Circular Dichroism Studies of Dihydrofolate Reductase from a Methotrexate-Resistant Strain of *Escherichia coli*†

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ABSTRACT: The circular dichroism of dihydrofolate reductase from a methotrexate-resistant strain of *Escherichia coli* B (MB 1428) and of a number of its liganded derivatives was measured from 190 to 375 nm. The enzyme itself exhibits little circular dichroism between 375 and 250 nm. Binding of dihydrofolate induces large extrinsic Cotton effects with a negative band centered at 332 nm, a large positive band centered at 292.5 nm and a negative shoulder at 270 nm. The dependence of ellipticity upon dihydrofolate concentration indicates one binding site with a dissociation constant of 1.1×10^{-6} M. Binding of folate induces a positive Cotton effect at 292 nm and a negative effect at 272.5 nm. There is also only one binding site indicated for folate, but the binding is weaker than that of dihydrofolate and the dissociation constant of the enzyme–folate complex is 2.8×10^{-5} M. At the enzyme concentrations used in this study, methotrexate binding is stoichiometric and induces Cotton effects similar to those produced by folate and dihydrofolate, but the bands are displaced. There is a negative band at 340 nm, a positive band at 305 nm, and a smaller positive band at 272 nm. NADPH addition to the enzyme causes only small changes in ellipticity. These changes seem to be due to a sharpening of tryptophan transitions at 282.5, 292, and 302 nm. In contrast there are large changes in ellipticity when NADPH is bound to a 1:1 enzyme–MTX complex to form a ternary complex with 1:1:1 stoichiometry. Below 250 nm the enzyme exhibits a negative band with a maximum at 220 nm and a slight shoulder at 210 nm, and a mean residue ellipticity $[\theta]_{220}$ equal to $-10{,}400 \pm 1000$ (deg cm²)/dmole. The spectrum suggests that the enzyme has very little helical content, about 10%, but perhaps considerable β structure, 50–60%. Addition of dihydrofolate to the enzyme does not change the spectrum below 250 nm, suggesting that there are no gross conformational changes of the peptide backbone upon dihydrofolate addition.

Dihydrofolate reductase [5,6,7,8-tetrahydrofolate:NADPH oxidoreductase, EC 1.5.1.3] is an enzyme of considerable pharmacological interest because it is the target for several potent drugs. For a review, see Hitchings and Burchall (1965). There have been relatively few physical studies on purified dihydrofolate reductase because it has only recently been isolated in homogeneous form. For a list of these isolations, see Poe *et al.* (1972). Moreover, the quantities of pure enzyme obtained have been small. In a previous paper we have described the large-scale purification of the enzyme from a methotrexate resistant strain of *Escherichia coli* B (Poe *et al.*, 1972). In this work we describe circular dichroism (CD) studies of this enzyme. We have used CD to obtain structural information on the pure enzyme in solution and to follow the binding behavior of the substrates and of some inhibitors to the enzyme. Freisheim and D'Souza (1971a,b) have described the circular dichroism of a dihydrofolate reductase from *Streptococcus faecium*. The CD of our *E. coli* dihydrofolate reductase in the presence and absence of NADPH differs markedly from their enzyme. Thus CD may also prove to be a convenient way to study some of the species specific differences between dihydrofolate reductases from different sources.

Materials and Methods

NADPH was purchased from Calbiochem, folic acid from Cyclo Chemical Corp., methotrexate (amethopterin, MTX)[1] from Nutritional Biochemical Corp., hen egg-white lysozyme from Schwarz-Mann, and chymotrypsin from Worthington. DEAE-Sephadex A-25M and Sephadex G-25M were purchased from Pharmacia. All other reagents were reagent grade.

Dihydrofolate was prepared from commercial folic acid by the dithionite method of Futterman (1957) as modified by Blakley (1960) and was stored at −20° in 5 mM HCl and 50 mM 2-mercaptoethanol. Buffer A is 0.05 M Tris-HCl (pH 7.2)–0.30 M NaCl.

Dihydrofolate reductase was prepared from *E. coli* B (Strain MB 1428) as described by Poe *et al.* (1972). As isolated, the enzyme contains bound dihydrofolate. The dihydrofolate was removed by passing 30 ml of enzyme solution containing 1 mg of protein/ml through a 2 × 80 cm column packed with DEAE-Sephadex, which had previously been equilibrated with buffer A to yield "stripped" enzyme. If one filtration through A-25 did not separate all dihydrofolate from the enzyme, the filtration was repeated. The column could be reused without repacking by flushing with buffer A until all dihydrofolate was eluted.

For CD measurements the purified enzyme with dihydrofolate removed was either dialyzed exhaustively against 0.1 M NaCl (pH 7.2) or 2 mg of enzyme in 2 ml of buffer A was desalted on a 2 × 15 cm Sephadex G-25M column equilibrated with 0.1 M NaCl.

Enzyme solutions were concentrated in a 10- or 50-ml capacity Amicon ultrafiltration apparatus at 4° equipped with Amicon UM-10 filters to the desired concentration of 1.0×10^{-5} to 4.0×10^{-5} M. The enzyme solutions were filtered through 0.1-μ Millipore filters directly before CD and uv spectral measurements.

Solutions of NADPH, MTX, folate, and dihydrofolate were made up in 0.05 M Tris-HCl (pH 7.2) at concentrations ranging from 1.0 to 5.0 mM. Thus addition of the sub-

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[1] Abbreviation used is: MTX, methotrexate or amethopterin.

strates to the enzyme resulted in very little dilution. The graphically presented titration data are all corrected for dilution.

The concentrations of NADPH, MTX, folate, and dihydrofolate were determined spectrophotometrically using a molar extinction of $\epsilon_{340\text{ nm}} = 6220$ for NADPH (Kornberg and Horecker, 1953), $\epsilon_{258\text{ nm}} = 23{,}250$ and $\epsilon_{302.5\text{ nm}} = 22{,}100$ for MTX at pH 13 (Seeger *et al.*, 1949); $\epsilon_{282\text{ nm}} = 27{,}000$ for folate (Dawson *et al.*, 1969), and $\epsilon_{282\text{ nm}} = 28{,}000$ for dihydrofolate (Dawson *et al.*, 1969).

The enzyme molar extinction coefficient was determined by measuring absorbance at 280 nm and then determining the protein concentration by three methods. The first method was the microbiuret protein determination method of Goa (1953) using lysozyme and chymotrypsin as standards. This gave protein concentrations in grams per liter. This value was divided by the enzyme molecular weight of 16,810 g/mol to give molar concentration. In the second method the dry weight and the percent nitrogen content of a known volume of enzyme solution which had been dialyzed exhaustively against water were measured. The number of grams dry weight was multiplied by the measured fractional nitrogen content, and this was then divided by 0.1545, the fractional nitrogen content of the pure protein, as calculated from the amino acid composition of the protein (Poe *et al.*, 1972). The resulting number of grams of protein in the known volume was divided by 16,810, as in the microbiuret procedure, to give the molar concentration. The third method employed methotrexate titration of the enzyme *via* loss of activity (Poe *et al.*, 1972) and difference uv spectroscopy. The binding of MTX is virtually stoichiometric at optical concentrations (near 10 μM) and induces an intense difference spectrum (Poe *et al.*).[2] The difference spectrum is saturated at a 1:1 methotrexate to enzyme ratio and thus the methotrexate concentration necessary to saturate the enzyme can be used to monitor the concentration of enzyme in solution. All three methods gave an average molar extinction, $\epsilon_M = 4.0 \pm 0.4 \times 10^4\ M^{-1}\ cm^{-1}$, which was then used for subsequent enzyme concentration determinations.

The dissociation constants (K_d) of the enzyme–ligand complexes were estimated by comparison of pairs of data points in a "difference" method similar to the one described for fluorometric data by Kurganov *et al.* (1972).

In the CD measurements, we define θ as the difference in the measured ellipticity between a given mixture of enzyme and ligand and the sum of the ellipticities of the components of the mixture. For the purpose of our analysis, it has been assumed that θ is linearly proportional to [EL], the concentration of complex (Deranleau, 1969a,b). From the definition of θ, when [EL] = 0, $\theta = 0$. The value of θ when [EL] = [E_t] is defined as θ_t. At the ith total ligand coocentration [L_{ti}], with total enzyme concentration [E_t]

$$[EL_i] = [E_t](\theta_i/\theta_t) \tag{1}$$

Putting eq 1 into the definition for K_d, along with the conservation of mass equation, gives

$$K_d = \frac{[1 - (\theta_i/\theta_t)]\{[L_{ti}] - [E_t](\theta_i/\theta_t)\}}{(\theta_i/\theta_t)} \tag{2}$$

For two different total ligand concentrations [L_1] and [L_2] at the same total enzyme concentration, the right-hand side of eq 2 should be equal for [L_{ti}] = [L_1] and [L_{ti}] = [L_2]. Therefore

$$\frac{[1 - (\theta_1/\theta_t)]\{[L_1] - [E_t](\theta_1/\theta_t)\}}{(\theta_1/\theta_t)} = \frac{[1 - (\theta_2/\theta_t)]\{[L_2] - [E_t](\theta_2/\theta_t)\}}{(\theta_2/\theta_t)} \tag{3}$$

Rearranging terms one obtains

$$\frac{\left(\frac{[L_1]}{\theta_1}\right) - \left(\frac{[L_2]}{\theta_2}\right)}{[L_1] - [L_2]} = \frac{1}{\theta_t} + \left(\frac{[E_t]}{(\theta_t)^2}\right)\left(\frac{\theta_1 - \theta_2}{[L_1] - [L_2]}\right) \tag{4}$$

Setting

$$y = \frac{\left(\frac{[L_1]}{\theta_1}\right) - \left(\frac{[L_2]}{\theta_2}\right)}{[L_1] - [L_2]} \qquad x = \left(\frac{\theta_1 - \theta_2}{[L_1] - [L_2]}\right)$$

the equation has the form of a straight line where the intercept on the y axis = $1/\theta_t$ and the slope is equal to the enzyme concentration divided by the square of θ_t.

By examining a series of total ligand concentrations and observing the ellipticity due to the formation of the complex one can determine the maximum ellipticity when all the enzyme is bound, θ_t, and the enzyme concentration, [E_t]. If there is only one binding site, these measurements give determinations of enzyme concentration and θ_t that do not depend upon any additional measurements. Once these parameters are obtained the K_d can be estimated at any point from eq 2.

In this laboratory, x and y were determined for eight to ten different ligand concentration pairs. The slope and intercept were determined by a least-squares fit of the data, and then the best value of K_d and its standard deviation were determined with all points assigned unit weight and using eq 2. The calculations were performed on a GE Mark II computer.

When the total enzyme concentration calculated from the CD data is the same as the enzyme concentration determined independently, one binding site is indicated.

Enzyme solutions were assayed as described by Poe *et al.* (1972). Enzyme activity did not decay during the course of the CD measurements.

CD measurements were obtained with a Cary 60 recording spectropolarimeter equipped with a Model 6001 CD attachment and set for a half-bandwidth of 1.5 nm. Measurements were made at the ambient temperature of 27°. The measurements of the ellipticity between 250 and 400 nm of the free enzyme and the enzyme bound to its substrates were obtained in cells with 1-cm light paths at enzyme concentrations ranging from 0.5×10^{-5} to 4.0×10^{-5} M. In this wavelength range the ellipticity of the enzyme and enzyme complexes are reported as molecular (molar) ellipticities [θ] in (deg cm^2)/dmol, where

$$[\theta] = \frac{100 \times \theta_{obsd}}{\text{light path (cm)} \times \text{concentration (mol of enzyme/l.)}}$$

From 190 to 250 nm, measurements were made in cells with 1-mm light paths and results were calculated as mean residue rotation in (deg cm^2)/dmol where the mean residue rotation, [θ], is equal to the molecular rotation divided by 151 (the

[2] Submitted for publication.

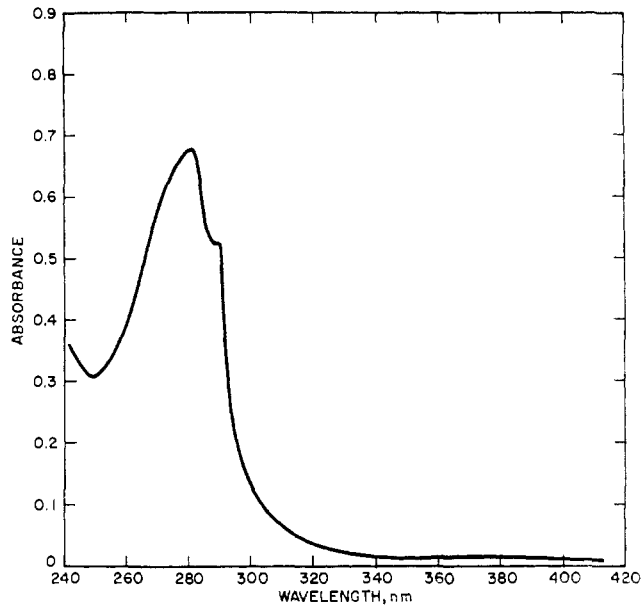

FIGURE 1: The ultraviolet absorption spectra of dihydrofolate reductase in a 1-cm path length cell at 1.8×10^{-5} M concentration in 0.10 M NaCl (pH 7.2) at 25°.

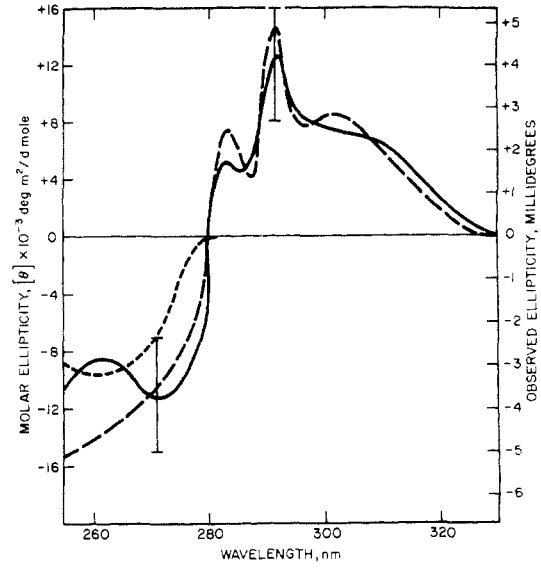

FIGURE 2: The circular dichroism of dihydrofolate reductase in 0.1 M NaCl (pH 7.2), 27° from 330 to 250 nm. Enzyme (——); enzyme plus an equimolar addition of NADPH (— —); NADPH alone (– – – –). The enzyme concentration is 3.2×10^{-5} M.

number of peptide residues in *E. coli* dihydrofolate reductase). Unless otherwise noted, all measurements have an uncertainty of ±10% due to the errors in measuring the enzyme concentration.

Results and Discussion

Circular Dichroism of Dihydrofolate Reductase from 375 to 250 nm. A typical ultraviolet absorption spectrum of dihydrofolate reductase used in these experiments is shown in Figure 1. The enzyme has a peak near 281 nm with a prominent shoulder at 290 nm. The lack of an absorbance band at 340 nm or a shoulder at 310 nm illustrates that the enzyme is free of the bound cofactors or substrates, sometimes found with the enzyme (Dunlap *et al.*, 1971; Gauldie and Hillcoat, 1972). The CD of the enzyme between 375 and 250 nm is shown as the solid line in Figure 2. The enzyme when stripped of cofactors has very low ellipticity in this region. The ellipticity maximum is at 291 nm, $[\theta] = 12{,}000 \pm 4000$ (deg cm²)/dmol, with a shoulder at 282.5 nm. The high uncertainty in this measurement has two principal causes. First, the ellipticity of the enzyme in the absence of cofactors is very low, about 3 mdeg for an enzyme solution with an absorbance of 1.5/cm. As the reproducibility of the instrument is only ±1 mdeg there is a large inherent error. Second, the enzyme is isolated *via* affinity chromatography and is eluted from a MTX-aminoethyl-Sepharose resin (see Poe *et al.*, 1972) by a large excess of dihydrofolate or folate. These ligands are very tightly bound to the enzyme and when bound they generate high extrinsic Cotton effects as shown below. Thus, as little as 1% of a dihydrofolate impurity could cause an error at 290 nm of +1600 (deg cm²)/dmol (~0.5 mdeg). Although great care was taken to remove all the bound ligands from the enzyme samples used in this study, trace impurities could remain bound to the enzyme which are below the limits of detectability *via* absorbance and activity criteria of purity. The estimation of the enzyme concentration has an error of less than 10%.

The fine structure of the enzyme CD spectrum near 281 and 290 nm suggests that the tryptophan residues may be principally responsible for the CD of this region as the bands are similar to the CD bands of tryptophan (Legrand and Viennet, 1965; Gill, 1967; Verbit and Heffron, 1967; Myer and MacDonald, 1967; Strickland *et al.*, 1969) and tryptophan containing model compounds (Edelhoch *et al.*, 1968). The profile of the enzyme bands are consistent with those of tryptophan models. In these studies, tryptophan has a molar ellipticity of approximately 2000 (deg cm²)/dmol in the region of 280–290 nm, depending on solvent and pH. Cyclic glycyl-L-tryptophan, a relatively rigid model compound, has an ellipticity near 290 nm, $[\theta] = 800$–3100 (deg cm²)/dmol, depending on solvent (Edelhoch *et al.*, 1968), and exhibits fine structure similar to that of our enzyme. Of course, the exact magnitude of the aromatic Cotton effects of proteins depends on the environment and conformation of the aromatic residues in the protein of interest.

The enzyme also has a band centered near 272 nm. Assignment of this band is complicated as there are many chromophores in proteins that contribute in this region including tyrosine, phenylalanine and tryptophan. Moreover, the position and magnitude of the ellipticity bands are strongly dependent upon the geometry and polarity of their environments (for a review, see Adler *et al.*, 1972).

The CD spectrum of dihydrofolate reductase from *E. coli* is quite distinct from the CD spectrum of the enzyme from *Streptococcus faecium*. Freisheim and D'Souza (1971) report a molar ellipticity of approximately 94,000 at 292 nm. The difference in molar ellipticities between the enzymes from *E. coli* and *S. faecium* may reflect large differences in tryptophan content and geometry.

Circular Dichroism of Dihydrofolate Reductase from 250 to 190 nm. Below 250-nm dihydrofolate reductase has a simple CD spectrum with a negative band centered at 220 nm with a mean residue ellipticity of $-10{,}400 \pm 1000$ (deg cm²)/dmole, a slight shoulder at 208 nm, $[\theta] = -6200$ (deg cm²)/dmole, and a positive ellipticity at 195 nm, $[\theta] = 8400 \pm 2000$ (deg cm²)/dmole. This spectrum is illustrated in Figure 3. The enzyme appears to have a peak at 195 nm. However, there is

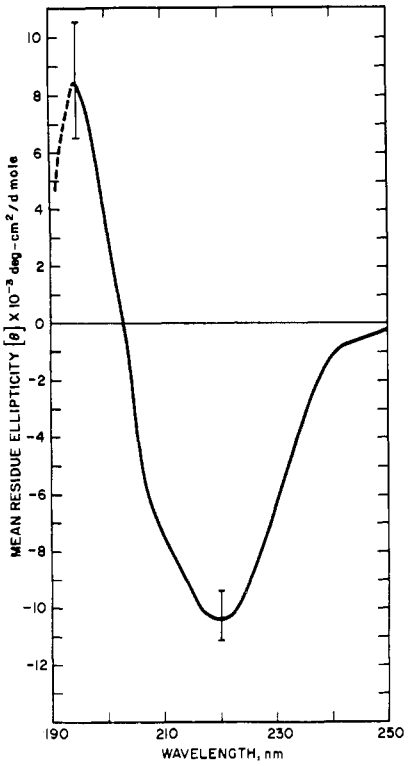

FIGURE 3: The circular dichroism of dihydrofolate reductase in 0.1 M NaCl (pH 7.2) at 27° from 250 to 190 nm.

some uncertainty in this result due to the high absorbancy of the sample near this wavelength. Comparison of the ellipticity to the graphs of Greenfield and Fasman (1969) suggests that the enzyme has a very low helical content. On the basis of comparison to those graphs, we suggest that the α-helical content is about 10%, but there may be considerable β structure (about 50–60%), with the remainder being "unordered."

Binding of Substrates and Inhibitors to Dihydrofolate Reductase. NADPH. The binding of NADPH to the enzyme has very little effect on its CD even though at the enzyme concentrations used, difference spectra and fluorescence results (manuscripts in preparation) indicate that the binding of NADPH should be very tight. The only effect noted upon addition of NADPH to the enzyme was a slight sharpening of transitions at 282, 290, and 302 nm. The CD of NADPH, enzyme, and a 1:1 mixture of NADPH and enzyme are shown in Figure 2. The changes are very difficult to quantitate precisely since the total changes seen were less than a millidegree for enzyme samples with optical densities of 1.5 at 282 nm. Moreover, at high concentrations NADPH itself contributes strongly to both the CD and absorbance of a 1:1 NADPH–enzyme mixture. The apparent changes at 260 are due to the contribution of free NADPH and are not significant. Nevertheless, the *E. coli* enzyme is clearly distinct from the *S. faecium* reductase. Freisheim and D'Souza (1971a,b) find that NADPH addition to *S. faecium* dihydrofolate reductase induces a Cotton effect at 340 nm and has a large effect on the transitions at 290 and 305 nm. No such changes are noted in the enzyme from *E. coli*.

FOLATE ANALOGS. Dihydrofolate. In contrast to the behavior with NADPH, the binding of dihydrofolate to dihydrofolate reductase induces extrinsic Cotton effects. Dihydrofolate has negligible ellipticity at the concentrations used in the experiment described below. Addition of dihydrofolate to the enzyme resulted in the appearance of strong ellipticity bands, with a negative band at 332 nm, $[\theta]_{molecular} = -60{,}000$ (deg cm²)/dmol, a large positive band at 292 nm, $[\theta] = 170{,}000$ (deg cm²)/dmol, and a negative band at 270 nm, $[\theta] = -40{,}000$ (deg cm²)/dmol. The spectrum of the enzyme–dihydrofolate complex is shown in Figure 4. The dependence of the induced ellipticity on the concentration of added dihydrofolate can be used to calculate a dissociation constant for the enzyme–dihydrofolate complex of $1.1 \pm 0.5 \times 10^{-6}$ M in 0.1 M NaCl pH 7.2 at 27° (see Figure 5), using the "difference" method described in Materials and Methods. In the experiment illustrated, an enzyme solution with an absorbance of 0.475 OD unit corresponding to a concentration of $1.19 \pm 0.12 \times 10^{-5}$ M was titrated with dihydrofolate. The calculated enzyme concentration from the difference method was $1.07 \pm 0.01 \times 10^{-5}$ M. There is clearly just one binding site.

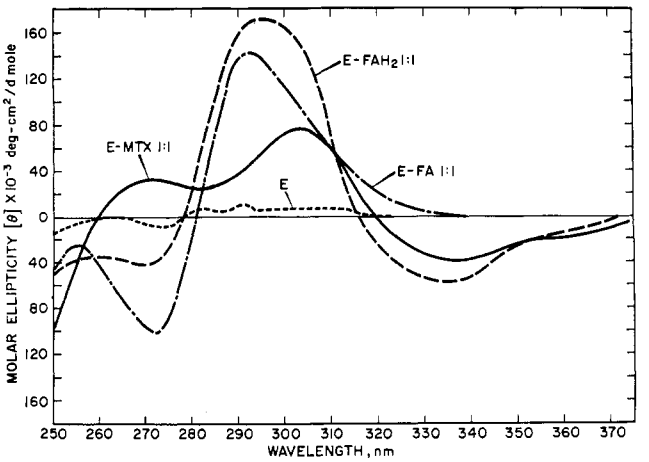

FIGURE 4: The molar circular dichroism of dihydrofolate reductase and 1:1 complexes with dihydrofolate, folate, and methotrexate in 0.1 M NaCl (pH 7.2) at 27° from 375 to 250 nm. The enzyme concentrations used were 3.0×10^{-5}, 1.8×10^{-5}, 1.0×10^{-5}, and 3.6×10^{-5} M, respectively.

It is difficult to draw conclusions as to which transitions of the dihydrofolate and the enzyme are contributing to the Cotton effects, as the transitions of dihydrofolate and those of the tyrosine and tryptophan residues of the enzyme over-

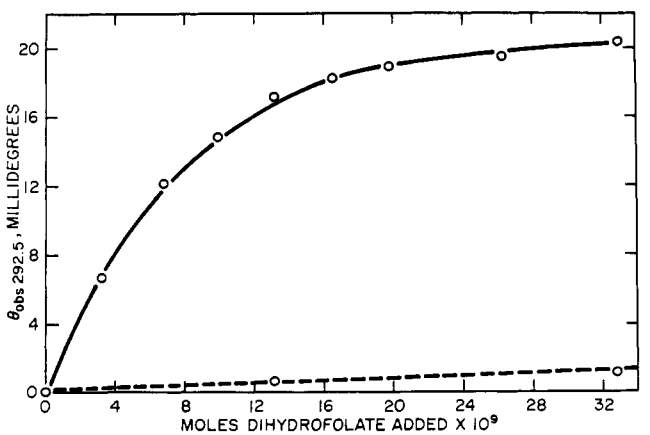

FIGURE 5: The variation of the ellipticity of dihydrofolate reductase with increasing dihydrofolate concentration at 292 nm in 0.1 M NaCl (pH 7.2) at 27°. Enzyme (0.9 ml) at 1.19×10^{-5} M was titrated. The data are corrected for dilution during substrate addition. (———) Enzyme plus dihydrofolate, (----) dihydrofolate alone.

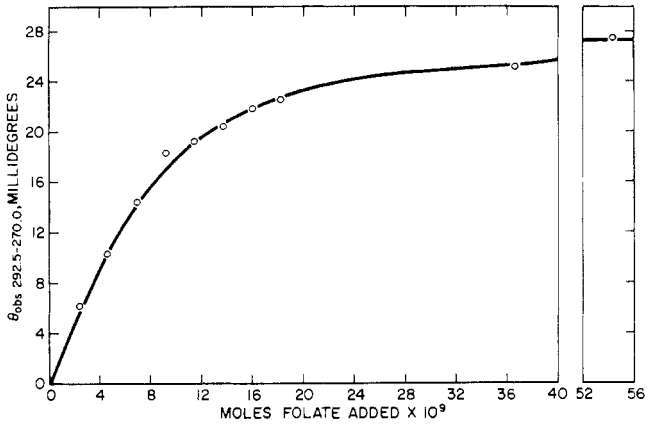

FIGURE 6: The variation of the difference in ellipticity between 292.5 and 270.0 nm of dihydrofolate reductase with increasing folate concentration. Enzyme (0.9 ml) 0.92 $\times$ 10^{-5} M in 0.1 N NaCl (pH 7.2) at 27° was titrated. The data are corrected for dilution due to folate addition. Folate in the absence of enzyme has a negligible difference in ellipticity at these wavelengths.

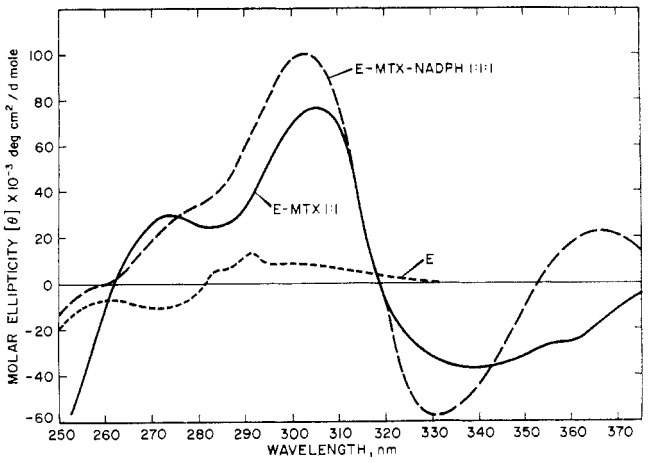

FIGURE 8: The circular dichroism of dihydrofolate reductase and the 1:1 complex with methotrexate and the 1:1:1 complex with methotrexate and NADPH in 0.1 N NaCl (pH 7.2) at 27° from 375 to 250 nm.

lap. Binding of dihydrofolate to dihydrofolate reductase does not affect the ellipticity of the enzyme below 250 nm; thus there are probably no large changes in the peptide backbone of dihydrofolate reductase upon dihydrofolate addition.

Folate. Folate, like dihydrofolate, binds quite strongly to dihydrofolate reductase and also induces large Cotton effects (see Figure 4). Folate is a substrate for many dihydrofolate reductases (for example, see Zakrzewski, 1960); it is also a substrate of the enzyme from *E. coli* B, MB 1428, but it is reduced at only $^1/_{27,000}$ the rate of dihydrofolate (unpublished results). The binding of folate induces a positive effect at 292.5 nm, $[\theta]$ = 140,000 (deg cm^2)/dmol, and a negative band at 272.5 nm, $[\theta]$ = $-100{,}000$ (deg cm^2)/dmol.

As is the case of dihydrofolate it is difficult to assign which transitions of the folate and the enzyme are responsible for the observed Cotton effects because the absorption bands of the folate and enzyme overlap. The absorption band of folate at 350 nm is not optically active and does not become so upon binding although this band is shifted to shorter wavelength upon binding to the enzyme (manuscript in preparation).

The appearance of ellipticity at 292.5 nm and 270 nm has been used to follow the binding of folate to the enzyme (see Figure 6). A dissociation constant for the enzyme-folate complex of 2.8 $\pm$ 0.5 $\times$ 10^{-6} M was determined using the difference method. As in the case of dihydrofolate, there appears to be only one folate binding site. In the study illustrated, the enzyme concentration was 0.92 $\pm$ 0.09 $\times$ 10^{-5} M using the extinction coefficient. The calculated enzyme concentration obtained was 0.79 $\pm$ 0.02 $\times$ 10^{-5} M.

Methotrexate. The binding of methotrexate (amethopterin, MTX) to the enzyme also induces large Cotton effects although the absolute magnitude of the effects are somewhat smaller than those induced by folate and dihydrofolate. The CD spectrum of the enzyme–MTX complex is shown in Figure 4. The E–MTX complex shows a broad complex band at 340 consisting of at least two transitions, $[\theta]_{340}$ = $-34{,}000$ (deg cm^2)/dmol. There is a peak at 305 nm, $[\theta]$ = 77,000 (deg cm^2)/dmol, and a second positive band at 272 nm, $[\theta]$ = 28,000 (deg cm^2)/dmol.

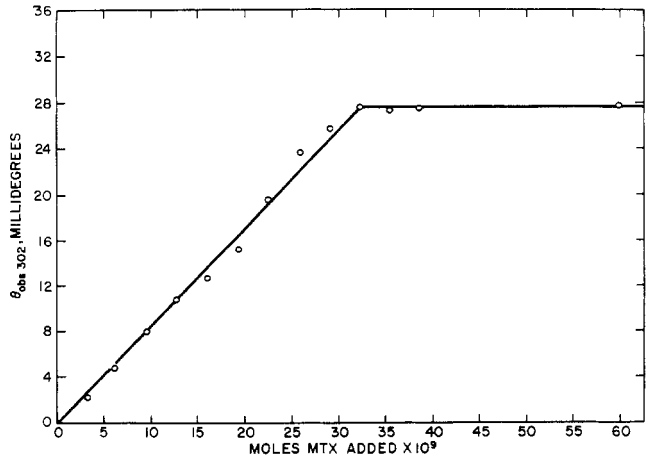

FIGURE 7: The variation of the ellipticity at 302 nm of dihydrofolate reductase with increasing MTX. Enzyme was 3.6 $\times$ 10^{-5} M in 0.9 ml of 0.1 N NaCl (pH 7.2) at 27°. The data are corrected for dilution due to MTX addition. MTX alone has negligible ellipticity at 302 nm at these concentrations.

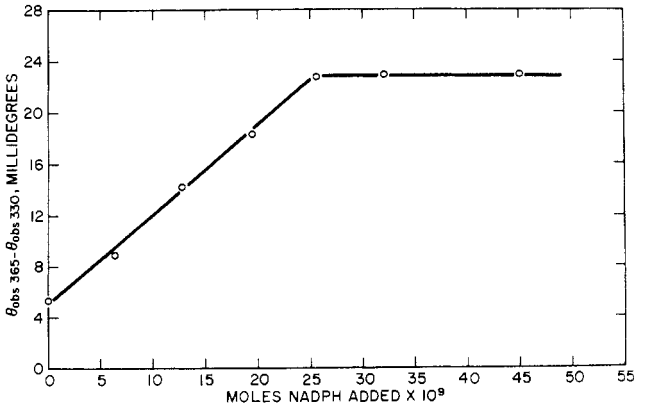

FIGURE 9: The variation of the difference in ellipticity between 365 and 330 nm of the 1:1 complex of 0.8 ml of dihydrofolate reductase and methotrexate at 3.25 $\times$ 10^{-5} M with increasing NADPH in 0.1 N NaCl (pH 7.2) at 27°. Data are corrected for dilution due to NADPH addition. NADPH alone has negligible ellipticity at these wavelengths in this concentration range.

Unfortunately, a precise constant for the binding of MTX to the enzyme cannot be obtained since the binding appears to be stoichiometric under the conditions of the experiment (enzyme = 3.6×10^{-5} M, 0.1 M NaCl, and pH 7.2 at 27°). Thus, the K_d is less than or equal to 1×10^{-7} M as weaker binding would give a noticeable turnover in the titration curve. The MTX titration of the enzyme is illustrated in Figure 7. The concentration of enzyme calculated from the extinction coefficient agreed exactly with the point of saturation of CD binding showing one binding site. The binding, performed in 0.05 M Tris-HCl (pH 7.2) at an enzyme concentration of 0.8×10^{-5} M, was also stoichiometric, within experimental error.

CD OF THE ENZYME–METHOTREXATE–NADPH TERNARY COMPLEX. While the addition of NADPH to the stripped enzyme causes only small effects, the addition of NADPH to 1:1 enzyme–MTX complex generates dramatic changes. The negative band at 340 nm of the E–MTX complex is replaced by a positive band at 365 nm, $[\theta] = 23{,}000$ (deg cm²)/dmol, and a negative band at 330 nm, $[\theta] = -54{,}000$ (deg cm²)/dmol. The band at 305 nm of the E–MTX complex is shifted to 302.5 nm and increases in magnitude to 100,000 (deg cm²)/dmol. The negative band of the E–MTX complex beginning at 260 nm is cancelled by the NADPH addition. The spectra of the complexes are shown in Figure 8.

The changes induced by NADPH addition to the E–MTX complex are linear with NADPH addition up to a 1:1 molar ratio. In the experiment illustrated in Figure 9, 0.8 ml of enzyme with an absorbance of 1.3 OD units equalling $3.25 \pm 0.33 \times 10^{-5}$ M or 2.60×10^{-8} mol were first titrated with MTX. Saturation of the increase in ellipticity at 305 nm occurred when 2.63×10^{-8} mol of MTX was added. This solution was then subsequently titrated with NADPH. Saturation of the change in ellipticity at 365 and 330 nm occurred when 2.59×10^{-8} mol of NADPH was added. This illustrates the formation of a 1:1:1 ternary complex. The dissociation constant of NADPH for the E–MTX–NADPH complex must be less than or equal to 1.0×10^{-7} M as the binding of NADPH to the E–MTX complex is virtually stoichiometric.

The ternary complex can also be formed by the addition of MTX to a 3×10^{-5} M solution of enzyme containing a 1-fold molar excess of NADPH, conditions under which all the enzyme should be bound to NADPH. The binding of MTX to this E–NADPH complex is also stoichiometric, within experimental accuracy, so K_d is again not greater than 1.0×10^{-7} M. The complex formed has the identical CD of the ternary complex formed by first adding MTX.

Summary and Conclusions

CD has been used to study the dihydrofolate reductase from a methotrexate-resistant strain of *E. coli* B. The CD observations of the enzyme indicate one binding site for MTX, dihydrofolate, and folate. Dissociation constants for the enzyme–folate and enzyme–dihydrofolate complexes were obtained. The binding of MTX has been shown to be stoichiometric in the presence and absence of NADPH at an enzyme concentration of about 3×10^{-5} M, and the binding of NADPH to the E–MTX complex has also been shown to be stoichiometric under these conditions. The formation of an enzyme–MTX–NADPH complex with 1:1:1 stoichiometry has been demonstrated. A preliminary estimate of the conformation of the enzyme in solution was made, and it has been demonstrated that binding of dihydrofolate produces no gross conformational changes in the peptide backbone of the protein.

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