Biosynthesis of Vitamin B_6: Enzymatic Conversion of 1-Deoxy-D-xylulose-5-phosphate to Pyridoxol Phosphate

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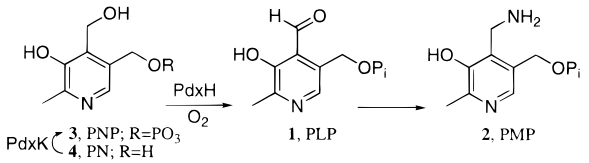
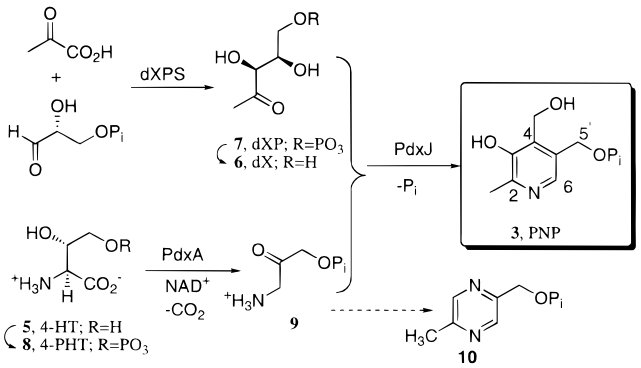
Pyridoxal 5′-phosphate (**1**, PLP) is a central coenzyme in amino acid metabolism,[1a] while its congener pyridoxamine 5′-phosphate (**2**, PMP) also plays an important role in the biosynthesis of deoxysugars.[1b] PMP is generated from PLP by transamination, while the latter is derived from pyridoxine 5′-phosphate (**3**, PNP) by an O_2-dependent oxidation catalyzed by the flavoenzyme known as PdxH.[2] (Scheme 1). Pyridoxine itself (**4**, PN, vitamin B_6) can be converted to PNP by an ATP-dependent kinase, PdxK,[3] which can also mediate the formation of PLP and PMP from the corresponding free alcohols.[4]

In *Escherichia coli* PNP is formed from two building blocks derived from 4-hydroxythreonine (**5**, 4-HT) and 1-deoxy-D-xylulose (**6**, dX).[5] (Scheme 2). The latter metabolite has attracted intensive interest with the recognition that 1-deoxy-D-xylulose-5-phosphate (**7**, dXP) is also a precursor of thiamin (vitamin B_1)[6a] as well as many bacterial and plant isoprenoids.[6b] Genetic studies have implicated two gene products, PdxA and PdxJ, as being responsible for the formation of PNP from 4-HT and dX or their derivatives.[7] Recently, we reported that PdxA is responsible for the NAD^+-dependent oxidation of 4-phosphohydroxy-L-threonine (**8**, 4-PHT) to a product tentatively identified as 3-phosphohydroxy-1-aminoacetone (**9**).[8,9] In the absence of additional substrates, **9** is unstable and undergoes dimerization and loss of phosphate to give the pyrazine derivative **10**. We now report that PdxJ catalyzes the condensation of 1-deoxy-D-xylulose-5-phosphate (**7**) and 3-phosphohydroxy-1-aminoacetone (**9**) to yield PNP.

Scheme 1



Scheme 2



The *E. coli* proteins PdxA and PdxJ were cloned, overexpressed, and purified as previously described.[8] The cosubstrate 4-phosphohydroxy-L-threonine (**8**) was also prepared as previously described by phosphorylation of synthetic 4-hydroxythreonine using homoserine kinase[8,10] (Scheme 2). Recombinant *E. coli* deoxyxylulose-5-phosphate synthase was used to prepare the requisite samples of dXP (**7**) and [2-^{14}C]dXP from pyruvate and [2-^{14}C]pyruvate, respectively, and glyceraldehyde-3-phosphate in the presence of thiamin diphosphate.[11] The corresponding samples of unlabeled deoxyxylulose (**6**) and [2-^{14}C]dX were readily obtained by treatment of the respective samples of dXP (**7**) with acid phosphatase.

A mixture of [2-^{14}C]-1-deoxy-D-xylulose-5-phosphate (**7**) and 4-phosphohydroxy-L-threonine (**8**) was incubated with desalted PdxA and PdxJ in the presence of NAD^+ in 100 mM Tris, pH 7.5, at 37 °C (Scheme 2). Aliquots of the mixture were periodically withdrawn at times up to 90 min and analyzed by silica gel TLC, visualizing the radioactivity on the plates by phosphoimaging. Within 5 min, the spot corresponding to the substrate [2-^{14}C]dXP had disappeared and was replaced by a spot with an R_F corresponding to pyridoxol-5′-phosphate (**3**, PNP). Control experiments showed that no PNP was formed in the absence of either enzyme or 4-PHT. By contrast, when [2-^{14}C]-deoxyxylulose was used in place of [2-^{14}C]dXP, no pyridoxol phosphate or pyridoxol could be detected. To gain insight into the mechanism of the reaction, we monitored a preparative-scale incubation by ^{31}P NMR over a period of 100 min at room

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(9) Interestingly, although use of PdxA as a query and either the BLAST or TFASTA algorithms to search the combined Genbank and EMBL databases turned up no significant matches to known proteins, use of the iterative PSI−BLAST algorithm (Altschul, S. F.; Madden, T. F.; Schäffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. *Nucleic Acids Res.* **1997**, *25*, 3389−3402; http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-psi_blast) to search the NCBI nonredundant (nr) peptide sequence database revealed after 2 iterations ∼30−35% similarity over 150−200 amino acids both to isocitrate dehydrogenases and to 3-isopropylmalate dehydrogenases, enzymes that also catalyze nicotinamide-dependent decarboxylative oxidations of *β*-hydroxy acids.

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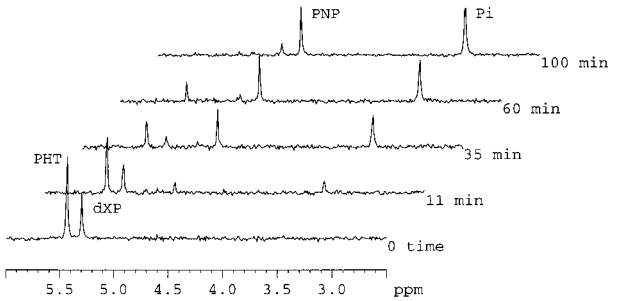


Figure 1. Time course of ^{31}P NMR spectra showing the conversion of DXP and PHT to PNP and P_i by PdxA and PdxJ in the presence of NAD. The reaction was carried out at room temperature in 0.7 mL of Tris/HCl buffer (0.1 M, pH 7.5) which contained D_2O (0.15 mL), DXP (1.21 mg, 4.19 μmol), PHT (1.05 mg, 4.91 μmol), NAD (9.85 μmol), desalted PdxA (0.6 mg), and PdxJ (0.6 mg).

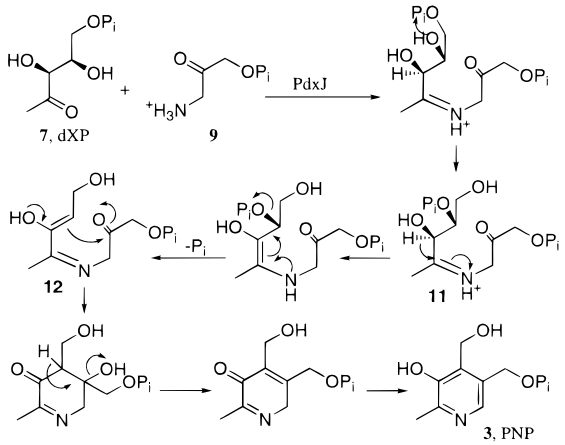
temperature using dXP (4.14 μmol), 4-PHT (4.91 μmol), and NAD^+ (9.85 μmol) in the presence of 600 μg of desalted PdxA and 600 μg of desalted PdxJ in 0.1 M Tris, pH 7.5, plus 0.15 mL of D_2O in a total volume of 0.7 mL. As illustrated in Figure 1, consumption of 4-PHT and dXP was accompanied by the simultaneous appearance of peaks of equal intensity at 4.79 and 3.42 ppm, corresponding to the stoichiometric formation of pyridoxol phosphate (**3**, PNP) and inorganic phosphate (P_i), the identities of both of which were verified by comparison with authentic samples.

To confirm further the identity of the enzymatic reaction product, we applied the preparative scale incubation mixture from the ^{31}P NMR experiment to an AG1 $\times$ 8 ion-exchange column, which was washed with water and then 6 mM HCl. The fractions containing pyridoxol phosphate contaminated with NAD^+ were reapplied to the AG1 $\times$ 8 column in a volume of 20 mL and eluted with 2 mM HCl, giving pure pyridoxol phosphate (**3**). The 400 MHz ^{1}H NMR spectrum of **3** (HCl salt, D_2O) showed a singlet at δ 2.49 (3 H, C*H*$_3$), a singlet at 4.88 ppm (2 H, C*H*$_2$O), a doublet at 4.90 ppm (2 H, J_{HP}=7.55 Hz, C*H*$_2$OP), and a singlet at 8.05 (1 H, *H*-6), and the 162 MHz ^{31}P NMR spectrum of **3** (HCl salt, D_2O) showed a single peak at 1.20 ppm (t, J = 7.55 Hz). Both the ^{1}H and ^{31}P spectra of enzymatically generated **3** were identical to those of an authentic sample of PNP. The TOF electrospray MS of enzymatically generated **3** also matched that of authentic PNP with peaks at *m*/*z* 249 ($C_8H_{12}O_6NP$), 248 (base peak), and 230. Finally the two samples were identical by UV (λ_{max} 324 nm) and TLC.

On the basis of the results of the ^{31}P NMR experiment, it was evident that the formation of pyridoxol phosphate catalyzed by PdxJ does not involve initial formation of either pyridoxal-4′,5′-diphosphate or the free alcohol deoxyxylulose, consistent with the fact that deoxyxylulose is not a substrate for the PdxJ-catalyzed reaction. In a separate ^{31}P NMR experiment, 4-phosphohydroxy-L-threonine (**8**) was initially incubated with NAD^+ and PdxA under the conditions described above, resulting in the appearance of a new ^{31}P signal at 4.90 ppm assumed to be due to 3-phosphohydroxy-1-aminoacetone (**9**), along with inorganic phosphate. Addition of 1-deoxyxylulose-5-phosphate (**7**) and PdxJ then resulted in depletion of **9** and concomitant formation of pyridoxol phosphate. Incubation of 1-deoxyxylulose-5-phosphate (**7**) alone with purified PdxJ for 5 h with monitoring by ^{31}P NMR did not lead to the formation of any detectable product. Taken together, the latter results are completely consistent with the postulated role of **9** as the cosubstrate for PdxJ and indicate that pyridoxol phosphate formation is catalyzed by PdxJ, not PdxA.

To determine the steady-state kinetic parameters of the PdxJ-catalyzed reaction, we used a coupled UV assay. Purified PdxA (400 μg, 11.4 nmol) and PdxJ (14 μg, 0.53 nmol) were incubated with 4-phosphohydroxy-L-threonine (400 μM), NAD^+ (500 μM) and lactate dehydrogenase (2.5 units) in 0.1 M Tris·HCl (pH 7.5)

Scheme 3



in a total vol of 1 mL at 37 °C in the presence of varying concentrations of 1-deoxy-D-xylulose-5-phosphate from 8 to 160 μM. Lactate dehydrogenase-catalyzed reduction of pyruvate consumed NADH as it was formed and allowed monitoring of the progress of the reaction by direct detection of pyridoxol phosphate (λ_{max} 324 nm, ϵ 7330 M^{-1} cm^{-1}; 0.1 M Tris, pH 7.5).[12] The observed initial rate data were fitted directly to the Michaelis−Menten equation, and the analysis was carried out in duplicate, giving a K_m(app) for DXP (**7**) of 26.9 $\pm$ 2.3 μM and a k_{cat} of 4.17 $\pm$ 0.12 min^{-1}.

The PdxA-catalyzed oxidation of 4-phosphohydroxythreonine[8] was inhibited by the addition of 1.0 mM EDTA. Addition of 1.0 mM Mn^{2+}, Co^{2+}, Mg^{2+}, or Ca^{2+} fully restored activity, while 1 mM Ni^{2+} or 2 mM Zn^{2+} restored only half of the original PdxA activity.

The formation of pyridoxol phosphate from 1-deoxy-D-xylulose-5-phosphate and **9** catalyzed by PdxJ can be explained by the mechanism illustrated in Scheme 3, which is a variant of mechanisms proposed earlier.[4,8] On the basis of the observations that pyridoxol phosphate and inorganic phosphate are formed in stoichiometric proportions, that the free alcohol deoxyxylulose is not a substrate for PdxJ, and that the enzyme does not release pyridoxol-4′,5′-diphosphate as an initial product, we propose that PdxJ has a phosphomutase activity which transfers the 5-phosphoryl group of dXP (**7**) to the 4-hydroxyl at some point in the reaction. The resulting 4-phospho intermediate **11** would then be able to eliminate phosphate rather than water to generate the enol **12** that is thought to undergo cyclization. The roles of PdxJ and PdxA in the formation of the pyridoxine ring of vitamin B_6 in *E. coli* have now been established. The substrates for PdxA and PdxJ are thus 4-phosphohydroxy-L-threonine (**8**) and 1-deoxy-D-xylulose-5-phosphate (**7**), rather than the corresponding free alcohols **6** and **5**, respectively, neither of which are involved in the biosynthesis of pyridoxol phosphate.[13] We suggest that the gene products PdxA and PdxJ should henceforth be known as 4-phosphohydroxy threonine dehydrogenase and pyridoxol phosphate synthase, respectively.

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(13) After completion of this work, we were informed by Dr. Bernd Laber of Hoechst Schering AgrEvo Gmbh that he and his colleagues had also independently observed the formation of PNP from DXP catalyzed by PdxJ and that their results were in press (Laber, B.; Maurer, W.; Scharf, S.; Stepusin, K.; Schmidt, F. S. *FEBS Lett.* **1999**, *449*, 45−48.)