J. Biosci., Vol. 11, Numbers 1-4, March 1987, pp. 265-274. © Printed in India.

Interaction of Cibacron Blue F3G-A and Procion Red HE-3B with sheep liver 5,10-methylenetetrahydrofolate reductase

K.VARALAKSHMI, H. S. SAVITHRI and N. APPAJI RAO*

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

Abstract. Cibacron Blue F3G-A, a probe used to monitor nucleotide binding domains in enzymes, inhibited sheep liver 5, 10-methylenetetrahydrofolate reductase competitively with respect to 5-methyltetrahydrofolate and NADPH. The K_i values obtained by kinetic methods and the K_d value for the binding of the dye to the enzyme estimated by protein fluorescence quenching were in the range 0·9-1·2 μM. Another triazine dye, Procion Red HE-3B interacted with the enzyme in an essentially similar manner to that observed with Cibacron Blue F3G-A. These results as well as the interaction of the dye with the enzyme monitored by difference spectroscopy and intrinsic protein fluorescence quenching methods indicated that the dye was probably interacting at the active site of the enzyme by binding at a hydrophobic region.

Keywords. Cibacron Blue F3G-A; Procion Red HE-3B; 5,10-methylenetetrahydrofolate reductase.

Introduction

5,10-Methylenetetrahydrofolate (5,10-CH_2-H_4 folate) reductase (EC 1·1·99·15), a flavoprotein catalyzes the reaction

$$5,10\text{-}CH_2\text{-}H_4 \text{ folate} + NADPH + H^+ \rightleftharpoons 5\text{-}CH_3\text{-}H_4 \text{ folate} + NADP^+.$$

The enzyme was earlier purified to homogeneity from sheep liver (Varalakshmi *et al.,* 1983) using affinity chromatography on Blue Sepharose matrix, a procedure similar to that described for the isolation of the enzyme from pig liver (Daubner and Matthews, 1982). The kinetic mechanism of the reaction catalyzed by the enzyme was established to be *bi-bi* ping-pong using initial velocity and product inhibition studies (Varalakshmi *et al.,* 1983) and this was in agreement with the mechanism suggested for the pig liver enzyme (Matthews and Haywood, 1979). The dye, Cibacron Blue F3G-A used as a ligand in the affinity matrix has been extensively employed to probe into the nucleotide binding domains of dehydrogenases and kinases (Rossmann *et al.,* 1974; Thompson *et al.,* 1975; Thompson and Stellwagen, 1976; Apps and Gleed, 1976; Stellwagen, 1977; Ashton and Polya, 1978; Lepo *et al.,* 1979). In addition, recent results seem to suggest that the dye could be interacting at additional sites on the protein especially in hydrophobic pockets (Chambers and Dunlap, 1979; Subramanian and Kaufman, 1980, Ramesh and Appaji Rao, 1980). The structural similarity of the dye with folate coenzymes as well as with antifolates used to probe the active site environment of dihydrofolate reductase (EC 1·5·1·3) and serine hydroxymethyltransferase (EC 2·1·2·1) suggested that the dye might interact in the region of the active site of enzymes requiring folate coenzymes as substrates. In

*To whom all correspondence should be addressed.

Abbreviations used: 5,10-CH_2-H_4 folate, 5,10-Methylenetetrahydrofolate; 5-CH_3-H_4 folate, 5-methyl-tetrahydrofolate.

the case of dihydrofolate reductase (Chambers and Dunlap, 1979; Subramanian and Kaufman, 1980), it was shown that the dye can bind at folate binding domain of the enzyme from chicken liver (Chambers and Dunlap, 1979) or in a manner which overlaps the pyridine nucleotide and dihydrofolate binding domains in the enzyme from *Lactobacillus casei* (Subramanian and Kaufman, 1980). Since 5,10-CH_2-H_4 folate reductase also binds to NADPH and 5-CH_3-H_4 folate (Matthews and Haywood, 1979; Daubner and Matthews, 1982; Varalakshmi *et al.,* 1983), it could be predicted that the dye might interact with one of the substrate binding sites or in a manner overlapping both the sites. Procion Red HE-3B, another triazine dye has been used as an affinity ligand to purify NADP-dependent dehydrogenases (Ashton and Polya, 1978). This paper reports the interaction of sheep liver 5,10-CH_2-H_4 folate reductase with Cibacron Blue F3G-A and Procion Red HE-3B monitored by kinetic, spectroscopic and fluorimetric methods.

Materials and methods

All the chemicals used were of analytical grade and purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Cibacron Blue F3G-A was a gift from CIBA-GEIGY (Basel, Switzerland).

Purification

5,10-CH_2-H_4 folate reductase was purified from sheep liver to homogeneity by ammonium sulphate fractionation, acid precipitation (pH 4·5), diethylaminoethyl Sephacel ion-exchange and Blue Sepharose affinity chromatography (Varalakshmi *et al.,* 1983).

Enzyme assays

Activity of 5,10-CH_2-H_4 folate reductase was measured either using radioactive [^{14}C]-5-CH_3-H_4 folate or by measuring the oxidation of NADPH spectrophoto metrically in the presence of a suitable electron acceptor such as dichlorophenolindo-phenol or menadione (Varalakshmi *et al.,*1983).

Difference spectrum

The enzyme (1 mg/ml) was taken in sample cuvette in 0·05 M potassium phosphate buffer (pH 7·2) containing 0·3 mM EDTA and the same volume of buffer was taken in reference cuvette and the base line was recorded using a Shimadzu spectrophoto meter. From a stock solution (1 mM) of Cibacron Blue or Procion Red, equal amounts of dye were added to both the cuvettes and the difference spectrum recor ded. Appropriate correction for dilutions were made. When the effect of ligands on dye difference spectra was studied, the ligand (at equal concentration) was added to both the cuvettes.

Fluorescence measurements

Fluorescence spectra were recorded using a Perkin-Elmer model 203 fluorescence spectrophotometer, at room temperature (26±2°C) in 3 ml cuvettes. Stern-Volmer plot (Lehrer, 1971) was used to calculate the K_d values for Cibacron Blue and Procion Red dyes in fluorescence quenching experiments. The concentration of Cibacron Blue F3G-A was determined using $\varepsilon = 13{\cdot}6 \times 10^3$ cm^{-1} M^{-1}. Care was taken to ensure that the absorbance of the reaction mixture was less than 0·1 at both the excitation and emission wavelengths to eliminate internal filter effects and non specific quenching.

Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Results

Kinetic analysis

Inhibition of the reductase by Cibacron Blue: The inhibition of the activity of the enzyme by the dye was examined by assaying the residual activity at different fixed concentrations of Cibacron Blue (0, 4, 6, 8 and 12 μM) at varying concentrations of 5-CH_3-H_4 folate (0-10 mM). The Lineweaver-Burk plots shown in figure 1 converged at a single point on the Y-axis and a replot of the slopes of the Lineweaver-Burk plots *v*s the concentration of Cibacron Blue gave a straight line indicating that the dye was a linear competitive inhibitor with a K_i value of 1·2 μM (inset figure 1). Similar kinetic analysis in the NADPH-menadione reductase assay (Varalakshmi *et al.,* 1983) using 0, 1, 2, 3 and 4 μM Cibacron Blue and varying concentrations of NADPH (20-150 μM) showed that the dye was a linear competitive inhibitor with respect to NADPH also. A K_i value of 0·9 μM was calculated from the slope replot (figure 2).

Inhibition by Procion Red: In order to compare the kinetics of the inhibition of the enzyme activity by Procion Red and Cibacron Blue, similar experiments were carried out using Procion Red. When 5-CH_3-H_4 folate was varier at different fixed concentrations of Procion Red (2, 4 and 6 μM), it showed competitive type of in hibition (figure 3). From the replot of slopes (inset figure 3), the K_i value for the dye was calculated to be 1 μM.

Difference spectral studies

Cibacron Blue F3G-A has a characteristic visible spectrum (– –) with a maximum at 610 nm (figure 4A) and the enzyme has little or no absorbance between 500-800 nm. It can be seen from figure 4A, that the difference spectrum (—) has a characteristic maximum at 690 nm and a minimum around 600 nm. This characteristic difference spectrum permitted a titration of the dye binding to the enzyme. It can be seen from the figure that Cibacron Blue caused a significant change in difference spectrum and the absorbance increased in a hyperbolic manner with increasing concentrations of

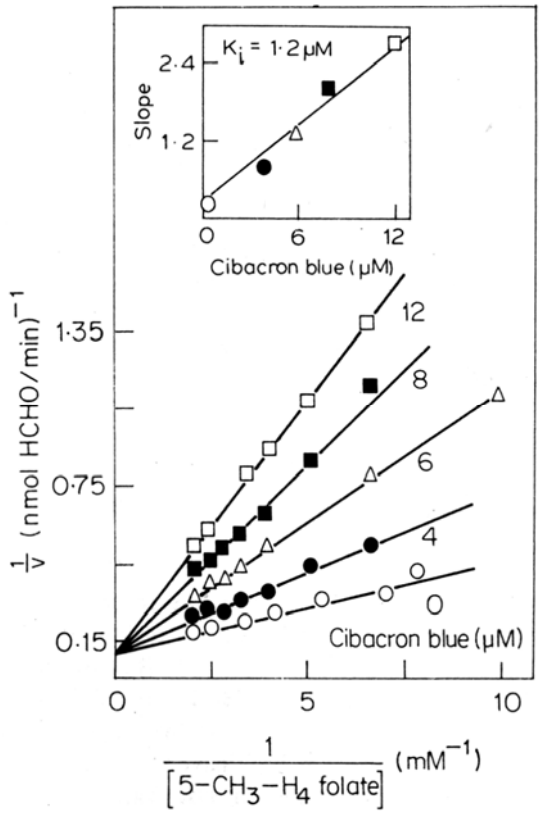

Figure 1. Competitive inhibition of the activity of 5,10-CH_2-H_4 folate reductase by Cibacron Blue F3G-A when 5-CH_3-H_4. folate was the varied substrate.

The enzyme (5 μg) in 0·2 M potassium phosphate buffer pH 7·2 containing 0·3 mM EDTA 0·2 M ascorbate, 50 μM FAD was preincubated with different fixed concentrations of the Cibacron Blue (O, none; ●, 4μM; Δ, 6μ M; ■, 8μM; □,12μM) at 37°C for 5 min and the reaction was started by the addition of various concentrations of [^{14}C]-5-CH_3-H_4 folate and the amount of [^{14}C]-HCHO formed was estimated (Varalakshmi *et al.,* 1983). Inset shows a replot of the slope of the reciprocal plots *vs* the corresponding concentration of Cibacron Blue F3G-A.

Cibacron Blue (figure 4) and the K_d value (14 μM) was calculated from a double reciprocal plot (figure 4B).

The kinetic studies (figure 1) revealed that 5-CH_3-H_4 folate was interacting competitively with Cibacron Blue. It was therefore of interest to examine whether the spectral changes induced by the dye (20 μ M) upon its binding to the enzyme could be reversed by 5-CH_3-H_4 folate. 5-CH_3-H_4 folate (0·6, 1·0 and 1·4 mM) was added to both the sample and reference cuvettes and the spectra were recorded. 5-CH_3-H_4 folate could reverse the difference spectrum caused by the binding of the dye to the enzyme (data not given). The concentrations of 5-CH_3-H_4 folate were approximately in the range used for the enzyme assays (0·5 mM). At this concentration of the dye (20 μM) and 5-CH_3-H_4 folate (0·5 mM) the enzyme activity was inhibited to more than 50% indicating that much higher concentrations may be necessary for complete reversal. Similarly the second substrate, NADPH could also partially reverse the

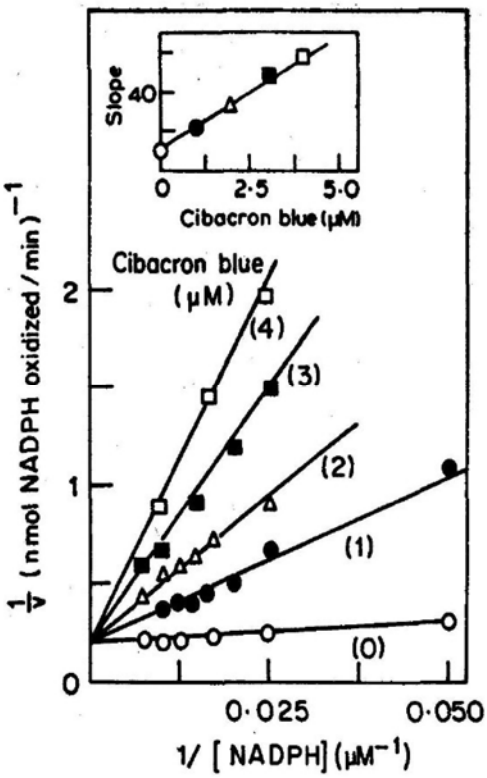

Figure 2. Inhibition of the enzyme by Cibacron Blue F3G-A when NADPH concentrations were varied.

The enzyme (2 μg) in 0·3 M potassium phosphate bufer pH 7·2 containing 0·3 mM EDTA, 0·2 mM ascorbate, 50 μM FAD was preincubated with different fixed concentrations of Cibacron Blue (O, none; ●, 1 μM; Δ, 2μM; ■, 3μM; □, 4 μM) and with various concentrations of NADPH (10-150 μM) for 5 min at 25°C. The reaction was started by adding a saturating concentration (125 μM) of menadione. The absorbance change was recorded at 343 nm using a Cary 219 Recording Spectrophotometer. The amount of NADPH consumed was calculated using an $\in$ value of $6{\cdot}22 \times 10^3 M^{-1} cm^{-1}$. Inset shows the replot of slope *vs* Cibacron Blue concentration.

spectral changes. The elution of the enzyme from the Blue Sepharose column by 2 M KCl suggested that electrostatic interactions may also be involved in dye binding. The addition of different concentrations (0·5-2 M) of KCl progressively decreased the intensity of the peak (690 nm) in the dye difference spectrum and at a concentration of 2 M, the signal disappeared completely (figure 4C).

The absorption spectrum of Procion Red exhibited two maxima at 510 nm and 540 nm whereas the difference spectrum of the dye in the presence of the enzyme showed a red shift with two peaks at 520 and 570 nm (data not given). This difference spectrum was partially reversed by NADPH.

Fluorescence studies

An additional convenient method for measuring macromolecular interactions with

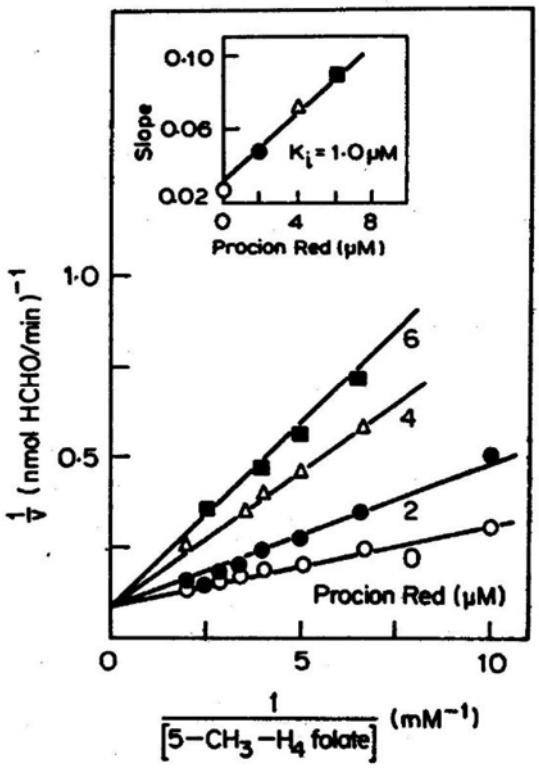

Figure 3. Procion Red, a competitive inhibitor for the activity of 5,10-CH_2-H_4 folate reductase.

The enzyme (6 μg) in 0·2 M potassium phosphate buffer pH 7·2 containing 0·3 mM EDTA, 0·2 M ascorbate, 50 μM FAD was preincubated with different fixed concentrations of Procion Red dye (O, none; ●, 2 μM; Δ, 4μM; ■, 6 μM) for 5 min at 37°C and the reaction was started by the addition of varying concentrations (0·05–0·5mM) of [^{14}C]-5-CH_3-H_4 folate and the [^{14}C]-HCHO formed was estimated (Varalakshmi *et al.,* 1983). Inset shows the replot of slopes *vs* Procion Red concentration.

ligands is the quenching of intrinsic protein fluorescence. The enzyme has a fluo rescence excitation maximum at 280 nm and an emission maximum at 330 nm due to the presence of tryptophan residues in a hydrophobic environment (figure 5). The dye binding was monitored by measuring the fluorescence quenching of the enzyme (50 μg/ml) upon the addition of different concentrations of Cibacron Blue. It can be seen from figure 5, that the dye causes a quenching in the intensity of the fluorescence at 330 nm. These data were used to construct a modified Stern-Volmer plot (figure 5A). A K_d value of 1·2 μ M from the slope and an *'n'* value of 1 per subunit from the Y-axis intercept were calculated. It can be seen from figure 5B that the increasing concentrations of KCl (0·02-0·4 M) reversed the fluorescence quenching caused by 2·5 μM Cibacron Blue.

From a similar fluorescence studies for the Procion Red dye (figure 6), a K_d value of 1·2 μM and an *'n'* valueof 1 were calculated (inset figure 6). KCl reversed the quenching caused by Procion Red dye also.

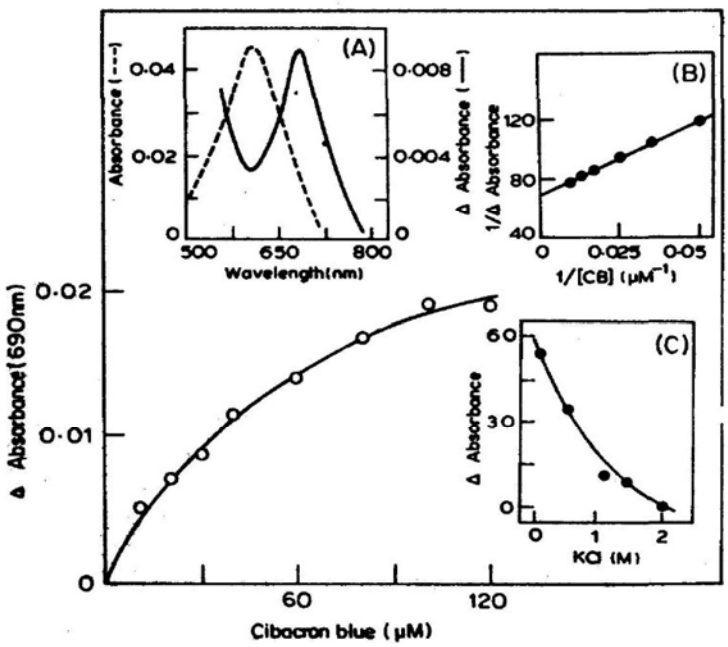

Figure 4. Binding of Cibacron Blue to 5,10-CH_2-H_4 folate reductase.

Enzyme (1 mg) in 1 ml of 0·05 M potassium phosphate buffer pH 7·2 containing 0·3 mM EDTA was taken in the sample cuvette and 1 ml of the same buffer was taken in the reference cuvette and the base line was recorded from 500-800 nm. Equal amounts of Cibacron Blue in the concentration range (10–120 μM) were added to both the cuvettes and the difference spectra were recorded. The absorbance change at 690 nm was plotted against Cibacron Blue concentration. The inset (A) shows the spectrum (– – –) of Cibacron Blue and (——) shows the difference spectrum when the enzyme was added to the dye solution. The inset (B) shows the double reciprocal plot for this data. The inset (C) shows the reversal of quenching of enzyme-dye difference spectrum by KCl.

Discussion

The sulphonated polyaromatic chlorotriazine dye, Cibacron Blue F3G-A has been extensively used for the purification of dehydrogenases and initially it was used as a probe to identify the nucleotide binding domains (Rossmann *et al.,* 1974; Thompson *et al.,* 1975; Thompson and Stellwagen, 1976; Apps and Gleed, 1976; Stellwagen, 1977; Ashton and Polya, 1978; Lepo *et al.,* 1979). But later work indicated that it was also interacting at hydrophobic regions of protein molecules (Chambers and Dunlap, 1979; Subramanian and Kaufman, 1980; Ramesh and Appaji Rao, 1980). A judicious combination of polyaromatic (nonpolar) and sulfonate (ionic) groups in the same molecule, enabled the use of the dye to obtain detailed information on the topology of interacting regions of several enzymes by employing a variety of physical and kinetic methods (Krakow, 1965; Jacobsberg *et al.,* 1975; Bornmann and Hess, 1977). The structural similarity of the triazine moiety with folate coenzymes prompted an

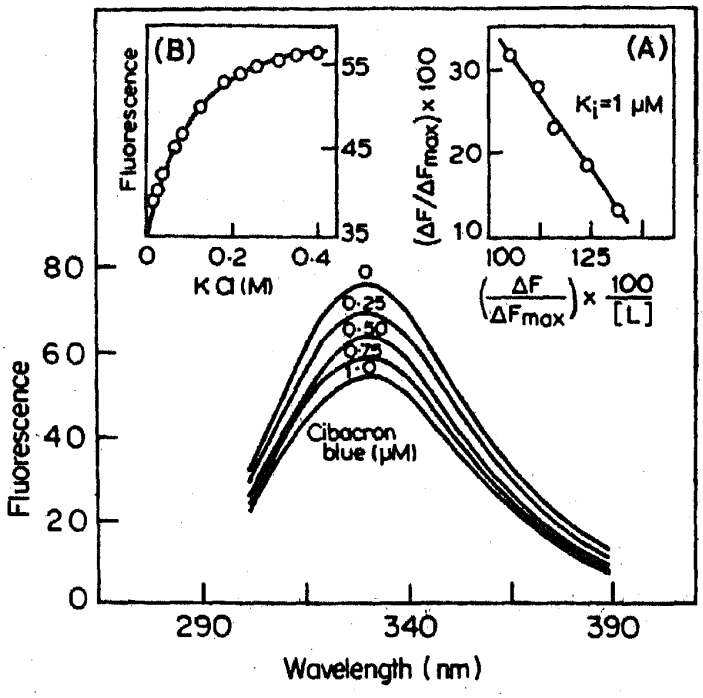

Figure 5. Quenching of the fluorescence of the enzyme by Cibacron Blue F3G-A.

To the enzyme (50 μg/ml) in 0·05M potassium phosphate buffer containing 0·3 mM EDTA, different concentrations of Cibacron Blue (0·25-1 μM) were added and the fluorescence emission spectra were recorded. The inset (A) shows the Stern-Volmer plot for the data. The inset (B) shows the reversal of fluorescence quenching by KCl.

examination of the interaction of the dye with dihydrofolate reductase (Chambers and Dunlap, 1979; Subramanian and Kaufman, 1980) and serine hydroxymethyl transferase (Ramesh and Appaji Rao, 1980) and these studies revealed that the dye binding was unique in each case. The *bi-bi* ping-pong mechanism for the reaction catalyzed by 5,10-CH_2- H_4 folate reductase with NADPH as the first substrate to add and 5-CH_3-H_4 folate as the last product to leave the enzyme (Varalakshmi *et al.,* 1983), enabled the prediction that the dye would be a competitive inhibitor with respect to both 5-CH_3-H_4 folate and NADPH. The results of kinetic analysis for the inhibition by the dye (figures 1 and 2) are in accordance with this prediction. Further evidence to suggest that the dye was binding to the free form of the enzyme was the characteristic differences spectrum of enzyme-dye complex (figure 4A). However, the spectrum was qualitatively difference from those obtained with other protein-Cibacron Blue complexes. The peak position in this case was at 690 nm, when compared to 700 nm in chicken liver dihydrofolate reductase (Subramanian and Kaufman, 1980), 645 nm in *L. casei* dihydrofolate reductase (Chambers and Dunlap, 1979) and 670 nm in monkey liver serine hydroxymethyltransferase (Ramesh and Appaji-Rao, 1980). The red shift indicates that the dye was probably binding in a hydrophobic region of the molecule. The position of spectral troughs were also difference in all the cases. These distinct difference spectra highlight the uniqueness in the dye binding environment of these enzymes. The mode of binding of the dye could be deduced from the characteristics of the differences spectrum. The difference spectrum of

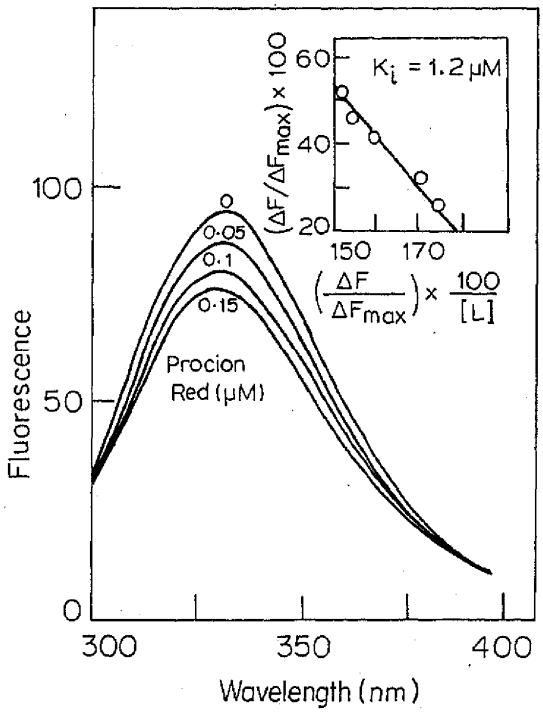

Figure 6. Fluorescence quenching by Procion Red dye.

To the enzyme (50 μg/ml) in 0·05 M potassium phosphate buffer pH 7·2 containing 0·3 mM EDTA, different concentrations of Procion Red dye were added and the fluorescence emission spectra were recorded. The inset shows the Stern-Volmer plot for this data.

Cibacron Blue closely resembles the dye difference spectrum in the presence of high salt which is called ionic spectrum (Subramanian, 1982). This ionic effect is caused by the shielding of sulfonate groups by the high concentration of sodium ions. This observation indicates that the dye might also be binding in an electrostatic manner to the enzyme.

The binding of the dye to the enzyme was confirmed by the quenching of the intrinsic protein fluorescence and a K_d value of 1·2 μM was calculated (figure 5A). The similarity in the *KK* values (1·2 and 0·9 μM) determined from the kinetic experiments (figures 1 and 2) and K_d value (1·2 μM) obtained by fluorescence studies (figure 5A) suggested that these probes were probably measuring a similar binding site on the enzyme.

The K_d value obtained by a double reciprocal plot of the spectroscopic data (figure 4B) was 14 μM compared to the values in the range of 0·9-1·2 μM obtained by other methods. The K_d value calculated for the same set of data by the method of Thompson and Stellwagen (1976) was 6 μM. Similar discrepancy was observed in the data of Chambers and Dunlap (1979). A recalculation of the constants from the data of Chambers and Dunlap (1979) for the binding of Cibacron Blue to dihydrofolate reductase by a double reciprocal plot analysis was 5 μM and the K_d value obtained by using the method of Thompson and Stellwagen (1976), was 0·13 μM indicating the inherent difficulties in the analysis of spectroscopic data for obtaining the K_d values for the binding of this dye to proteins.

The competitive inhibition (figures 1-3), almost identical K_i and K_d values (figures 5 and 6) and the reversal of spectral changes by NADPH and 5-CH_3-H_4 folate strongly suggest that Cibacron Blue and Procion Red are probably interacting at the active site of the reductase. However, there was no enhancement in the reversal when NADPH and 5-CH_3-H_4 folate were added together suggesting that the dye, NADPH and 5-CH_3-H_4 folate were probably binding at the same domain in the enzyme but not to identical residues. The bulky atomatic groups in the dye and its structural similarity with both the substrates might direct the dye to the hydrophobic region of the active site of 5,10-CH_2-H_4 folate reductase and binding could be further strengthened by electrostatic interaction between the charged groups of the dye and the amino acid residues of the enzyme.

Acknowledgements

We are grateful to the Department of Science and Technology, New Delhi, for financial support.

References

Apps, D. K. and Gleed, C. D. (1976) *Biochem. J.,* **159,** 441
Ashton, A. R. and Polya, G. M. (1978) *Biochem. J.,* **175,** 501.
Bornmann, L. and Hess, B. (1977) *Z. Naturforsch.,* **C32,** 756.
Chambers, B. B. and Dunlap, R. B. (1979) *J. Biol. Chem.,* **254,** 6515.
Daubner, S. C. and Matthews, R. G. (1982) *J. Biol. Chem.,* **257,** 140.
Jacobsberg, L. B., Kantrowitz, E. R. and Lipscomb, W. N. (1975) *J. Biol. Chem.,* **250,** 9238.
Krakow, J. S. (1965) *Biochim. Biophys. Acta,* **95,** 532.
Lehrer, S. S. (1971) *Biochemistry,* **10,** 3254.
Lepo, J. E., Stacey, G., Wyss, O. and Tabita, F. R. (1979) *Biochim. Biophys. Acta,* **568,** 428.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.,* **193,** 265.
Matthews, R. G. and Haywood, B. J. (1979) *Biochemistry,* **18,** 4845.
Ramesh, K. S. and Appaji Rao, N. (1980) *Biochem. J.,* **187,** 249.
Rossmann, M. G., Moras, D. and Olsen, K. W. (1974) *Nature (London),* **250,** 194.
Stellwagen, E. (1977) *Acc. Chem. Res.,* **10,** 92.
Subramanian, S. (1982) *Arch. Biochem. Biophys.,* **216,** 116.
Subramanian, S. and Kaufman, B. T. (1980) *J. Biol. Chem.,* **255,** 10587.
Thompson, S. T., Cass, K. H. and Stellwagen, E. (1975) *Proc. Natl. Acad. Sci. USA,* **72,** 669.
Thompson, S. T. and Stellwagen, E. (1976) *Ptoc. Natl. Acad. Sci. USA,* **73,** 361.
Varalakshmi, K., Savithri, H. S. and Appaji Rao, N. (1983) *J. Biosci.,* **5,** 287.