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Purification and Characterization of Pyridoxine 5′-Phosphate Phosphatase from *Sinorhizobium meliloti*

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Here we report the purification and biochemical characterization of a pyridoxine 5′-phosphate phosphatase involved in the biosynthesis of pyridoxine in *Sinorhizobium meliloti*. The phosphatase was localized in the cytoplasm and purified to electrophoretic homogeneity by a combination of EDTA/lysozyme treatment and five chromatography steps. Gel-filtration chromatography with Sephacryl S-200 and SDS/PAGE demonstrated that the protein was a monomer with a molecular size of approximately 29 kDa. The protein required divalent metal ions for pyridoxine 5′-phosphate phosphatase activity, and specifically catalyzed the removal of Pi from pyridoxine and pyridoxal 5′-phosphates at physiological pH (about 7.5). It was inactive on pyridoxamine 5′-phosphate and other physiologically important phosphorylated compounds. The enzyme had the same Michaelis constant (K_m) of 385 μM for pyridoxine and pyridoxal 5′-phosphates, but its specific constant [maximum velocity (V_{max})/K_m] was nearly 2.5 times higher for the former than for the latter.

Key words: phosphatase; pyridoxine 5′-phosphate phosphatase; vitamin B_6; pyridoxine; *Sinorhizobium* (*Rhizobium*) *meliloti*

When pyridoxine (PN), which is also known as vitamin B_6[***], and other compounds in this family are taken up by living organisms, they are converted into pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP). These act as cofactors of vitamin B_6 enzymes that are active in the metabolism of amino acids[1,2] and deoxysugars.[3] Many microorganisms (bacteria, yeasts, fungi, and algae) and plants synthesize their own vitamin B_6. The biosynthetic pathway in *Escherichia coli* is well understood (Fig. 1). It contains two key intermediates, 4-phosphohydroxy-L-threonine and 1-deoxy-D-xylulose 5-phosphate. The former is synthesized from D-erythrose 4-phosphate in three reaction steps catalyzed by Epd, PdxB, and SerC (PdxF) proteins in that order.[4–7] The latter is formed from pyruvate and D-glyceraldehyde 3-phosphate by 1-deoxy-D-xylulose 5-phosphate synthase (Dxs).[8] The two intermediates are then combined by PdxA and PdxJ proteins to generate the first vitamin B_6 compound: pyridoxine 5′-phosphate (PNP).[9] PNP is finally oxidized to the active form, PLP, by a PNP/PMP oxidase (PdxH).[10] PLP and PMP are easily interconverted by ubiquitous transaminases.

Sinorhizobium (formerly known as *Rhizobium*) *meliloti* IFO 14782 produces large quantities of PN.[11] Since two compounds, 1-deoxy-D-xylulose (1-DX) and 4-hydroxy-L-theronine (4-HT), have been found to act as precursors of PN in a study of the biosynthetic pathway of vitamin B_6[12] we examined the biosynthesis of PN from 1-DX and 4-HT *in vitro*,[13] and purified the enzymes involved in its synthesis from the two precursors (M. Tazoe, K. Ichikawa, and T. Hoshino, unpublished results). BLAST searches of the five purified enzymes required for the formation of PN from the two compounds identified two putative xylulose and homoserine kinases, 4-phosphohydroxy-L-threonine dehydrogenase (PdxA), PNP synthase (PdxJ), and a conserved hypothetical protein, based on analysis of the chromosome sequence of *S. meliloti* strain 1021.[14] This suggests that PN is formed in the following sequence of reactions (Fig. 2): Initially, 1-DX and 4-HT are converted to 1-deoxy-D-xylulose 5-phosphate and 4-phosphohydroxy-L-threonine by xylulose kinase[15] and homoserine kinase[16,17] respectively. The latter product is then oxidized by PdxA, followed by condensation with 1-deoxy-D-xylulose 5-phosphate by a PdxJ protein to yield PNP, as it is in *E. coli*. The final step requires a hypothetical protein that dephosphorylates PNP to PN and an enzyme with the required

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Abbreviations: 1-DX, 1-deoxy-D-xylulose; 4-HT, 4-hydroxy-L-threonine; *p*-NPP, *p*-nitrophenylphosphate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PN, pyridoxine; PNP, pyridoxine 5′-phosphate

[***] In this paper, the term vitamin B_6 is used to denote the six compounds, pyridoxine, pyridoxal, pyridoxamine, and their 5′-phosphate esters.

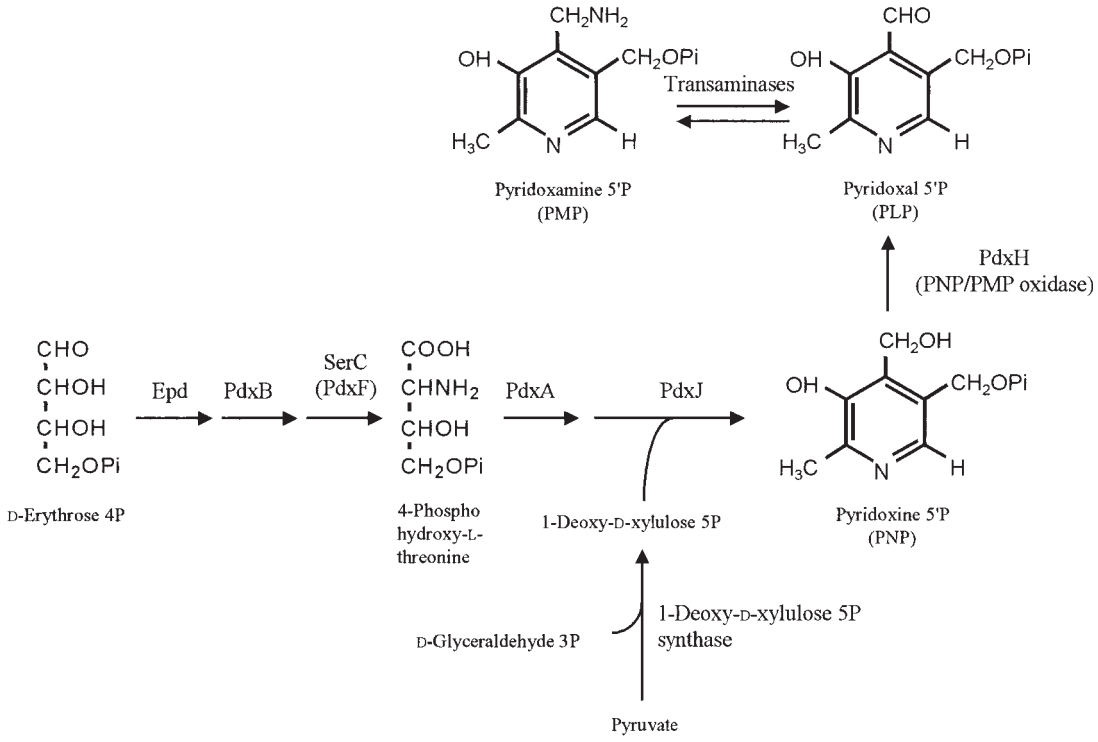

Fig. 1. Currently Proposed Biosynthetic Pathway for Vitamin B_6 in *Escherichia coli*.

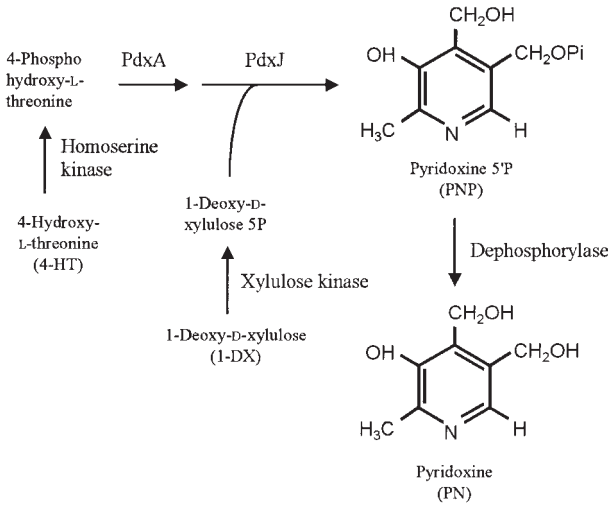

Fig. 2. Proposed Pathway for Pyridoxine from 1-Deoxy-D-xylulose and 4-Hydroxy-L-threonine in *S. meliloti*.

properties, which is the subject of this paper. In vitamin B_6 biosynthesis in microorganisms, an enzyme catalyzing dephosphorylation of PNP has not yet been identified. With a view to understanding the dephosphorylation step in the biosynthesis of PN in *S. meliloti* and our ultimate aim of gene engineering PN production, it is important to characterize the enzyme catalyzing the dephosphorylation of PNP. Here we show that this enzyme is a cytoplasmic PNP/PLP-specific phosphatase that is active as a monomer at physiological pH.

Materials and Methods

Materials. ATP, ADP, AMP, malate, and phospho-L-serine were purchased from Wako Pure Chemical (Osaka, Japan). PN, PLP, PMP, *p*-nitrophenylphosphate (*p*-NPP), and most of the other biochemicals were obtained from Sigma Chemical (St. Louis, MO.). PNP was synthesized by reducing PLP with sodium borohydride according to the method of Stock *et al.*[18] Most of the chromatographic media and prepacked columns were purchased from Amersham Biosciences (Uppsala, Sweden), and Ether Toyopearl was purchased from Toyo Soda Manufacturing (Tokyo). All other chemicals were of reagent grade and were purchased from commercial sources.

Microorganisms, media, and cell growth. S. meliloti IFO 14782 was cultured in a Pi-rich production medium composed of 4% glucose, 2% polypeptone, 0.2% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $MnSO_4 \cdot 5H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$ (pH 6.8) at 28 °C. The Pi-limited chemically defined medium for the induction of periplasmic marker proteins comprised the following: 1% glucose and L-arginine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-threonine, L-valine, L-aspartic acid, L-glutamic acid, L-phenylalanine, and L-tyrosine; pantothenic acid, inositol, nicotinic acid, thiamine, and biotin; and $MnSO_4 \cdot 5H_2O$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, KCl, and KH_2PO_4, which was limited to 14 mg/l (pH 7.0).

Fractionation of cellular proteins. S. meliloti IFO 14782 was grown in the production medium for 3 d. After harvesting by centrifugation, the cells were subcultured in the chemically defined medium and in the production medium for an additional 6 h. Then they were pelleted, washed twice with 0.85% NaCl, and suspended in buffer A [30 mM Tris–HCl (pH 8.0) containing 20% (w/v) sucrose and 1 mM EDTA]. Then lysozyme (0.05%, w/v) was added at 23 °C with slow stirring for 20 min, and the suspensions were separated into periplasmic proteins and cell pellets by centrifugation. The periplasmic fractions were dialyzed against buffer B [10 mM Tris–HCl (pH 7.5) containing 15% (w/v) sucrose, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride], and alkaline phosphatase activity was measured by recording the hydrolysis of *p*-NPP.[19] The activity from the Pi-limited culture was 1.56 μmol/min/mg protein, and that from the Pi-rich culture was about 36-fold lower. When the cytoplasmic marker enzyme malate dehydrogenase was assayed by monitoring the rate of oxidation of NADH (A_{340}),[20] no activity was found in the periplasmic fractions of either culture.

To obtain the cytoplasmic proteins, pelleted cells were resuspended in buffer B and passed through a French pressure cell, and the suspensions were centrifuged at 37,500 × *g* for 1 h. Cytoplasmic extracts of both cultures had substantial malate dehydrogenase activity (0.55 and 0.58 μmol/min/mg protein in Pi-limited and Pi-rich cultures respectively).

Enzyme assays. Dephosphorylase activity was initially assayed with NADP, NAD, ATP, $MnCl_2$, and $MgCl_2$ as cofactors. A mixture containing 0.38 mM NADP, 0.38 mM NAD, 5 mM ATP, 8.4 mM $MnCl_2$, 32 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.0), and enzyme solution was pre-incubated for 1 min at 28 °C. The reaction in a total volume of 125 μl was then started by adding 40 μM PNP, and was incubated at the same temperature for 30 min and then cooled in ice water. Once a cofactor necessary for the enzyme activity and its optimum concentration had been determined, the mixture contained 1 mM $MnCl_2$ as a cofactor. Enzyme activity was calculated by quantifying the PN produced, or the Pi released. First, the amount of PN was measured using the turbidity method with *Saccharomyces carlsbergensis* ATCC 9080, whose growth is supported with PN but not with PNP in vitamin B_6 assay medium, as described previously.[11] One unit of PNP dephosphorylase (or phosphatase) activity produced 1 μmol of PN in 30 min. Second, Pi was determined by the malachite-green method of Geladopoulos *et al.*[21] This method was used to determine substrate specificity, the Michaelis constant (K_m), and the maximum velocity (V_{max}).

Purification of PNP dephosphorylase. Unless otherwise stated, all operations were performed at 0–4 °C.

Step 1. Preparation of cell extract containing PNP dephosphorylase. The cell cake (59.5 g) formed by centrifugation of a 3-d culture broth (3.4-liter) in production medium was washed twice with 300 ml of 0.85% NaCl and resuspended in 340 ml of buffer A. The cellular proteins were divided into periplasmic and cytoplasmic fractions using the procedure described above.

Step 2. Q Sepharose column chromatography (I). The lysate of the cytoplasmic fraction was centrifuged, and the supernatant was dialyzed against buffer B and applied to a Q Sepharose column (4.4 × 17 cm). It was developed with 600 ml of 400 mM KCl at a flow rate of 0.57 ml/min.

Step 3. Q Sepharose column rechromatography (II). The active fractions, which were dialyzed against buffer B, were then applied to a Q Sepharose column (4.4 × 12.5 cm). Elution was carried out at a flow rate of 0.72 ml/min with a linear gradient of KCl (0–400 mM) in buffer B (total volume, 2,200 ml).

Step 4. Ether Toyopearl column chromatography. The sample was dialyzed against buffer B and adjusted to 1.3 M $(NH_4)_2SO_4$, and then applied to an Ether Toyopearl column (2.5 × 15 cm) previously equilibrated with buffer B containing 1.3 M $(NH_4)_2SO_4$. Elution was carried out at a flow rate of 0.7 ml/min with a linear gradient of $(NH_4)_2SO_4$ (1.3–0.5 M) in buffer B (total volume, 1,000 ml).

Step 5. Resource ISO column chromatography. The sample was dialyzed against buffer B and applied to a Resource ISO column (6 ml) equilibrated with buffer B containing 1.2 M $(NH_4)_2SO_4$. Elution was carried out at a flow rate of 0.8 ml/min with a linear gradient of $(NH_4)_2SO_4$ (1.2–0.5 M) in buffer B (total volume, 120 ml).

Step 6. Sephacryl S-200 column chromatography. The sample was dialyzed against buffer C [50 mM Tris–HCl (pH 7.5), 15% (w/v) sucrose, and 150 mM KCl]. Finally, the dialysate was concentrated to 300 μl by ultrafiltration. The solution was applied to a Sephacryl S-200 column (1.6 × 60 cm), which was developed with buffer C.

Molecular size estimation and amino-terminal sequence analysis. The molecular size of the native dephosphorylase was calculated using the Sephacryl S-200 column calibrated with molecular mass standards (Bio-Rad, Richmond, CA). SDS/PAGE was carried out according to the method of Laemmli[22] with 12% acrylamide gels. Protein bands on the gels were visualized with Coomassie brilliant blue R-250. The apparent molecular size of the protein was estimated by comparison with molecular mass standards (Amersham Biosciences, Uppsala, Sweden). A purified protein was identified on SDS/PAGE gels by the method of Matsudaira,[23] and the amino-terminal sequence was determined by an amino acid sequence system.

Removing divalent metal ions to test for divalent

Table 1. Localization of PNP Dephosphorylase

Cellular proteins	PNP dephosphorylase (nmol/min/mg protein)	Alkaline phosphatase (μmol/min/mg protein)	Malate dehydrogenase (μmol/min/mg protein)
(Cells from Pi-limited culture)			
Periplasmic proteins	2	1.56	0.01
Cytoplasmic proteins	137	0.07	0.55
(Cells from Pi-rich culture)			
Periplasmic proteins	1	0.04	0.03
Cytoplasmic proteins	138	0.11	0.58

Reaction mixtures contained 40 μM PNP, 0.38 mM NADP, 0.38 mM NAD, 5 mM ATP, 8.4 mM $MnCl_2$, 32 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.0), and periplasmic proteins (3.23 mg) or cytoplasmic proteins (3.19 mg) in 125 μl.

metal requirements. Purified enzyme solution (1 mg/ml) was dialyzed twice against buffer B containing 1 mM EDTA for 15 h, and subsequently twice against buffer B for 15 h to remove the remaining EDTA.

Identification of the reaction product. A reaction mixture (5 ml) consisting of 640 μM PNP, 1 mM $MnCl_2$, 50 mM Tris–HCl (pH 7.5), and enzyme solution was incubated at 28 °C for 1 h and heated for 3 min in boiling water, and then the denaturated proteins were removed by centrifugation. The supernatant was applied to an Amberlite CG-120 column (1.6 × 11 cm; Rohm and Haas Co., Philadelphia, PA), which was washed with water and developed with a 5% (w/v) ammonium hydroxide solution. The eluate was concentrated under reduced pressure, and the residue was dissolved in a small volume of methanol and analyzed by high-pressure liquid chromatography.[11] The product was identified as PN by reference to a pure sample.

Results and Discussion

A protein involved in the last step of PN synthesis in S. meliloti

PN was formed when 1-DX and 4-HT were incubated with extracts of *S. meliloti* IFO 14782 in buffer with 15% (w/v) sucrose, but not when they were incubated with extracts made in the absence of sucrose.[13] PNP was identified by high-pressure liquid chromatography in the latter mixture; when treated with potato acid phosphatase (Boehringer Mannheim, Munich, Germany), it supported the growth of a vitamin B_6 indicator, *S. carlsbergensis* ATCC 9080, which requires phosphate-free vitamin B_6, PN, pyridoxal, or pyridoxamine for growth in vitamin B_6 assay medium (data not shown). These observations suggest that sucrose extracts of *S. meliloti* IFO 14782 generate PN by dephosphorylation of PNP and that the enzyme responsible is inactivated by extraction in the absence of sucrose.

Localization of a PNP dephosphorylase

S. meliloti IFO 14782 accumulates large amounts of extracellular PN, and the result presented above suggests that a dephosphorylase participates in the final step of PN synthesis. To determine the location of this enzyme, extracts of Pi-limited and Pi-rich cultures were divided into periplasmic and cytoplasmic fractions after EDTA/lysozyme treatment (see "Materials and Methods"). Alkaline phosphatase and malate dehydrogenase were assayed as standard periplasmic and cytoplasmic marker proteins respectively. The cytoplasmic fractions of both the Pi-limited and the Pi-rich cultures had high dephosphorylase activity. By contrast, there was no activity in the periplasmic fractions of either culture (Table 1). It appears likely that the PNP dephosphorylase involved in the *de novo* synthesis of PN is cytoplasmic.

Purification of PNP dephosphorylase

Protein samples for purification of PNP dephosphorylase were prepared initially by rupture of whole cells from Pi-rich cultures, but the enzyme was difficult to purify because of the high concentrations of other proteins and its instability. Before the application of chromatographic procedures, periplasmic proteins were removed by treatment with EDTA/lysozyme and centrifugation. PNP dephosphorylase was then purified to apparent homogeneity from the cytoplasm in five chromatographic steps. The purification procedure, starting with the cytoplasmic fraction of *S. meliloti*, is summarized in Table 2. The protein was purified 2,090-fold with a yield of 18% based on the PNP dephosphorylase activity in cytoplasmic extracts. Since it was enriched about two-fold with a yield of about 80% by isolating the cytoplasmic proteins, the overall purification was approximately 4,180-fold with a yield of 14.4%.

Molecular size estimation of PNP dephosphorylase and amino-terminal amino acid sequence

In the final gel-filtration step described in the previous section, PNP dephosphorylase activity eluted as a single peak between chicken ovalbumin (44 kDa) and equine myoglobin (17 kDa) (to the left in Fig. 3). The position of the peak corresponded to a molecular size of 29 kDa, which was similar to that seen on SDS/PAGE gels (to the right in Fig. 3). The protein is thus a monomer with a molecular size of approximately 29 kDa.

The amino-terminal amino acid sequence was determined to be M(amino-terminal)–K–K–L–D–R–M–P–T–H. The NCBI databases were predicted to be a

Table 2. Purification of PNP Dephosphorylase Starting from Cytoplasmic Extracts

Steps	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg protein)	Purification (-fold)	Yield (%)
Cytoplasmic extracts	8,570	4.1	0.00048	1.0	100
Q Sepharose (I)	5,700	3.4	0.0006	1.3	83
Q Sepharose (II)	316	2.5	0.0079	16.5	62
Ether Toyopearl	74	1.6	0.022	45.8	39
Resource ISO	4.2	1.2	0.29	605	28
Sephacryl S-200	0.71	0.74	1.0	2,090	18

PNP dephosphorylase was assayed in mixtures containing (in 125 µl): 40 µM PNP, 0.38 mM NADP, 0.38 mM NAD, 5 mM ATP, 8.4 mM $MnCl_2$, 32 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.0), and desalted fraction in each chromatography.

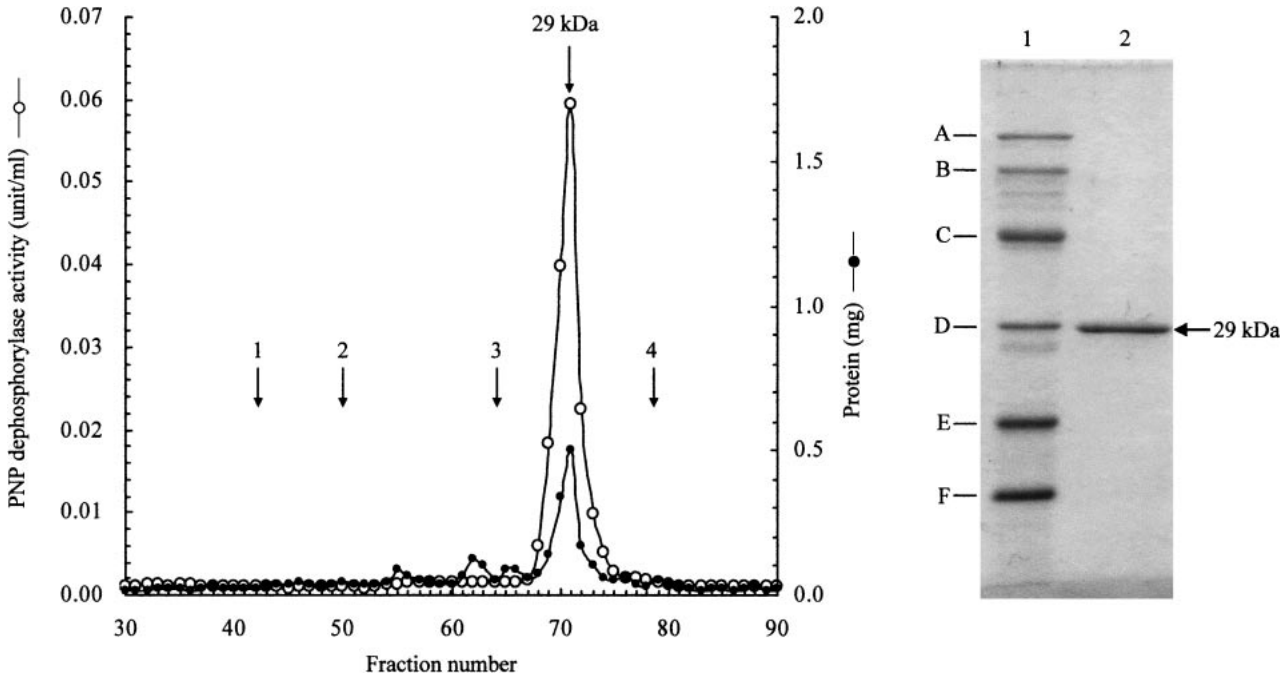

Fig. 3. Molecular Size Estimation of PNP Dephosphorylase by Sephacryl S-200 Chromatography (left) and SDS–PAGE (right).
Left figure: molecular mass standard markers correspond to (1) thyroglobulin (669 kDa), (2) bovine gamma globulin (158 kDa), (3) chicken ovalbumin (44 kDa), and (4) equnine myoglobin (17 kDa). Right figure: lanes correspond to (1) molecular mass markers: A, phosphorylase B (92.5 kDa); B, bovine serum albumin (66.2 kDa); C, ovalbumin (45 kDa); D, carbonic anhydrase (31 kDa); E, soybean trypsin inhibitor (21.5 kDa); and F, α-lactalbumin (14.4 kDa) and (2) 2.7 µg of purified PNP dephosphorylase.

conserved hypothetical protein consisting of 258 amino acids [*Sinorhizobium meliloti* strain 1021] with 100% identity from this sequence.[14] A further search was conducted using the 258 amino acid-sequence. It resulted in predictions of putative hydrolases (many bacteria), phosphoglycolate phosphatase (*Brucella melitenis*), and putative haloacid dehalogenase superfamily hydrolase (many bacteria) with 40–70% identities. These results suggest that our enzyme is yet unspecified.

Characterization of PNP dephosphorylase

PNP dephosphorylase activity did not require NAD, NADP, or ATP, which are essential for PN formation from 1-DX and 4-HT, but it did require either Mn^{2+} or Mg^{2+} (Table 3). Activation by $MnCl_2$ or $MgCl_2$ followed saturation kinetics (Fig. 4). Activity was highest with Mn^{2+} (100%), followed by Mg^{2+} (88%), Co^{2+} (65%), Sn^{2+} (11%), and Ni^{2+} (7%). The enzyme should thus be classified either as a phosphohydrolase or as a phosphotransferase, depending on its reaction mechanism. In the former case, Pi is released as PN is formed. Hence we used the malachite-green method to determine whether Pi is released upon incubation of PNP with a purified protein. Pi was indeed released, indicating that the reaction is hydrolytic. The enzyme is therefore likely to be a phosphatase.

Many phosphatases have divalent metal ions,[24] which play an important role in protein structures to maintain activity.[19] An alkaline phosphatase of *R. leguminosarum*, which incorporates divalent metal ions and forms dimers, releases the metal ions upon EDTA treatment and breaks down to inactive fragments, and its activity is restored completely by adding an appropriate concentration of divalent metal ions. Our PNP phosphatase was

Table 3. Cofactor Requirements of PNP Dephosphorylase

Cofactors present	PNP dephosphorylase activity (unit/ml)
Complete	0.32
minus NAD	0.32
minus NADP	0.31
minus ATP	0.32
minus NAD, NADP, ATP	0.32
minus $MnCl_2$	0.30
minus $MgCl_2$	0.32
minus $MgCl_2$ and $MnCl_2$	0
minus NAD, NADP, ATP and $MnCl_2$	0.32
minus NAD, NADP, ATP and $MgCl_2$	0.33
minus all	0

Mixtures for the complete system contained 40 µM PNP as substrate, 0.38 mM NADP, 0.38 mM NAD, 5 mM ATP, 8.4 mM $MnCl_2$, and 32 mM $MgCl_2$ as cofactors, 50 mM Tris–HCl (pH 7.0), and a purified protein (1.53 µg) in 125 µl.

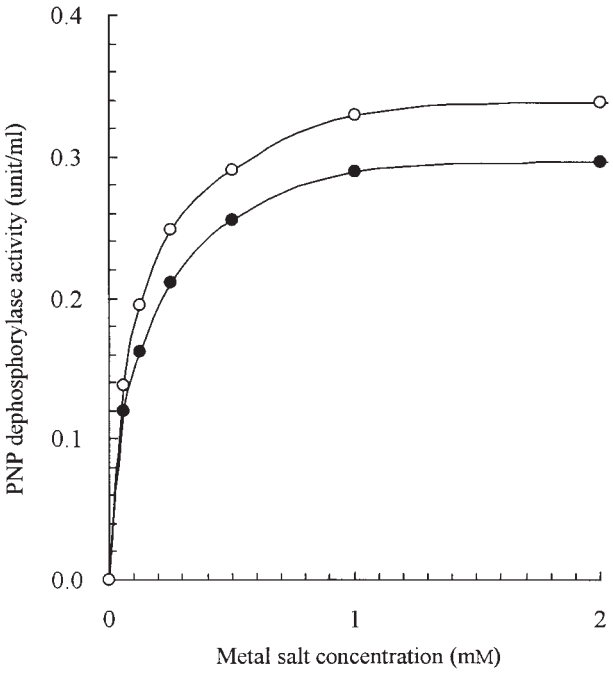

Fig. 4. Dependence of PNP Dephosphorylase Activity on $MnCl_2$ and $MgCl_2$.

Reaction mixtures contained 40 µM PNP, $MnCl_2$, or $MgCl_2$, 50 mM Tris–HCl (pH 7.0), and a purified protein (1.53 µg) in 125 µl. Metal salts were tested at concentrations of 0.625–2 mM $MnCl_2$ (○) and 0.625–2 mM $MgCl_2$ (●).

purified in a buffer without metal ions, and its molecular size was estimated using gel-filtration chromatography in a buffer without metal ions. To determine the actual molecular size of the active enzyme, we performed chromatography on a Sephacryl S-200 column in a buffer containing 1 mM $MnCl_2$. The protein eluted at the same position as that purified in buffer without a divalent metal ion (to the left in Fig. 3). From these results, we infer that this enzyme is likely to act as a monomer. Our PNP/PLP phosphatase differs from the human erythrocyte vitamin B_6 phosphate phosphatase, which is a dimer of 64 kDa.[24)]

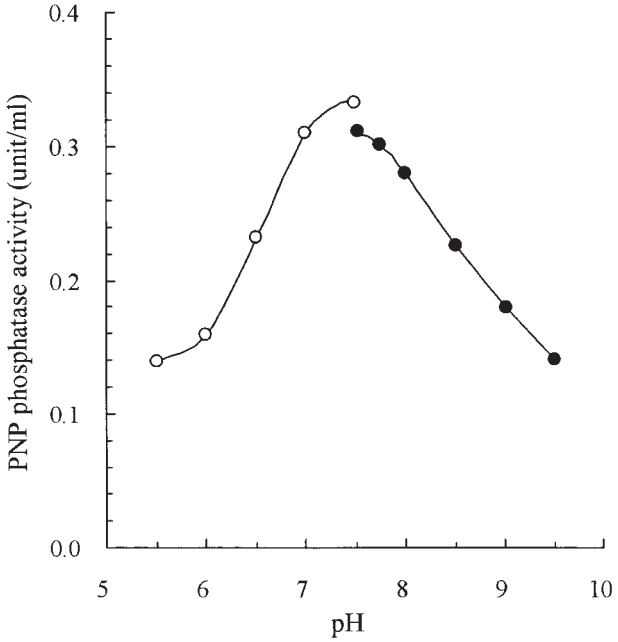

Fig. 5. Effect of pH on PNP Phosphatase Activity.

Activity was measured in 50 mM Tris–maleate buffer (pH 5.5–7.5) (○) and 50 mM Tris–HCl buffer (pH 7.5–9.5) (●) containing 40 µM PNP, 1 mM $MnCl_2$, and a purified protein (1.53 µg) in 125 µl.

Table 4. Kinetic Parameters of PNP Phosphatase with Potential Substrates

Compound	K_m (µM)	V_{max} or activity (µmol/min/mg protein)	V_{max}/K_m (min/mg)
Pyridoxine 5′-phosphate	385	105[a]	2,180
Pyridoxal 5′-phosphate	385	42[a]	870
Pyridoxamine 5′-phosphate		<0.73[b]	
D-Glucose 6-phosphate		<0.73[b]	
D-(−)-3-Phosphoglyceric acid		<0.73[b]	
2-Phosphoglycolic acid		<0.73[b]	
ATP		<0.73[b]	
ADP		<0.73[b]	
AMP		<0.73[b]	
Phospho-L-serine		<0.73[b]	

The reaction was conducted in mixtures containing (in 125 µl) the indicated compounds, 1 mM $MnCl_2$, 50 mM Tris–HCl (pH 7.0), and a purified protein (1.53 µg).

[a] V_{max} values were calculated using the indicated compounds at concentrations of 40, 80, 160, 320, and 640 µM.

[b] Activity with 640 µM of the indicated compounds.

Properties of PNP phosphatase

Next we examined the pH dependence of PNP phosphatase activity. The optimal pH was about 7.5 (Fig. 5). The enzyme catalyzed the removal of Pi from PNP and PLP, but not from PMP or other physiologically important phosphorylated compounds, such as phosphosugars, nucleotides, and a phosphoamino acid (Table 4). Phosphoglycolic acid, a substrate for phosphoglycolate phosphatase predicted in a NCBI database search, was not a substrate for our phosphatase. This substrate specificity indicates that it is a PNP/PLP

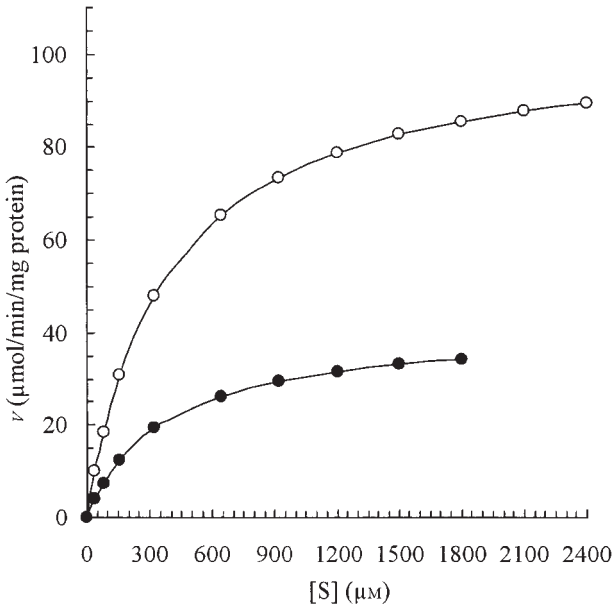

Fig. 6. Kinetics of Dephosphorylation of Pyridoxine 5′-Phosphate and Pyridoxal 5′-Phosphate.

The reaction mixtures contained substrate, 1 mM $MnCl_2$, 50 mM Tris–HCl (pH 7.0), and a purified protein (1.53 μg) in 125 μl. Substrate concentrations were 40–2,400 μM pyridoxine 5′-phosphate (○) and 40–1,800 μM pyridoxal 5′-phosphate (●).

phosphatase. The velocities with PNP and PLP followed Michaelis–Menten kinetics (Fig. 6). The K_m and V_{max} (μM, μmol/min/mg protein) values for PNP and PLP were 385 and 105, and 385 and 42, respectively (Table 4). Thus it had the same K_m for PNP and PLP, but had a V_{max} that was nearly 2.5-times higher for PNP than for PLP; therefore the specific constant (V_{max}/K_m) for PNP was higher than that for PLP. The human erythrocyte vitamin B_6 phosphate-specific phosphatase shows substrate specificity similar to our enzyme, but it has a lower K_m value for PLP than for PNP.[24)]

To differentiate our phosphatase from human erythrocyte phosphatase, the effects of inhibitors tested by Fonda[24)] on our enzyme were examined in the presence of 1 mM $MnCl_2$, which gave a maximum activity for PNP phosphatase (Fig. 4). EDTA was an effective inhibitor ($I_{50} = 16$ μM). From these results, we infer that the Mn ion is not tightly bound to this enzyme. Sodium molybdate was a poor inhibitor. Potassium phosphate, *N*-ethylmaleimide, idoacetate, sodium fluoride, and levamisole were even less inhibitory, and L-phenylalanine and L-(+)-tartarate showed no inhibition to concentration of 5 mM (Table 5). These features of inhibition to our enzyme were similar to those of the human phosphatase except for sulfhydryl reagents, *N*-ethylmaleimide, and idoacetate, which are very sensitive inhibitors of the latter phosphatase.[24)]

There has been one previous report of an acidic phosphatase specific for PLP in an anaerobic photosynthetic bacterium, but the enzyme was periplasmic.[25)] An interesting aspect of our present work was the finding that the highly specific PNP/PLP phosphatase of *S. meliloti* is localized in the cytoplasm rather than in the periplasm, where the well-known non-specific alkaline and acid phosphatases are found. This is the first report of a microbial phosphatase with this unique characteristic. There have been many previous studies of the regulation of PLP levels by interactions between PLP phosphatase and PL kinase in mammals, which can not synthesize their own vitamin B_6.[24,26–28)] In *Sinorhizobium*, which possesses a complete vitamin B_6 biosynthetic pathway, PNP is the first vitamin B_6 compound synthesized *de novo* and represents the point of divergence of the pathways leading to PN and PLP. PNP phosphatase might play a major role together with a PNP oxidase in the control of intracellular PLP levels.

Table 5. Effect of Various Compounds on PNP Phosphatase Activity

compounds	Relative activity (%)
None	100
Sodium molybdate	21
K-PO_4	44
N-Ethylmaleimide	54
Idoacetate	48
NaF	76
Levamisole	74
L-Phenylalanine	100
L-(+)-Tartarate	100

Reaction mixtures contained 40 μM PNP, 1 mM $MnCl_2$, 50 mM Tris–HCl (pH 7.0), a purified protein (1.53 μg), and 5 mM of each compound in 125 μl.

In conclusion, we believe the results obtained in this study will provide information that will help develop approaches to genetic engineering of the PNP phosphatase of *S. meliloti* in order to produce PN.

References

1) Bender, D. A., The role of vitamin B_6 in amino acid metabolism. In "Amino Acid Metabolism", John Wiley and Sons, New York, pp. 75–94 (1985).
2) Dakshinamurti, K., "Annals of the New York Academy of Sciences, Vol. 585, Vitamin B_6", New York Academy of Sciences, New York, pp. 128–144 (1990).
3) Burns, K. D., Pieper, P. A., Liu, H.-W., and Stankovich, M. T., Studies of the redox properties of CDP-6-deoxy-L-*threo*-D-glycero-4-hexulose 3-dehydrase (E1) and CDP-6-deoxy-L-*threo*-D-glycero-4-hexulose 3-dehydrase reductase (E3), two important enzymes involved in the biosynthesis of ascarylose. *Biochemistry*, **35**, 7879–7889 (1996).
4) Dempsey, W. B., Synthesis of pyridoxal phosphate. In "*Escherichia coli* and *Salmonella typhimurium*", "Cellular and Molecular Biology", eds. Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., and Umbarger, H. E., American Society for Microbiology, Washington D.C., pp. 539–543 (1987).
5) Dreke, C., Notheis, C., Hansen, U., Leistner, E., Hemscheidt, T., Hill, R. E., and Spenser, I. D., Growth response to 4-hydroxy-L-threonine of *Escherichia coli* mutants blocked in vitamin B_6 biosynthesis. *FEBS Lett.*, **318**, 125–128 (1993).

6) Lam, H.-M., and Winkler, M. E., Metabolic relationships between pyridoxine (vitamin B_6) and serine biosynthesis in *Escherichia coli* K-12. *J. Bacteriol.*, **172**, 6518–6528 (1990).
7) Lam, H.-M., and Winkler, M. E., Characterization of the complex *pdxH-tyrS* operon of *Escherichia coli* K-12 and pleiotropic phenotypes caused by *pdxH* insertion mutants. *J. Bacteriol.*, **174**, 6033–6045 (1992).
8) Hill, R. E., Sayer, B. G., and Spenser, I. D., Biosynthesis of vitamin B_6: incorporation of D-1-deoxyxylulose. *J. Am. Chem. Soc.*, **111**, 1916–1917 (1989).
9) Laber, B., Maurer, W., Scharf, S., Stepusin, K., and Schmidt, F. S., Vitamin B_6 biosynthesis: formation of pyridoxine 5′-phosphate from 4-(phosphohydroxy)-L-threonine and 1-deoxy-D-xylulose 5-phosphate by PdxA and PdxJ protein. *FEBS Lett.*, **449**, 45–48 (1999).
10) Zhao, G., and Winkler, M. E., Kinetic limitation and cellular amount of pyridoxine (pyridoxamine) 5′-phosphate oxidase of *Escherichia coli* K-12. *J. Bacteriol.*, **177**, 883–891 (1995).
11) Tazoe, M., Ichikawa, K., and Hoshino, T., Production of vitamin B_6 in *Rhizobium*. *Biosci. Biotechnol. Biochem.*, **63**, 1378–1382 (1999).
12) Tazoe, M., Ichikawa, K., and Hoshino, T., Biosynthesis of vitamin B_6 in *Rhizobium*. *J. Biol. Chem.*, **275**, 11300–11305 (2000).
13) Tazoe, M., Ichikawa, K., and Hoshino, T., Biosynthesis of vitamin B_6 in *Rhizobium*: *in vitro* synthesis of pyridoxine from 1-deoxy-D-xylulose and 4-hydroxy-L-threonine. *Biosci. Biotechnol. Biochem.*, **66**, 934–936 (2002).
14) Capela, D., Barloy-Hubler, F., Gouzy, J., Bothe, G., Ampe, F., Batut, J., Boistard, P., Becker, A., Boutry, M., Cadieu, E., Dreano, S., Gloux, S., Godrie, T., Goffeau, A., Kahn, D., Kiss, E., Lelaure, V., Masuy, D., Pohl, T., Portetelle, D., Puehler, A., Purnelle, B., Ramsperger, U., Renard, C., Thebault, P., Vandenbol, M., Weidner, S., and Galibert, F., Analysis of the chromosome sequence of the legume symbiont *Sinorhizobium meliloti* strain 1021. *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 9877–9882 (2001).
15) Wungsintaweekul, J., Herz, S., Hecht, S., Wisenreich, W., Feicht, R., Rordich, F., Bacher, A., and Zenk, M. H., Phosphorylation of 1-deoxy-D-xylulose by D-xylulokinase of *Escherichia coli*. *Eur. J. Biochem.*, **268**, 310–316 (2001).
16) Shames, S. L., Ash, D. E., Wedler, F. C., and Villafranca, J. J., Interaction of aspartate and aspartate-derived antimetabolites with the enzymes of the threonine biosynthetic pathway of *Escherichia coli*. *J. Biol. Chem.*, **259**, 15331–15339 (1984).
17) Farrington, G. K., Kumar, A., Shames, S. L., Ewaskiewicz, J. I., Ash, D. E., and Wedle, F. C., Threonine synthase of *Escherichia coli*: inhibition by classical and slow-binding analogues of homoserine phosphate. *Arch. Biochem. Biophys.*, **307**, 165–174 (1993).
18) Stock, A., Ortanderl, F., and Pfleiderer, G., Darstellung von radioakiv markiertem pyridoxal 5′-phosphat. *Biochem. Z.*, **344**, 353–360 (1966).
19) Glenn, A. R., and Dilworth, M. J., The effect of metal ions on the alkaline phosphatase of *Rhizobium leguminosarum*. *Arch. Microbiol.*, **126**, 251–256 (1980).
20) Deng, S., Summers, M. L., Kahn, M. L., and McDermott, T. R., Cloning and characterization of a *Rhizobium meliloti* nonspecific acid phosphatase. *Arch. Microbiol.*, **170**, 18–26 (1998).
21) Geladopoulos, T. P., Sotiroudis, T. G., and Evangelopoulos, A. E., A malachite green colorimetric assay for protein phosphatase activity. *Anal. Biochem.*, **192**, 112–116 (1991).
22) Laemmli, U. K., Cleavage of structural proteins during the assembly of the band of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
23) Matsudaira, P., Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.*, **232**, 10035–10038 (1987).
24) Fonda, M. L., Purification and characterization of vitamin B_6-phosphate phosphatase from human erythrocytes. *J. Biol. Chem.*, **267**, 15978–15983 (1992).
25) Doi, M., and Shioi, Y., A novel phosphatase specific for pyridoxal 5′-phosphate in an aerobic photosynthetic bacterium, *Erythrobacter sp.* OCh 114. *FEMS Microbiol. Lett.*, **55**, 309–313 (1988).
26) Gao, G. J., and Fonda, M. L., Identification of an essential cysteine residue in pyridoxal phosphatase from human erythrocytes. *J. Biol. Chem.*, **269**, 8234–8239 (1994).
27) Gao, G. J., and Fonda, M. L., Kinetic analysis and chemical modification of vitamin B_6 phosphatase from human erythrocytes. *J. Biol. Chem.*, **269**, 7163–7168 (1994).
28) Jang, Y. M., Kim, D. W., Kang, T.-C., Won, M. H., Baek, N.-I., Moon, B. J., Choi, S. Y., and Kwon, O.-S., Human pyridoxal phosphatase. *J. Biol. Chem.*, **278**, 50040–50046 (2003).