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Purification and Characterization of Purine Nucleoside Phosphorylase (PNP) in *Micrococcus luteus*

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Purine nucleoside phosphorylase (PNP) was purified in *Micrococcus luteus (M. luteus)* using streptomycin sulfate and ammonium sulfate fractionation, three times by a Sephadex G-100 gel filtration and a DEAE-Sephadex A-50 ion exchange chromatography. The enzyme was purified 72 folds with a 11% recovery and showed a single band in a nondenaturing gel electrophoresis. The M.W. of PNP turned out to be 1.35×10^5 dalton in G-150 gel filtration chromatography. The stability of the enzyme was increased by treatment with both substrates, $MgCl_2$ or $CaCl_2$, but not significantly with DTT or BSA. PNP showed maximum activity at pH 6.7 and an activation energy of 17.2 kcal/mol. *M. luteus* PNP catalyzed the phosphorolysis of inosine, deoxyinosine, guanosine and deoxyguanosine with the Km value of 1.5×10^{-3} M, 3.0×10^{-3} M, 5.0×10^{-4} M, 5.0×10^{-4} M, respectively. The enzyme was reacted with adenosine, 1-methylinosine and 1-methylguanosine as substrates, which were shown to be poor substrates for mammalian enzyme.

Key words: Purine nucleoside phosphorylase, *Micrococcus luteus*, inosine, guanosine, adenosine, 1-methylinosine, purification

Purine nucleoside phosphorylase (PNP) catalyzes the reversible phosphorolysis reaction of ribonucleosides or 2'-deoxyribonucleosides:

$$\text{B-Nucleoside} + \text{Pi} \rightleftarrows \text{Base} + \text{L-Ribose 1-phosphate}$$

The equilibrium constant favors nucleoside synthesis. In mammalian cells PNP functions primarily in the direction of nucleoside breakdown. PNP was first recognized as a distinct group of enzyme through the classic studies of Kalckar (14). The glycosidic bonds of nucleosides were cleaved enzymatically in a reversible manner, generating base and pentose 1-phosphate. There would be an increase in the intracellular pools of free bases and pentoses that could be reused for biosynthetic processes. PNP is widely distributed in nature. It has been identified and studied in various sources such as mammals, chicken and microorganisms (2, 10, 15, 16, 18, 19, 20, 33). The importance of PNP has been a focus after finding that there is a relationship between the deficiency of PNP and certain immunological disease (24, 27, 29). Absence of PNP is associated with severe T cell immunodeficiency while B cell immunity remains intact. This made PNP as a primary target of chemotherapy for selective immunosuppressant (6, 9, 17, 28, 32, 35, 37). X-ray crystallography has been attempted to elucidate the three dimensional structure of mammalian PNP and the reaction mechanisms involved (4, 7, 8). In addition, the ability to use adenosine as a substrate for bacterial PNP has encouraged us to screen the bacteria which showed generous enzyme activity (21, 22). From amino acid sequence analysis a group of plant stress-inducible proteins were found to share unexpected sequence similarity with mammalian and bacterial PNP (26). PNP is known to play an essential role in the salvage pathway, but it appeared to share other important functions possibly by regulating the cellular nucleoside and nucleotide levels or by some other mechanisms. In this study, we have purified PNP in *Micrococcus luteus* and characterized some of its properties.

Materials and Methods

Chemicals

Inosine, guanosine, guanine, hypoxanthine, xanthine

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oxidase from butter milk, Tris, DEAE-Sephadex A-50, Sephadex G-100, Sephadex G-150, streptomycin sulfate, ammonium sulfate were purchased from Sigma Chemical Co. (U.S.A.). PM-10 membrane filter was supplied from Amicon (U.S.A.). All the other chemicals used in the study were of the highest quality.

Cultivation of *Micrococcus luteus*

Micrococcus luteus KCTC 1071 was purchased and maintained in nutrient agar (peptone, 5 g; beef extract, 3 g; NaCl, 8 g; agar, 15 g in 1 L). The organism was inoculated using a loop in nutrient broth and incubated at 30℃ for 3-4 days (200 ml medium in a 1 L Erlenmeyer flask).

Enzyme purification

Each time two liters of cultured broth were centrifuged at 10,000×*g* for 10 min to harvest the cell. The cells were washed with distilled water twice, resuspended with small amount of 10 mM Tris-Cl, pH 7.0 containing 1 mM $MgCl_2$ (buffer A) and stored frozen at −28℃.

Frozen cells were thawed, incubated for 30 min with lysozyme, prepared from egg white and centrifuged at 10,000×*g* for 10 min to remove lysozyme protein. The precipitate was suspended in buffer A and disrupted twice for 30 min with pulse in an ice-chilled bead beater (Biospec Products, U.S.A.) with 0.5 mm diameter glass beads. The homogenate was centrifuged at 10,000×*g* for 40 min in a refrigerated centrifuge, T-324 using an A-8.24 rotor (Kontron Instruments).

The supernatant was precipitated with 30~80% ammonium sulfate saturation. The precipitate was collected after centrifugation and dissolved with buffer A. A portion of precipitate was loaded on a Sephadex G-100 column (3×100 cm) equilibrated with 20 mM Tris-Cl, pH 7.0 containing 1 mM $MgCl_2$ (Buffer B). After elution with buffer B, the active fraction was pooled and lyophilized to reduce the volume. Lyophilization did not reduce the enzyme activity and it was further concentrated and stored. The powder was dissolved with distilled water and applied to a DEAE-Sephadex A-50 column (2×25 cm) equilibrated with buffer A. The column was washed with 3 bed volumes of buffer A until the absorbance at 280 nm was decreased below the value of 0.1. A linear gradient from 0 to 1.5 M NaCl in buffer A was applied and fractions of 3 ml each were collected. The fractions containing enzyme activity were pooled and concentrated with a PM 10 membrane filter (Amicon, U.S.A.). The buffer of the enzyme solution was exchanged with buffer A. The concentrated enzyme was chromatographed on a Sephadex G-100 column (1.8×68 cm) equilibrated with buffer B. Fractions of 1.3 ml each were collected and the active fractions were pooled and lyophilized. The final purification was accomplished by rechromatography on a Sephadex G-100 column (1.8×68 cm) under the same condition. The active fraction was lyophilized and stored at −70℃.

Determination of molecular weight

The molecular weight of the purified enzyme was determined by Sephadex G-150 column chromatography. A portion of purified enzyme (1.5 U) was applied on the gel filtration column (1.8×65 cm), equilibrated with 20 mM Tris-Cl, pH 7.0 containing 1 mM $MgCl_2$. The gel was eluted with the same buffer and protein concentration and PNP activity were measured for the M.W. standard and sample, respectively. The reference proteins were supplied from Sigma Chemical Co. (U.S.A.) and contained alcohol dehydrogenase (150,000), bovine serum albumin (67,000) carbonic anhydrase (29,000) and lysozyme (14,300).

Measurement of protein concentration

Protein concentrations were measured by the methods of Lowry *et al.* (23) with bovine serum albumin as a standard or the methods in which the absorbances at 280 and 260 nm were multiplied by factors of 1.55 and 0.76, respectively and the latter was subtracted to obtain the protein concentration in mg per ml.

Purine nucleoside phosphorylase assay

All the enzyme assays were carried out at 30℃ if the conditions are not specified. The assay was performed using the coupled xanthine oxidase methods (14) which was based on the measurement of the increase in absorbance at 293 nm due to the formation of uric acid. A temperature controlled spectrophotometer (Kontron, UVKON 860) equipped with RS 232C-interface was used. The reaction mixture for standard assay contained, in a final volume of 1 ml, 50 mM Tris-Cl, pH 7.0, 10 mM inosine, 20 mM potassium phosphate, 0.02 unit of xanthine oxidase and a measured amount of enzyme, PNP. All reaction mixture except PNP was preincubated for 3 min to remove any trace amount of hypoxanthine or xanthine as a contaminant in the commercially available inosine preparation. The reaction was initiated by the addition of the enzyme, and continued for about 2 min to get the linear region. One unit of PNP is defined as the amount of enzyme which gives an optical density increase of 12.5 per min at 293 nm under standard assay conditions. The phosphorolysis of guanosine or deoxyguanosine was monitored at 257 nm with $\Delta E = -5 \times 10^3\ M^{-1}\ cm^{-1}$. The reaction mixture contained 50 mM Tris-Cl, pH 7.0, 20 mM potassium phosphate

Table 1. Purification of purine nucleoside phosphorylase in *Micrococcus luteus*

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude Homogenate	504	800	0.63	100	1.0
1% Streptomycin sulfate precipitation	453	254	1.78	90	2.8
30~80% ammonium sulfate fractionation	474	206	5.48	94	8.7
Sephadex G-100 filtration I	262	86.5	12.48	52	19.8
DEAE-Sephadex A-50 chromatography	170	9.1	18.68	34	29.7
PM 10 Ultra-filtration	158	9.1	17.36	31	27.6
Sephadex G-100 filtration II	115	3.7	31.08	23	49.3
Sephadex G-100 filtration III	54.6	1.2	45.5	11	72.2

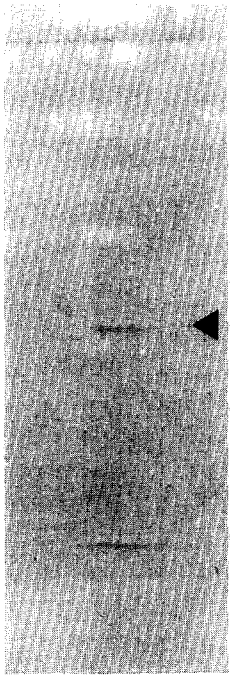

Fig. 1. Nondenaturing PAGE analysis of purine nucleoside phosphorylase (PNP). The active fraction after final Sephadex G-100 column chromatography was applied on a 10% polyacrylamide gel. A gel was run as described in Materials and Methods.

and various concentrations of guanosine or deoxyguanosine in a 1 ml cuvette. The reaction was carried out at 30℃ and continued for 2 min. The values of Km and Vm were calculated from Lineweaver-Burk plot.

Polyacrylamide gel electrophoresis

Nodenaturing discontinuous polyacrylamide gel (10% acrylamide) was performed by the methods of Davis (5). The gel was stained for proteins with Coomasie brilliant blue G-250, destained, and dried under air using a cassette.

Determination of equilibrium constant

All the procedures were followed by the some modification of the methods described by Choi (3). The reaction mixture contained 50 mM Tris-Cl, pH 7.0, 0.1 mM guanosine and different concentrations (1, 2 and 5 mM) of phosphate in a final volume of 1 ml.

Results

Enzyme purification

The purification procedures are summarized in Table 1. The enzyme was bound on a DEAE-Sephadex A-50 column and the activity was found in the elution buffer containing 0.5~0.7 M NaCl with a single peak. Lyophilization of the enzyme with 1 M NaCl reduce the enzyme activity significantly, whereas that in buffer A showed no change on the PNP activity. After removing the salts and concentration through an ultrafiltration, the major fraction of PNP was applied to a gel filtration column two times. The active fractions after final gel filtration column was lyophilized for storage. The final preparation of PNP was purified 72 folds with a 11% recovery. The identity of enzyme was tested by using other nucleoside substrate. The production of guanine from guanosine with the final purified enzyme was observed by the change of absorption spectrum. The purity of enzyme was analyzed by nondenaturing PAGE (Fig. 1), showing a single protein band. The molecular weight of nondenatured enzyme was estimated to be 1.35×10^4 dalton from Sephadex G-150 chromatography (Fig. 2).

Stability

The activity of purified PNP remained 80% of total activity in dilute buffer (10 mM Tris-Cl, pH 7.0) after 1 day, but in 50 mM Tris, pH 7.0 after 8 days. When PNP was incubated for 60 min at different temperatures, the enzyme was stable up to 37℃. After 15 min incubation at 42℃ and 50℃, 50% and 90% of the total activity was lost, respectively. The effect of ionic strength on the stability of PNP was tested in the range of 20 mM to 500 mM of Tris buffer. When the enzyme was incubated at 42℃ for 30 min, 95% activity was remaining in 500 mM, whereas only 3% was left in 20 mM buffer.

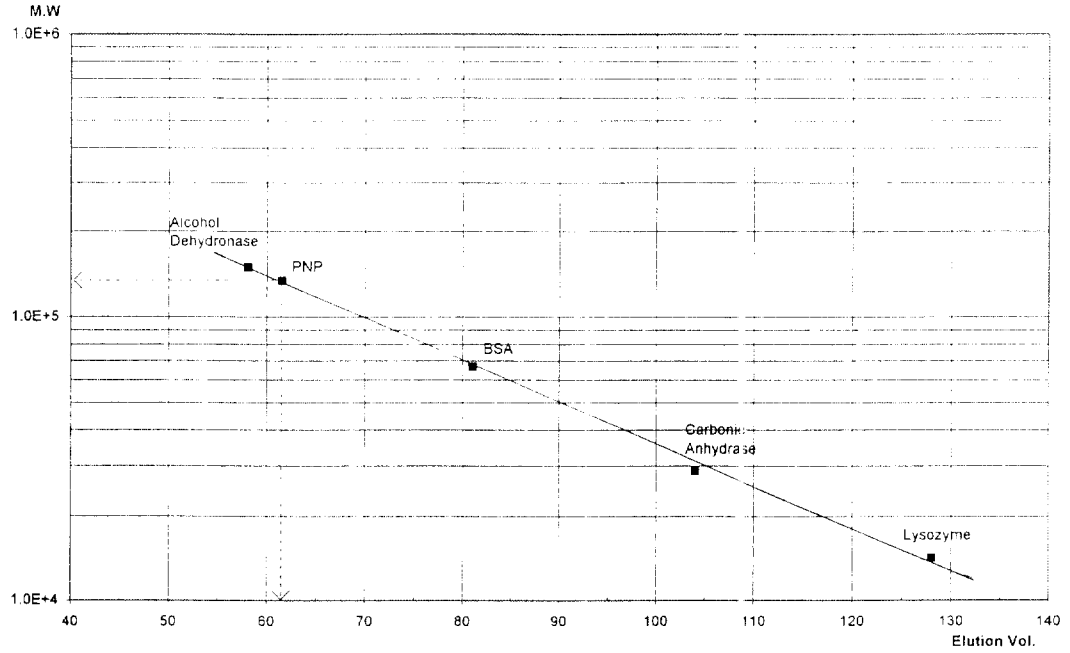

Fig. 2. Estimation of molecular weight of purine nucleoside phosphorylase (PNP) by Sephadex G-150 column chromatography. The M.W. standards consisted of alcohol dehydrogenase (150,000), bovine serum albumin (67,000), carbonic anhydrase (29,000) and lysozyme (14,300). The M.W. of PNP was calculated to be 135,000 dalton.

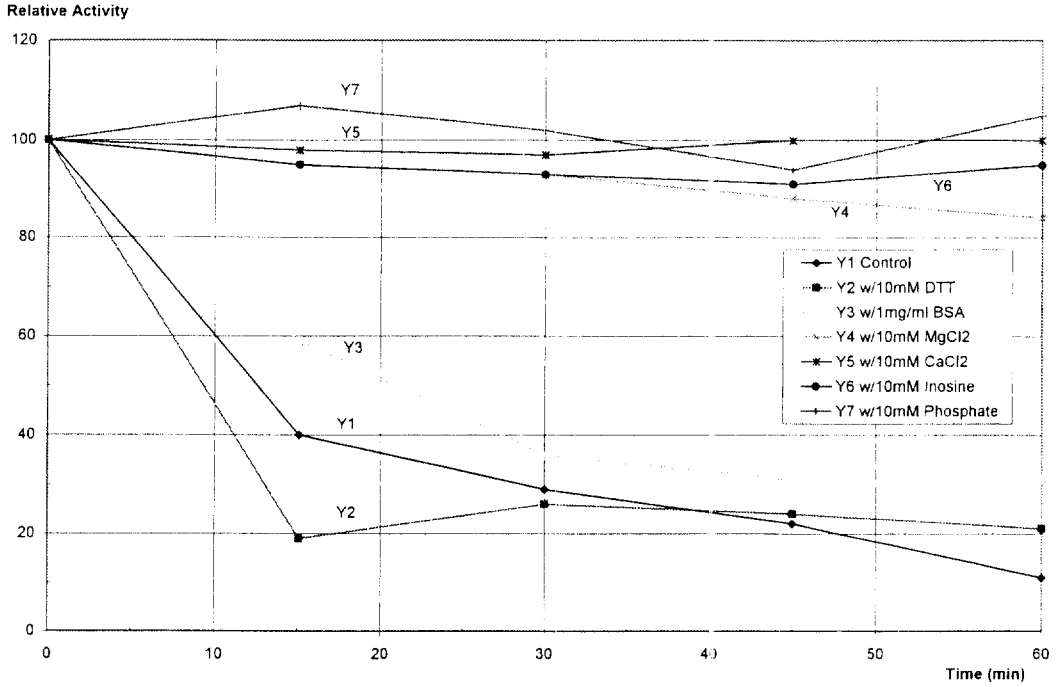

Fig. 3. Protection of purine nucleoside phosphorylase activities from heat inactivation. The purified enzyme was treated without (Y1) or with 10 mM of DTT (Y2), 1 mg/ml of BSA (Y3), 10 mM of $MgCl_2$ (Y4), $CaCl_2$ (Y5), inosine (Y6) and phosphate (Y7). The reaction mixture was put at 42℃ and then aliquots were collected at the indicated time points.

However, more than 100 mM of buffer reduced the initial enzyme activity. Under the same condition, the effects of substrates, divalent cations and other possible stabilizers were tested. As shown in Fig. 3, the saturating concentrations of inosine or phosphate or $CaCl_2$ protected the enzyme completely. The incubation with 10 mM $MgCl_2$ showed slightly less protection of PNP activity. The binding of substrate and the divalent cation to the enzyme appeared to stabilize the protein. However, almost no protection was observed with the addition of

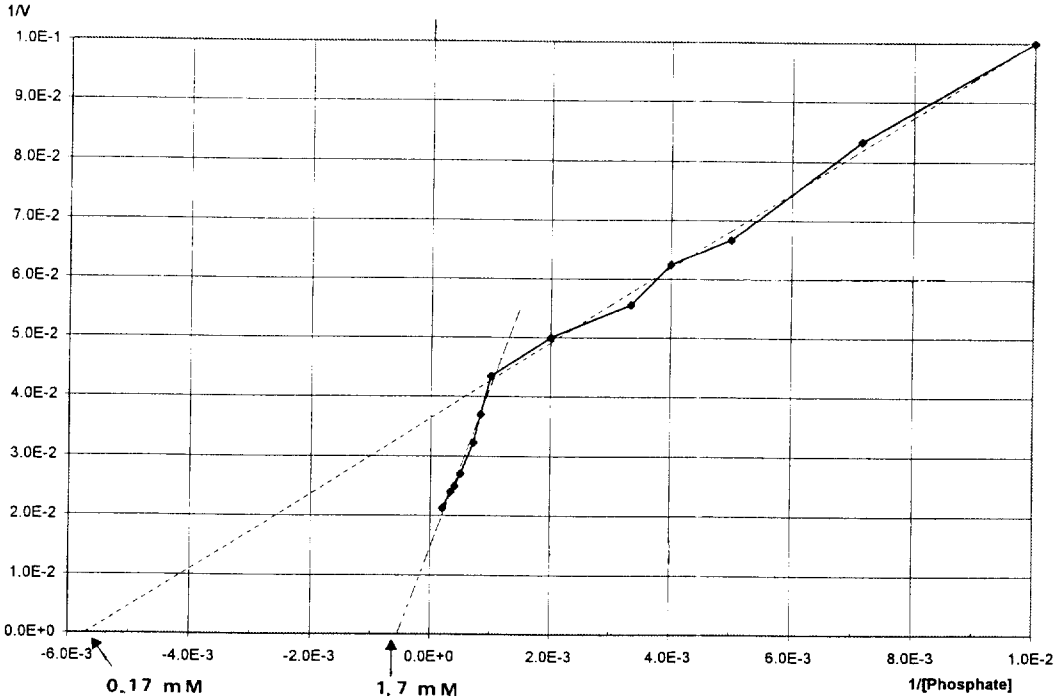

Fig. 4. Lineweaver-Burk plot of purine nucleoside phosphorylase (PNP) in *Micrococcus luteus* with phosphate as a variable substrate. The reaction mixture contained in a volume of 1 ml; 50 mM Tris-Cl, pH 7.0, 10 mM inosine, 0.02 units of xanthine oxidase, variable concentrations of 0.1~5 mM phosphate and PNP. The values of Km of phosphate were 1.7×10^{-4} M and 1.7×10^{-3} M for the concentration of phosphate of 0.1~1 mM and 1~5 mM, respectively.

Table 2. Kinetic parameters of purine nucleosides for PNP in *M. luteus*

Substrate	Km (mM)	Relative k_{cat}	Relative k_{cat}/Km
Inosine	1.5	1.0	1.0
Deoxyinosine	3.0	0.31	0.16
Guanosine	0.5	0.28	0.83
Deoxyguanosine	0.5	0.14	0.42

10 mM DTT or 1 mg/ml of bovine serum albumin, both of which were demonstrated to have stabilizing effect on PNP from other sources (2, 33).

Effects of pH and temperature

PNP showed highest activity at pH 6.7 and more than 90% of maximal activity in the range of pH 5.5 to 7.0. Effect of temperature on initial velocity of PNP was observed at 8.5℃ to 42℃ of the temperature range. The Arrhenius plot of PNP showed an activation energy of 17.2 kcal/mol and no inactivation occurred in the tested range.

Effects of various compounds

PNP activity was not inhibited by 10 mM of Cs^+, Na^+, K^+, NH_4^+, Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+} and EDTA when the enzyme was preincubated for 5 min with the effector and assayed. Among the tested metal ions, 1 mM of Cu^{2+} and Hg^{2+} reduced the PNP activity to 6 and 4% of the total activity, respectively. Among the sulfhydryl group reactive agents, 1 mM of p-chloromercuribenzoate and mersalyl acid inactivated PNP completely. However, PNP was insensitive to 1 mM 5,5'-dithiobis(2-nitrobenzoate) and N-ethylmaleimide. Alkylating agents, iodoacetic acid and iodoacetamide did not show any effect on PNP at the tested concentration of 1 mM.

Kinetic parameters

The substrate specificities of PNP from *M. luteus* was tested with various nucleosides. All substrates were used at 100 uM of final concentration and the changes of absorption spectrum from 220 to 300 nm were measured, resulting from the generation of respective base by phosphorolysis of tested nucleosides. Besides the known substrates such as inosine, deoxyinosine, guanosine and deoxyguanosine, the nucleosides, adenosine, 8-aminoguanosine, 6-mercaptopurine riboside, thymidine, 1-methylinosine, 7-methylinosine, 1-methylguanosine and 7-methylguanosine showed the substrate activity.

The affinities of purine nucleosides for PNP from *M. luteus* were estimated. The values of Km were determined in Lineweaver-Burk plot to be 1.5×10^{-3} M, 3.0×10^{-3} M, 5.0×10^{-4} M and 5.0×10^{-4} M for inosine, deox-

yinosine, guanosine and deoxyguanosine, respectively. Table 2 showed the kinetic parameters for above substrates. The ratio of relative k_{cat}/Km for inosine was the highest among the tested nucleosides, representing that inosine was the best substrate. The rate of phosphorolysis of guanosine was two times faster than that of deoxyguanosine. The values of relative k_{cat} for guanosine and deoxyguanosine were lower than that for deoxyinosine. Deoxyinosine seemed to be better substrate than guanosine or deoxyguanosine at high concentrations when each reaction was performed separately. When the substrates are mixed together, the order of substrate specificity was considered to be inosine, guanosine, deoxyguanosine and deoxyinosine from the ratio of k_{cat}/Km.

When the effect of phosphate was measured as the concentration of inosine was fixed at 10 mM, the downward curvature of double reciprocal plot was observed. As shown in Fig. 4, two values of Km for phosphate were estimated to be 1.7×10^{-4} M and 1.7×10^{-3} M for the concentraion range of 0.1~1.0 mM and 1.0~5.0 mM, respectively.

The equilibrium constant of the reaction:

Guanosine + Phosphate $\rightleftarrows$ Guanine + Ribose 1-Phosphate

was determined from the ratio of the equilibrium concentrations and estimated to be 0.0081. Each reaction was performed with different concentrations of phosphate until it reached equilibrium point and the value was averaged.

Discussion

The M.W. of purified PNP was found to be 1.35×10^5 dalton in *M. luteus*. This value was consistent with that of bacterial enzyme such as *Escherichia coli* (*E. coli*) (13). *M luteus* PNP showed substrate activity to adenosine which was known to be a poor substrate for mammalian PNP. Stabilization of the enzyme by substrates was found. Divalent cation such as Ca^{2+} and Mg^{2+} and high ionic strength also stabilized the enzyme. The interesing feature of PNP reaction was shown when the effect of phosphate on the initial velocity was measured. The biphasic nature of a double reciprocal plot with phosphate as the variable substrate indicated the substrate activation. The deviation of linearity of the plot was apparent at phosphate concentrations greater than 1.0 mM. This phenomena seemed to be caused by a cooperativity between the subunits. The similar substrate activation at the high concentration of phosphate has been demonstrated in PNP from *Saccharomyces cerevisiae* (*S. cerevisiae*) (3), PNP from *E. coli* and *Salmonella typhimurium* (*S. typhimurium*) (13), human and rabbit erythrocytic PNP (31), bovine liver (11) and spleen (30). Kinetic binding studies reported for human erythrocytic enzyme (34) indicated that while nucleosides and purine were strongly bound by the enzyme, inorganic phosphate and ribose 1-phosphate were only weakly associated. It was also found in PNP from *Bacillus subtilis* (12), *S. typhimurium* (13) and *S. cerevisiae* (3). The values of affinity constants for inosine was about 10 folds lower than those for phosphate. However, the reverse was found in PNP from *M.luteus*. The Km value of inosine was 1.5×10^{-3} M and those of phosphate were 1.7×10^{-4} M and 1.7×10^{-3} M for low and high affinity sites. Similar phenomena was reported in Proteus vulgaris (Km of inosine: 3.9×10^{-5} M and Km of phosphate: 1.3×10^{-5} M) (36) and *Klebsiella* sp. (Km of inosine: 6.6×10^{-4} M and Km of phosphate: 5.6×10^{-4} M) (21). These observations seemed to be related to different reaction mechanism of PNP from various sources. This will be further investigated.

PNP from *M. luteus* catalyzed the phosphorolysis of inosine, deoxyinosine, guanosine and deoxyguanosine. From the value of relative k_{cat}, deoxyinosine was better substrate than guanosine or deoxyguanosine, since it reacted with the enzyme faster than guanosine or deoxyguanosine. However, the ratio of rates for each nucleoside could be considered from relative k_{cat}/Km under physiological conditions, in which all the substrates were present in one compartment. Therefore, inosine was 6 times faster than deoxyinosine. Guanosine or deoxyguanosine was better substrate than deoxyinosine.

The equilibrium constant for phosphorolytic direction was estimated to be 0.0081. Equilibrium favored inosine synthesis, the synthetic direction for the reversible reaction. The reported K_{eq} value for the phosphorolysis of inosine at pH values near neutrality, ranged from 0.018 in PNP from *E. coli* (13) and human erythrocyte (33) to 0.048 in *S. cerevisiae* (3) and chicken liver PNP (25).

The substrate activities of various nucleosides were determined by the absorption spectrum. *M. luteus* PNP showed broader substrate specificity than mammalian PNP. Inosine, guanosine, adenosine and their deoxynucleosides generated the corresponding bases by the phosphorolytic reaction, though PNP reacted slowly with adenosine. Inosine analog, formycin B was not cleaved. Among pyrimidine nucleosides, thymidine acted as a substrate, but uridine did not. It was reported that 7-methylinosine and 7-methylguanosine were excellent substrates for calf spleen PNP, but 1-methylinosine and 1-methylguanosine were neither substrates nor inhibitors of this enzyme. Furthermore, kinetic analysis demonstrated that

both 1-methylinosine and 1-methylguanosine were as good substrates as the parent nucleosides for *E. coli* PNP (1). In contrast to the mammalian enzyme, PNP from *M. luteus* also reacted with 1-methylinosine and 1-methylguanosine as well as 7-methylinosine and 7-methylguanosine. Those results suggested that the ring N-7 and ring N-1 were excluded as a binding site for bacterial enzyme.

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