A Kinetic Study of Thymidylate Synthase from Lactobacillus casei*

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The kinetics of the reaction catalyzed by thymidylate synthase from Lactobacillus casei were investigated at pH 6.8 and 30° with the natural isomer of 5,10-methylene-5,6,7,8-tetrahydrofolate ((+)-CH₂-H₇folate). An apparatus for adding the enzyme to the reaction mixture and rapidly mixing without opening the sample compartment allowed the acquisition of spectrophotometric rate data within a few seconds after initiating the reaction. Initial velocity data in the absence of products with phosphate buffer gave linear, intersecting double reciprocal plots consistent with a sequential mechanism. The inability to obtain similar plots with 1,4-piperazinediethanesulfonic acid buffer is ascribed to the lack of sufficient sensitivity for rate measurements at the low deoxyuridylate (dUMP) concentrations required. A double reciprocal plot of data when both substrates, in constant proportion, were simultaneously varied was parabolic and thus consistent with a sequential mechanism. Product inhibition by thymidylate (dTMP) was competitive when dUMP was the varied substrate and noncompetitive when (+)-CH₂-H₇folate was the varied substrate. 7,8-Dihydrofolate (H₇folate) inhibited noncompetitively with either substrate. The data support an ordered reaction sequence where dUMP adds to the enzyme before (+)-CH₂-H₇folate and H₇folate is released before dTMP. Michaelis constants for dUMP and (+)-CH₂-H₇folate were 0.70 μM and 14.0 μM, respectively. Dissociation constants of dUMP and dTMP from the binary enzyme complexes were 0.32 μM and 2.37 μM, respectively.

The kinetics of the reaction catalyzed by thymidylate synthase from amethopterin-resistant L. casei grown in a 400-liter fermentor at Oak Ridge National Laboratories on medium described by Dunlap et al. (5). The enzyme was purified in the presence of 10 mM 2-mercaptoethanol by ammonium sulfate fractionation of cell-free extracts of sonicated cells, then by successive chromatography on carboxymethyl-Sepha- dex (C-50) and hydroxylapatite (11). Enzyme preparations, which were homogeneous by analytical polyacrylamide gel electrophoresis (12, 13), had specific activities of 3.2 to 3.7 units/mg when assayed by the usual spectrophotometric procedure (5). The enzyme was activated prior to use by dialysis at 5° for 12 to 24 h against 0.1 M potassium phosphate buffer, pH 6.8, containing 25 mM 2-mercapto- ethanol. One unit of thymidylate synthase is defined as that amount of enzymatic activity catalyzing the formation of 1 μmol of H₇folate per min.

Epimeric (∓)-H₇folate was prepared by the catalytic hydrogenation of folic acid in acetic acid (14) and was stored at −50° as a lyophilized powder under argon in sealed serum bottles (15). The active diastereoisomer ((−)-H₇folate) was prepared by reducing H₇folate with NADPH using dihydrofolate reductase from L. casei (16) and was purified by chromatography on DEAE-cellulose according to the procedure of Zakrzewski and Sansone (17) as modified by Donato et al. (18). The spectrum of the column eluate had absorption

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1 Thymidylate synthase is used as the recommended name rather than thymidylate synthetase since the latter, though widely used, violates rule 29 (1) which restricts the use of "synthetase" to ligases. Rule 23 (1) suggests that synthase be used for enzymes in other classes when the synthetic aspect of the reaction is to be emphasized.

2 The abbreviations used are: CH₂-H₇folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; Pipes, 1,4-piperazinediethanesulfonic acid; H₇folate, 5,6,7,8-tetrahydrofolate; H₇folate, 7,8-dihydrofolate.
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FIG. 1. Effect of 2-mercaptoethanol and formaldehyde on reaction rate. The reactions were carried out at 30° in 1 ml total volume containing 0.2 ml of 0.5 M potassium phosphate buffer, pH 6.8, and 0.1 ml of a modified cofactor solution that contained (+)-H$_2$folate, one-fifth the normal amount of HCHO, and no mercaptoethanol. The final concentrations of mercaptoethanol and HCHO are shown above. Each reaction mixture contained 0.02 nmol of thymidylate synthase. The reactions were started by adding 0.1 ml of 1 mM dUMP.

FIG. 2. Double reciprocal plot of initial velocity data with CH$_2$H$_2$folate the variable substrate. The reactions were conducted as described under "Methods." The concentrations of (+)-CH$_2$H$_2$folate were 4.17 μM (○), 6.06 μM (●), 9.19 μM (▲), 16.7 μM (■), and 91.9 μM (▲). The lines were drawn from analyses of each data subset by the program HYPER (24). The inset is a replot of intercepts versus the reciprocal of the corresponding (+)-CH$_2$H$_2$folate concentrations.

maxima at 272 and 292 nm and was characteristic of (+)-H$_2$folate (19). Analysis of the methylene derivative of the nearly white final product with excess dUMP and thymidylate synthase showed it to be essentially pure (9). H$_2$folate was produced by reducing folic acid with dithionite (20), purified by DEAE-cellulose chromatography and precipitated, washed, lyophilized, and stored as described by Donato et al. (18). Spectra of this material were identical with those of H$_2$folate (20, 21) and indicated that the material was 90% pure based on H$_2$folate. The purity may have been greater than this since the material was not dried over P$_2$O$_5$ and may have had a greater degree of hydration. The concentration of the H$_2$folate solution used in the product inhibition experiments was determined spectrophotometrically at 283 nm with a molar absorptivity of 28,500 M$^{-1}$cm$^{-1}$ (20, 21).

Other chemicals were obtained as follows: folic acid, dUMP, dTMP, NADPH from the Sigma Chemical Co.; carboxymethyl-sephadex (C-50) from Pharmacia Fine Chemicals, Inc.; hydroxylapatite from Bio-Rad Laboratories; Pipes from Calbiochem; formaldehyde, from Fisher Scientific Co.

Methods—Reaction rates were determined spectrophotometrically by measuring the increase in absorbance at 340 nm due to the formation of H$_2$folate (5, 22). Most rate measurements were obtained with reaction mixtures containing 100 pmol of Pipes buffer, pH 6.8, variable amounts of dUMP and CH$_2$H$_2$folate, and 0.0355 nmol of thymidylate synthase in a volume of 1 ml. The concentration of CH$_2$H$_2$folate was conveniently varied by making an appropriate dilution of a standard cofactor solution (51, which contained 250 μmol of sodium bicarbonate, pH 8.1, 337.5 μmol of formaldehyde, 1.25 mmol of 2-mercaptoethanol, and 3 mg of (-)-H$_2$folate in 5 ml, with an identical solution lacking the H$_2$folate. In this way, concentrations of bicarbonate, formaldehyde, and 2-mercaptoethanol were kept essentially constant in the reaction mixture at 5 mM, 6.75 mM, and 25 mM, respectively. The equilibrium of the nonenzymatic reaction of formaldehyde with H$_2$folate to form CH$_2$H$_2$folate is such ($K_{eq} = 32,000$ M$^{-1}$ (19)) that under the conditions employed, 99.9% of
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The lines were drawn from an analysis of each data subset by the program HYPER (24). The inset is a replot of intercepts versus the reciprocal of the corresponding (+)-CH₂-H₃folate concentrations.

The H₂folate added was converted to the methylene derivative. The concentration of HCHO was sufficiently greater than that of H₂folate so that the formation of CH₂-H₃folate did not change the concentration of free HCHO by more than 3%, and no correction was made. The concentration of (+)-CH₂-H₃folate in the cofactor solution was determined spectrophotometrically with thymidylate synthase and excess dUMP; cofactor solutions were used the same day they were prepared. Before use, activated thymidylate synthase solutions were diluted 1 to 20 with 0.1 M Pipes buffer, pH 6.8, containing 25 mM 2-mercaptoethanol. Enzyme concentrations were determined by the absorbance at 278 nm with absorptivity coefficients of 1.05 x 10⁴ M⁻¹ cm⁻¹ or 1.53 ml mg⁻¹ cm⁻¹ (11).

The reactions were initiated by adding 20 µl of the diluted enzyme preparation to the reaction mixture with an apparatus that allowed addition and mixing without opening the sample compartment (23). In this manner, acquisition of rate data began within 2 s after starting the reaction. The reactions were carried out at 30°C and measurements were made with a Gilford model 200 spectrophotometer (Gilford Instruments, Inc.) with the recorder set at 0.05 A full scale deflection and a chart speed of 12 inches/min. Any deviations from this procedure are noted in the figure legends.

Data were analyzed with BASIC language versions of Clandel's programs HYPER, SEQUEN, COMP, and NONCOMP (24), and a computer program for nonlinear least square analysis according to Marquardt's method (25).

RESULTS

Mercaptoethanol is a usual constituent of assays of thymidylate synthase since it has been reported to stimulate the enzyme activity (5, 8). To determine the desirability of including it in reaction mixtures for the kinetic investigation, the influence of mercaptoethanol concentration on rate was examined. The results are shown in Fig. 1. The concentration which gave the optimum rate was found to depend on the HCHO concentration. The two substances seemed to act antagonistically, i.e. high mercaptoethanol concentrations relieved the inhibition by high HCHO concentrations, and high HCHO concentrations relieved the inhibition by high mercaptoethanol concentrations. The latter effect suggests that mercaptoethanol may inhibit by reducing the HCHO concentration as a result of hemithioacetal formation (19). Although nearly full activity was found in reaction mixtures devoid of mercaptoethanol when the HCHO concentration was sufficiently low, mercaptoethanol was included in reaction mixtures for the kinetic study because it was difficult to compensate for the very rapid nonenzymatic rate of absorbance change that occurred in its absence.

The reaction was also examined with pure formaldehyde, because commercial formaldehyde contains methanol as a preservative and may also contain various oxidation products which might cause the inhibition seen at high HCHO concentrations. Pure formaldehyde was made by heating paraformaldehyde and passing the gaseous HCHO into deaerated water with argon gas. The resulting HCHO solution was used and analyzed (26) the same day it was prepared. Pure formaldehyde behaved exactly like commercial formaldehyde, so the commercial material was used in the kinetic investigation.

A plot of v⁻¹ versus [dUMP]⁻¹ at several constant concentrations of (+)-CH₂-H₃folate is shown in Fig. 2. Although the pattern appears to be a series of parallel lines, which would indicate a ping-pong reaction sequence, all other kinetic data (see below) suggest a sequential mechanism. The reason for this apparent discrepancy may be that the point of intersection
Fig. 6. Double reciprocal plot of product inhibition data with (+)-CH₂-H⁺folate the variable substrate and dTMP the product inhibitor. The concentration of (+)-CH₂-H⁺folate was 17.7 μM in all reaction mixtures. The concentrations of dTMP were 0 μM (O), 25 μM (■), 60 μM (△), and 100 μM (●). The lines were drawn from an analysis of the data by the program NONCOMP (24).

The data presented in Figs. 5, 6, 7, and 8 show that dTMP was a competitive inhibitor when dUMP was the variable substrate but inhibited noncompetitively when (+)-CH₂-H⁺folate was the variable substrate. H⁺folate, on the other hand, was a noncompetitive inhibitor when either dUMP or (+)-CH₂-H⁺folate was the variable substrate. These results are consistent with an ordered reaction sequence under steady state conditions in which dUMP adds to the enzyme before (+)-CH₂-H⁺folate and H⁺folate is released from the enzyme before dTMP.

All of the data in Figs. 2, 3, 5, 6, 7, and 8 were combined and used to calculate the values of the kinetic constants. A computer program based on Marquardt's method of nonlinear least squares (25) was used to fit the data to Equation 1, which is the rate equation for an Ordered Bi Bi reaction sequence (27) with the PQ terms omitted. Equation 1 accounts quite well for the variation in velocity; the multiple coefficient of determination is 0.99.

\[
V = \frac{V_{\text{max}}[A][B]}{K_v K_s + K_v A + K_p B + AB + \frac{K_v K_s Q}{K_p} + \frac{K_v K_s P}{K_p} + \frac{K_v K_p A P}{K_p} + \frac{K_v B Q}{K_p} + \frac{AB P}{K_p}}
\]  

Values for the kinetic constants are given in Table I. The value of 10.9 milliunits for \(V_{\text{max}}\) corresponds to a specific activity of 4.39 units/mg, which is comparable to the highest previously reported values obtained under similar reaction conditions. This specific activity corresponds to a molecular activity of 307 units μmol⁻¹. The values of 0.7 μM and 14 μM for the Michaelis constants of dUMP and (+)-CH₂-H⁺folate agree with those reported previously (5). \(K_v\) and \(K_p\) do not occur independently in Equation 1 but only as the ratio \(K_v/\)
The concentration of dUMP was 20 μM in all reaction mixtures. The concentrations of H₄folate were 0 μM (●), 25 μM (●), 60 μM (▲), and 100 μM (●). The lines were drawn from an analysis of the data by the program NONCOMP (24).

**Table 1**

<table>
<thead>
<tr>
<th>Kinetic constant</th>
<th>Estimate</th>
<th>Asymptotic standard error</th>
<th>95% Confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{\text{max}} )</td>
<td>10.85 nmoths</td>
<td>0.25</td>
<td>10.96-11.34</td>
</tr>
<tr>
<td>( K_a )</td>
<td>0.70 μM</td>
<td>0.10</td>
<td>0.49-0.90</td>
</tr>
<tr>
<td>( K_b )</td>
<td>14.0 μM</td>
<td>0.75</td>
<td>12.6-15.5</td>
</tr>
<tr>
<td>( K_{i\alpha} )</td>
<td>0.32 μM</td>
<td>0.10</td>
<td>0.11-0.52</td>
</tr>
<tr>
<td>( K_{i\beta} )</td>
<td>193.0 μM</td>
<td>52.6</td>
<td>88.0-297.0</td>
</tr>
<tr>
<td>( K_{\alpha} )</td>
<td>2.37 μM</td>
<td>0.24</td>
<td>1.88-2.86</td>
</tr>
<tr>
<td>( K_{\beta}/K_{\alpha} )</td>
<td>0.098</td>
<td>0.008</td>
<td>0.021-0.055</td>
</tr>
</tbody>
</table>

\( a = \text{dUMP}, \ b = \text{CH}_2\text{H}_4\text{folate}, \ p = H_4\text{folate}, q = \text{dTMP} \)

\( K_{\alpha} \) and \( K_{\beta} \) correspond to the dissociation constants for the binary enzyme complexes with dUMP and dTMP, respectively. The value of \( K_{\alpha} \) in Table I is essentially the same as that of Leary et al. (8) (4 × 10⁻⁷ M) for the dissociation constant of dUMP measured by circular dichroic spectral changes. Their values ranged from 0.13 to 0.45 μM, which are well within the 95% confidence limits given in Table I. By means of equilibrium dialysis, Galivan et al. (9) reported values of 1.80 and 5.75 μM for the dissociation constants of the dUMP and dTMP complexes, respectively. These agree rather well with 0.32 and 2.37 μM for \( K_{\alpha} \) and \( K_{\beta} \), when one considers that the former are based on equilibrium measurements and the latter are obtained from initial velocity measurements.

**REFERENCES**

A kinetic study of thymidylate synthase from Lactobacillus casei.
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