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CIRCULAR DICHROISM STUDIES OF DIHYDROFOLATE REDUCTASE FROM A METHOTREXATE-RESISTANT STRAIN OF *ESCHERICHIA COLI* B, MB 1428: TERNARY COMPLEXES

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Summary

Circular dichroism has been used to monitor the binding of pyridine nucleotide cofactors to enzyme-folate analog complexes of dihydrofolate reductase from *Escherichia coli* B (MB 1428). The enzyme binds one molar equivalent of many folate analogs and two molar equivalents of several pyridine nucleotide cofactors. The apo-enzyme has very low optical activity. The binding of folate analogs including folate, dihydrofolate, methotrexate, trimethoprim and pyrimethamine induce large Cotton effects. Pyridine nucleotides when bound to the enzyme-folate analog complexes also induce new optically active bands; all the effects being due to the first molar equivalent of cofactor bound. NADPH and $NADP^+$ induce very similar bands when bound to the enzyme-methotrexate complex suggesting that the geometry of the complexes formed are very similar. The oxidized and reduced cofactor likewise have similar effects on the enzyme-folate complex. However, NADPH and $NADP^+$ addition to both the enzyme-trimethoprim and enzyme-pyrimethamine complexes have significantly different effects on the circular dichroism spectra, suggesting that the inhibitors which are less homologous to the natural dihydrofolate substrate allow more conformational freedom in the enzyme-inhibitor-cofactor complex. In most cases the prior binding of the folate analog greatly increases the binding of the first molar equivalent of cofactor so that at concentrations of approx. 5—20 μM the binding appears stoichiometric. Pyrimethamine is an exception in that it apparently has no effect on the binding of NADPH to the enzyme.

Introduction

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase,

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Abbreviations: Ado-*PP*-Rib, adenosine-diphosphoriboside.

EC 1.5.1.3) is the target of several clinically useful drugs. Inhibitors of the enzyme include methotrexate (amethopterin), an antineoplastic agent; pyrimethamine, an antimalarial compound; and trimethoprim, an antibiotic. The latter two compounds owe their effectiveness to the fact that they inhibit the enzyme from plasmodial and bacterial sources more strongly than they inhibit the enzyme from mammalian sources, i.e. that they are species specific inhibitors [1].

In a previous publication [2] we described the circular dichroism (CD) of dihydrofolate reductase from a methotrexate-resistant strain of *Escherichia coli* B (MB 1428). The enzyme from *E. coli* has very low ellipticity between 250 and 400 nm. The binding of folate, dihydrofolate and methotrexate all induce large extrinsic circular dichroism bands which have been used to determine the dissociation constants of the enzyme-ligand complexes. The dissociation constant for the enzyme-dihydrofolate complex is approximately 1 μM, and for the enzyme-folate complex is about 3 μM. Methotrexate binds to the enzyme very tightly and the binding constant cannot be determined from the CD data.

NADPH addition to the enzyme in the absence of folate analogs has very little effect on the CD of the enzyme [2]. When NADPH is bound to the enzyme-methotrexate complex, however, new bands are generated. The appearance of the new bands are linear with NADPH addition to the complex and saturate at an apparent 1 : 1 addition of NADPH to enzyme-methotrexate. It was speculated from this CD titration that the enzyme-methotrexate complex has one NADPH binding site [2]. Fluorescence spectroscopy [3] and equilibrium ultrafiltration measurements [3] subsequently demonstrated that the enzyme has two non-equivalent NADPH binding sites both in the presence and absence of methotrexate. In the presence of methotrexate the binding of the first molar equivalent of NADPH is much tighter than the second and is responsible for all the effects of NADPH on the circular dichroism and fluorescence properties of the enzyme-methotrexate complex.

Ultraviolet difference spectroscopy [4] and equilibrium ultrafiltration [4] subsequently demonstrated that the enzyme has two NADH and $NADP^+$ binding sites as well. In all cases the major effect on the spectrum of the enzyme and enzyme-methotrexate complex was attributed to the binding of one molar equivalent of pyridine nucleotide cofactor at the tighter binding site. The binding of methotrexate to the enzyme greatly increases the affinity of the enzyme for NADH and $NADP^+$ as well as NADPH [4].

In this work the circular dichroism studies have been extended. First, the effects of several pyridine nucleotides on the enzyme-methotrexate complex have been examined. Second, the ternary complexes of the enzyme with pyridine nucleotides and folate have been studied. Finally, the interaction of the enzyme with trimethoprim and pyrimethamine and the effects of NADPH and $NADP^+$ on the spectra of these inhibitor-enzyme complexes have been monitored.

Materials and Methods

NADPH, NADP, 3-acetylpyridine-NAD^+, nicotinamide mononucleotide (NMN^+), adenosine-diphosphoribose (Ado-*PP*-Rib) and NADH were purchased

from P-L Biochemicals Inc. Folic acid was purchased from Cyclo Chemical Corp. and methotrexate from Nutritional Biochemical Corp. Trimethoprim and pyrimethamine were obtained from the Merck and Co. Inc. sample collection.

Dihydrofolate was prepared from commercial folic acid by the dithionite method of Futterman [5] as modified by Blakley [6] and was stored at −20° C in 5 mM HCl and 50 mM 2-mercaptoethanol.

Dihydrofolate reductase was prepared from *E. coli* B (strain MB 1428) as described by Poe et al. [7] and modified by Williams et al. [8]. For CD measurements the enzyme was either dialyzed exhaustively against 0.05 M Tris · HCl, 0.05 M NaCl, pH 7.2, or 2 mg of enzyme were exchanged into the above buffer by passing the enzyme solution through a 2 × 15 cm Sephadex G-25M column equilibrated with the buffer. Enzyme solutions were concentrated as described previously [2].

The concentrations of NADPH, methotrexate, folate and dihydrofolate were determined as described previously [2]. The concentration of NADH, NADP, NMN^+, Ado-*PP*-Rib and 3-acetylpyridine-$NADP^+$ were determined spectrophotometrically. The following extinction coefficients were used: NADH ϵ_{340nm} = 6220 $cm^{-1} \cdot M^{-1}$ [9], $NADP^+$ ϵ_{260nm} = 18 000 $cm^{-1} \cdot M^{-1}$ [10], NMN^+ ϵ_{266nm} = 4600 $cm^{-1} \cdot M^{-1}$ [11], Ado-*PP*-Rib ϵ_{259nm} = 15 400 $cm^{-1} \cdot M^{-1}$ [12] and 3-acetylpyridine-$NADP^+$ ϵ_{260nm} = 16 400 $cm^{-1} \cdot M^{-1}$ [13]. At pH 7.2 the extinction of trimethoprim was ϵ_{278nm} = 5800 $cm^{-1} \cdot M^{-1}$ and pyrimethamine was ϵ_{278nm} = 8200 $cm^{-1} \cdot M^{-1}$. Trimethoprim and pyrimethamine were determined gravimetrically.

The enzyme concentration was determined using the extinction coefficient at 280 nm of 40 000 $cm^{-1} \cdot M^{-1}$ [2].

Enzyme solutions were assayed as described by Poe et al. [7] in 0.05 M Tris · HCl, 0.05 M NaCl, pH 7.2, in the presence of 10^{-3} M dithiothreitol. The standard assay concentrations were 100 μM for both NADPH and dihydrofolate.

Enzyme inhibition studies were performed in two different fashions. In the first method the NADPH and dihydrofolate concentrations were kept constant and the inhibitor concentration was varied. Several fixed concentrations of dihydrofolate and NADPH ranging from 50 to 250 μM were used in each set of assays. In the second method the inhibitor concentrations were held constant and either the NADPH or dihydrofolate concentrations were varied. In both methods the effects of preincubation of the enzyme with substrates and inhibitors were tested. First the substrate, cofactor and inhibitor were mixed and the assay was initiated with enzyme. Second the enzyme was preincubated for 5 min with the inhibitor and cofactor and the assay was initiated with the substrate. The data were analyzed as described by Williams et al. [8].

CD measurements were obtained with a Cary 60 recording spectropolarimeter equipped with a Model 6001 CD attachment and set for a half band width of 1.5 nm. The measurements were made at the ambient temperature of 27° C. The measurements of ellipticity between 250 and 400 nm of the free enzyme and the enzyme ligand complexes were made in cells with 1-cm lightpaths at enzyme concentrations ranging from $0.5 \cdot 10^{-5}$ to $4 \cdot 10^{-5}$ M. The ellipticity of the enzyme and enzyme-ligand complexes are reported as molecular (molar) ellipticities [Θ] in degrees · cm^2 · $dmol^{-1}$ where:

$$[\Theta] = 100 \times \Theta_{observed}/\text{lightpath (cm)} \times \text{concentration (molar)}$$

The spectra were all corrected for the contribution of free ligand.

Equilibrium ultrafiltration measurements were performed as described by Williams et al. [3].

The changes in ellipticity of the enzyme upon ligand binding were used to estimate dissociation constants. For one ligand binding site, Scatchard [14] plots and the "difference method" of Kurganov et al. [15] as adapted for CD analyses [2] were used. The method of Williams et al. [3] was used to analyze the data in the cases where two molar equivalents of ligand bind to the enzyme.

Results

Circular dichroism measurements

(1) *Methotrexate complexes.* Table I shows the number of molar equivalents of various pyridine nucleotide cofactors bound to the enzyme-methotrexate complex at the concentrations used for the CD experiments.

NADPH, NADH and NADP$^+$. In agreement with results obtained with NADPH [2], the cofactors enumerated in Table I when added to dihydrofolate reductase generate virtually no changes in the CD spectrum of the folate free enzyme.

When the enzyme-methotrexate complex is titrated with NADH spectral changes identical to those generated by NADPH addition occur. At saturation the spectrum shown in Fig. 1 is the same in shape and magnitude as the spectrum of the enzyme-methotrexate-NADPH complex reported previously [2]. The binding of NADH is much weaker, however, and the titration curve shows considerable curvature. The titration, monitored by the difference in ellipticity between 365 and 330 nm, is shown in Fig. 2. When the data are analyzed either by the difference method of Kurganov et al. [15] or Scatchard [14] plots the titration curve can be analyzed as if all the spectral perturbations are due to NADH binding at one site with a K_d = 9.3 μM. The equilibrium ultrafiltration

TABLE I

THE BINDING OF PYRIDINE NUCLEOTIDE COFACTORS BY THE COMPLEX OF DIHYDROFOLATE REDUCTASE WITH METHOTREXATE IN 0.05 M TRIS · HCl, 0.05 M NaCl, pH 7.2 AT 25°C

The concentration of free ligand was determined spectrophotometrically from the ultrafiltrate.

Ligand	Enzyme-methotrexate concentration (μM)	Total ligand: enzyme-methotrexate	Free ligand concentration (μM)	Bound ligand: enzyme-methotrexate
NADPH	11.5	5.9 : 1	45.8	1.9 ± 0.2
NADP$^+$	11.5	5.1 : 1	40.3	1.8 ± 0.2
NADH	11.5	5.1 : 1	41.8	1.5 ± 0.2
NMN$^+$	13.8	5.9 : 1	67.4	0.96 ± 0.1
		11.5 : 1	143.5	1.2 ± 0.2
Ado-*PP*-Rib	13.8	1.4 : 1	13.7	0.38 ± 0.1
		4.3 : 1	57.5	0.65 ± 0.2
3-Acetylpyridine-NADP$^+$	16.0	3.8 : 1	38.2	1.3 ± 0.2

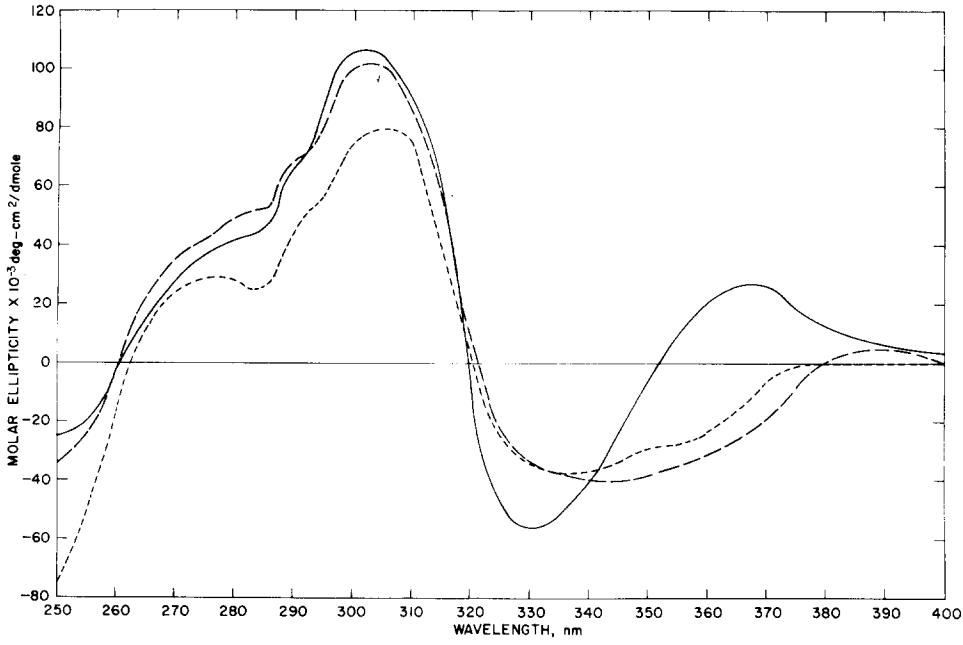

Fig. 1. The circular dichroism of dihydrofolate reductase complexes with methotrexate in 0.05 M Tris · HCl, 0.05 M NaCl, pH 7.2, at 27° C. The spectra are the composites of several sets of data taken at enzyme concentrations ranging from 10 to 20 μM. The enzyme was first titrated with inhibitor until there were no spectral changes and then titrated with cofactor until there were no spectral changes. The spectra are corrected for the contributions of free ligands. - - - - - -, enzyme-methotrexate; ———, enzyme-methotrexate-NADH; — — —, enzyme-methotrexate-$NADP^+$.

measurements show that the enzyme-methotrexate complex has multiple NADH binding sites, however. If the circular dichroism data are analyzed assuming that the complex has two NADH binding sites, with all of the induced CD spectrum due to the first site, as described in Williams et al. [3], the first site has a dissociation constant of approximately 9 μM and the second site is weaker, with a K_d of approx. 100 μM in agreement with the spectral titrations of Poe et al. [4]. The theoretical binding curve, assuming all the CD changes are due to the first molar equivalent of NADH bound, for K_{d1} = 9 μM and K_{d2} = 100 μM is also shown in Fig. 2.

When the enzyme-methotrexate complex is titrated with $NADP^+$ spectral changes very similar to those induced by NADPH binding are observed below 320 nm, while $NADP^+$ binding has very little effect on the CD spectrum above 320 nm. These spectral changes are also shown in Fig. 1. As in the case of NADPH and NADH all the spectral perturbations are associated with the binding of the first molar equivalent of $NADP^+$. At an enzyme concentration of 18 μM the binding of the first molar equivalent of $NADP^+$ to the enzyme-methotrexate complex binds very tightly and saturates at a 1 : 1 ratio, and additional $NADP^+$ does not perturb the enzyme's CD spectrum even though equilibrium ultrafiltration results (see Table I) show that two molar equivalents of $NADP^+$ bind to the enzyme at concentrations comparable to those used for the CD measurements.

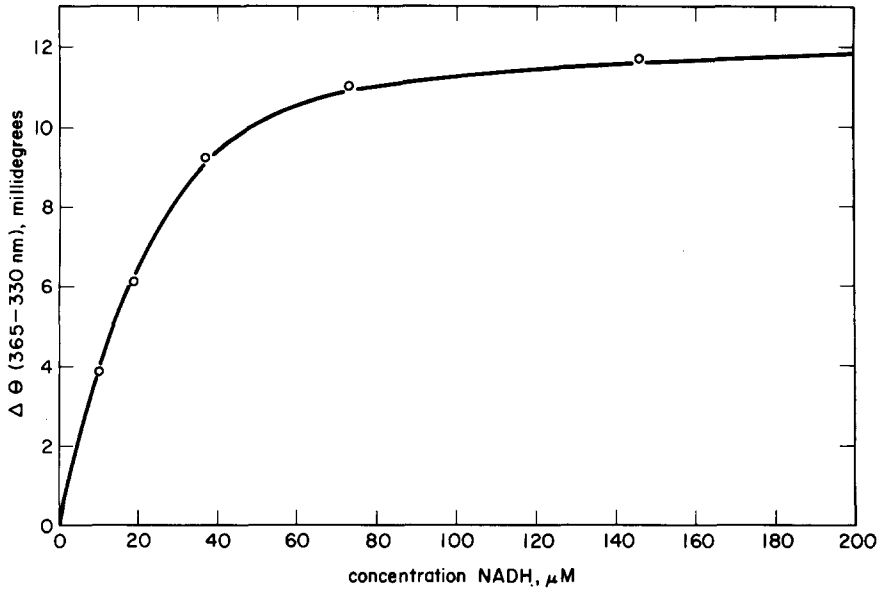

Fig. 2. Titration of the complex of dihydrofolate reductase and methotrexate with NADH as measured from the difference in the induced circular dichroism between 365 and 330 nm. The solid line is the theoretical curve for K_{d1} = 9 μM and K_{d2} = 100 μM. The enzyme concentration is 18 μM.

Ado-PP-Rib, NMN$^+$ and 3-acetylpyridine-NADP$^+$. In contrast to the effects of NADPH, NADP and NADH the other pyridine nucleotides listed in Table I have no apparent effect on the circular dichroism spectrum of the enzyme-methotrexate complex even though ultrafiltration experiments demonstrate binding at the concentration range used in the CD measurements.

Methotrexate binds to the enzyme stoichiometrically at concentrations of 5—40 μM. Thus the effect of prior binding of coenzymes to the enzyme upon the binding strength of methotrexate cannot be determined from the CD measurements.

(2) *Folate complexes.* The circular dichroism spectrum of the complex of dihydrofolate reductase with folate has been reported in 0.1 M NaCl at pH 7.2 previously [2]. The spectrum of the enzyme-folate complex in 0.05 M Tris · HCl, 0.05 M NaCl, pH 7.2 is substantially the same and is shown in Fig. 3. When one molar equivalent of NADPH is added to the enzyme-folate complex the spectrum is perturbed slightly. The spectral peak at 292 nm is shifted to 295 nm but there are no other changes. Further additions of NADPH have no effect on the CD spectrum of the complex. In a similar manner when NADP$^+$ is added to the enzyme-folate complex the spectrum of the peak at 292 nm is also shifted to 295 nm. Both the positive band at 295 nm and the negative band at 272 nm are increased slightly in magnitude relative to the enzyme-folate spectrum. Once again the spectral changes saturate with the addition of one molar equivalent of NADP$^+$. Thus in the presence of folate the binding constant for the first molar equivalent of NADPH and NADP$^+$ becomes too tight to measure from CD spectral analysis. The spectra of the enzyme-folate-NADPH and -NADP$^+$ complexes are shown in Fig. 3.

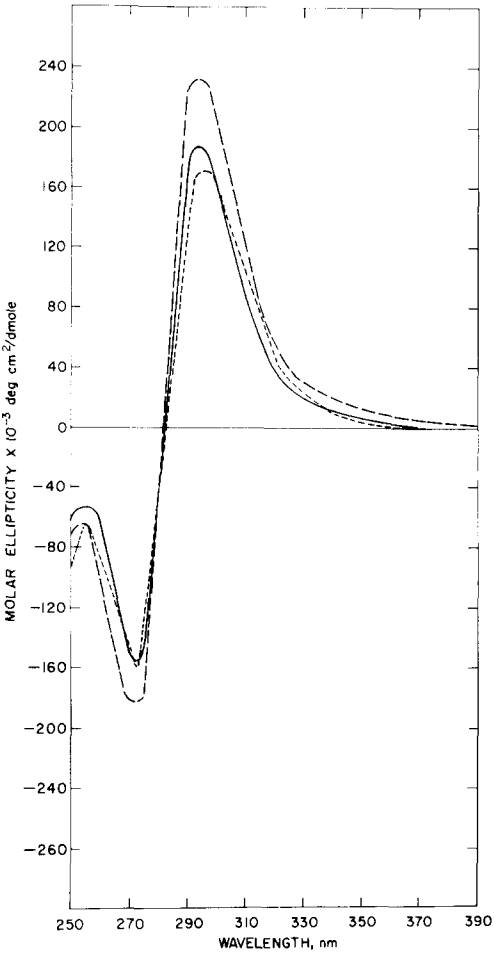

Fig. 3. The circular dichroism of dihydrofolate reductase complexes with folate. For conditions see Fig. 1. ———, enzyme-folate; - - - - - -, enzyme-folate-NADPH; — — —, enzyme-folate-$NADP^+$.

TABLE II

THE DISSOCIATION CONSTANTS OF DIHYDROFOLATE REDUCTASE-FOLATE COMPLEXES IN THE PRESENCE AND ABSENCE OF PYRIDINE NUCLEOTIDE COFACTORS

The dissociation constants were determined from circular dichroism data using the methods of Kurganov et al. [15] and Scatchard [14]. The ratio cofactor: enzyme is the total molar ratio of cofactor added to enzyme.

Cofactor	Ratio of cofactor to enzyme	Dissociation constant
None	—	2.8 ± 0.4
NADPH	1 : 1	2.2 ± 1.1
	3 : 1	2.3 ± 1.8
$NADP^+$	1 : 1	0.5 ± 0.4
	3 : 1	0.6 ± 0.2
	4 : 1	0.5 ± 0.1

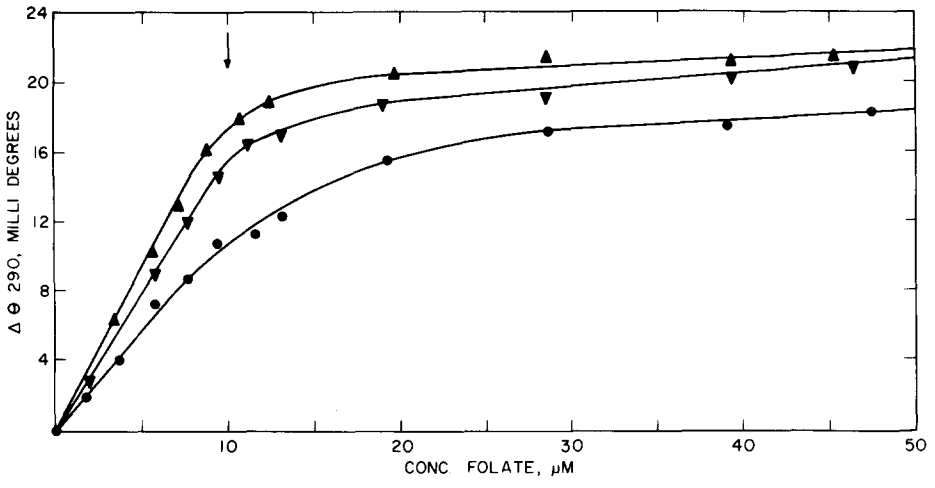

Fig. 4. The titration of dihydrofolate reductase with folate in the presence and absence of $NADP^+$ as measured from changes in the circular dichroism band at 290 nm. ●———●, no $NADP^+$; ▼———▼, 1 : 1 $NADP^+$; ▲———▲, 4 : 1 $NADP^+$. The arrow shows the enzyme concentration.

The apparent dissociation constants for the enzyme-folate complexes in the absence and presence of NADPH and $NADP^+$ are summarized in Table II. In the presence of a 1—4-fold equivalence of $NADP^+$ the dissociation constant of folate seems to decrease from 2.8 to 0.5 μM as determined from both the difference method of Kurganov et al. [15] and Scatchard [14] plots. This figure, however, is only an apparent dissociation constant. In the absence of folate analogs the binding of $NADP^+$ to the enzyme is quite weak [4] and thus only a part of the enzyme binds $NADP^+$ when a 1 : 1 addition of cofactor to enzyme is made. The equilibrium between the enzyme, folate and $NADP^+$ is therefore quite complicated. The titration curves of the addition of folate to dihydrofolate reductase in the presence and absence of NADP are shown in Fig. 4.

(3) *Dihydrofolate complexes.* The spectrum of the enzyme-dihydrofolate complex and its dissociation constant in 0.05 M Tris · HCl, 0.05 M NaCl, pH 7.2, are almost identical to those reported previously in 0.1 M NaCl, pH 7.2 [2]. It was attempted to study the effect of $NADP^+$ on the binding of dihydrofolate to the enzyme. Unfortunately the enzyme catalyzes the oxidative cleavage of dihydrofolate [16]. In the presence of $NADP^+$, dihydrofolate breakdown is accelerated. The spectrum of the enzyme-dihydrofolate-$NADP^+$ complex is not stable within the time course of a spectrometer scan, thus no analysis of the data has been attempted.

(4) *Trimethoprim complexes.* The spectrum of dihydrofolate reductase for *E. coli* MB 1428, and its complexes with trimethoprim, trimethoprim and NADPH, and trimethoprim and $NADP^+$ are shown in Fig. 5.

At pH 7.2 trimethoprim has an absorption maxima at 278 nm. When dihydrofolate reductase is titrated with trimethoprim positive CD bands at approximately 283 and 268 nm appear. The CD spectral changes saturate at a 1 : 1 addition of trimethoprim to the enzyme. Trimethoprim has no effect on

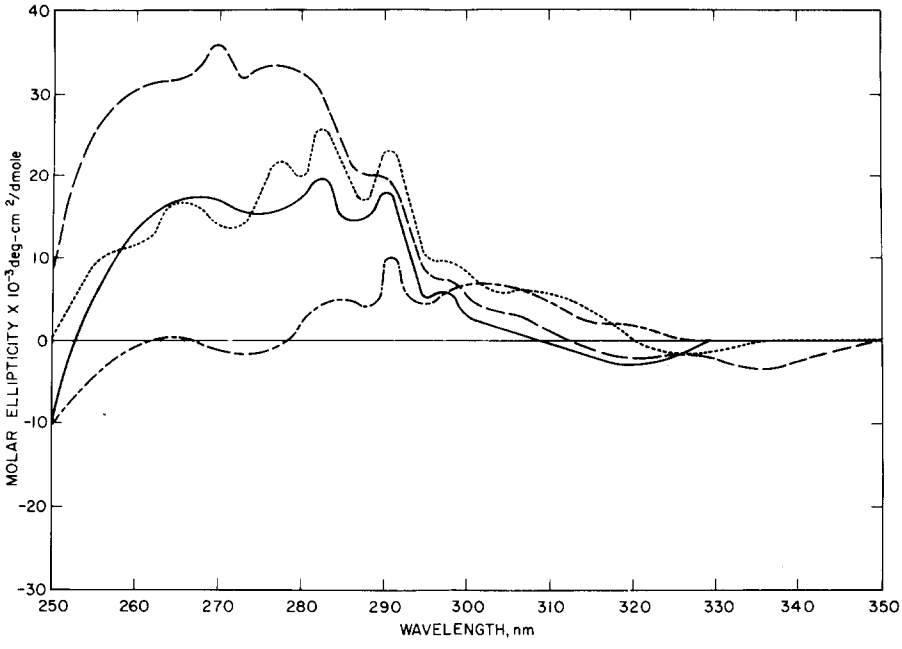

Fig. 5. The circular dichroism of dihydrofolate reductase complexes with trimethoprim. For conditions see Fig. 1. -.-.-., apoenzyme; ———, enzyme-trimethoprim; - - - - - -, enzyme-trimethoprim-NADPH; . . . . . ., enzyme-trimethoprim-$NADP^+$.

the spectrum of the enzyme-methotrexate complex, and methotrexate removes the effect of trimethoprim on the enzyme suggesting that they are bound in the same site.

When NADPH is bound to the enzyme-trimethoprim complex, a small negative band at 335 nm with a molar ellipticity of approx. —4000 degrees · cm^2 · $dmol^{-1}$ and a large positive band near 270 nm with a molar ellipticity of about 35 000 degrees · cm^2 · $dmol^{-1}$ appear. As in the case of the folate and methotrexate complexes, all the spectral changes saturate at a one molar equivalent addition of NADPH to enzyme. $NADP^+$ addition to the enzyme-trimethoprim complex generates small changes which are not the same as those generated by NADPH. Essentially the changes are a sharpening of the fine structure of the enzyme-trimethoprim spectrum at 277, 283 and 291 nm. Once again all the changes are caused by the first molar equivalent addition of $NADP^+$ to the enzyme complex.

(5) *Pyrimethamine complexes.* At pH 7.2 pyrimethamine also has an ultraviolet absorption band at approx. 278 nm. The binding of pyrimethamine to the enzyme induces a sharpening of the transitions of the enzyme at 291 and 284 nm. In addition, positive shoulders are induced at 277 and 270 nm and a large negative band is induced below 260 nm. The CD changes induced by the binding of pyrimethamine saturate at a 1 : 1 molar ratio at optical concentrations of 10—20 μM. The CD spectrum of the enzyme-pyrimethamine complex is shown in Fig. 6.

When NADPH is added to the enzyme-pyrimethamine complex large posi-

tive bands are induced centered at 275 and 340 nm. Pyrimethamine is the first compound studied which induces substantial optical activity in the 340 nm band of NADPH in a case where the enzyme-"folate analog" complex is not itself optically active in that region. In contrast with the other "folate analogs" heretofore reported, the binding of pyrimethamine does not appear to increase the binding constants of the first molar equivalent of NADPH to the enzyme and the optical activity change does not saturate at a 1 : 1 addition of NADPH to the enzyme. The increase in ellipticity at 340 nm as a function of pyrimethamine concentration is shown in Fig. 7. As in the case of the other compounds studied only one pattern of spectral changes are seen (i.e. there are isobestic points) suggesting that all the changes are due to NADPH binding in one site. The binding curve, however, is inconsistent with one binding site and is similar to the curve seen by Poe et al. [4] for the difference spectral titration of the native (unliganded) enzyme with NADPH. The curvature is due to competition for NADPH by the two binding sites rather than by weak binding. Apparently pyrimethamine does not change the binding constant of NADPH to dihydrofolate reductase. Unfortunately, the binding of pyrimethamine is too tight for determination of a dissociation constant from circular dichroism studies, therefore the influence of NADPH on the binding of pyrimethamine cannot be examined.

When $NADP^+$ is bound to the enzyme-pyrimethamine complex there is a slight sharpening of the transitions of 291 and 284 nm as well as the induction of a slight shoulder at 302 nm. All the changes in optical activity appear with a 1 : 1 addition of $NADP^+$ to the enzyme-pyrimethamine complex. The binding

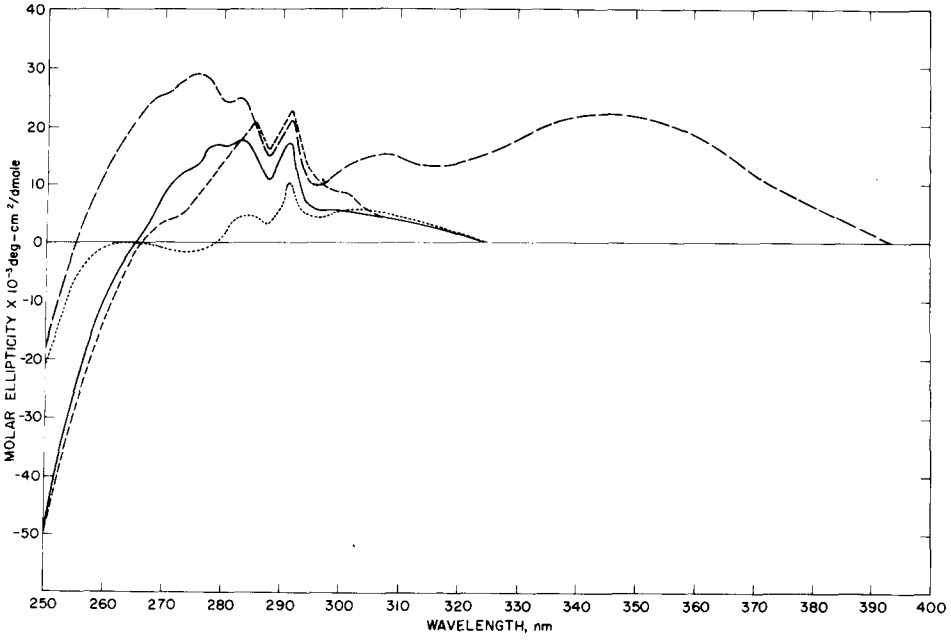

Fig. 6. The circular dichroism of dihydrofolate reductase complexes with pyrimethamine. For conditions see Fig. 1. . . . . ., apoenzyme; ———, enzyme-pyrimethamine; - - - - - -, enzyme-pyrimethamine-NADPH; — — —, enzyme-pyrimethamine-$NADP^+$.

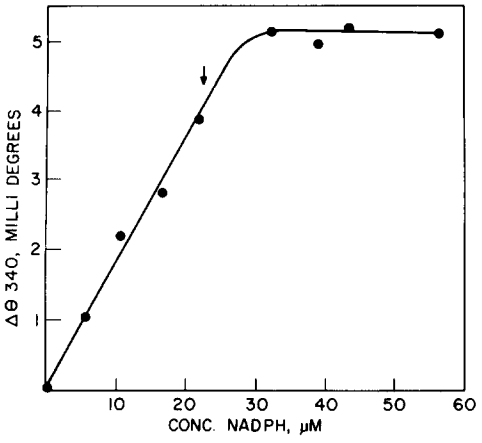

Fig. 7. The titration of the complex of dihydrofolate reductase and pyrimethamine with NADPH as monitored by changes in the circular dichroism at 340 nm. The arrow shows the enzyme concentration.

of NADPH and $NADP^+$ to the enzyme-pyrimethamine complex thus induce quite different effects in the CD spectrum in contrast to the results with the enzyme-folate and enzyme-methotrexate complexes. The spectra of the enzyme-pyrimethamine complexes in the presence and absence of NADPH and $NADP^+$ are shown in Fig. 6.

Kinetics

(1) *Pyridine nucleotide cofactors.* A comparison of the Michaelis constants and inhibition constants of various pyridine nucleotide cofactors and cofactor fragments for dihydrofolate reductase is shown in Table III. The results obtained were independent of preincubation conditions. The nucleotides listed are competitive inhibitors of the enzyme with respect to NADPH.

(2) *Folate analogs.* A comparison of the binding constants obtained from CD measurements, the Michaelis constants and inhibition constants of various folate analog substrates and inhibitors of dihydrofolate reductase is shown in Table IV.

Trimethoprim is a very strong competitive inhibitor of dihydrofolate

TABLE III

KINETIC CONSTANTS FOR DIHYDROFOLATE REDUCTASE: PYRIDINE NUCLEOTIDES

Measurements were performed in 0.05 M Tris · HCl, 0.05 M NaCl, pH 7.2, with 0.001 M dithiothreitol present to stabilize the enzyme, at 23°C.

Cofactor	K_m	K_i	Reference
NADPH	6.5 ± 0.9 μM		[7]
NADH	320 ± 30 μM		[7]
$NADP^+$		18 ± 9 μM	
3-Acetylpyridine-$NADP^+$		21 ± 4 μM	
Ado-*PP*-Rib		2.2 ± 1.2 mM	
NMN^+		No inhibition at 1.5 mM	

TABLE IV

KINETIC CONSTANTS FOR DIHYDROFOLATE REDUCTASE: FOLATE ANALOGUES

Kinetic measurements were performed in 0.05 M Tris · HCl, 0.001 M dithiothreitol, 0.05 M NaCl, pH 7.2, at 23°C. CD measurements were made in 0.1 M NaCl at 27°C.

Substrate or inhibitor	K_d from CD	K_i or (K_m)	Effect of preincubation of inhibitor and cofactor on K_i
Folate	2.8 μM [2]	21 ± 9 μM	none
Trimethoprim		3.6 ± 0.2 nM	stronger inhibition non-linear assays
Pyrimethamine		53 ± 3 nM	none
Methotrexate		0.36 nM [8]	0.01 nM [8]
Dihydrofolate	1.1 μM [2]	(1.9 μM) [7]	

reductase towards dihydrofolate with a K_i of 3.6 ± 0.2 nM when the assay is initiated with enzyme. When the enzyme is preincubated with dihydrofolate and trimethoprim and the assay is initiated with NADPH the inhibition appears much weaker, however, accurate constants could not be determined because the assays were non-linear and the rate decreased rapidly during the course of the assay. When the enzyme was preincubated with NADPH and trimethoprim and the assay was initiated with dihydrofolate the inhibition of the enzyme by trimethoprim appeared to be at least an order of magnitude tighter than the case with no preincubation. Unfortunately accurate inhibition constants once again could not be obtained because the assays were non-linear. In this case the reaction rate increased during the course of the assays.

Discussion

When NADPH or NADH are bound to the complex of dihydrofolate reductase with methotrexate there are large changes in the circular dichroism spectrum, associated with the addition of one molar equivalent of cofactor. In contrast when the reduced pyridine nucleotides are bound to the apoenzyme there are virtually no CD perturbations. The CD changes induced by NADPH / NADH binding to the complex may have several distinct origins. First, the binding may change the conformation of the enzyme and thus change the orientation of the bound methotrexate. Some of the CD changes could therefore be due to a change in the enzyme groups which interact with the methotrexate. Alternatively, the configuration of the bound methotrexate itself might change, leading to changes in the sign and position of the extrinsic CD bands as the orientation of the pteridine rings of the inhibitor with respect to the *p*-aminobenzoate ring of the inhibitor alters. Also the transitions of the electrons of the bound NADPH/NADH might interact with the transitions of the electrons of the bound methotrexate to give new bands [17].

NADH addition to the enzyme-methotrexate complex generates CD changes virtually identical to those generated by NADPH. This similarity would suggest that conformations of the enzyme-methotrexate complexes with NADPH and NADH are homologous. The difference in binding strength of the first molar equivalent NADPH and NADH to the enzyme complex (K_{d1} is less

than 10^{-10} M for NADPH [3] but is 9 μM for NADH) is therefore probably electrostatic in origin, being due to the extra charged phosphate of NADPH, rather than due to an environmental difference of the two bound cofactors.

$NADP^+$ binding also induces CD changes very similar to those induced by NADPH above 320 nm suggesting that the geometry of the complex with the oxidized cofactor is similar in geometry to that of the reduced. Above 320 nm, $NADP^+$ only induces slight perturbations in the CD spectrum of the enzyme methotrexate complex. This would suggest that the changes in the CD bands above 320 nm upon NADPH addition to the enzyme-methotrexate complex might be due to direct interaction between the electrons of the pyridine ring of NADPH and the pterin ring of methotrexate because NADPH has an absorption band at 340 nm which $NADP^+$ lacks. It is of interest, therefore, to compare the changes generated in the CD spectrum by NADPH and $NADP^+$ binding to the enzyme-methotrexate complex with those generated in the difference spectra. The difference spectra of the mixture of NADPH or $NADP^+$ with the enzyme-methotrexate complex compared to the unmixed cofactors and complex are virtually identical above 300 nm, but differ below 300 nm [4]. Thus the changes monitored by the two different techniques are distinct and may reflect different effects, and the situation may be more complicated than the CD results alone would suggest.

NMN^+ and Ado-*PP*-Rib generate virtually no changes in the circular dichroism spectrum of the enzyme-methotrexate complex even though they bind at the concentration measured. These results suggest that either the cofactor fragments bind in a different orientation than the whole coenzyme, or that the binding of the intact cofactors is necessary to generate a conformational change which causes the perturbation of the CD spectrum of the enzyme-methotrexate complex. The possibility that NMN^+ binds in a different orientation is enhanced by the fact that NMN^+ does not inhibit the enzyme at concentrations up to 1.5 mM.

3-Acetylpyridine nucleotide when added to the enzyme-methotrexate complex, also generates no circular dichroism changes. Replacing the amide group of $NADP^+$ with an acetyl group is therefore sufficient to change the precise interactions which lead to changes in the optical activity of the enzyme-methotrexate complex even though 3-acetylpyridine-$NADP^+$ is as good an inhibitor as $NADP^+$ itself.

Folate is a poor substrate for dihydrofolate reductase from *E. coli* B MB 1428 at pH 7.2 and is reduced at only 1/27 000 the rate of dihydrofolate [2]. As in the case of methotrexate, when folate is bound to the enzyme the binding constant for both the first molar equivalent of NADPH and $NADP^+$ is increased so that the binding appears to be very tight with 1 : 1 stoichiometry by circular dichroism criteria. Moreover, the effect of NADPH and $NADP^+$ on the CD spectrum of the enzyme-folate complex are similar suggesting that they are bound in homologous conformations.

In contrast to methotrexate, however, prior binding of NADPH to the enzyme has only a small effect on the binding constants of folate. In the absence of cofactor, methotrexate has a binding constant of approx. 4 nM in 0.05 M Tris · HCl, 0.05 M NaCl, pH 7.2 [8]. If the enzyme is assayed without preincubation (i.e. assays are initiated with enzyme) the binding of methotrex-

ate is competitive with dihydrofolate and gives a K_i = 0.36 nM. When 1 : 1 NADPH is added to the enzyme the binding of methotrexate becomes stoichiometric. Moreover, if the enzyme is preincubated with NADPH and methotrexate and assays are initiated with dihydrofolate the inhibition of the enzyme by methotrexate seems stoichiometric and only an upper limit of the K_i of 0.01 nM can be determined [8]. In contrast, preincubation conditions have no effect on the K_i of folate for the enzyme. It is apparently a competitive inhibitor of the enzyme with respect to dihydrofolate with a $K_i = 2 \pm 1 \times 10^{-5}$ M. The results with folate provide further confirmation that the increased affinity of the enzyme for methotrexate in the presence of NADPH enhances the effectiveness of methotrexate as an antimetabolite [8].

NADPH and $NADP^+$ have very similar effects on the spectrum of the enzyme-folate complex. No new bands are generated by the addition of the coenzymes. The small changes that are observed may be due to an effect by the cofactors on the enzyme's conformation rather than direct folate-cofactor interactions. The similarity of the shifts induced by both cofactors suggest that they may be bound in similar configurations.

Trimethoprim is a potent inhibitor of bacterial dihydrofolate reductase [1]. As in the case of methotrexate [8] the strength of inhibition depends upon preincubation conditions. When the enzyme is titrated with trimethoprim the CD spectral changes saturate at a 1 : 1 molar addition to the enzyme and the binding is too tight to estimate binding constants for the CD data. When the enzyme-trimethoprim complex is titrated with either NADPH or NADP CD spectral changes occur which also saturate at a 1 : 1 addition of cofactor to the enzyme-antifole complex. Thus trimethoprim has the same effect as folate and methotrexate in increasing the affinity of the enzyme for the first molar equivalent of coenzyme. However, unlike the complexes of folate and methotrexate, NADPH and NADH induce significantly different changes in the circular dichroism spectrum of the enzyme-trimethoprim complex. It is possible that the conformation of the enzyme is less constrained when trimethoprim binds than when folate or methotrexate bind as trimethoprim is a smaller molecule. Possibly, therefore, NADPH and $NADP^+$ are not restricted to similar binding configurations when they bind to the enzyme-trimethoprim complex. Alternatively, the binding site or configuration of the trimethoprim might be distinct in the presence of the two different cofactors.

Pyrimethamine is commonly used as an antimalarial agent [1]. However, it is also a very strong competitive inhibitor of the reductase from *E. coli* B (MB 1428) with a K_i = 53 nM. Pyrimethamine binding appears stoichiometric at optical concentrations and saturates at a 1 : 1 addition of inhibitor to enzyme as monitored by induced CD spectral changes. Pyrimethamine is quite different from the other antifoles studies herein because it has no apparent effect on the binding affinity of NADPH to the enzyme. Moreover, the prior binding of pyrimethamine induces optical activity in the 340 nm band of NADPH when the coenzyme is bound to the enzyme-pyrimethamine complex. No other folate analog heretofore studied induces activity in this band unless the enzyme-analog complex itself has an optically active band in the same wavelength range. Prior binding of pyrimethamine apparently changes either the binding site or the geometry of bound NADPH but it does not seem to

affect the binding constant of the cofactor to the enzyme. As in the case of the enzyme-pyrimethamine complex, the binding of NADPH and $NADP^+$ to the enzyme-pyrimethamine complex have different effects on the complexes CD spectrum. The spectral differences suggest that the oxidized and reduced cofactor may not be constrained to bind in the same conformation in the presence of a "small" inhibitor. Poe et al. [4] have shown that in the absence of methotrexate, NADPH, $NADP^+$ and NADH all have quite distinct effects on the difference spectra of dihydrofolate reductase. Thus in the absence of substrate it is possible that the three cofactors bind in different orientations. Alternatively, the orientation of the pyrimethamine may be different in the presence of NADPH then it is in the presence of $NADP^+$.

The inhibition of dihydrofolate reductase by pyrimethamine does not increase upon preincubation of the enzyme with the inhibitor and cofactor in contrast to the results with methotrexate and trimethoprim. It would be of interest to extend these studies to other inhibitors of the enzyme to see if there is a correlation between the mutual enhancements of the binding of cofactors and inhibitors and the changes in degree of enzyme inhibition depending upon preincubation conditions.

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