolated tryptic peptide was labeled with aminoketone **16** which should be stable. The formation of imine **17** occurs using high concentrations of ammonia or using ammonia generated by the Pdx2-catalyzed hydrolysis of glutamine. The deprotonation of **18** to give **19** involves removal of the pro-*R* proton at C5. This

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deprotonation reaction shows a primary deuterium isotope effect of 2.4 indicating that it is a partially rate-limiting step in the conversion of **16** to **22**. Phosphate elimination occurs after C5 deprotonation because we observe a primary deuterium isotope effect on phosphate formation. The addition elimination mechanism for phosphate removal is consistent with the absence of ^{18}O incorporation into the phosphate as determined by ^{31}P NMR analysis. A similar mechanism of phosphate removal has been previously reported for the *E. coli* PLP synthase which uses 1-deoxy-D-xylulose-5-phosphate and 1-amino-acetone-3-phosphate and makes chemical sense in that it provides an easy way to control the point at which phosphate is lost during the reaction sequence. We propose structure **22** for I_{320} based on the following: Formation of I_{320} requires high concentrations of ammonia suggesting that noncovalent interactions of ammonia with Pdx1 are weak. In contrast Pdx1−I_{320} can be purified by gel filtration and reacts with G3P to give PLP in the absence of ammonia. This suggests that ammonia is covalently bound to I_{320}. I_{320} must be a highly conjugated system to account for its long wavelength absorption. The observation of a primary deuterium isotope effect on the formation of I_{320} from **16** suggests that the C5 pro-*R* proton of R5P is absent in I_{320}. Phosphate elimination is stoichiometric with the production I_{320} demonstrating that I_{320} does not contain phosphate. MS analysis of a tryptic peptide modified with I_{320} is consistent with this structure. However this peptide did not contain the amino group suggesting that it had been hydrolytically removed during sample preparation. Finally, analysis of the formation of I_{320} from **16** by ESI-FTMS is consistent with intermediate **22** and its hydration product. This technique requires minimal protein manipulation and therefore minimizes the possibility of I_{320} decomposition. The mechanistic details for the conversion of I_{320} to PLP have not yet been experimentally verified. G3P is the preferred substrate suggesting the formation of imine **23**. In addition 2′-hydroxypyridoxol-5′-phosphate, a previously proposed intermediate, is not a substrate for Pdx1. This supports our proposal for early loss of water from the C1 of ribose (**14** to **15**).

The mechanism described in Scheme 4 is the fourth generation PLP synthase mechanism. As additional experimental facts on the mechanism of this fascinating enzyme emerge, the mechanism will no doubt continue to evolve. In particular, experimental evidence for the proposed linkage shift from C1 to C5 needs to be established as well as the steps involved in the conversion of I_{320} to PLP and the role of K149. As **16** and **22** are stable intermediates they should be amenable to NMR and crystallographic characterization.

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