Circular-Dichroism Studies of Ligand Binding to Dihydrofolate Reductase from *Lactobacillus casei* MTX/R

By KEVIN HOOD, PETER M. BAYLEY and GORDON C. K. ROBERTS*

*Divisions of Molecular Pharmacology and Biophysics, National Institute for Medical Research,
Mill Hill, London NW7 1AA, U.K.*

(Received 20 June 1978)

Circular-dichroism spectra (200–450 nm) were recorded for *Lactobacillus casei* MTX/R dihydrofolate reductase and its complexes with substrates, inhibitors and coenzymes. These spectra are compared with those reported by others for dihydrofolate reductase from other sources. The binding of NADP⁺ or NADPH is associated with the perturbation of one or more aromatic amino acid residues, and there is marked enhancement of the negative c.d. band at 340 nm arising from the dihydronicotinamide chromophore of NADPH. The substrates folate and dihydrofolate give rise to substantial extrinsic c.d. bands on binding, which show a number of specific differences between enzymes from different sources. The binary complexes between the enzyme and the inhibitors methotrexate or trimethoprim also show strong c.d. bands, and these are qualitatively very similar for all dihydrofolate reductases studied so far. The ternary complexes between enzyme, NADPH and trimethoprim or methotrexate are very different from the sum of the spectra of the binary complexes. Trimethoprim leads to the disappearance of the 340 nm c.d. band of bound NADPH, whereas in the methotrexate–NADPH–enzyme ternary complex a ‘couplet’ c.d. spectrum is observed at long wavelengths. Analysis of this latter feature suggests that it arises from a direct interaction between the dihydronicotinamide and pteridine rings in the ternary complex.

The importance of dihydrofolate reductase as the site of action of a number of clinically useful drugs, such as methotrexate and trimethoprim, has stimulated many studies of the binding of substrates and inhibitors to the enzyme (for reviews see Blakley, 1969; Bertino, 1971; Roberts, 1977, 1978). Among other techniques, c.d. has been widely used to study ligand binding to this enzyme, since for many ligands large extrinsic Cotton effects are observed (D’Souza & Freisheim, 1972; Greenfield *et al.*, 1972; Gupta *et al.*, 1977; Reddy *et al.*, 1978). This work has shown that when ligand binding to dihydrofolate reductase from different species is compared by c.d., there are a number of constant features, but there is also a marked species-to-species variation in many of the spectral changes observed. This is of interest in view of the therapeutic importance of the species-specificity of some inhibitors of the enzyme (Hitchings & Burchall, 1965). We have therefore studied the binding of ligands to the reductase from *Lactobacillus casei* MTX/R (Dann *et al.*, 1976) using c.d. spectroscopy, and compared the effects observed with those reported for the reductases of *Streptococcus faecium* (Freisheim & D’Souza, 1971; D’Souza & Freisheim, 1972), *Escherichia coli* MB 1428 (Green-

field *et al.*, 1972; Greenfield, 1975), another strain of *L. casei*, referred to here for brevity as *L. casei* RBF (Reddy *et al.*, 1978), and L1210 cells (Gupta *et al.*, 1977). In addition we propose a molecular explanation of one of the common features in the c.d. spectra of ligand binding to dihydrofolate reductases, namely that the marked spectral changes accompanying addition of the coenzyme, NADPH, to the enzyme–methotrexate complex arise from a direct interaction between the dihydronicotinamide and methotrexate chromophores.

Experimental

Materials

Dihydrofolate reductase was purified from *L. casei* MTX/R as described by Dann *et al.* (1976). Its concentration was determined by assaying its catalytic activity, from its A_{280} and by fluorimetric titration with methotrexate (by using procedures and absorption coefficients given by Dann *et al.*, 1976). The sources and absorption coefficients of the substrates and inhibitors used are given by Hood & Roberts (1978). NADPH and NADP⁺ were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

* To whom reprint requests should be addressed.

Methods

C.d. spectra were recorded at 20°C by using a Jouan Dichrographe II fitted with a 150W xenon arc. Enzyme solutions were 20–50 μM in a buffer of 100mM-KCl/50mM-Bis-tris {2-[bis-(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol}/HCl, pH 6.50, and were contained in cells of path-length 1 cm for the wavelength range 250–450 nm, or path-length 1 mm for the range 200–260 nm. The total absorbance was close to 1.0. The operating voltage was 560–760 V for the near-u.v. and up to 1100 V for the far-u.v. measurements.

Spectra were recorded simultaneously on the chart recorder and in digital form on punched paper tape. Points were taken at 0.5 nm intervals, at a scanning rate of 3.75 nm/min and a nominal time constant of 10 s. When necessary (particularly in the far-u.v.) the signal-to-noise ratio was improved by averaging several scans and/or by applying a 20-point second-order polynomial smoothing function, though all the spectra shown in the Figures are unsmoothed. Instrumental baselines were subtracted and difference spectra for ligand binding calculated by appropriate computer programs. Near-u.v. spectra are expressed in terms of the molar dichroic absorbance, $\Delta\epsilon$, based on a mol.wt. of 18000 (Dann *et al.*, 1976), and peptide-region spectra are given as $\Delta\epsilon$ in terms of residue

molar concentration (mean residue mol.wt. = 109).

Results and Discussion

Fig. 1 (curve A) shows the c.d. spectrum of *L. casei* MTX/R dihydrofolate reductase. In the peptide region (Fig. 1a) the spectrum ($\Delta\epsilon_{217} = -2.1 \text{ M}^{-1} \cdot \text{cm}^{-1}$) closely resembles that of the enzyme from *E. coli* (Greenfield *et al.*, 1972) for which an approximate secondary-structural content of 10% α -helix and 50–60% β -structure was calculated. The determination of the crystal structure of the *E. coli* enzyme in its complex with methotrexate (Matthews *et al.*, 1977) indicates 18% α -helix and 30% β -sheet. In the wavelength range 250–350 nm the enzyme shows relatively weak c.d. (Fig. 1b) originating from overlapping and probably oppositely signed contributions from several aromatic chromophores, principally tyrosine and tryptophan. The very small negative band at 303 nm most likely arises from a red-shifted tryptophan transition, probably from a 'buried' residue. The absence of c.d. at wavelengths longer than 310 nm confirms the absence of significant amounts of bound pteridines (see Dann *et al.*, 1976). The maximum at 265 nm is similar to the $^1\text{L}_a$ type of tryptophan spectrum found in simple derivatives (Strickland, 1974). This spectrum is comparable in amplitude with those

Fig. 1. Circular-dichroism spectra of *L. casei* MTX/R dihydrofolate reductase and its complexes with NADP⁺ and NADPH

(a) Far-u.v. and (b) near-u.v. regions of the spectrum. Curve A, enzyme alone; curve B, enzyme-NADP⁺ complex; curve C, enzyme-NADPH complex; curve D, difference (on the same scale) between curves C and A. Note the different scales for the two regions of the spectrum.

observed for the enzymes from *E. coli* (Greenfield *et al.*, 1972) and *L. casei* RBF (Reddy *et al.*, 1978), though those from *S. faecium* (Freisheim & D'Souza, 1971; D'Souza & Freisheim, 1972) and L1210 cells (Gupta *et al.*, 1977) show 4–8-fold greater intensity. The spectra of the various enzymes differ quite noticeably in this wavelength range, presumably owing to specific differences in the distribution and environment of the tyrosine and tryptophan residues.

Coenzyme binding

The spectrum of the enzyme–NADP⁺ complex (Fig. 1, curve B) shows very little additional effect at 260nm, the $\lambda_{\max}$ of the absorption spectrum of bound NADP⁺, but there is an increase in c.d. ($\Delta\Delta\epsilon = +3.5\text{M}^{-1}\cdot\text{cm}^{-1}$) centred on 285–290nm, which presumably arises from a ligand-induced change in the environment and asymmetry of aromatic amino acid residues. ¹H and ¹⁹F n.m.r. studies (Feeney *et al.*, 1977a,b; Kimber *et al.*, 1977) have shown that NADP⁺ binding does affect the environment of several tyrosine and tryptophan residues. Addition of the coenzyme analogue 2'-phospho-adenosine-5'-diphosphoribose fails to produce a comparable increase in c.d. at 285–290nm, suggesting that the observed change in the environment of aromatic residues is dependent on the presence of the nicotinamide ring.

The c.d. spectrum of the enzyme–NADPH complex (Fig. 1, curve C) is quite distinct from that observed with NADP⁺. The negative band at 340nm clearly arises from the dihydronicotinamide chromophore of bound NADPH. The enhancement of this band by approx. 10-fold relative to the free coenzyme is typical of the effects seen with, for example, alcohol dehydrogenase (Ulmer & Vallee, 1965). Similar negative bands at 340nm are seen for the NADPH complexes of dihydrofolate reductase from other sources, with the exception of *E. coli*, for which no c.d. at 340nm was observed (Greenfield *et al.*, 1972). The substantial increase in c.d. at about 270nm on NADPH binding shown in Fig. 1 is also observed for the enzymes from *L. casei* RBF (Reddy *et al.*, 1978) and L1210 cells (Gupta *et al.*, 1977), but not for those from *E. coli* (Greenfield *et al.*, 1972) or *S. faecium* (Freisheim & D'Souza, 1971). This increase could arise from immobilization of the adenine chromophore on binding, or from a change in the environment of an aromatic residue of the protein. Simple immobilization of the adenine ring seems an unlikely explanation: n.m.r. experiments (Feeney *et al.*, 1975, 1977a,b) show that the environment of the adenine-ribose 2'-phosphate part of the bound coenzyme is very similar for NADP⁺ and NADPH, yet no c.d. band at 270nm is seen in the NADP⁺ complex. This is supported by the absence of the 270nm band in the NADPH complexes of the *E. coli* and *S. faecium* enzymes. On the

other hand, involvement of a tryptophan residue does seem likely. Oxidation of tryptophan-21 with *N*-bromosuccinimide leads to loss of catalytic activity and a marked decrease in the binding constant for NADPH (Liu & Dunlap, 1974; Freisheim *et al.*, 1977; K. Hood, J. Thomson & G. C. K. Roberts, unpublished work). N.m.r. studies of 6-fluoro-tryptophan-containing dihydrofolate reductase showed that the environment of one tryptophan residue was markedly altered by NADPH binding, but much less so by NADP⁺ binding (Kimber *et al.*, 1977). A significantly different perturbation of aromatic amino acid residues by NADPH and NADP⁺ is also evident from the c.d. spectrum (Fig. 1) and the u.v. difference spectrum (not shown) at 295–305nm; in both spectra the effects of oxidized and reduced coenzymes are of opposite sign.

It appears, then, that coenzyme binding is associated with the perturbation of one or more aromatic residues, including a tryptophan residue, and that this perturbation differs both between NADP⁺ and NADPH and between enzymes from different sources. In particular, a clear difference in the binding of NADPH between the dihydrofolate reductases from *E. coli* and *L. casei* MTX/R is indicated.

In addition to these effects in the 250–400nm region, NADPH binding has significant effects below 250nm (Fig. 1a). There is enhanced negative c.d. centred at 215nm, due either to interaction of chromophores with ordered peptide structures or even to an increased amount of secondary structure. The clear positive effect at 235nm is interpreted as arising from a strong perturbation of aromatic residues by NADPH. If only a single residue is involved, the magnitude would amount to $\Delta\epsilon = +100\text{M}^{-1}\cdot\text{cm}^{-1}$, indicating a highly specific and asymmetric interaction.

Binding of folate and dihydrofolate

The c.d. spectra of the complexes between *L. casei* MTX/R dihydrofolate reductase and the substrates dihydrofolate and folate are shown in Fig. 2; in both cases large-amplitude c.d. bands are generated. Two mechanisms must be considered as the source of the c.d. bands of this size: (a) immobilization of a chromophore within the molecule in a stable chiral conformation, with c.d. generated by an intramolecular polarizability mechanism localized essentially within the chromophoric ligand, and (b) interaction of amino acid residues with the chromophores of the ligand, including the possibility of coupling (in the sense of the Born–Kirkwood coupled oscillator mechanism; see Bayley, 1973) between the strongly allowed electronic transitions of the chromophore and those of nearby amino acids. Both mechanisms can of course contribute simultaneously, though not necessarily additively.

Fig. 2. Circular-dichroism difference spectra of the enzyme-folate (curve A) and enzyme-dihydrofolate (curve B) complexes

In both cases the c.d. spectrum of the enzyme alone (curve A of Fig. 1b) has been subtracted from that of the complex.

The spectrum of the folate complex consists of a negative band ($\Delta\epsilon = -3 \text{ M}^{-1} \cdot \text{cm}^{-1}$) at approx. 340 nm, a large positive band ($\Delta\epsilon = +28 \text{ M}^{-1} \cdot \text{cm}^{-1}$), probably consisting of at least two transitions, centred at 290–295 nm, and a negative band ($\Delta\epsilon = -12 \text{ M}^{-1} \cdot \text{cm}^{-1}$) at 270 nm. An essentially identical spectrum has been reported for the enzyme from *L. casei* RBF (Reddy *et al.*, 1978). By contrast, the enzymes from *E. coli*, *S. faecium* and L1210 cells (D'Souza & Freisheim, 1972; Greenfield *et al.*, 1972; Gupta *et al.*, 1977) show no negative band at long wavelength; rather there is a long positive 'tail' to long wavelength of the 290 nm band, suggesting that the c.d. associated with the 330 nm transition (a $\pi \rightarrow \pi^*$ transition of the pteridine ring) may have opposite sign in the last three enzymes. This suggests a specific difference in the environment of folate in the *L. casei* enzyme from that in enzymes from other sources, and is perhaps most easily explained if the c.d. at 330 nm arises primarily by mechanism (b). The 290–295 nm band is observed in all cases (though its amplitude varies over a 3-fold range), but the *S. faecium* enzyme lacks the band at 270 nm (D'Souza & Freisheim, 1972).

For the *L. casei* MTX/R enzyme, the dihydrofolate complex shows essentially only a positive band centred at 292 nm, though there is probably also a very weak negative feature at approx. 325 nm. This resembles in form the spectrum obtained with the *S.*

faecium enzyme (D'Souza & Freisheim, 1972), though in the latter case the amplitude of the 292 nm band was an order of magnitude greater. Enzymes from other sources show a negative band at 330 nm, and also a band at 270 nm which is negative in *L. casei* RBF and *E. coli*, but positive in L1210 cells.

It is apparent that the only consistent feature of the c.d. spectra of the folate and dihydrofolate complexes is the positive band at 290–295 nm, though even this varies substantially in amplitude from one enzyme to another. Both the pteridine and *p*-aminobenzoyl-L-glutamate chromophores of folate and dihydrofolate have $\pi \rightarrow \pi^*$ transitions at around 280 nm, and it is clear from the c.d. spectra, particularly of the folate complex, that there are two transitions contributing to this c.d. band. In dihydrofolate, these two chromophores are essentially independent (Whiteley & Huennekens, 1967), but in folate they will be coupled, at least to some degree, by hyperconjugation through C-9 and N-10. This coupling should make the observed c.d. very sensitive to the relative disposition of the two rings in bound folate. This might account for the variation in intensity of the 290–295 nm band between enzymes from different sources, although any such variation in the conformation of bound folate would seem likely to be small, and the alternative explanation, that the intensity variation reflects differing contributions from mechanism (b), is just as plausible. With dihydrofolate the very marked differences in intensity of the 290–295 nm band are almost certainly due to differences in coupling with transitions of neighbouring aromatic amino acids. The binding of *p*-aminobenzoyl-L-glutamate itself gives rise to only a slight ($\Delta\Delta\epsilon = +0.5 \text{ M}^{-1} \cdot \text{cm}^{-1}$) increase in c.d. at 285 nm. We cannot differentiate between the contributions of the dihydropteridine and benzoyl chromophores to the 290 nm band, although the fact that there is no parallel between the behaviour of this band in the different enzymes and that of the 330 nm pteridine band might suggest that the major variation is in the intensity of the benzoyl contribution.

Binary and ternary complexes with trimethoprim

The binding of trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] to the *L. casei* MTX/R reductase leads to an increase in (positive) c.d. centred at about 280 nm (Fig. 3) and extending to about 295 nm. A similar, though rather smaller, increase is seen for the enzyme from *E. coli* (Greenfield, 1975). Much of it is presumably due to immobilization of the trimethoprim (λ_{max} 271 nm in the protonated form, which binds preferentially; Hood & Roberts, 1978) on binding (i.e. mechanism a), although contributions from aromatic amino acids, which are known to be affected by trimethoprim binding (Feeney *et al.*, 1977a; Hood & Roberts

Fig. 3. Circular-dichroism spectra of enzyme-trimethoprim complexes

Curve A; enzyme-trimethoprim complex; curve B, enzyme-trimethoprim-NADP⁺ complex; curve C, enzyme-trimethoprim-NADPH complex.

1978) cannot be ruled out. Addition of NADP⁺ to the enzyme-trimethoprim complex has very little effect (Fig. 3), and in fact the c.d. spectrum of the ternary complex can be largely accounted for by simple addition of the effects seen in the two binary complexes.

This is also true for NADPH (Fig. 3) at the shorter wavelengths (below 300nm), but the presence of trimethoprim leads to the disappearance of the negative band at 340nm observed in the binary enzyme-NADPH complex (see Fig. 1). A marked effect of trimethoprim on the dihydronicotinamide chromophore is also observed in the absorption spectrum, which reveals that the 340nm absorption band is shifted to 334nm in the presence of trimethoprim. It is notable that with the *E. coli* reductase (Greenfield, 1975), which does not show a c.d. band at 340nm in the binary complex with NADPH, a weak negative band at this wavelength appears on addition of trimethoprim. There is clearly a substantial effect of trimethoprim on coenzyme binding which is, interestingly, apparently species-specific.

Binary and ternary complexes with methotrexate

The peptide and aromatic regions of the c.d. spectrum of the binary complex of methotrexate with *L. casei* MTX/R dihydrofolate reductase are shown in Fig. 4. It is notable that there is a marked increase

in the intensity of the negative c.d. in the peptide region, centred around 220nm, when methotrexate binds. This could reflect some reorganization of secondary structure on methotrexate binding [some conformational changes are known to occur (Roberts, 1977, 1978; Roberts *et al.*, 1977)], but it could also be due to a far-u.v. transition of methotrexate itself. In the latter case its intensity on a molar basis would be $\Delta\epsilon = -200\text{M}^{-1}\cdot\text{cm}^{-1}$, indicating an unusually strong coupling, possibly with an extended peptide structure.

In the aromatic region of the spectrum of the enzyme-methotrexate complex there are large positive bands at 280 and 305nm and a broad negative band at approx. 340nm, with indications of a shoulder at 360–365nm. Qualitatively very similar spectra have been reported for all the other dihydrofolate reductases studied, except for that from *S. faecium* (D'Souza & Freisheim, 1972), for which the published difference c.d. spectrum gives no indication of a negative band at long wavelengths. The absorption spectrum of bound methotrexate has been studied in some detail (Hood & Roberts, 1978). At neutral pH methotrexate binds in the form protonated at N-1, with pteridine $\pi \rightarrow \pi^*$ transitions at 342nm and at 362nm (red-shifted on binding from 321nm in the free cation). Both of these transitions appear to give negative c.d. bands. The transition at 305nm most probably arises primarily from the benzoyl residue, and there is a further pteridine $\pi \rightarrow \pi^*$ transition at about 280nm (the very weak long-wavelength $n \rightarrow \pi^*$ transitions have been ignored for present purposes). The c.d. band at 270–280nm is comparable in intensity with that at 305nm in the complexes of methotrexate with the reductases from *L. casei* MTX/R and L1210 cells, but only about half the intensity with those from *L. casei* RBF and *E. coli*. Some at least of this difference presumably reflects differing contributions from the aromatic amino acid residues of the protein. Here again the enzyme from *S. faecium* differs from all the others, having bands at 295 and 315nm some 4 times as intense as those observed with the other enzymes; it appears that the environment of methotrexate bound to *S. faecium* dihydrofolate reductase is in some way unique among this group of reductases.

Aminopterin differs from methotrexate only in the absence of the N-10 methyl group; it shows, when bound to the enzyme, a c.d. spectrum very similar to that given by methotrexate. The long-wavelength bands are identical, and the band at approx. 305nm is blue-shifted slightly to approx. 295nm. A small blue shift of the absorption band of the *p*-amino-benzoyl chromophore on removal of the N-10 methyl group would be expected from the behaviour of model compounds (cf. Jaffé & Orchin, 1962) and indeed the 307nm absorption maximum of methotrexate shifts to 290nm in aminopterin (both in the cationic form).

Fig. 4. Circular-dichroism spectra of the enzyme-methotrexate (curve A) and enzyme-methotrexate-NADP⁺ (curve B) complexes

(a) Far-u.v. and (b) near-u.v. regions of the spectrum. In (a), curve C is the spectrum of the enzyme alone, and curve D the difference (on the same scale) between curves C and A. Note the different scales for the two regions of the spectrum.

Addition of NADP⁺ to the enzyme-methotrexate complex to form the ternary complex has relatively little effect on the c.d. spectrum (Fig. 4). The long-wavelength negative c.d. bands arising from the methotrexate are wholly unaffected, but there is an increase in the intensity of the 280nm and 300nm bands, the increase (of about $6\text{ M}^{-1}\cdot\text{cm}^{-1}$) appearing to be centred at about 280nm. This increase is rather too large to be accounted for simply by addition of the enzyme-NADP⁺ c.d. spectrum (Fig. 1). Comparison of the difference between the c.d. spectra of the ternary complex and the enzyme-NADP⁺ complex with the spectrum of the enzyme-methotrexate complex shows that the small 'extra' intensity in the ternary complex is centred around 275nm, and could thus arise from either ligand or from the protein. From the c.d. point of view, then (though not in other respects: Way *et al.*, 1975; Feeney *et al.*, 1977a,b), the binding of NADP⁺ and methotrexate to the *L. casei* MTX/R reductase is almost independent. This is also true of the *E. coli* enzyme (Greenfield, 1975).

By contrast, addition of NADPH to the enzyme-methotrexate complexes produces major qualitative changes in the spectrum with all dihydrofolate reductases studied to date. The spectrum of the ternary complex with *L. casei* MTX/R enzyme is shown in Fig. 5(a, curve B). The shorter-wavelength part of the

spectrum (below 310nm) is only slightly affected, much of the increased intensity around 280nm being attributable simply to the binding of NADPH (cf. Fig. 1). However, the long-wavelength transitions of bound methotrexate are substantially perturbed, clearly indicating an electronic interaction between the pteridine and dihydronicotinamide chromophores of the two ligands.

Closely similar c.d. spectra are observed for the ternary complexes of methotrexate and NADPH with the reductases from *E. coli* and L1210 cells (Greenfield *et al.*, 1972; Gupta *et al.*, 1977). However, the spectrum observed with the enzyme from *L. casei* RBF (Reddy *et al.*, 1978) is totally different, showing a very large positive band at 360nm, only a very small negative band at 320nm and a negative band at 260nm in place of the positive bands at 280 and 300nm seen with the other enzymes. Since this enzyme shows a spectrum for the binary complex with methotrexate that is closely similar to that reported here, it appears that the mutual effects of coenzyme and methotrexate binding must be significantly different in the enzyme from *L. casei* RBF compared with those from other sources.

It should be noted that the long-wavelength (over 300nm) transitions of both the pteridine and dihydronicotinamide chromophore are very broad (cf. Figs. 1 and 4). Furthermore, analysis of the u.v. difference

Fig. 5. Circular-dichroism spectra of the enzyme-methotrexate-NADPH complex

(a): Comparison of the c.d. difference spectra (enzyme c.d. spectrum subtracted) of the enzyme-methotrexate (curve A) and the enzyme-methotrexate-NADPH (curve B) complexes. (b): curve A: difference between curves A and B of part (a) (enzyme-methotrexate-NADPH minus enzyme-methotrexate). Curve B is the corresponding difference spectrum between complexes involving aminopterin rather than methotrexate.

spectra shows that none of these long-wavelength bands have shifted appreciably (less than 2 nm) on forming the ternary complex. Thus the marked changes in the c.d. spectrum cannot be explained by changes in the relative position of oppositely signed c.d. bands. It is clear that in the ternary complex there must be an electronic interaction between the dihydronicotinamide chromophore and that of methotrexate which gives rise to the complex c.d. profile in the region 300–400 nm of Fig. 5(a). The difference spectrum between the enzyme-methotrexate-NADPH and enzyme-methotrexate complexes (Fig. 5b) shows an approximately equal and opposite pair of bands, with a crossover at 347 nm. Fig. 5(b) also shows that the corresponding difference spectrum between the ternary and binary complexes with aminopterin is, apart from a small difference at

305 nm, virtually identical with that observed with methotrexate. The similarity of these spectra to the 'couplet' c.d. spectrum characteristic of exciton effects in stacked dinucleotides, such as ApA (see Bayley, 1973), suggests as a possible mechanism the direct coupling of the transitions of NADPH and methotrexate.

This coupling of transitions occur when two chromophores are sufficiently close in space for the excitation of one to affect the excitation of the other. It arises from an interaction between the bound chromophores which may be considered independently of their individual interactions with the enzyme. The spectroscopic effects therefore represent an additional contribution from interaction of the chromophores in the ternary complex: it should be noted that this additional rotational strength is of the same order as the total observed in the binary enzyme-methotrexate complex.

This mechanism corresponds to a non-degenerate analogue of the Born-Kirkwood 'coupled' oscillator model (Bayley, 1973), which shows how optical activity is manifested by equal and opposite bands in the c.d. spectrum with, in the simplest case, the crossover being approximately midway between the wavelengths of the transitions that are coupled. Exciton interactions are significant for strongly allowed transitions of similar energy in chromophores with suitable orientations with respect to one another. The long-wavelength $\pi \rightarrow \pi^*$ bands of the bound pteridine chromophore at 342 and 363 nm and the 343 nm band of the bound dihydronicotinamide chromophore are strongly allowed transitions that are suitably close in energy. It is difficult to be certain which of the two methotrexate transitions is involved, but the observations that the amplitude of the positive and negative components of the 'couplet' are only made approximately equal by subtraction of the main negative c.d. band of methotrexate at 340 nm (see Fig. 5b), together with the fact that the crossover is clearly nearer 350 nm than 340 nm, suggests that it is the 363 nm transition that couples to the 343 nm transition of the dihydronicotinamide.

The idea of a direct interaction between the pteridine and dihydronicotinamide chromophores is supported by the spectrum of the enzyme-methotrexate-thioNADPH ternary complex (Fig. 6). ThioNADPH in its binary complex with the enzyme shows a very broad negative c.d. band ($\Delta\epsilon = 8 \text{ M}^{-1} \cdot \text{cm}^{-1}$), clearly consisting of more than one transition, centred at 395 nm. The ternary complex again clearly shows a 'couplet' feature, weaker and substantially broader than for NADPH, with a crossover at approx. 370 nm.

According to the simple degenerate exciton model, the spectroscopic splitting, σ , is critically dependent on r , the distance between μ_1 and μ_2 (the two transition moment vectors) with $\sigma \propto r^{-3}$, and the magnitude

Fig. 6. Circular-dichroism spectra of the enzyme-thioNADPH (curve A) and enzyme-thioNADPH-methotrexate (curve B) complexes

of the couplet is proportional to $\mu_1^2\mu_2^2 \sin \alpha \cos \alpha$ and thus depends on absorption coefficients and on the stacking angle α between μ_1 and μ_2 . In molecular terms, the most suitable orientation is achieved by stacking of the two chromophores at distances close to van der Waals contact; to produce the observed effects the transition dipoles would be oriented with right-handed chirality, $\sin \alpha \cos \alpha$ having a maximum for $\alpha = 45^\circ$. Thus this interpretation of the c.d. spectrum of the ternary complex strongly predicts that the pteridine ring of methotrexate (or aminopterin) and the dihydronicotinamide ring of NADPH are stacked in close proximity on the enzyme surface in the ternary complex. A more precise specification of their mutual orientation must await a fuller understanding of the methotrexate absorption spectrum, specifically the origins and orientations in the molecular frame of the transition moments giving rise to the 342 and 363 nm absorption bands. However, this arrangement of the two ligands is entirely consistent with what one would expect to occur in the enzyme-folate-NADPH complex, where a close approach of the dihydronicotinamide ring to the pyrazine portion of the pteridine ring of the substrate would be required for direct hydride-ion transfer.

We are most grateful to Ms Gill Ostler for excellent technical assistance.

References

- Bayley, P. M. (1973) *Prog. Biophys. Mol. Biol.* **27**, 1-77
 Bertino, J. R. (ed.) (1971) *Ann. N.Y. Acad. Sci.: Folate Antagonists as Chemotherapeutic Agents* **186**

- Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines*, chapter 5, North Holland, Amsterdam
 Dann, J. G., Ostler, G., Bjur, R. A., King, R. W., Scudder, P., Turner, P. C., Roberts, G. C. K., Burgen, A. S. V. & Harding, N. G. L. (1976) *Biochem. J.* **157**, 559-571
 D'Souza, L. & Freisheim, J. H. (1972) *Biochemistry* **11**, 3770-3774
 Feeney, J., Birdsall, B., Roberts, G. C. K. & Burgen, A. S. V. (1975) *Nature (London)* **257**, 564-566
 Feeney, J., Roberts, G. C. K., Birdsall, B., Griffiths, D. V., King, R. W., Scudder, P. & Burgen, A. S. V. (1977a) *Proc. R. Soc. London Ser. B* **196**, 267-290
 Feeney, J., Birdsall, B., Roberts, G. C. K. & Burgen, A. S. V. (1977b) in *NMR in Biology* (Dwek, R. A., Campbell, I. D., Richards, R. E. & Williams, R. J. P., eds.), pp. 111-124, Academic Press, London
 Freisheim, J. H. & D'Souza, L. (1971) *Biochem. Biophys. Res. Commun.* **45**, 803-808
 Freisheim, J. H., Ericsson, L. H., Bitar, K. G., Dunlap, R. B. & Reddy, A. V. (1977) *Arch. Biochem. Biophys.* **180**, 310-317
 Greenfield, N. J. (1975) *Biochim. Biophys. Acta* **403**, 32-46
 Greenfield, N. J., Williams, M. N., Poe, M. & Hoogsteen, K. (1972) *Biochemistry* **11**, 4706-4711
 Gupta, S. V., Greenfield, N. J., Poe, M., Makulu, D. R., Williams, M. N., Moroson, B. A. & Bertino, J. R. (1977) *Biochemistry* **16**, 3073-3079
 Hitchings, G. L. & Burchall, J. J. (1965) *Adv. Enzymol.* **27**, 417-483
 Hood, K. & Roberts, G. C. K. (1978) *Biochem. J.* **171**, 357-366
 Jaffé, H. H. & Orchin, M. (1962) *Theory and Applications of Ultraviolet Spectroscopy*, pp. 242-287, Wiley, New York
 Kimber, B. J., Griffiths, D. V., Birdsall, B., King, R. W., Scudder, P., Feeney, J., Roberts, G. C. K. & Burgen, A. S. V. (1977) *Biochemistry* **16**, 3492-3500
 Liu, J.-K. & Dunlap, R. B. (1974) *Biochemistry* **13**, 1807-1814
 Matthews, D. A., Alden, R. A., Bolin, J. T., Freer, S. T., Hamlin, R., Xuong, N., Kraut, J., Poe, M., Williams, M. N. & Hoogsteen, K. (1977) *Science* **197**, 452-455
 Reddy, A. V., Behnke, W. D. & Freisheim, J. H. (1978) *Biochim. Biophys. Acta* **533**, 415-427
 Roberts, G. C. K. (1977) in *Drug Action at the Molecular Level* (Roberts, G. C. K., ed.), pp. 127-150, Macmillan, London
 Roberts, G. C. K. (1978) in *CIBA Found. Symp.: Molecular Interactions and Activity in Proteins* in the press
 Roberts, G. C. K., Feeney, J., Birdsall, B., Kimber, B., Griffiths, D. V., King, R. W. & Burgen, A. S. V. (1977) in *NMR in Biology* (Dwek, R. A., Campbell, I. D., Richards, R. E. & Williams, R. J. P., eds.), pp. 95-109, Academic Press, London
 Strickland, E. H. (1974) *Crit. Rev. Biochem.* **2**, 113-175
 Ulmer, J. B. & Vallee, B. L. (1965) *Adv. Enzymol.* **27**, 37
 Way, J. L., Birdsall, B., Feeney, J., Roberts, G. C. K. & Burgen, A. S. V. (1975) *Biochemistry* **14**, 3470-3475
 Whiteley, J. & Huennekens, F. M. (1967) *Biochemistry* **6**, 2620-2625