Deoxythymidylate Phosphohydrolase Induced by Bacteriophage PBS2 during Infection of Bacillus subtilis*

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SUMMARY

The deoxythymidylate phosphohydrolase (dTMPase) induced by Bacillus subtilis bacteriophage PBS2 (whose DNA contains uracil instead of thymine) has been partially purified and shown to possess deoxyuridylate phosphohydrolase (dUMPase) activity. The similarities of induction period, pH dependence, heat and trypsin inactivation, sulfhydryl reagent and fluoride inhibition, metal ion effects, kinetic constants for substrates and products, and apparent molecular weight suggest that a single enzyme catalyzes both dTMPase and dUMPase reactions. Although the PBS2 phosphohydrolase is active on many different deoxyribonucleotide derivatives of 4-hydroxypyrimidine and 6-hydroxypurine, it displays a strong preference for dTMP (apparent \(K_m\) of \(10^{-4}\) M versus \(10^{-5}\) M for dUMP). This phage-induced phosphohydrolase may be responsible for the exclusion of thymine from PBS2 DNA during PBS2 phage infection, by eliminating the substrate (dUMP) and the product (dTMP) of the R. subtilis thymidylate synthetase.

Bacteriophage PBS2 which infects Bacillus subtilis belongs to a small class of viruses, unique because their DNA contains uracil instead of thymine (1-4). Thus PBS2 provides a tool for the study of the role of thymine versus uracil in DNA. The present work is part of an investigation of the induction of phage enzymes which allow the synthesis of uracil-containing DNA in a cell which normally makes thymine-containing DNA. Kahan (5) has reported that PBS2 phage induces a dUMP kinase and a thymidylate synthetase. Tomita and Takahashi (6) have described a phage-induced dCTP deaminase, and we have found a dTMP phosphohydrolase (dTMPase) activity. The similarities of induction period, the substrate (dUMP) and the product (dTMP) of the PBS2 DNA polymerase (4). This report describes studies on the induction and the properties of the PBS2 dTMP phosphohydrolase. Tomita and Takahashi (6) have described a phage-induced dCTP deaminase, and we have found a dTMP phosphohydrolase (dTMPase) activity. This phage-induced dTMPase-dUMPase may be responsible for excluding thymine from PBS2 DNA.

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† The abbreviations used are: dTMPase, dTMP phosphohydrolase; dUMPase, dUMP phosphohydrolase; MSH, 2-mercapto-ethanol; p-HMB, p-hydroxymercuribenzoate; DTNB, 5,5'-di-thiobis(2-nitrobenzoate); and MES, 2-(N-morpholino)ethane-sulfonate.

MATERIALS AND METHODS

Cell and Phage—The methods for the growth of B. subtilis SB19 and PBS2 phage have been described (4). Temperature-sensitive phage mutants were the generous gift of I. Takahashi (McMaster University).

Enzyme Assays—Several assay methods were employed to measure the enzymatic hydrolysis of dTMP (or dUMP) to Pi and deoxythymidine (or deoxyuridine), depending on the enzyme and substrate preparations involved.

Assay A is similar to that of Aphoshian (7). Reaction mixtures (125 p1) contained 0.3 \(\mu\)moles of substrate, 2 \(\mu\)moles of MgCl\(_2\), 0.05 \(\mu\)moles of EDTA, 10 \(\mu\)moles of sodium glycylglycine buffer (pH 7.2), and enzyme. After incubation for 15 min at 37°C, assay tubes were heated at 100°C for 1 min. Their contents were spotted on Whatman No. 40 paper for descending chromatography for 18 hr in solvent 1 (isobutyric acid-1 M NH\(_4\)OH-0.1 M EDTA, 100:60:1.6, v/v/v) or in Solvent E (95% ethanol-1 M ammonium acetate (pH 7.5), 7:3, v/v). Substrates and products were eluted from the paper in 0.1 N HCl and quantitated by their ultraviolet absorbance (8).

Assay B employed the same conditions as Assay A, except that 0.5 \(\mu\)moles of MgCl\(_2\) and 10 \(\mu\)moles of MES buffer (pH 6.2), were used. The boiled reaction mixtures were assayed in 1.25-ml incubations for Pi (9), measuring the absorbance at 750 nm (0.1 \(\mu\)moles of Pi gave an absorbance of 1.1 in a 1-cm cuvette). None of the inhibitors of enzyme induction or enzyme activity employed (see below) affected color development in the Pi assay.

Assay C used the reaction conditions of Assay B, except that tritium-labeled substrates were employed, generally 0.3 \(\mu\)Ci per assay. When the substrate concentration was less than 1 mm, 0.2 \(\mu\)moles of unlabeled substrate was added to the reaction mixture immediately prior to boiling. The mixtures were diluted with 1 ml of 1 N ammonium formate (pH 3.5), and applied to columns (0.3 \(\times\) 2 cm) of Bio-Rad AG1-X8 formate resin (200 to 400 mesh). After collecting 2 ml more of ammonium formate solution, the combined eluates containing the nucleoside product were mixed with 15 ml of scintillation fluid, consisting of 7 parts of toluene containing 0.3% 2,5-diphenyloxazole and 0.01% p-bis-(o-methylstyryl)-benzene plus 6 parts of Triton X-100 (Research Products International) for counting in a Beckman LS-230 instrument at an efficiency of 25%.

For each assay, appropriate blanks were employed: no enzyme, no substrate, no incubation time, and/or no substrate until after the incubation period. The results of the three assay
methods were essentially identical, being linear with respect to incubation time and the enzyme concentration until at least 25% conversion of the substrate to product. When low substrate concentrations were employed in Assay C, conditions were chosen to ensure only 1 to 5% substrate hydrolysis.

Enzyme preparations were diluted, when necessary, in Buffer D (10 mM Tris-Cl buffer (pH 7.5), containing 1 mM EDTA, 0.1 mM dithiothreitol, and 1 mg of bovine serum albumin per ml). One unit of enzyme is defined as the amount of enzyme which hydrolyzes 1 μmol of dTMP per hour at 37° in Assay B. For specific activity determinations, protein concentrations were measured by the method of Lowry et al. (10).

Nucleotides and Nucleotides—The following nucleotides were gifts: β-d-arabinosyluracil from William Wechter (Upjohn Company); β-d-arabinosylthymine from I. Wempen and J. Fox; 2'-fluoro-deoxyuridine and 2'-fluoro-deoxycytidine from J. G. Coddington; 2-thio-deoxythymidine from Alex Lezius; and 5-ethyl-deoxyxuridindine from D. Shugar.

The following nucleotides were gifts: 5-fluoro-dUMP from G. R. Greenberg and from Peter Danenberg; 2'-O-methyl-UMP from Frits Rottman; 4-thio-dTMP from Karl Scheit; O4, 4,5,6-tetrahydro-deUMP from Frank Maley; and 5-trifluoromethyl-dUMP from Peter Danenberg and C. Heidelberger.

The following compounds were obtained from the indicated commercial sources: 5-hydroxymethyl-deoxyuridine, 6-aza-deoxythymidine, 3'-dCMP, 3'-dTMP, and 5'-dTMP-3'-P from P.I. Biochemicals; d(pTpT), d(TpT), 3',5'-dTMP, 5'-dTMP-p-nitrophenyl ester, 5-methyl-dCMP, and dIMP from Sigma; ribothymidine, 5-chloro-deoxyuridine, 5-bromo-deoxyuridine, 5-iodo-deoxyuridine, and 5-methamino-deoxyuridine from Calbiochem.

Deamination by NaNO2 in acetic acid (11) or in acetate buffer (pH 4), was used to prepare 3'-dUMP from 3'-dCMP or dXMP from dGMP, respectively.

All nucleosides were phosphorylated by use of the wheat seedling phosphotransferase with AMP as donor (12) or with p-nitrophenylphosphate as donor (13). These nucleotides were further purified by chromatography in Solvent E, characterized by their ultraviolet absorption spectra in acid and alkali (8), and confirmed to possess a 5'-phosphate residue (when applicable) by treatment with snake venom 5'-nucletidase to produce a compound with the chromatographic mobility in Solvent I and the spectral properties of the known nucleotides. The pApA was prepared and characterized as described previously (14). Other nucleosides and nucleotides were purchased from Sigma or Calbiochem; tritium-labeled nucleotides were from Schwarz-Mann.

RESULTS

Enzyme Induction—The time course of dTMPase and dUMPase induction was determined in PBS2-infected cells using extracts prepared by several previously described methods (4). Both activities were present at a very low level (0.05 unit per mg) in uninfected B. subtilis strain SB19, and they increased in parallel from 7.5 min to a maximum at 45 min after phage infection (15 to 20 units per mg). The PBS2 DNA polymerase is induced during the same time period (4). The simultaneous appearance of dTMPase and dUMPase suggests that one phage-induced enzyme may possess both activities.

The low activity in uninfected cell extracts does not appear to result from the presence of an excess of a diffusible inhibitor, since the dTMPase-dUMPase activity of infected cell extracts (9 μg of protein) was inhibited less than 5% by the addition of uninfected cell extracts (30 μg of protein).

By using the conditions described previously (4), the effect of various antibiotics on PBS2 dTMPase-dUMPase induction was determined. Induction was prevented by the addition of chloramphenicol (100 μg per ml) or actinomycin D (10 μg per ml) at the time of infection, indicating that protein and RNA synthesis are required for the induction of enzyme during PBS2 infection (see also Reference 4).

Treatment of uninfected cells with mitomycin C (3 μg per ml for 10 min) under conditions known to cause the appearance of defective phages in B. subtilis SB19 (15) did not result in an increase in dTMPase-dUMPase activity. This mitomycin-treated culture lysed several hours after removal of the antibiotic, but no plaque-forming particles were released. This suggests that the observed PBS2 dTMPase-dUMPase induction is not the result of the production of defective phage in the host cell.

When added directly to assay mixtures at levels of 100 μg per ml, the above antibiotics did not inhibit the PBS2 dTMPase-dUMPase activity, suggesting that their effects described above are due to their inhibitory action on macromolecule biosynthesis.

A mock infection performed with a lysate from which the phage had been removed by centrifugation (4) also did not result in increased dTMPase-dUMPase activity, eliminating the possibility that a low molecular weight inducer in the lysate was responsible for enzyme induction.

Finally, rifampicin at 20 μg per ml was added at the time of infection to prevent B. subtilis RNA polymerase activity (16). Rifampicin inhibits the synthesis of RNA hybridizable to B. subtilis DNA, while permitting the synthesis of RNA hybridizable to phage DNA3 and permitting a normal burst of progeny phage (17). We have found that the level of dTMPase-dUMPase and DNA polymerase induced by PBS2 phage is identical in the presence and absence of rifampicin (17). These results suggest that phage RNA is responsible for the increase in dTMPase-dUMPase activity. The basis for the resistance of PBS2 infection to rifampicin is now under investigation.

Enzyme Purification—By using methods described previously (4), B. subtilis SB19 cells (19.2 liters) infected with PBS2 phage for 25 min at 37° were harvested, lysed, and centrifuged at 15,000 x g for 4° for 20 min to prepare a crude extract (Fraction I in Table I). All subsequent operations were performed at 4°. Three-tenths volume of 5% streptomycin sulfate was added in drops while stirring the extract for 5 min. The supernatant fluid following centrifugation at 15,000 x g for 15 min was made 1 mM in MSH to give Fraction II.

A column (4 x 36 cm) of DEAE-cellulose (previously washed in 0.1 N HCl, 0.1 N NaOH, and water) was packed under air pressure and equilibrated with Buffer T (10 mM Tris-HCl buffer (pH 7.5), containing 1 mM EDTA and 1 mM MSH). Fraction II was applied, followed by 375 ml of Buffer T, at a rate of 120 ml per hr. A linear 4-liter gradient from 0 to 400 mM NaCl in Buffer T was employed to elute the PBS2 dTMPase-dUMPase activity. The peak fractions (200 to 240 mM NaCl) were pooled to give Fraction III.

A column (1 x 12 cm) of hydroxyapatite was poured in Buffer P (10 mM potassium phosphate buffer (pH 7.0), containing 0.1 mM EDTA and 1 mM MSH). Fraction III was applied, and

1 Nomenclature as recommended by the IUPAC-IUB Commission in J. Biol. Chem. 246, 5171-5176 (1970).

2 B. Rima and I. Takahashi, personal communication.
TABLE I
Purification of PBS2 phage-induced dTMPase-dUMPase
See text for descriptions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>dTMPase</th>
<th>dUMPase</th>
<th>Ratio dTMPase: dUMPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>Activity</td>
<td>Recovery</td>
<td>Activity</td>
</tr>
<tr>
<td>I. Crude extract</td>
<td>102</td>
<td>1050</td>
<td>18</td>
<td>100</td>
<td>18</td>
</tr>
<tr>
<td>II. Streptomycin</td>
<td>123</td>
<td>851</td>
<td>22</td>
<td>99</td>
<td>21</td>
</tr>
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<td>III. DEAE-cellulose</td>
<td>299</td>
<td>135</td>
<td>82</td>
<td>61</td>
<td>94</td>
</tr>
<tr>
<td>IV. Hydroxylapatite</td>
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<td>34</td>
<td>340</td>
<td>61</td>
<td>400</td>
</tr>
<tr>
<td>V. Sephadex G-100</td>
<td>19</td>
<td>5.8</td>
<td>2000</td>
<td>62</td>
<td>2000</td>
</tr>
<tr>
<td>VI. Electrofocusing</td>
<td>3.4</td>
<td>4.5</td>
<td>550</td>
<td>13</td>
<td>510</td>
</tr>
<tr>
<td>VII. DEAE-Sephadex</td>
<td>2.4</td>
<td>1.0</td>
<td>1340</td>
<td>7</td>
<td>1380</td>
</tr>
<tr>
<td>VIII. Sephadex G-100</td>
<td>3.0</td>
<td>0.2</td>
<td>6800</td>
<td>7</td>
<td>7150</td>
</tr>
</tbody>
</table>

the column was washed with 600 ml of Buffer P at 120 ml per hr. A linear 600-ml gradient from 0 to 200 mm potassium phosphate in Buffer P eluted the dTMPase-dUMPase activity. The peak fractions (30 to 90 mm P) were pooled, dialyzed in Buffer G (50 mm Tris-HCl buffer (pH 7.5), containing 10 mm NaCl, 0.1 mm EDTA, and 0.1 mm dithiothreitol), and concentrated by ultrafiltration in a collodion bag in Buffer G to give Fraction IV.

Fraction IV was applied to a column (1.4 × 100 cm) of Sephadex G 100 and eluted with Buffer G at 27 ml per hr. The fractions containing dTMPase-dUMPase activity (corresponding to a molecular weight of 35,000 to 45,000) were pooled to give Fraction V.

Fraction V was dialyzed in 0.5% glycine containing 0.1 mM EDTA and 0.1 mM dithiothreitol. Electrofocusing was performed in 110 ml of 2% ampholines (pH 3 to 6), as described in the LKB 8100 manual. Fractions containing dTMPase-dUMPase activity were pooled, dialyzed, and concentrated in Buffer G to give Fraction VI.

Fraction VI was applied to a column (2.5 × 7.5 cm) of DEAE-Sephadex in Buffer G at 20 ml per hr. Following 70 ml of Buffer G, a 400-ml gradient from 0 to 1 M NaCl in Buffer G was applied. Fractions containing dTMPase-dUMPase activity (0.4 to 0.6 M NaCl) were pooled and concentrated by ultrafiltration in Buffer G to give Fraction VII.

Fraction VII was applied to the Sephadex G-100 column described above, eluted with Buffer G, and concentrated to give Fraction VIII. This procedure removed the ampholines which contaminated Fractions VI and VII.

The 350-fold over-all purification is summarized in Table I. Only a single peak with coincident dTMPase and dUMPase activities in constant ratio was observed throughout the purification. The enzyme has not been purified to homogeneity (see below).

Enzyme Stability—Fraction I dTMPase-dUMPase activity is stable for at least 1 year at -20°. It loses about 25% of its activity on storage at 4° for a month. It loses all of its activity during this period when stored in the absence of MSH or dithiothreitol; addition of 1 mM MSH or dithiothreitol (5 or 60 min before assaying) restores about 20% of the activity. Fraction VIII enzyme could be stored at 4° or in 50% glycerol at -20° for at least 1 year with full retention of activity.

pH Optimum—Fig. 1 shows the influence of pH on the activity of dTMPase and dUMPase by using various buffers. The curve for dTMPase is rather broad, with an optimum in the range of pH 6 to 7. The curve for dUMPase is much narrower but shows a similar pH optimum. The enzyme is inactivated below pH 4.5 and above pH 10.5, but it could be partially reactivated by dialysis in Buffer G. Increasing the substrate concentration 10-fold in assays using buffer at pH 8.5 did not affect the dTMPase activity, but resulted in a 3-fold increase in the dUMPase activity, suggesting a low affinity for dUMP at high pH.
Heat Inactivation—When Fraction VIII dTMPase-dUMPase (0.2 unit of enzyme in 5 μl of Buffer G) activity was measured in Assay B by using various incubation temperatures, the optimum was about 42° for dTMPase and about 37° for dUMPase. The ratio of activities at 32°/22° was 1.4 for both dTMPase and dUMPase, but the ratio at 42°/32° was 1.4 for dTMPase and 0.9 for dUMPase in 15-min incubations. Thus, under assay conditions, the dTMPase activity is more heat-stable than the dUMPase activity.

Fig. 2 indicates the rate of enzyme inactivation during incubation at 52°. The dTMPase and dUMPase activities decrease in parallel, with 50% inactivation in 2.5 to 3 min. When this 52° incubation was performed in the presence of the components of Assay B (110 μl, without substrate), parallel inactivation of dTMPase and dUMPase was also observed, with 50% inactivation in 1 min. Thus, under incubation conditions without substrates, dTMPase and dUMPase activities are equally sensitive to heat inactivation.

Trypsin Inactivation—Fig. 3 indicates the parallel inactivation of dTMPase and dUMPase activities which occurs by treatment with trypsin, before assaying in the presence of trypsin inhibitor. When 40 μg trypsin inhibitor was added with 20 μg trypsin, 90% of the inactivation of dTMPase-dUMPase seen in Fig. 3 was prevented.

In other experiments, trypsin was added directly to assay mixtures for 15 min incubations. Relative to untreated controls, there was 46% dUMPase and 93% dTMPase activity in the presence of 2 μg trypsin, and 16% dUMPase and 43% dTMPase with 8 μg trypsin in Assay B. Thus, dTMP protects the enzyme more than dUMP does from proteolytic inactivation.

Inhibition by p-HMB and DTNB—Fraction VIII (0.4 unit) dTMPase-dUMPase was inactivated by 4 × 10⁻⁴ M p-HMB or by 8 × 10⁻⁴ M DTNB. This inactivation was prevented or immediately reversed by the addition of 4 × 10⁻³ M dithiothreitol. The dithiothreitol had no stimulatory effect on dTMPase-dUMPase in the absence of p-HMB and DTNB.

Effect of Monovalent Cations—Fig. 4 indicates the sensitivity of dUMPase activity to inhibition by NaCl, in contrast to the relative resistance of dTMPase activity. The inhibition by KCl, NaCl, and LiCl (2 M in Assay B) was 16, 18, and 35%, respectively, for dTMPase, and 87, 91, and 95%, respectively, for dUMPase. The 60% inhibition of dUMPase by 0.4 M NaCl seen in Fig. 6 was completely eliminated by increasing the dUMP concentration in Assay B from 2.4 to 24 mM (also see below).

Effect of Divalent and Trivalent Cations—Both dTMPase and dUMPase activities were stimulated by Mg²⁺, with a $K_m$ for...
Hydrolysis greater than that for dTMP were observed for several 2-thio, or 4-thio derivatives. The addition of a 3'-phosphate dTMP analogues with modified sugar residues (ribose, arabinose, or 2-fluoro-2-deoxyribose) or with modified thymine rings (6-aza, 2-thio, or 4-thio derivatives). The addition of a 3'-phosphate residue to dTMP reduces its activity 14-fold; and 3'-dTMP itself is only slightly active. Similarly, the dinucleotide d(pTpT), is slowly hydrolized to d(TpT), which is resistant to attack. The cyclic nucleotide, 3',5'-dTMP is inactive, as are other modifications of the 5'-phosphate (dTDP, dTTP, and the p-nitropheryl ester of dTMP). Thus, the PBS2 phosphorylase appears to require a free phosphate in the 5'-position of a nucleotide, but it does not distinguish between several sugar and ring modifications of pyrimidine nucleotides (at least at the concentrations employed).

In the deoxyuridine series, the enzyme is much more sensitive to sugar residue modification, since the ribose, arabinose, 2-fluoro-2-deoxyribose, and 2-O-methylribose analogues have very low activity. However, as found above, considerable variation in ring structure is tolerated, with many derivatives at the 5-position of the uracil ring (halo, hydroxymethyl, trifluoromethyl, ethyl, methylamino, and methyl) showing high rates of nucleotide hydrolysis. Even a reduced ring (tetrahydro-dUMP) is acceptable in a substrate. Again, the 3'-phosphate analogue is only slightly active, and the 5'-diphosphate and triphosphate derivatives are inactive.

All cytosine-containing nucleotides tested were poor substrates. The 4-amino derivative of dTMP (5-methyl-dCMP) was only 3% as active as dTMP; this activity did not result from prior denaturation of the substrate to form dTMP, since the product was 5-methyl-deoxycytidine. Likewise, the low level of activity on dCMP did not result from prior denaturation to dUMP. Although the activity was low, 3' dCMP was a better substrate (forming deoxyctydine) than 3'-dTMP or 3'-dUMP.

The activity on all adenine-containing nucleotides was low or undetectable. Only dAMP of those tested had measurable activity. The dinucleotide, pApA, was not attacked, showing that the PBS2 phosphorylase does not have general activity on all dinucleotides.

Considerable activity was observed with other purine deoxyribonucleotides (dGMP, dIMP, and dXMP). As above, the ribonucleotide (GMP) and the triphosphate (dTTP) were poor substrates.
TABLE II

Nucleotide substrate specificity of PBS2 dTMPase-dUMPase

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Concentration</th>
<th>Activity</th>
<th>Nucleotide</th>
<th>Concentration</th>
<th>Activity</th>
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</thead>
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<tr>
<td>dTMP</td>
<td>2.4</td>
<td>100</td>
<td>5-Ethyl-dUMP</td>
<td>1.3</td>
<td>100</td>
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<tr>
<td>ribo-TMP</td>
<td>2.6</td>
<td>110</td>
<td>5-Methylamino-dUMP</td>
<td>2.4</td>
<td>120</td>
</tr>
<tr>
<td>Arabino-TMP</td>
<td>2.1</td>
<td>120</td>
<td>O-4,5,6-Tetrahydro-dUMP</td>
<td>4.8</td>
<td>400</td>
</tr>
<tr>
<td>2'-Fluoro-dTMP</td>
<td>2.7</td>
<td>100</td>
<td>3'-dUMP</td>
<td>3.1</td>
<td>0.7</td>
</tr>
<tr>
<td>6-Aza-dTMP</td>
<td>2.6</td>
<td>110</td>
<td>dUDP</td>
<td>2.6&lt;0.5</td>
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<tr>
<td>2-Thio-dTMP</td>
<td>1.6</td>
<td>120</td>
<td>dUTP</td>
<td>2.5&lt;1.0</td>
<td></td>
</tr>
<tr>
<td>4-Thio-dTMP</td>
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<td>140</td>
<td>dCMP</td>
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<td>0.4</td>
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<td>5'-dTMP-3'-P</td>
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<td>7.3</td>
<td>5-Methyl-dCMP</td>
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<td>3.0</td>
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<td>0.2</td>
<td>5-Hydroxymethyl-dCMP</td>
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<td>0.3</td>
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<td>5'-dTMP-3'-P</td>
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<td>2.1</td>
<td>dCDP</td>
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<td>0.2</td>
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<td>d(TpT)</td>
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<td>&lt;0.2</td>
<td>dCTP</td>
<td>2.0</td>
<td>&lt;0.2</td>
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<tr>
<td>d(TpT)</td>
<td>3.1</td>
<td>&lt;0.2</td>
<td>dAMP</td>
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</tr>
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<td>&lt;0.2</td>
<td>dAMP</td>
<td>2.5&lt;0.2</td>
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<td>5'-dTMP p-nitrophenyl ester.</td>
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<tr>
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<td>dAMP</td>
<td>2.5&lt;0.2</td>
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<tr>
<td>UMP</td>
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<td>2.8</td>
<td>dAMP</td>
<td>2.5&lt;0.2</td>
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<tr>
<td>Arabino-UMP</td>
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<td>1.6</td>
<td>dAMP</td>
<td>2.0&lt;0.2</td>
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<tr>
<td>2'-Fluoro-dUMP</td>
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<td>100</td>
<td>dAMP</td>
<td>2.5&lt;0.2</td>
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<td>0.4</td>
<td>dAMP</td>
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<tr>
<td>5'-Fluoro-dUMP</td>
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<td>100</td>
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<td>dAMP</td>
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</tr>
<tr>
<td>5-Hydroxymethyl-dUMP</td>
<td>0.5&lt;4</td>
<td>110</td>
<td>dAMP</td>
<td>2.5&lt;0.2</td>
<td></td>
</tr>
<tr>
<td>5-Trifluoromethyl-dUMP</td>
<td>2.4&lt;4</td>
<td>130</td>
<td>dAMP</td>
<td>2.5&lt;0.2</td>
<td></td>
</tr>
</tbody>
</table>

* The product using tetrahydro-dUMP as substrate (which contained less than 0.7% dUMP) was isolated by ion exchange chromatography as in Assay C, and it was quantitated by the indole-hydrochloric acid method for deoxyribose (38).

TABLE III

Apparent Km and Ki values for certain substrates and products of PBS2 phosphohydrolase activity

The values presented represent the average from two to five experiments.

| Constant | Substrate | Kinetic constant of compound
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dTMP</td>
<td>dThd</td>
</tr>
<tr>
<td></td>
<td>dTMP</td>
<td>dThd</td>
</tr>
<tr>
<td>K</td>
<td>(substrate)</td>
<td>0.011</td>
</tr>
<tr>
<td>K</td>
<td>dTMP</td>
<td>0.021</td>
</tr>
<tr>
<td>K</td>
<td>dUMP</td>
<td>0.022</td>
</tr>
<tr>
<td>K</td>
<td>dGMP</td>
<td>0.028</td>
</tr>
</tbody>
</table>

* Abbreviations are: dThd, deoxythymidine; dUrd, deoxyuridine; and dGuo, deoxyguanosine. All the compounds tested behaved as competitive inhibitors of each substrate.
substrates. These kinetic data support the proposal that a single phosphohydrolase is involved, with dTMP, dUMP, and dGMP competing for one active site. The apparent affinity of dTMP is about 100-fold greater than that for dUMP or dGMP, suggesting that the enzyme's major role in vivo may be to hydrolyze dTMP.

The phosphohydrolase reactions appear to proceed to completion. Incubation of dTMP or dUMP in Assay A for 6 hours with 2 units of Fraction VIII enzyme resulted in over 99% conversion to deoxythymidine or deoxyuridine, respectively, with no residual substrate detectable.

The presence of 1 mM NaCl in Assay C causes a 5- to 7-fold increase in the apparent $K_m$ for each substrate: to 0.06 mM for dTMP, to 5 mM for dUMP, and to 5 mM for dGMP. The ionic environment in infected cells would be expected to be much lower than that employed in these assays.

**Enzyme Inhibition by Nucleotides**—Table IV presents the results of assays conducted with various nucleotides as inhibitors of dTMPase activity. The dTMP concentration employed equalled one-tenth the apparent $K_m$ for dTMP, in order to maximize any inhibitory properties of the nucleotides. It is apparent that dTMP itself is by far the most potent inhibitor of the PBS2 phosphohydrolase. Other nucleotides required much higher concentrations to significantly affect dTMPase activity.

Most of the nucleotides tested (except dCMP and CMP) significantly inhibited dTMPase when added at high concentrations (4 to 14 mM, which was 3,000- to 10,000-fold greater than the dTMP concentration). Some of the inhibition observed may result from an ionic effect (see above). Most of these nucleotides would not be expected to occur naturally in the cell. However, if the role of the PBS2 phosphohydrolase is to eliminate dTMP from infected cells, then the inhibition by AMP and ATP at low dTMP concentrations (see Table IV) may have some significance in vivo.

Many of the dTMP analogues with modified sugar residues or rings which were shown in Table II to be excellent substrates (at high concentrations) are rather weak inhibitors, as are many nonsubstrate nucleotides. In addition, all the nucleoside diphosphates and triphosphates tested at high concentrations gave 50 to 90% inhibition of dTMPase. This may indicate that all nucleotides compete for a phosphate-binding site on the enzyme (which, however, fails to bind dCMP, CMP, or deoxyribose-5-P). The inhibition of dTMPase-dUMPase by Pi itself (0.65 mM Pi) tested in Assay C is no greater than that observed for the same concentration of NaCl (see Fig. 4).

It is noteworthy that relatively minor modifications of dTMP structure cause a sharp decrease in inhibitory properties. The phosphohydrolase appears to possess a recognition site for the 2'-position of nucleotides, since deoxyribose is highly preferred over ribose or arabinos. Similarly, replacement of the 4'-oxygen in dTMP by sulfur significantly reduces its apparent affinity for the enzyme. The data demonstrate a strong preference of the PBS2 phosphohydrolase for dTMP over all other nucleotides tested.

**Molecular Size of dTMPase-dUMPase**—Fig. 6 shows the results of Sephadex chromatography of the PBS2 phosphohydrolase with protein standards to estimate the enzyme's molecular size as approximated by its Stokes' radius (19). The dTMPase and dUMPase activities eluted in coincident peaks corresponding to a molecular weight of about 40,000. When the enzyme was applied to this column after calibration by protein standards, a similar result was obtained (38,000, which is equal within the experimental error to the above value). Similar values have been obtained for the following phosphohydrolase preparations: Fraction I extract; a crude extract subjected to DNase-RNase digestion and dialysis; and a crude extract prepared and chromatographed in Buffer T containing 2 mM phenylmethylsulfonyl fluoride, a protease inhibitor (20), which
Gels sliced and incubated electrophoresis in polyacrylamide gels by the method of Ornstein may become more "unfolded" on inactivation by p-HMB.

When Fraction VIII enzyme was subjected to chromatography with protein standards in Buffer G containing 1 mM dTMP, the elution position of the standard proteins had not been changed by the addition of p-HMB. We have shown to inhibit B. subtilis subtilisin and subtilopeptidase.

When Fraction VIII enzyme was subjected to chromatography with proteins in Buffer G containing 1 mM dTMP, the apparent molecular weight (41,000) was similar to that in the absence of dTMP.

When chromatographed in Buffer G containing 0.2 mM p-HMB with protein standards, Fraction VIII enzyme was inactive. On storage of the fractions in 10 mM dithiothreitol overnight at 4°C, about 60% of the dTMPase-dUMPase activity applied was recovered, migrating as a single peak (apparent molecular weight: 46,000) slightly ahead of ovalbumin. The elution position of the fractions (1 ml) were assayed for dTMPase (●) and dUMPase (○) activity (nmol of Pi in 30 min in Assay B) and for absorbance at 280 nm or 415 nm to locate the standard protein peaks (□).

In the presence of 1 M NaCl was also observed during sucrose density gradient centrifugation.

Gel Electrophoresis—When subjected to discontinuous pH electrophoresis in polyacrylamide gels by the method of Ornstein (21) and Davis (22), Fraction VIII enzyme gave at least five protein bands (migrating 0.54, 0.64, 0.66, 0.72, and 0.76 relative to the bromophenol blue tracking dye). Gels sliced and incubated in Assay B for 1 hour showed dTMPase activity in the region 0.7 to 0.8 relative to the front, but could not be positively identified with one of the protein bands.

Upon electrophoresis in gels containing sodium dodecyl sulfate by the method of Weber and Osborn (23), Fraction VIII enzyme showed eight protein bands corresponding to molecular weights of 7,000 to 73,000. The relationship of the protein bands to possible dTMPase-dUMPase subunits is not yet clear, and will require further large scale purification of the enzyme.

Enzyme Induction by Phage Mutants—In an attempt to find a phage mutant inducing a defective dTMPase-dUMPase, extracts were prepared using cells at 45°C infected by the 16 available PBS1 ts mutants (whose efficiency of plating at 45°C was less than 3% that at 30°C). None of the 16 ts mutants induced a dTMPase-dUMPase more heat-labile than wild type enzyme under our conditions.

FIG. 6. Estimation of PBS2 dTMPase-dUMPase molecular weight by Sephadex G-100 column chromatography. Fraction VIII enzyme (22 units) in 1 ml of Buffer G containing 10 mg of bovine serum albumin (BSA; mol wt 68,000), 10 mg of ovalbumin (mol wt 45,000), and 2 mg of horse cytochrome c (cyto c; mol wt 13,000) was applied to a column (1.4 X 85 cm) of Sephadex G-100. Elution with Buffer G was at 5 ml per hour at 4°C. Aliquots (90 μl) of the fractions (1 ml) were assayed for dTMPase (●) and dUMPase (○) activity (nmol of Pi, in 30 min in Assay B) and for absorbance at 280 nm or 415 nm to locate the standard protein peaks (□).

The evidence presented here indicates that PBS2 phage induces a single phosphohydrolase possessing both dTMPase and dUMPase activities. Various physical and chemical treatments of the enzyme inhibit both activities in parallel. In the presence of the substrates, however, there are qualitative similarities but quantitative differences in the effects of various agents, with dTMPase activity being more resistant to inhibition than dUMPase activity. These differences are apparently attributable to the large difference in K_m for dTMP (10^4 M) versus dUMP (10^-3 M), which was close to the assay concentration used in these experiments.

The PBS2 phosphohydrolase utilized many 4-hydroxyxypirimidine and 6-hydroxypurine deoxyribonucleotides at high concentrations, but inhibition studies indicated a strong preference for dTMP over any other nucleotide tested. Determination of the specificity constant (the turnover constant divided by the K_m) for each substrate would be the most accurate measure of specificity (24) but is not currently possible with our limited supply of most dTMP analogues.

The present study does suggest some possibilities about the mechanism of enzyme recognition. Since 4-thio-dTMP was about 10-fold less effective a dTMPase inhibitor than dTMP, and since the 4-thione forms hydrogen bonds considerably less well than the 4-oxone (25), we suggest that the PBS2 phosphohydrolase forms hydrogen bonds with the oxygen on the 4-position of some pyrimidine deoxyribonucleotides (and perhaps on the 6-position of some purine deoxyribonucleotides). This may explain the very weak substrate activity and inhibition properties of cytosine and adenine nucleotides, which have an amino group in this position which could not form the proposed hydrogen bond to the enzyme. We believe that this is a strong recognition site, since 5-methyl-dCMP (which is identical to dTMP except for the 4-amino group versus the 4-oxo group) was a very poor substrate and inhibitor. Furthermore, because of the acceptability of 5-fluoro-dUMP and 5-trifluoromethyl-dUMP as substrates (although less potent inhibitors than dTMP), we suggest that the N—H bond in the 3-position of pyrimidine deoxyribonucleotides does not bind to a cationic site on the enzyme. We believe that this is a strong recognition site, since 5-methyl-dCMP (which is identical to dTMP except for the 4-amino group versus the 4-oxo group) was a very poor substrate and inhibitor. Furthermore, because of the acceptability of 5-fluoro-dUMP and 5-trifluoromethyl-dUMP as substrates (although less potent inhibitors than dTMP), we suggest that the N—H bond in the 3-position of pyrimidine deoxyribonucleotides does not bind to a cationic site on the enzyme.

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A. R. Price, unpublished data.
Although we have not yet characterized the low level of uninfected B. subtilis phosphohydrolase activity, and we have not yet found phage mutants with defective phosphohydrolase activity, our induction studies suggest that the dTMPase-dUMPase is unique to PBS2 infection. In addition, the PBS2 phosphohydrolase activity appears to be different from the other known B. subtilis phage-induced activities. The SP5C phage dTMPase differs (27) markedly from the PBS2 enzyme in its stability, metal requirement, pH optimum, substrate specificity, and Km for dTMP (10⁻⁴ M). The SP3 phage dTMPase in crude extracts was inhibited by ATP (28), in contrast to the PBS2 enzyme. Other known 5’-nucleotide phosphohydrolases from snake venom, bull semen, prostate gland, and Ehrlich ascites tumor cells are somewhat similar to the PBS2 dTMPase in their response to metal ions, sulfhydryl reagents, and changes in the phosphate residue, but they are far less specific than the PBS2 dTMPase (29–31).

It is also of interest that other phage-induced enzymes involved in deoxyribonucleotide metabolism have been shown to utilize multiple substrates. The Escherichia coli phage T5-induced deoxyribonucleotide kinase phosphorylated dTMP, dAMP, and dGMP (32). The E. coli T4-induced deoxyribonucleotide kinase was active on dTMP, dGMP, and 5-hydroxymethyl-dCMP (33). The phage T4-induced nucleotidohydrolase could utilize dUTP, dCDP, dCTP, and dUDP (34). Finally, the B. subtilis phage φ8-induced nucleotidohydrolase appeared to be active on dTTP and dUTP (35).

We propose that during PBS2 infection the PBS2 phosphohydrolase functions to hydrolyze the dTMP produced by the B. subtilis dTMP synthetase, whose specific activity is less than 1% of the PBS2 dTMPase activity (36) and remains constant throughout phage infection. The PBS2 phosphohydrolase may also hydrolyze dUMP, the substrate of dTMP synthetase. This dTMPase-dUMPase activity would effectively shut off the pathway to dTTP and presumably would prevent thymine from entering PBS2 DNA. The other pathways from UDP to dUDP to dUTP and from CDP to dCDP to dCTP to dTTP would presumably supply enough dUTP for the synthesis of phage DNA containing uracil. However, there is evidence (see also Reference 4) that PBS2 may induce a dUTPase activity producing dUDP, which could assist in the exclusion of thymine from PBS2 DNA. Any evaluation of the in vivo role of the PBS2 dTMPase-dUMPase must await a study of the changes in the pool sizes of dUMP, dUTP, dTMP, and dTTP following PBS2 infection and the isolation of phage mutants defective in the relevant enzymes.

REFERENCES


Deoxythymidylate Phosphohydrolase Induced by Bacteriophage PBS2 during Infection of *Bacillus subtilis*

Alan R. Price and Suzanne M. Fogt


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