Purification and Properties of Purine Nucleoside Phosphorylase from Salmonella typhimurium*  

(Received for publication, July 21, 1972)

Benjamin C. Robertson‡ and Patricia A. Hoffee§

From the Department of Microbiology, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15213

SUMMARY

Purine nucleoside phosphorylase (inosine + P\textsubscript{i} → hypoxanthine + α-D-ribose 1-phosphate, EC 2.4.2.1) from Salmonella typhimurium LT-2 has been purified 230-fold. High speed sedimentation equilibrium studies showed that the molecular weight of the native enzyme was 141,000. A molecular weight of 130,000 ± 10% was determined by Sephadex G-150 filtration. Disc gel electrophoresis of the enzyme in the presence of sodium dodecyl sulfate revealed a subunit molecular weight of 23,500, indicating that the enzyme is composed of six subunits. The purified enzyme was found to be specific for the purine ribonucleosides and deoxyribonucleosides, inosine, deoxyinosine, guanosine, deoxyguanosine, adenosine, and deoxyadenosine. The Michaelis constants for inosine, deoxyinosine, and phosphate ion were 50 μM, 47 μM, and 0.37 mM, respectively.

The purification of purine nucleoside phosphorylase from Bacillus cereus var. terminalis has been reported by Gardner and Kornberg (1). The enzyme from both vegetative cells and spores has been shown to catalyze the phosphorolysis of inosine, deoxyinosine, guanosine, and deoxyguanosine, but not of adenosine or deoxyadenosine. Genetic evidence from studies with Salmonella typhimurium and Escherichia coli, however, indicate that the enzymes from these organisms are specific for all six of the above purine ribonucleosides and deoxyribonucleosides. Karlström (2) reported that mutants of E. coli which lose purine nucleoside phosphorylase (293 nm. An extinction coefficient of 12.5 was used. One unit of activity is defined as the number of nanomoles of uric acid formed per min at 26°C.

The above findings, a study was undertaken to determine whether only one enzyme was induced in S. typhimurium and if it was capable of catalyzing the phosphorolysis of all six purine nucleosides. This paper reports some of the physical and catalytic properties of the enzyme purified during the investigation.

EXPERIMENTAL PROCEDURE

Materials

Commercial preparations were used unless otherwise specified. Inosine, deoxyinosine, guanosine, deoxyguanosine, adenosine, and deoxyadenosine were obtained from Calbiochem; xanthine oxidase from Boehringer and Sons, New York; calcium phosphate gel, acrylamide, N,N'-methylenebisacrylamide, and N,N',N'',N'''-tetramethylenediamine from Bio-Rad Laboratories; Coomassie blue from Allied Chemicals, Morristown, N. J.; ribonuclease A, chymotrypsinogen A, ovalbumin, and fructose diphosphate aldolase from Pharmacia; carboxypeptidase from Nutritional Biochemicals Corp. Deoxyribose 5-phosphate aldolase was prepared from S. typhimurium by the method of Hoffee (6).

Methods

S. typhimurium LT-2 strain PH 4021 (4) was used as enzyme source. This strain requires 2 μg per ml of thymine for growth and is deficient in deoxyribose 5-phosphate aldolase, making it phenotypically constitutive for purine nucleoside phosphorylase (7-10).

Growth of Organism—S. typhimurium strain PH 4021 was grown to late log phase in Casamino acids buffered with potassium phosphate at pH 7.0 and containing 10 μg per ml of thymine. The cells were then centrifuged and washed in 1 mM ethylenediaminetetraacetate (EDTA), pH 7.4, and stored at -10°C.

Assay of Purine Nucleoside Phosphorylase—Purine nucleoside phosphorylase was assayed by the coupled xanthine oxidase method of Kalekar (11). One milliliter of reaction mixture contained inosine, 0.5 μmole; potassium phosphate buffer, pH 7.5, 50 μmole; and xanthine oxidase, 0.02 unit. The increase in absorbance, due to the formation of uric acid, was measured at 293 nm. An extinction coefficient of 12.5 was used. One unit of activity is defined as the number of nanomoles of uric acid formed per min at 26°C.

A modification of the Park-Johnson method for glucose determination was employed (12) when purine nucleoside phosphorylase was assayed for substrate specificity. The reaction...
mixture, 0.5 ml, contained 20 mm glycine HCl at pH 7.5, 10 mm sodium arsenate, and 10 mm substrate. Enzyme was added to each of three tubes containing 0.5 ml of reaction mixture. The tubes were placed in a 37° water bath and incubated for 0, 1, and 2 min. The reaction was stopped by placing the tubes in boiling water for 1 min. Ribose or deoxyribose was used as a standard.

One unit of activity is defined as the number of micromoles of substrate converted to reducing sugar per min at 37°.

**Protein Determination**—Protein was determined by the method of Lowry et al. (13) or that of Bücher (14) with fructose diphasate aldolase purified from rabbit muscle as a standard, or by the method of Waddell (15).

**Molecular Weight Determination Using High Speed Sedimentation Equilibrium**—Purine nucleoside phosphorylase which had a specific activity of 160,000 was examined at a concentration of 0.22 mg per ml in a Spinco model E analytical centrifuge in 100 mM KCl plus 5 mM potassium phosphate at pH 7.5 by the method of Yphantis (16) and Raleigh interference optics. The enzyme solution was spun at 24,630 rpm for 23 hours at 14°. In the calculation of molecular weight, a partial specific volume of 0.75 was assumed for the purified enzyme.

**Molecular Weight Determination by Filtration on Sephadex G-150**—A column, 2.5 × 40.3 cm, of Sephadex G-150 packed in 250 ml potassium phosphate buffer, pH 7.0, 10 mm in 2-mercaptoethanol, was eluted under a pressure of 15 cm of water at 4°. The column was calibrated with 10 mg each of ribonuclease, chymotrypsinogen, ovalbumin, and FDP aldolase dissolved in equilibrating buffer. Purine nucleoside phosphorylase was added separately to the column and eluted with equilibrating buffer. The column was calibrated with 10 mg each of ribonuclease, chymotrypsinogen, ovalbumin, and FDP aldolase dissolved in equilibrating buffer. Purine nucleoside phosphorylase was added separately to the column and eluted with equilibrating buffer. The molecular weight of purine nucleoside phosphorylase was calculated according to the procedure of Andrews (17).

**Submit Molecular Weight by Gel Electrophoresis**—Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate was performed according to the method of Weber and Osborn (18). Ten micrograms each of bovine serum albumin, ovalbumin, carboxylypeptidase, chymotrypsigen, trypsin, and ribonuclease were used as standards. The mobility of each protein relative to the dye front was measured with a micro-comparator. A linear graph was obtained when the log molecular weight was plotted with respect to the relative mobility of the standards. The submit molecular weight of purine nucleoside phosphorylase was estimated from its mobility at various gel concentrations.

**RESULTS**

**Purification of Purine Nucleoside Phosphorylase**

**Crude Extracts**—Twenty-five grams of frozen *S. typhimurium* strain PH 4021 were ground with twice their wet weight of alumina. The mixture was suspended in 40 ml of triethanolamine HCl at pH 7.0, 1 μm in EDTA, and centrifuged for 45 min at 27,000 × g. The supernatant fluid was reserved for further purification (Table I).

**Protein Sulfate Treatment**—Of 1% protamine sulfate, 0.25 ml was added per ml of crude extract. After 5 min at 4°, the precipitate was removed by centrifugation.

**Ammonium Sulfate Fractionation**—The protamine sulfate supernatant fluid was brought to 45% ammonium sulfate saturation by the addition of 254 mg per ml of solid (NH₄)₂SO₄. After 30 min at 4°, the suspension was centrifuged as before. The precipitate was dissolved in 5 ml of 50% potassium phosphate at pH 7.5 and dialyzed overnight against 500 volumes of the same buffer.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total units</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>19</td>
<td>1,670,000</td>
<td>700</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>100</td>
<td>1,820,000</td>
<td>1,400</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>fraction I</td>
<td>19</td>
<td>1,030,000</td>
<td>3,350</td>
<td>4.8</td>
</tr>
<tr>
<td>CaPO₄-gel</td>
<td>20</td>
<td>550,000</td>
<td>18,000</td>
<td>26</td>
<td>33</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>4.5</td>
<td>545,000</td>
<td>77,000</td>
<td>110</td>
<td>32.5</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>fraction II</td>
<td>4.5</td>
<td>360,000</td>
<td>160,000</td>
<td>230</td>
</tr>
</tbody>
</table>

**Calcium Phosphate Gel Treatment**—Calcium phosphate gel, suspended in 5 mm potassium phosphate at pH 7.5, was added to the dialyzed fraction from the previous step until all purine nucleoside phosphorylase activity was absorbed. Since different preparations of calcium phosphate gel vary in absorption properties, a series of test tubes containing a known amount of purine nucleoside phosphorylase was titrated with increasing amounts of calcium phosphate gel to determine the amount necessary to absorb 95% of the enzyme. The suspension was centrifuged and the supernatant fluid discarded. The pellets were washed several times with 10 mm potassium phosphate at pH 7.5.

**Physical Properties of Purine Nucleoside Phosphorylase**

**Molecular Weight Determination**—The molecular weight of purine nucleoside phosphorylase was determined using the sedimentation equilibrium method of Yphantis (16). The molecular weight, calculated by assuming a partial specific volume of 0.75 for purified purine nucleoside phosphorylase, was 141,000.

**Table I**

<p>| Purification of purine nucleoside phosphorylase from <em>Salmonella typhimurium</em> |
|-------------------------------|-------------|---------|---------|----------|---------|
| Enzyme assays were performed as described under “Methods,” using inosine as substrate. |</p>
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total units</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>91</td>
<td>1,670,000</td>
<td>700</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>100</td>
<td>1,820,000</td>
<td>1,400</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>fraction I</td>
<td>19</td>
<td>1,030,000</td>
<td>3,350</td>
<td>4.8</td>
</tr>
<tr>
<td>CaPO₄-gel</td>
<td>20</td>
<td>550,000</td>
<td>18,000</td>
<td>26</td>
<td>33</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>4.5</td>
<td>545,000</td>
<td>77,000</td>
<td>110</td>
<td>32.5</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>fraction II</td>
<td>4.5</td>
<td>360,000</td>
<td>160,000</td>
<td>230</td>
</tr>
</tbody>
</table>
The molecular weight determined by gel filtration on a Sephadex G-150 column was 130,000 ± 10%.

Subunit Molecular Weight by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—A subunit molecular weight of 23,500 was determined on a 9% polyacrylamide gel in the presence of sodium dodecyl sulfate by the method of Weber and Osborn (18). The same value was obtained with 10 and 8% gels. Since only a single band appeared on the gels at each concentration, it appears that the subunits have either identical or very similar molecular weights.

Chemical Properties of Purine Nucleoside Phosphorylase

Effect of Substrate—The effects of inosine and deoxyinosine concentration on enzyme activity were determined in 50 mM potassium phosphate at pH 7.5. The Michaelis constants determined by the method of Lineweaver and Burk (19) are 50 and 47 μM for inosine and deoxyinosine, respectively. The effect of phosphate on enzyme activity was determined in 5 mM glycine HCl at pH 7.5 containing 0.5 mM inosine. A Michaelis constant of 0.37 mM was calculated for phosphate.

Effect of pH—Enzyme activity of purine nucleoside phosphorylase was assayed over the pH range 5.5 to 8.5 in 50 mM potassium phosphate buffers by the coupled xanthine oxidase assay of Kalckar (11). Optimum activity was observed at pH 7.5 with a rapid decrease in activity both below and above this value.

Effect of Cations—No significant inhibition of enzyme activity was observed with the chloride salts of the cations Zn++, Mg++, Ni++, or Na++ at concentrations up to 100 μM. (Above this concentration some cations precipitated.) Cobalt, however, had a stimulatory effect of 1.65-fold at 1 mM.

Substrate Specificity—Purified purine nucleoside phosphorylase was capable of catalyzing the conversion of inosine, deoxyinosine, guanosine, deoxyguanosine, adenosine, and deoxyadenosine to their respective bases and pentose 1-phosphates. Deoxyinosine showed maximum activity and was twice as active as the other substrates (4). No activity was observed with the pyrimidine nucleosides uridine, cytidine, deoxyuridine, or deoxycytidine.

To determine whether more than one purine nucleoside phosphorylase is present in wild type cells, crude extracts from cells grown in the presence of deoxyribose or inosine were assayed for their activity toward the ribonucleoside, inosine, and the deoxyribonucleoside, deoxyinosine (Table II). The deoxyinosine to inosine activity ratio of 2.2 was observed in uninduced cells, cells induced by inosine or deoxyribose, and with the purified enzyme. This suggests that only one enzyme in S. typhimurium is responsible for catalyzing the phosphorolysis of purine ribo- and deoxyribonucleosides.

### DISCUSSION

Purine nucleoside phosphorylase has been purified about 230-fold from a strain of *S. typhimurium* which contains fully induced levels of the enzyme. This enzyme is significantly different from the enzymes isolated from vegetative cells and spores of *Bacillus cereus* T. The purified enzyme from vegetative cells of *Bacillus* has been shown to have a molecular weight between 80,000 (18) and 92,000 (19). Gulpin and Sadoff (20) have reported that the *Bacillus* enzyme is composed of four subunits with a molecular weight of 24,000. Purine nucleoside phosphorylase from *S. typhimurium*, however, has been shown by ultracentrifuge and gel filtration techniques to have a molecular weight between 130,000 and 141,000. Gel electrophoresis in the presence of sodium dodecyl sulfate suggests that this enzyme is composed of six subunits with a molecular weight of 23,500.

Substrate specificity studies reported here clearly show that the enzyme from *S. typhimurium* is capable of catalyzing the phosphorolysis of the purine ribonucleosides, inosine, guanosine, and adenosine, as well as of their corresponding deoxyribonucleoside derivatives. It appears that only one enzyme is present with the above specificity since the ratio of activity between the deoxyribonucleoside, deoxyinosine, and the ribonucleoside, inosine, remains constant at 2.2 throughout the purification. The same ratio exists whether wild type cells are induced by a purine ribonucleoside or by deoxyribose. Robertson *et al.* (4) have previously shown that when wild type *S. typhimurium* is grown in the presence of a purine ribonucleoside, purine nucleoside phosphorylase, and phosphodeoxyribomutase are coordinately induced. These enzymes are specific for both ribo and deoxyribo derivatives (3). On the other hand, when cells

---

**Table II**

Deoxyinosine to inosine activity ratios using crude extracts and purified enzyme

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Deoxyinosine</th>
<th>Inosine</th>
<th>Ratio doi1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninduced cells</td>
<td>151</td>
<td>70</td>
<td>2.2</td>
</tr>
<tr>
<td>Inosine-induced cells</td>
<td>835</td>
<td>384</td>
<td>2.2</td>
</tr>
<tr>
<td>Deoxyribose-induced cells</td>
<td>1,330</td>
<td>655</td>
<td>2.1</td>
</tr>
<tr>
<td>Purified enzyme</td>
<td>357,000</td>
<td>167,000</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Enzyme activity is reported as nanomoles of substrate converted per min per mg of protein at 26°.
are grown in the presence of deoxyribose, four enzymes, each possessing a specificity for a deoxy compound, are induced. These include thymidine phosphorylase, purine nucleoside phosphorylase, phosphodeoxyribo nucleosidase, and deoxyribose 5-phosphate aldolase. The demonstration that the purified enzyme is specific for both purine ribo and deoxyribonucleosides is consistent with its unusual induction pattern.

REFERENCES

Purification and Properties of Purine Nucleoside Phosphorylase from Salmonella typhimurium
Benjamin C. Robertson and Patricia A. Hoffee


Access the most updated version of this article at http://www.jbc.org/content/248/6/2040

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/248/6/2040.full.html#ref-list-1