volving the second cysteine residue is suggested by the instability of the inactive disulfide enzyme. Oxidation of the enzyme under aerobic conditions could produce the inactive enzyme, which, in effect, would terminate glycolytic activities. This possible role among others can be studied in yeast cell-free systems, by employing the yeast dehydrogenase which contains only cysteines-149 and -153.

References

Allison, W. S. (1968), *Ann. N. Y. Acad. Sci. 151*, 180.

Allison, W. S., and Kaplan, N. O. (1964), *J. Biol. Chem. 239*, 2140.

Boross, L. (1969), *Acta Biochim. Biophys. Acad. Sci. Hung. 4*, 57.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Conway, A., and Koshland, D. E., Jr. (1968), *Biochemistry 7*, 4011.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Fenselau, A. (1968), 156th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept, Abstract BIOL 104.

Fenselau, A. (1970), *J. Biol. Chem. 245*, 1239.

Fenselau, A, and Weigel, P. (1970), *Biochim. Biophys. Acta 198*, 192.

Harris, J. I., and Perham, R. N. (1965), *J. Mol. Biol. 13*, 876.

Harris, J. I., and Perham, R. N. (1968), *Nature (London) 219*, 1025.

Hoagland, V. D., Jr., and Teller, D. C. (1969), *Biochemistry 8*, 594.

Johansen, J. T., and Vallee, B. L. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2532.

Kirtley, M. E., and Koshland, D. E., Jr. (1970), *J. Biol. Chem. 245*, 276.

Krimsky, I., and Racker, E. (1963), *Biochemistry 2*, 512.

McConnell, H. M. (1971), *Annu. Rev. Biochem. 40*, 227.

McMurray, C. H., and Trentham, D. R. (1969), *Biochem. J. 115*, 913.

Moore, J., Jr., and Fenselau, A. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1158.

Moore, J., Jr., and Fenselau, A. (1972), *Biochemistry 11*, 3753.

Moore, T. B., and Baker, C. G. (1958), *J. Chromatogr. 1*, 513.

Parker, D. J., and Allison, W. S. (1969), *J. Biol. Chem. 244*, 180.

Pihl, A., and Lange, R. (1962), *J. Biol. Chem. 237*, 1356.

Rafter, G. W. (1957), *Arch. Biochem. Biophys. 67*, 267.

Smyth, D. G., Blumenfeld, O. O., and Konigsberg, W. (1964), *Biochem. J. 91*, 589.

Vas, M., and Boross, L. (1970), *Acta Biochim. Biophys. Acad. Sci. Hung. 5*, 203.

Wassarman, P. M., and Major, J. P. (1969), *Biochemistry 8*, 1076.

Wassarman, P. M., and Watson, H. C. (1970), *Enzymes and Isoenzymes: Struct. Prop. Funct., Fed. Eur. Biochem. Soc. Meet., 5th, 1968*, 51.

Circular Dichroic Studies of the Interaction of Dihydrofolate Reductase with Substrates, Coenzymes, and Inhibitors†

Leo D'Souza and James H. Freisheim*,‡

ABSTRACT: Streptococcal dihydrofolate reductase shows a marked aromatic side-chain Cotton effect in the 260–310 nm region of its circular dichroic spectrum. This effect consists of at least three distinct ellipticity bands with maxima centered at 305, 292, and 270 nm. Interaction of the enzyme with TPNH results in the generation of an extrinsic Cotton effect at *ca.* 340 nm and a decrease in the magnitude of the aromatic side-chain Cotton effect. Titration of the enzyme with TPN^+ results in a sixfold lower decrease in the side-chain Cotton effect of the protein as compared to that observed in the TPNH complex. Binding of dihydrofolate, folate, or amethopterin generates a large enhancement of the molar ellipticity of the protein in the 270–315 region but does not alter the far-ultraviolet dichroic spectrum of the enzyme. The increase in ellipticity is proportional to the extent of complex formed. The maximum total change in molar ellipticity at 290 nm for the dihydrofolate–enzyme complex is +828,000 (deg cm^2) $dmole^{-1}$. This enhancement is, to our knowledge, the largest change in magnitude observed in an enzyme–substrate complex. From enzyme–ligand *vs.* enzyme difference circular dichroic spectra the dihydrofolate–enzyme complex exhibits a single maximum at 290 nm whereas the oxidized compounds, folate and amethopterin, show double maxima in the 290 to 315 nm region. The enhancement of molar ellipticity at 290 nm is 30–40% smaller in the folate and amethopterin complexes as compared to that of the dihydrofolate complex. The binding of amethopterin at pH 5.9 appears to be stoichiometric from circular dichroic titration studies, whereas the affinity of the enzyme for the inhibitor is reduced at pH 7.5. The ellipticity differences observed in the various folate analog complexes are considered to reflect different modes of attachment of these ligands to dihydrofolate reductase. The possible involvement of aromatic amino acid residues in the binding of these folate analog and pyridine nucleotide coenzymes is discussed in terms of a mutually facilitated orientation of the ligand with functional groups on the enzyme.

There have been an increasing number of communications describing Cotton effects and dichroic bands associated with optical activity in the aromatic side chains of proteins (reviewed by Beychok, 1968). The presence of such effects

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provides a useful approach for investigating conformational changes in proteins which are not primarily associated with an alteration in the polypeptide backbone, but rather are due to localized conformational changes at particular sites. In addition the asymmetric binding of small chromophoric molecules to macromolecules which results in the generation of an extrinsic Cotton effect in the absorption bands of the bound chromophores (Ulmer and Vallee, 1965) has proven useful in gaining insight into the nature of ligand–protein interactions.

Streptococcal dihydrofolate reductase which catalyzes the TPNH-dependent reduction of dihydrofolate to tetrahydrofolate shows a marked aromatic side-chain Cotton effect in the 260- to 310-nm region of its circular dichroic (CD) spectrum (Freisheim and D'Souza, 1971b; D'Souza *et al.*, 1972). This effect consists of at least three distinct ellipticity bands with maxima centered at 305, 292, and 270 nm and is qualitatively similar to the near ultraviolet CD spectrum observed for egg white lysozyme (Glazer and Simmons, 1966; Ikeda *et al.*, 1967; Ikeda and Hamaguchi, 1969). In the case of lysozyme, the transitions due to tryptophyl residues contribute most to the magnitude of the aromatic side-chain Cotton effect (Teichberg *et al.*, 1970). Previous chemical modification studies by Freisheim and Huennekens (1969) have indicated the functional importance of tryptophyl residues in the binding of substrates to chicken liver dihydrofolate reductase. The positive ellipticity bands in the 260- to 310-nm region of the CD spectrum of streptococcal dihydrofolate reductase are most probably determined by the aromatic side-chain transitions due to tyrosyl or tryptophyl residues, since the reductase under study does not contain cystine residues (D'Souza *et al.*, 1972).

Complex formation between dihydrofolate reductase and TPNH resulted in the generation of a negative extrinsic Cotton effect at *ca.* 340 nm (Freisheim and D'Souza, 1971b), an absorption maximum of the reduced pyridine nucleotide. Concomitantly, a decrease in the magnitude of the aromatic side-chain Cotton effect was observed. These effects most probably occurred as a result of the induced asymmetry in the coenzyme molecule upon binding to an aromatic amino acid rich asymmetric site on the enzyme. The present communication extends these observations to include the CD spectra of complexes of dihydrofolate reductase with TPN^+, dihydrofolate, folic acid, and amethopterin, a potent inhibitor of the enzyme.[1]

Experimental Section

Dihydrofolate reductase used in these studies was purified, from an amethopterin-resistant strain of *Streptococcus faecium* according to the procedure of D'Souza *et al.* (1972). Dihydrofolate was prepared by the reduction of folic acid (Calbiochem) employing the dithionite method of Futterman (1957) as modified by Blakley (1960). TPNH and TPN^+ were obtained from P-L Biochemicals. Tris (Ultra Pure) was obtained from Schwarz/Mann. Stock solutions of enzyme (2–5 mg/ml) were stored at −20°. Enzyme assays were performed as described by D'Souza *et al.* (1972).

CD spectra were measured at 25° in a Cary Model 60 spectropolarimeter with a Model 6001 CD attachment. Molar ellipticities, $[\theta]$, expressed in units of (deg cm^2) dmole^{-1} were determined from the relationship: $[\theta] = (\theta/10)(M_w/lc)$, where

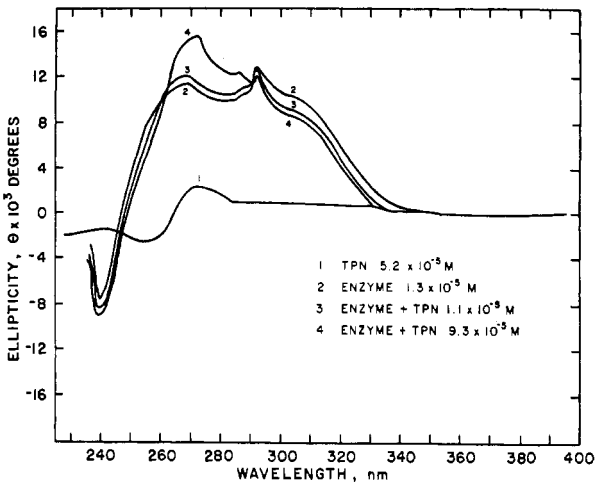

FIGURE 1: CD titration of dihydrofolate reductase with TPN. Increments of TPN were added to a 3-ml solution of enzyme in 0.05 M Tris-HCl (pH 7.5) at the concentrations shown.

θ = the measured ellipticity; M_w = the molecular weight of dihydrofolate reductase; l = cell path length in cm; c = concentration of the enzyme in g ml^{-1}. All calculations of molar ellipticity of dihydrofolate reductase and its complexes are based on a molecular weight of 20,000 for the enzyme (D'Souza *et al.*, 1972).

Dissociation constants for enzyme–substrate, enzyme–coenzyme and enzyme–inhibitor complexes were determined from Hill plots of the data by relating changes in molar ellipticity at a given wavelength to substrate, coenzyme, or inhibitor concentration.

Results

Coenzyme Binding Studies. Results of the CD titration of dihydrofolate reductase with TPN^+, a competitive inhibitor for TPNH, are shown in Figure 1. Addition of successive increments of TPN^+ to the enzyme solution (curves 3 and 4) results in a decrease in the magnitude of the aromatic side-chain Cotton effect. At wavelengths below *ca.* 290 nm the spectrum is complicated by the presence of the negative ellipticity band of TPN^+ at 260 nm (curve 1, Figure 1). This transition is thought to be due to the adenine moiety of TPN (Tinoco, 1970). The binding of the oxidized coenzyme to the reductase does not result in any apparent changes in the 220-nm region (not shown in Figure 1), suggesting that no gross conformational alterations in the polypeptide backbone occur. The greatest change in magnitude in the aromatic region of the CD spectrum upon enzyme–coenzyme complex formation occurred in the 305-nm region. Decreases in ellipticity of the enzyme at 305 nm upon addition of increments of TPN are indicated in Figure 2A. The data were evaluated from Hill plots and a K_D of $(6.4 \pm 0.8) \times 10^{-6}$ M was obtained with one binding side of TPN per mole of enzyme. The difference CD spectrum (Figure 2B) shows a single negative band centered at *ca.* 305 nm with a maximum molar ellipticity $[\theta]_{305}$ of −800 (deg cm^2) dmole^{-1}.

Folate Analog Binding Studies. Complex formation with the substrate, dihydrofolate, results in an increase in the ellipticity values in the aromatic side-chain region of the CD spectrum of dihydrofolate reductase. At low enzyme concentrations where the side-chain Cotton effect is not detected, additions of dihydrofolate result in an increase in the 270- to 300-nm

[1] A preliminary account of this work has been published (Freisheim and D'Souza, 1971a).

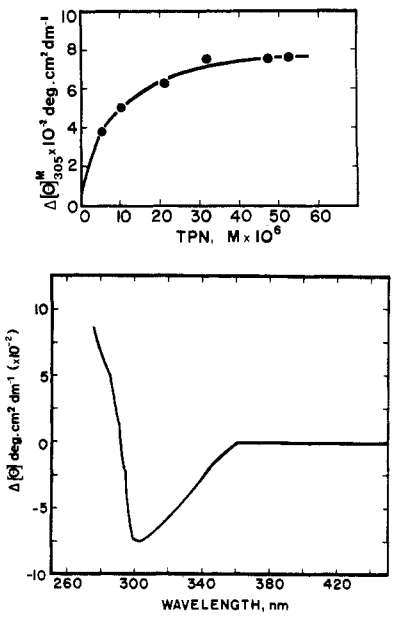

FIGURE 2: (A, top) Changes in the molar ellipticity at 305 nm of dihydrofolate reductase as a function of the concentration of TPN added. The decreases in ellipticity are plotted as $\Delta[\theta]_{305}$ against TPN concentration. (B, bottom) CD difference spectrum of TPN–dihydrofolate reductase complex *vs.* dihydrofolate reductase. The difference in molar ellipticity, $\Delta[\theta]$, obtained by subtraction, is plotted against wavelength. The maximum difference molar ellipticity, $\Delta[\theta]_{305}$, is -800 (deg cm²) dmole^{-1}.

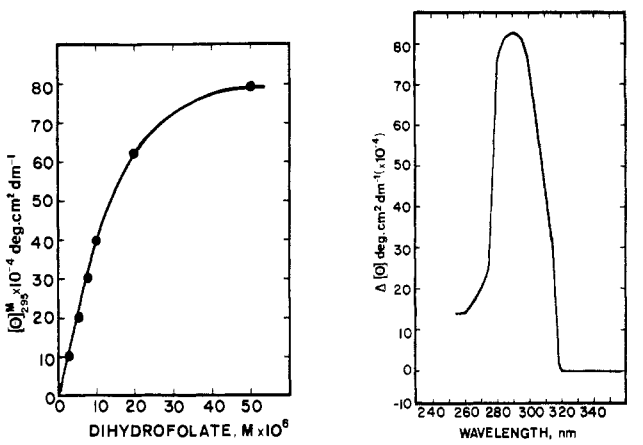

FIGURE 4: (A, left) Changes in the molar ellipticity at 295 nm upon dihydrofolate–dihydrofolate reductase complex formation. The increases in molar ellipticity, $[\theta]_{295}$, are plotted against dihydrofolate concentration. (B, right) CD difference spectrum of dihydrofolate–dihydrofolate reductase complex *vs.* dihydrofolate reductase. The difference in molar ellipticity, $\Delta[\theta]$, obtained by subtraction, is plotted against wavelength. The maximum difference molar ellipticity, $\Delta[\theta]_{290}$, is $+828{,}000$ (deg cm²) dmole^{-1}.

region with a maximum centered at *ca.* 290 nm (Figure 3). The enhancement in this spectral region generated by substrate binding increases in proportion to the extent of complex formed. The increases in molar ellipticity at 290 nm, due to complex formation upon addition of increments of dihydrofolate to the enzyme, are shown in Figure 4A. A K_D of $(8.6 \pm 1.1) \times 10^{-6}$ M for dihydrofolate was calculated from the data from Hill plots with a single binding site for the substrate. This value is in fairly good agreement with the K_M of the enzyme for dihydrofolate of 7×10^{-6} M (D'Souza *et al.*, 1972). The difference circular dichroic spectrum (Figure 4B) shows a single positive band centered at 290 nm with a maximum molar ellipticity $[\theta]_{290}$ of $+828{,}000$ (deg cm²) dmole^{-1}.

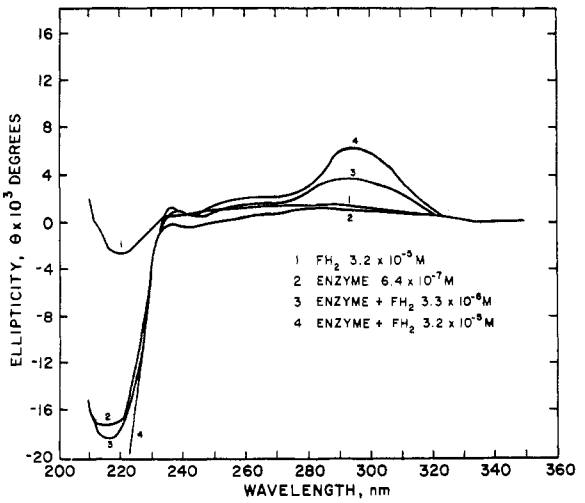

FIGURE 3: CD titration of dihydrofolate reductase with dihydrofolate. The spectra were taken using 10-mm cells containing 3.0 ml of the indicated solutions (in 0.05 M Tris-HCl, pH 7.5). Dihydrofolate was added to enzyme solutions at the concentrations indicated.

Since dihydrofolate absorbs maximally at 282 nm (Blakley, 1960), it would be tempting to interpret the observed effects in this spectral region solely as a result of an enhanced absorption of the substrate due to its asymmetric binding on the enzyme (*i.e.*, an extrinsic Cotton effect). However, since the enzyme also displays a Cotton effect in the 260- to 310-nm region, one could conclude that the observed ellipticity enhancement may be due to a *mutually facilitated* orientation of dihydrofolate with one or more of the aromatic side-chain residues upon complex formation. An inseparable consequence of the binding of dihydrofolate may be to effect an enhancement of the ellipticity of the side-chain Cotton effect by inducing a localized conformational change in one or more of the aromatic amino acid residues of the enzyme.

The negative Cotton effect at *ca.* 220 nm, due to dehydrofolate alone (curve 1, Figure 3), is presumably due to the peptide bond involving the L-glutamate and *p*-aminobenzoyl moieties. When increasing concentrations of dihydrofolate are added to the enzyme a decrease in ellipticity in the 220-nm region is observed. These effects are, however, due entirely to the effect of the additive contributions of the substrate in this spectral region and no changes in the intrinsic Cotton effect of the protein at 220 nm occur upon complex formation with dihydrofolate.

The binding of folic acid to dihydrofolate reductase also resulted in changes in the near-ultraviolet CD spectrum of dihydrofolate reductase. The total enhancement of the ellipticity values in the 270- to 350-nm region was, however, less than that observed for the enzyme–dihydrofolate complex (Figure 5A). Since folate is a competitive inhibitor for the dihydrofolate binding site on the enzyme, it is not unexpected that the effect of folate binding to the enzyme results in changes in the same region of the CD spectrum as occurs upon substrate binding. The difference CD spectrum (Figure 5B), however, reveals changes in $[\theta]$ which are both qualitatively and quantitatively distinct from that observed for the dihydro-

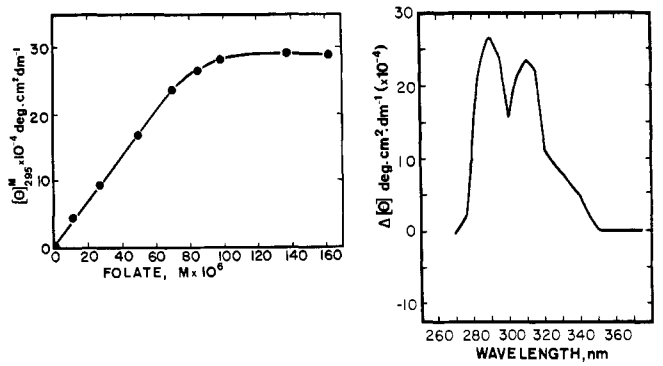

FIGURE 5: (A, left) Increases in molar ellipticity at 295 nm upon formation of the folate–dihydrofolate reductase complex. The enzyme concentration is 6.4×10^{-7} M. (B, right) CD difference spectrum of folate–dihydrofolate reductase complex *vs.* dihydrofolate reductase. The difference in molar ellipticity, $\Delta[\theta]$, obtained by subtraction, is plotted against wavelength. The two maxima in difference molar ellipticity shown are $\Delta[\theta]_{290} = +265{,}000$ (deg cm²) dmole^{-1} and $\Delta[\theta]_{310} = +235{,}000$ (deg cm²) dmole^{-1}.

folate complex. Maxima at 290 and 310 nm as well as a shoulder between 320 and 350 nm are apparent from the difference spectrum. The maximum molar ellipticity observed in the folate–enzyme complex at 290 nm of +280,000 (deg cm²) dmole^{-1} is about 32% of that observed for the dihydrofolate complex at the same wavelength (*vide supra*).

Amethopterin is a potent inhibitor of dihydrofolate reductase. At neutral pH values the affinity of the enzyme for the inhibitor is less than that at more acid pH values; in fact, at pH values of 6 and below, amethopterin is a "stoichiometric" inhibitor of dihydrofolate reductase (Bertino *et al.*, 1964; Werkheiser, 1961). The observation that this inhibitor interacts with the enzyme on an equimolar basis at low pH has led to the use of this agent as an active-site titrant in the determination of molecular weights of purified preparations of the reductase (reviewed by Blakley, 1969, and by Huennekens *et al.*, 1972). The binding of amethopterin to dihydrofolate reductase results in increased ellipticity values in the 270- to 320-nm region of the CD spectrum as was observed in the dihydrofolate and folate complexes. The difference CD spectra for the amethopterin–enzyme complex at pH 7.5 and 5.9

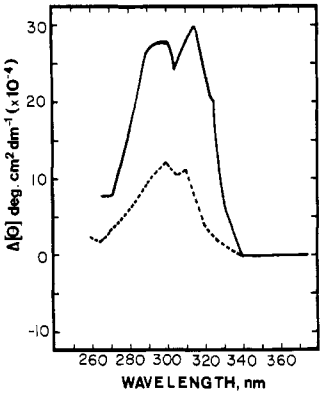

FIGURE 6: CD difference spectra of amethopterin–dihydrofolate reductase *vs.* dihydrofolate reductase complexes at pH 7.5 (——) and at pH 5.9 (- - - - -). At pH 7.5 the two maxima in difference molar ellipticity are $\Delta[\theta]_{300} = +280{,}000$ (deg cm²) dmole^{-1} and $\Delta[\theta]_{315} = 296{,}000$ (deg cm²) dmole^{-1}. The corresponding maxima at pH 5.9 are $\Delta[\theta]_{300} = +120{,}000$ (deg cm²) dmole^{-1} and $\Delta[\theta]_{310} = +118{,}000$ (deg cm²) dmole^{-1}.

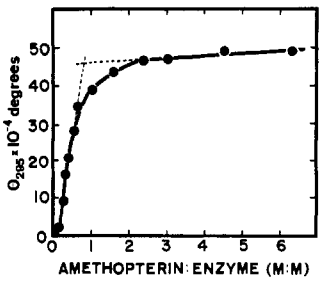

FIGURE 7: CD titration of dihydrofolate reductase with amethopterin at pH 5.9. Increments of amethopterin were added to solutions of enzyme (1.05×10^{-5} M) in 0.05 M KPO_4 (pH 5.9). The extrapolated linear portions of the titration curve intersect at 0.9 mole of amethopterin/mole of enzyme.

are indicated in Figure 6. As was observed in the folate–enzyme complex two maxima for amethopterin binding are evident at both pH values. At pH 7.5 the maxima occur at 300 and 315 nm, while at pH 5.9 the maxima occur at 330 and 310 nm. At pH 7.5 the maximum $\Delta[\theta]_{300}$ in the amethopterin complex is +280,000 (deg cm²) dmole^{-1}, approximately the same as that for the $\Delta[\theta]_{290}$ observed in the folate complex. However, the $\Delta[\theta]_{300}$ at 5.9 for the amethopterin complex is only 43% of that observed at the higher pH. The latter effect may result from a localized conformational change in one or more of the aromatic amino acid residues as the pH is decreased. Lowering the pH from 7.5 to 5.9 increases the affinity of the enzyme for the inhibitor. At pH 7.5 the K_D for the amethopterin–enzyme complex is $(1.0 \pm 0.2) \times 10^{-6}$ M, whereas the binding of the inhibitor at pH 5.9 is stoichiometric (Figure 7).

The dissociation constants (K_D values) for the coenzyme, substrate, and inhibitor complexes of dihydrofolate reductase calculated from Hill plots of the respective data are summarized in Table I. The K_D values were calculated at 305 nm

TABLE I: Dissociation Constants of Pyridine Nucleotide Coenzyme and Folate Analog Complexes of Dihydrofolate Reductase.

	Enzyme–X ⇌ Enzyme + X		
X[a]	K_D (M)	$\Delta[\theta]_\lambda$[b] (deg cm² dmole^{-1})	λ (nm)
TPNH[c]	$(4.0 \pm 0.6) \times 10^{-7}$	4,700	305
TPN^+	$(6.4 \pm 0.8) \times 10^{-6}$	800	305
Dihydrofolate	$(8.6 \pm 1.1) \times 10^{-6}$	828,000	290
Folate	$(3.8 \pm 0.4) \times 10^{-5}$	265,000	290
		235,000	310
Amethopterin	$(1.0 \pm 0.2) \times 10^{-6}$	280,000	300
		296,000	315
Amethopterin[d]		120,000	300
		118,000	310

[a] Except where noted the buffer employed in each of these studies was 0.05 M Tris-HCl (pH 7.5) containing between 6.4×10^{-7} and 1.3×10^{-5} M dihydrofolate reductase. Measurements were made in cells of 1.0-cm path length. [b] Maxima of circular dichroic difference spectra of various dihydrofolate reductase–ligand complexes *vs.* dihydrofolate reductase in the same buffer. [c] Data of Freisheim and D'Souza, 1971b. [d] Samples in 0.05 M potassium phosphate (pH 5.9).

for TPN^+ and TPNH complexes and at 295 nm for folate analog complexes; the values for each ligand were determined over an enzyme concentration range of 6.4×10^{-7} M to 1.3×10^{-5} M. The affinity of TPN^+ for the enzyme is *ca.* two orders of magnitude less than that of the TPNH complex and the maximum change in $[\theta]_{305}$ is only 17% of that which occurs in the TPNH–enzyme complex. Dihydrofolate is bound much more tightly than folate, which contains the fully aromatic pteridine ring, whereas the amethopterin complex exhibits a lesser degree of dissociation than in either the folate or dihydrofolate complexes at the same pH. The molar ellipticity increase at 290 nm was the greatest for the dihydrofolate complex. The enhancement in molar ellipticity at 290 nm was *ca.* 32% in the folate complex and 43% in the amethopterin complex at pH 7.5 of that observed in the dihydrofolate complex.

Discussion

Interaction of dihydrofolate reductase with TPN^+, a competitive inhibitor for the TPNH binding site, results in a sixfold lower decrease in the region of the aromatic side-chain Cotton effect than that which occurs in the TPNH complex (*cf.* Table I). Such variations in the magnitude of the respective Cotton effects may be attributed to steric differences in the mode of binding of the oxidized and reduced forms of the pyridine nucleotide coenzymes.

The binding of dihydrofolate to the reductase produces a maximum change in molar ellipticity at 290 nm of +828,000 (deg cm^2) $dmole^{-1}$ (Table I). This extremely large positive effect is clearly dependent on complex formation with the substrate, but occurs in a region in which the protein displays an aromatic side-chain Cotton effect. One might, therefore, conclude that this rather large enhancement in molar ellipticity results from a *mutually oriented* asymmetric stacking of the reduced pteridine ring moiety of the substrate on one or more of the aromatic amino acid side chains of the protein.

By contrast, the lower molar ellipticities observed in the aromatic side-chain region upon folate or amethopterin binding (Table I) might suggest a somewhat different mode of binding of these oxidized compounds. The presence of two maxima in the difference CD spectrum of the folate complex (Figure 5B) and a single maximum in the dihydrofolate complex (Figure 4B) further suggests a difference in the mode of binding in the respective complexes.

The binding of amethopterin to the enzyme at pH 7.5 results in a difference CD spectrum (Figure 6) qualitatively similar to that observed in the folate complex. The affinity of the reductase for amethopterin is greater than that for folate by one order of magnitude, but the total enhancements in molar ellipticity in the 290- to 310-nm region are nearly identical (Table I). At pH 5.9 the binding of amethopterin to the streptococcal reductase appears to be "stoichiometric" from inhibition studies (D'Souza *et al.*, 1972). At the same pH the enhancement of molar ellipticity in the 290- to 315-nm region is less than one-half that observed at pH 7.5 (Figure 6) although the affinity of the enzyme for the inhibitor is greater at the lower pH.

Although the physical basis for the observed qualitative and quantitative changes in these CD spectral data remains to be elucidated, the results strongly suggest the involvement of aromatic amino acid residues in the binding of these ligands. Chemical modification studies by Freisheim and Huennekens (1969) and more recently in this laboratory (Warwick *et al.*, 1972) have indicated the involvement of tryptophan residues in the catalytic function of dihydrofolate reductase. The available evidence from these chemical modification and fluorescence[2] studies suggests that tryptophan residues play an important role in the binding of substrates, coenzymes, and inhibitors to dihydrofolate reductase.

Acknowledgments

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References

Bertino, J. R., Booth, B. A., Beiber, A. L., Cashmore, A., and Sartorelli, A. C. (1964), *J. Biol. Chem. 239*, 479.

Beychok, S. (1968), *Annu. Rev. Biochem. 37*, 437.

Blakley, R. L. (1960), *Nature (London) 188*, 231.

Blakley, R. L. (1969), *Biochem. Folic Acid Relat. Pteridines 13*, 464.

D'Souza, L., Warwick, P. E., and Freisheim, J. H. (1972), *Biochemistry 11*, 1528.

Freisheim, J. H., and Huennekens, F. M. (1969), *Biochemistry 8*, 2271.

Freisheim, J. H., and D'Souza, L. (1971a), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 870.

Freisheim, J. H., and D'Souza, L. (1971b), *Biochem. Biophys. Res. Commun. 45*, 803.

Futterman, S. (1957), *J. Biol. Chem. 228*, 1031.

Glazer, A. N., and Simmons, N. S. (1966), *J. Amer. Chem. Soc. 88*, 2335.

Huennekens, F. M., Dunlap, R. B., Freisheim, J. H., Gunderson, L. E., Harding, N. G. L., Levison, S. A., and Mell, G. P. (1972), *Ann. N. Y. Acad. Sci. 186*, 85.

Ikeda, K., Hamaguchi, K., Imanishi, M., and Amano, T. (1967), *J. Biochem. (Tokyo) 62*, 315.

Ikeda, K., and Hamaguchi, K. (1969), *J. Biochem. (Tokyo) 66*, 513.

Teichberg, V. I., Kay, C. M., and Sharon, N. (1970), *Eur. J. Biochem. 16*, 55.

Tinoco, I., Jr. (1970), *Methods Biochem. Anal. 18*, 81.

Ulmer, D. D., and Vallee, B. L. (1965), *Advan. Enzymol. 27*, 37.

Warwick, P. E., D'Souza, L., and Freisheim, J. H. (1972), *Biochemistry 11*, 3775.

Werkheiser, W. C. (1961), *J. Biol. Chem. 236*, 888.

[2] Unpublished observations from this laboratory.