The Effect of Substrate Analogs on the Circular Dichroic Spectra of Thymidylate Synthetase from *Lactobacillus casei*†

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ABSTRACT: Circular dichroism studies from 290 to 400 nm with the thymidylate synthetase from *Lactobacillus casei* revealed characteristic Cotton effects in the presence of various folate analogs plus 5-fluoro-2′-deoxyuridylate. Omission of either substrate analog prevented the appearance of the Cotton effects. When 5-fluoro-2′-deoxyuridylate and (±)-5,10-methylenetetrahydrofolate are mixed with the synthetase, a ternary complex results which yields distinctive minor negative ellipticity bands at 285 and 332 nm and a major positive band at 305 nm. Similar results were obtained with the ternary complex containing (+)-5,10-methylenetetrahydrofolate, but the enzymically inactive (−) diastereoisomer induced only the positive band at 305 nm. More intense Cotton effects were elicited by (±)-5,11-methylenetetrahydrohomofolate with a major positive ellipticity band at 308 nm and a minor negative band at 335 nm. A ternary complex was also formed with dihydrofolate, which provided a major circular dichroic band at 305 nm and a broad minor negative band in the region of 335 nm. Deoxyuridylate and thymidylate also formed ternary complexes with dihydrofolate, but their ellipticity bands were much less intense. Other folate analogs that formed ternary complexes with 5-fluoro-2′-deoxyuridylate to provide characteristic circular dichroic spectra were tetrahydrofolate, tetrahydrohomofolate, 10-methyltetrahydrofolate, and a 2-amino-4-hydroxyquinazoline derivative. By measuring the increment in ellipticity at 305 nm on addition of specific ligands to enzyme solutions, it was determined that the *L. casei* thymidylate synthetase contains two binding sites for 5-fluoro-2′-deoxyuridylate and for each of the diastereoisomers of 5,10-methylenetetrahydrofolate. An improved procedure is presented for the large-scale purification and crystallization of *L. casei* thymidylate synthetase.

Studies of the mechanism involved in the reductive transfer of the methylene group from 5,10-CH_2H_4folate[1] to dUMP by thymidylate synthetase have stimulated many imaginative and interesting approaches to this elusive problem. Following the finding of Pastore and Friedkin (1962) that the reducing equivalents required for the conversion of the methylene to a methyl group are provided by the pyrazine ring of (±)-5,10-CH_2H_4folate, evidence was presented by Lorenson et al. (1967) that the hydrogens are derived from the 6 position exclusively.

On the basis of enzymic and model studies with compounds designed to represent intermediates in the reaction, it was proposed that a ternary complex is formed between dUMP, (±)-5,10-CH_2H_4folate, and thymidylate synthetase during the course of methyl transfer (Santi and McHenry, 1972; Langenbach et al., 1972). Recent studies have established (+)-5,10-CH_2H_4folate as the active or biologic diastereoisomer (Leary et al., 1974). A key step in the reaction is believed to involve an attack on C-6 of dUMP by a nucleophilic group in the enzyme which promotes a covalent linkage between the methylene carbon of 5,10-CH_2H_4folate and C-5 of dUMP. Partial support for this concept is derived from kinetic studies (Santi and Brewer, 1973) and from binding studies with the quasisubstrate FdUMP, a compound that forms a stable covalent linkage with thymidylate synthetase in the presence of 5,10-CH_2H_4folate (Santi et al., 1974; Danenberg et al., 1974; Galivan et al., 1974a). Evidence for the ternary complex has also been obtained from difference spectra, where a 5,6-dihydro-FdUMP enzyme complex (Santi et al., 1974; Danenberg et al., 1974) and H_2folate (Sharma and Kisliuk, 1973) have been proposed as possible intermediates.

While covalently linked intermediates may be involved in the reaction, folate analogs that cannot form bridge complexes also provide support for the tight binding of FdUMP. Evidence for the latter effect was reported initially for studies measuring the binding of [^{3}H]FdUMP to the *Lactobacillus casei* (Santi et al., 1974) and [2-^{14}C]FdUMP to the T2 bacteriophage thymidylate synthetases (Galivan et al., 1974a). Binding data obtained through the use of circular dichroic (CD) spectra and presented in this paper demonstrate that the spectra obtained are characteristic of the folate analog employed and that the increments at specific wavelengths can be used to determine the number of binding sites on thymidylate synthetase for both FdUMP and the (+) and (−) diastereoisomers of 5,10-CH_2H_4folate. A preliminary account of these findings has been presented (Galivan et al., 1974b).

Materials and Methods

Chemicals. 5-Fluoro-2′-deoxyuridine, purchased from the Aldrich Chemical Co., was converted to FdUMP (ϵ = $8.08 \times 10^3\ M^{-1}\ cm^{-1}$ at 265 nm) (Mukherjee and Heidelberger, 1962) by the cyanoethyl phosphate procedure of

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[1] Abbreviations used are: H_4folate, 5,6,7,8-tetrahydrofolate; 5,10-CH_2H_4folate, 5,10-methylenetetrahydrofolate; 5-CH_3H_4folate, 5-methyltetrahydrofolate; 10-CH_3H_4folate, 10-methyltetrahydrofolate; H_2folate, 7,8-dihydrofolate; H_4homofolate, 5,6,7,8-tetrahydrohomofolate; 5,11-CH_2H_4homofolate, 5,11-methylenetetrahydrohomofolate; FdUMP, 5-fluoro-2′-deoxyuridylate.

Tener (1961). Folic acid, dTMP, and dUMP were obtained from the Sigma Chemical Co. The folic acid exhibited a single fluorescent spot on cellulose thin-layer chromatography (Polygram Cel 300 UV_{254}; Machery-Nagel & Co.) with 0.1 *M* potassium phosphate (pH 6.0) as developer. H_2folate ($\epsilon = 28.4 \times 10^3\ M^{-1}\ cm^{-1}$ at 282 nm) was prepared from folic acid by reduction with sodium dithionite (Blakley, 1960b) and H_4folate by the catalytic hydrogenation of folic acid according to Lorenson et al. (1967), except that 2,3-dimercaptopropanol was omitted.

(±)-5,10-CH_2H_4folate was synthesized by the method of Osborn et al. (1960), and its diastereoisomers were resolved by a modification of the procedure of Kaufman et al. (1963). The triethylaminoethylcellulose column was eluted with a linear gradient of 0.04 to 0.4 *M* triethylamine bicarbonate (pH 9.1) containing 0.02 *M* 2-mercaptoethanol. Each diastereoisomer was then rechromatographed with the same gradient to ensure complete resolution. The products were lyophilized and dissolved in a solution containing 0.02 *M* potassium bicarbonate (pH 9.1), 0.02 *M* 2-mercaptoethanol, and 0.02 *M* formaldehyde. The concentration of 5,10-CH_2H_4folate, calculated with an $\epsilon = 32.0 \times 10^3\ M^{-1}\ cm^{-1}$ at 294 nm (Blakley, 1960a), agreed with the concentration of the (+) diastereoisomer determined enzymically with thymidylate synthetase and an excess of dUMP.

The biologic diastereoisomers of H_4folate and of 5,10-CH_2H_4folate were synthesized according to Mathews and Huennekens (1960) and purified on triethylaminecellulose as described above. 5-CH_3H_4folate was synthesized by the technique of Gupta and Huennekens (1967). Methotrexate was a generous gift of Lederle Laboratories. 10-Methylfolate was synthesized from methotrexate by alkali treatment according to Seeger et al. (1949). 10-Methylfolate and homofolate were converted to their tetrahydro derivatives by catalytic hydrogenation (Lorenson et al., 1967) in the absence of 2,3-dimercaptopropanol. The 2-amino-4-hydroxyquinazoline derivative (inhibitor II; Bird et al., 1970) was a generous gift of Dr. R. B. Dunlap of the Department of Chemistry, University of South Carolina.

CD Measurements. CD spectra were recorded with a Cary 61 spectropolarimeter calibrated with a standard solution of 10-camphorsulfonic acid (Cassim and Yang, 1969). Spectra were scanned at 3 nm/min, with a pen period of 10 sec and a spectral band width of 3 to 3.5 nm. The full-scale ellipticity in all measurements was 0.05°. In no case were ellipticity values included where the dynode voltage exceeded 0.4. Samples were examined in a 1.0-cm cuvet at room temperature (25°). The results are expressed in terms of molar ellipticity, $[\theta]$, in deg $cm^2\ dmol^{-1}$ and were calculated from the equation:

$$[\theta] = \frac{100 \times \theta_{obsd}}{\text{light path (cm)} \times \text{concentration } (M)}$$

In studies involving enzyme–ligand interaction, the concentration of enzyme was converted to moles based on an A_{280} of 1.35/mg of protein per ml and a molecular weight of 70,000 (Dunlap et al., 1971). The protein concentration was determined from an amino acid analysis of the crystalline enzyme. Except for Figure 1, the CD spectra of the enzyme in the presence of various ligands were corrected for the spectra of FdUMP and the folate derivatives.

Large-Scale Purification of Thymidylate Synthetase from L. casei. Partially purified enzyme was purchased from the New England Enzyme Center as a dialyzed ammonium sulfate concentrate (fraction no. 2; Leary and Kisliuk, 1971) and was diluted with an equal volume of cold 40 m*M* 2-mercaptoethanol. The resulting solution (200 ml) was applied to a phosphocellulose column (3.4 × 25 cm) equilibrated previously with 10 m*M* potassium phosphate (pH 7.1)–20 m*M* 2-mercaptoethanol. The column was washed successively with 250-ml portions of 0.05, 0.075, and 0.09 *M*, and 500 ml of 0.1 *M* potassium phosphate (pH 7.1), containing 20 m*M* 2-mercaptoethanol. Fractions of about 20 ml were collected, and the enzyme was eluted in the last 750 ml.

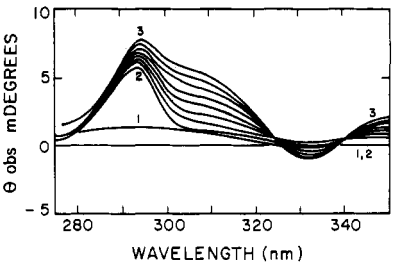

FIGURE 1: Titration of a solution of (±)-5,10-CH_2H_4folate and thymidylate synthetase with FdUMP. To a 2.7-ml solution containing 125 μmol of potassium phosphate (pH 7.0) and 100 μmol of 2-mercaptoethanol was added 150 nmol (0.03 ml) of 5,10-CH_2H_4folate (curve 1), which was followed by 17 nmol of thymidylate synthetase (curve 2). Successive additions of 5 nmol of FdUMP in 5-μl aliquots yielded a series of spectra which increased in observed ellipticity (θ_{obsd}) until saturation was reached at 35 nmol of FdUMP (curve 3).

The enzyme was concentrated by adding solid ammonium sulfate to 80% saturation. After centrifugation, the precipitated enzyme was redissolved in 0.05 *M* potassium phosphate (pH 7.1)–20 m*M* 2-mercaptoethanol, and neutral saturated ammonium sulfate was added dropwise until a faint opalescence appeared. The preparation was allowed to stand overnight at 0–4°, during which time the enzyme crystallized. The crystals were collected by centrifugation and recrystallized by the same procedure. The crystalline enzyme yielded a single band on disc gel electrophoresis in 7% gels by the method of Davis (1964) and also in 9% sodium dodecyl sulfate gels according to Weber and Osborn (1969). The specific activity of the crystalline enzyme was 3.2 to 3.3 units/mg of protein, as determined by amino acid analysis, with a unit defined as the amount of enzyme required to produce 1 μmol of H_2folate per min at 30°.

The above procedure is preferred to the final hydroxylapatite step in the original procedure (Leary and Kisliuk, 1971), since larger quantities of enzyme can be handled at one time and higher yields of pure enzyme are obtained. Thus, from 500 units of enzyme, 400–450 have been recovered, 50% of which was in a crystalline state. Comparable results were obtained starting with 1000 units of enzyme and a 7.5 × 15 cm phosphocellulose column.

Results

CD Spectra of Thymidylate Synthetase in the Presence of FdUMP and 5,10-CH_2H_4folate. The CD spectrum of *L. casei* thymidylate synthetase exhibits a single band at 292 nm (Figure 1, curve 2), which calculates to a $[\theta]$ of 80×10^3 deg $cm^2\ dmol^{-1}$, when corrected for the contribution of (±)-5,10-CH_2H_4folate (curve 1). Although the CD spectrum of the enzyme is unaffected between 350 and 290 nm by FdUMP or (±)-5,10-CH_2H_4folate, neither of which possesses a significant spectrum of its own in this region, large sequential changes in ellipticity occur when successive increments of FdUMP are made to a solution of (±)-5,10-

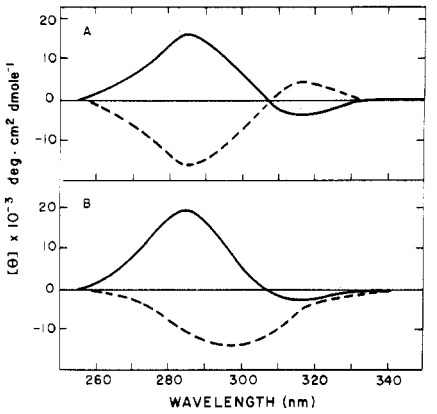

FIGURE 2: CD spectra of the (+) and (−) isomers of 5,10-CH_2H_4folate. Each cuvet contained a 2.7-ml solution with 125 μmol of potassium phosphate (pH 7.0) and 100 μmol of 2-mercaptoethanol. The spectra in A were obtained on bringing the volume to 3.0 ml with 85 nmol of (+)-5,10-CH_2H_4folate (—) or 85 nmol of (−)-5,10-CH_2H_4folate (- - -). The isomers were chemically synthesized as a diastereoisomeric mixture and resolved as described under Materials and Methods. The spectra in B were obtained as in A but with 80 nmol of enzymically synthesized H_4folate (- - -). On addition of 24 μmol of formaldehyde, the spectrum designated by (—) was obtained.

Table I: Summary of Molar Ellipticities of CD Bands Obtained with *L. casei* Thymidylate Synthetase in the Presence of Various Folate Analogs and FdUMP.

Folate Analog	λ (nm)	$[\theta] \times 10^{-4}$
(±)-5,10-CH_2H_4folate	305	7.0
	332	−2.0
(+)-5,10-CH_2H_4folate	265	2.0
	285	−1.0
	305	7.0
	332	−2.0
(−)-5,10-CH_2H_4folate	305	5.0
H_4folate	305	12.2
H_4homofolate	305	13.6
(±)-5,11-CH_2H_4homofolate	308	15.6
	335	−4.0
H_2folate	305	19.0
	335	−5.0
10-CH_3H_4folate	305	12.4
2-Amino-4-hydroxyquinazoline	315	13.0

CH_2H_4folate and enzyme. Saturating levels of FdUMP (Figure 1, curve 3) effect a large positive ellipticity at 305 nm, a negative ellipticity at 332 nm, and a broad positive band from 340 to 400 nm with a maximum at about 350 nm (for $[\theta]$ values, see Table I).

CD Spectra of the Separated (+) and (−) Diastereoisomers of 5,10-CH_2H_4folate Alone and Their Contribution to the Ternary Complex. 5,10-CH_2H_4folate is composed of two diastereoisomers due to the asymmetric carbon at position 6 of the pyrazine ring. To determine the extent to which each contributes to the ternary complex (Figure 1, curve 3), it was necessary to separate the diastereoisomers by chromatographic and enzymic procedures (see Materials and Methods). As indicated in Figure 2A, the enzymically active (+) diastereoisomer has a negative ellipticity band at 313 nm, $[\theta] = -3 \times 10^3$, and a positive band at 285 nm, $[\theta] = 18 \times 10^3$. The (−) diastereoisomer, which has been shown to be enzymically inactive with the *L. casei* thymidylate synthetase (Leary et al., 1974), contributes a CD spectrum (dashed line) which is equal in magnitude but opposite in sign to that of the (+) diastereoisomer. Figure 2B

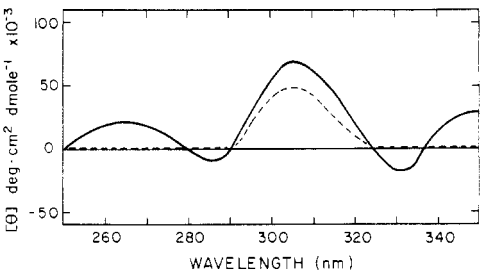

FIGURE 3: Effect of FdUMP on the CD spectra of thymidylate synthetase in the presence of (+)- or (−)-5,10-CH_2H_4folate. CD difference spectra were obtained with both cuvets containing the following components in 3 ml: potassium phosphate (pH 7.0), 130 μmol; 2-mercaptoethanol, 100 μmol; thymidylate synthetase, 14.2 nmol; formaldehyde, 5.2 μmol; and (+)-5,10-CH_2H_4folate (—) or (−)-5,10-CH_2H_4folate (- - -), 35 nmol; but with FdUMP in the sample cuvet at a final concentration of 1.75×10^{-5} *M*. Each spectrum is corrected for the contribution of FdUMP alone.

demonstrates that while the CD spectrum of enzymically synthesized (−)-H_4folate possesses a negative $[\theta]$ at 298 nm of -15×10^3 deg cm^2 dmol^{-1}, the addition of formaldehyde to this compound converts its CD spectrum rapidly to that of (+)-5,10-CH_2H_4folate[2] (solid line). These data thus support the identity of the diastereoisomers of 5,10-CH_2H_4folate isolated by column chromatography and by enzymic synthesis. If the spectrum of (+)-5,10-CH_2H_4folate is taken in 40 m*M* $KHCO_3$ (pH 9.5), the solution used for its chromatographic purification, $[\theta]$ at 285 nm is increased to 28×10^3. Similar CD spectra for (+)- and (−)-5,10-CH_2H_4folate were presented recently by Leary et al. (1974), but their reported molar ellipticities were much lower.

With purified diastereoisomers, it was possible to determine the contribution of each to the CD spectrum of the (±)-5,10-CH_2H_4folate–enzyme–FdUMP complex. For this purpose the CD difference spectrum of the ternary complex for each diastereoisomer was obtained by comparing a sample cell containing enzyme, (+)- or (−)-5,10-CH_2H_4folate, and FdUMP with a reference cell containing only enzyme, and the respective (+) or (−) diastereoisomer of 5,10-CH_2H_4folate (Figure 3). With the (−) diastereoisomer, only a single positive band centered at 305 nm was observed, which differs from the spectrum of the ternary complex containing the (±) diastereoisomeric mixture (Figure 1). In contrast, the (+) diastereoisomer yielded a positive $[\theta]$ at 305 nm in addition to two broad positive ellipticity bands (400–340 nm, 270–255 nm) and negative bands at 332 and 285 nm (for $[\theta]$ values, see Table I). The latter CD transitions correspond closely to those obtained with the diastereoisomeric mixture of 5,10-CH_2H_4folate (Figure 1) and suggest that the enzyme preferentially binds the enzymically active (+) diastereoisomer in the ternary complex but does not exclude the possibility that some of the (−) diastereoisomer is also bound. The fact that (−)-5,10-CH_2H_4folate can contribute to ternary complex formation supports the contention of Leary et al. (1974) that this compound is not an inert substrate analog.

Neither diastereoisomer produced any change in the CD spectrum of thymidylate synthetase in the absence of

[2] As noted by Blakley (1969) and Mathews and Huennekens (1960), (−)-H_4folate is the biochemically active isomer. Addition of formaldehyde to this compound generates an enzymically active form of 5,10-CH_2H_4folate which has a positive rotation and thus is the (+) isomer (Blakley, 1969).

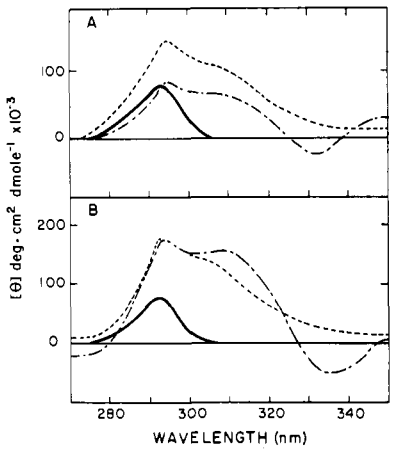

FIGURE 4: Effect of H_4folate and H_4homofolate on the CD spectra of FdUMP saturated thymidylate synthetase. (A) To 2.7-ml solutions containing potassium phosphate (pH 7.0) (130 μmol), 2-mercaptoethanol (100 μmol), thymidylate synthetase (14 nmol), and FdUMP (75 nmol), the following additions were made: none (—); H_4folate (- - -), 0.12 μmol in 0.01 ml; H_4folate, 0.12 μmol plus 5 μmol of formaldehyde in 0.1 ml (---). (B) The same basic solution as in A was used but with the following additions: none (—); H_4homofolate (- - -), 0.15 μmol in 0.02 ml; H_4homofolate, 0.15 μmol plus 5 μmol of formaldehyde in 0.1 ml (---). In both A and B, the final CD measurement was made 15 min after the addition of formaldehyde.

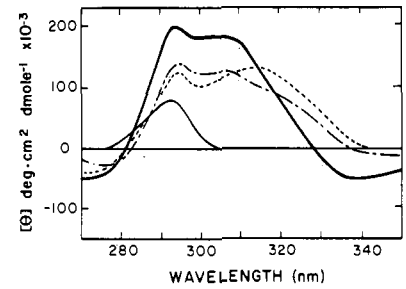

FIGURE 5: CD spectra of thymidylate synthetase in the presence of FdUMP and folate analogs. To 2.8-ml solutions containing potassium phosphate (pH 7.0) (130 μmol), 2-mercaptoethanol (100 μmol), FdUMP (50 nmol), and thymidylate synthetase (14 nmol), the following additions were made: none (—); H_2folate (—), 0.20 μmol in 0.3 ml; 10-CH_3H_4folate (---), 0.10 μmol in 0.05 ml; 2-amino-4-hydroxyquinazoline (- - -), 0.20 μmol in 0.2 ml.

FdUMP. However, FdUMP, when added in increasing amount to the enzyme in the absence of the reduced folate analogs, effected a slight change in ellipticity between 285 and 260 nm. With the amount of FdUMP employed in Figure 3 (17.5×10^{-6} *M*), a negative ellipticity of -12×10^3 deg cm^2 dmol^{-1} centered around 275 nm was generated. When greater quantities of FdUMP were added, a further decrease occurred, but due to the high sample absorbance at 275 nm the change in ellipticity could not be accurately measured. No effect on the CD spectrum above 290 nm was noted. Leary and Gaumont (1973) have reported a similar but more intense CD spectral shift by dUMP in the presence of *L. casei* thymidylate synthetase.

Effects with Other Folate Derivatives. Previous reports from this laboratory (Galivan et al., 1974a) and others (Santi et al., 1974; Danenberg et al., 1974) indicated that analogs of 5,10-CH_2H_4folate, such as H_4folate and H_4homofolate, can promote the binding of radioactive FdUMP to the thymidylate synthetase from T2 bacteriophage and *L. casei*. Because these results suggested that ternary complex formation could be obtained with other folate derivatives, the effect of these compounds on the CD pattern of the resultant ternary complex was examined. As shown in Figure 4, H_4folate and H_4homofolate induced large Cotton effects at 305 nm with FdUMP and enzyme (for [θ] values, see Table I).

It has been proposed that H_4folate forms a ternary complex with FdUMP and thymidylate synthetase only if converted to 5,10-CH_2H_4folate by formaldehyde (Santi and McHenry, 1972; Danenberg et al., 1974). The unique CD spectrum observed with (±)-H_4folate in place of (±)-5,10-CH_2H_4folate (Figure 4A vs. Figure 1, curve 3) does not support this proposal. The spectra are interconvertible, however, as evidenced by the rapid spectral shift of the ternary complex containing H_4folate to that of (±)-5,10-CH_2H_4folate on addition of formaldehyde (Figure 4A). It should be emphasized that formaldehyde addition in the absence of H_4folate did not alter the CD spectrum. Similarly, on treatment of the H_4homofolate-FdUMP ternary complex with formaldehyde, a CD spectrum almost identical in shape (Figure 4B) with that of the ternary complex containing 5,10-CH_2H_4folate-FdUMP is obtained, but with increased ellipticities at 308 nm (for [θ] values, see Table I). The similarity in shape of these spectra is not surprising since both (±)-5,10-CH_2H_4folate and (±)-5,11-CH_2H_4homofolate are substrates for the *L. casei* thymidylate synthetase (Crusberg et al., 1970), most probably as a consequence of the presence of the (+) diastereoisomers (Leary et al., 1974).

The possibility that a considerable portion of the H_4folate-enzyme-FdUMP spectrum could have been contributed by contaminating 5,10-CH_2H_4folate was ruled out by the absence of synthetase activity in the presence of H_4folate and dUMP, and by reduction of the H_4folate solution with $NaBH_4$. If present, contaminating 5,10-CH_2H_4folate would have been converted to 5-CH_3H_4folate, a compound that only weakly contributes to the CD spectrum as indicated below. Since the spectrum at 305 nm of the H_4folate ternary complex was not altered following $NaBH_4$ reduction, while that of the 5,10-CH_2H_4folate complex was, it is doubtful whether significant amounts of the latter were present in the H_4folate (J. H. Galivan, unpublished data).

Several other nonsubstrate folate derivatives were also able to induce large, characteristic Cotton effects with the *L. casei* thymidylate synthetase in the presence of FdUMP. As indicated in Figure 5, H_2folate induces a large positive ellipticity band at 305 nm and a negative ellipticity band at 335 nm (for [θ] values, see Table I), which were unaffected by the subsequent addition of formaldehyde. 2-Amino-4-hydroxyquinazoline (Bird et al., 1970) and 10-CH_3H_4folate (Danenberg et al., 1974), both inhibitors of the synthetase, also interact with the enzyme and FdUMP to yield unique spectra.

The latter compound induces a positive ellipticity band centered at 305 nm, and the former compound a broad positive band with a maximum at 315 nm (for [θ] values, see Table I). Under similar conditions, 5-CH_3H_4folate, methotrexate, and folic acid produce only small ellipticity changes. The latter results are consistent with the reduced ability of these compounds to induce the binding of FdUMP to the thymidylate synthetases from T2 bacteriophage (Galivan et al., 1974a) and *L. casei* (Santi et al., 1974).

Replacement of FdUMP with dUMP or dTMP. The production of extrinsic Cotton effects is not absolutely dependent on FdUMP, since ellipticity changes can also be obtained with dUMP or dTMP in the presence of H_2folate and enzyme (Figure 6). With either nucleotide the peak is

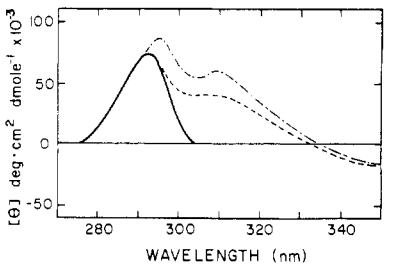

FIGURE 6: CD spectra of thymidylate synthetase in the presence of H_2folate and dUMP or dTMP. To 2.68-ml solutions containing potassium phosphate (pH 7.0) (130 μmol), 2-mercaptoethanol (100 μmol), thymidylate synthetase (14.5 nmol), and H_2folate (0.10 μmol), the following additions were made: none (—); dUMP (···), 0.5 μmol in 0.1 ml; or dTMP (- - -), 0.5 μmol in 0.1 ml.

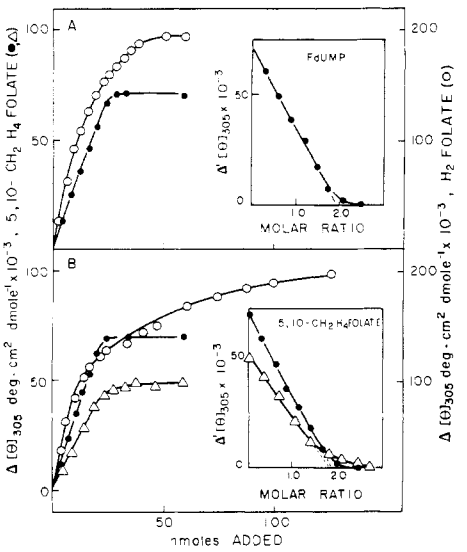

FIGURE 7: Titration of thymidylate synthetase with FdUMP as determined by CD difference spectra at 305 nm. Sample cuvets contained the following components in 2.7 ml: potassium phosphate (pH 7.0) (130 μmol), 2-mercaptoethanol (100 μmol), formaldehyde (5 μmol) (omitted in H_2folate studies), and thymidylate synthetase (14 nmol). Enzyme was omitted from the reference cuvet. To both reference and sample cuvets in A were added 0.10 μmol of (+)-5,10-CH_2H_4folate, in 0.07 ml (●), or 0.20 μmol of H_2folate, in 0.3 ml (○); and increasing amounts of FdUMP to a final concentration of 2.1×10^{-5} *M*. In B the sample and reference solutions were as in A, but contained 50 nmol of FdUMP and were titrated with increasing amounts of (+)-5,10-CH_2H_4folate (●); (−)-5,10-CH_2H_4folate (△); or H_2folate (○). The molar ellipticities were corrected for dilution, although the volumes did not increase by more than 10% during titrations. The data obtained in A for FdUMP and B for the diastereoisomers of 5,10-CH_2H_4folate are replotted in the inserts to determine the stoichiometry of binding for each ligand. $\Delta[\theta]$ represents the increase in molar ellipticity at 305 nm on the addition of ligand and $\Delta'[\theta]$ the difference between $\Delta[\theta]$ at each ligand concentration and the maximal $\Delta[\theta]$ obtained at saturating levels of ligand. The molar ratio was determined from the ligand and enzyme concentrations at each point on the graph.

centered around 310 nm, but the $[\theta]$ for the respective ternary complexes are not nearly as pronounced as with FdUMP at concentrations that are at least 5× higher than the latter (Figure 5). Equilibrium dialysis studies indicate that the level of these nucleotides was well above saturation (J. H. Galivan, unpublished data).

Determination of the Number of Binding Sites. By measuring the increment in ellipticity in the 305-nm region on ternary complex formation, the number of binding sites for FdUMP and for each of the diastereoisomers of 5,10-CH_2H_4folate could be determined. Thus, on saturation of the enzyme with (+)-5,10-CH_2H_4folate and addition of increasing amounts of FdUMP, a linear curve was obtained (Figure 7A) which titrated to a maximum of 2 mol of FdUMP/mol of enzyme. The hyperbolic nature of the curve obtained in the presence of H_2folate (Figure 7A) suggests that it forms a weaker ternary complex with FdUMP and enzyme.

Titration of the synthetase with increasing quantities of (+)- or (−)-5,10-CH_2H_4folate in the presence of a constant saturating level of FdUMP yields an extrapolated value of 1.90 mol/mol of enzyme for each diastereoisomer (Figure 7B). As in the case of FdUMP, the enzyme appears to possess two stoichiometric binding sites for (+)- and (−)-5,10-CH_2H_4folate in the saturated ternary complex, although their CD difference spectra (Figure 3) suggest that the mode of binding of these diastereoisomers is not identical. Similar conclusions can be derived from Sephadex G-25 filtration studies, where it was found that the ternary complex containing the (+) diastereoisomer is more stable than that containing the (−) diastereoisomer. Thus, on passage of the FdUMP–enzyme–(+)-5,10-CH_2H_4folate complex through the column, the original spectrum of the complex was retained, while that for the complex containing the (−) diastereoisomer was characteristic of the enzyme alone. These results indicate that the latter complex dissociated during Sephadex passage while the former did not.

Due to the apparent stoichiometric binding of each compound in the 5,10-CH_2H_4folate–enzyme–FdUMP complex, accurate binding constants could not be determined. However, in the case of the H_2folate–enzyme–FdUMP ternary complex, both the number of binding sites and the dissociation constant for each ligand in the ternary complex could be calculated by a modification (Wu and Hammes, 1973) of the procedure of Murphy and Morales (1970). By this procedure, 1.2 binding sites for both H_2folate and FdUMP were obtained, with respective dissociation constants of 1.5 $\times 10^{-6}$ and 8×10^{-7} *M*. That there may be a second weaker binding site for FdUMP in the presence of H_2folate was revealed recently in equilibrium dialysis studies (manuscript in preparation).

Discussion

CD spectra have become increasingly useful in detecting conformational transitions associated with the binding of ligands to proteins. Since most of the optical transitions occur between 250 and 310 nm, they probably reflect perturbations in the microenvironment of tyrosine and tryptophan (Beychok, 1966). Alternatively, these transitions may result from the asymmetric binding of chromophoric ligands or in the more extreme cases from combinations of both phenomena.

Comparison of the CD spectra of dihydrofolate reductases from *S. faecium* (D'Souza and Freisheim, 1972) and *Escherichia coli* (Greenfield et al., 1972) showed the qualitative nature of the Cotton effects and the magnitude of their ellipticities to be dependent on the ligands and the source of the reductase. In the case of the *E. coli* reductase (Greenfield et al., 1972), extrinsic Cotton effects were obtained at 270, 292, and 332 nm with H_2folate, but only a single strong positive band at 290 nm was obtained under similar conditions with the *S. faecium* reductase.

Thymidylate synthetase poses a more complex situation, since two ligands, a pyrimidine nucleotide analog and a folate derivative, are required before Cotton effects become evident above 285 nm. Yet here too, the qualitative and

quantitative transitions induced are dependent on the specific ligands employed (Figures 3–5) and it is significant that even the diastereoisomers of 5,10-CH_2H_4folate complex with enzyme and FdUMP to yield dissimilar difference spectra (Figure 3). These results suggest that the (+) and (−) diastereoisomers affect different conformational transitions in the aromatic amino acid region responsible for the observed Cotton effects. Another possible explanation is that the diastereoisomers may have different modes of binding. The resemblance of the CD pattern produced by the ternary complex containing the (+) diastereoisomer to that of the diastereoisomeric mixture indicates that the (+) diastereoisomer is bound to the synthetase in preference to the (−) diastereoisomer. These findings are consistent with those of Leary et al. (1974), which reveal that the (−) or inactive diastereoisomer of 5,10-CH_2H_4folate is a competitive inhibitor ($K_i = 5.5 \times 10^{-5}$ M) of the (+) diastereoisomer ($K_m = 1.5 \times 10^{-5}$ M).

Even more distinctive CD spectral differences were obtained with other folate analogs (Figures 4–6) suggesting that the spectra can at times be used as a means of characterization. For example, the ternary complex of 5,11-CH_2H_4homofolate, although only one methylene group longer, yielded more intense Cotton effects and a small bathochromic shift of the ellipticity bands. Surprisingly, the spectrum for the 5,11-CH_2H_4homofolate ternary complex was more like that obtained with H_2folate, both in the molar ellipticity of its CD bands and their location. While the observed CD differences in the 270–310 nm region with the various folate analogs can be attributed to differences in their modes of binding and the nature of their interactions with the aromatic chromophores, the Cotton effects induced at 332 and 335 nm by 5,10-CH_2H_4folate and 5,11-CH_2H_4homofolate, respectively, are more difficult to explain. The only other compound that induces a similar but broader transition in this region in the presence of FdUMP is H_2folate. Although Santi et al. (1974) propose, on the basis of differential extinction coefficients, that the ultraviolet (uv) difference peak at 330 nm is not due to H_2folate, the potential modulating effect of the enzyme protein on the difference spectra was not considered. As indicated (Santi et al., 1974), 5,10-CH_2H_4folate could rearrange when present in a ternary complex to yield the following equilibrium mixture:

FdUMP-[enzyme]-5,10-CH_2H_4folate

$$\overset{H^+}{\rightleftarrows} \text{FdUMP-[enzyme}\cdot CH_3\text{]-}H_2\text{folate}$$

Because of the complexity of overlapping spectra that might result from the various components present at equilibrium it would be premature at this time to rule out the negative Cotton effect in the 330-nm region as being due to H_2folate or even to an iminium cation derivative of H_4folate (Santi et al., 1974). Of significance in this regard is the fact that the inactive diastereoisomer, (−)-5,10-CH_2H_4folate, does not induce a Cotton effect at 332 nm, although it appears to bind to the enzyme. The lack of a definitive Cotton effect in this region with the ternary complex containing dUMP or dTMP and H_2folate also suggests that an active enzymically involved 5,10-methylene group is required for this transition. Studies with the synthetase from animal sources (Lorenson et al., 1967; Bonney and Maley, 1974) indicate that dUMP and H_2folate combine to stabilize this enzyme and suggest that they would do the same for the *L. casei* synthetase. The characteristic CD spectrum produced in the presence of these compounds and the latter enzyme strongly suggests that a ternary complex is formed (Figure 6).

From equilibrium dialysis studies with labeled FdUMP (J. Galivan, unpublished data) and from the small negative Cotton effect produced at 275 nm by a mixture of FdUMP and thymidylate synthetase, it is evident that this nucleotide will bind to the synthetase in the absence of folate analogs. However, the extent to which the folate analogs will bind to the enzyme in the absence of FdUMP is not known, although the apparent absence of CD spectral changes suggests that little or no binding occurs.

In any event, the concentration-dependent change in the 305-nm Cotton effect can be used in a modified Scatchard plot (Wu and Hammes, 1973) to determine the number of binding sites for FdUMP (Figure 7A) and for the (+) and (−) diastereoisomers of 5,10-CH_2H_4folate (Figure 7B). The excellent agreement of our results with those of previous studies on FdUMP (Santi and Brewer, 1973; Galivan et al., 1974a) and (±)-5,10-CH_2H_4folate binding (Aull et al., 1974) (2 mol of each compound bound/mol of enzyme) again emphasizes the value of CD studies in characterizing enzyme–ligand interactions.

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Tyrosyl-tRNA Synthetase from *Escherichia coli.* Stoichiometry of Ligand Binding and Half-of-the-Sites Reactivity in Aminoacylation†

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ABSTRACT: The tyrosyl-tRNA synthetase from *Escherichia coli* binds only 1 mol of tRNA, tyrosine, and tyrosyl adenylate per mol of enzyme dimer. However, like the enzyme from *Bacillus stearothermophilus,* once one active site is occupied by tyrosyl adenylate the other becomes accessible to bind a further molecule each of tyrosine and ATP. Both bacterial enzymes show biphasic kinetics with respect to tyrosine in the aminoacylation of tRNA. Equilibrium dialysis experiments show that this is due to 2 mol of tyrosine binding in the presence of ATP and tRNA. A method is given for a correction for the effects of hydrolysis of the charged tRNA on the aminoacylation kinetics.

The derivation of general principles of enzymic catalysis is greatly assisted by structure–reactivity studies. In the case of enzymes of broad steric specificity, such as chymotrypsin, the study of the nature of the protein–ligand interactions and the individual chemical steps is simplified by the ease of preparing substrates of varying structure. On the other hand, this is not possible for enzymes of high specificity such as the aminoacyl-tRNA synthetases. We are therefore undertaking a comparative study of several of these enzymes in order to derive similar information.

We have shown so far (Fersht et al., 1975a,b; Fersht, 1975) that there are similarities between the valyl activating enzyme which is a typical member of the monomeric class of enzymes of molecular weight 110,000 and the tyrosyl enzyme from *Bacillus stearothermophilus* which is a symmetrical dimer of mol wt 2 × 47,500 (Koch, 1974; Reid et al., 1973). Both enzymes exhibit negative cooperativity of ligand binding and half-of-the-sites reactivity in binding only one amino acid and apparently only one aminoacyl adenylate although possessing two sets of binding sites. The phenomena of half-of-the-sites reactivity and negative cooperativity are now well documented (Seydoux et al., 1974) but their catalytic importance is as yet unknown. We have suggested a possible catalytic mechanism, involving three limiting cases, which could explain this (Fersht, 1975).

In this context we extend the studies on the partial reactions of the tyrosyl-tRNA synthetase to cover the complete reaction of aminoacylation of tRNA. Most of the measurements concern the enzyme from *Escherichia coli* which is also a dimer of mol wt 95,000. We first show that it is mechanistically very similar to that from *B. stearothermophilus,* and then attempt to resolve the controversy between Chousterman and Chapeville (1973b) and Krajewska-Grynkiewicz et al. (1973) concerning the stoichiometry of ligand binding. Before tackling the rapid reaction studies described in the following paper (Fersht and Jakes, 1975) it is necessary to establish unambiguously the relevant stoichiometries and the relevant dissociation constants.

Experimental Section

Materials and apparatus are described by Fersht et al. (1975a) and Fersht (1975). The tyrosyl-tRNA synthetase from *E. coli* (EM 20031) was purified in the presence of phenylmethanesulfonyl fluoride (to prevent proteolysis). A detailed description will be given elsewhere (Atkinson et al., 1975). The resultant highly purified material was homogeneous by the accepted criteria showing a single band of mol

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