Communication

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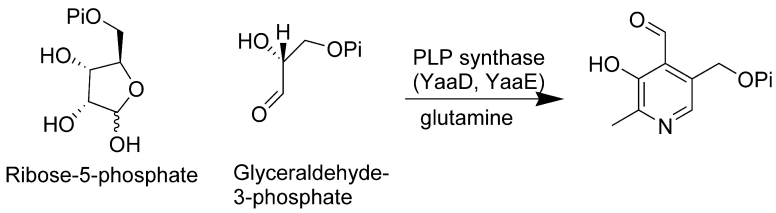


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# Reconstitution and Biochemical Characterization of a New Pyridoxal-5′-Phosphate Biosynthetic Pathway

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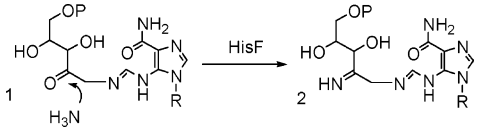
Pyridoxal-5′-phosphate (PLP, vitamin $B_6$) is an essential cofactor in all living systems.[1] It plays an important role in amino acid and carbohydrate metabolism and has recently been implicated in singlet oxygen resistance.[2] The biosynthesis of PLP in *Escherichia coli* has been well studied. The enzymes PdxA and PdxJ catalyze the conversion of 1-deoxy-D-xylulose-5-phosphate and 4-hydroxy-L-threonine to pyridoxine phosphate, which is then oxidized to PLP by PdxH.[3] This pathway, however, is restricted to a relatively small number of bacteria, and most bacteria, archaebacteria, fungi, and plants do not contain PdxA/J/H homologues.[4] In these organisms, the highly conserved SNZ and SNO family of genes has been implicated in PLP biosynthesis.[4] Precursor labeling studies in yeast suggest that carbons 2, 2′, 3, 4, and 4′ of PLP are derived from an unidentified five carbon sugar, that carbons 5, 5′, and 6 are derived from an unidentified triose, and that the pyridine nitrogen is derived from the amide nitrogen of glutamine.[5] In this communication, we identify the substrates for the SNZ and SNO family of proteins in *Bacillus subtilis* (YaaD and YaaE; PLP synthase), describe the first reconstitution of PLP biosynthesis by the major pathway, and identify three new partial reactions catalyzed by the YaaD subunit of PLP synthase.

The active site regions of the YaaE subunit of PLP synthase shows high sequence similarity (41%) to glutamine amidotransferase, an enzyme that catalyzes the hydrolysis of glutamine to glutamate and ammonia. This functional assignment has been confirmed by the recent structure of YaaE[6] as well as by the reconstitution of its biochemical activity.[7] A structural model for the YaaD/YaaE complex has also been proposed in which the ammonia generated at the YaaE active site is channeled to the active site of YaaD where PLP formation occurs.[6] The structural model of YaaD shows a high level of similarity to imidazole glycerol-phosphate synthase (HisF), the enzyme that catalyzes the formation of the imidazole ring of histidine. One of the steps catalyzed by this enzyme involves an amine addition to the C2 carbonyl of a 1-amino-ribulose-5-phosphate analogue (Scheme 1).[8] This similarity suggested that YaaD might also catalyze an amine addition to the carbonyl group of ribulose phosphate. In addition, we have recently found that 1-deoxy-D-xylulose-5-phosphate forms an imine with thiazole synthase and that this imine is cleaved by an intramolecular transimination later in the reaction sequence.[9] Analogous imine formation was a reasonable possibility for the YaaD catalyzed PLP formation.

***Scheme 1***




ESI-FTMS analysis of freshly isolated YaaD demonstrated the presence of unmodified YaaD (34 209.0 Da) as well as an adduct (34 421.6 Da) that was 212 Da heavier than native YaaD (Figure 1a). The mass of this adduct is consistent with that expected for a YaaD/pentulose phosphate imine. Treatment of the mixture with ribose-5-phosphate **3** or with ribulose-5-phosphate **4** converted most of the unmodified YaaD to the adduct as shown in Figure 1b. This suggests that ribulose-5-phosphate is the bound carbohydrate. MS−MS analysis of the YaaD adduct[9] localized the imine to lysine 149, which is absolutely conserved in the SNZ family of proteins. The results were confirmed by demonstrating that the K149A mutant did not form an adduct with ribulose-5-phosphate, suggesting that the YaaD/ribulose-5-phosphate imine is an intermediate in the PLP forming reaction.

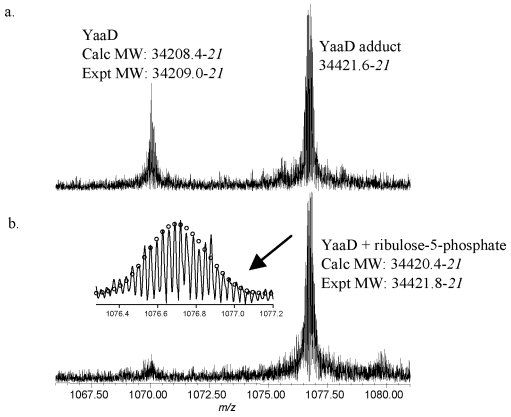



***Figure 1.*** ESI-FTMS of freshly purified YaaD (charge state +32). (a) YaaD as isolated. (b) Ribulose-5-phosphate treated YaaD.

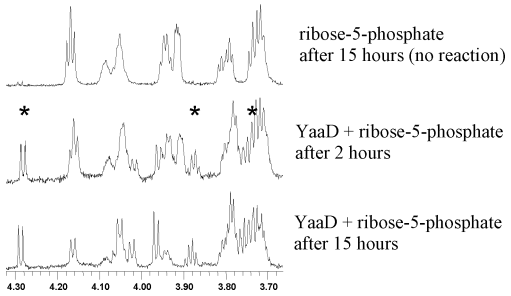



***Figure 2.*** $^1$H NMR of the YaaD catalyzed isomerization of ribose-5-phosphate to ribulose-5-phosphate **4**. * indicates signals from ribulose-5-phosphate. The triplet at 4.16 (H2 of **3**) collapses to a doublet due to deuterium exchange at C1.

The observation that the YaaD adduct could be reconstituted with equal ease from both ribulose-5-phosphate **4** and ribose-5-phosphate **3** suggested that YaaD catalyzed the interconversion of these two compounds. This was confirmed by NMR analysis of a reaction mixture containing ribose-5-phosphate and YaaD (Figure 2). Signals with chemical shifts at 3.75 (m, 2H, H5), 3.88 (m, 1H, H4), and 4.29 (d, $J$ = 5.8 Hz, 1H, H3) confirm the formation of ribulose-5-phosphate. It has been previously demonstrated that a ribose-5-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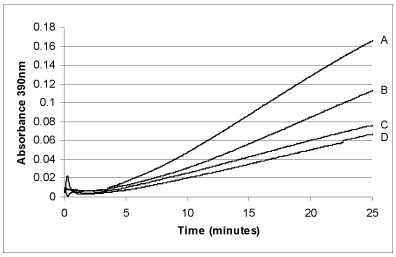



***Figure 3.*** Rate of formation of PLP under various conditions. Ribose-5-phosphate and glyceraldehyde-3-phosphate (A), ribulose-5-phosphate and glyceraldehyde-3-phosphate (B), ribose-5-phosphate and dihydroxyacetone phosphate (C), ribulose-5-phosphate and dihydroxyacetone phosphate (D).

phosphate isomerase mutant in yeast requires PLP for growth,[10] suggesting that the yeast ortholog of YaaD (SNZ) may not have this activity.

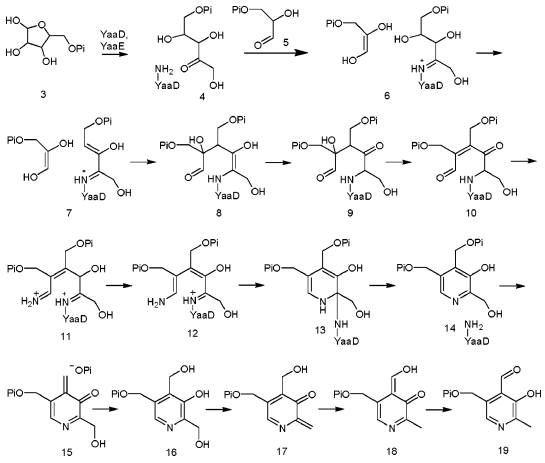
While previous labeling studies did not identify the triose precursor to carbons 5, 5′, and 6 of PLP,[5] dihydroxyacetone phosphate or glyceraldehyde-3-phosphate **5** were the most likely precursors and the stage was now set for attempting the in vitro reconstitution of the biosynthesis. In the event, when ribulose-5-phosphate, glyceraldehyde-3-phosphate, and glutamine were incubated with PLP synthase, the reaction mixture turned yellow after 30 min at 37 °C and showed a UV−visible spectrum with an absorbance maximum at 390 nm. HPLC analysis demonstrated the formation of two reaction products that comigrated with PLP and pyridoxal (PL) standards. A sample for NMR analysis was generated by treating the reaction mixture with alkaline phosphatase, followed by HPLC purification. Signals with chemical shifts of 2.35 (s, 3H, H2′), 4.86 (d, $J$ = 14.2 Hz, 1H, H5′), 5.10 (d, $J$ = 14.2 Hz, 1H, H5′), 6.41 (s, 1H, H6), and 7.45 (s, 1H, H4′) unambiguously identified the isolated product as pyridoxal.

The rate of formation of PLP, under various conditions, was monitored by measuring the absorbance increase at 390 nm (Figure 3). Both ribulose-5-phosphate and ribose-5-phosphate, as well as glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, are substrates for PLP synthase. The absorption at 390 nm is dependent on the YaaD concentration. The optimal pH for this reaction is 6−6.5. The initial lag of about 3 min cannot be explained currently; however, it is important to note that it is observed under all conditions tested so substrate isomerization does not play a role in the lag.

The observation that dihydroxyacetone phosphate and glyceraldehyde-3-phosphate both were substrates suggested that PLP synthase also has triose phosphate isomerase activity. To test this, we incubated dihydroxyacetone phosphate with YaaD in $D_2O$. NMR analysis of this sample indicated that YaaD catalyzed complete H−D exchange at C-1 of dihydroxyacetone phosphate, and no glyceraldehyde-3-phosphate was detected. The absence of glyceraldehyde-3-phosphate from the reaction mixture is not unexpected as the equilibrium constant lies in favor of dihydroxyacetone phosphate.[11] In addition, triose phosphate isomerase activity was confirmed when we detected the formation of dihydroxyacetone phosphate from YaaD and glyceraldehyde-3-phosphate by NMR.

We have demonstrated the first successful reconstitution of PLP biosynthesis from glutamine, ribose-5-phosphate, and glyceraldehyde-3-phosphate. The identification of three partial reactions, pentose isomerization, triose isomerization, and imine formation, as well as the previous observation that dephospho-**16** is a PLP precursor[12] allows us to propose a mechanism for PLP formation as outlined in Scheme 2. In this proposal, imine formation between ribulose-5-phosphate **4** and lysine 149 followed by enolization of glyceraldehyde-3-phosphate gives **6**. Loss of water followed by a

***Scheme 2***




conjugate addition gives **8**. Tautomerization to **9** followed by loss of water and imine formation using ammonia, generated at the glutaminase active site of YaaE, gives **11**. Tautomerization followed by a transimination gives **14**. Substitution of the C4′ phosphate, loss of water from C2′, and two tautomerization reactions complete the biosynthesis.

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**Supporting Information Available:** Cloning and expression of YaaD and YaaE, isomerization of ribose-5-phosphate, identification of PL by NMR, rate of PLP formation, isomerization of dihydroxyacetone phosphate. This material is available free of charge via the Internet at http://pubs.acs.org.

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