Inhibition of Purine Nucleoside Phosphorylase (PNP) in *Micrococcus* luteus by Phenylglyoxal

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***Micrococcus luteus* purine nucleoside phosphorylase (PNP) has been purified and characterized. The physical and kinetic properties have been described previously. Chemical modification of the enzyme was attempted to gain insight on the active site. The enzyme was inactivated in a time-dependent manner by the arginine-specific modifying reagent phenylglyoxal. There was a linear relationship between the observed rate of inactivation and the phenylglyoxal concentration. At 30℃ the bimolecular rate constant for the modification was 0.015 $min^{-1}mM^{-1}$ in 50 mM $NaHCO_3$ buffer, pH 7.5. The plot of logk versus log phenylglyoxal concentration was a straight line with a slope value of 0.9, indicating that modification of one arginine residue was needed to inactivate the enzyme. Preincubation with saturated solutions of substrates protected the enzyme from inhibition of phenylglyoxal, indicating that reactions with phenylglyoxal were directed at arginyl residues essential for the catalytic functioning of the enzyme.**

Key words: Purine nucleoside phosphorylase, phenylglyoxal, arginine

PNP catalyzes the phosphorolysis of inosine and guanosine to hypoxanthine and guanine. It also cleaves the respective deoxynucleoside.

Purine nucleoside+phosphate ⇌ purine base+pentose 1-phosphate

PNP plays the key role in the purine salvage pathway. The direction of nucleoside synthesis is favorable at equilibrium, but under physiological conditions, reaction proceeds in the catabolic direction.

Various PNPs have been purified and studied in various mammalian cells and microorganisms (3, 6, 9, 10). Most bacterial PNPs consist of six subunits with a subunit molecular weight of 25,000~28,000 (6, 9), whereas mammalian enzymes consist of three subunits with a molecular weight of 32,000 (10). Since bacterial PNP has been shown to be useful in biosynthesis of clinically useful purine nucleoside and its analogs due to its broad substrate specificity (6, 9), particular attention was paid to getting information about the structure of the enzyme.

The present communication presents data on the inhibition of purified *Micrococcus luteus* PNP by phenylglyoxal. The residue modified by the reagent seemed to be located at or near the active site of the enzyme, since both substrates protected the enzyme.

These findings will help to elucidate the mechanism of action and structure of the enzyme.

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Methods and Materials

Chemicals

Inosine, guanosine, guanine, hypoxanthine, xanthine oxidase from butter milk, Tris, DEAE-Sephadex A-50, Sephadex G-100, Sephadex G-150, streptomycin sulfate, ammonium sulfate, $NaHCO_3$ were purchased from Sigma Chemical Co. (U.S.A.). PM-10 membrane filter was supplied by Amicon (U.S.A.). Phenylglyoxal was obtained from Aldrich Chemical Co.

Enzyme Purification

Micrococcus luteus KCTC 1071 was purchased and used as an enzyme source. All purification procedures were followed as described in the previous paper (3). Aliquots of Purified PNP fraction were lyophilized and stored frozen at -70℃.

Inactivation of PNP

Enzyme was incubated with 0.67~4 mM phenylglyoxal in the dark at room temperature in 50 mM of $NaHCO_3$

buffer, pH 7.5. At each time point, diluted aliquot was removed and assayed at 30℃ for remaining activity. The phosphorolysis of inosine was measured by a spectrophotometric assay coupled to xanthine oxidase as described previously (2). 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, pH 7.0, 10 mM inosine, 20 mM potassium phosphate, 0.02 unit of xanthine oxidase and an appropriate 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. Data was fitted to the equation for a pseudo first order inactivation, $-\ln(E/E_o)=k_{obs}\,t$ (8). Protection against inactivation was studied by preincubation of the enzyme with various concentrations of phosphate or inosine for 10 min.

Results and Discussions

M. luteus PNP has been purified electrophoretically homogenous in our laboratory (3). Chemical modification of enzyme was tried to get the information of amino acid residues as being essential to the catalytic activity of PNP. The enzyme was inactivated in a time-dependent manner by the arginine-specific modifying reagent phenylglyoxal. The observed rate of inactivation showed a linear dependence on the concentration of phenylglyoxal added as shown in Fig. 1-A. There was a linear relationship between the observed rate of inactivation

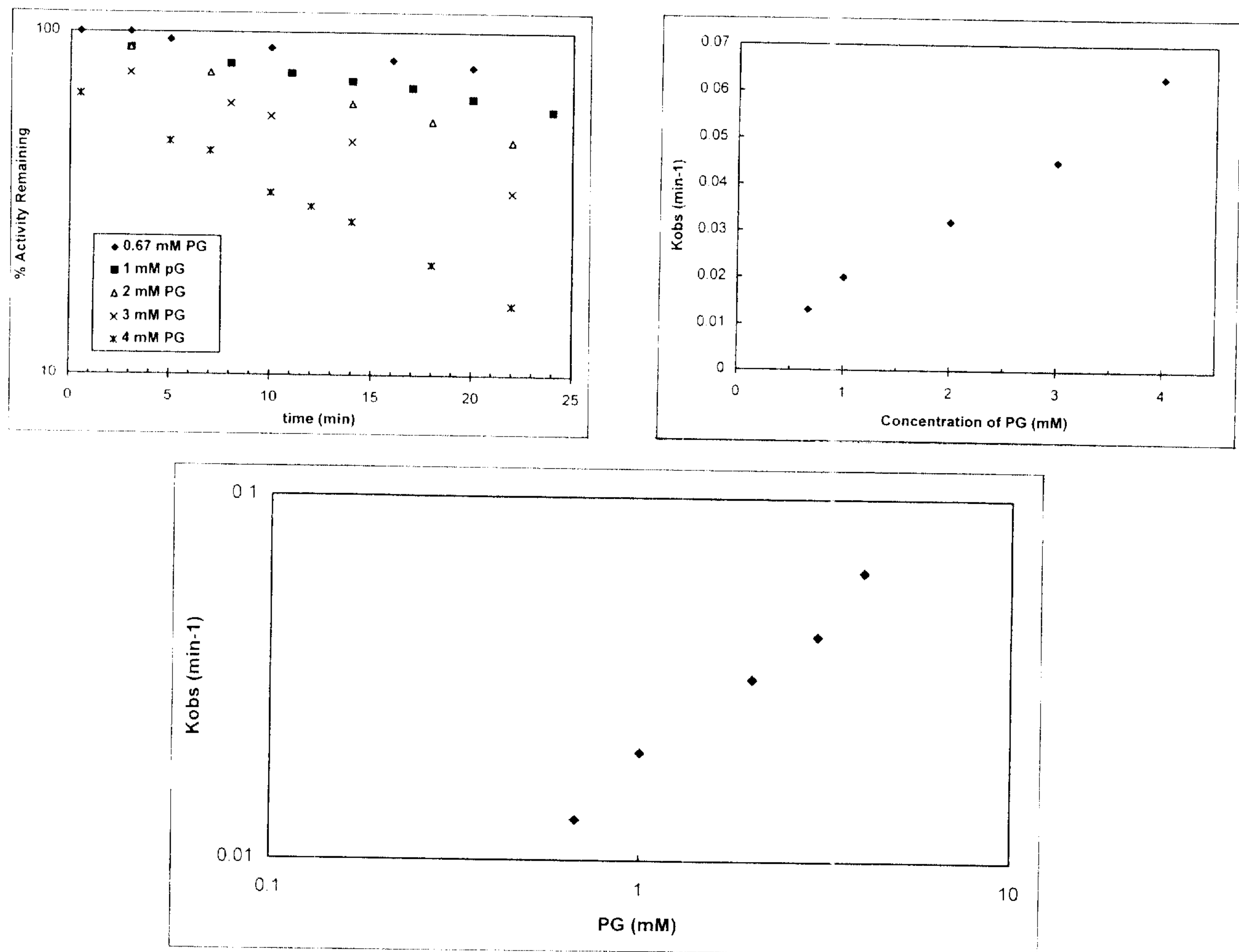

Fig. 1. Inactivation of M.luteus PNP by phenylglyoxal The enzyme was incubated with the following concentrations of Phenylglyoxal; 0.67 mM, 1 mM, 2 mM, 3 mM and 4 mM in 50 mM $NaHCO_3$ buffer, pH 7.5. A. The logarithm of fractional residual activity versus time of reaction. B. The apparent first order inactivation rate versus phenylglyoxal concentration. C. The logarithm of the apparent first order inactivation rate constant (kobs) versus the logarithm of phenylglyoxal concentration.

Table 1. The effect of pH on the apparent first order rate constants of purine nucleoside phosphorylase inactivated by phenylglyoxal

pH	K_{obs} (min^{-1})
7.0	0.017
7.5	0.029
8.0	0.048
8.5	0.069
9.0	0.301

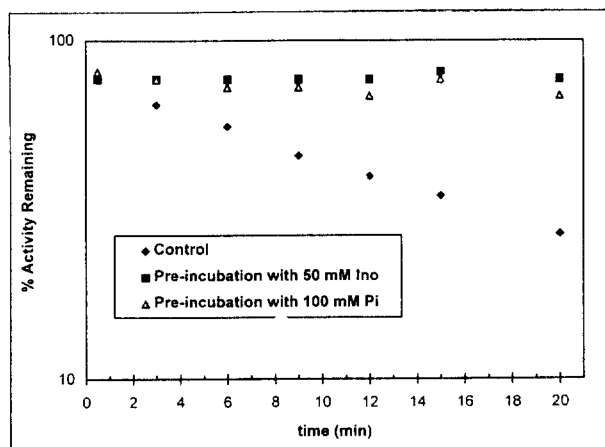

Fig. 2. Phenylglyoxal inactivation of PNP in the presence and absence of substrates. Each enzyme was incubated with 3 mM phenylglyoxal in 100 mM $NaHCO_3$, pH 7.5 after preincubation without substrate and with 100 mM Pi and 50 mM inosin.

and the phenylglyoxal concentration as can be seen in Fig. 1-B. At 30°C, the bimolecular rate constant for the modification was 0.015 $min^{-1}mM^{-1}$ in 50 mM $NaHCO_3$ buffer, pH 7.5. There was no saturation at high concentrations of phenylglyoxal, indicating that an enzyme-inhibitor complex did not form before inactivation. The number of modifications carried out per enzyme molecule was determined from the replot. The plot of logk versus log phenylglyoxal concentration was a straight line with a slope value of 0.9 (Fig. 1-C), indicating that modification of about one arginine residue was needed to inactivate the enzyme. The stoichiometry of phenylglyoxal to arginine could be due to steric restriction by the localized environment of the specific arginine residue (5)

The rate at which phenylglyoxal inactivated PNP was markedly influenced by the pH at which the reaction was carried out. Table 1 shows that as the pH was increased from 7.0 to 9.0, there was a pronounced increase in the reaction rate. The marked increase in reaction rate could not be the result of ionization of an arginine since the pK of free NH_3 group is normally above 12.

Since measurements could not be made at higher pH due to instability of phenylglyoxal and the enzyme, the value of pK of this rate effect was impossible to determine. The effect of pH on the rate could be due to ionization of other groups in the vicinity of the arginine residue or the reagent itself. Similar pH phenomena has been demonstrated with aspartate transcarbamylase (7) and phospholipase A2 (11) treated with phenylglyoxal. The rate of inactivation of PNP by phenylglyoxal showed a specificity for the buffer present. At pH 7.5, inactivation by phenylglyoxal was significant in bicarbonate buffer, but reactivation of enzyme activity occurred in Tris buffer. The modification of glutamate apodecarboxylase (1) and alkaline phosphatase (4) was shown to be enhanced by bicarbonate or to be more effective in bicarbonate than in other buffers. However, the concentration of bicarbonate did not show any effect on rate of inactivation between 10 mM and 500 mM of concentration range.

If the reaction with phenylglyoxal was directed at arginyl residues essential for the catalytic functioning of the enzyme, substrates would be expected to protect the enzyme from inactivation. Each of the saturated concentrations of inosine and phosphate showed protection as shown in Fig. 2. Protection experiments with substrates of PNP indicated that the essential arginine was involved in the substrate binding site. Arginine residues have been shown to be essential for catalytic activity in a number of proteins (5, 12). In all cases, the positively charged arginine side chain has been postulated to interact with the phosphate portion of the substrate. It seemed likely that the arginine residue was capable of directly interacting with the phosphate portion of PNP. Protection by nucloside substrate could be because this arginine residue is located in a position that interacts with both.

Since the inherent instability of the phenylglyoxal-arginine derivative, identification of the exact position of this essential arginine residue in the enzyme's primary sequence has not been tried. However, our chemical modification results with phenylglyoxal suggest that there is an arginine residue essential for the activity of PNP from *M. luteus*.

Acknowledgements

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