QUENCHING OF THYMIDYLATE SYNTHETASE FLUORESCENCE BY SUBSTRATE ANALOGS*

R.K. Sharma** and R.L. Kisliuk

Department of Biochemistry and Pharmacology
Tufts University School of Medicine
Boston, Massachusetts 02111

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SUMMARY: Quenching of fluorescence occurs when Lactobacillus casei thymidylate synthetase is titrated with fluorodeoxyuridylate in the presence of l-L-methylenetetrahydrofolate to form a ternary complex. Neither fluorodeoxyuridylate nor l-L-methylenetetrahydrofolate added singly has any effect on enzyme fluorescence but d-L-methylenetetrahydrofolate alone causes quenching. Thus ternary complex formation and interaction with d-L-methylenetetrahydrofolate alter the environment of tryptophan residues in thymidylate synthetase in a similar manner.

Thymidylate synthetase catalyzes the reaction of methylenetetrahydrofolate with deoxyuridylate to form thymidylate and dihydrofolate (1). Tetrahydrofolate serves as the reductant to convert the methylene group to a methyl group which displaces the hydrogen on the 5 carbon of dUMP. The pyrimidine nucleotide analog FdUMP, a potent inhibitor of thymidylate synthetase, is known to form a ternary complex with the enzyme in the presence of methylenetetrahydrofolate (2,3,4). We here report studies on the quenching of tryptophan fluorescence in thymidylate synthetase brought about by interaction with substrate analogs.

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** Present address: Department of Biochemistry, University of South Alabama School of Medicine, Mobile, Alabama 36688

Abbreviations: $1\text{-}CH_2\text{-}THF$ = natural diastereoisomer of methylenetetrahydrofolate at carbon 6
$d\text{-}CH_2\text{-}THF$ = unnatural diastereoisomer of methylenetetrahydrofolate at carbon 6
FdUMP = 5-fluoro-2'-deoxyuridylate

MATERIALS AND METHODS

Folic acid from Nutritional Biochemicals was used to prepare d,l-L-THF by catalytic hydrogenation (5) over platinum catalyst. l-L-THF was prepared enzymatically from dihydrofolate by the method of Mathews and Huennekens (6) as modified (7). It was desalted by gel filtration on a Sephadex G-10 (2 x 52 cm) using 0.02 M potassium phosphate, 0.2 M 2-mercaptoethanol, pH 7.4 as eluent. The concentration of THF was determined spectrophotometrically (8). FdUMP was obtained from Terra-Marine Bioresearch, La Jolla, California, and purified on a 0.5 by 8 cm DEAE cellulose column equilibrated with 0.01 M NH_4HCO_3 pH 7.4 as suggested by Dr. Daniel Santi. One mg FdUMP in 1 ml 0.01 M NH_4HCO_3 pH 7.4 was applied to the column and eluted with a linear gradient generated from 75 ml 0.01 M NH_4HCO_3 in the mixing chamber and 75 ml 0.2 M NH_4HCO_3 in the reservoir. FdUMP emerges at 0.12 M NH_4HCO_3. The NH_4HCO_3 was removed by lyophilization and the FdUMP dissolved in 1 mM potassium phosphate pH 7.3. The purified material had the absorption spectrum and ability to inhibit thymidylate synthetase expected of FdUMP (1,9).

Crude bacterial extracts containing thymidylate synthetase were prepared at the New England Enzyme Center (10) from a strain of methotrexate-resistant Lactobacillus casei developed in this laboratory (11). The crude extract was treated with RNAse and DNAse (12), concentrated with $(NH_4)_2SO_4$, and dialyzed at 4° overnight vs. six liters of 0.08 M potassium phosphate, pH 6.8. It was purified using phosphocellulose according to a method developed by Dr. Gladys Maley (personal communication). 500 gms phosphocellulose (Schleicher and Schuell Co.) was washed with the following: 7 l 0.5 M NaOH, (15 min); water to neutrality; 0.5 M HCl (15 min); water to neutrality; 0.1 M potassium phosphate, pH 7.1, until the pH of the effluent was the same as the washing buffer, 0.01 M potassium phosphate, pH 7.1 until the pH and conductivity of the effluent were the same as the washing buffer. 1,120 ml of the dialyzed enzyme preparation (12 mg protein/ml) in 0.08 M potassium phosphate, pH 6.8, was diluted to 2,240 ml with water and placed on a 10 x 64

cm phosphocellulse column. The column was eluted with 3 l each of potassium phosphate pH 7.1 of increasing concentration 0.025 M, 0.05 M, 0.075 M, 0.09 M and 6 l of 0.125 M. Fractions of 240 ml were collected at a rate of 1 l per hour. Dihydrofolate reductase elutes with the first wash. Thymidylate synthetase elutes with 0.125 M potassium phosphate. The pooled fractions were concentrated by $(NH_4)_2SO_4$ precipitation and dialyzed overnight against a solution containing 0.08 M potassium phosphate, 0.1 mM KCl, 1 mM disodium ETDA, pH 6.8. Further purification was obtained by gel filtration on Sephadex G-100. 100 mg protein in 4-6 ml was applied to the column (2.6 x 86 cm) and eluted with the same solution (13). Fractions having a specific activity of 190-210 were pooled and concentrated by $(NH_4)_2SO_4$ precipitation. This protein (yield 36 mg) showed a single band when 180 µg were subjected to acrylamide gel electrophoresis at pH 8.5. Tryptophan analysis of the enzyme by magnetic circular dichroism (14) carried out by Dr. Barton Holmquist (Biophysics Research Laboratory, Harvard Medical School, Boston, Mass.) showed 12 tryptophan residues per MW 68,000. The extinction coefficient of enzyme at 280 nm was taken as 108,000 based on amino acid analysis performed by Dr. B.H. Davis and Dr. Juris Ozols of the University of Connecticut.

The spectrophotometric assay for thymidylate synthetase was carried out as described (15). Specific activity is defined as the number of micromoles of thymidylate formed per hour per mg of protein at 30°.

Fluorescence measurements were made in Perkin-Elmer MPF-2A fluorescence spectrophotometer. All fluorescence measurements were performed at 25° $\pm$ 1 in 1.0 cm quartz cells, using a 2 ml sample. Emission spectra were obtained with excitation at 291 nm with 6 nm band slit, 6 nm emission slit and sensitivity setting 5.

RESULTS

The protein emission spectrum for thymidylate synthetase activated at 291 nm is shown in Fig. 1A. The emission band at 338 nm is typical of tryptophan fluorescence reported for other proteins (16). Addition of in-

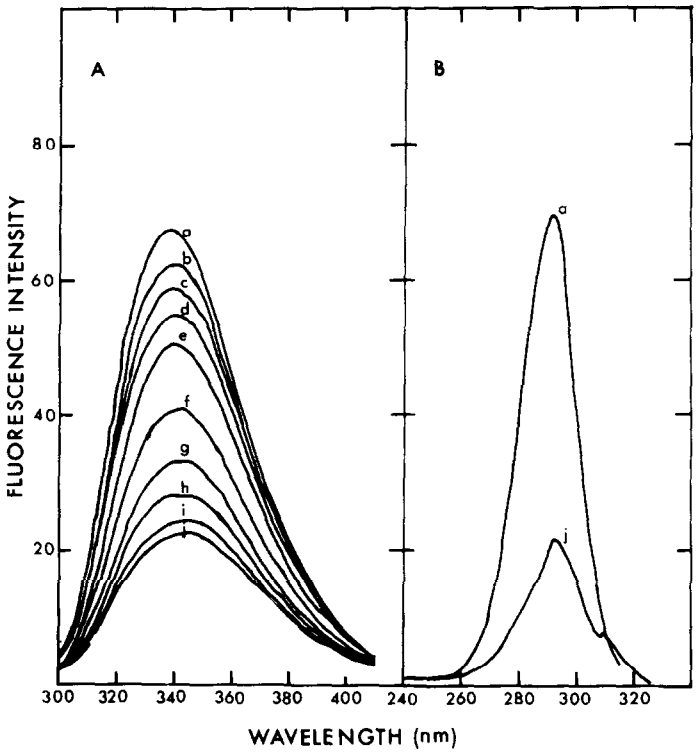

Figure 1 Fluorescence spectra of thymidylate synthetase and the ternary complex formed from enzyme, 1-CH_2-THF and FdUMP. The incubating medium contained 0.1 M 2-mercaptoethanol, 0.02 M $MgCl_2$, 3 x 10^{-5} M formaldehyde, 0.08 M potassium phosphate, 0.1 M KCl, 10^{-3} M Na_2 EDTA, pH 6.8. A. Fluorescence emission spectra of thymidylate synthetase 3.6 x 10^{-7} M (a), plus 1-CH_2-THF 3.8 x 10^{-6} M (a) plus FdUMP 0.5 x 10^{-7} M (b), 10^{-7} M (c), 1.5 x 10^{-7} M (d), 2.25 x 10^{-7} M (e), 3 x 10^{-7} M (f), 3.6 x 10^{-7} M (g), 4.5 x 10^{-7} M (h), 6 x 10^{-7} M (i), 7.5 x 10^{-7} M (j). B. Excitation spectra of thymidylate synthetase 3.6 x 10^{-7} M (a) plus 1-CH_2-THF 3.8 x 10^{-6} M (a) plus FdUMP 7.5 x 10^{-7} M (j).

crements of FdUMP to a solution containing enzyme and 1-CH_2-THF results in quenching of fluorescence (Fig. 1A) and a shift in the emission maximum to 348 nm. These changes are stable for at least 1 hr. The same data plotted in Fig. 2 show that the titration curve follows the form expected for single site binding. Control experiments showed that the presence of 1-CH_2-THF, FdUMP and dUMP individually at concentrations up to 10^{-4} M did not alter the fluorescence emission spectrum of the enzyme shown in Fig. 1A. Additional

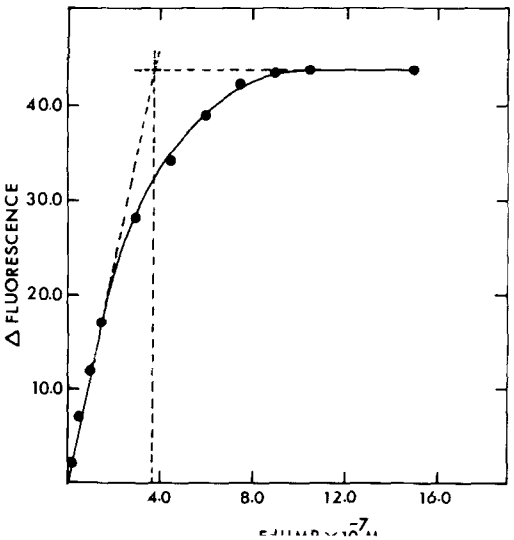

Figure 2 Data of Figure 1A plotted as the change in fluorescence at 338 nm versus FdUMP concentration (●—●).

controls demonstrated that the fluorescence emission spectra neither of tryptophan 4.4 x 10^{-6} M nor bovine serum albumin 3.6 x 10^{-7} M were altered by addition of 1-CH_2-THF 3.6 x 10^{-6} M or FdUMP 3.6 x 10^{-6} M singly or in combination.

Fig. 1B illustrates the excitation spectrum of the enzyme with emission monitored at 338 nm. Addition of 1-CH_2-THF does not alter the spectrum. The excitation maximum is at 291 nm as expected. With formation of the ternary complex (Fig. 1B) the excitation maximum remains at 291 nm but fluorescence is quenched and a new peak appears at 310 nm.

When 10^{-4} M d-CH_2-THF was added to enzyme the fluorescence was quenched and the emission maximum shifted from 338 nm to 335 nm(Fig. 3A). Unlike the fluorescence changes described in Fig. 1 for the ternary complex, the quenching brought about by d-CH_2-THF was not stable and the excitation peak at 310 nm (Fig. 1B) observed in the ternary complex was absent. Over a period of 2.5 hours fluorescence increased somewhat (Fig. 3A). Incubation

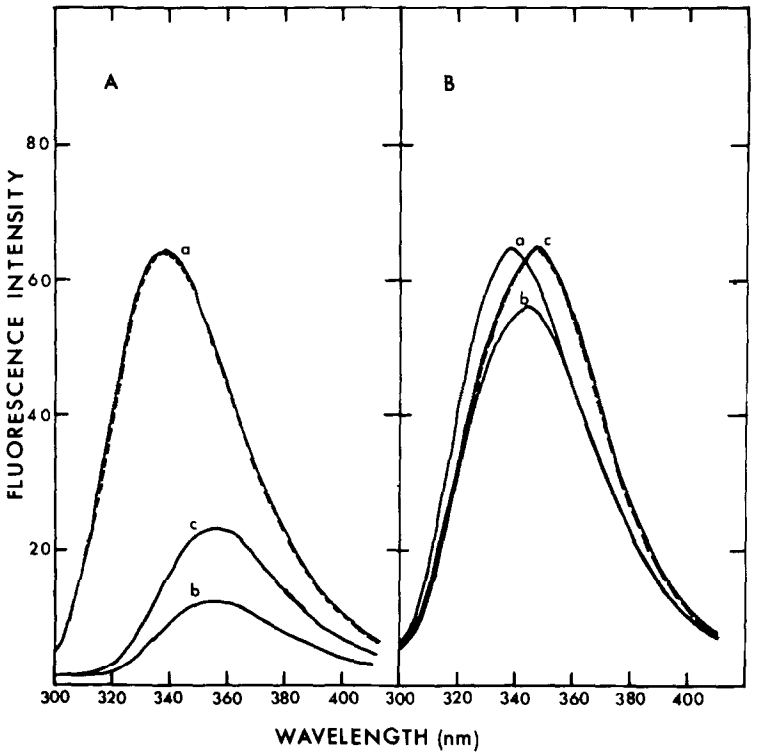

Figure 3 Effect of d-CH_2-THF on thymidylate synthetase fluorescence. Incubation medium as in Fig. 1. A. Thymidylate synthetase 3.6 x 10^{-7} M (a), plus d-CH_2-THF 10^{-4} M (b), same solution 2.5 hr. later (c). B. Thymidylate synthetase 3.6 x 10^{-7} M (a), plus d-CH_2-THF 3.7 x 10^{-6} M (b), same solution after 15 min (c), plus FdUMP 3.6 x 10^{-6} M (----).

for an additional 2.5 hours caused no further change. After dialysis against the incubation medium described in the legend of Fig. 1 for 24 hours the fluorescence of the original enzyme solution returned.

When 10^{-6} M d-CH_2-THF was added to enzyme less quenching occurred (Fig. 3B) and the original fluorescence intensity returned after 15 minutes but the emission maximum shifted to 348 nm. Addition of 3.6 x 10^{-6} M FdUMP caused no further change.

When 10^{-4} M dihydrofolate was added to enzyme 20% of the fluorescence was quenched without any change in the emission maxima. Addition of 3.6 x 10^{-6} M FdUMP resulted in no further change.

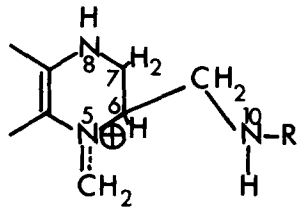

Figure 4 Hypothetical iminium ion intermediate.

DISCUSSION

The results obtained suggest that during catalysis by thymidylate synthetase the environment of tryptophan residues is altered. L. casei thymidylate synthetase has been reported to consist of two subunits (17) each with the same amino acid composition. Our data do not permit a conclusion as to whether the fluorescence quenching occurs to changes in only one or in both subunits but maximum quenching occurs when one mole of FdUMP interacts with one mole of enzyme (Fig. 2).

The fact that d-CH_2-THF, which is a competitive inhibitor with respect to 1-CH_2-THF (18), by itself causes fluorescence quenching similar to that observed on ternary complex formation suggests that carbon 6 of 1-CH_2-THF may invert its configuration as the ternary complex is formed. An intermediate with a structure related to the iminium ion postulated for this reaction (19) (Fig. 4) might account for the observed fluorescence changes in the ternary complex in that the 5,6 bond might be strained, altering the configuration at carbon 6.

The change of fluorescence with time observed with d-CH_2-THF in the presence of thymidylate synthetase (Fig. 3) indicates that more than one type of binary complex exists between these two substances.

The appearance of a peak in the excitation spectrum at 310 nm (Fig. 1B) probably reflects the conversion of 1-CH_2-THF to a new form as observed in ultraviolet (4,20,21) and laser-Raman (22) spectroscopic studies.

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