Biosynthesis of Vitamin B_6: The Oxidation of 4-(Phosphohydroxy)-L-threonine by PdxA

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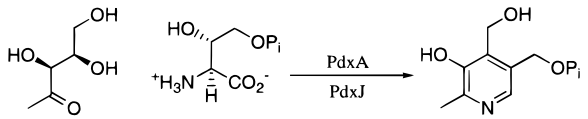
Received December 12, 1997

The two immediate precursors of Vitamin B_6 (pyridoxol phosphate) (**3**) have been identified as 1-deoxy-D-xylulose (**1**) and 4-(phosphohydroxy)-L-threonine (HTP) (**2**).[1] In *Escherichia coli* only two genes (*PdxA* and *PdxJ*) have been implicated in the condensation process between **1** and **2** leading to the formation of pyridoxol phosphate (**3**) (Scheme 1).[2] The exact roles played by each of the corresponding gene products, PdxA and PdxJ, have not previously been determined and neither has the redox cofactor responsible for the required 2-electron oxidation been identified. Since neither PdxA nor PdxJ show any significant similarity to any other protein in the existing DNA and protein databases, no clues are available as to their possible mode of action. In this paper we present results which demonstrate that PdxA is an NAD-dependent dehydrogenase responsible for the oxidation of HTP (**2**), prior to its incorporation into pyridoxol phosphate (**3**).

The *PdxA* and *PdxJ* genes were subcloned from the Kohara phage *E. coli* DNA library.[3] PCR was used to amplify each gene while simultaneously introducing a ribosome binding site and restriction sites before ligation into the pLM1 expression vector.[4] After transformation of the resultant plasmids into *E. coli* BL21-(DE3),[5] the overexpressed target proteins were purified to homogeneity using a three-step procedure involving hydroxyapatite, anion exchange, and gel filtration columns. The purified PdxA and PdxJ proteins were characterized by electrospray mass spectroscopy and N-terminal peptide sequencing. Both proteins behaved as monomers of the predicted M_r upon gel filtration, and no evidence for association was observed when a mixture of the two proteins was analyzed by nondenaturing PAGE.

1-Deoxy-D-xylulose (**1**) was prepared by the method of Backstrom et al.[6] The instability of the free pentulose required that it be freshly prepared for each screening experiment. The procedure described by Wolf et al.[7] was used to prepare [2,3-$^{13}C_2$]-4-hydroxy-L-threonine from [1,2-$^{13}C_2$]acetylene. Unlabeled 4-hydroxy-L-threonine was synthesized by the same method after basic hydrolysis of commercially available (2*S*,3*R*)-3-(benzyloxymethyl)oxirane 2-methanol-*p*-nitrobenzoate (Fluka). The corresponding 4-(phosphohydroxy)-L-threonine (**2**) was readily prepared using recombinant homoserine kinase and ATP or [γ-^{32}P]-ATP, respectively.[8]

Scheme 1. Biosynthesis of Pyridoxol Phosphate (**3**)



(**1**) (**2**) (**3**)

Desalted crude protein extracts of recombinant PdxA and PdxJ were used to test the in vitro activity of the two proteins with [^{32}P]-4-(phosphohydroxy)-L-threonine (**2**) and/or 1-deoxy-D-xylulose (**1**) as substrates in the presence of a range of different redox cofactors and other additives. The incubation mixtures were analyzed by reverse phase TLC, visualizing the radioactivity on the plates using a phosphoimager. In this manner it was found that incubation of PdxA with HTP (**2**) in the presence of NAD led to the formation of new products. Whereas NADP could serve in place of NAD, neither 4-hydroxy-L-threonine nor 1-deoxy-D-xylulose (**1**) was a substrate for the reaction; PdxJ was also not required for the transformation. The retention of the phosphate moiety in the product was evident from the presence of the ^{32}P label. The PdxA oxidation of HTP (**2**) was assayed by measuring the increase in absorbance at 340 nm as NAD is reduced to NADH during the oxidation of substrate. Assays were carried out with purified[9] PdxA and NAD at concentrations of HTP (**2**) ranging from 1 to 200 μM at 37 °C in 0.1 M Tris-HCl, pH 7.5. The rates derived from the above experiment were plotted against substrate concentrations and fitted directly to the Michaelis−Menten equation to give a K_M of 85 μM and V_{max} of 2.8 μmol/(min mg) (k_{cat} 1.66 s^{-1}). Similar kinetic experiments performed in 0.1 M phosphate buffer, pH 7.5, gave a K_M of 113 μM and V_{max} of 0.66 μmol/(min mg) (k_{cat} 0.39 s^{-1}).

To identify the product of the PdxA-catalyzed oxidation of HTP (**2**), a preparative scale reaction was carried out, using lactate dehydrogenase (LDH) reduction of pyruvate to recycle NAD. A typical 1-mL incubation contained 75 mM HTP (**2**), 2.5 mM NAD and 75 mM pyruvate in 0.1 M KH_2PO_4 buffer (pH 7.5). The reaction was initiated by the addition of desalted PdxA (in 100 μL 0.1 M KP_i buffer, 4 μg/μL) and LDH (3 μL, 1 U/μL). After 1 h at 37 °C, an additional batch of PdxA was added, and the incubation was continued until TLC showed that all of the starting material had been consumed. The mixture was acidified to pH 4.0 by the addition of 1 M HCl and freeze-dried. The ^{1}H NMR spectrum of the crude incubation mixture confirmed that all HTP (**2**) had been consumed. Several new signals were observed including two singlets at 8.49 and 8.39 ppm, a doublet at 4.91 ppm (J_{HP} = 8.0 Hz), and a singlet at 2.46 ppm. The pyrazine structure **4** was assigned to the new product on the basis of NMR analysis of PdxA incubations with [2,3-$^{13}C_2$]-L-threonine 4-phosphate, including HMQC and HMBC experiments. In the aromatic region of the ^{13}C NMR spectrum of [$^{13}C_4$]-**4**, three of the four aromatic signals appeared as a doublet of doublet of doublets, while the multiplet at 150.2 ppm had an additional coupling, J_{CP} = 7.5 Hz. (Figure 1). In final confirmation of the structure, unlabeled **4** was converted to the known 5-methylpyrazine-2-methanol (**8**)[10] by treatment with acid phosphatase.

These results suggest that the initial product of the PdxA reaction with **2** is zwitterionic 2-amino-3-oxo-4-(phosphohydroxy)butyric acid (**5**) (Scheme 2), which then undergoes decar-

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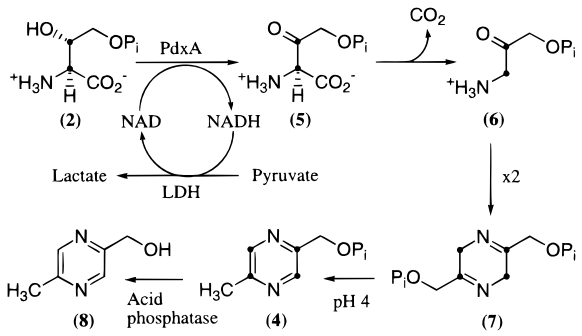
S0002-7863(97)04208-X CCC: $15.00
Published on Web 03/04/1998

Scheme 2. In Vitro Oxidation of 4-(Phosphohydroxy)-L-threonine (**2**) Catalyzed by Recombinant PdxA



boxylation, either concurrently or after release from the enzyme, to give protonated 1-amino-3-(phosphohydroxy)propan-2-one (**6**).[11] On standing, **6** forms a dimer **7** that aromatizes by loss of

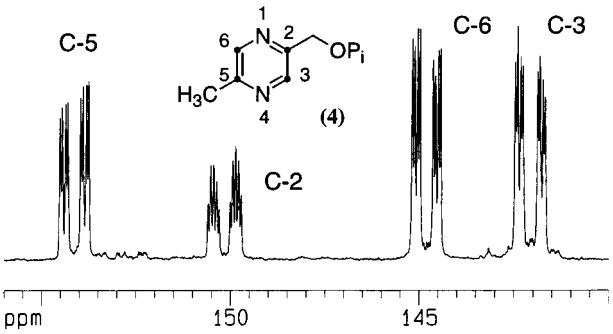
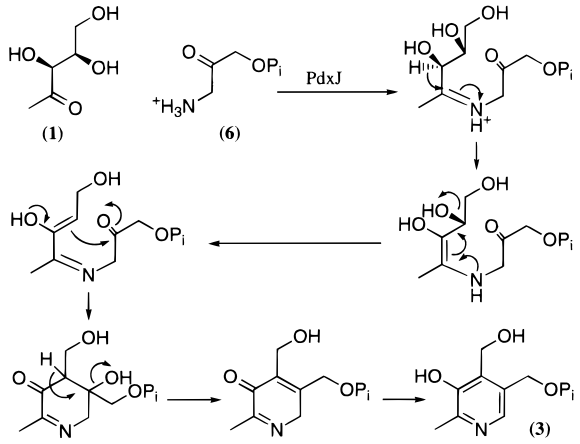


Figure 1. The enriched signals in the ^{13}C NMR spectrum from incubation of PdxA with [2,3-$^{13}C_2$]-4-(phosphohydroxy)-L-threonine (**2**).

P_i upon acidification. It has previously been reported that synthetic 1-amino-3-hydroxypropan-2-one dimerizes in aqueous solution.[12]

On the basis of the above results, we can now propose a possible mechanism (Scheme 3) for the last step in the biosynthesis of pyridoxol phosphate (**3**) involving a ring closure reaction between 1-deoxy-D-xylulose (**1**) and the amino ketone **6**, formed by the oxidation and decarboxylation of 4-(phosphohydroxy)-L-threonine (**2**) catalyzed by PdxA. This final condensation and ring closure reaction would be catalyzed by PdxJ.

Scheme 3. The Proposed Final Steps in the Biosynthesis of Vitamin B_6



In summary we have shown that PdxA catalyzes the oxidation, and possibly the decarboxylation, of 4-(phosphohydroxy)-L-threonine (**2**) to form protonated 1-amino-propan-2-one 3-phosphate **6**. Dimerization and acid-induced aromatization gives the pyrazine derivative **4**. Future work will be directed toward investigating the nature and timing of the decarboxylation of **5**, the reaction of intermediate **6** with 1-deoxy-D-xylulose (**1**), and the role played by PdxJ.

Acknowledgment. This work was supported by a grant from the NIH, GM22172. We thank Dr. Bernd Laber at Schering AG (Berlin, Germany) for a gift of the homoserine kinase overproducing strain (JM103/pHK1), Dr. Edmund Graziani for assistance with 2D NMR spectra, Dr. James Van Epp for mass spectral analysis, and Gregory Schmidt for synthesis of additional substrate.

Supporting Information Available: Kinetic data on PdxA and NMR data for **4** and **8** (7 pages). See any current masthead page for ordering and Web access instructions.

JA9742085

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