Inhibition of Monkey Liver Serine Hydroxymethyltransferase by Cibacron Blue 3G-A

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Cibacron Blue 3G-A inhibited monkey liver serine hydroxymethyltransferase competitively with respect to tetrahydrofolate and non-competitively with respect to L-serine. NADH, a positive heterotropic effector, failed to protect the enzyme against inhibition by the dye and was unable to desorb the enzyme from Blue Sepharose CL-6B gel matrix. The binding of the dye to the free enzyme was confirmed by changes in the dye absorption spectrum. The results indicate that the dye probably binds at the tetrahydrofolate-binding domain of the enzyme, rather that at the 'dinucleotide fold'.

Serine hydroxymethyltransferase (EC 2.1.2.1) catalyses the reaction:

$$\text{L-Serine} + \text{H}_4\text{folate} \rightleftharpoons \text{glycine} + 5,10\text{-CH}_2\text{-H}_4\text{folate}$$

and was purified from monkey liver by Blue Sepharose affinity chromatography (Ramesh & Appaji Rao, 1980). The enzyme exhibited positive homotropic co-operative interactions with H_4folate, and reduced nicotinamide nucleotides were positive heterotropic effectors (Ramesh & Appaji Rao, 1978). The similarities in the structure of this dye (Cibacron Blue 3G-A) and NAD^+ suggested that the dye was interacting at the NAD^+-binding domain ('dinucleotide fold') of several dehydrogenases (Rossmann *et al.*, 1974; Thompson *et al.*, 1975; Thompson & Stellwagen, 1976; Stellwagen, 1977) and at the ATP-binding site of kinases (Apps & Gleed, 1976; Ashton & Polya, 1978; Lepo *et al.*, 1979). However, other studies have demonstrated that the dye may be interacting at additional sites on the protein (Wilson, 1976; Beissner & Rudolph, 1978).

In the present paper we report the interaction of Cibacron Blue 3G-A at the H_4folate-binding domain of serine hydroxymethyltransferase, a pyridoxal phosphate-dependent enzyme.

Experimental

Materials

Cibacron Blue 3G-A was a gift from CIBA–GEIGY (Basel, Switzerland). Blue Sepharose CL-6B

Abbreviations used: H_4folate, ($\pm$)-L-tetrahydrofolate; H_2folate, dihydrofolate; 5,10-CH_2-H_4folate, N^5N^{14}-methylenetetrahydrofolate.

was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). DL-[3-¹⁴C]Serine (specific radioactivity 48.5 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). H_4folate, prepared by the method of Hatefi *et al.* (1960), was generously given by Dr. J. H. Mangum (Brigham Young University, Provo, UT, U.S.A.). Pyridoxal phosphate, EDTA (disodium salt) and 2-mercaptoethanol were products of Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Methods

Serine hydroxymethyltransferase from Bonnet-monkey (*Macaca radiata*) liver was purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation (25–50% saturation), adsorption on and elution from CM-Sephadex, gel filtration on Ultrogel AcA34 and affinity chromatography on Blue Sepharose CL-6B. The enzyme (3.3 units/mg) was homogeneous on polyacrylamide-gel disc electrophoresis, isoelectrofocusing, ultracentrifugation, gel filtration, immunodiffusion and immunoelectrophoresis. The enzyme was assayed as described previously (Ramesh & Appaji Rao, 1978), and 1 unit of enzyme activity was defined as the amount that catalysed the formation of 1 μmol of formaldehyde/min at 37°C at pH 7.4. Protein concentrations was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

The concentration of Cibacron Blue 3G-A was determined spectrophotometrically at 610 nm, a molar absorption coefficient of 13.6 mM⁻¹·cm⁻¹ being used. Difference absorption spectra were recorded at 25°C in a Beckman model 26 spectrophotometer. Exactly 1 ml of protein solution and 1 ml of protein solvent were placed in the sample and

reference cuvettes (1 cm path length) respectively and the baseline difference spectrum was recorded. Identical volumes of a concentrated solution of Cibacron Blue 3G-A were added to both the cuvettes and the difference spectrum was recorded.

Enzyme solutions were extensively dialysed against 50 mM-potassium phosphate buffer (pH 7.4)/1 mM-EDTA/1 mM-2-mercaptoethanol and passed through Millipore filters (type HA; 0.45 μm) before spectral measurements.

Results

Serine hydroxymethyltransferase from monkey liver binds to Blue Sepharose columns very strongly (Ramesh, 1979). The positive allosteric heterotropic effectors of this enzyme, namely NADH, NADPH and L-serine, up to 10 mM failed to desorb the enzyme from the affinity-gel matrix. Elution with H₄folate could not be attempted in view of the extreme instability of this compound to oxidation in air. However, KCl (0.4–0.6 M) eluted the enzyme from the affinity-gel matrix.

It is evident from the absorption spectrum of the dye bound to serine hydroxymethyltransferase (Fig. 1) that a red-shift has occurred, with a maximum at 670 nm. The dye absorbs maximally at 610 nm and the absorbance at this wavelength was decreased on binding to the enzyme. The absorbance at 670 nm increased linearly with increasing concentration of the dye. The binding of the dye to the enzyme was
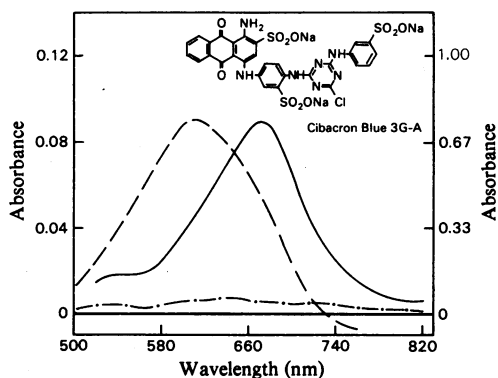

Fig. 2. *Competitive inhibition of serine hydroxymethyltransferase by Cibacron Blue 3G-A*

The enzyme (1 μg) in 0.05 M-potassium phosphate buffer, pH 7.4, containing 1 mM-2-mercaptoethanol and 1 mM-EDTA, was preincubated with L-[3-^{14}C]-serine (3.6 mM) for 5 min at 37°C, followed by different fixed concentrations of the dye for an additional 5 min: ●, none; ○, 5 μM; ▲, 10 μM; △, 20 μM; ■, 40 μM; □, 72 μM. The reaction was started by the addition of various concentrations (0.7–2.25 mM) of H₄folate. After incubation for 15 min, [^{14}C]formaldehyde was determined (Taylor & Weissbach, 1965). Inset (i) shows a secondary replot of slope versus Cibacron Blue 3G-A concentrations. Inset (ii) shows the inhibition of the enzyme activity at saturating concentrations of H₄folate (1.8 mM) and L-serine (3.6 mM) by Cibacron Blue 3G-A (0–0.5 mM).

Fig. 1. *Difference spectra of Cibacron Blue 3G-A in the presence of serine hydroxymethyltransferase from monkey liver*

The sample cuvette contained enzyme (1 mg/ml), and both sample and reference cuvettes contained (1) no Cibacron Blue 3G-A (baseline, ·——·—) or (2) 0.25 mM-Cibacron Blue 3G-A (————) (left-hand scale). The spectrum of free Cibacron Blue 3G-A (0.05 mM) is shown (– – –) for comparison (right-hand scale). Inset shows the structure of Cibacron Blue 3G-A.
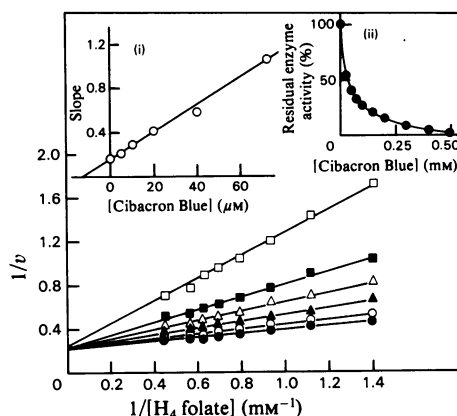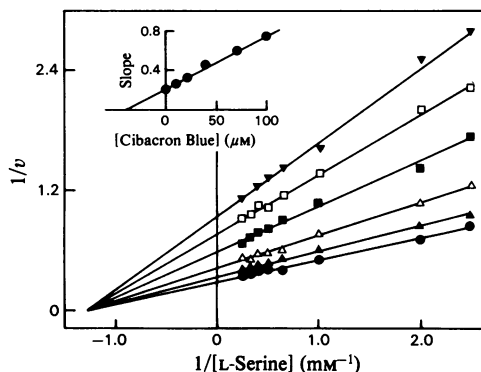

Fig. 3. *Non-competitive inhibition of serine hydroxymethyltransferase by Cibacron Blue 3G-A*

The enzyme was preincubated with various concentrations of L-serine (0.4–4 mM), followed by different fixed concentrations of the dye (●, none; ▲, 10 μM; △, 20 μM; ■, 40 μM; □, 70 μM; ▼, 100 μM), and enzyme activity was assayed at saturating concentration of H₄folate (1.8 mM) as described in the legend for Fig. 2. The inset shows the replot of slopes of the double-reciprocal plot versus Cibacron Blue 3G-A concentrations.

indicated by alterations in the c.d. spectrum of the dye as well as of the enzyme and by changes in the electrophoretic mobility of the enzyme (results not given).

Cibacron Blue 3G-A completely inhibited the activity of monkey liver serine hydroxymethyltransferase (inset to Fig. 2). This inhibition was not time-dependent and was completely reversible by dialysis against buffer containing 0.2 M-KCl. Allosteric effectors, namely NADH, NADPH and L-serine (10 mM), did not decrease the extent of the inhibition. Steady-state kinetic analysis revealed that Cibacron Blue 3G-A competitively inhibited the enzyme when H_4folate was the varied substrate (Fig. 2) and non-competitively when L-serine was the varied substrate (Fig. 3). Secondary slope replots were linear, and K_i values of 13 μM and 40 μM were calculated when H_4folate and L-serine were the varied substrates respectively.

Discussion

Cibacron Blue 3G-A, a reactive sulphonated polyaromatic chlorotriazine dye, has been attached to various inert supports for the purification of a variety of enzymes and proteins (Baird et al., 1976; Seelig & Colman, 1977; Stellwagen, 1977; Reyes & Sandquist, 1978; Young & Webb, 1978; Appukuttan & Bachhawat, 1979; Chauvin et al., 1979; Erickson & Paucker, 1979; Kulbe & Schuer, 1979; Lepo et al., 1979; Reddy et al., 1979). It is well documented that Cibacron Blue 3G-A binds at the protein supersecondary structure known as the 'dinucleotide fold' (Thompson et al., 1975; Thompson & Stellwagen, 1976; Wilson, 1976; Stellwagen, 1977). A number of dyes and conjugated ring systems (e.g. Beibrich Scarlet, Bromophenol Blue and tetraiodofluorescein) have been used to explore the hydrophobic environment of catalytic and effector sites of proteins [e.g. α-chymotrypsin (Glazer, 1967), lysozyme (Rossi et al., 1969). Aspartate aminotransferase (Harruff & Jenkins, 1976), aspartate carbamoyltransferase (Jacobsberg et al., 1975)]. The observation that Blue Sepharose affinity chromatography could be used to purify serine hydroxymethyltransferase, the inability to elute the enzyme bound to Blue Sepharose with NADH and the failure of NADH to reverse or diminish inhibition by the dye suggested that Cibacron Blue 3G-A may be interacting at a site(s) other than a 'dinucleotide fold' or the allosteric site. The dye absorption spectral studies at least indicate the presence of a hydrophobic pocket, which the dye occupies in order to produce a perturbation in the absorption spectrum of the dye chromophore. Competitive inhibition with H_4folate and non-competitive inhibition with L-serine suggested that the H_4folate-binding domain on the enzyme may be overlapping with the Cibacron Blue 3G-A-inter-

acting site. It is noteworthy in this connection that Cibacron Blue 3G-A inhibited bovine liver dihydrofolate reductase (EC 1.5.1.3) competitively with respect to H_2folate and non-competitively with respect to NADPH (Wilson, 1976). Chambers & Dunlap (1979) indicated that Cibacron Blue 3G-A interacted with the aminopterin-resistant Lactobaccilus casei dihydrofolate reductase in a manner partially overlapping both the H_2folate-binding site and the nicotinamide nucleotide-binding site. Chauvin et al. (1979) showed that folate competes with Cibacron Blue 3G-A for the NADH-binding site of bovine liver quinonoid dihydropterin reductase. All these folate reductases require nicotinamide nucleotide as a co-substrate. Even in the case of yeast hexokinase (EC 2.7.1.1), where no 'dinucleotide fold' exists, Cibacron Blue 3G-A interacts probably at the adenine nucleotide-binding site (Steitz et al., 1976; Wilson, 1976).

Our observations demonstrate for the first time that Cibacron Blue 3G-A binding occurs to an enzyme that does not utilize either nicotinamide nucleotide or adenine nucleotide as a co-substrate. The results presented clearly show that the interaction of Cibacron Blue 3G-A occurs at the H_4folate-binding domain of serine hydroxymethyltransferase and not at the NADH-binding site or at the 'dinucleotide fold'.

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