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Preliminary Communication

Biosynthesis of Vitamin B_6 in *Rhizobium*: *In Vitro* Synthesis of Pyridoxine from 1-Deoxy-D-xylulose and 4-Hydroxy-L-threonine

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Pyridoxine (vitamin B_6) in *Rhizobium* is synthesized from 1-deoxy-D-xylulose and 4-hydroxy-L-threonine. To define the pathway enzymatically, we established an enzyme reaction system with a crude enzyme solution of *R. meliloti* IFO14782. The enzyme reaction system required NAD^+, $NADP^+$, and ATP as coenzymes, and differed from the *E. coli* enzyme reaction system comprising PdxA and PdxJ proteins, which requires only NAD^+ for formation of pyridoxine 5′-phosphate from 1-deoxy-D-xylulose 5-phosphate and 4-(phosphohydroxy)-L-threonine.

Key words: vitamin B_6 *in vitro* synthesis; *Rhizobium*; 1-deoxy-D-xylulose; 4-hydroxy-L-threonine; pyridoxine

The biosynthetic study of vitamin B_6 has been done intensively in *Escherichia coli* from the '70s. In the past fifteen years, rapid progress was made both through the genetic investigation of Winkler *et al.*[1] and through the tracer investigations of Spenser *et al.*[2–4] It was found by the tracer experiments that pyridoxine (PN) was synthesized from two compounds, 1-deoxy-D-xylulose (1-DX) and 4-hydroxy-L-threonine (4-HT). The mechanism of PN formation from 1-DX and 4-HT has been studied as the most interesting pathway in the biosynthesis of vitamin B_6, which is assumed to be advanced by a hypothetical seven-step chemical sequence.[3] Recently, researchers of two groups reported that pyridoxine 5′-phosphate (PNP) was synthesized *in vitro* from 1-deoxy-D-xylulose 5-phosphate (1-DXP) and 4-(phosphohydroxy)-L-threonine (4-PHT) as substrates in the presence of only one coenzyme, NAD^+, by two purified recombinant proteins, PdxA (4-PHT dehydrogenase)[5] and PdxJ (PNP synthase)[6,7] of *E. coli*. We previously reported that *R. meliloti* IFO 14782 was a PN overproducer[8] and that PN (**3**) was formed by ring closure of two precursors, 1-DX (**1**) and 4-HT (**2**), which entered the C_5 (C-2′, 2, 3, 4 and 4′) and NC_3 (N-1, C-6, 5 and 5′) units, respectively, of the PN molecule (Fig. 1).[9] To identify the enzymes responsible for the pathway leading to PN from 1-DX and 4-HT in *Rhizobium*, we established an *in vitro* system of PN synthesis from the two precursors. Here, we present the first evidence that a crude enzyme solution prepared from a cell-free extract of *R. meliloti* IFO 14782 catalyzed synthesis of PN (**3**) from 1-DX (**1**) and 4-HT (**2**) in the presence of three coenzymes, NAD^+, $NADP^+$, and ATP, and that the coenzymes required were different from those of the *E. coli* enzyme reaction system containing PdxA and PdxJ, which requires only NAD^+ in PNP formation from 1-DXP and 4-PHT.[6,7]

First, *in vitro* synthesis of vitamin B_6 from 1-DX and 4-HT was examined in the presence of a range of different coenzymes using a crude enzyme solution prepared from cells in a buffer without a stabilizer,

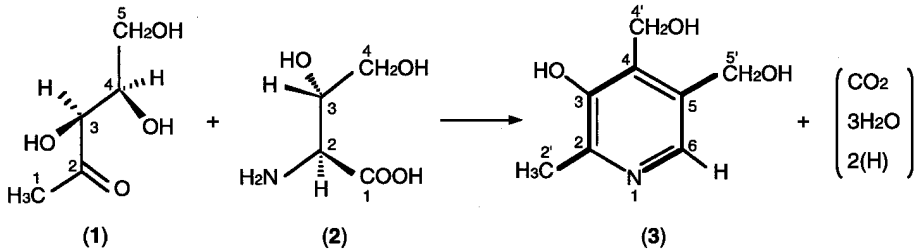

Fig. 1. Minimum Reaction Scheme for the Synthesis of Pyridoxine (**3**) from 1-Deoxy-D-xylulose (**1**) and 4-Hydroxy-L-threonine (**2**) in *Rhizobium*.

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Abbreviations: PN, pyridoxine; 1-DX, 1-deoxy-D-xylulose; 4-HT, 4-hydroxy-L-threonine; PNP, pyridoxine 5′-phosphate; 1-DXP, 1-deoxy-D-xylulose 5-phosphate; 4-PHT, 4-(phosphohydroxy)-L-threonine

however, synthesis of vitamin B_6 did not occur at all. Therefore, we tried adding a stabilizer such as glycerol or sucrose to the buffer for cell disruption. *R. meliloti* IFO 14782 was cultured at 28°C in a production medium described in a previous paper,[8] then 3-d cells were harvested and washed twice with 0.85% (w/v) NaCl solution. The resulting cells were suspended in 10 mM Tris-HCl, pH 7.5, with glycerol [0, 5, 10, or 20% (w/v)] or sucrose [3.75, 7.5, or 15% (w/v)] (final $A_{600}=250$) and passed through a French pressure cell. The cell debris was removed by centrifugation, and then the supernatant was fractionated with 80% (w/v) ammonium sulfate. The precipitate was resuspended in 10 mM Tris-HCl, pH 7.5, desalted with the same buffer, and used as an enzyme source of the following reaction mixture. The reaction mixture contained 1-DX and 4-HT as substrates, NAD^+, $NADP^+$, and ATP as coenzymes, and the crude enzyme solution in Tris-HCl buffer, pH 7.5. After incubation of the reaction mixture at 28°C for 2 h, the reaction was stopped by heating the mixture for 3 min in a boiling water bath. The vitamin B_6 synthesized was measured by the turbidity method with *S. carlsbergensis* ATCC 9080 as described in a previous paper.[8] From the study, apparent synthesis of vitamin B_6 was observed only when the reaction mixture contained crude enzyme solution prepared from cells suspended in 10 mM Tris-HCl, pH 7.5, with 15% sucrose as an enzyme source. Furthermore, in experiments with concentrations of sucrose (zero to 17.5%) in 10 mM Tris-HCl, pH 7.5, for cell disruption, the maximum vitamin B_6 synthesis was observed at the concentration of 15% sucrose, and could not be replaced by addition of 5–20% glycerol to the buffer. Adding a high concentration of sucrose, 15%, to the buffer for cell disruption was effective for the synthesis of vitamin B_6 from 1-DX and 4-HT, and might be for stabilization of enzymes involved in the synthesis, but such a stabilizer was not added to the buffer for cell disruption in isolation of the *E. coli* PdxA and PdxJ proteins.[7]

The requirement of NAD^+, $NADP^+$, and ATP and the optimum concentrations were examined using a crude enzyme solution prepared from cells suspended in 10 mM Tris-HCl, pH 7.5, with 15% sucrose. From the study, three coenzymes, NAD^+, $NADP^+$, and ATP were essential for *in vitro* synthesis of vitamin B_6 from 1-DX and 4-HT, and their optimum concentrations were 0.38 mM for NAD^+ and $NADP^+$ and 5 mM for ATP. The result suggested that at least three enzymes might be involved in formation of vitamin B_6 from 1-DX and 4-HT in *R. meliloti*.

Effects of $MnCl_2$ and $MgCl_2$ on vitamin B_6 formation were tested in 500 μl of reaction mixture containing 80 mM Tris-HCl, pH 7.5, 2.5 mM 1-DX, 2.5 mM 4-HT, 0.38 mM NAD^+, 0.38 mM $NADP^+$, 5 mM ATP, and crude enzyme solution (1.1 mg protein, as a complete enzyme system). As shown in Table 1, addition of 8.4 mM $MnCl_2$ or 32 mM $MgCl_2$ stimulated vitamin B_6 formation. The concentration of vitamin B_6 formation was increased by the addition of both 8.4 mM $MnCl_2$ and 32 mM $MgCl_2$ to a six-fold higher yield than that in the complete enzyme system without the supplement. The vitamin B_6 synthesized was analyzed by thin-layer chromatography (Silica gel 60, Merck, chloroform/methanol = 3/1, v/v) and identified as PN by bioautograms on an agar plate with *S. carlsbergensis* ATCC 9080 (data not shown).

Table 1. Effects of $MnCl_2$ and $MgCl_2$ in the Complete Enzyme System on Synthesis of Vitamin B_6 from 1-Deoxy-D-xylulose and 4-Hydroxy-L-threonine

The complete enzyme system contained 80 mM Tris-HCl, pH 7.5, 2.5 mM 1-DX, 2.5 mM 4-HT, 0.38 mM $NADP^+$, 0.38 mM NAD^+, 5 mM ATP, and crude enzyme solution (1.1 mg protein). Incubation was done without shaking at 28°C for 2 h in a glass tube (13 × 100 mm).

Reaction mixture	Vitamin B_6 (nmol)
Complete enzyme system	0.46
plus 8.4 mM $MnCl_2$	0.56
plus 32 mM $MgCl_2$	0.58
plus 8.4 mM $MnCl_2$ and 32 mM $MgCl_2$	2.77

In *E. coli*, PNP is synthesized from 1-DXP and 4-PHT in the presence of only one coenzyme, NAD^+, by PdxA and PdxJ proteins, as reported by Cane and Laber *et al.*[5–7] However, in *Rhizobium*, three coenzymes, NAD^+, $NADP^+$, and ATP, are essential for *in vitro* synthesis of PN from 1-DX and 4-HT, although substrates and the product were dephosphorylated forms of 1-DXP, 4-PHT, and PNP, respectively. Establishment of the *in vitro* system in *R. meliloti* will be valuable not only for isolation of enzymes involved in formation of PN from 1-DX and 4-HT but also for definition of the PN synthetic pathway from 1-DX and 4-HT in *Rhizobium*.

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