Purification and Substrate Specificity of Pyrimidine Nucleoside Phosphorylase from Haemophilus influenzae*

JOHN J. SCOCCA

From the Department of Biochemistry, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland 21205

SUMMARY

A single pyrimidine nucleoside phosphorylase was found to comprise both uridine phosphorylase and thymidine phosphorylase activities in Haemophilus influenzae. The enzyme has been purified 400-fold, and was stabilized by dithiothreitol and glycerol. Evidence that a single enzyme cleaves both ribonucleosides and deoxynucleosides was obtained from gel electrophoresis, heat inactivation studies, and kinetic experiments.

The relative $V_{	ext{max}}$ values (at pH 7.4) for the nucleosides tested were: uridine, 1.0; 5-bromodeoxyuridine, 0.75; 5-bromouridine, 0.40; 5-methyluridine, 0.27; thymidine, 0.21; and deoxyuridine, 0.12. Uracil arabinoside was split approximately 10% as well as uridine. Cytidine was not cleaved either by crude extracts or by the purified enzyme.

The pyrimidine nucleoside phosphorylases from a variety of sources have been resolved into a thymidine phosphorylase (thymidine:orthophosphate deoxyribosyltransferase, EC 2.4.2.4) absolutely specific for deoxyribosyl substrates, and a uridine phosphorylase (uridine:orthophosphate ribosyltransferase, EC 2.4.2.3) (1–5). In many cases, the latter enzyme retains significant activity in the cleavage of deoxyuridine or thymidine (2–5); the major exception appears to be the uridine phosphorylase from Escherichia coli, which was reported to be absolutely specific for ribonucleosides (6). This enzyme will tolerate rearrangement at position 2', however; Tono and Cohen (7) demonstrated that uracil arabinoside was cleaved by uridine phosphorylase from E. coli. More recently, it was reported that in Bacillus stearothermophilus, and possibly in Bacillus subtilis, a single pyrimidine nucleoside phosphorylase possesses both thymidine- and uridine-cleaving activities (8).

In the course of studies on the utilization of exogenous pyrimidine nucleosides by Haemophilus influenzae, the uridine phosphorylase activity was purified and characterized. The enzyme was found to possess thymidine phosphorylase activity, and the ratio of specific activities toward ribonucleosides and deoxynucleosides had remained unchanged during fractionation. This paper presents evidence that a single pyrimidine nucleoside phosphorylase comprises both uridine and thymidine phosphorylase activities in this bacterial species.

MATERIALS AND METHODS

Uracil arabinoside ([β-D-arabinofuranosyl]uracil), prepared by the enzymatic deamination of the corresponding cytosine nucleoside, was the generous gift of Dr. C. D. Steuart of Baltimore City Hospitals, Baltimore, Maryland. DEAE-cellulose (DE-52), obtained from H. Reeve Angel and Company, Inc., New York, New York, was treated according to the manufacturer’s suggestions before use. Calcium phosphate gel was prepared by the method of Kelin and Hartree (9). Dowex 1-X8, (200 to 400 mesh) was washed successively with 1 N NaOH, 1 N HCl, and H2O, until the effluents were free of ultraviolet-absorbing material. Tris base was recrystallized from aqueous acetone; orcinol was recrystallized from hot benzene. Norit A was washed and degassed in 5 N HCl, washed with H2O to neutrality, and stored as a 20% (v/v) suspension. Dialysis tubing was boiled in 2% Na2CO3, washed with H2O, and stored in 1 mM EDTA in the cold. Polyacrylamide gel electrophoresis at pH 8.9 was done by the method of Davis (10), except that the sample gel was omitted. Gels were stained for protein using Coomassie brilliant blue. Other chemicals were obtained from standard commercial sources.

Protein was measured by the method of Lowry et al. (11) with bovine serum albumin as the standard. Ribose was measured by the orcinol reaction (12). Inorganic phosphate was assayed by the method of Lowry and Lopez (13). Haemophilus influenzae strain Rd was from the collection of Dr. R. M. Herriott of this Department. Inocula were prepared by adding sterile glycerol to logarithmically growing cultures to a final concentration of 15% (v/v), and freezing aliquots at −70°C.

For enzyme preparation, bacteria were grown overnight at 37°C in 3.7% Brain Heart Infusion (Difco) supplemented with 1 mg per liter of hemin and 0.4 mg per liter of DPN. Cells were harvested by centrifugation and washed with 0.1 culture volume of cold 0.15 M NaCl.

Pyrimidine nucleoside phosphorylase activity was assayed by spectrophotometric measurement of the free pyrimidine base produced (1). In Assay I, the reaction mixture (0.35 ml) contained 0.14 M KPO4 buffer, pH 8.2, 5 mM dithiothreitol, 12 mM uridine, and enzyme. Incubation was for 20 min at 37°C; the reaction was stopped by addition of 3 ml of 0.01 N NaOH.

* This study was supported in part by United States Atomic Energy Commission Contract AT(30-1)1371 (NYO-1371-65).

(Received for publication, June 25, 1971)
Cleavage of deoxynucleosides was assayed similarly, except that the pH was 7.4, and dithiothreitol was omitted. One unit of enzyme produced 1 μmole of base under these conditions; the assay gave a linear response over the range of 0.02 to 0.1 unit.

Assay II was used when initial rates were measured. The reaction mixtures (0.35 ml) contained 0.14 M KPO₄ buffer, pH 7.4, 1.2 mM nucleoside, and enzyme. The reaction was stopped by the addition of 1.5 ml of 0.02 N NaOH after incubation at 37°C for times between 2 and 5 min. Several time points were taken to ensure that the initial rate was in fact being measured.

For either assay, the absorbance was determined and corrected for a blank which had been stopped at zero time. The wave length and extinction coefficients used for the various bases were (1): uracil, 290 nm, ε₉₄ = 5.7; 5-methyluracil (thymine), 300 nm, ε₉₄ = 37; 5-bromouracil, 312 nm, ε₉₄ = 2.2 (determined in this laboratory).

RESULTS

Purification of Uridine Phosphorylase

All centrifugations were at 10,000 × g for 20 min, and all operations were carried out at 0–4°C. All solutions used in the purification contained 2 mM dithiothreitol.

Preparation of Crude Extract—To 40 g of packed H. influenzae cells, 80 g of olumin A 305 (Sigma) were added, and the mass was triturated until a homogeneous tacky paste was obtained. The homogenate was extracted with 100 ml of 0.02 M potassium phosphate buffer, pH 7.4, and centrifuged. The pellet was re-extracted with 80 ml of the same buffer and centrifuged. The pooled supernatant solutions constituted Fraction I, the crude extract.

Precipitation with Streptomycin Sulfate—Solutions of streptomycin sulfate (10% in H₂O) were prepared just before use. Fraction I (230 ml) was stirred while 0.2 volume of streptomycin sulfate solution was added dropwise. The suspension was stirred for an additional 30 min; the precipitate was centrifuged and discarded. The supernatant solution was Fraction II.

Treatment with Acid and Adsorption to Calcium Phosphate Gel—Sodium acetate buffer (1 M), pH 4.8, was added dropwise with stirring to Fraction II to a final concentration of 0.05 M. Stirring was continued for 20 min; the suspension was centrifuged and the precipitate was discarded. Calcium phosphate gel (50 mg of solids per ml) was added to the supernatant solution (0.15 ml of gel per ml); the suspension was stirred for 10 min and centrifuged, and the packed gel was discarded. Uridine phosphorylase was adsorbed by adding 0.2 ml of gel per ml of supernatant solution, stirring for 10 min, and centrifuging as before. The packed gel was washed twice with 50-ml portions of cold H₂O, and collected by centrifugation. Enzymatic activity was eluted by suspending the gel in 50 ml of 0.1 M potassium phosphate buffer, pH 7.4, stirring for 10 min, and centrifuging. The packed gel was re-extracted with 25 ml of the same buffer, stirred, and centrifuged as before. The combined supernatant solutions constituted Fraction III.

Fractionation with Ammonium Sulfate—A solution of 1 M EDTA-2 mM dithiothreitol saturated with (NH₄)₂SO₄ at 25°C was used. To 75 ml of Fraction III, 49.5 ml of ammonium sulfate solution were added slowly with continuous stirring. Stirring was continued for 20 min; the precipitate was collected by centrifugation and discarded. To the supernatant solution, 13.0 ml of ammonium sulfate solution were added; the mixture was stirred for 20 min and centrifuged. The precipitate (Fraction IV) was dissolved in 10 ml of 0.02 M potassium phosphate buffer (pH 7.4) containing 2 mM dithiothreitol and 20% (v/v) glycerol. In all subsequent operations, the buffers used contained both dithiothreitol and glycerol at these concentrations.

Chromatography on DEAE-cellulose—A column (1.4 × 25 cm) of DEAE-cellulose was equilibrated with 0.02 M Tris-Cl, pH 7.4 (Buffer I). Fraction IV, previously dialyzed for 18 hours against 15 volumes of this buffer, was applied to the column at a flow rate of 0.02 ml per min. The column was washed with 50 ml of Buffer I, and eluted with a linear gradient established between 400 ml of 0.05 M KCl in Buffer I and 400 ml of 0.4 M KCl in the same buffer. The enzymatic activity appeared after 300 ml of eluant had passed through the column. The active fractions were pooled to give Fraction V.

Chromatography on Hydroxylapatite—Fraction V was dialyzed for 18 hours against 0.01 M potassium phosphate buffer, pH 6.8, and applied to a column of hydroxylapatite (1 × 5 cm) which had been equilibrated with the same buffer. The column was washed with 50 ml of Buffer I, and eluted with a linear gradient established between 400 ml of 0.05 M KCl in Buffer I and 400 ml of 0.4 M KCl in the same buffer. The enzymatic activity appeared after 200 ml of eluant had passed through the column. The active fractions were pooled to give Fraction VI.

The results of this purification procedure are summarized in Table I.

Stability of Enzyme—Uridine phosphorylase from H. influenzae was found to be quite labile during the course of purification. The use of dithiothreitol and glycerol was necessary to obtain acceptable recoveries in the various steps; in addition, repeated cycles of freezing and thawing inactivated the enzyme if phosphate was absent. When these precautions were taken, Fractions IV to VI were stable for 6 months when stored at −20°C.

Products of Reaction—Since P₁ stabilized the enzyme, it was important to establish that the reaction was a phosphorolysis and not a P₁-stabilized nucleoside hydrolysis reaction. To do this, the reaction products were separated on a column (0.9 × 10 cm) of Dowex 1-X8 (Cl⁻) (200 to 400 mesh). A scaled-up reaction mixture (0.5 ml) with sufficient enzyme (Fraction IV in glycerol-free buffer) to produce approximately 1 μmole of uracil was incubated for 60 min and diluted to 10 ml with 0.02 M KPO₄ buffer (pH 7.4). The reaction was stopped by the addition of 1.5 ml of 0.02 N NaOH after incubation at 37°C for times between 2 and 5 min. Several time points were taken to ensure that the initial rate was in fact being measured.
had passed through; uridine emerged immediately afterward. Ribose (1.25 μmoles), as a phosphate ester, eluted with 0.1 M KCl. This material released 1.3 μmoles of inorganic phosphate when incubated with 0.1 N HCl for 60 min at 37°, conditions sufficient to hydrolyze ribose-1-P. The stoichiometry and nature of the products demonstrate that the enzyme is a uridine phosphorylase.

Table II

**Substrate specificity**

Activity was measured by Assay I, with 0.1 to 0.2 unit of Fraction VI as the enzyme. Nucleosides were included at the indicated concentrations; incubation was for 20 min at 37°. Reaction mixtures containing cytidine, adenosine, or guanosine were acidified, the nucleosides were adsorbed to Norit, the mixtures were centrifuged, and ribose was determined in the supernatant solution. All other reaction mixtures were stopped by the addition of 0.01 N NaOH, and the free pyrimidine base was determined spectrophotometrically.

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Concentration</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>max</td>
<td>units/ml</td>
</tr>
<tr>
<td>Uridine</td>
<td>8.3</td>
<td>20.0</td>
</tr>
<tr>
<td>5-Bromouridine</td>
<td>5.6</td>
<td>4.1</td>
</tr>
<tr>
<td>5-Methyluridine</td>
<td>5.6</td>
<td>3.8</td>
</tr>
<tr>
<td>Uracil arabinoside</td>
<td>1.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td>10.0</td>
<td>5.4</td>
</tr>
<tr>
<td>Thymidine</td>
<td>8.0</td>
<td>5.3</td>
</tr>
<tr>
<td>5-Bromodeoxyuridine</td>
<td>12.0</td>
<td>32.0</td>
</tr>
<tr>
<td>Adenosine</td>
<td>6.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Guanosine</td>
<td>6.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Cytidine</td>
<td>0.0</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table II presents the results of a study of the abilities of various nucleosides to serve as substrates. The enzyme cleaved all the 4-keto pyrimidine nucleosides tested, regardless of the nature of the pentose moiety. Purine- and 4-amino pyrimidinenucleosides were not split. Assays of the crude extract and of less purified fractions showed that the ratio of uridine phosphorylase activity to thymidine phosphorylase activity had remained constant at a value of approximately 3.8 throughout the purification. The effects of varying the substituent at position 5 of the pyrimidine ring were dissimilar for ribo- and deoxyribonucleosides; uridine was the best ribonucleoside substrate tested, whereas 5-bromodeoxyuridine was the deoxyribo nucleoside most readily cleaved. This pattern of substrate specificity did not change during purification.

**Effect of pH**—The initial rates of phosphorolysis of ribonucleosides and of uracil arabinoside were maximal at pH 7.4. The optimum pH found with deoxynucleoside substrates was slightly more acidic, with a maximum at pH 6.9. At pH 7.4, deoxyribo nucleoside cleavage proceeded at 75% of the rate observed at pH 6.9.

Two alternatives could explain the observed association between nucleoside and deoxynucleoside phosphorylase activities; the enzyme from *H. influenzae* could be a single pyrimidine nucleoside phosphorylase with limited specificity for the pentose moiety of the substrate, or the purification procedure used could have failed to resolve two similar but distinct enzymes.

**Electrophoresis on Polyacrylamide Gel**—In order to obtain direct evidence on the number of nucleoside phosphorylase activities present in the purified preparation, Fraction VI was analyzed by gel electrophoresis, and the distribution of thymi-
the enzyme from heat inactivation. Table III presents data obtained at different temperatures, and in a phosphate-free solvent. Further evidence for a single enzyme was obtained by studying the abilities of various nucleoside substrates to protect the enzyme from heat inactivation. Table III presents data showing that some, but not all, ribonucleoside substrates protected both uridine and thymidine phosphorylase activities to the same extent. Curiously, the pyrimidine deoxynucleosides tested failed to protect either activity. This failure is not related to the relative susceptibilities of the nucleosides to enzymatic cleavage (cf. Table II).

Table III

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Enzyme remaining</th>
<th>Thymidine phosphorylase</th>
<th>Uridine phosphorylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Uridine</td>
<td>6</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>5-Methyluridine</td>
<td>23</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Thymidine, deoxyuridine, 5-Br-uridine, 5-Br-deoxyuridine, Cytidine</td>
<td>5.7</td>
<td>5.7</td>
<td></td>
</tr>
</tbody>
</table>

Heat Inactivation Studies—The rates of inactivation of uridine and thymidine phosphorylase activities were found to be identical (Fig. 2). The ratio between the two activities was unaltered during the loss of 90% of the input activity. Similar results were obtained at different temperatures, and in a phosphate-free solvent. Further evidence for a single enzyme was obtained by studying the abilities of various nucleoside substrates to protect the enzyme from heat inactivation. Table IV presents data showing that some, but not all, ribonucleoside substrates protected both uridine and thymidine phosphorylase activities to the same extent. Curiously, the pyrimidine deoxynucleosides tested failed to protect either activity. This failure is not related to the relative susceptibilities of the nucleosides to enzymatic cleavage (cf. Table II).

**Table IV**

Summary of kinetic parameters of pyrimidine nucleoside phosphorylase with various nucleoside substrates

Initial rates were measured at pH 7.4 with Assay II, described under "Materials and Methods." Fraction VI was used as the enzyme source; the measured $V_{\text{max}}$ with uridine as substrate was 83.5 nmol per min per mg of protein. The data were treated by the method of Lineweaver and Burk (14).

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>$K_m$ (mM)</th>
<th>Relative $V_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridine</td>
<td>0.24</td>
<td>1.0</td>
</tr>
<tr>
<td>5-Methyluridine</td>
<td>0.07</td>
<td>0.27</td>
</tr>
<tr>
<td>5-Bromouridine</td>
<td>0.03</td>
<td>0.40</td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td>0.13</td>
<td>0.12</td>
</tr>
<tr>
<td>Thymidine</td>
<td>0.11</td>
<td>0.21</td>
</tr>
<tr>
<td>5-Bromodeoxyuridine</td>
<td>0.10</td>
<td>0.74</td>
</tr>
</tbody>
</table>

* The equation used (16) was:

$$v = \frac{V_{\text{max}}(S/K_m) + V'_{\text{max}}(S'/K'_m)}{1 + [S]/K_m + [S']/K'_m}$$

**Table V**

Competitive inhibition between ribonucleoside and deoxynucleoside substrates

The reaction mixtures were 1 mM in each of the indicated substrates; the pH was 7.4, and the enzyme used was Fraction VI (0.1 unit). Other conditions were those of Assay II.

<table>
<thead>
<tr>
<th>Substrate pair</th>
<th>Predicted rate</th>
<th>One enzyme</th>
<th>Two enzymes</th>
<th>Observed rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridine plus deoxyuridine</td>
<td>5.2</td>
<td>9.6</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>5-Bromouridine plus 5-bromodeoxyuridine</td>
<td>4.5</td>
<td>10.1</td>
<td>4.5</td>
<td></td>
</tr>
</tbody>
</table>

* Sum of the rates observed with substrates present singly.

Measurements of the initial rate of production of uracil or of 5-bromouracil from mixtures of the corresponding ribo- and deoxynucleosides were made and compared with the rates expected for a single enzyme or for two independent enzymes; the results are presented in Table V. The experimental values are in good quantitative agreement with the expectations for a single enzyme. Additional evidence on this point is presented in Fig. 3. In this experiment initial rates of cleavage of 5-bromodeoxyuridine as a function of uridine concentration were measured at two levels of 5-bromodeoxyuridine, and the results were plotted by the method of Dixon (15). Uridine inhibited the cleavage of 5-bromodeoxyuridine competitively, since the intersection point of the lines is at an ordinate value approximately equal to $1/V_{\text{max}}$; in addition the $K_i$ for uridine (0.25 mM) in this experiment agrees well with the $K_m$ for uridine (0.24 mM) obtained independently.

Discussion

The conclusion that a single pyrimidine nucleoside phosphorylase cleaves both uridine and thymidine in *H. influenzae* rests on several lines of evidence. First, the two activities maintained a constant ratio over a 400-fold purification, and were not resolved...
by analytical polyacrylamide gel electrophoresis. Second, the activities showed identical kinetics of heat inactivation, and were identically protected against heating by several nucleoside substrates. Third, kinetic studies with ribo- and deoxynucleoside substrate pairs gave results which conformed quantitatively with expectation for a single enzyme comprising both activities.

The pattern of substrate specificity observed is quite similar to that of the uridine phosphorylases purified from several mammalian sources, which were observed to retain significant activity toward deoxynucleosides (2–5). B. stearothermophilus was found to possess a single pyrimidine nucleoside phosphorylase which was 1.5 times as active with thymidine as with uridine (8); this ratio for the H. influenzae enzyme was 0.25. The enzyme described in the present paper shows a striking preference for 5-bromodeoxyuridine as a substrate; the initial rate observed with this substrate was 5 times that found with any other deoxy nucleoside. This is of interest in the light of the results of Fangman (17), who observed that strains of E. coli mutant in thymidine phosphorylase had retained the ability to degrade 5-bromodeoxyuridine to the corresponding base, although the ability to cleave thymidine had been lost. It is conceivable that the uridine phosphorylase of E. coli may possess significant activity toward halogenated pyrimidine deoxynucleosides.

The patterns of substrate specificity and of protection against heat inactivation observed in the present work suggest that the enzyme interacts with and is influenced by many structural features of the substrate; this property of the pyrimidine nucleoside phosphorylases has been emphasized by several authors (18, 19). It seems likely that these enzymes might furnish useful model systems for the study of the recognition of specific nucleic acid sequences by protein molecules.

REFERENCES

Purification and Substrate Specificity of Pyrimidine Nucleoside Phosphorylase from *Haemophilus influenzae*
John J. Scocca


Access the most updated version of this article at http://www.jbc.org/content/246/21/6606

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/246/21/6606.full.html#ref-list-1