Dihydrofolate Reductase from a Resistant Subline of the L1210 Lymphoma. Purification by Affinity Chromatography and Ultraviolet Difference Spectrophotometric and Circular Dichroic Studies†

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ABSTRACT: Dihydrofolate reductase has been isolated in 80% yield and purity >99% from a methotrexate-resistant subline of the L1210 lymphoma (L1210/MTX) by the use of affinity chromatography. Purity has been established by titrating enzymic activity and protein fluorescence with a stoichiometric inhibitor methotrexate (MTX) and confirmed by polyacrylamide gel electrophoresis. The apparent molecular weight calculated from filtration through a Sephadex G-100 column is 21 000 ± 3%. This reductase contains four tryptophan residues per molecule. The binary complexes of enzyme with folate, dihydrofolate, MTX, NAD^+, and NADPH resulted in a changed ultraviolet absorption spectra compared to the unmixed components. The changes observed upon binding of dihydrofolate, MTX, and NADPH in the range 240 to 400 nm in the spectra were similar to those reported for the reductase of *Escherichia coli* (Poe, M., Greenfield, N. J., Hirschfield, J. M., and Hoogsteen, K. (1974a), *Cancer Biochem. Biophys. 1*, 7; Poe, M., Greenfield, N. J., and Williams, M. N. (1974b), *J. Biol. Chem. 249*, 2710). This indicates that the chemical environment of the bound substrate, inhibitor, and coenzyme may be the same in the two enzymes. Absorbance changes at 280 and 290 nm suggest that one (or more) tryptophan residues are perturbed upon binding of these ligands. The intrinsic circular dichroic (CD) spectra of dihydrofolate reductase from L1210/MTX and *E. coli* B (Greenfield, N. J., Williams, M. N., Poe, M., and Hoogsteen, K. (1972), *Biochemistry 11*, 4706) are very different. Both the "backbone" ellipticity and aromatic region are quite distinctive. However, the CD of the enzyme–substrate and inhibitor complexes as well as enzyme–MTX–NADPH ternary complexes exhibit some homologies as compared with the *E. coli* B enzyme. This suggests that the ligands may be constrained in similar conformation on the two enzymes.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3), which catalyzes the reduction of dihydrofolate to tetrahydrofolate in the presence of NADPH,[1] is of considerable pharmacological interest. It is the target enzyme for a number of chemotherapeutic agents (Hitchings and Burchall, 1965) and the species specific differences have been exploited for therapeutic purposes (Thomas and Starb, 1971; Condit, 1971; Hitchings, 1971). Previous communications from this laboratory have described purification and some of the properties of dihydrofolate reductase from a subline of L1210 murine leukemia resistant to methotrexate[2] (Perkins and Bertino, 1966; Perkins et al., 1967; Hillcoat et al., 1967). This paper describes an improved method for the isolation of pure enzyme in high yield by the use of affinity chromatography[3] and the spectral and circular dichroism (CD) characteristics upon interaction with substrates, pyridine nucleotide coenzymes, and some inhibitors. The purpose of spectral and circular dichroism studies was 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. The comparison of properties of difference spectra and CD curves may also prove a simple and convenient method to study some of the species specific differences between dihydrofolate reductases from different sources, which may be useful in the design of drugs for selective treatment of neoplasms.

Experimental Section

Materials

Folic acid was purchased from Nutritional Biochemical Corp; methotrexate (MTX) from Lederle Laboratories; NADPH from Sigma Chemical Co; Tris (tris(hydroxymethyl)aminomethane) from Schwarz/Mann; and 2-mercaptoethanol from Eastman Chemical Corp. Sephadex G-25 and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Inc., Bio-Rex (300–400 mesh) was from Bio-Rad Laboratories, and electrophoresis chemicals were from Can-

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[1] Abbreviations used are: FA, folic acid; FAH_2, 7,8-dihydrofolic acid; FAH_4, tetrahydrofolic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate; MTX, methotrexate; MTX-Sepharose, the affinity resin, methotrexate aminoethyl-Sepharose; CD, circular dichroism; AA, amino acid.

[2] A subline of the L1210 murine leukemia resistant to MTX contains approximately 12 times the level of FAH_2 reductase as compared to the parent strain and is a rich source of the enzyme (Schrecker et al., 1962).

[3] Affinity chromatography using MTX covalently bound to an agarose matrix via an aminoalkyl bridge (Kaufman and Pierce, 1971; Chello et al., 1972; Gauldie and Hillcoat, 1972; Poe et al., 1972) has been used recently for the isolation of FAH_2 reductase from a number of sources.

alcoCo. Carbodiimide [1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate] was purchased from Aldrich Chemical Co.

Dihydrofolate (FAH_2) was synthesized from folic acid by dithionite reduction using the method of Blakley (1960) and stored under nitrogen at −60 °C. The concentrations of folate, FAH_2, MTX, and NADPH were determined spectrophotometrically, using molar extinction coefficients of $\epsilon_{282\ nm}$ 27 000 for folate at pH 7.0 (Dawson et al., 1969), $\epsilon_{282\ nm}$ 28 000 for FAH_2 at pH 7.0 (Dawson et al., 1969), $\epsilon_{258\ nm}$ 23 250 and $\epsilon_{302.5\ nm}$ 22 100 for MTX at pH 13 (Seegar et al., 1949), and $\epsilon_{340\ nm}$ 6220 for NADPH at pH 7.0 (Kornberg and Horecker, 1953). The enzyme molar extinction coefficient was estimated by measuring absorbance at 280 nm. Protein concentration was determined by the ultraviolet absorption method of Waddell (1956) and also by titration with MTX assuming stochiometric binding of inhibitor (Werkheiser, 1961; Bertino et al., 1964).

Enzyme and ligands were dissolved in buffer B. The extinction coefficient of 30 000 at 280 nm was used to determine the enzyme concentration of the purified enzyme.

Methods

Absorption spectra were recorded using a Cary spectrophotometer (Model 15). Difference spectra were recorded on an Aminco DW-2 UV-vis spectrophotometer (American Instrument Co., Silver Springs, Md.) using the split compartment cells (Yankeelov, 1963). Each compartment had a path length of 0.44 cm. The instrument was set in the split beam mode and difference spectra were recorded from 240 to 400 nm at a scan rate of 1 nm/s and band-pass of 2 nm on either the 0.05 or 0.1 absorbance scale at 20 °C. Circular dichroism (CD) measurements were performed using 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 °C.

Calculation of Circular Dichroism. The intrinsic ellipticity of the peptide backbone (250–190 nm) is reported as the mean residue ellipticity in deg cm^{-2} $dmol^{-1}$. The results are calculated as follows:

$$[\theta]_{\text{mean residue}} = \frac{100 \times \theta_{\text{obsd}}\ (\text{deg})}{\text{path length (cm)} \times \text{concn (M)} \times \text{no. of AA in protein}}$$

The ellipticity of the aromatic portion of the enzyme and the bound chromophores (from 250 to 400 nm) was reported as molar ellipticity where:

$$[\theta]_{\text{molar}} = \frac{100 \times \theta_{\text{obsd}}}{l\ (\text{cm}) \times \text{concn (M)}}$$

The spectra above 250 nm were corrected for free ligand.

Buffers. Standard buffers used were: buffer A, sodium citrate (0.05 M, pH 6.0), and buffer B, Tris-HCl (0.05 M, pH 7.0) containing KCl (0.1 M).

Enzyme Assay. The reductase activity was determined by measuring the decrease in absorbance at 340 nm by a previously published method (Perkins et al., 1967) using a Gilford spectrophotometer (Model 2000 or 2400) at 37 °C. The assay mixture contained 100 μmol of Tris-HCl (pH 7.0), 150 μmol of KCl, enzyme, 0.1 μmol of NADPH, and 0.05 μmol of FAH_2 containing 10 μmol of 2-mercaptoethanol in a total volume of 1.0 mL. The reaction was initiated by the addition of substrate. Specific activity is defined as μmol of substrate reduced min^{-1} $(mg\ of\ protein)^{-1}$. A molar extinction coefficient of 12 000 (Mathews and Huennekens, 1963) at 340 nm was used for calculation of activity.

Synthesis of Methotrexate–Sepharose Complex. The procedure described below is a modification of a method previously reported (Chello et al., 1972). The present method gives material with a high MTX to Sepharose ratio.

After washing with 800 mL of distilled water to remove azide and "fines", 200 g of Sepharose-4B was suspended in a final volume of 400 mL of water, previously adjusted to pH 11 with 1 N NaOH. With constant stirring, a solution of cyanogen bromide (24 g in 40 mL of water adjusted to pH 11) was added. The pH of the reaction mixture was maintained between 10 and 11 by constant addition of small amounts of 10 N NaOH and the temperature of the reaction mixture was maintained between 20 and 25 °C by addition of ice around the reaction vessel. When the reaction was complete as indicated by the stability of pH at 11 (approximately 30 to 40 min) the temperature of the reaction mixture was immediately lowered to 4 °C by adding ice. The mixture was rapidly filtered and washed with 5 to 6 L of ice-cold water (pH 11) and finally with 3 L of cold borate buffer (0.125 M; pH 10.0). The final product was suspended in a final volume of 600 mL of cold borate buffer. An ice-cold solution containing 60 mL of ethylenediamine[4] (adjusted to pH 10 with 5 N HCl) was added to the activated Sepharose slurry. The mixture was stirred for 2 h at 25 °C and then for 18 h at 4 °C, filtered, and washed extensively with cold water (8–10 L). The Sepharose–aminoethylene slurry was then suspended in 800 mL of water (pH 6.5). Methotrexate (1 g) in 100 mL of water adjusted to pH 6.5 was added to the slurry and the mixture was stirred for 15 min at 25 °C. Carbodiimide (5 g dissolved in 100 mL of water adjusted to pH 6.5) was added to the reaction mixture in 5 equal portions during the next 60 min and the mixture was stirred for 20 h at 25 °C.

After filtration, the bright yellow MTX–Sepharose complex was washed exhaustively with water (ca. 20 L) to remove most of the unreacted MTX. The MTX–Sepharose was loaded into a column (2.5 × 40 cm) and washed continuously with sodium bicarbonate (0.2 M, 3 L) followed with water (6–8 L) and finally with 1 M KCl (15–20 L) until the column effluent was free from unbound MTX as indicated by the lack of inhibition by the effluent solution upon enzyme in the standard enzyme assay system. This assay can detect as little as 10^{-6} μmol of MTX/mL. The column was finally washed with water (4–6 L) and the slurry was stored in 0.2 M NaCl solution at 4 °C protected from light. Approximately 0.54 μmol of MTX was bound per mL of Sepharose as indicated by release of inhibitor by potassium borohydride in the presence of 0.1 M NaOH (Failla and Santi, 1973).

Preparation of Bio-Rex Resin. The resin was equilibrated with 1 M HCl for 20 h, washed with water exhaustively (until free from acid), and then suspended in buffer B. After loading into the column, it was continuously washed until the effluent showed no absorbance at 280 nm.

Isoelectric Focusing. Pure enzyme was applied to the 110-mL Ampholine electrofocusing column (Model 8100, LKB Instruments, Inc.) using carrier ampholyte mixture to

[4] Contrary to previous reports that coupling of ethylenediamine to activated Sepharose must be accomplished within 2.5 min of Sepharose activation (Cuatrecasas, 1970), recent work (Wagner et al., 1971) has shown that activated matrix is stable for an extended period of time. In the present studies no differences were observed in the quantity of MTX coupled nor in the efficiency of the MTX–Sepharose complex when activated Sepharose was used immediately or 5 days after activation.

establish a pH gradient (from 3 to 10) during the focusing in a sucrose gradient (0–50%). The electrofocusing procedure was carried out for 48 h at 300 V at 4 °C. Fractions (1.5 mL) were collected and stoppered immediately to avoid changes of pH due to absorption of CO_2 from atmosphere. Enzyme activity and pH of each fraction were determined.

Electrophoresis. Polyacrylamide gels (7.5%) were used and electrophoresis was conducted using Tris–glycine buffer (pH 8.3) at 4 °C at a constant current (250 V, 11 mA/6 gels) using the method of Davis (1964).

Molecular Weight. Gel filtration on Sephadex G-100 as described by Whitaker (1963) was used to determine the molecular weight of the enzyme. Cytochrome *c* (mol wt 12 384), chymotrypsin A (mol wt 25 000), ovalbumin (mol wt 45 000), and aldolase (mol wt 158 000) were employed as standards.

Tryptophan Content. The tryptophan content of reductase was measured by the methods of Goodwin and Morton (1946) and Edelhoch (1967).

TABLE I: Purification of Dihydrofolate Reductase from L1210/MTX Cells.[a]

Step	Vol (mL)	Total protein (mg)	Total enzyme act. (units)	Sp act. (units/mg)	Recovery (%)
Crude extract	90	4500	675	0.15	100
pH 5.1 supernate	82	2050	615	0.3	91
MTX-Sepharose	93		615		91
Sephadex G-25	100	12.0	605	50	89
Bio-Rex	125	11.0	545	50	80.7
Amicon ultrafiltration	11.4	11.0	500	50	74

[a] Experimental details are given in the Experimental Section. Because of the presence of FAH_2 and 2-mercaptoethanol, protein concentration could not be determined in the enzyme extract following MTX-Sepharose chromatography.

Results and Discussion

Enzyme Purification. All purification steps described below were carried out at 4 °C.

Step 1: Preparation of the Crude Extract. An MTX-resistant subline of the L1210 mouse lymphoma (L1210/MTX) was maintained in ascites form in BDF_1 male mice as described previously (Perkins et al., 1967; Chello et al., 1972).

This subline has been shown to contain higher reductase activity than the parent MTX-sensitive line (Perkins et al., 1967; Hillcoat et al., 1967) and serves as a rich source of the enzyme. Mice bearing a 7-day growth of tumor were sacrificed and cells harvested from the peritoneal cavity with 0.9% NaCl. Packed cells were obtained after sedimentation at 8000*g* for 20 min. The supernatant fluid was decanted, and the cells were frozen and stored at −60 °C until used. The cells from a total of 600 mice were thawed by the addition of 3 vol of distilled water and stirred for 1 h at 4 °C. One volume of 3.6% NaCl was then added to restore isotonicity, and the cell lysate was centrifuged at 27 000*g* for 20 min. The supernatant containing the enzyme was decanted through two layers of cheesecloth to remove lipid material. The residue was suspended in 3 vol of 0.9% NaCl, centrifuged, and decanted through cheesecloth as before. The two supernatant fractions were pooled to yield the "crude extract".

Step 2: pH 5.1 Supernatant Fraction. The pH of the crude extract was adjusted to 5.1 by cautious addition of 1 N HCl at 4 °C with constant stirring. After 15 min the mixture was centrifuged at 27 000*g* for 20 min and the precipitate discarded. The pH of the supernatant solution was adjusted to 6.0 by the addition of 1 N NaOH. This solution is designated as "pH 5.1" extract.

Step 3: MTX-Sepharose Affinity Chromatography. The pH 5.1 extract was allowed to adsorb on a MTX-Sepharose column (1.8 × 18 cm) previously equilibrated with buffer A. After all the extract had gone through the column, it was washed with buffer B until no absorbance at 280 nm was observed in the wash. Elution of enzyme from the column was with buffer B (40 mL) containing FAH_2 (0.2 mM) and mercaptoethanol (10 mM) followed by buffer B. Fractions of 5 mL were collected at a flow rate of 2 mL/min and enzymatic activity was measured in the fractions by the method described above (Perkins et al., 1967). Fractions containing reductase activity were combined and stored frozen at −60 °C. No loss of activity was observed up to 3 months. The enzyme could not be eluted from the MTX-Sepharose column by simply raising the pH of the buffer up to 8.5 or by increasing the salt (KCl) concentration in the buffer up to 0.4 M. However, as reported by others (Kaufman and Pierce, 1971; Chello et al., 1972; Gauldie and Hillcoat, 1972) a nonspecific component from the crude extract bound to the MTX-Sepharose column could be eluted with high salt concentration. Therefore, after use the column was washed with 5 L of 1 M KCl.

Step 4: Sephadex G-25. Separation of enzyme from FAH_2 was partially accomplished by G-25 filtration. The enzyme mixture from the affinity column was applied to a Sephadex column and eluted with buffer B. Fractions (2 mL) were collected and assayed for enzyme activity. When no more than 30 mL of enzyme extract was applied to a G-25 column with a bed volume of 120 mL, approximately 60 to 70% of FAH_2 was removed as judged by complete spectra. Active fractions were pooled.

Step 5: Bio-Rex. The pooled fractions were applied to a small column (1.8 × 15 cm) of Bio-Rex previously washed and equilibrated with buffer B. The column was eluted with same buffer and 2.1-mL fractions were collected. The ultraviolet spectrum of each fraction was scanned on a Cary spectrophotometer and the enzyme activity determined. Dihydrofolate was strongly adsorbed on the column and active fractions were stored frozen in individual tubes. This step resulted in complete removal of bound FAH_2 as judged by the UV spectrum (Perkins et al., 1967) and by the fluorescence spectrum (Perkins and Bertino, 1966).

Concentration of Purified Enzyme. For further enzyme studies, excess buffer was removed from the enzyme solution by use of the Amicon on-line column eluate concentrator (Model CECI, Amicon Corporation) using a PM10 ultrafiltration membrane with a molecular weight retention of 10 000. Representative results of the purification procedure are illustrated in Table I. In this case about 12 mg of homogeneous enzyme was obtained in pure form (sp act. 50) from L1210/MTX cells, with an overall yield of about 80%.

Enzyme Purity. The purified enzyme (100 μg) when subjected to analytical electrophoresis on polyacrylamide moved as a homogeneous band. Titration of enzyme activity using MTX (Werkheiser, 1961; Perkins et al., 1967) and measurement of the quenching of the fluorescence of the free enzyme using MTX (Perkins and Bertino, 1966; Williams et al., 1973) also showed that the isolated enzyme was pure (>99%).

Isoelectric Focusing. Electrofocusing was performed using

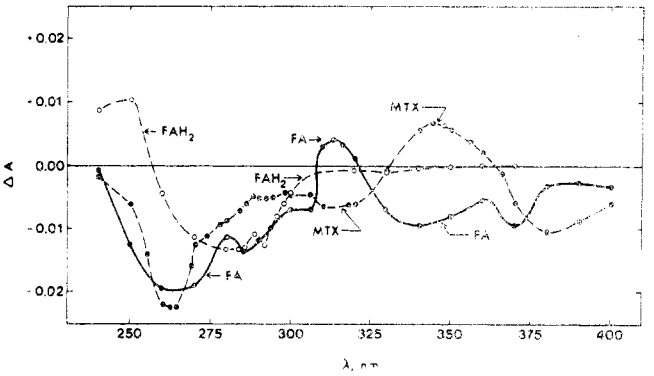

FIGURE 1: Difference spectrum generated upon binding of folate, dihydrofolate, and methotrexate to the reductase. Prior to mixing the ratios of enzyme:ligand concentrations were: 3.0 μM; MTX, 3.0 and 5.0 μM; FA, 18.18 and 5.5 μM; FAH_2, 14.14 μM. The difference spectra are: (●- - -●) MTX; (●—●) FA; (○- - -○) FAH_2.

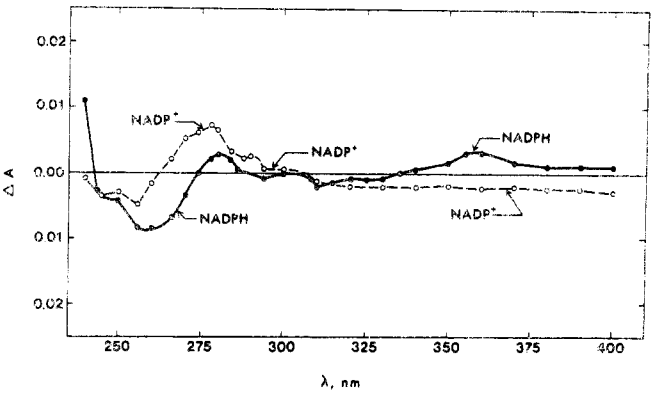

FIGURE 2: Difference spectrum generated upon binding of pyridine nucleotides to the reductase. Prior to mixing the ratio of enzyme was 8.10 μM, NADPH was 10.73 μM, and $NADP^+$ was 11.30 μM. The difference spectra are: (●—●) NADPH; (○- - -○) $NADP^+$.

a pH gradient from 3 to 10. The purified enzyme always moved as a single symmetrical peak and recovery of the activity from the column was essentially complete (>90%) in different runs. The isoelectric point (pI = 8.1) of the enzyme is approximately 1.5 units higher than chicken liver reductase (Huennekens et al., 1970). However, after incubation with NADPH, two peaks of enzyme activity resulted with two acidic optima (pI = 5.6 and 6.0) and disappearance of the peak at 8.1. The isoelectric point (8.2) was not significantly altered upon incubation of purified protein with FAH_2. Thus, NADPH, but not FAH_2, appears to change the isoelectric point of the enzyme.

Tryptophan Content. Because of their possible involvement in substrate and coenzyme binding, tryptophan residues were determined by two spectrophotometric methods. Values of 3.9 and 4.1 residues per molecule were obtained, respectively, by the Goodwin and Morton (1946) and the Edelhoch (1967) procedures. The amino acid composition of this reductase has been reported previously (Perkins, 1966; Huennekens et al., 1970). The aromatic amino acid content of this enzyme is very similar to reductases of bacteria (Gundersen et al., 1972; Bennet, 1974; Gleisner et al., 1974) as well as chicken liver (Huennekens et al., 1970). From the amino acid composition a mol wt of 19 230 is calculated for the enzyme which is in good agreement with a mol wt of 21 000 ± 3% determined by gel filtration (vide infra).

Molecular Weight. The molecular weight of the enzyme was also determined by filtration through Sephadex G-100 standardized with proteins of known molecular weight. Three separate determinations gave molecular weights in the range of 20 500 to 21 200. An apparent mol wt of 21 000 ± 3% is suggested for this enzyme. This value is in the range reported for reductase from bacterial and mammalian enzymes (Huennekens, 1968; Blakley, 1969).

Ultraviolet Difference Spectra. (1) Enzyme plus Folates. The difference in absorbance between mixed L1210/MTX reductase and FA, FAH_2, and MTX and the unmixed components as measured directly is shown in Figure 1. The concentrations were selected to assure virtually complete enzyme complex formation. Therefore, the difference spectra reported are of the saturated enzyme–ligand complex alone vs. a corresponding amount of unmixed enzyme and ligand. Excess ligand in the sample cuvette is balanced by ligand in the reference cuvette. The difference spectrum of the L1210/MTX enzyme–FA complex is similar to that of the *Escherichia coli* reductase–FA complex (Poe et al., 1974a) except that the minimum in the difference spectrum at 280 nm is at about 8 nm longer wavelength in the *E. coli* enzyme difference spectrum. This possibly reflects some differences between the enzymes in their binding of FA. Similarly, the difference spectrum of the L1210/MTX reductase–FAH_2 complex over the range of 240 to 400 nm is almost superimposable on the difference spectrum reported for reductase of the *E. coli*–FAH_2 complex (Poe et al., 1974a), but there is a more pronounced, but still small, local minimum at 292 nm. This indication of multiple bands in the FAH_2 difference spectrum between 260 and 300 nm suggests that one or more aromatic residues are perturbed in their environment upon binding of substrate. However, the homologies suggest that the chemical environment of the FAH_2 must be very similar in the two enzymes. The L1210/MTX enzyme–MTX complex difference spectrum is also almost superimposable on the difference spectrum noted for *E. coli* reductase upon binding of MTX (Poe et al., 1974a) with the exception of the negative band at 315 nm. The origin of this band is unknown; however, it was also observed upon binding of MTX to T_4-phage reductase (Erickson and Mathews, 1972). The *E. coli* and T_4 enzyme–MTX difference spectra have been interpreted as preferential binding of the acidic form of MTX (protonated at N-1 of the 2,4-diaminopteridine moiety) (Erickson and Mathews, 1972; Poe et al., 1974a) by the enzyme. The N-1 protonated form of MTX to the *E. coli* enzyme is almost 10^5 times smaller than the N-1 unprotonated form. The binding of MTX to enzyme perturbs the protonated ⇌ unprotonated equilibrium in favor of the protonated form of inhibitor and results in tighter binding. The similarity of the difference spectra suggests that L1210/MTX reductase also preferentially binds N-1 protonated MTX better than N-1 unprotonated MTX and in this respect is similar in behavior to the *E. coli* enzyme.

(2) Enzyme plus Pyridine Nucleotides. The difference spectra resulting from the binding of $NADP^+$ and NADPH to the enzyme are shown in Figure 2. The difference spectra were recorded with sufficient nucleotide to essentially saturate the binding site. The difference spectra of L1210/MTX reductase obtained upon binding with NADP and $NADPH^+$ are virtually identical with those reported for the first or tighter pyridine nucleotide binding site for the *E. coli* enzyme (Poe et al., 1974b). However, this is in contrast to the T_4-phage reductase (Erickson and Mathews, 1972). In this enzyme, NADPH addition had virtually no effect on the spectrum. In analogy with malate dehydrogenase (Fisher et al., 1969) the difference spectrum maximum at approximately 355 nm may indicate that the stereospecificity with respect to utilization

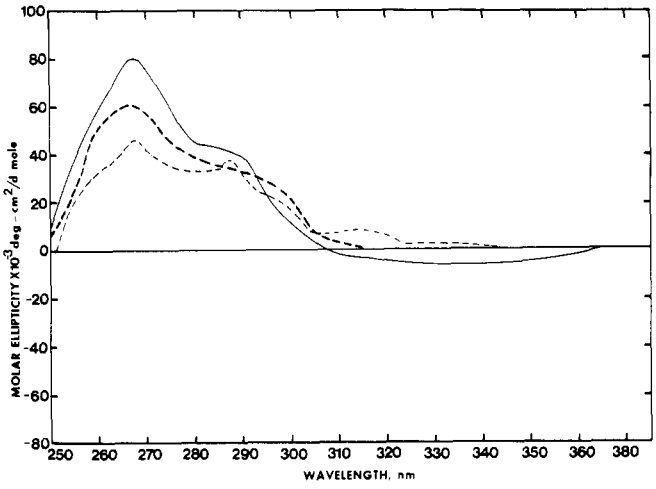

FIGURE 3: The molar circular dichroism of the reductase and 1:1 complexes with $NADP^+$ and NADPH at 27 °C from 375 to 250 nm: (-·-) enzyme; (- - -) enzyme plus $NADP^+$; (—) enzyme plus NADPH. The spectra are corrected for the spectra of free ligands.

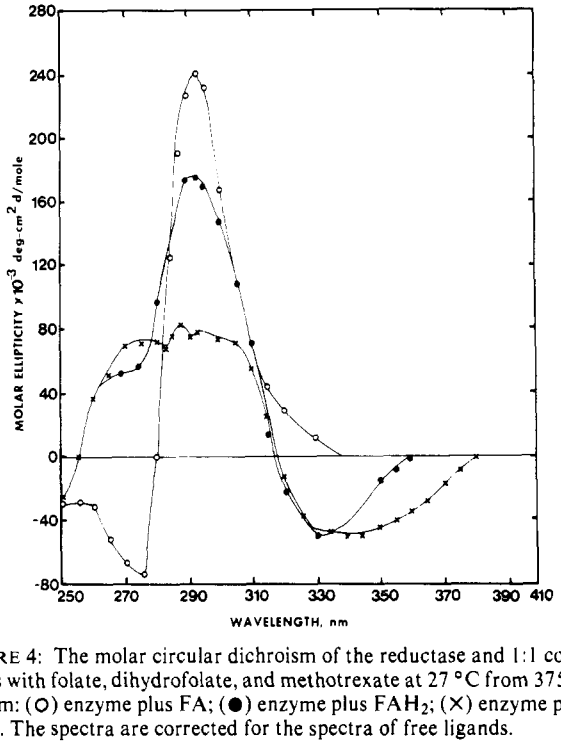

FIGURE 4: The molar circular dichroism of the reductase and 1:1 complexes with folate, dihydrofolate, and methotrexate at 27 °C from 375 to 250 nm: (○) enzyme plus FA; (●) enzyme plus FAH_2; (×) enzyme plus MTX. The spectra are corrected for the spectra of free ligands.

of C-4 protons of NADPH by this enzyme is A, i.e. the A proton at C-4 of the reduced nicotinamide moiety is transferred to FAH_2. This correlation of difference spectrum band position with stereospecificity in enzymatic activity was first demonstrated for mitochondrial malate dehydrogenase (Fisher et al., 1969) and was also seen with *E. coli* dihydrofolate reductase (Poe and Hoogsteen, 1974). The dihydrofolate reductases from mouse L1210 cells (Pastore and Friedkin, 1962), *Streptococcus faecalis* (Blakley et al., 1963), Sarcoma 180 strain AT/300 (Zakrzewski and Sansone, 1967; Zakrzewski, 1969), and chicken liver and wild-type *E. coli* B (Pastore and Williamson, 1968) have been also demonstrated to use the A proton of NADPH. The E-NADPH difference spectrum between 270 and 310 nm exhibits a pair of maxima at approximately 280 and 290 nm. The intensity of maxima at 280 nm was much higher than *E. coli* reductase (Poe et al., 1974b). The spectral changes observed in the region 270–310 nm are presumably due to changes in the local electronic environment of one (or more) tryptophan residues. In the case of the *E. coli* enzyme, it has been suggested that the binding of the first mole of NADPH to the enzyme altered the local environment of at least one tryptophan residue.

The greater intensity of the bands upon binding of NADPH to L1210/MTX reductase may indicate perturbation of the environment of more than one tryptophan residue. The difference spectrum seen upon the binding of $NADP^+$ to the L1210/MTX reductase in the presence of a slight excess of cofactor (see Figure 2) is quite different from the corresponding spectrum for NADPH. The bands between 240 and 300 nm are also quite different from *E. coli* reductase (Poe et al., 1974b). The L1210/MTX reductase showed two positive bands at approximately 278 and 290 nm. Presumably, these bands also arise from alteration of tryptophan residues upon binding of $NADP^+$ to the enzyme.

Circular Dichroism Studies. (1) Peptide Backbone. The L1210/MTX reductase showed a negative band at 210 nm with an ellipticity of approximately 8000 deg cm^2 dmol^{-1} and a shoulder at 230 nm with an ellipticity of approximately 3000 deg cm^2 dmol^{-1}. Comparisons with model curves using poly(L-lysine) as a standard (Greenfield and Fasman, 1969) suggest this enzyme has very low α helix, approximately 15%, with 60–70% random and 15–25% β structure. Binding of FA had no effect on the ellipticity. Interaction with MTX, on the other hand, caused a decrease in ellipticity at 210 nm and a sharpening of the shoulder at 230 nm. These changes could be either due to small side chain structural changes, or due to low-wavelength extrinsic Cotton effects of the bound MTX.

NADPH also had a slight effect on the CD spectrum of the enzyme. There was a slight sharpening of the shoulder at 225 nm. This could be due either to a small structural change or movement of an aromatic chromophore on binding of the coenzyme. Addition of MTX to the E-NADPH complex caused further small changes in the CD spectrum. As in the case of MTX binding alone, these changes may be due to generation of extrinsic bands arising from the band chromophores or to shifts in the side chain aromatic amino acid chromophores of the enzyme. There is no evidence that these bands are due to a major conformational change in "backbone" ellipticity of the enzyme.

The "backbone" CD pattern of L1210/MTX reductase is distinctly different from *E. coli* enzyme (Greenfield et al., 1972). This bacterial reductase had a maximum at 220 nm and a shoulder at 210 nm. The magnitude of the bands of the enzyme from *E. coli* suggested that the bacterial enzyme had a very low α helix (~10%) but considerably more β structure (50–60%).

(2) Ellipticity in the Aromatic Region and Ellipticity Induced by Added Ligands. The spectrum of the unliganded enzyme is shown in Figure 3. The reductase has bands at 315, 287.5, 277, and 267.5 nm and the ellipticity is fourfold greater than that of the *E. coli* enzyme (Greenfield et al., 1972). However, the CD spectrum of reductase of L1210/MTX is very similar to reductase of *S. faceium* (Freisheim and D'Souza, 1971a,b). Binding of MTX (Figure 4) to the enzyme generates several new CD bands. If the ellipticity of the free enzyme is subtracted from that of the complex, the spectrum is quite similar to that generated by MTX binding to the enzyme of *E. coli*. However, the reductase of L1210/MTX shows two additional bands at 282 and 290 nm which suggests that a tryptophan residue is perturbed when MTX binds to this enzyme. The positions and magnitude of other bands are

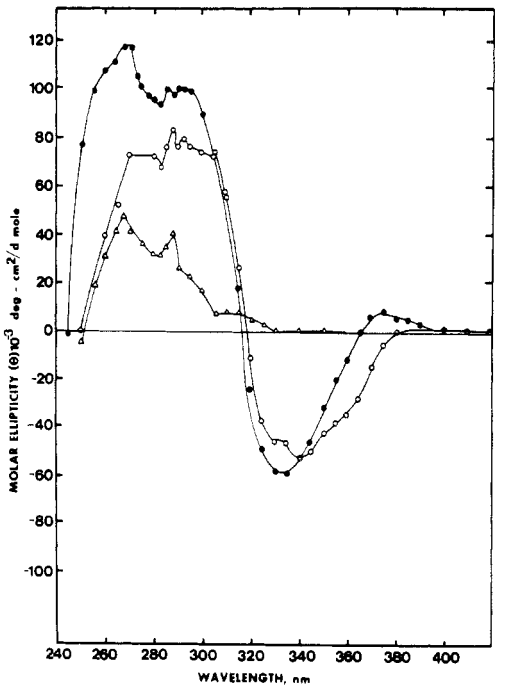

FIGURE 5: The molar circular dichroism of the reductase (enzyme, △) and the 1:1 complex with MTX (○) and the 1:1:1 complex with MTX and NADPH (●) at 27 °C from 375 to 250 nm. The spectra are corrected for the spectra of free ligands.

similar. The homologies of the E-MTX spectra of reductase from two different sources suggest that most of the ellipticity is due to placing the MTX in a rigid asymmetric conformation and that the geometries of the MTX-enzyme complexes are very similar.

The spectra of the enzyme complexes with FA and FAH_2 are shown in Figure 4. The binding of these ligands results in CD spectra essentially similar to those reported for *E. coli* enzyme (Greenfield et al., 1972). Both NADPH and $NADP^+$ generate extrinsic Cotton effects on binding to the L1210/MTX reductase (Figure 3). The binding of NADPH resulted in the generation of bands at 335 and 265 nm. The position of these new bands of the enzyme-NADPH complex suggests that they are due to the coenzyme chromophore rather than a perturbation of the aromatic residues of the protein. These results are in contrast to the *E. coli* enzyme, where pyridine nucleotides have no effect on the CD of the enzyme when it is free of inhibitors.

When NADPH is bound to the enzyme-MTX complex or MTX is bound to the E-NADPH complex, a new CD spectrum characteristic of the ternary complex appears (Figure 5). In this case the spectral changes are essentially similar to those reported for the reductase of *E. coli* (Greenfield et al., 1972). The spectra above 280 nm are not simply additive and suggest that the nicotinamide portion of the pyridine nucleotide is interacting directly with the pteridine ring of MTX to generate new bands. A comparison of the spectral maxima of L1210/MTX and *E. coli* reductase is shown in Table II. The results suggest that the geometries of the ternary complexes of enzyme-MTX-NADPH are very similar for both the *E. coli* and L1210/MTX enzymes.

In summary, the present studies considerably extend information on the interaction of this enzyme with coenzymes, substrates, and the analogue MTX. The availability of this mammalian enzyme of high purity and in good yield should facilitate additional studies, and possibly allow sequencing of the protein, thus adding to information on this pharmacologically important enzyme, already sequenced from bacterial sources (Bennet, 1974; Gleisner et al., 1974).

TABLE II: Comparison of Bands of Circular Dichroism of Dihydrofolate Reductase of MTX Resistant L1210 Lymphoma Cells and *E. coli* B MB 1428.

	Wavelength	[θ] L1210/MTX	[θ] *E. coli* B MB 1428
E-MTX	340	−50 000	−34 000
	305	74 000	77 000
	272	62 000	28 000
$E\text{-}FAH_2$	332	−50 000	−60 000
	292	170 000	170 000
	270	50 000	−40 000
E-FA	292.5	240 000	140 000
	270	−70 000	−100 000
E-MTX-NADPH	375	7 900 (365)	23 000
	335	−59 500 (330)	−54 000
	290	100 000 (302.5)	100 000
	267.5	119 000 (280)	+ shoulder

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