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STUDIES ON THE INTERACTION OF CIBACRON BLUE AND PROCION RED WITH DOPAMINE β-MONOOXYGENASE *

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Summary

1. The interaction of Procion Red HE3B and three isomers of Cibacron Blue with the water-soluble form of the copper enzyme dopamine β-monooxygenase (3,4-dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1) was studied by enzyme inhibition, difference spectroscopy and binding of enzyme to the immobilized dyes.

2. Cibacron Blue 3GA gave noncompetitive inhibition with both tyramine and ascorbate as the variable substrates (competitive inhibitor constants of 1—4 μM and uncompetitive inhibitor constants of 5—7 μM).

3. Difference spectral titration of the apoenzyme (Cu-depleted) with Cibacron Blue 3GA indicated binding of four dye molecules per enzyme tetramer and gave Scatchard plots with upward curvatures, which implies that the dye either interacts at different classes of sites or that there is a negative cooperativity in the binding. The holoenzyme binds about eight dye molecules per tetramer. Addition of $CuSO_4$ to the apoenzyme indicated that the enzyme must attach five or slightly fewer copper atoms per tetramer to obtain the maximal binding of eight dye molecules per tetramer. The dissociation constant for the dye binding to the four Cu-dependent sites is in the same range as the competitive inhibitor constants, thus indicating that this binding of the dye is at the active site. These results indicate that dopamine β-monooxygenase contains four active sites per tetramer with one copper atom per active site.

4. Different isomers of Cibacron Blue gave dissimilar difference spectra with dopamine β-monooxygenase, thus emphasizing the importance of using pure isomers of the dye when studying the interactions with proteins.

5. The results presented indicate that Cibacron Blue can bind strongly to

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* Dopamine β-monooxygenase is more commonly known as dopamine β-hydroxylase.
Abbreviation: Mes, 2-(*N*-morpholino)ethanesulfonic acid.

proteins which do not contain nucleotide binding sites and that electrostatic interactions are important for the binding of this dye to dopamine β-monooxygenase.

Introduction

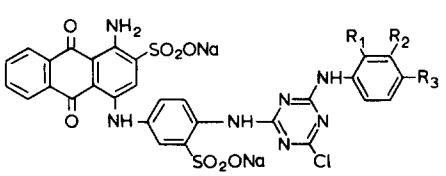
The dye Cibacron Blue F3GA, which is a mixture of two isomers (Fig. 1), has a high affinity to many proteins. The dye has therefore been coupled to agarose to obtain affinity-chromatographic gels, which have been frequently used in the purification of proteins. See Ref. 1 for a recent review on protein purification using immobilized Cibacron Blue and other triazine dyes.

Cibacron Blue is structurally similar to NAD, and it has been postulated that both molecules bind to proteins at the same site [2]. In agreement with this proposal, Biellmann et al. [3] recently showed by X-ray crystallography that the *para*-isomer of the dye binds mainly to the nucleotide binding domain of alcohol dehydrogenase (EC 1.1.1.1) in a manner partly similar to the binding of NAD^+; the *meta*-isomer showed, however, only a very weak binding if any.

Procion Red HE3B (see structure in Ref. 1) is another triazine dye which has been used as an affinity absorbent. This red dye has been suggested to be complementary to Cibacron Blue such that Procion Red is $NADP^+$-specific, whereas Cibacron Blue is NAD^+-specific [4].

Many dehydrogenases and kinases possess a supersecondary structure-folding associated with the binding sites of nucleotides. This folding is known as the dinucleotide binding fold, and it has been postulated that Cibacron Blue may be used as a probe for the presence of such a binding fold [2]. Other investigators have, however, claimed that the dye interacts less specifically with proteins by hydrophobic forces [5,6] or a combination of hydrophobic and electrostatic forces [3,7].

In the present study, the interactions of different Cibacron Blue isomers and Procion Red HE3B with the enzyme dopamine β-monooxygenase (3,4-dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase (β-hydroxylating),



(1) $R_1 = SO_2ONa$; $R_2 = R_3 = H$
(2) $R_2 = SO_2ONa$; $R_1 = R_3 = H$
(3) $R_3 = SO_2ONa$; $R_1 = R_2 = H$

Fig. 1. Structures of Cibacron Blue isomers. Cibacron Blue F3GA is a mixture of isomer (2) and (3) with the sulfonate group in the *meta* or *para* position of the -NH- bridge. In Blue Dextran and in gels used for affinity chromatography, the chloride group of these isomers is replaced by sugar alcohols. Cibacron Blue 3GA, which is the dye currently produced by Ciba Geigy, is the isomer having the sulfonate group in the *ortho* position, i.e., isomer (1).

EC 1.14.17.1) (see Ref. 8 for a review) were studied by enzyme inhibition, differences spectroscopy and the binding of enzyme to the immobilized dyes. The holoenzyme binds about eight molecules of Cibacron Blue per enzyme tetramer, whereas the apoenzyme (Cu-depleted) binds only four molecules of the dye per tetramer. The results presented indicate that dopamine β-monooxygenase contains four active sites per tetramer with one copper atom per active site. It is concluded that electrostatic interactions are important for the binding of Cibacron Blue to this enzyme, which is not known to contain nucleotide binding folds.

Materials and Methods

Materials. Cibacron Blue 3GA (*ortho*-isomer) and both the *para*- and *meta*-isomers of Cibacron blue F3GA (named Cibacron Blue GH 3138/1 and Cibacron Blue GH 3134/1, respectively) were gifts from Dr. G. Höltzle, Ciba Geigy, Basle. Procion Red HE3B was a gift from Dr. C.V. Stead, I.C.I. Organics Division, Manchester. Blue Sepharose CL-6B (Cibacron Blue F3GA coupled to agarose) was from Pharmacia; Matrex Gel Red A (Procion Red HE3B coupled to agarose) from Amicon; Chelex-100 from Bio-Rad; catalase, ATP, tyramine and octopamine from Sigma; fusaric acid from ICN Pharmaceuticals and NADH from P-L Biochemicals. All the other chemicals were of analytical grade.

Preparation of dopamine β-monooxygenase. Dopamine β-monooxygenase was purified from bovine adrenal medulla as described [9], with the modifications as given [10]. This purification method, which includes chromatography on DEAE-cellulose and Con A-Sepharose and ultrafiltration against Cu-free 20 mM potassium phosphate, pH 7.0, gives only the water-soluble form of the enzyme [11], with a copper content of about four copper atoms per enzyme tetramer of 290 000 daltons [10]. The apoenzyme was prepared by dialysis against EDTA [12] and contains no detectable copper (i.e., less than 0.04 copper atoms per tetramer) when analyzed by the bathocuproine disulfonate method [12]. The concentrations of holoenzyme and apoenzyme were estimated assuming an absorbance of 1.24 at 280 nm for a solution of 1 mg/ml with a 10 mm light path [12,13].

Enzyme assay. The enzymic activity of dopamine β-monooxygenase was assayed at 25°C using ascorbate and tyramine as the substrates. The consumption of O_2 was measured with a Clark electrode using a high sensitivity membrane (Yellow Springs Instruments Co.).

The incubation mixture of 3.0 ml contained: 10 mM Mes/20 mM fumarate/2 μM $CuSO_4$/10 000 units catalase/0.07 μM dopamine β-monooxygenase (20 μg/ml)/varying amounts of the dye (0—8 μM)/tyramine (0.3—10 mM)/ascorbate (0.3—3 mM); the final pH was 6.0. The reaction was started by addition of 30 μl tyramine, and the oxygen consumption then obtained was corrected for the small contribution measured before addition of tyramine.

Purification and quantitation of the dyes. All the Cibacron Blue isomers were purified by precipitation from methanol solution with dry diethyl ether [14] and then passed through a column with Chelex-100 to remove copper. The concentrations of Cibacron Blue were estimated spectrophotometrically at 610 nm using an extinction coefficient of 13.6 $mM^{-1} \cdot cm^{-1}$ [2]. Procion Red

HE3B concentrations were estimated spectrophotometrically at 512 nm using an extinction coefficient of 30 $mM^{-1} \cdot cm^{-1}$. Beer-Lambert's law was followed at the dye concentrations used in the present study.

Spectroscopy. The absorption measurements were performed with a Cary 219 recording spectrophotometer at 25°C. The light path of the cuvette was 10 mm, and the spectra were recorded at a scan rate of 1 nm/s with a constant slit of 0.4 nm.

The difference spectra were obtained in the following way: 0.8 ml of a dopamine β-monooxygenase solution (containing 0.3—1.5 nmol holoenzyme or apoenzyme tetramer) and 0.8 ml of the protein solvent were placed in the sample and reference cuvettes, respectively. The protein solvent, 20 mM sodium phosphate (pH 7.0), was passed through a column of Chelex-100. The baseline feature of the instrument was used to obtain a smooth baseline. The dye was then added from a concentrated solution to give identical volume increments (5 or 10 μl) in both cuvettes, and the difference spectrum after each addition was recorded. The experiments where the enzyme-dye complex was titrated with different ligands, were performed in a similar manner by addition of identical volume increments of a concentrated ligand solution to both cuvettes.

The Scatchard plots with upward curvature were resolved into two components using the graphical method of Rosenthal [15].

Affinity chromatography. Columns (0.5 × 5.0 cm) containing either Blue Sepharose CL-6B or Matrex Gel Red A were equilibrated with 20 mM sodium phosphate, pH 7.0. About 0.3 mg purified dopamine β-monooxygenase (in 0.8 ml of the equilibration buffer) were applied to each of the columns, following by washing with 20 ml equilibration buffer and elution with 0.2 or 0.8 M NaCl in the equilibration buffer. The eluate was analyzed with polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) [16] using gels that contained 6% (w/w) acrylamide and 0.16% (w/w) *N,N'*-methylenebisacrylamide. The gels were stained for protein with Coomassie brillant blue R.

Results

Inhibition of dopamine β-monooxygenase

The data in Fig. 2 show that both the three Cibacron Blue isomers and Procion Red HE3B are strong inhibitors of dopamine β-monooxygenase, and that all these dyes give a similar percent of inhibition at the same dye concentration. Dilution experiments (data not shown) with the *ortho*-isomer of Cibacron Blue showed that this inhibition was reversible. The type of inhibition obtained with the *ortho*-isomer of Cibacron Blue was analyzed by varying the ascorbate and tyramine concentrations. Although the data (data not shown) varied somewhat between different experiments, they indicated a noncompetitive type of inhibition with either ascorbate or tyramine as the variable substrate. The following inhibitor constants were estimated: K_{ii} (the uncompetitive inhibitor constant; obtained from replots of the intercepts in double-reciprocal plots) = 6—7 μM and K_{is} (the competitive inhibitor constant; obtained from replots of the slopes in double-reciprocal plots) = 1—2 μM with tyramine as the varibale substrate, and K_{ii} = 5—7 μM and K_{is} = 2—4 μM with ascorbate as the variable substrate.

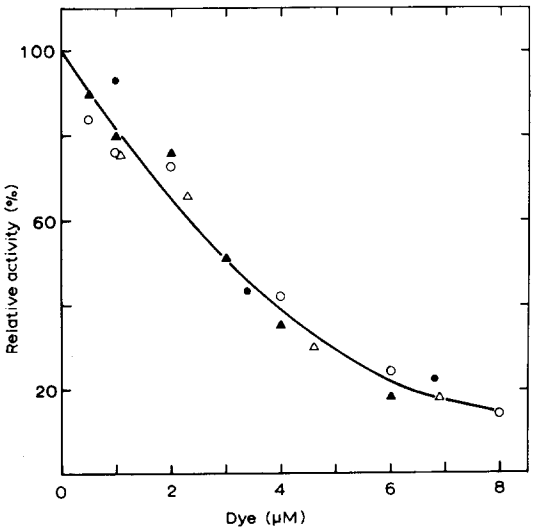


Fig. 2. Inhibition of dopamine β-monooxygenase by Cibacron Blue isomers and Procion Red HE3B. The enzyme was preincubated with the dye for 5 min at 25°C (the final concentrations of the dyes are given on the figure), and the activity was measured with a Clark electrode at 25°C using 1.0 mM tyramine and 1.0 mM ascorbate as described in Methods. The uninhibited activity (corresponding to 100% on the figure) was 2.0 μmol O_2 consumed/min per mg enzyme. The *ortho*-isomer of Cibacron Blue (○); the *meta*-isomer of Cibacron Blue (●); the *para*-isomer of Cibacron Blue (△) and Procion Red HE3B (▲).

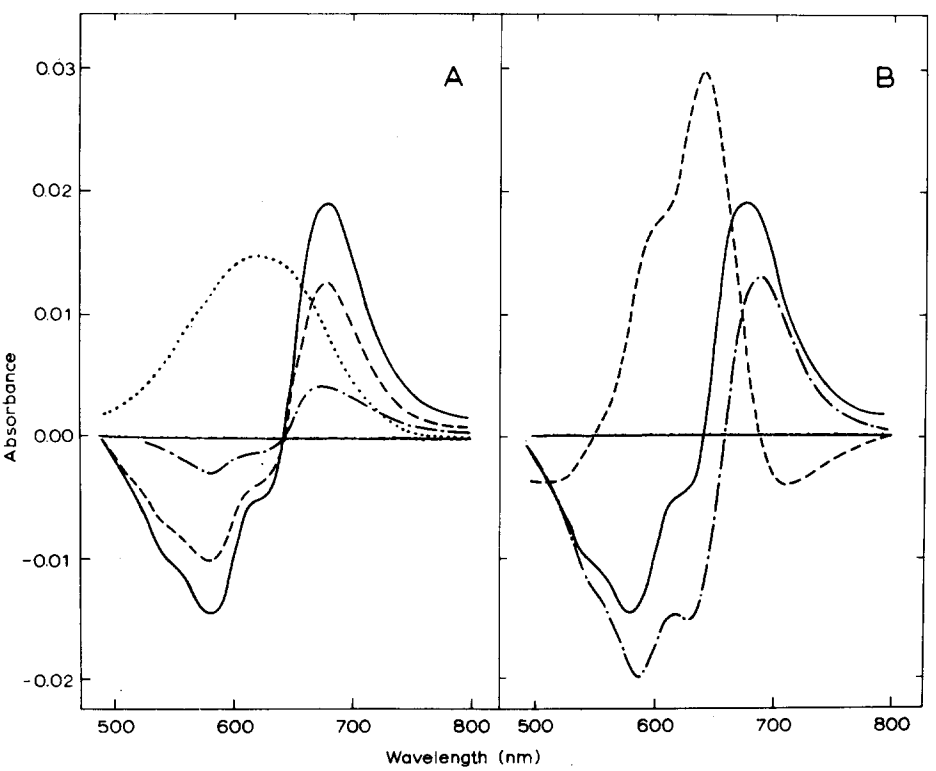


Fig. 3. Spectrum of the *ortho*-isomer of Cibacron Blue and difference spectra obtained with this dye. A: The difference spectra obtained with dopamine β-monooxygenase (1.2 nmol holoenzyme tetramer in 0.8 ml 20 mM sodium phosphate, pH 7.0) after addition of 1.6 nmol dye (· — · — ·), 7.2 nmol dye (— — —) and 16.0 nmol dye (———). The spectrum of 1.1 μM dye in 20 mM sodium phosphate, pH 7.0, is also shown (· · · · · ·). B: The difference spectrum obtained with 14 μM dye in 10 mM sodium phosphate, pH 7.0, with 50% (v/v) ethylene glycol in the sample cuvette, but not in the reference cuvette (— — —). The difference spectrum obtained with 14 μM dye in 10 mM sodium phosphate, pH 7.0, with 0.15 M KCl in the sample cuvette, but not in the reference cuvette (· — · — ·). The third spectrum (———) is that obtained with dopamine β-monooxygenase and 16 nmol dye (from Fig. 3A).

Difference spectroscopy with the ortho- and the meta-isomers of Cibacron Blue

Some of the difference spectra obtained when dopamine β-monooxygenase was titrated with the *ortho*-isomer of Cibacron Blue (i.e., Cibacron Blue 3GA) are shown in Fig. 3A. The difference spectra at the highest dye concentrations showed an absorption maximum at 676 nm and a minimum at 580 nm with an isobestic point at 642 nm. The difference spectra obtained at low dye concentrations were slightly different in the region 650—700 nm (Fig. 3A), indicating that the enzyme may contain different binding sites for the dye. The difference spectra obtained with the dye in 50% (v/v) ethylene glycol and 0.15 M KCl are shown in Fig. 3B for comparison. The binding of dye to the enzyme was fast as the final values of the absorbances at 676 and 580 nm (the maximum and the minimum of the difference spectra) were reached within 8 s after addition of 14 nmol dye to 0.8 ml of a solution containing 1.1 nmol enzyme tetramer.

The amplitudes of the difference spectra obtained with dopamine β-monooxygenase are shown as a function of the dye concentration in Fig. 4, and the same data are displayed as Scatchard plots in Fig. 5. These two figures also show the data when the titration was performed in the presence of about 350 μM fusaric acid (5-butylpicolinic acid), which is a strong inhibitor of dopamine β-monooxygenase [17]. The titration in the presence of fusaric acid was performed, because this inhibitor seemed to compete with the dye for half of the binding sites on the enzyme. The results in Fig. 7A, thus show that fusaric acid lowered the amplitude of the difference spectrum to 53% of its maximal value. Moreover, eight similar experiments performed with 0.4—1.4 nmol enzyme tetramer gave a final amplitude of 51—56% (average 54%) of its maximal value.

The Scatchard plots obtained in the presence and in the absence of fusaric acid (Fig. 5) both show an upward curvature, indicating that the dye in both cases either binds at different sites or that there is a negative cooperativity in the dye binding [18]. When these Scatchard plots were resolved into their components to estimate the possible number of 'different' binding sites and the K_d (dissociation constant) for the binding to these sites, the following results were obtained from the data in Fig. 5: (a) in the absence of fusaric acid, each enzyme tetramer binds 2.0 dye molecules with $K_d = 0.03$ μM and 5.6 dye molecules with $K_d = 2.5$ μM, and (b) in the presence of fusaric acid, each enzyme tetramer binds 1.6 dye molecules with $K_d = 0.02$ μM and 2.1 dye molecules with $K_d = 2.8$ μM. The results obtained from other similar experiments are shown in Table I.

Fusaric acid is assumed to inhibit dopamine β-monooxygenase by binding to the active site copper [17]. The effect of fusaric acid on the binding of Cibacron Blue to dopamine β-monooxygenase thus promted the investigation of dye binding to the Cu-depleted enzyme (apoenzyme), and it appeared (Fig. 4) that the apoenzyme bound the dye in a manner similar to that observed with the holoenzyme in the presence of fusaric acid. Addition of $CuSO_4$ to the apoenzyme in the presence of an excess of dye increased the amplitude of the difference spectra from a value corresponding to the binding of 3.9 dye molecules per tetramer to 8.4 dye molecules per tetramer (Fig. 6). Moreover, the results indicate that the enzyme must bind 4.9 copper atoms per tetramer (or slightly less, as the two first additions do not give a linear increase in the

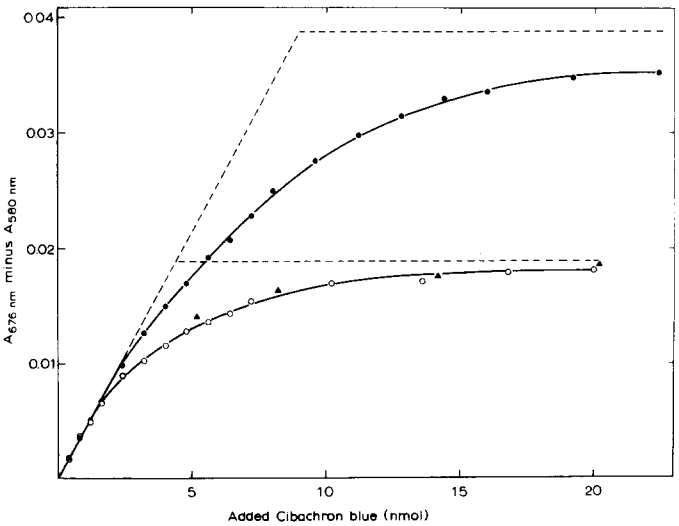


Fig. 4. Difference spectral titration of dopamine β-monooxygenase with the *ortho*-isomer of Cibacron Blue. The titrations were performed with 1.2 nmol enzyme tetramer (in 0.8 ml 20 mM sodium phosphate, pH 7.0) in the absence (●——●) or presence (○——○) of fusaric acid. The initial fusaric acid concentration of 380 μM decreased to 345 μM during the titration. The amplitude of the difference spectra (absorbance maximum at 676 nm less the absorbance minimum at 580 nm; see Fig. 3) were corrected for dilution during the titration. The broken line drawn through the origin was used to calculate the amount of free and bound dye for the Scatchard plots (Fig. 5), and the broken horizontal line corresponds to the asymptotes estimated from these Scatchard plots. Some data from a titration of 1.1 nmol apoenzyme tetramer with the dye are also shown (▲——▲); see also Fig. 6 for further experiments with this apoenzyme.

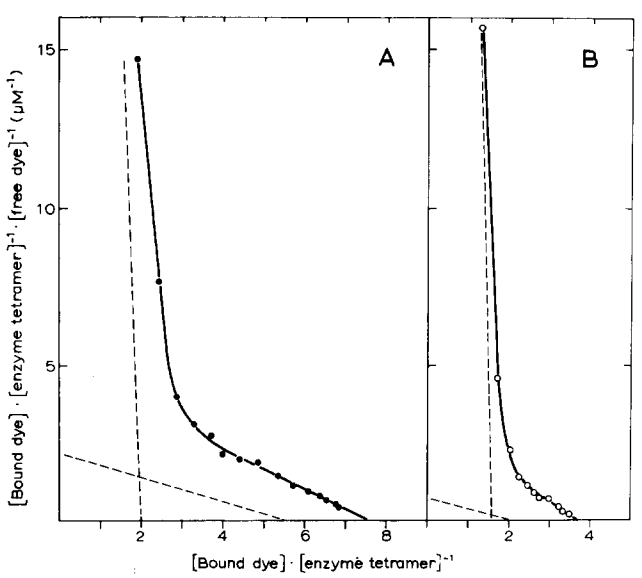


Fig. 5. Scatchard plots of the difference spectral titration of dopamine β-monooxygenase with the *ortho*-isomer of Cibacron Blue. The data in Fig. 4 were used to produce these plots. A: Absence of fusaric acid; 1.2 nmol holoenzyme tetramer. B: Presence of about 350 μM fusaric acid; 1.2 nmol holoenzyme tetramer. The broken lines are those used to estimate the number of binding sites (from the interception with the x-axis) and the dissociation constant (K_d) of the enzyme-dye complex (from the slope).

TABLE I

NUMBER OF BINDING SITES AND DISSOCIATION CONSTANTS (K_d) FOR THE BINDING OF THE *ORTHO*-ISOMER OF CIBACRON BLUE TO DOPAMINE β-MONOOXYGENASE

The data were estimated from Scatchard plots as shown in Fig. 5. Two different holoenzyme preparations were used to perform a total of four experiments in the absence of fusaric acid and three experiments in the presence of fusaric acid. The average values are given outside the parenthesis with the lowest and highest values within the parenthesis.

	Absence of fusaric acid	Presence of 300—400 μM fusaric acid
Total binding sites	7.5 (7.0 —7.8)	3.9 (3.6 —4.4)
'High affinity' binding		
Sites per tetramer	2.0 (1.6 —2.6)	1.7 (1.5 —1.9)
K_d (μM)	0.04 (0.01—0.07)	0.03 (0.01—0.06)
'Low affinity' binding		
Sites per tetramer	5.4 (5.1 —5.8)	2.2 (2.1 —2.5)
K_d (μM)	2.2 (1.8 —2.5)	2.0 (1.5 —2.8)

amplitude) to obtain the maximal amplitude of the difference spectra. Another similar experiment showed that 5.2 copper atoms per tetramer were necessary to increase the number of dye molecules bound per tetramer from 4.4 to 8.8. Addition of fusaric acid to the apoenzyme in the presence of an excess of both dye and copper (after the final addition of $CuSO_4$ in Fig. 6), decreased the amplitude in both these experiments to exactly 50% of its maximal value.

The difference spectra obtained with the *ortho*-isomer of Cibacron Blue and dopamine β-monooxygenase indicates a $\Delta\epsilon_{(676-580nm)}$ of 3.8 $mM^{-1} \cdot cm^{-1}$ (average of 12 analyses varying from 3.4 to 4.1 $mM^{-1} \cdot cm^{-1}$).

The effects of KCl on the amplitude of the difference spectra both in the presence and absence of fusaric acid are shown in Fig. 7A, and the effects of ATP, NADH, tyramine and octopamine (the enzyme product after β-hydroxylation of tyramine) on this amplitude are shown in Fig. 7B. Addition of SDS to a final concentration of 0.1% (w/w) completely abolished the difference spectrum.

The binding of the *meta*-isomer of Cibacron Blue to dopamine β-monooxygenase seems to be very similar to that of the *ortho*-isomer, as the difference spectra, the effect of fusaric acid on these spectra, the number of 'high and low affinity' binding sites, and their K_d values, were all similar for these two isomers (the data for the *meta*-isomer are not shown).

Difference spectroscopy with the para-isomer of Cibacron Blue

The difference spectra obtained with the *para*-isomer of the dye (Fig. 8) were different from that obtained with the two other isomers (see Fig. 3 for comparison). Although the difference spectra obtained at high concentrations of the *para*-isomer showed some similarities with the spectra obtained using other isomers, the difference spectra with the *para*-isomer were quite different both at low dye concentrations and in the presence of fusaric acid. These spectra thus indicate that the *para*-isomer binds at two or more different sites, with the spectral difference from the two other isomers due to the Cu-independent sites.

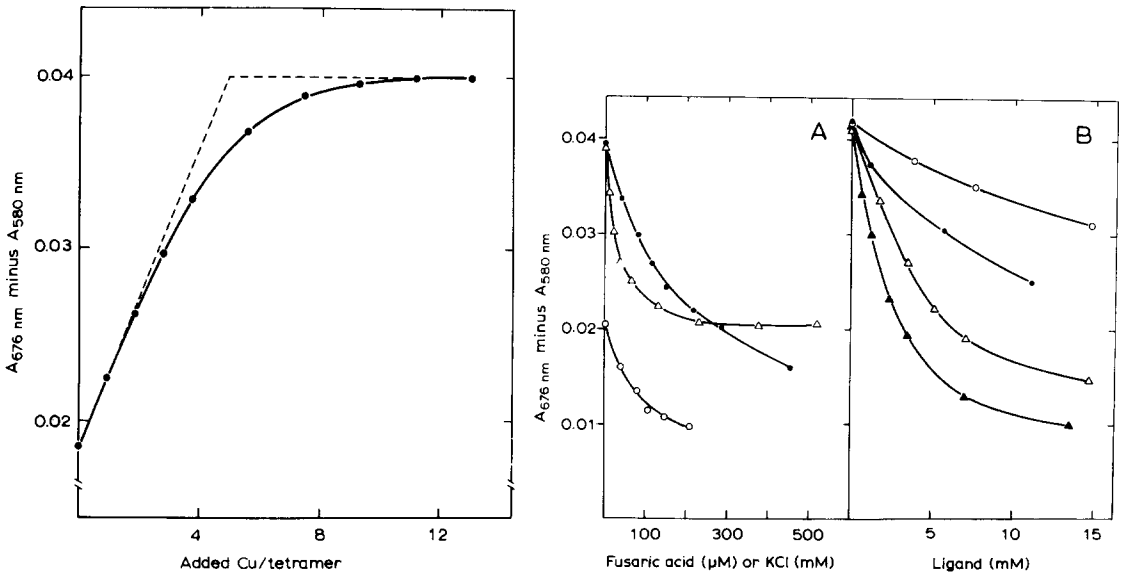


Fig. 6. Effect of $CuSO_4$ on the amplitude of the difference spectra of the *ortho*-isomer of Cibacron Blue with Cu-depleted dopamine β-monooxygenase. Up to 20.4 nmol dye were added to the apoenzyme (1.1 nmol tetramer in 0.8 ml 20 mM sodium phosphate, pH 7.0); the amplitudes of the difference spectra then obtained are shown in Fig. 4. The amplitudes shown in the present figure are those obtained after addition of $CuSO_4$ to the cuvette containing 1.1 nmol apoenzyme tetramer and 20.4 nmol dye. The amplitudes were corrected for dilution during the titration.

Fig. 7. Effect of fusaric acid, KCl, ATP, NADH, tyramine, and octopamine on the amplitude of the difference spectra of dopamine β-monooxygenase with the *ortho*-isomer of Cibacron Blue. The amplitude of the difference spectra of 1.3 nmol holoenzyme tetramer (in 0.8 ml 20 mM sodium phosphate, pH 7.0) with 20 nmol dye corresponds to the points with no additions of ligands. A: The amplitude of the difference spectra measured after addition of fusaric acid (△———△) or KCl (●———●). KCl was also added to the solution which previously had been titrated with up to 520 μM fusaric acid (○———○). B: The amplitude of the difference spectra measured after addition of ATP (○———○), NADH (●———●), octopamine (△———△) and tyramine (▲———▲). The amplitudes of the difference spectra were corrected for dilution during the titration.

Difference spectroscopy with Procion Red HE3B

Addition of Procion Red HE3B to dopamine β-monooxygenase gave difference spectra with a maximum at 558 nm and a minimum at 496 nm (data not shown). Although the wavelength for the maximum and the minimum of the difference spectra did not change during the titration, the shape of the spectra changed. The amplitudes of the difference spectra obtained with increasing amount of the dye (Fig. 9) indicate that about 17 molecules of the dye bind per enzyme tetramer, i.e., about twice that obtained with Cibacron Blue. Fig. 9 also shows the effect of KCl on the amplitude of the difference spectra and the lack of effect of fusaric acid on this amplitude.

Affinity chromatography

Purified dopamine β-monooxygenase was adsorbed to both Blue Sepharose CL-6B and Matrex Gel Red A. Addition of 0.2 M NaCl to the buffer (20 mM sodium phosphate, pH 7.0) eluted the enzyme from the blue gel, but not from

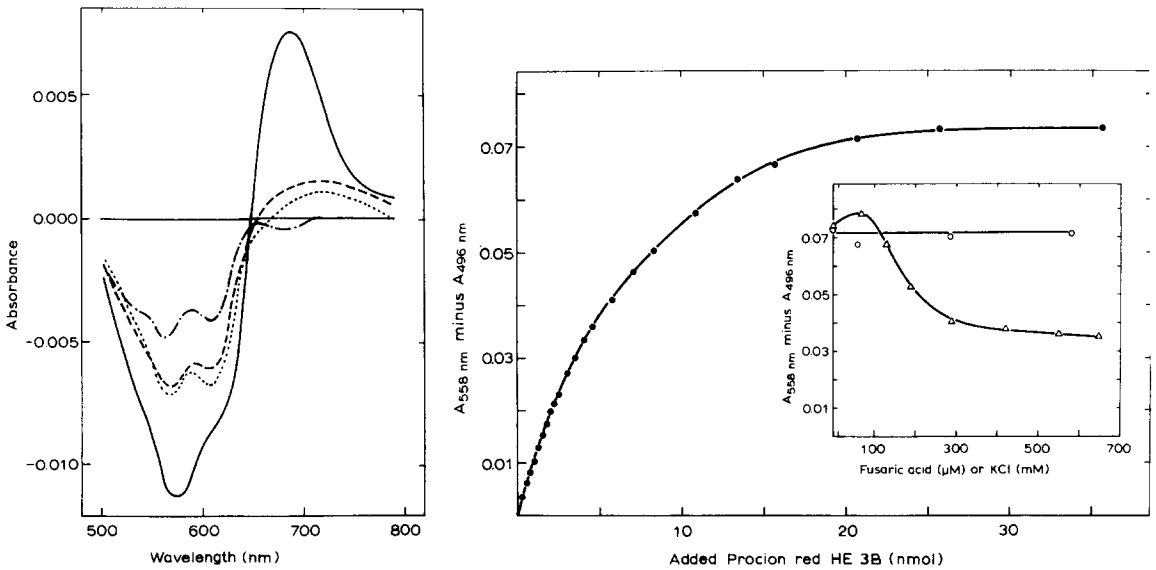


Fig. 8. Difference spectra of dopamine β-monooxygenase with the *para*-isomer of Cibacron Blue. The difference spectra were obtained with 1.0 nmol holoenzyme (in 0.8 ml sodium phosphate, pH 7.0) after addition of 5.1 nmol dye (· — · — ·), 8.5 nmol dye (— — —), and 20.4 nmol dye (———). Addition of 300 μM fusaric acid to the cuvette containing 20.4 nmol dye gave the spectrum marked (· · · · · ·).

Fig. 9. Amplitudes of the difference spectra of dopamine β-monooxygenase with Procion Red HE3B. The difference spectra were obtained with 0.35 nmol holoenzyme tetramer (in 0.8 ml 20 mM sodium phosphate, pH 7.0) and increasing amounts of the dye (●———●). The effects on the amplitude of the difference spectrum (0.35 nmol holoenzyme and 25 nmol dye) after the addition of fusaric acid (○———○) or KCl (△———△) are shown in the inset. The amplitudes were corrected for dilution during the titration.

the red gel, whereas addition of 0.8 M NaCl eluted the enzyme from the red gel.

Discussion

The present results show that both Procion Red HE3B and all three isomers of Cibacron Blue interact with dopamine β-monooxygenase. The difference spectral titration curves with the red dye (Fig. 9) and the *para*-isomer of Cibacron Blue (Fig. 8) indicated that quantitative interpretations with these two dyes are complicated. The two other isomers of Cibacron Blue gave similar difference spectra for all the binding sites, and the *ortho*-isomer (i.e., Cibacron Blue 3GA) was selected for the most detailed studies, because only this isomer is currently produced by Ciba Geigy.

Quantitation of the dye binding to the enzyme is based on the assumptions that (a) the dye is quantitatively bound at low dye concentrations and (b) binding of the dye to the enzyme gives the same difference spectrum with the same extinction coefficient throughout the titration. These assumptions seem reasonable as (a) the amplitude of the difference spectra increased linearly (within the uncertainty) at low dye concentrations (Fig. 4), and (b) the shapes of the difference spectra were almost identical throughout the titration (Fig. 3).

The difference spectral titration of the *ortho*-isomer of Cibacron Blue with the holoenzyme in the presence of about 350 μM fusaric acid (which is assumed to interact with the active-site copper [17]) and with the apoenzyme (Fig. 5 and Table I) both indicate binding of four dye molecules per apoenzyme tetramer. The holoenzyme seems, however, to interact with almost eight dye molecules per tetramer (Table I) and a similar amount of dye binding was observed after addition of $CuSO_4$ to the apoenzyme (Fig. 6). It should be noted that a little less Cibacron Blue is bound to the holoenzyme preparations containing four copper atoms per tetramer (Table I) than that obtained bound to the apoenzyme after addition of an excess of $CuSO_4$ (Fig. 6). This indicates, in agreement with an earlier report [12], that the copper binding to the active site is not extremely tight.

The data from the difference spectral titrations mentioned above gave Scatchard plots with upward curvatures (Fig. 5). This means that the dye either binds at different classes of sites with different dissociation constants (K_d) or that there is a negative cooperativity in the binding [18], and it is not possible in the present case to determine between these two possibilities. Nevertheless, the Scatchard plots were resolved into their components to estimate the number of possible 'different' binding sites and their K_d values. With this in mind, the results in Table I indicate two 'high affinity' sites per tetramer both on the holoenzyme and on the apoenzyme. It should be noted that the data in Fig. 5 and Table I do not show whether these two 'high affinity' sites have the same K_d, or if the K_d for the binding of the first molecule to the enzyme is lower than that estimated for the binding of the second molecule, i.e., the value given for the 'high affinity' sites in Table I. The binding to the four Cu-dependent sites all seem to have the same K_d, and this K_d is in the same range as that obtained for the binding to the 'low affinity' sites on the apoenzyme (Table I).

It cannot be ascertained whether the copper effect is to bind the dye in the active site (e.g., by electrostatic interaction to the negatively charged dye), or if the copper causes some structural changes in the protein leading to the extra dye binding apart from the active sites. However, the observation that the competitive inhibitor constants for the dye are in the same range as the K_d for the binding of the dye to the Cu-dependent sites, indicates that the dye binds at the active site. An attempt to clarify this point with titration of the enzyme-dye complex with the substrate tyramine was of no help, because of effects in addition to those concerning the Cu-dependent sites (Fig. 7B). Moreover, octopamine (the product after β-hydroxylation of tyramine) had a similar effect on the amplitude of the difference spectra as that observed for tyramine (Fig. 7B). This was observed even though octopamine binds less strongly than tyramine to the active site, because an octopamine concentration of 3-times that of tyramine did not inhibit the tyramine hydroxylation of the enzyme (unpublished data).

Knowledge of the number of copper atoms per active site is of the utmost importance for the discussion of the enzyme mechanism [8]. The present observation that five or slightly fewer copper atoms are needed to give the maximal amplitude of the difference spectra with Cibacron Blue (Fig. 6), supports our previous conclusion that four copper atoms bind to active sites

per enzyme tetramer [12]. As mentioned above, the present results indicate that the four Cu-dependent binding sites for Cibacron Blue are at the active sites. Because these dye molecules are expected to be too large for the binding of more than one dye molecule per active site, these results indicate that dopamine β-monooxygenase contains four active sites per tetramer with only one copper atom per active site. This conclusion contradicts the recent claim of Blackburn et al. [19] for binuclear copper sites in dopamine β-monooxygenase. They concluded, based on measurements of the relationship between enzyme activity and the copper/protein ratio, that the enzyme-catalyzed reaction is second-order with respect to protein-bound copper. However, they varied the copper/protein ratio only in the range 0.4—2.0 copper atoms per tetramer, and alternative explanations for their results are equally plausible, such as dissociation of enzyme-bound copper from the active sites or binding of copper outside the active sites (see above and Ref. 10), and/or interactions between Cu-containing and Cu-depleted active sites.

Why do two molecules of Cibacron Blue bind so tightly ($K_d < 0.1$ μM) to the apoenzyme of dopamine β-monooxygenase? It may be speculated that the enzyme has some binding sites for nucleotides. Although there was no large effect of, for example, ATP on the amplitude of the difference spectra (Fig. 7B), it must be remembered that the concentration of ATP in the environment of the enzyme inside the catecholamine storage vesicles is as high as 120—150 mM [20]. Even though ATP should bind to dopamine β-monooxygenase in these storage vesicles, such a binding would be expected to play only a minor role in the stabilization of the ATP-complexes which exist within these vesicles [20]. It should be noted that Tachikawa et al. [21] recently reported that several nucleotides activate dopamine β-monooxygenase, and that their preliminary studies (data not shown) with circular dichroism indicated that ATP induced conformational changes of the enzyme. The enzyme activation reported by Tachikawa et al. [21] is, however, difficult to evaluate as they did not indicate the copper concentration in their incubation mixtures, even though Laduron [22] has pointed out that activation of dopamine β-monooxygenase by some ATP preparations was due to contamination of these preparations by copper. In addition, we have not been able to observe any stimulation of the enzyme activity by up to 10 mM ATP when the enzyme was assayed with a Clark electrode in the presence of optimal copper concentration (the assay conditions mentioned in Methods with 2 μM $CuSO_4$/1 mM ascorbate/3 mM tyramine; copper was removed from the ATP solutions with passage through columns with Chelex-100; unpublished data).

Previous studies on the interaction of Cibacron Blue with proteins have usually been performed with Cibacron Blue F3GA, which is a mixture of two isomers (Fig. 1). The observation that these two isomers (*meta* and *para*) did not give identical difference spectra with dopamine β-monooxygenase show that it is important to use pure isomers of the dye when studying the interactions of Cibacron Blue with proteins. The same conclusion has been drawn by Biellmann et al. [3] based on their X-ray crystallographic studies on alcohol dehydrogenase.

The nature of the interaction of Cibacron Blue with proteins has been much debated in the literature. The postulate that the dye should bind only to

nucleotide binding sites, has been argued against by several groups in recent years [3,5—7,23]. The observation that dopamine β-monooxygenase contains four strong (K_d = 1—3 μM) Cu-dependent binding sites for Cibacron Blue per tetramer, is important in this respect. There is no reason to believe that binding of four copper atoms per tetramer of this enzyme should cause the formation of four nucleotide binding sites. It is much more likely that these copper atoms bind at the active site and thus, cause the binding of Cibacron Blue to this site by hydrophobic and/or electrostatic interactions.

The observation that the difference spectrum of Cibacron Blue F3GA with lactate dehydrogenase (EC 1.1.1.27) is very similar to the difference spectrum obtained with the dye in 50% (v/v) ethylene glycol, has been interpreted as evidence for the binding of the dye to a hydrophobic pocket on this enzyme [2]. The difference spectrum of the *ortho*-isomer of the dye with dopamine β-monooxygenase is quite different from that obtained with the dye in 50% (v/v) ethylene glycol, but shows great similarities with that obtained with the dye in 0.15 M KCl (Fig. 3B). This, together with the large effects of KCl on the amplitude of the difference spectra compared to the effects of the nucleotides (Fig. 7), indicates that electrostatic interactions are more important for the binding of the dye to dopamine β-monooxygenase than to, for example, lactate dehydrogenase [2]. It should be mentioned that the difference spectra observed for the binding of Cibacron Blue to other proteins (see e.g. Refs. 14, 23, 24) are also far more similar to that obtained for the dye in KCl than for the dye in ethylene glycol. The present results thus indicate that Cibacron Blue interactions with proteins are due to a combination of electrostatic and hydrophobic interactions.

The interaction of the enzyme with Procion Red HE3B seems to be stronger than with Cibacron Blue as evaluated from the difference spectroscopy and the experiments with the affinity gels, although all these dyes gave a similar degree of inhibition at the same dye concentration (Fig. 2). Further studies should be carried out to decide whether the red or blue gel will be useful in the purification of dopamine β-monooxygenase; the data in Fig. 7A indicate that fusaric acid may be utilized to obtain biospecific elution of the enzyme from such blue gels.

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