Purification and Properties of Hypoxanthine Phosphoribosyltransferase from *Streptomyces cyanogenus*

Tatsuhiko Ohe and Yasuto Watanabe

The Osaka Municipal Technical Research Institute, Ogimachi, Kita-ku, Osaka 530, Japan

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Hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) of a strain of *Streptomyces cyanogenus* was purified 1,900-fold to an apparent homogeneity from cell-free extracts. The enzyme had a molecular weight of 150,000 and consisted of eight identical subunits with a molecular weight of 18,000. The isoelectric point was at pH 4.4. The enzyme required Mg$^{2+}$ or Mn$^{2+}$ for activity and had a pH optimum at 8.5. Hypoxanthine and guanine were good substrates for the enzyme. Xanthine was a very poor substrate and adenine was not a substrate. Apparent Km values of the enzyme for hypoxanthine, guanine and 5-phosphoribose-1-pyrophosphate were $1.6 \times 10^{-6}$, $2.7 \times 10^{-6}$ and $6.3 \times 10^{-6}$ M, respectively. All purine nucleotides tested inhibited the activity significantly, apparently by competing with 5-phosphoribose-1-pyrophosphate.

Hypoxanthine phosphoribosyltransferase (HPRT) (EC 2.4.2.8), one of the purine salvage enzymes, catalyzes 5-phosphoribose-1-pyrophosphate (PRPP) dependent conversion of the purine bases, hypoxanthine and guanine, to the corresponding nucleotides, IMP and GMP. This enzyme is thought to be involved in controlling the rate of incorporation of these purine bases supplied exogenously into the nucleotides, irrespective of differences in their uptake mechanisms. The enzyme has been purified and characterized from higher animals and yeasts. Although the enzyme of bacteria has been studied intensively in intact cells and in crude extracts, few paper has been reported on purification.

It was found previously that a strain of *Streptomyces cyanogenus* which metabolized exogenous hypoxanthine either to the anabolic or to the catabolic route utilized it almost exclusively for the synthesis of nucleotides and nucleic acids in early phase of growth. When the cell growth reached stationary phase, or when the cells growing logarithmically were transferred to a nitrogen free medium, however, the incorporation was stopped and hypoxanthine was oxidized to xanthine or 6,8-dihydroxypurine and then to uric acid. Nevertheless, the amount of intracellular HPRT was constant regardless of the growth phase and of the medium conditions.

It may be assumed that the changes in hypoxanthine incorporation are, at least in part, dependent on changes in HPRT activity in vivo, and, therefore, the study on properties of HPRT are required to elucidate the regulation of hypoxanthine metabolism of this organism. The present paper describes the purification of HPRT from *Streptomyces cyanogenus* and properties of the purified enzyme, and discusses the relationship between the properties of the enzyme and the regulation of hypoxanthine metabolism.

**MATERIALS AND METHODS**

Organism and cultivation. The organism used in this study was a strain of *Streptomyces cyanogenus* isolated from soil and maintained on potato-glucose-agar slants as described previously.

Seed cultures were obtained by cultivating the organism in a growth medium containing 2% peptone, 3% glucose, 0.1% K$_2$HPO$_4$, 0.05% MgSO$_4$·7H$_2$O and 0.05% NaCl and adjusted to pH 7 (60 ml portions in
500-ml shaking flasks) at 27°C for 36 hr with shaking. The organism was then grown to obtain it in a large mass in the same medium (15 liters in a 30-liter jar fermentor) at 27°C for 30 hr with aeration and agitation. Cells were collected by continuous centrifugation, washed three times with water and frozen at −20°C until use.

**Enzyme assay.** Phosphoribosyltransferase activities were determined by measuring the formation of 3H-nucleotides from appropriate 3H-purine bases. A standard reaction mixture contained 0.35 mM 3H-purine base (0.1 μCi), 0.75 mM PRPP, 3 mM MgCl₂ and 100 mM Tris-HCl buffer (pH 8.0) in a final volume of 250 μl. Reactions were initiated by addition of enzyme. Reaction mixtures were incubated at 30°C for 3 to 10 min and reactions were stopped by addition of 50 μl of 80 mM EDTA (pH 7.8). In control, EDTA was added prior to the addition of enzyme.

All aliquots (20 μl) of the reaction mixtures were applied to DEAE ion exchange paper (Whatman DE 81) which had previously been spotted with the unlabeled 5'-ribonucleotide corresponding to the substrate to make the detection of spots of product easy. Chromatograms were developed by dipping the top end of the paper in 20 mM Tris-HCl buffer (pH 8.0). After the chromatographic separation of the substrate and product, UV absorbing spots corresponding to the product were cut off from the paper, immersed in 10 ml of scintillation fluid (0.5 % 2, 5-diphenyloxazole (PPO) and 0.03 % 2, 2'-p-phenylene-bis (5-phenyloxazole) (POPOP) in toluene) and counted in a Packard 3310 liquid scintillation counter.

One unit of the enzyme activity was defined as an amount catalyzing the production of 1 μmol of nucleotide per min under the conditions. Specific activity was defined as units per milligram of protein.

**Protein assay.** Protein concentrations were estimated by the method of Lowry et al. using bovine serum albumin as a standard. In chromatographic procedures, the absorbance at 280 nm was used.

**Determination of Mg and Mn in the cell.** The organism was grown in the growth medium in shaking flasks at 27°C for 36 hr with shaking. Cells were collected on filter paper, washed with water 3 times and digested with a mixture of nitric and perchloric acids. Determination of Mg and Mn in the digests was carried out with a Shimadzu AA-610S atomic absorption spectrophotometer.

**Affinity chromatography column.** 3, 3'-Iminobispropylamine-GMP-Sepharose was prepared according to the method of Gilham and used for affinity chromatographic purification of HPRT.

**Polyacrylamide gel electrophoresis.** Analytical polyacrylamide gel electrophoresis was carried out according to Williams et al. with columns of 7.5% polyacrylamide gel with the pH 7.0 buffer system. Protein samples (50 μg) were loaded on the columns and a constant current of 3 mA per column was applied for 2 hr.

**SDS-polyacrylamide gel electrophoresis.** SDS-polyacrylamide gel electrophoresis was carried out according to Weber and Osborn with columns of 9% polyacrylamide gel containing 0.1% SDS. Electrophoresis was conducted at a constant current of 5 mA per column at room temperature for 3 hr. Cytochrome c (MW 13,000), chymotrypsinogen A (25,000), egg white albumin (45,000) and bovine serum albumin (67,000) were used as standards.

**Gel filtration.** Gel filtration was performed according to the method of Andrews with a column of Sephadex G-200 (1.5 × 96 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.8) containing 0.5 mM KCl, 10 mM MgCl₂ and 1 mM mercaptoethanol. Elution was performed with the same buffer at a flow rate of 4.5 ml per hr and the eluate was collected in 2-ml fractions. Lysozyme (MW 14,400), bovine serum albumin (67,000), γ-globulin (160,000) and apoferritin (480,000) were used as standards.

**Isoelectric focusing.** Isoelectric focusing was carried out according to Vesterberg and Svensson. The enzyme protein was applied to a column of carrier ampholyte (110 ml) with a pH range of 3 to 6. A constant potential of 300 V was applied to the column at 4°C for 40 hr and then the column was drained and fractionated into 2-ml fractions.

**Materials.** “Polypepton” of Daigo Eiyo Kagaku Co. was used in the growth medium. 3H-labeled purine bases were obtained from New England Nuclear Co. Unlabeled purine bases were obtained from Wako Junyaku Co. PRPP was purchased from Kyowa Hakko Co. The concentration of PRPP at the use was determined using the purified HPRT and 3H-hypoxanthine. Other reagents used were of the highest grade commercially available.

**RESULTS AND DISCUSSION**

**Extraction and purification.**

Unless otherwise stated, all operations of extraction and purification were carried out at 4°C in 50 mM Tris-HCl buffer (pH 7.8) containing 10 mM MgCl₂ and 1 mM mercaptoethanol (buffer A). Cells were suspended in buffer A in a concentration of 200 g (wet weight) per liter and disrupted by a Dyno
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Extracts (5,000 ml) were centrifuged at 20,000×g for 20 min and supernatant solution obtained was used as cell-free extracts. The extracts contained phosphoribosyltransferase activities to hypoxanthine, guanine and xanthine but no activity to adenine. The ratio of the activities to hypoxanthine and to guanine was about one.

Solid ammonium sulfate was added to the cell-free extracts and precipitate formed between 0.35 and 0.75 saturations were collected by centrifugation. The precipitates were dissolved in and dialyzed against buffer A. To the dialyzed solution was added protamine sulfate, 4.5 ml of 3% solution per g of protein, and precipitates were removed by centrifugation.

The supernatant solution was loaded on a column of DEAE-Sephadex A-50 (300 ml) and the enzyme was eluted by a linear gradient of KCl concentration prepared with 500 ml of buffer A and 500 ml of the buffer containing 1 M KCl. Elution was performed at a flow rate of 25 ml per hr and eluates were collected in 5-ml fractions. Phosphoribosyltransferase activities to hypoxanthine and guanine were eluted as one peak, followed by an overlapping peak of the activity to xanthine. Active fractions to hypoxanthine and guanine (fraction Nos. 49–54) were combined, concentrated by means of ultrafiltration and dialyzed.

The dialyzed solution was subjected to gel filtration with a column of Sephadex G-200 (320 ml). Buffer A was allowed to flow at a rate of 20 ml per hr and eluates were collected in 5-ml fractions. Phosphoribosyltransferase activities to hypoxanthine and guanine were eluted as one peak, the ratio of these activities being about one that in the crude extracts. The activity to xanthine was eluted next.

Active fractions to hypoxanthine and guanine (fraction Nos. 37–41) were combined and loaded on a column of 3, 3′-iminobispropylamine-GMP-Sepharose (8 ml) equilibrated with buffer A. The column was washed with the buffer containing 0.45 M KCl and the enzyme was then eluted with the buffer containing 1.05 M KCl at a flow rate of 5 ml per hr. An elution profile of the enzyme from the affinity column is shown in Fig. 1. HPRT activity was eluted just behind a large protein peak without activity. Active fractions (fraction Nos. 19–22) of the eluate were combined and dialyzed against buffer A and the dialyzed solution was used as the purified enzyme.

The purification of HPRT from the cell-free extracts of Streptomyces cyanogenus is summarized in Table I. The enzyme was purified about 1,900 folds from the extracts at a yield of 8.4%.

**Homogeneity**

The purified enzyme preparation gave a single protein band in both polyacrylamide gel electrophoresis and SDS-polyacrylamide...
The enzyme preparation showed the activity to both hypoxanthine and guanine. The ratio of activities to the purine bases did not change throughout all the purification steps (Table I). The purified enzyme in 50 mM phosphate buffer (pH 7.4) was placed in a water bath at 52°C and aliquots were withdrawn at appropriate time intervals and cooled on ice. The heating caused a similar reduction in the activities to hypoxanthine and guanine (Fig. 4). These results suggested that the activities could be attributed to the same enzyme.

**Molecular weight**

Molecular weight of the enzyme was estimated to be about 150,000 by gel filtration on Sephadex G-200 (Fig. 5). This value is much larger than those reported for HPRT from other sources.\(^3\)\(^-\)\(^7\)

In SDS-polyacrylamide gel electrophoresis, a single protein band corresponding to a molecular weight of 18,000 was obtained as shown in Fig. 3. It was then suggested that the enzyme consisted of eight identical subunits. HPRT from *Saccharomyces cerevisiae*\(^7\) was reported to consist of one subunit and from human erythrocytes\(^8\) and mouse liver\(^3\) to
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**TABLE II. REQUIREMENT OF DIVALENT CATIONS FOR HPRT ACTIVITY**

The activity of the enzyme previously dialyzed against 50 mM Tris-HCl buffer (pH 7.8) was measured under the standard conditions, except that the divalent cations were added as indicated.

<table>
<thead>
<tr>
<th>Cation</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca²⁺</td>
<td>3.0</td>
<td>16.3</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>3.0</td>
<td>35.1</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>3.0</td>
<td>0</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>3.0</td>
<td>0</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.2</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>25.8</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>59.4</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>100</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>0.2</td>
<td>32.1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>80.3</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>189</td>
</tr>
<tr>
<td>Sn²⁺</td>
<td>3.0</td>
<td>0</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>3.0</td>
<td>69.0</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Table II, some divalent cations such as Mn²⁺ and Mg²⁺ were required for the enzyme activity. The optimum concentration of the cations was found to be in a range of 3~10 mM. Although the activating effect of Mn²⁺ was higher than that of Mg²⁺, the activity and the characteristics of the enzyme were studied in the presence of Mg²⁺, because the ion appeared to be of physiological significance. Intracellular concentrations of Mg²⁺ and Mn²⁺ were determined by atomic absorption, calculated assuming that cells contained 80% water and found to be 40 mM and less than 0.01 mM, respectively.

Effect of various compounds on the activity was studied at a concentration of 1 mM. The activity was inhibited by EDTA, but not by cyanide, PCMB or moniodoacetate.

**Stability**

When the enzyme was kept at 30°C in 50 mM phosphate buffer (pH 7.4), there was no decrease in the activity, but a gradual decrease was observed at 40°C. The addition of PRPP (2 mM) and MgCl₂ (2 mM) to the enzyme solution was found to increase the stability slightly.

isolectric point

Isoelectric point of the enzyme was found to be at pH 4.4 by isoelectric focusing as shown in Fig. 6.

**Effect of pH and various compounds on the activity**

Effect of pH on the enzyme activity was studied over a range of pH 5.8~9.7. The maximum activity was at pH 8.5 (Fig. 7).

Effect of various divalent cations was studied with the enzyme previously dialyzed against 50 mM Tris-HCl buffer (pH 7.8). As shown in

Fig. 6. Determination of Isoelectric Point.

Isoelectric focusing was carried out according to Vesterberg and Svensson. A column (110 ml) of carrier ampholytes with a pH range of 3~6 was used. O—O, HPRT activity; ---, pH.

Fig. 7. Effect of pH on the Activity.

The enzyme activity was measured under the standard conditions, except that pH was altered as indicated. □—□, phosphate buffer; O—O, Tris-HCl buffer; Δ—Δ, glycine buffer.

consist of three subunits.

**FIG. 6.** Determination of Isoelectric Point.

**FIG. 7.** Effect of pH on the Activity.
TABLE III. SUBSTRATE SPECIFICITY
The enzyme activity was measured under the standard conditions, except that the purine bases were added as indicated.

<table>
<thead>
<tr>
<th>Substrate (0.35 mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanine</td>
<td>100</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>95.2</td>
</tr>
<tr>
<td>Xanthine</td>
<td>0.09</td>
</tr>
<tr>
<td>Adenine</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

The enzyme could be stored without loss of the activity for at least a month in 50 mM Tris-HCl buffer (pH 7.8) containing 1 M KCl and 10 mM MgCl₂ at 4°C. The activity was lost completely if the enzyme was kept in the buffer without the salts at 4°C for 4 days.

Substrate specificity
The activities of the enzyme to hypoxanthine, guanine, xanthine and adenine were tested. Hypoxanthine and guanine were found to be good substrates and the enzyme showed almost the same activity to these purine bases. The enzyme was much less active to xanthine and had no activity to adenine (Table III). The similar substrate specificity has been reported for the enzyme from other sources.⁵,⁶

Effect of substrate concentration
Fig. 8 shows double-reciprocal plots of reaction velocities of the enzyme against concentrations of hypoxanthine, guanine or PRPP.

Enzyme reactions were carried out at 30°C at pH 8.0 in the presence of 3 mM MgCl₂ and excess of the co-substrate. Apparent Km values obtained were 1.6 × 10⁻⁶, 2.7 × 10⁻⁶ and 6.3 × 10⁻⁵ M for hypoxanthine, guanine and PRPP, respectively. Although the specific activity of the purified enzyme is several times higher than those reported for the enzyme from other sources, the Km values observed for this enzyme are similar to the values for the other enzymes.⁴,⁸

Effect of nucleotides and pyrophosphate
Effect of various purine nucleotides on the activity is shown in Table IV. All nucleotides tested caused appreciable inhibition and the effects of guanosine mono-, di- and triphosphates were the most intensive among these nucleotides. The inhibitions caused by any series of purine tri-, di- and mononucleotides were stronger in this order.

Double reciprocal plots of activities against PRPP concentrations in the presence or absence of various purine nucleotides and pyrophosphate are shown in Fig. 9. All nucleotides tested caused an increase in the apparent Km values without affecting the Vmax, thus they are apparently competitive with respect to PRPP. Ki values calculated from the data were 1.3 × 10⁻⁴, 9.6 × 10⁻⁴ and 8.0 × 10⁻⁶ M for IMP, ATP and GTP, respectively. Inhibition by pyrophosphate was also apparently

Fig. 8. Double-Reciprocal Plots of the Initial Velocity against the Substrate Concentration.
The enzyme activity was measured under the standard conditions except the substrate concentration.
A: PRPP, 1.2 mM; ○—○, hypoxanthine; ●—●, guanine. B: hypoxanthine, 0.56 mM; ○—○, PRPP.
TABLE IV. EFFECT OF PURINE NUCLEOTIDES ON HPRT ACTIVITY

The enzyme activity was measured under the standard conditions, except that the purine nucleotides were added as indicated.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 mm</td>
</tr>
<tr>
<td>none</td>
<td>100</td>
</tr>
<tr>
<td>AMP</td>
<td>—</td>
</tr>
<tr>
<td>ADP</td>
<td>—</td>
</tr>
<tr>
<td>ATP</td>
<td>—</td>
</tr>
<tr>
<td>IMP</td>
<td>69.3</td>
</tr>
<tr>
<td>IDP</td>
<td>62.0</td>
</tr>
<tr>
<td>ITP</td>
<td>56.2</td>
</tr>
<tr>
<td>GMP</td>
<td>34.9</td>
</tr>
<tr>
<td>GDP</td>
<td>24.2</td>
</tr>
<tr>
<td>GTP</td>
<td>19.0</td>
</tr>
</tbody>
</table>

FTC. 9. Competitive Inhibition of HPRT by Nucleotides and Pyrophosphate.

The enzyme activity was measured under the standard conditions, except that the concentration of PRPP was altered and nucleotides and pyrophosphate were added as indicated. ○, control; ●—●, GTP (0.04 mM); △—△, ATP (1.5 mM); □—□, IMP (0.2 mM); ■—■, pyrophosphoric acid (0.2 mM).

competitive with respect to PRPP, Ki value being $2.5 \times 10^{-4}$ M.

It may be interesting that the activity of HPRT is subjected to the competitive regulation with respective to levels of the substrate, PRPP, and the products, nucleotides and pyrophosphate. The co-substrates, hypoxanthine and guanine, have nothing to do with the regulation. Because the purine bases are substrates not only for the anabolic but also for the catabolic pathways, the regulatory mechanism would have employed the exclusive substrate, PRPP, as a regulatory signal for the anabolic pathway. If the changes in the growth phase or in the medium conditions, which caused the changes in the metabolic pathways of hypoxanthine, brought about changes in intracellular concentrations of these nucleotides and of PRPP, the activity of HPRT may be changed in vivo and the regulation may result in the changes in the pathways observed. Effect of the growth conditions on intracellular concentrations of the nucleotides and PRPP, therefore, has remained to be studied to elucidate the regulation of hypoxanthine metabolism in this organism.

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