A Kinetic Study of Thymidylate Synthase from \textit{Lactobacillus casei}*

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The kinetics of the reaction catalyzed by thymidylate synthase from \textit{Lactobacillus casei} were investigated at pH 6.8 and 30°C with the natural isomer of 5,10-methylene-5,6,7,8-tetrahydrofolate (\(+\)-\(\text{CH}_2\text{-H}_2\text{folate}\)) and \(\text{H}_2\text{folate}\) with NADPH using dihydrofolate reductase from \textit{L. casei}. 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 a linear, intersecting double reciprocal plots consistent with a sequential mechanism. The inability to obtain similar plots with 1,4-piperazine diethanesulfonic 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 \((+\)-\(\text{CH}_2\text{-H}_2\text{folate}\)) was the varied substrate. The dissociation constants of dUMP and \((+\)-\(\text{CH}_2\text{-H}_2\text{folate}\)) were 0.70 \(\mu\text{M}\) and 14.0 \(\mu\text{M}\), respectively. Dissociation constants of dUMP and dTMP from the binary enzyme complexes were 0.32 \(\mu\text{M}\) and 2.37 \(\mu\text{M}\), respectively.

Thymidylate synthase\(^1\) catalyzes the reductive methylation of 2'-deoxyuridylate by \((+\)-5,10-methylene-5,6,7,8-tetrahydrofolate) to form thymidylate and 7,8-dihydrofolate (2, 3) as shown. The reaction is of special interest because oxidation of the methyl donor accompanies methyl transfer and also be

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\(\text{dUMP} + (++\)-\(\text{CH}_2\text{-H}_2\text{folate}\)) \(\rightarrow\) dTMP + \(\text{H}_2\text{folate}\)

The discovery of a amethopterin-resistant strain of \textit{Lactobacillus casei} that produces relatively large quantities of thymidylate synthase (5) and the development of purification procedures (5, 6) have made thymidylate synthase readily available in apparently pure form. The enzyme from this source is a dimer composed of subunits that seem identical (7). However, recent evidence suggests that the subunits associate asymmetrically so that the dimeric enzyme has only one catalytic site (8-10). Although Michaelis constants and inhibitor constants for a number of substrate analogs have been determined for thymidylate synthasen from various sources, no extensive kinetic studies with only the active isomer of \(\text{CH}_2\text{-H}_2\text{folate}\) have been reported. The reaction has been investigated in only the forward direction because the equilibrium is far toward thymidylate synthesis. This report describes a kinetic investigation of the reaction catalyzed by thymidylate synthase from amethopterin-resistant \textit{L. casei}.

**EXPERIMENTAL PROCEDURES**

Materials—Thymidylate synthase was purified from amethopterin-resistant \textit{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-Sepharose (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°C for 12 to 24 h against 0.1 M potassium phosphate buffer, pH 6.8, containing 25 mM 2-mercaptoethanol. One unit of thymidylate synthase is defined as that amount of enzymatic activity catalyzing the formation of 1 \(\mu\text{mol}\) of \(\text{H}_2\text{folate}\) per min.

Epimeric \((\pm)\text{-H}_2\text{folate}\) was prepared by the catalytic hydrogenation of folic acid in acetic acid (14) and was stored at \(-50^\circ\)C. L-5,10-Methylene-5,6,7,8-tetrahydrofolate, \((+\)-\(\text{CH}_2\text{-H}_2\text{folate}\)), \((\pm)\text{-H}_2\text{folate}\), 5,6,7,8-tetrahydrofolate: Pipes, 1,4-piperazine diethanesulfonic acid; \(\text{H}_2\text{folate}\), 5,6,7,8-tetrahydrofolate; \(\text{H}_2\text{folate}\), 7,8-dihydrofolate.

\(\text{CH}_2\text{-H}_2\text{folate}\), 5,10-methylene-5,6,7,8-tetrahydrofolate; Pipes, 1,4-piperazine diethanesulfonic acid; \(\text{H}_2\text{folate}\), 5,6,7,8-tetrahydrofolate; \(\text{H}_2\text{folate}\), 7,8-dihydrofolate.

\(^1\) 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.
Kinetics of Thymidylate Synthase

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 dUMP the variable substrate. The reactions were conducted as described under "Methods." The concentrations of (+)-CH$_2$-H$_2$folate were 4.17 $\mu$M (V), 6.06 $\mu$M (C), 9.19 $\mu$M (A), 16.7 $\mu$M (M), and 91.9 $\mu$M (B). The lines were drawn from analyses of each data subset by the program HYPHER (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 showed it to be essentially pure (9). H$_2$folate was produced by reducing folic acid with dithionite (20), purified by DEAE-cellulose chromatography (17), 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. 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 pmol of sodium bicarbonate, pH 8.1; 337.5 pmol of formaldehyde, 1.25 $\mu$mol 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 bicarbonates, 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...
942 Kinetics were as described under "Methods." The lines were drawn from the variable substrate. The reactions were conducted in 0.1 M potassium phosphate buffer, pH 6.8, with cofactor solution prepared with (±)-HCHO folate. The reactions were started by adding 0.139 nmol of thymidylate synthase (specific activity 3.7 units/mg). The concentrations of (±)-CH₂-H₄folate were 4.81 μM (●), 6.62 μM (▼), 9.33 μM (▲), 16.7 μM (Δ), 34.3 μM (■), and 85.8 μM (□). All other conditions were as described under "Methods." 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 (±)-CH₂-H₄folate concentrations.

The H₄folate added was converted to the methylene derivative. The concentration of HCHO was sufficiently greater than that of H₄folate so that the formation of CH₂-H₄folate did not change the concentration of free HCHO by more than 3%, and no correction was made. The concentration of (±)-CH₂-H₄folate in the cofactor solution was determined spectrophotometrically with thymidylate synthase and excess dUMP (9); 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 measuring absorbance at 278 nm with absorptivity coefficients of 1.05 x 10⁴ M⁻¹cm⁻¹ or 1.55 ml mg⁻¹cm⁻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 Cleland'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-H₄folate 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 (+)-CH$_2$H$_4$folate the variable substrate and dTMP the product inhibitor. The reactions were conducted as described under "Methods." The concentration of dUMP was 20 $\mu$M in all reaction mixtures. The concentrations of dTMP were 0 $\mu$M (O), 25 $\mu$M (■), 60 $\mu$M (▲), and 100 $\mu$M (●). The lines were drawn from an analysis of the data by the program NONCOMP (24).

The data lies far to the left of the graph shown and the data needed to show the intersecting pattern would have required obtaining rates at dUMP concentrations too low for the method employed to accurately measure.

Ping-pong and sequential mechanisms can also be distinguished by measuring rates when both substrates are varied while the ratio of their concentrations is kept constant. Plots of $v^{-1}$ versus $[S]^{-1}$ are linear for ping-pong mechanisms and parabolic for sequential mechanisms. A graph of this type is shown in Fig. 3, where it is seen that the data are consistent with a sequential mechanism.

Other data which tend to confirm the interpretation of the mechanism as sequential were obtained with phosphate, a competitive inhibitor of dUMP, to increase the value of the apparent Michaelis constant ($K_{app}$) and thus increase the range of substrate concentration influencing the reaction rate. The Pipes buffer in the reaction mixture was replaced with phosphate buffer, and initial velocity studies were conducted at dUMP concentrations which appreciably affected the rate and were large enough that accurate rate data could be easily obtained. The intersecting pattern in Fig. 4 is consistent with a sequential mechanism.

There are several sequential mechanisms which can be distinguished on the basis of the type of inhibition caused by each product when each substrate was the variable substrate. $^3$ Unpublished observations.

Fig. 7. Double reciprocal plot of product inhibition data with dUMP the variable substrate and H$_4$folate the product inhibitor. The reactions were conducted as described under "Methods." The concentration of (+)-CH$_2$H$_4$folate was 17.7 $\mu$M in all reaction mixtures. The concentrations of H$_4$folate were 0 $\mu$M (●), 25 $\mu$M (■), 60 $\mu$M (▲), and 100 $\mu$M (●). 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 (+)-CH$_2$H$_4$folate was the variable substrate. H$_4$folate, on the other hand, was a noncompetitive inhibitor when either dUMP or (+)-CH$_2$H$_4$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 (+)-CH$_2$H$_4$folate and H$_4$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.

$$V = \frac{V_{max} AB}{K_v K_s + K_s A + K_B + AB + \frac{K_v K_Q}{K_s}}$$

Values for the kinetic constants are given in Table I. The value of 10.9 milliunits for $V_{max}$ 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 $\mu$mol$^{-1}$. The values of 0.7 $\mu$M and 14 $\mu$M for the Michaelis constants of dUMP and (+)-CH$_2$H$_4$folate agree with those reported previously (5). $K_v$ and $K_s$ do not occur independently in Equation 1 but only as the ratio $K_v/K_s$. $^3$ Unpublished observations.
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The concentrations of $H_4$folate were 0 PM (○), 25 PM (●), 60 PM (△), and 100 PM (◆). The lines were drawn from an analysis of the data by the program NONCOMP (24).

<table>
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<th>Kinetic constant</th>
<th>Estimate</th>
<th>Asymp. standard error</th>
<th>95% Confidence limits</th>
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<td>$V_{\text{max}}$</td>
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<td>0.008</td>
<td>0.021</td>
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</table>

The kinetic data, taken all together, are consistent with an ordered reaction sequence that can be diagrammed as shown in Fig. 9. Such a sequence is in agreement with the substrate binding studies of Galivan et al. (9) where thymidylate synthase bound dUMP and dTMP in the absence of folates, but folates were not bound in the absence of nucleotides. The data are not consistent with a random reaction sequence under equilibrium conditions, where only competitive inhibition would be observed for all combinations of product inhibitor and variable substrate. Neither are the data consistent with a ping-pong mechanism, which would predict competitive inhibition when the product inhibitor and the variable substrate were both methylated (i.e., $H_4$folate and dTMP) and thus competing for the demethyl form of the enzyme, and also when neither the product inhibitor nor the variable substrate was methylated (i.e., dUMP and $H_4$folate) and thus competing for the methylated enzyme. The data also exclude a nonclassical ping-pong mechanism where competitive inhibition between nucleotides and competitive inhibition between folate derivatives would be expected. A Thorell-Chance mechanism is also excluded since it predicts that two of the four combinations above would show competitive inhibition; which two would depend on the order of addition. Two other possible mechanisms, viz., a random reaction sequence under steady state conditions and an ordered reaction sequence under equilibrium conditions, are eliminated by the data from the initial velocity studies.

$K_{i_d}$ and $K_{i_p}$ correspond to the dissociation constants for the binary enzyme complexes with dUMP and dTMP, respectively. The value of $K_{i_d}$ in Table I is essentially the same as that of Leary et al. (8) ($4 \times 10^{-7}$ μM) 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 $K_{i_d}$ and $K_{i_p}$, 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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