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Biosynthesis of vitamin B_6: direct identification of the product of the PdxA-catalyzed oxidation of 4-hydroxy-L-threonine-4-phosphate using electrospray ionization mass spectrometry

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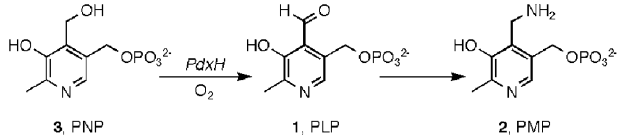
Abstract—PdxA (E.C. 1.1.1.262) catalyzes a key step in the biosynthesis of vitamin B_6: the nicotinamide-dependent oxidation of 4-hydroxy-L-threonine-4-phosphate (HTP) to a product tentatively identified as 3-amino-1-hydroxyacetone 1-phosphate (AHAP). To date, the evidence for the formation of AHAP, while self-consistent, has been largely circumstantial, and does not exclude the possibility that the actual product of the enzyme-catalyzed oxidation of HTP might be 2-amino-3-oxo-4-hydroxybutyric acid 4-phosphate which could undergo rapid, non-enzyme-catalyzed decarboxylation once released from the protein. Use of negative ion electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometric analysis (MS-MS) confirms that AHAP is the product of the PdxA-catalyzed reaction.

Pyridoxal 5′-phosphate (**1**, PLP, vitamin B_6) is an essential cofactor for many of the enzymes that mediate the central biochemical transformations of amino acid metabolism.[1a] Pyridoxamine 5′-phosphate (**2**, PMP), a congener of PLP, is also a critical cofactor in deoxysugar biosynthesis.[1b] PMP is generated from PLP by transamination, while the latter is derived from pyridoxol 5′-phosphate (**3**, PNP) by an O_2-dependent oxidation catalyzed by the flavoenzyme known as PdxH (Scheme 1).[2]

The de novo biosynthesis of PLP in *Escherichia coli* and many other eubacteria involves six committed steps from erythrose-4-phosphate.[3–7] Four of the enzymes, erythrose-4-phosphate dehydrogenase (Epd, GapB), erythronate-4-phosphate dehydrogenase (PdxB), 4-hydroxythreonine-4-phosphate dehydrogenase (PdxA), and pyridoxine-5′-phosphate synthase (PdxJ) are unique to this pathway.[4] While none of these enzymes are present in mammals, they are essential for the eubacteria that employ this pathway. Thus, each of the four proteins represents an attractive target for the design of new antibiotics.



3, PNP 1, PLP 2, PMP

Scheme 1. Biosynthesis of the catalytically active vitamers of B_6.

We have previously reported that PdxA (E.C. 1.1.1.262) catalyzes the nicotinamide-dependent oxidation of 4-hydroxy-L-threonine-4-phosphate (**4**, HTP) to a product tentatively identified as 3-amino-1-hydroxyacetone 1-phosphate (**5**, AHAP) (Scheme 2).[7] The enzyme carries a tightly bound Zn^{2+} that can be replaced by other divalent cations such as Mg^{2+}.[7,8] Although AHAP was not directly observed, our results suggested that formation of AHAP involves an oxidative decarboxylation in which 2-amino-3-oxo-4-hydroxybutyric acid 4-phosphate (**10**, APBA) may be formed as an intermediate. In the presence of PdxJ and deoxyxylulose-5-phosphate (**6**, dXP), the AHAP (**5**) is converted to PNP (**3**) and inorganic phosphate. In the absence of PdxJ, however, the reactive amino ketone **5** undergoes rapid dimerization through formation of the Schiff's base, loss of phosphate, and aromatization to yield the pyrazine **7**.[7] Recent crystallographic studies have established the structure of PdxA with bound HTP (**4**).[8] Comparison with known structures in the Protein Database revealed significant structural homologies to both isocitrate

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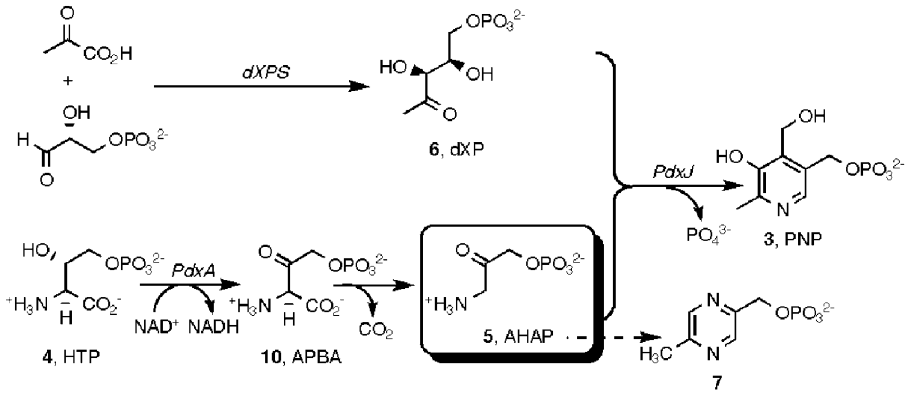


Scheme 2. Final steps in the biosynthesis of pyridoxol 5′-phosphate.

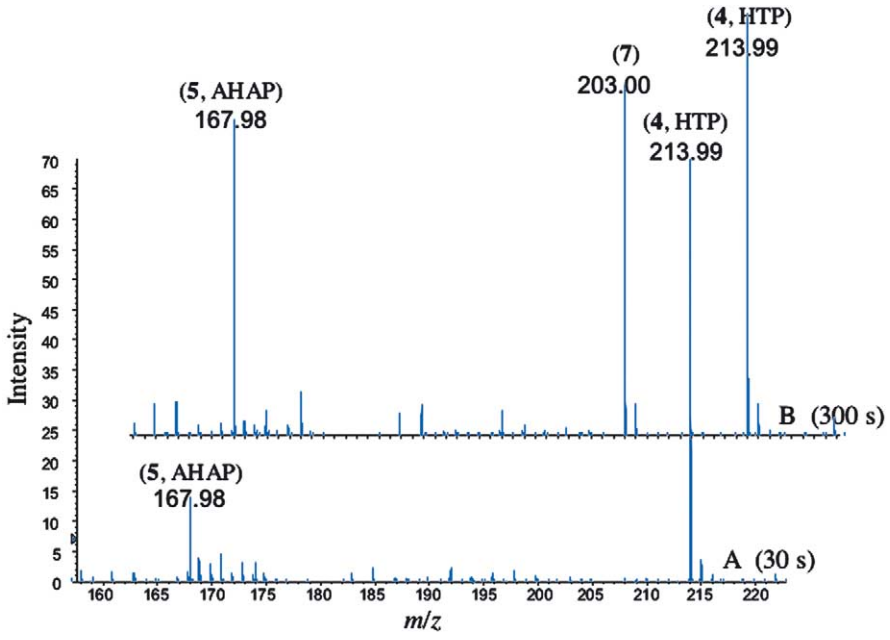


Figure 1. Partial time course of negative ion ESI-QTOF mass spectra showing the conversion of HTP (**4**) to AHAP (**5**) by PdxA and the subsequent formation of the pyrazine dimer **7**. A. 30 s incubation. B. 5 min incubation.

dehydrogenase and isopropylmalate dehydrogenase, each of which is known to catalyze a nicotinamide- and Mg^{2+}-dependent oxidative decarboxylation of a 3-hydroxy carboxylic acid substrate.[8,9]

To date, the evidence for the formation of AHAP (**5**) as the product of the PdxA reaction, while self-consistent, has been largely circumstantial, and does not exclude the possibility that the actual product of the enzyme-catalyzed oxidation of HTP might be APBA (**10**) which could undergo rapid, non-enzyme-catalyzed decarboxylation once released from the protein, analogous to the formation and release of 2-amino-3-ketobutyrate by L-threonine dehydrogenase.[10] We now report the use of negative ion electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometric analysis (MS-MS) to confirm that AHAP (**5**) is the product of the PdxA-catalyzed reaction.

The *E. coli* protein PdxA was overexpressed and purified as previously described.[7] The substrate, 4-hydroxy-L-threonine-4-phosphate (**4**, HTP), was prepared by the previously described chemoenzymatic procedure.[7]

In order to identify the product of the PdxA-catalyzed reaction a series of incubations were directly analyzed by negative ion ESI-MS of the entire reaction mixture after removal of protein. A typical 1-mL incubation contained 4.8 mM HTP (**4**) and 9.3 mM NAD^+ in 0.1 M ammonium acetate buffer (pH 7.5). The reaction was initiated by the addition of 300 μg (85.4 nmol) of desalted PdxA and the mixture was incubated at 37 °C. Incubations were conducted for times ranging from 30 s to 0.5 h and the reactions were quenched by addition of 65 μL of 1 M HCl, conditions designed to retard dimerization of the reactive amino ketone. The protein was removed by ultrafiltration (5000 NMWL) and the resulting mixtures were immediately analyzed by ESI-MS. Two distinct peaks were observed after 30 s (Fig. 1A). In addition to the peak for unreacted HTP (**4**) (*m*/*z* 213.99), the second peak displayed the MW of the expected amino ketone product AHAP (**5**) (*m*/*z*

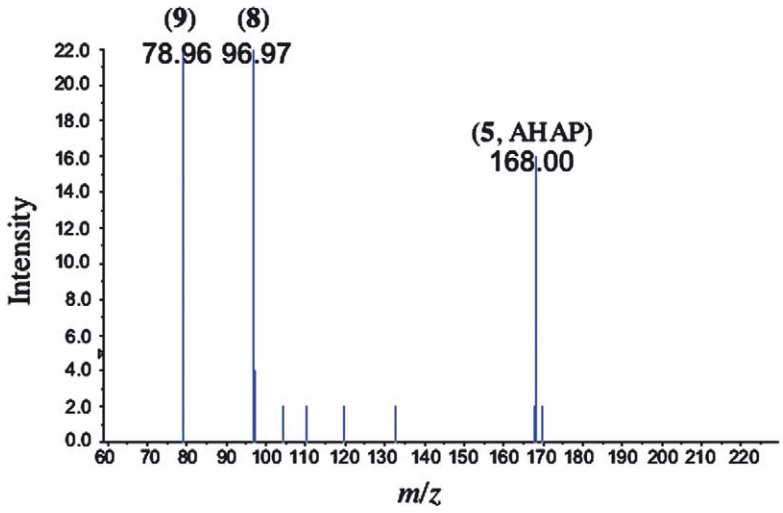
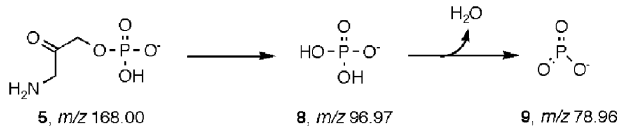


Figure 2. Negative ion ESI-QTOF MS-MS showing the fragmentation pattern of AHAP (**5**).



Scheme 3. MS-MS fragment ions from AHAP.

167.98).[11] After 5 min of incubation, the proportion of AHAP (*m*/*z* 167.98) increased, accompanied by a third peak (*m*/*z* 203.00) corresponding to the previously reported pyrazine derivative **7** (Fig. 1B).[7,11] In the spectra obtained after 0.5 h, the only detectable peak was that for pyrazine **7** (*m*/*z* 203.00) (data not shown). The structure of the putative AHAP ion (*m*/*z* 167.98) was confirmed by coupled MS-MS analysis (Fig. 2), which revealed a parent for AHAP (**5**) (*m*/*z* 168.00) as well as two fragment ions corresponding to inorganic phosphate, P_i (**8**) (*m*/*z* 96.97), and its dehydration product, metaphosphate (**9**) (*m*/*z* 78.96) (Scheme 3).[11]

The same results were obtained when the incubations were quenched under neutral conditions with either EDTA (8.9 mM, pH 7.8) or by protein ultrafiltration alone, except that dimerization of the AHAP product to pyrazine **7** was more rapid. Most significantly, none of the putative intermediate oxidation product, 2-amino-3-oxo-4-hydroxybutyric acid 4-phosphate (**10**, APBA), was detected in the ESI-mass spectra of any of the incubation mixtures (Fig. 1), even under the neutral quench conditions. The closely related 2-amino-3-ketobutyrate, the product of the oxidation of threonine by threonine dehydrogenase, shows increasing stability at higher pH values, with reported half-lives of 8.57 min and 15.15 min at pH 5.9 and 8.1, respectively.[10] Using a conservative value of 10 min for the half-life of the keto-acid **10** at pH 7.8, were this compound to have been formed initially, it would have been easily detected using the neutral quench since less than 4% would have undergone decarboxylation to AHAP after 30 s of incubation, and no more than 30% would have decomposed after 5 min. We therefore can conclude that AHAP (**5**) is the primary product of the PdxA-catalyzed oxidation of 4-hydroxythreonine phosphate (**4**, HTP). Analysis of the early time points demonstrates that AHAP (**5**) is the only product released from the enzyme, and the MS-MS results provide further support for assignment of the structure of AHAP (**5**). At later time points, the reactive amino ketone **5** is seen to undergo dimerization to the previously reported pyrazine derivative **7**.

In summary, we have described the first direct evidence for the formation of AHAP (**5**) in the PdxA-catalyzed oxidation of HTP (**4**). We have shown that consumption of HTP is coupled to the formation of a phosphate-containing compound with the M-1 mass of the proposed reaction product AHAP (**5**) and that the putative intermediate APBA (**10**) is not released from the enzyme during catalysis. We have also reconfirmed that formation of AHAP (**5**) leads to the accumulation of the previously reported 2-phosphomethyl-5-methyl pyrazine dimer **7**.[7] Whether enzyme-catalyzed oxidation and decarboxylation take place in a concerted or sequential manner is still not known, although it is interesting to note that the mechanistically and structurally related isocitrate dehydrogenase is known to catalyze a stepwise oxidative decarboxylation.[9]

Acknowledgements

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