Purification and Comparative Properties of a Pyrimidine Nucleoside Phosphorylase from *Bacillus stearothermophilus*  

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**SUMMARY**

The pyrimidine-nucleoside: orthophosphate ribosyltransferase (EC 2.4.2.2), or pyrimidine nucleoside phosphorylase, from *Bacillus stearothermophilus* has been purified and compared with similar enzymes from *Bacillus subtilis* and *Escherichia coli*. The enzyme is stable at 60°, appears to be induced by either uridine or thymidine, and has a molecular weight of approximately 78,000. Unlike nucleoside phosphorylases from most other sources, the thermophile enzyme catalyzes the phosphorolysis of both thymidine (apparent $K_m = 3.8 \times 10^{-4}$ M) and uridine (apparent $K_m = 2.5 \times 10^{-4}$ M) and, in the synthetic direction, does not distinguish between ribose 1-phosphate and deoxyribose 1-phosphate. It differs from *E. coli* and *B. subtilis* nucleoside phosphorylases in many properties including molecular weight, substrate specificity, thermostability, and induction properties.

The existence of thermophilic microorganisms in nature is a profound example of environmental influence on macromolecular evolution. Such cells have, presumably through mutation, developed means of stabilizing those proteins essential to life to high temperatures and, in many cases, rendered them dependent on relatively high temperatures. Only a few extensive studies have been carried out on enzymes purified from thermophilic bacteria; these include $\alpha$-amylase (1-3), glyceraldehyde 3-phosphate dehydrogenase (4, 5), and malate dehydrogenase (6). These studies indicate that the property of thermophilicity is induced by either uridine or thymidine, and has a molecular weight of approximately 78,000. Unlike nucleoside phosphorylases from most other sources, the thermophile enzyme catalyzes the phosphorolysis of both thymidine (apparent $K_m = 3.8 \times 10^{-4}$ M) and uridine (apparent $K_m = 2.5 \times 10^{-4}$ M) and, in the synthetic direction, does not distinguish between ribose 1-phosphate and deoxyribose 1-phosphate. It differs from *E. coli* and *B. subtilis* nucleoside phosphorylases in many properties including molecular weight, substrate specificity, thermostability, and induction properties.

**MATERIALS AND METHODS**

**Bacterial Strains**—In order to obtain reasonably high levels of the nucleoside phosphorylase of *B. subtilis*, a strain of this organism which requires uracil and thymine was used. This strain, *B. subtilis* GSY 568 (b), and its growth medium have been previously described (7). Mid-log cells of *E. coli* B were purchased from Miles Laboratories or grown as described for *B. subtilis* without addition of uracil and thymine. *B. stearothermophilus* strain NCA 10 was the source of the thermophile enzyme.

**Growth of *B. stearothermophilus***—Strain 10 was grown in 24-liter batches in a minimal medium, TCG (8), at 60° to an optical density of 420 nm of 1.2 as read on a Beckman DU spectrophotometer. The cells were chilled with ice, harvested by continuous flow centrifugation, washed once with 0.05 M Tris-HCl, pH 7.5, and stored at -20°.

**Preparation of Crude Extracts**—Mid-log cells were suspended in 0.05 M Tris-HCl (pH 7.5), 2 ml per g, and disrupted by three or four 15-second bursts with a Branson sonifier, model W 185 C. Cell debris was removed by centrifugation at 10,000 x g, and the supernatant solution was dialyzed overnight against 50 volumes of the same Tris buffer. When desired, the extracts were fractionated by the addition of ammonium sulfate to 75% saturation. The precipitate was collected, dissolved in a minimum of Tris buffer as above, and dialyzed against the same buffer for 4 to 8 hours.

**Enzyme Assays**—Nucleoside phosphorylase activity was measured by two different methods, both of which have been described (9).  
1. The optical method is based on the fact that at pH 12 the ratio of absorbances at 260 nm and 260 nm is a direct measure of the composition of a mixture of a pyrimidine base and its nucleoside. Standard curves were constructed for each base and its nucleoside. Reaction mixtures contained 0.5 $\mu$ mole of nucleoside, 75 $\mu$ moles of potassium phosphate buffer (pH 7.5), and extract in a final volume of 1.0 ml and were incubated for 15 min at 60° for the thermophile enzyme and at 37° for the mesophile enzymes unless otherwise specified. The reaction was terminated by the addition of 1.0 ml of cold 10% perchloric acid, and then the tubes were allowed to stand at 0° for at least 15 min followed by centrifugation to remove the precipitate. One milliliter of the supernatant solution was added to 1 ml of 1 N NaOH, and the absorbances at 260 and 290 nm were read.
2. The isotope method of assay involves measuring the degradative direction of the reaction. Incubation mixtures contained 0.25 $\mu$ mole of riboside including 0.5 $\mu$C of tritium-labeled riboside, 37.5 $\mu$ moles of potassium phosphate buffer (pH 7.5), and extract in a final volume of 0.5 ml. The incubation was carried...
Whatman 3MM paper strips, 57 out as in the previous assay and the reaction was stopped by placing the tubes in a boiling water bath for 5 min. The precipitate was removed by centrifugation, and 20–50 µl aliquots of the supernatant solution were spotted for electrophoresis on Whatman 3MM paper strips, 57 × 2 cm. Electrophoresis was carried out at 1500 to 2000 volts in 0.05 M sodium borate buffer, pH 9.5, for 2 hours.

For measuring the reaction in the synthetic direction, incubation mixtures contained 0.25 µmol of pyrimidine base containing 2 µC of tritium per µmol or 1 µC of 14C-labeled base per µmol; 37.5 µmoles of Tris-HCl buffer, pH 7.5; 0.25 µmol of pentose 1-phosphate in a final volume of 0.5 ml. Incubations and analyses were carried out as above.

**Induction Experiments**—Bacteria were grown in 500-ml shake flasks, each containing 250 ml of the appropriate minimal medium, at 37° or 60° to an absorbance of 0.3 to 0.4 as measured with a Bausch and Lomb Spectronic 20. The desired additions were made and incubation was continued for 1 hour. The cells were then harvested and washed once with 0.05 M Tris-HCl, pH 7.5, and crude extracts were prepared as described above. For this experiment a prototrophic strain of *B. subtilis* was employed, strain 168.

**Sucrose Density Gradient Centrifugation**—Linear gradients of sucrose from 5 to 20% made up in 0.05 M Tris-HCl, pH 7.5, were centrifuged in a SW 27 or SW 50 rotor in a Spinco model L2-65 preparative ultracentrifuge at 4°C. Upon termination of the run, the gradients were fractionated either by piercing the bottom of the tube and dripping out the contents or by the use of an Isco density gradient fractionator. With the Isco fractionator the first fractions correspond to the top of the gradient. The fractions were then analyzed as described.

**RESULTS**

Before initiating a detailed study of this thermophile enzyme, it seemed pertinent to establish, with crude extracts, whether basic differences exist between this enzyme (or enzymes) and comparable ones from mesophilic organisms. Of the two bacterial species chosen for comparison, *B. subtilis* represents a phylogenetically related organism. On the other hand, *E. coli* provides pyrimidine nucleoside phosphorylases which have previously been studied in some detail (10–13). The following two experiments illustrate that dramatic differences do indeed exist between the pyrimidine nucleoside phosphorylases of these organisms.

**Thermostability of Nucleoside Phosphorylases in Crude Extracts**

The relative heat stability of the crude thermophile nucleoside phosphorylase, compared with similar enzymes from other bacteria, is shown in Fig. 1. In contrast to the enzymes from *B. subtilis* and *E. coli*, the thermophile enzyme was relatively stable at 60°, the optimal growth temperature of the organism, but was rapidly inactivated at slightly higher temperatures. This phenomenon has been shown to be characteristic of several thermophile enzymes (14).

**Induction Properties**

Table I shows the data from an experiment designed to compare both the levels and the control of uridine phosphorylase and thymidine phosphorylase activities in *E. coli*, *B. subtilis*, and *B. stearothermophilus*. As previously shown by others (11, 12), the thymidine phosphorylase of *E. coli* is induced by high levels of thymidine. The uridine phosphorylase of this organism, however, does not appear to be induced by either uridine or thymidine, as evidenced by the constant specific activity seen under various growth conditions in Table I. In *B. subtilis* both the

![Graph](http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Present during Induction</th>
<th>Specific activity</th>
<th>U/Ta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TdR</td>
<td>Uridine</td>
<td>TdR</td>
</tr>
<tr>
<td><strong>B. stearothermophilus</strong></td>
<td>0.5</td>
<td>2.05</td>
<td>1.54</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2.35</td>
<td>1.83</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2.32</td>
<td>1.61</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.08</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>B. subtilis 108</strong></td>
<td>0.5</td>
<td>0.44</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.20</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.36</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.17</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>0.5</td>
<td>5.72</td>
<td>2.50</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.02</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>3.78</td>
<td>2.43</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.75</td>
<td>2.10</td>
</tr>
</tbody>
</table>

a Uridine phosphorylase/thymidine phosphorylase.
thymidine phosphorylase and the uridine phosphorylase were induced (2- to 3-fold) by thymidine, but neither was induced by uridine. The enzymes behaved similarly in \textit{B. stearothermophilus}; however, in this case induction (again 2- to 3-fold) resulted from exposure to either uridine or thymidine.

These data are suggestive that the three enzyme systems vary markedly with regard to both structure and regulation. The remainder of this report will thus be devoted to the purification and comparative properties of the thermophile enzyme.

\section*{Purification of Enzyme}

Uridine was employed as substrate in all enzyme assays throughout purification. One unit of enzyme activity is defined as that amount catalyzing the formation of 1 \( \mu \text{mole} \) of free base in 15 min under the conditions described for the optical assay.

\textbf{Step I}—The cells were suspended in 0.05 M Tris-HCl (pH 7.5), 2 ml per g, and disrupted with a Branson sonifier, model W-185-C. Cell debris was removed by centrifugation for 20 min at 27,000 \( \times \) g in a Sorvall RC2-B refrigerated centrifuge. The supernatant solution was termed the crude extract (Fraction 1).

\textbf{Step II}—To Fraction 1 was added 1 ml of 1 M MnCl\(_2\) per 20 ml of extract, dropwise at 4\(^\circ\)C with stirring. After 30 min in the cold with stirring, the precipitate was removed by centrifugation for 15 min at 27,000 \( \times \) g. The supernatant solution was dialyzed for 4 to 6 hours against 20 volumes of 0.05 M Tris-HCl (pH 7.5).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Fraction} & \textbf{Protein} & \textbf{Volume} & \textbf{Units/mg} & \textbf{Activity} \\
\hline
1. Crude & 4488 & 165 & 1155 & 0.25 & 0.64 \\
2. MnCl\(_2\) & 2208 & 120 & 720 & 0.32 & \\
3. DEAE-cellulose I & 288 & 390 & 702 & 2.43 & 0.25 \\
4. Ultrafiltration & 154 & 6.2 & 638 & 4.14 & 0.20 \\
5. Sephadex G-100 & 38 & 36 & 676 & 10.47 & \\
6. DEAE-cellulose II & 25.2 & 63 & 397 & 15.75 & 0.31 \\
7. Ultrafiltration & 12.2 & 4.2 & 306 & 25.08 & 0.41 \\
8. Sucrose gradient & 6.2 & 47 & 173 & 27.9 & 0.43 \\
\hline
\end{tabular}
\caption{Purification of pyrimidine nucleoside phosphorylase from \textit{B. stearothermophilus}}
\end{table}

If a precipitate had appeared, it was removed by centrifugation. The resulting supernatant solution was Fraction 2.

\textbf{Step III}—Fraction 2 was applied to a DEAE-cellulose column (2.5 \( \times \) 80 cm) which had previously been washed with acid and base (15) and equilibrated with 0.05 M Tris-HCl (pH 7.5). Chromatography was carried out at room temperature with a linear gradient of NaCl (1500 ml of 0.05 M NaCl in 0.05 M Tris-HCl, pH 7.5, and 1500 ml of 0.35 M NaCl in the same buffer) as shown in Fig. 2. Fractions of approximately 15 ml were collected and assayed for nucleoside phosphorylase activity. Those fractions containing activity were pooled (Fraction 3), concentrated, and then dialyzed by ultrafiltration against 0.05 M Tris-HCl (pH 7.5) (Fraction 4).

\textbf{Step IV}—Fraction 4 was then applied to a Sephadex G-100 column (2.5 \( \times \) 85 cm), and elution was carried out at room temperature with 0.05 M Tris-HCl (pH 7.5). Five- to 10-ml fractions were collected and assayed for enzyme activity (Fig. 3). Those containing nucleoside phosphorylase activity were pooled (Fraction 5).

\textbf{Step V}—A second DEAE-cellulose column (1.5 \( \times \) 33 cm) was prepared as described, and Fraction 5 was applied directly onto the column. A linear NaCl gradient was again employed, 250 ml of 0.05 M NaCl and 250 ml of 0.25 M NaCl, in the buffer previously used, and 4-ml fractions were collected. The elution pro-
Effect of pH on nucleoside phosphorylase activities with uridine (A) and thymidine (B). The enzyme preparation employed was Fraction 8. Potassium phosphate buffers were used throughout.

**TABLE III**

Nucleoside specificity of *B. stearothermophilus* nucleoside phosphorylase

<table>
<thead>
<tr>
<th>Assay method</th>
<th>Experiment</th>
<th>Nucleoside</th>
<th>Amount of base formed</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical</td>
<td>I</td>
<td>Uridine</td>
<td>0.176 µmol/ml</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thymidine</td>
<td>0.230 µmol/ml</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Deoxyuridine</td>
<td>0.256 µmol/ml</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cytidine</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Deoxycytidine</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Uridine</td>
<td>0.184 µmol/ml</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-Bromouridine</td>
<td>0.176 µmol/ml</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bromodeoxyuridine</td>
<td>0.176 µmol/ml</td>
<td>95</td>
</tr>
<tr>
<td>Isotope</td>
<td>III</td>
<td>Uridine</td>
<td>0.184 µmol/ml</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thymidine</td>
<td>0.280 µmol/ml</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenosine</td>
<td>0.082 µmol/ml</td>
<td>28</td>
</tr>
</tbody>
</table>

Fig. 5 shows the effect of pH on the conversion of uridine and thymidine to their respective free bases. Although more activity was observed with thymidine, the pH curves looked nearly identical, and the optimum in each case was approximately pH 7.2.

**Step VI**—Preparative sucrose density gradient centrifugation was carried out in an attempt to increase further the specific activity of the preparation. Two 32-ml gradients were layered with 1.6 ml of Fraction 7 and centrifuged for 60 hours at 26,000 rpm in an SW 27 rotor at 4°C. Twenty-seven fractions were collected, and those containing activity were pooled, dialyzed, and concentrated as described. As shown in Table II, only a slight increase in specific activity was obtained by this procedure.

Fig. 6. Comparative sedimentation of uridine phosphorylase and thymidine phosphorylase through sucrose density gradients. Five-milliliter gradients were layered with 0.2 ml of (NH₄)₂SO₄-treated *E. coli* extract containing 3.2 mg of protein (A), (NH₄)₂SO₄-treated *B. subtilis* GS 508 (b) extract containing 3.3 mg of protein (B), and a concentrated preparation of Fraction 8 of the *B. stearothermophilus* enzyme (C). The gradients were centrifuged for 12 hours at 45,000 rpm, and 0.2-ml fractions were collected. Seventy-five-microliter aliquots of each fraction were assayed for enzyme activity by the optical method.
Nucleoside Specificity

The pyrimidine deoxynucleosides, thymidine and deoxyuridine, appear to be the best substrates for this enzyme (Table III). Good activity was also observed toward uridine, 5-bromouridine, and 5-bromodeoxyuridine; but neither cytidine nor deoxycytidine could serve as substrates. Thus, it appears that a change in the substituent group at the 4 position in the pyrimidine ring has a greater influence on binding to the enzyme than does a change at the 5 position.

The possibility was considered that the activities observed toward uridine and thymidine could, in fact, reflect the presence of two different enzymes. In an attempt to separate the two activities, a sample of the enzyme preparation was subjected to sucrose density gradient centrifugation. The fractions obtained were assayed for both uridine and thymidine phosphorylase activity. Fig. 6C shows that the activities could not be separated in this way in contrast to the results of a similar experiment with an E. coli extract (Fig. 6A). These enzyme activities in E. coli extracts have previously been shown to result from the presence of two separable enzyme (10, 13). The enzyme activities in a B. subtilis extract (Fig. 6B) behaved similarly to those of the thermophile in that they did not separate on the gradient. The comparative size of the protein (or proteins) as judged by sedimentation velocity appears, however, to be somewhat less.

Michaelis Constants

The Michaelis constants for uridine and thymidine were determined from Lineweaver-Burk plots as shown in Fig. 7. The apparent $K_m$ for uridine was $2.5 \times 10^{-4}$ M while that for thymidine was $3.8 \times 10^{-4}$ M.

Pentose Specificity

By measuring the conversion of $^{14}$C-uracil to uridine or deoxyuridine as described under "Materials and Methods," it was possible to compare the relative efficiencies of ribose 1-phosphate and deoxyribose 1-phosphate in the reaction. Table IV shows that although a slight preference for ribose 1-phosphate was observed, the enzyme would readily accept either as a substrate with uracil. This observation provides further suggestive evidence that the uridine phosphorylase and thymidine phosphorylase reactions are catalyzed by one enzyme in B. stearothermophilus.

Thermostability of Purified Nucleoside Phosphorylase

In view of the apparent gradual loss in activity of the thermophile enzyme between 40° and 60° in a crude extract (Fig. 1), it was of interest to determine whether this was due to protein alteration as a result of temperature or whether it was due to proteolytic activity in the extract during preincubation. Prolonged incubation at 60° (Fig. 8) resulted again in a slight inactivation of the enzyme in a crude extract; however, a purified preparation lost no activity in 60 min at this temperature. Thus, the enzyme is indeed stable in vitro at the growth temperature of the organism.

If the uridine phosphorylase and thymidine phosphorylase reactions are catalyzed by one enzyme, the two activities should
**TABLE V**

**Effect of heat denaturation on nucleoside phosphorylase activities**

For incubation, 0.06-ml aliquots of Fraction 8 were diluted to 0.4 ml with 0.05 M Tris-HCl (pH 7.5) and treated as described for Fig. 1.

<table>
<thead>
<tr>
<th>Incubation temperature</th>
<th>Remaining activity</th>
<th>U/T^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uridine</td>
<td>Thymidine</td>
</tr>
<tr>
<td></td>
<td>phosphorylase</td>
<td>phosphorylase</td>
</tr>
<tr>
<td>0°</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>40</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>104</td>
</tr>
<tr>
<td>60</td>
<td>105</td>
<td>104</td>
</tr>
<tr>
<td>65</td>
<td>57</td>
<td>45</td>
</tr>
<tr>
<td>70</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

^a Uridine phosphorylase/thymidine phosphorylase.

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**DISCUSSION**

Pyrimidine nucleoside phosphorylases have been studied from a variety of sources including *E. coli* (10-13), Ehrlich ascites tumor cells (18), horse liver (19), human leukocytes (20), guinea pig kidney (21), hamster liver (21), and mouse tissues (22). Several of these cell types, such as *E. coli* (10, 13), human leukocytes (20), and mouse tissues (22), have been shown to contain two distinct pyrimidine nucleoside phosphorylases, a thymidine phosphorylase and a uridine phosphorylase. The enzyme described here presents a different situation. Several criteria have indicated that the uridine phosphorylase and the thymidine phosphorylase activities in *B. stearothermophilus* can be attributed to one enzyme. These observations include: (a) during purification the ratio of the two activities remained essentially constant, (b) the activities sedimented together through sucrose density gradients, (c) the pH optima were essentially identical, (d) during heat denaturation the ratio of the two activities remained constant, and (e) the ratio of the two activities also remained constant under a variety of cultural conditions including induction by uridine or thymidine, or both. Although there was a marked difference in thermostability between the thermophile pyrimidine nucleoside phosphorylase and that of *B. subtilis*, a few experiments (induction, sucrose gradient sedimentation) did suggest that, in this organism as well, one enzyme catalyzes both the uridine phosphorylase and the thymidine phosphorylase reactions. Purification of the enzyme will, however, be necessary to establish this conclusion.

The induction of thymidine phosphorylase in *E. coli* has been suggested by Razzell and Casshyap (11) to be the result of deoxyribose 1-phosphate accumulation. The data presented here on the induction of the nucleoside phosphorylases of *B. stearothermophilus* and *B. subtilis* are thus suggestive that the thermophile enzyme may be induced by either ribose 1-phosphate or deoxyribose 1-phosphate while synthesis of the *B. subtilis* enzyme is influenced only by the latter.

In view of the pronounced differences between the enzymes described in this communication, further studies on the physicochemical characteristics of the enzymes should yield interesting information with regard to evolutionary processes and the molecular basis of thermophily.

**REFERENCES**

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