Communications to the Editor

Biosynthesis of Vitamin B_6: Origin of the Oxygen Atoms of Pyridoxol Phosphate

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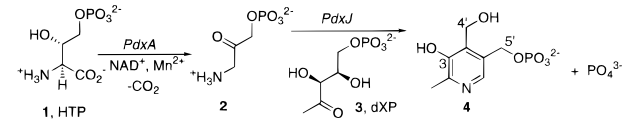
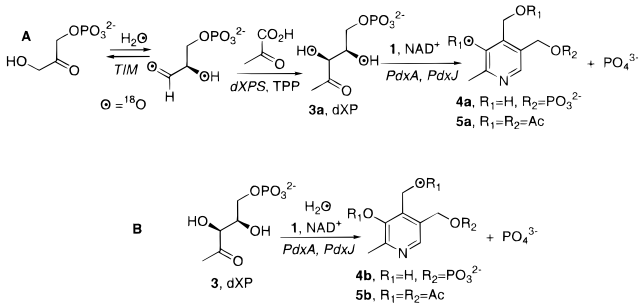
In the biosynthesis of vitamin B_6 in *Escherichia coli*,[1,2] pyridoxol 5′-phosphate (**4**) is derived from two acyclic building blocks, 1-deoxy-D-xylulose 5-phosphate (dXP, **3**) and 4-hydroxy-L-threonine 4-phosphate (HTP, **1**) (Scheme 1). We have shown that the first step in this process involves the oxidative decarboxylation of HTP by the NAD^+-dependent enzyme 4-hydroxythreonine 4-phosphate dehydrogenase (PdxA)[3] to give an unstable intermediate tentatively identified as 3-hydroxy-1-aminoacetone 3-phosphate (**2**).[4] More recently, it was found that pyridoxol phosphate synthase (PdxJ)[5] catalyzes the condensation of **2** with dXP (**3**) to give pyridoxol phosphate and 1 equiv of inorganic phosphate.[6,7] The reaction has a K_m(app) for dXP of 26.9 μM and a k_{cat} of 4.2 min^{-1}. The free alcohol, 1-deoxy-D-xylulose, is not a substrate. Formation of **4** has been postulated to involve initial formation of a Schiff's base between **2** and **3**, followed by a sequence of steps in which the key cyclization reaction is the result of nucleophilic attack of a transiently generated enol on the carbonyl carbon derived from the ketone moiety of **2**. Various mechanistic proposals differ in the precise timing and mechanism suggested for the loss of the phosphate group of dXP.[6,7] The results here reported provide further evidence for the mode of action of PdxA and of PdxJ, and provide insight into the mechanism of formation of pyridoxol 5′-phosphate (**4**), based on the origin of its oxygen atoms.

Our previous experiments had indicated that **2**, the product of the PdxA-catalyzed reaction, serves as the substrate for pyridoxol phosphate synthase (PdxJ).[4,6] To demonstrate unequivocally that PdxA and PdxJ act independently, HTP (**1**) was incubated with PdxA[4] in the presence of NAD^+ at 37 °C for 5 min, using lactate dehydrogenase and pyruvate to recycle NADH. These conditions were sufficient to consume all the starting HTP. Protein was then

Scheme 1



Scheme 2



removed by ultrafiltration (5000 NMWL). When the filtrate was mixed with **3** in the presence of PdxJ,[4] pyridoxol phosphate (**4**) was formed.[6] The yield of **4** was comparable to that in a control incubation in which dXP and HTP were mixed in the presence of both enzymes, PdxA and PdxJ. Furthermore, when the filtrate containing **2** from the PdxA-catalyzed reaction was incubated with PdxJ, which had been preincubated with 43.4 mM EDTA in 23 μL of 0.1 M Tris (pH 7.5), at room temperature for 10−15 min in a total volume of 1 mL, with the formation of **4** monitored at 37 °C by UV at 324 nm,[6,8] no difference was observed in the rate of formation of **4** compared to that in a parallel control in which the PdxJ had been preincubated in the absence of EDTA. These results establish that the pyridoxol phosphate synthase reaction does not require a metal ion, in contrast to the HTP dehydrogenase reaction which requires a divalent cation such as Mn^{2+}.[6] When PdxJ was incubated for 24 h at room temperature with stoichiometric quantities of dXP, the substrate was recovered unchanged, indicating that pyridoxol phosphate synthase does not catalyze a partial reaction of dXP in the absence of the cosubstrate **2**. Treatment of a mixture of dXP and PdxJ with $NaBH_3CN$ or $NaBH_4$ gave unchanged synthase, as indicated by ESMS analysis of recovered protein, thereby ruling out the formation of a Schiff's base intermediate between **3** and the protein, PdxJ.

The mechanism by which the inorganic phosphate is cleaved from dXP in the course of the formation of pyridoxol phosphate was studied by observing the PdxJ-catalyzed conversion of [3,4-$^{18}O_2$]dXP (**3a**) into pyridoxol phosphate. The requisite sample of **3a** was prepared from [1,2-$^{18}O_2$]glyceraldehyde 3-phosphate and pyruvate using recombinant *E. coli* deoxyxylulose 5-phosphate synthase[4,9] in the presence of thiamin diphosphate (TPP) (Scheme 2A). The [1,2-$^{18}O_2$]glyceraldehyde 3-phosphate was in turn generated in situ from dihydroxyacetone phosphate and triose phosphate isomerase (TIM) after preincubation of dihydroxyacetone phosphate in 45 atom % [^{18}O]water at room temperature for 30 min. The presence of ca. 45% ^{18}O label at both C-3 and C-4 of dXP (**3a**) was evident from the ^{13}C NMR

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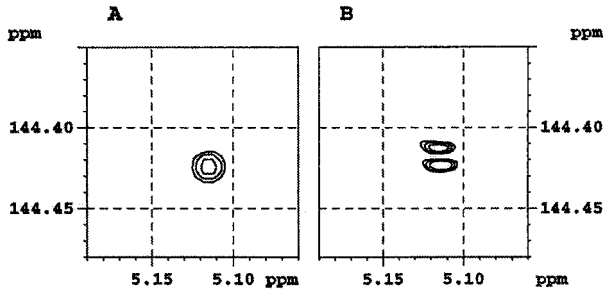


Figure 1. Partial HMBC spectra of (A) unlabeled authentic pyridoxine triacetate and (B) ^{18}O-labeled pyridoxine triacetate (**5a**) derived from [3,4-$^{18}O_2$]dXP (**3a**). The spectra were acquired on a Bruker AM 400 spectrometer at 100.61 MHz (^{13}C) and 400.13 MHz (^{1}H).

spectrum, which showed the expected ^{18}O-induced upfield shifts[10] of 0.019 and 0.021 ppm for the C-3 and C-4 ^{13}C NMR signals, respectively.

HTP (**1**) and [3,4-$^{18}O_2$]dXP (**3a**) were incubated with purified recombinant PdxA and PdxJ[4] in the presence of NAD^+ in 0.1 M Tris/HCl, pH 7.5, with 30% D_2O (Scheme 2A). The formation of inorganic phosphate was monitored by ^{31}P NMR during the course of the reaction. Only unlabeled P_i was observed, with no evidence for formation of any [^{18}O]P_i as indicated by the absence of isotopically shifted ^{31}P NMR signals.[11] This observation ruled out our earlier suggestion[6] that phosphate is eliminated following migration of the phosphoryl moiety from the C-5 to the C-4 hydroxyl of dXP or in any derived intermediate. To determine the actual distribution of ^{18}O label in the resulting pyridoxol 5′-phosphate, the purified product was treated with acid phosphatase and was then acetylated by reaction with acetic anhydride in pyridine. Both the chemical ionization (CI) and electrospray ionization (ESI) mass spectra of the resulting pyridoxine triacetate (**5a**) established the presence of a single ^{18}O atom. In the ^{13}C NMR spectrum of **5a**, no isotopically shifted peaks were evident for either C-4′ or C-5′. The signal for C-3, which is a quaternary carbon sandwiched between two other quaternary carbon atoms, was too weak to be detected. Instead, the sample of **5a** was analyzed by heteronuclear multiple bond correlation (HMBC) using a 5.0 ppm ^{13}C sweep width centered on the signal for C-3 at 145 ppm and inverse detection via the H-4′ proton signal (Figure 1), clearly revealing a cross-peak between H-4′ and C-3 for the ^{18}O−^{13}C-labeled species, thereby confirming the retention at C-3 of pyridoxol phosphate of the ^{18}O from the [3,4-$^{18}O_2$]dXP precursor.

We next incubated dXP and HTP with HTP dehydrogenase and pyridoxol phosphate synthase in [^{18}O]water (30 atom % ^{18}O) (Scheme 2B). The resulting pyridoxol phosphate (**4b**) was isolated by an AG 1 × 8 anion exchange column, and converted to pyridoxine triacetate (**5b**). The ESI mass spectrum of **5b** showed a pair of molecular ion (MH^+) peaks at *m*/*z* 296.3 and 298.3 with an intensity ratio of 7:3, corresponding to the ^{18}O content of the labeled water and indicating the presence of one water-derived oxygen atom in pyridoxol phosphate **4b**. The ^{13}C NMR spectrum (Figure 2) of the triacetate **5b** confirmed the presence of

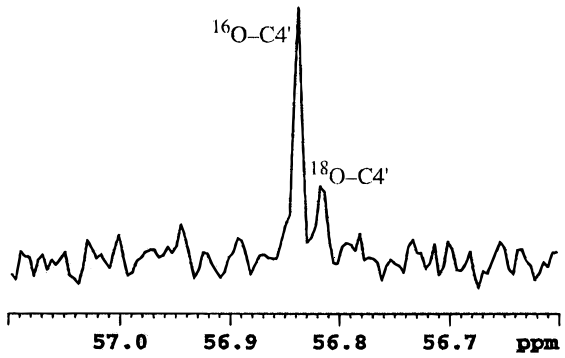
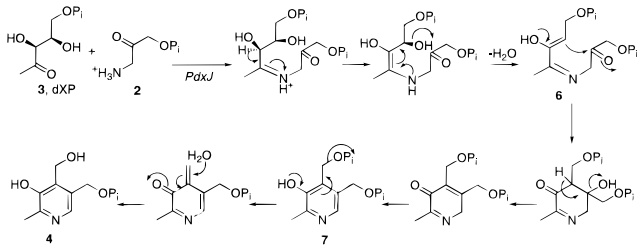


Figure 2. Partial proton-decoupled ^{13}C NMR spectrum of [4′-^{18}O]-pyridoxine triacetate (**5b**) (0.4 Hz/data point).

Scheme 3



30% ^{18}O at the 4′-oxygen atom, based on the observation of a ^{13}C−^{18}O signal shifted 0.022 ppm upfield of the C-4′ signal at 56.838 ppm due to the natural abundance ^{13}C−^{16}O species. The observed ^{13}C NMR, GC-MS, and ESMS data for the enzymatically derived [4′-^{18}O]pyridoxine triacetate **5b** were identical with those of a reference sample of [4′-^{18}O]pyridoxine triacetate that had been prepared by incubation of pyridoxal in [^{18}O]H_2O (30 atom %) at room temperature for 30 min to effect exchange of the carbonyl oxygen atom, followed by room-temperature reduction with $NaBH_4$ at pH 7−8, and treatment of the resulting [4′-^{18}O]pyridoxol with acetic anhydride in pyridine. These results establish that the 4′-OH oxygen atom of pyridoxol 5′-phosphate originates from water and that elimination of the phosphate group derived from C-5 of dXP takes place by C−O, distinct from P−O, bond cleavage.

The mechanism shown in Scheme 3 is consistent with the ^{18}O-labeling results. According to this proposal, the phosphate is cleaved from the hypothetical enzyme-bound intermediate, pyridoxol 4′,5′-diphosphate (**7**), by an elimination−addition sequence, taking advantage of the enol-like hydroxyl at C-3. The results can be rationalized equally well by the mechanism suggested by Laber,[7] in which the phosphate elimination−water addition sequence takes place at a stage (**6**) preceding ring formation. Experiments are in progress to determine the precise timing of the removal of the phosphate group in the pyridoxol phosphate synthase reaction.

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