Interaction of Deoxyuridylate with Thymidylate Synthetase*

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SUMMARY

The reaction of deoxyuridylate with Lactobacillus casei thymidylate synthetase (methyleneetetrahydrofolate:deoxyuridylate C-methyltransferase) in the absence of folate or thiols can be followed by changes in the circular dichroic spectra at 267 nm. These changes at the maximum absorption wavelength of the pyrimidine suggest that on binding the interaction of the pyrimidine ring and the deoxoribose moiety is altered. The binding curve follows the form expected for single site binding. There was no change in the ultraviolet absorption spectra. The dissociation constant of the deoxyuridylate enzyme complex was calculated to be $4 \times 10^{-7}$ M.

Iodoacetamide also reacts stoichiometrically with one of the two subunits of thymidylate synthetase and inactivates the enzyme. This reaction is blocked by the presence of deoxyuridylate or chloromercuribenzoate. Complement fixation and immunodiffusion studies gave no indication that iodoacetamide treatment caused conformational changes or breakdown of the enzyme into subunits. Treatment with 0.1 M mercaptoethanol restored activity to one-half the value obtained with native enzyme, suggesting that mercaptoethanol enabled the unreacted subunit to become functional.

Thymidylate synthetase catalyzes the reaction:

Deoxyuridylate $\rightarrow$ 5,10 methylenetetrahydrofolate $\rightarrow$ thymidylate + dihydrofolate

The reaction is of special interest because of the unique role of tetrahydrofolate which serves as the carrier of the single carbon unit and as a reductant and because the enzyme is a target in cancer chemotherapy with fluorouracil (1). Lactobacillus casei thymidylate synthetase has a molecular weight of approximately 68,000 (2, 3) and is composed of apparently identical subunits (4). In the presence of methylenetetrahydrofolate and thiols, 2 mol of fluordeoxyuridylate react per mol of enzyme (5, 6), presumably one with each subunit. It has been reported, however, that the enzyme is inactivated with 1 mol of p-CMB (5, 6). We now report that in the absence of folates or thiols both deoxyuridylate and iodoacetamide react stoichiometrically with one subunit of thymidylate synthetase.

METHODS

p-CMB, DTNB, and iodoacetamide were purchased from Sigma Chemical Co., St. Louis, Missouri. [14C]Iodoacetamide was purchased from New England Nuclear, Boston, Massachusetts. dt+1-Tetrahydrofolate was prepared by catalytic hydrogenation in neutral aqueous solution (7).

Homogeneous thymidylate synthetase was prepared as described (8) from a methotrexate-resistant strain of Lactobacillus casei as well as by an alternative procedure as follows: 90 mg of protein from the hydroxylapatite fraction (8) in 5 ml of a solution containing 100 mM KCl, 1 mM EDTA, and 50 mM KPOd (pH 6.8) were applied to a Sephadex G-100 column (2.6 X 86 cm) and eluted with the same solution at 0-3°C. Activity emerged between 180 and 230 ml. The enzyme was concentrated by (NH₄)₂SO₄ precipitation (8). Starting with material having a specific activity of 84 pmol/mg of protein/hour, this method, repeated twice, resulted in a 60% yield of enzyme-specific activity 200 showing a single peak on gel electrophoresis (8). This laboratory has prepared the enzyme from dichloromethotrexate as well as methotrexate-resistant strains and it has been determined that enzymes from both strains as well as from the parent strain (ATCC 7469) behave identically in immunodiffusion and complement fixation tests.

Enzyme concentration was determined using an extinction coefficient of 108/1000 at 280 nm based on amino acid analysis of a homogeneous sample prepared by Dr. R. K. Sharma of this laboratory. The amino acid analysis was carried out by Dr. Bruce H. Davis and Dr. Jurio Otsu of the University of Connecticut.

Thymidylate synthetase activity was measured by the spectrophotometric assay (9). Minus thiol assays were carried out in the same way as with 2-mercaptoethanol excluded. Tetrahydrofolate for the minus thiol assays was dissolved in 0.05 M Tris, pH 7.4, 0.03 M formaldehyde in small hydrogen-filled test tubes fitted with a rubber serum cap and introduced into the assay mixture with a microsyringe. It did not decompose appreciably over the 5-min assay period as judged by ultraviolet absorption spectrum.

Absorption measurements were obtained with either a Gilford model 2000 multiple absorbance recorder attached to a Beckman DU spectrophotometer or a Gilford model 230 photometer indicator unit attached to a Beckman DU spectrophotometer. Circular dichroic spectra were obtained using a Jasco J 20 recording spectropolarimeter. The points plotted in Fig. 2 were obtained by summing ellipticity values for dUMP and enzyme and then subtracting the ellipticity obtained after mixing the two.

The DTNB titrations (10) and p-CMB titrations (11) were carried out as described.

Antithymidylate synthetase serum was obtained by immunization of two New Zealand White rabbits with homogeneous enzyme. Two 1-ml doses of antigen (containing 200 pg of enzyme) in Freund's adjuvant were given 1 week apart at multiple intradermal and subcutaneous sites. An intravenous injection of en-

1 The abbreviations used are: p-CMB, p-chloromercuribenzoate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

2 R. P. Leary, unpublished data.
zyme alone was given a week later and blood was withdrawn at weekly intervals and stored at -20°.

Two-dimensional immunodiffusion was performed (12) in 0.87 M agar gels. Complement fixation was performed (13) in a buffer of 0.14 M NaCl, 0.01 M Tris (pH 7.4), containing 0.5 M MgCl₂, 0.15 M CaCl₂, and 1% bovine serum albumin.

Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrophotometer (model 3330) using Aquasol (New England Nuclear) as the scintillation fluid.

Metal analysis of homogeneous thymidylate synthetase was carried out by Dr. Charles F. Dolloff, Biophysics Research Laboratory, Harvard Medical School, Boston, Mass., utilizing an emission spectrograph equipped with Wadsworth gratings and Jarrell-Ash varisources (14).

RESULTS

Circular Dichroism Studies—The interaction of dUMP with thymidylate synthetase was studied using circular dichroism (Fig. 1). dUMP has a peak at 267 nm and the enzyme at 292 nm. Complex formation resulted in a substantial decrease in ellipticity at 267 nm. Under the same conditions the ultraviolet absorption spectrum of a mixture of dUMP and thymidylate synthetase is identical with the sum of the spectra of the individual components.

The titration curve obtained by adding increments of dUMP to the enzyme (Fig. 2) demonstrates a stoichiometry of 1 mol of dUMP binding per mol of enzyme as indicated by the reaction

\[ E + \text{dUMP} \rightleftharpoons (E-\text{dUMP}) \] (1)

The dissociation constant for reaction (1) is given by equation (2)

\[ K = \frac{[E][\text{dUMP}]}{[E-\text{dUMP}]} \] (2)

where \([E] \), [dUMP], and [E-dUMP] are the equilibrium concentrations of enzyme, dUMP, and complex, respectively. Defining the binding ratio, \( \varphi \), as the ratio of the change in ellipticity observed in the presence of a given dUMP concentration to the maximum observable change in ellipticity, the following expression can be derived

\[ \frac{1}{\varphi} = 1 + \frac{K}{([\text{dUMP}]_o - E_0 \varphi)} \] (3)

where \([\text{dUMP}]_o \) and \( E_0 \) are total dUMP and enzyme concentrations, respectively. A plot of \( 1/\varphi \) versus \( 1/([\text{dUMP}]_o - E_0 \varphi) \) is presented in Fig. 3. The points fall on a line as expected for a single binding site. The dissociation constant calculated from the slope of the line is \( 4.0 \times 10^{-7} \) M. A single binding site per mole of enzyme is also indicated by the fact that values of \( K \) show no significant trend when calculated from equation (3) at individual values of [dUMP] (Table I).

The circular dichroic experiments were carried out without added divalent metal ions and analysis of the enzyme revealed

![Fig. 1. CD spectra of dUMP, thymidylate synthetase, and the dUMP-thymidylate synthetase complex in 0.05 M Tris and 10\(^{-3}\) M EDTA, pH 7.4. A, dUMP; 4.8 \times 10^{-5} M (Curve 1) and 3.4 \times 10^{-5} M (Curve 2). B, thymidylate synthetase, 5.6 \times 10^{-6} M (Curve 3); plus dUMP, 4.8 \times 10^{-4} M (Curve 1); plus dUMP, 3.4 \times 10^{-4} M (Curve 2).]

![Fig. 2. CD titration of thymidylate synthetase 5.6 \times 10^{-6} M with dUMP in 0.05 M Tris and 10^{-3} M EDTA, pH 7.4. Dashed lines indicate equivalence point obtained by extrapolation (5.6 \times 10^{-6} M).]

![Fig. 3. Reciprocal plot of the single site binding equation (Equation (3)) for the binding of dUMP with thymidylate synthetase. Data obtained from those used in Fig. 2.]

**TABLE I**

<table>
<thead>
<tr>
<th>([\text{dUMP}]_o ) ( \times 10^{-5} ) (M)</th>
<th>( \varphi )</th>
<th>( K ) ( \times 10^{-6} ) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.8</td>
<td>0.70</td>
<td>0.37</td>
</tr>
<tr>
<td>6.4</td>
<td>0.81</td>
<td>0.45</td>
</tr>
<tr>
<td>8.0</td>
<td>0.88</td>
<td>0.42</td>
</tr>
<tr>
<td>9.6</td>
<td>0.97</td>
<td>0.13</td>
</tr>
<tr>
<td>11.2</td>
<td>0.96</td>
<td>0.28</td>
</tr>
</tbody>
</table>

\( a \) \([\text{dUMP}]_o \) = total dUMP concentration.

\( b \) \( \varphi \) calculated as \( \Delta \sigma/\Delta \sigma_{\text{max}} \) where \( \Delta \sigma \) = change in CD at the concentration of dUMP indicated and \( \Delta \sigma_{\text{max}} \) = maximum \( \Delta \sigma \) observed.
none (14). The titration described in Fig. 2 was not altered by the presence of 0.02 M MgCl₂. Thus, Mg²⁺ which is known to stimulate Lactobacillus casei thymidylate synthetase (2) is not required for dUMP binding.

Interaction of Thymidylate Synthetase and Iodoacetamide—Iodoacetamide treatment resulted in complete inactivation of the enzyme when assayed in the absence of mercaptoethanol (Table II). The stoichiometry of the reaction was investigated by incubating [¹⁴C]iodoacetamide with enzyme for 10 min, at which time inactivation was complete. Incubation for 1 hour did not result in any increased incorporation of radioactivity. The mixture was then subjected to gel filtration on a Sephadex G-15 column (Fig. 4). The inactivated enzyme contained 1 mol of iodoacetamide per mol of enzyme. Treatment of enzyme with p-chloromercuribenzoate before incubation with [¹⁴C]iodoacetamide blocked the incorporation of all but a trace of ¹⁴C (Fig. 4).

Immunoelectrophoretic studies showed that the iodoacetamide treatment did not result in either a major conformational change or breakdown of the enzyme into subunits. Immunodiffusion patterns obtained between antithymidylate synthetase serum and native or iodoacetamide-treated enzyme were identical (Fig. 5). The same results were obtained with p-CMB- and DTNB-treated enzyme.

Complement fixation tests (Fig. 6) could also detect no difference between native- and iodoacetamide-treated enzyme.

dUMP protected the enzyme from inactivation by iodoacetamide (Fig. 7). The degree of protection was dependent on the dUMP concentration, substantial protection being observed at molar ratios of dUMP to enzyme of 0.9 and 1.7. Control experiments showed that untreated enzyme lost no activity under similar conditions in the presence or absence of dUMP.

Thymidylate synthetase treated with iodoacetamide could be partially reactivated by including 0.11 M mercaptoethanol in the assay system (Table II). An average value of 45% of the activity of untreated enzyme was obtained on repetition of this experiment five times. Values ranged from 40 to 55%.

Inactivation and Reactivation of Thymidylate Synthetase Treated with DTNB and p-CMB—One mole of DTNB rapidly reacts with 1 mol of thymidylate synthetase, causing inactivation as determined in the minus thiol assay procedure (Fig. 8). However, 3 mol of p-CMB react per mol of thymidylate synthetase (Fig. 8). With p-CMB there is a rapid initial reaction (within 1 min) followed by a slower reaction complete within 10 min. The enzyme is inactive after 1 min of incubation with p-CMB in the absence of thiols. Both the DTNB- and p-CMB-treated enzyme regain full activity when tested in the assay system containing 0.11 M mercaptoethanol (Table II).

Effect of Pretreatment of Thymidylate Synthetase with Mercaptoethanol—It is common practice to include mercaptoethanol in solutions of thymidylate synthetase during purification (2, 3, 9, 15). This can be a complicating factor because hydroxyethyl-disulfide, an oxidation product of mercaptoethanol, inhibits the enzyme (3). We have omitted mercaptoethanol from buffers used during the purification procedure without diminishing the yield of active enzyme (8). When enzyme so prepared is assayed in the absence of mercaptoethanol, activity is routinely 30% that obtained in the standard assay (mercaptoethanol 0.1 M, Fig. 9, Table II) even with enzyme preparations stored for several months at 3° in 0.01 M potassium phosphate, pH 6.8. Pretreatment of enzyme in 0.1 M mercaptoethanol, pH 7.4, for periods up to 2 hours followed by 10-fold dilution into the minus mercaptoethanol assay mix still yielded only 30% maximum activity (Table II). At this dilution mercaptoethanol exerts no activating effect (Fig. 9). Thus, pretreatment with mercaptoethanol is not sufficient to restore full activity.

DISCUSSION

Nucleotides have a preferred anti conformation in solution, that is the oxygen atom on C-2 of the pyrimidine ring is directed

Fig. 4 (left). Sephadex G-15 gel filtration of [¹⁴C]iodoacetamide-treated thymidylate synthetase; 1 × 10⁻⁴ M enzyme was treated with 1 × 10⁻⁸ M [¹⁴C]iodoacetamide 400 cpm/nmol. Eluting buffer was 0.05 M Tris and 10⁻³ M EDTA, pH 7.4. Column was 2.5 × 30 cm. Each fraction was 2 ml. ——, absorbance at 280 nm; Δ, radioactivity; ○, radioactivity obtained after pretreatment of enzyme with p-CMB.

Fig. 5 (center). Immunodiffusion of native iodoacetamide-treated thymidylate synthetase versus antithymidylate synthetase serum. Well 1, untreated enzyme; Well 2, iodoacetamide-treated enzyme; Well 3, antithymidylate synthetase serum.

Fig. 6 (right). Complement fixation curves. Δ, untreated enzyme; ○, iodoacetamide-treated enzyme. Serum at 1:15,000.
FIG. 7. Effect of dUMP on the rate of iodoacetamide inactivation of thymidylate synthetase. Thymidylate synthetase \(5.6 \times 10^{-6} \text{ M}\); iodoacetamide \(2.8 \times 10^{-6} \text{ M}\;\text{dUMP concentration} \) \(0 \text{ M} (\bullet)\); \(10^{-6} \text{ M} (\Delta); \) \(10^{-5} \text{ M} (\bigtriangleup); \) \(10^{-4} \text{ M} (\bigtriangledown); \) \(3 \times 10^{-4} \text{ M} (\triangle)\). Carried out in 0.05 M Tris and 10 mM EDTA, pH 7.4. Numerals in parentheses indicate the molar ratio of dUMP to enzyme.

**TABLE II**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minus mercaptoethanol</td>
</tr>
<tr>
<td>1. None ...............</td>
<td>30</td>
</tr>
<tr>
<td>2. 0.1 M mercaptoethanol</td>
<td>30*</td>
</tr>
<tr>
<td>3. p-CMB ..............</td>
<td>0</td>
</tr>
<tr>
<td>4. DTNB ...............</td>
<td>0</td>
</tr>
<tr>
<td>5. Iodoacetamide ......</td>
<td>0</td>
</tr>
</tbody>
</table>

* Concentration of mercaptoethanol in final assay \(10^{-4} \text{ M}\).

away from the furanose ring (Fig. 10) (16). This is due to the restricted rotation about the glycosyl bond. It seems likely that the observed decrease in ellipticity upon formation of the enzyme-substrate complex (Fig. 1) results from a breakdown in the ring-ring interaction. Emerson et al. (17) observed that the amplitude of the Cotton effect of a series of uridine derivatives depended on the orientation of the pyrimidine to the sugar. A change in conformation from anti to syn resulted in a reversal in the sign of the Cotton effect. Cyclic derivatives which are held in fixed orientation displayed increased amplitudes compared to noncyclic derivatives whose rotation about the glycosidic bond is restricted but not prevented. Therefore, a reasonable interpretation of the CD data would be that upon formation of the enzyme-substrate complex, the pyrimidine ring rotates about the glycosidic bond to a syn conformation and is held in this new conformation. An alternative explanation for the decrease in ellipticity is that complex formation brings about a negative band in the protein spectrum at 267 nm. This seems unlikely because...
the ellipticity of the protein at 292 nm showed no change on addition of dUMP (Fig. 1).

The effects of mercaptoethanol on thymidylate synthetase are complex. Earlier results from this laboratory (2) which showed no activity in an assay mix lacking mercaptoethanol probably came about because the enzyme was prepared in buffers containing mercaptoethanol which on aging accumulated the inhibitor hydroxethylidissulfide (3). This substance inhibits the enzyme completely at $4 \times 10^{-5} \text{M}$. Excess mercaptoethanol in the assay mix reverses this effect. When inactive preparations were pretreated with fresh mercaptoethanol and diluted $10^4$-fold into assay mix lacking mercaptoethanol, the activity obtained was 30% of that obtained in the presence of mercaptoethanol (2), the same value as was obtained with enzyme purified in the absence of mercaptoethanol reported in the present work (Table 1). Dunlap et al. (3) reported that extensively dialyzed preparations of thymidylate synthetase assayed in the absence of mercaptoethanol show 40% of the activity of preparations assayed with added mercaptoethanol. Thus, mercaptoethanol is not an absolute requirement for thymidylate synthetase activity but exerts a stimulatory effect which is readily reversible.

One to one stoichiometry is observed when either dUMP, iodoacetamide, or DTNB interacts with thymidylate synthetase even though the enzyme consists of two identical subunits (4). This asymmetry could result because the subunits are linked in an asymmetric manner or alternatively ligand binding induces asymmetry. The fact that complement fixation studies detect no conformational change in the iodoacetamide-treated enzyme indicates that the former explanation is more probable.

The observation that iodoacetamide-inactivated enzyme can be restored to one-half its maximum activity by the addition of mercaptoethanol suggests that this substance activates the iodoacetamide-free subunit to full activity. Mercaptoethanol would not be expected to cause removal of $[\text{I}^\text{14}]$iodoacetamide (18) which is bound to the enzyme as carboxymethylcysteine (19). The low dissociation constant of $4 \times 10^{-5} \text{M}$ for the formation of the dUMP-thymidylate synthase complex calculated from the circular dichroic titration is substantiated by the considerable protection afforded by low concentrations of dUMP against iodoacetamide inactivation of the enzyme (Fig. 7). This protection, as previously reported for Ehrlich ascites thymidylate synthetase (20), is consistent with the hypothesis that a sulfhydryl group is involved at the active center (21). However, recent studies have shown that after reaction of the enzyme with fluorodeoxyuridyldiphosphate and methylenetetrahydrofolate both ligands are attached to a peptide which does not contain sulfur (22). This peptide was prepared by pronase digestion after denaturation with trichloroacetic acid and it may be that the initial reaction of pyrimidine nucleotide with enzyme involves a sulfhydryl group but on denaturation transfer takes place.

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