CIRCULAR DICHROIC TITRATION OF DIHYDROFOLATE REDUCTASE WITH TPNH[1]

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SUMMARY

Dihydrofolate reductase from Streptococcus faecium shows a marked aromatic side chain Cotton effect in the 260-310 nm region of its circular dichroic spectrum. This effect consists of three distinct ellipticity bands with maxima centered at 305 nm, 295 nm and 270 nm. Titration of the enzyme with TPNH to a 1:1 stoichiometry results in the generation of an extrinsic Cotton effect at ca. 340 nm and a decrease in the magnitude of the side chain Cotton effect. This is the first such example of a TPNH-generated extrinsic Cotton effect. The data suggest the involvement of tryptophyl residues in coenzyme binding.

Dihydrofolate reductase which catalyzes the TPNH-dependent reduction of dihydrofolate to tetrahydrofolate has been purified from an amethopterin-resistant strain of Streptococcus faecium (1,2). The near ultraviolet circular dichroic (CD) spectrum of the native enzyme shows a positive band system in the 260-310 nm region which is similar to that observed for egg white lysozyme (3-5). In the case of lysozyme, the transitions due to tryptophan contribute most to the magnitude of the aromatic side chain Cotton effect (6). Previous chemical modification studies by Freisheim and Huennekens (7) have indicated the functional importance of tryptophyl residues in the binding of substrates to chicken liver dihydrofolate reductase.

The present work describes the interaction of TPNH with dihydrofolate reductase as measured by circular dichroic titration. Enzyme-coenzyme complex

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formation results in marked changes in the near ultraviolet CD spectrum of dihydrofolate reductase. This technique provides a powerful tool for examining the three-dimensional interaction of functional groups on the enzyme with chromophoric substrates, coenzymes and inhibitors.

MATERIALS AND METHODS

Dihydrofolate reductase was isolated from an amethopterin-resistant strain of Streptococcus faecium and was purified ca. 400-fold using conventional methods (1,2).

TPNH (P-L Biochemicals) was dissolved in 0.05 M Tris (Mann, ultrapure) buffer previously adjusted to pH 7.5 with HCl. Enzyme was diluted with the same Tris buffer and the molar concentrations determined based on a molecular weight of 19,500 (1,2).

CD measurements were made at 25° with a Cary model 60 spectropolarimeter with a circular dichroism attachment using 10 mm cells. Scans of the CD spectra were made at the slowest possible speed using the slit program in order to supply constant energy. Aliquots of TPNH were added directly to the enzyme solution and mixed well before the spectra were recorded. The TPNH additions were made such that dilution of the enzyme solutions never exceeded 3%. Molar ellipticity values, $[\theta]_\lambda$, were calculated from the following equation:

$$[\theta]_\lambda = \frac{\theta}{10} \times \frac{MW}{lc},$$ where θ = the measured ellipticity; MW = the molar weight of dihydrofolate reductase; l = cell path length in cm; c = concentration of the enzyme in g/ml.

RESULTS AND DISCUSSION

The near ultraviolet CD spectrum of dihydrofolate reductase shows a positive band system as indicated in curve 3 of Fig. 1. The side chain Cotton effect in the 260-310 nm region shows three distinct ellipticity bands centered around 305 nm, 295 nm and 270 nm, respectively. These features most probably are determined by the aromatic side chain transitions of tyrosyl or tryptophyl

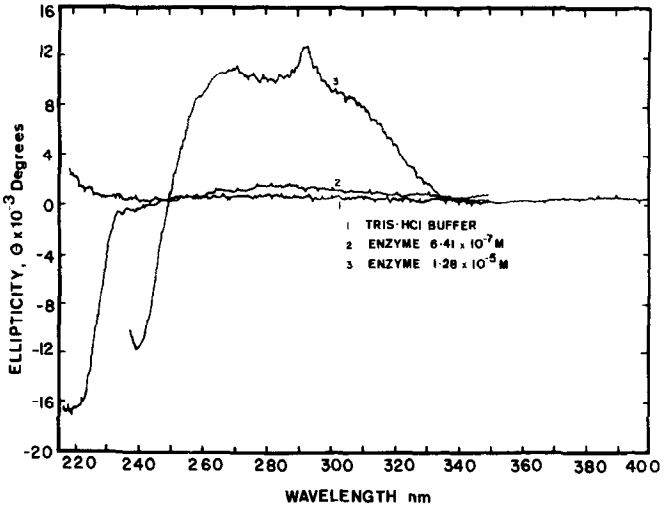

FIGURE 1. The circular dichroic spectrum of dihydrofolate reductase. The spectra were taken using 10 mm cells containing 3 ml of the indicated solutions.

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residues, since the reductase under study does not contain cystine residues (2). In addition to the positive aromatic side chain bands, the enzyme shows a negative CD band centered at ca. 220 nm, presumably due mainly to the intrinsic polypeptide backbone absorption (curve 2, Fig. 1). Another negative band centered around 240 nm has also been observed at relatively high enzyme concentrations. The transition involved at 240 nm is unknown, although phenylalanyl residues might be considered (8).

Studies by Glazer and Simmons (5,9) on egg white lysozyme have indicated that tryoptophyl residues contribute very highly in the 260-330 region of the CD spectrum. Studies by Kronman (10) and by Ikeda et al. (3,4) have shown that the aromatic side chain Cotton effect of lysozyme is composed of three distinct ellipticity bands, similar to the fine structure observed for dihydrofolate reductase in this region. Furthermore, Teichberg et al. (6) have shown that of the six tryptophans in lysozyme, tryptophan-108 is the main contributor to the CD pattern in the 190-300 nm range. Tyrosines were found to contribute very little to this region of the CD spectrum except at high pH when ionization occurred.

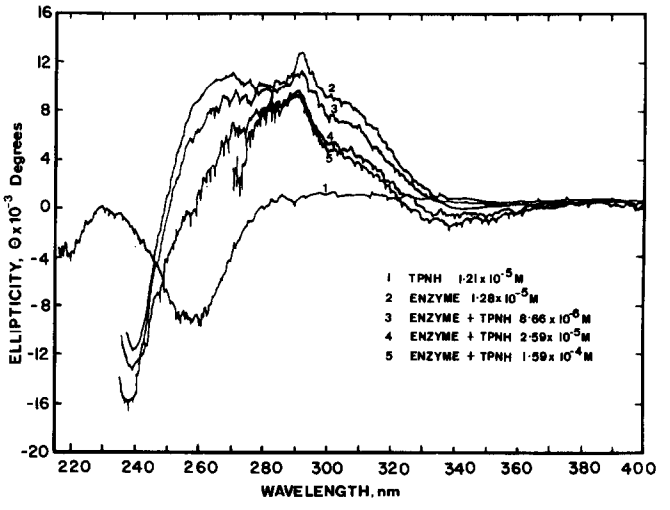

FIGURE 2. Circular dichroic titration of dihydrofolate reductase with TPNH. Increments of TPNH were added to a 3 ml solution of enzyme at the concentrations shown.

Results of the circular dichroic titration of dihydrofolate reductase with TPNH are shown in Fig. 2. Addition of successive increments of TPNH to the enzyme solution (curves 3-5) resulted in the generation of a negative extrinsic Cotton effect centered at ca. 340 nm, an absorption maximum of TPNH. Concomitantly, a decrease in the aromatic side chain Cotton effect was observed. At wavelengths below ca. 290 nm the spectrum is complicated by presence of the negative ellipticity band of TPNH at 260 nm (curve 1). This transition is thought to be due to the adenine moiety of TPNH (11). The binding of the coenzyme to the reductase does not result in any apparent changes in the 220 nm region (not shown in Fig. 2), 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 at a 1:1 stoichiometry. Decreases in ellipticity of the enzyme at 305 nm upon addition of increments of TPNH are indicated in Fig. 3. From these titration data a dissociation constant of 4×10^{-7} M for TPNH was obtained. This value agrees quite well with that determined by fluorescence studies of

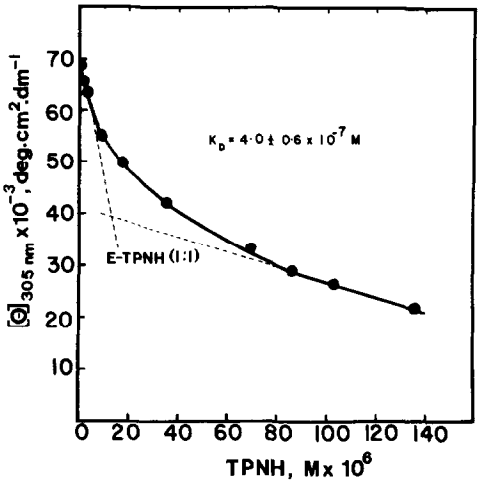

FIGURE 3. Decreases in the molar ellipticity at 305 nm of dihydrofolate reductase as a function of the concentration of TPNH added.

the enzyme-TPNH complex at much lower enzyme concentrations (D'Souza and Freisheim, unpublished results). These results suggest that tryptophyl residues may plan an important functional role in the binding of TPNH to dihydrofolate reductase, although the degree to which tyrosyl residues contribute to each of the three aromatic side chain ellipticity bands remains to be determined.

Ulmer et al. (12) and Li et al. (13) have shown that the binding of certain chromophoric molecules to asymmetric sites on enzyme molecules induces an extrinsic Cotton effect in the absorption bands of the bound chromophores.

The extrinsic Cotton effect generated at 340 nm upon addition of TPNH to dihydrofolate reductase appears to be solely dependent upon the concentration of the enzyme-coenzyme complex (cf. Fig. 2). It appears to be the first example of such a TPNH-generated extrinsic Cotton effect. Such an effect most probably occurs as a result of the induced asymmetry in the coenzyme molecule upon binding to a tryptophan-rich asymmetric site on the enzyme macromolecule.

The fine structure observed for dihydrofolate reductase in the 260-310 nm

region of the CD spectrum is similar to that obtained for native egg white lysozyme by Glazer and Simmons (5), Kronman (10) and Ikeda and Hamaguchi (3). In addition, the binding of saccharides has been shown to alter the aromatic region of the CD spectrum of lysozyme (3,5). This technique will also enable us to assess the contribution of tyrosyl residues to the aromatic side chain Cotton effect after suitable modification of tryptophyl residues in dihydrofolate reductase. In addition, the interaction of substrate analogues and inhibitors with the enzyme can be more closely examined.

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