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DESIGN AND APPLICATIONS OF BIOMIMETIC ANTHRAQUINONE DYES

II. THE INTERACTION OF C.I. REACTIVE BLUE 2 ANALOGUES BEARING TERMINAL RING MODIFICATIONS WITH HORSE LIVER ALCOHOL DEHYDROGENASE

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SUMMARY

A number of terminal ring analogues of the anthraquinone dye C.I. Reactive Blue 2 were synthesised and characterised. The interaction of these dye analogues with horse liver alcohol dehydrogenase was investigated by difference spectroscopy and analytical affinity chromatography. Studies by difference spectroscopy showed that anionic terminal ring substituents or an unsubstituted phenyl ring promoted tight binding of the dye to the enzyme, whereas neutral or cationic terminal ring substituents reduced the affinity of the dye. Terminal ring analogues of C.I. Reactive Blue 2 with *ortho*- or *meta*-orientated groups were bound more tightly than those with *para*-orientated substituents. The observed differences in the affinity of the dye analogues towards horse liver alcohol dehydrogenase could be accounted for by proposing that the terminal ring of C.I. Reactive Blue 2 is bound in a relatively apolar pocket lateral to the principal coenzyme binding site and located in the catalytic domain of the enzyme. The 2900-fold difference in affinity between the C.I. Reactive Blue 2 analogues and horse liver alcohol dehydrogenase in solution was not translated into significant differences in chromatographic behaviour when the dyes were immobilised to beaded agarose. However, an *ortho*-orientated sulphonate substituent promoted exceptionally tight binding of the enzyme to the immobilised dye. Immobilisation of the dye analogues via the reactive chlorotriazine was identified as the most likely explanation for the relatively minor influence of the terminal ring substituent of the immobilised dye on enzyme binding.

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INTRODUCTION

Immobilised textile dyes are being used increasingly for protein purification[1–4]. The low cost of reactive dyes coupled with their ease of immobilisation and resistance to chemical and biological degradation, has led to dye adsorbents that are invariably less expensive and more stable than those based on natural biological ligands[1–2]. Furthermore, textile dyes have been shown to interact with a large number of seemingly unrelated proteins[5–7], in some cases, with remarkable degrees of specificity[8–10].

To date, however, almost all studies have been performed with commercially available textile dyes. The selectivity of these affinity ligands could, in principle, be improved by designing synthetic dyestuffs which mimic the structure and binding of natural biological ligands. Such "biomimetic" dyes should not only display high specificity for the target protein, but also retain most of the advantages of the commercial dyes. As a first step towards designing entirely novel dyes, the interaction between analogues of C.I. Reactive Blue 2 and horse liver alcohol dehydrogenase was investigated. The structure of this enzyme has been studied extensively[11–13] and its interaction with dyes has been established by X-ray crystallography[14] and affinity labelling[15]. The dye binds to the NAD^+-binding site with the anthraquinone, diaminobenzene sulphonate and triazine rings apparently adopting similar positions with respect to the adenine, adenyl-ribose and pyrophosphate groups of NAD^+. In contrast, the terminal aminobenzene sulphonate ring of the dye is bound in a lateral cleft not normally occupied by NAD^+, being some 10Å distant from the nicotinamide binding site[14]. It was deemed likely, therefore, that the interaction between C.I. Reactive Blue 2 and horse liver alcohol dehydrogenase could be improved by selective modification of the terminal ring moiety of the dye. Previous work has shown that even relatively minor modifications to the structure of reactive dyes may markedly influence their capacity to bind proteins[8]. Consequently, it was expected that alterations to the charge, hydrophobicity and spatial arrangement of substituents on the terminal ring of C.I. Reactive Blue 2 would lead to dye analogues with altered specificity and affinity for horse liver alcohol dehydrogenase. Thus, a number of terminal ring analogues of C.I. Reactive Blue 2 have been synthesised and their interaction with the enzyme investigated in free solution and by analytical affinity chromatography[16]. This report demonstrates that modification of the terminal ring moiety of C.I. Reactive Blue 2 influences protein binding in a manner entirely consistent with the postulated interaction between the dye and horse liver alcohol dehydrogenase.

EXPERIMENTAL

Chemicals

Bromamine acid (1-amino-4-bromoanthraquinone-2-sulphonic acid), 2,5-diaminobenzenesulphonic acid, cyanuric chloride (2,4,6-trichloro-*s*-triazine) and the terminal ring forming groups *o*-, *m*- and *p*-aminobenzenesulphonic acid, *o*-, *m*- and *p*-aminobenzoic acid, *p*-aminobenzylphosphonic acid, *m*-aminobenzamide, *m*-aminobenzylalcohol and *m*- and *p*-aminobenzenetrimethylammonium chloride were supplied by ICI Organics Division. Dried acetone and 1,6-diaminohexane were obtained from Fisons (Loughborough, U.K.), whilst 1,1'- carbonyldiimidazole (CDI) was pur-

chased from Sigma (Poole, U.K.). Horse liver alcohol dehydrogenase [alcohol: NAD^+ oxidoreductase: E.C.1.1.1.1. (2.7 U mg^{-1})] and the coenzymes NAD^+ (free acid, grade 1) and NADH (disodium salt; grades I and II) were from Boehringer (Lewes, U.K.). Sepharose 4B was obtained from Pharmacia Biotechnology (Uppsala, Sweden). All other reagents and solvents were of analytical grade and were obtained from U.K. suppliers.

Synthesis of terminal ring analogues of C.I. Reactive Blue 2

The terminal ring analogues of C.I. Reactive Blue 2 were synthesised, characterised and purified according to previously published procedures[17].

Molecular models

A single subunit α-carbon model of horse liver alcohol dehydrogenase (scale 1 cm = 1 Å) was constructed from a kit of parts supplied by Shapely Models (Milton Keynes, U.K.). The model was built according to the method of Fletterick and Matela[18] using atomic coordinates stored in the Brookhaven Protein Data Bank. Models of NAD^+ and C.I. Reactive Blue 2 analogues (scale 1 cm = 1 Å) were constructed from components purchased from Labquip (Reading, U.K.).

Difference spectral titration

The dissociation constants (K_d) of the analogues of C.I. Reactive Blue 2 with horse liver dehydrogenase were determined by difference spectral titration at 25°C. A stock solution (approximately 1 mg ml^{-1}) of enzyme (clarified by filtration through a 0.45 μm pore size filter) in tricine–sodium hydroxide buffer, pH 8.5 (0.1 *M*) was prepared and the subunit concentration determined from the absorbance at 280 nm using an extinction coefficient of 0.455 ml mg^{-1} cm^{-1} and a subunit M_r of 40 000[19,20]. A solution of stock enzyme (5.0–12.5 μ*M* subunits; 1 ml) in tricine–sodium hydroxide buffer, pH 8.5 (0.1 *M*) was added to a black-walled silica cuvette (10 mm pathlength) and placed in the sample beam of a Perkin-Elmer Lambda 5 spectrophotometer. Tricine–sodium hydroxide buffer, pH 8.5 (0.1 *M*; 1 ml) was placed in a similar cuvette in the reference beam. A background-absorbance correction was performed between 850–500 nm, whereupon identical aliquots (2–10 μl) of purified dye solution (0.5–1.0 m*M*) were added to both cells and the difference absorption spectrum recorded after each pair of additions.

The maximum difference absorption ($\Delta A_{\lambda,max}$) was obtained either directly from the titration results, or calculated from a double reciprocal plot of dye concentration against difference absorbance (ΔA_λ) using linear regression analysis. A difference extinction coefficient ($\Delta\varepsilon_\lambda$) was calculated, from which the concentration of enzyme–dye complex ([ED]) formed with each dye addition could be estimated. The K_d was calculated using non-linear regression analysis[21] (with bi-square weighting) by fitting values of total dye concentration ($[D_T]$) and [ED] to the expression:

$$[ED] = \frac{[E_T]\,([D_T] - [ED])}{K_d + ([D_T] - [ED])}$$

where $[E_T]$ is the total enzyme subunit concentration and $[D_T]$ is the total dye concentration.

Synthesis of 6-aminohexyl derivatives of C.I. Reactive Blue 2 analogues

To a stirred solution of 1,6-diaminohexane (12.5 mmol) in water (20 ml) was added a solution of purified dye (1.25 mmol) in water (30 ml) and the temperature increased to 60°C. Stirring was continued for 3 h and the reaction followed by analytical thin-layer chromatography (TLC)[17] using a propan-2-ol–ammonia–water (7:2:1, v/v/v) solvent. Sodium chloride solution (3%, w/v) was added and the mixture allowed to cool. The pH was reduced to 1.0 by the addition of hydrochloric acid and the product precipitate filtered off, washed with hydrochloric acid (1 *M*, 100 ml), acetone (50 ml) and dried under vacuum. Typically, yields of 90–95% were achieved by this procedure.

Immobilisation of 6-aminohexyl C.I. Reactive Blue 2 analogues to Sepharose 4B

Sepharose 4B was activated with CDI[22] to facilitate the immobilisation of 6-aminohexyl C.I. Reactive Blue 2 analogues. Exhaustively washed Sepharose 4B (3 g moist weight) was washed sequentially with water–acetone (2:1, v/v; 20 ml), water–acetone (1:2, v/v; 20 ml), acetone (20 ml) and dried acetone (50 ml). The gel was resuspended in dried acetone (5 ml) to which CDI (0.06–0.12 g) was added and the mixture agitated for 15 min at 20–25°C. The activated gel was washed with dried acetone (100 ml) and used immediately.

A solution of 6-aminohexyl dye (0.25 mmol) in dimethyl sulphoxide (DMSO)–water (50%, v/v; 8 ml) was adjusted to pH 10.0 with 2 *M* sodium carbonate solution, whereupon CDI-activated Sepharose 4B (3 g) was added. The mixture was tumbled overnight (4°C) and washed sequentially with water (200 ml), sodium chloride solution (1 *M*, 100 ml), water (100 ml), DMSO solution (50% v/v; 20 ml) and water (200 ml). Dyed gels were stored in sodium azide solution (0.02% w/w; 4°C) until required.

Determination of immobilised dye concentration

Dyed Sepharose (30 mg moist weight) was hydrolysed (60°C; 5 min) following the addition of hydrochloric acid (5 *M*; 0.6 ml). The hydrolysate was neutralised by the addition of sodium hydroxide (10 *M*; 0.3 ml) and potassium phosphate buffer, pH 7.6 (1 *M*; 2.1 ml). The absorbance (620 nm; 25°C) of the hydrolysate was read against a Sepharose 4B blank treated in an identical manner. Molar extinction coefficients of purified 6-aminohexyl dyes were determined (620 nm; 25°C) from dye solutions (20 μM) made up in a medium identical to that of the hydrolysed gel. Immobilised dye concentrations were calculated as μmol dye per g moist weight gel.

Affinity chromatography of horse liver alcohol dehydrogenase on immobilised C.I. Reactive Blue 2 analogues

The interaction of horse liver alcohol dehydrogenase with C.I. Reactive Blue 2 analogues immobilised to Sepharose 4B was determined by analytical affinity chromatography at 4°C. A linear gradient of NADH was used to displace adsorbed horse liver alcohol dehydrogenase, the eluting concentration of the coenzyme being taken as a measure of the affinity of the enzyme for the immobilised dye.

Glass minicolums (0.5 × 10 cm) packed with adsorbent (1.0 g moist weight)

were equilibrated with 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)–sodium hydroxide buffer, pH 7.5 (20 m*M*) at a flow-rate of 30 ml $h^{-1}cm^{-2}$. Horse liver alcohol dehydrogenase (10 mg ml^{-1}) was dialysed overnight against 2000 vol. equilibration buffer and 200 μl of the dialysed enzyme (approximately 3 units) run into the gel bed. The sample was incubated with the adsorbent (10 min) by halting the buffer flow, following which the gel bed was washed with equilibration buffer (6 ml) and adsorbed enzyme eluted with a linear NADH (grade II) gradient (20 ml; 0–0.5 m*M*). Fractions (1 ml) were collected and samples (20 μl) assayed for enzyme activity.

Alcohol dehydrogenase was assayed by following the reduction of NAD^+ at 340 nm (25°C). In a total volume of 1 ml were: glycine (180 μmol)–sodium pyrophosphate (61 μmol) buffer, pH 9.0; semicarbazide (73 μmol); ethanol, 99.7% (v/v) (0.56 mmol); reduced glutathione (10 μmol) and NAD^+ (1.8 μmol). The molar extinction coefficient of NADH at 340 nm was taken to be 6220 l mol^{-1} cm^{-1}.

RESULTS

A series of analogues of C.I. Reactive Blue 2 were synthesised which bore anionic, cationic, neutral and hydrophobic groups substituted at various positions (*ortho, meta* or *para*) on the terminal aminobenzene ring of the dye (Fig. 1). These analogues were synthesised by a method similar to that for the parent dye[16,17]. The desired terminal ring structure was achieved by reacting the blue dichlorotriazinyl precursor of C.I. Reactive Blue 2 with the relevant substituted arylamine.

The reactive dyes used in this study were extensively purified as minor impurities in reactive dye preparations are known to interact with proteins[23,24]. C.I. Reactive Blue 2 analogues with terminal ring modifications were purified by Sephadex LH-20 column chromatography[17]. A 60:40 (v/v) methanol–water solvent was used for dyes possessing anionic and neutral terminal ring substituents, whereas less soluble dyes bearing cationic trimethylammonium groups were chromatographed in a 50:50 (v/v) methanol–water solvent. The degree of purification of the dyes was assessed by both analytical TLC and high-performance liquid chromatography (HPLC) as described previously[17]. Greater resolution of dyes with cationic terminal ring substituents was achieved by TLC using a solvent system comprising butanone–ammonia–water (3:4:3, v/v/v) rather than the butan-1-ol–propan-2-ol–ethylacetate–water (2:4:1:3, v/v/v) system used for anionic and neutral dyes. In all cases, the puri-

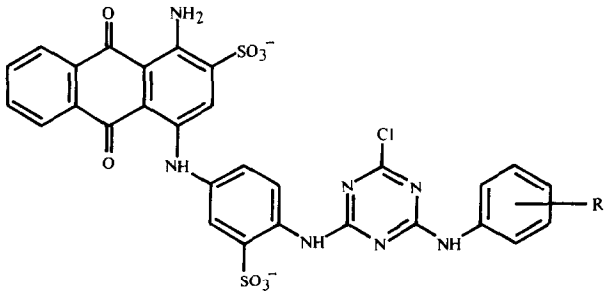


Fig. 1. The structure of C.I. Reactive Blue 2 analogues with terminal ring modifications.

TABLE I

SPECTRAL DATA DETERMINED FOR TERMINAL RING ANALOGUES OF C.I. REACTIVE BLUE 2

*C.I. Reactive Blue 2 Analogue**	*Terminal ring substituent (R)*	M_r *(sodium salt)*	λ_{max}** *(nm)*	ε_{620}** *(l mol^{-1} cm^{-1})*
I	*ortho*-SO_3^-	839.5	617	12 600
II	*meta*-SO_3^-	839.5	620	11 100
III	*para*-SO_3^-	839.5	624	11 000
IV	*ortho*-COO^-	803.5	618	10 600
V	*meta*-COO^-	803.5	624	11 100
VI	*para*-COO^-	803.5	624	10 300
VII	*para*-$CH_2PO_3H^-$	853.5	626	11 200
VIII	*meta*-CH_2OH	767.5	620	9800
IX	*meta*-$CONH_2$	780.5	616	9700
X	-H	737.5	618	9000
XI	*meta*-$N^+(CH_3)_3Cl^-$	831.0	624	7500
XII	*para*-$N^+(CH_3)_3Cl^-$	831.0	617	9000

* See Fig. 1.
** Data obtained from 100 μM aqueous solutions.

fied dyes chromatographed as single blue bands and were estimated by peak integrations from HPLC to be 96% pure, with the majority having a purity >99%.

The spectral properties of the C.I. Reactive Blue 2 analogues with terminal ring modifications were determined from 100 μM aqueous solutions of purified dye (Table I). A characteristic broad absorption peak with a λ_{max} centred around 620 nm was observed for all dye analogues. The molar extinction coefficients at 620 nm (ε_{620}) were found to be in the range of 7500–12 600 l mol^{-1} cm^{-1}, even though all of the dyes possessed the same chromogen. This phenomenon is likely to be due to dye stacking, since the relatively hydrophobic dye molecules aggregate in aqueous solution in a concentration-dependent manner. The degree of dye stacking (as determined by ε_{620}) appeared to be dependent upon the overall anionic charge carried by the dye and thus the solubility of the dye. Dyes with anionic terminal ring substituents (Table I: I–VII; net charge −3) exhibited notably higher molar extinction coefficients than dyes with neutral (Table I: VIII–X) or cationic (Table I: XI–XII) terminal ring substituents which possessed net charges of −2 and −1, respectively. Plots of dye concentration against A_{620} (not shown) suggested that dyes with neutral and cationic terminal ring substituents exhibited a greater deviation from Beer's law compared to dyes with anionic terminal ring functions.

The 1,4-diaminoanthraquinone chromophore of C.I. Reactive Blue 2 exhibits marked spectral changes upon interacting with ligand binding sites of proteins [25,26]. Thus, the binding of free C.I. Reactive Blue 2 analogues to horse liver alcohol dehydrogenase was readily assessed by difference spectroscopy in the 500–850 mm region. Fig. 2 shows a typical example in which the dye displayed difference spectra composed of multiple peaks, from which the most prominent peak (typically in the region 650–670 nm) was used to calculate the K_d. Peak heights were measured either relative to a zero-absorbance reference point (usually 850 nm) or an isosbestic point. The practice of measuring one peak height relative to that of another was not fol-

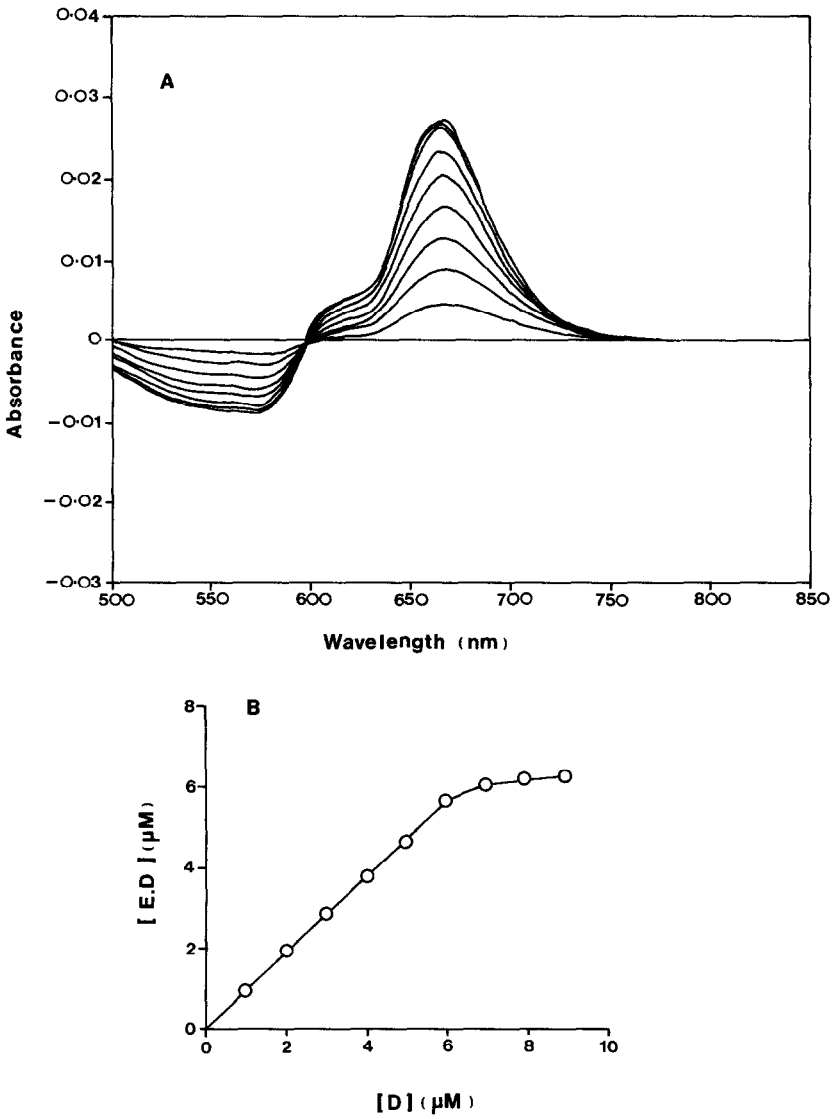


Fig. 2. Titration of horse liver alcohol dehydrogenase with V. A solution of enzyme (6.25 μmol subunits) in 1 ml tricine–sodium hydroxide buffer, pH 8.5 (0.1 *M*) and a buffer reference were titrated with 2 μl aliquots of V (0.5 m*M*) and the difference absorption spectrum recorded at 25°C following each pair of additions (A). A $\Delta\varepsilon_{666}$ of 4200 l · mol^{-1} cm^{-1} was calculated, from which the concentration of enzyme–dye complex formed with each pair of dye additions could be estimated (B).

lowed since peaks were occasionally observed to increase in intensity in a disproportionate manner. Non-linear regression analysis was used to calculate K_d values from spectral data since previously reported methods[25] proved unreliable for tightly bound dyes. Furthermore, the higher dye concentrations required for K_d determinations of the lower affinity dyes often gave higher than expected ΔA_λ values, particularly at

TABLE II

SPECTRAL DIFFERENCE TITRATION OF HORSE LIVER ALCOHOL DEHYDROGENASE WITH C.I. REACTIVE BLUE 2 ANALOGUES

*C.I. Reactive Blue 2 analogue**	λ_{max}** *(nm)*	$\Delta\varepsilon_\lambda$ *($l\ mol^{-1}\ cm^{-1}$)*	K_d *(μM)*
I	672	5400	0.42 ± 0.05
II	656	4800	1.60 ± 0.08
III	668	5100	9.30 ± 1.60
IV	674	3800	0.20 ± 0.04
V	666	4200	0.06 ± 0.02
VI	660	4500	5.90 ± 0.10
VII	660	4500	10.5 ± 1.90
VIII	664	7000	4.50 ± 0.15
IX	660	6500	5.70 ± 0.20
X	654	4000	0.19 ± 0.04
XI	720	–	–
XII	700	3900	172 ± 5.00
XIII	676	5000	3.00 ± 0.10
XIV	668	6400	6.40 ± 0.50

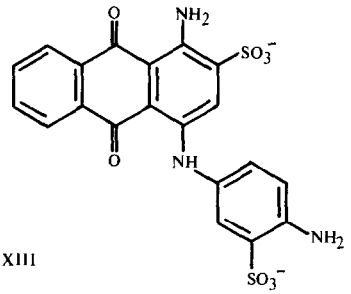
* See Fig. 1.

** Mean value determined for the major difference absorption peak.

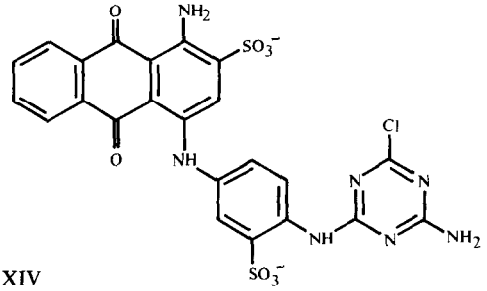
dye concentrations above 60 μM. This effect was probably attributable to non-specific dye–protein interaction which is known to occur at high dye:protein ratios[27]. Thus, K_d values were only calculated from data obtained at dye concentrations below 60 μM.

C.I. Reactive Blue 2 analogues bearing carboxylate terminal ring substituents (Table I: IV, V) displayed higher affinities for horse liver alcohol dehydrogenase than dyes with more bulky sulphonate (Table I: I–III) or methylphosphonate (Table I: VII) groups at equivalent positions on the terminal ring (Table II). Furthermore, dyes with *para*-orientated anionic groups were bound with a reduced affinity compared to dyes with equivalent *ortho*- or *meta*-orientated anionic groups. Thus, of the C.I. Reactive Blue 2 analogues containing anionic terminal ring groups, dye VII (*p*-methylphosphonate substituent) exhibited the lowest affinity (K_d = 10.5 μM) whereas dye V (*m*-carboxylate substituent) had the highest affinity (K_d = 0.06 μM). Dyes with *meta*-orientated anionic groups (II, V) were bound more tightly than dyes with *meta*-orientated neutral hydrophilic terminal ring substituents (VIII, IX). For example, exchange of a *m*-aminobenzoate terminal ring (dye V) for a *m*-aminobenzamide terminal ring (dye IX), resulted in a decrease in binding energy equivalent to 11.3 kJ mol^{-1}. This observation suggests that electrostatic interaction is important for the binding of the terminal ring of C.I. Reactive Blue 2 to horse liver alcohol dehydrogenase.

The introduction of a cationic trimethylammonium substituent on the terminal ring produced a marked decrease in affinity for the enzyme. Thus, dye XII (*p*-trimethylammonium substituent) exhibited the lowest affinity of all the C.I. Reactive Blue 2 analogues investigated (K_d = 172 μM). The K_d of dye XI (*m*-trimethylammonium substituent) could not be estimated with any accuracy due to interferences caused by dye stacking.



XIII



XIV

Fig. 3. The structure of C.I. Reactive Blue 2 intermediate compounds XIII and XIV.

A terminal ring devoid of substituent groups (dye X) facilitated tight dye binding (K_d = 0.19 μM). Thus, with the exception of *ortho-* and *meta*-orientated carboxyl groups, the addition of substituent groups to an otherwise unsubstituted phenyl ring lowers the affinity of the dye for the enzyme binding site. This observation would suggest that hydrophobic interaction and steric hindrance also influence the binding of the terminal ring of C.I. Reactive Blue 2 to horse liver alcohol dehydrogenase.

The affinity for the enzyme of two partial structures of C.I. Reactive Blue 2 were also determined. The purified chromophoric base compound (Fig. 3, XIII), 1-amino-4-(4′-amino-3′-sulphoanilino)-anthraquinone-2-sulphonic acid[17], exhibited a comparatively high affinity for the enzyme (K_d = 3.0 μM). This compound is also bound avidly by other C.I. Reactive Blue 2 binding proteins, particularly dehydrogenases, where it is thought to mimic the binding of the adenosine monophosphate (AMP) moiety of NADH[23].

The purified intermediate (Fig. 3, XIV), 1-amino-4-[4′-(2″-chloro-4″-amino-*s*-triazin-6″-ylamino)-3′-sulphoanilino]-anthraquinone-2-sulphonic acid (synthesised by reacting the dichlorotriazinyl precursor compound[17] with ammonia at 20–25°C) was bound with a slightly reduced affinity (K_d = 6.4 μM) compared to dye XIII. Thus, the introduction of a 2-chloro-4-amino-*s*-triazine group onto dye XIII lowers its affinity for horse liver alcohol dehydrogenase by 1.9 kJ mol^{-1}. The addition of a terminal arylamine ring to XIV had a variable effect on the overall affinity of the dye for the enzyme. In general, the addition of a substituted terminal phenyl ring increased the affinity of the dye for the enzyme. However, the introduction of phenyl

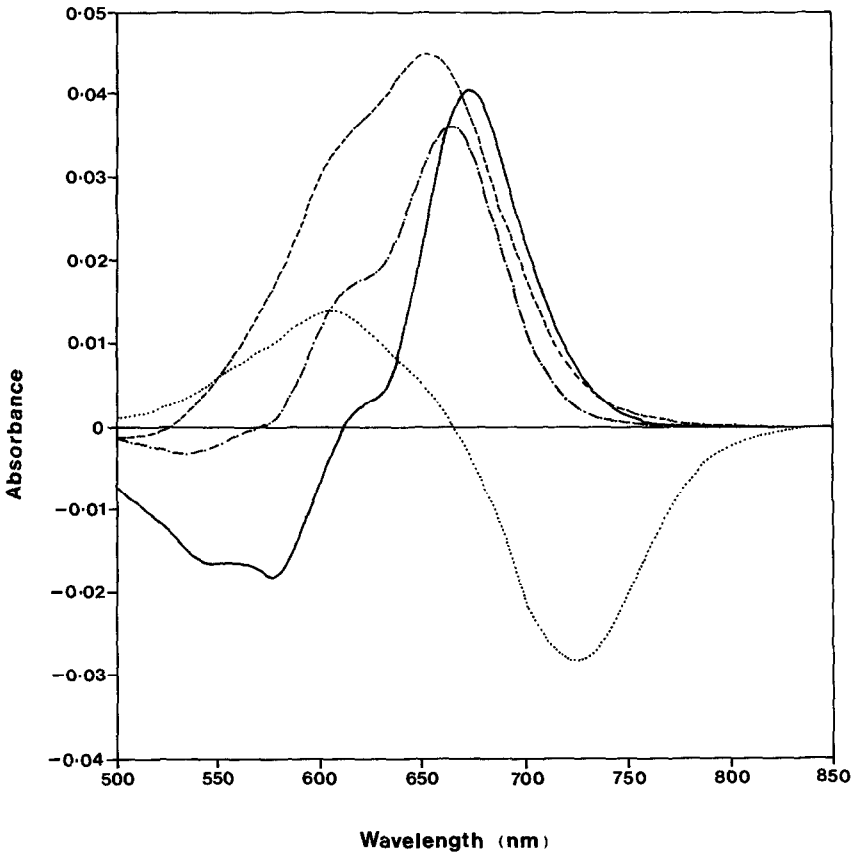


Fig. 4. Difference spectra profiles of C.I. Reactive Blue 2 analogues bound to horse liver alcohol dehydrogenase. Sample solutions contained in 1 ml total volume: tricine–sodium hydroxide buffer, pH 8.5 (99 μmol); enzyme (0.5 mg; 12.5 nmol) and 10 nmol of the C.I. Reactive Blue 2 analogues I (———), II (· – · – ·), X (– – –) and XI (·····). Difference spectra were recorded against a tricine–sodium hydroxide buffer, pH 8.5 (0.1 *M*, 1 ml) blank (25°C), containing dye (10 nmol).

rings bearing bulky *para*-orientated substituents (dyes III, VII and XII) lowered the affinity of the dyes.

The shape of the difference spectra generated when analogues of C.I. Reactive Blue 2 were bound to horse liver alcohol dehydrogenase was influenced by the structure of the terminal ring of the dye (Fig. 4). From a study of the spectral properties of Cibacron Blue F3G-A (dye I) in ionic and apolar solution, Subramanian[26] suggested that a negative difference peak at 585 nm is characteristic of electrostatic dye–protein interaction, whereas a positive peak shoulder at 610 nm and a slight negative peak below 550 nm are characteristic of hydrophobic interaction. On the basis of these observations it may be inferred that the anthraquinone chromogen of I is located in a largely electrostatic environment, whilst that of II is located in a more hydrophobic environment. Thus, alterations to the terminal ring of C.I. Reactive Blue 2 may well influence the binding conformation of the whole dye molecule. The difference spec-

trum observed for XI bore no resemblance to spectra obtained for other C.I. Reactive Blue 2 analogues, since a positive peak at 608 nm and a strong negative peak at 720 nm was observed (Fig. 4). The paradoxical difference spectrum observed for XI, a relatively insoluble dye, was probably due to dye aggregation phenomena, rather than the mode in which the anthraquinone chromophore was bound to the enzyme.

The binding of C.I. Reactive Blue 2 analogues to horse liver alcohol dehydrogenase was competitive with coenzyme binding, since the addition of NADH (grade 1) to enzyme–dye mixtures reduced the intensity of the difference spectrum. The concentration of NADH required to displace 50% of a bound dye was proportional to the affinity of the dye for the enzyme.

The affinity of horse liver alcohol dehydrogenase for immobilised analogues of C.I. Reactive Blue 2 was assessed by analytical affinity chromatography. Dyes were immobilised to beaded agarose by a 1,6-diaminohexane spacer arm to promote optimal protein–ligand interaction (Fig. 5). Adsorbent performance was compared at an immobilised dye concentration of approximately 2.0 μmol g^{-1} moist weight gel, achieved by adjusting the concentration of CDI used in the matrix activation step. The molar extinction coefficients observed for purified 6-aminohexyl dyes in hydrolysed gel media were approximately 14% lower than those obtained for the unsubstituted dyes in distilled water. This reduced extinction was probably due to stacking of the 6-aminohexyl dyes in strongly ionic solutions, and highlights the need to consider such effects when determining the concentrations of reactive dyes in free solution by spectral methods.

With the exception of dye I, the affinity of horse liver alcohol dehydrogenase for the immobilised dyes (as determined by the concentration of NADH required for elution, see Table III) paralleled the affinity of the enzyme for the dye in free solution (Table II). Thus, immobilised dyes V and X bound the enzyme more tightly than dyes III and VII. Furthermore, immobilised dyes XI and XII merely retarded the enzyme, which was eluted prior to the application of the linear NADH gradient. However, the apparent differences in affinity to the immobilised dyes for horse liver alcohol dehydrogenase were less than expected from the $2.9 \cdot 10^3$-fold difference in affinity displayed by the dyes in free solution (Table II). This suggested that the method of dye immobilisation to the matrix sterically hindered dye binding to the enzyme and thus obfuscated intrinsic differences in affinity of the soluble dyes. Not surprisingly, therefore, dye analogues coupled directly to beaded agarose via the chlorotriazine

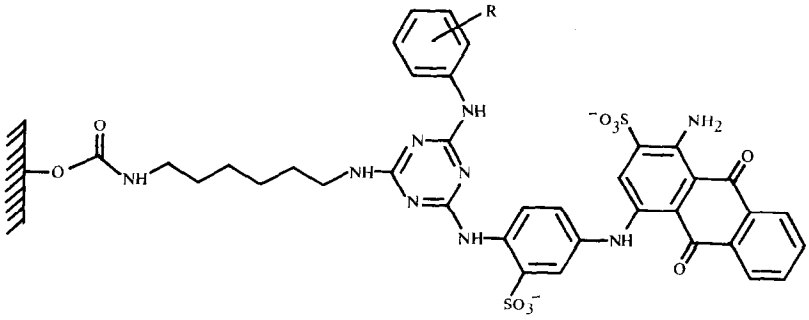


Fig. 5. The structure of 6-aminohexyl-C.I. Reactive Blue 2 analogues coupled to CDI-activated agarose.

TABLE III

AFFINITY CHROMATOGRAPHY OF HORSE LIVER ALCOHOL DEHYDROGENASE ON C.I. REACTIVE BLUE 2 ANALOGUES COUPLED TO SEPHAROSE 4B WITH A 1,6-DIAMINOHEXANE SPACER ARM (4°C; pH 7.5)

C.I. Reactive Blue 2 analogue	*Immobilised dye concentration ($\mu mol\ g^{-1}$ moist weight)*	*[NADH] required for elution*	*Activity recovered (%)*
I	2.2	245	54
II	2.1	78	100
III	2.2	73	90
IV	2.1	81	87
V	1.8	90	80
VI	2.1	80	83
VII	1.9	78	83
VIII	2.3	83	78
X	1.9	89	83
XI	2.2	0	81
XII	2.3	0	84

ring[2] behaved similarly, with very little difference in enzyme binding behaviour being observed between the various analogues (data not shown).

The *ortho*-sulphonate isomer of C.I. Reactive Blue 2 (I) bound horse liver alcohol dehydrogenase considerably more tightly than other C.I. Reactive Blue 2 analogues with higher affinities in free solution. A substantial amount of enzyme was still bound to the column at the end of the elution gradient. An alternative elution strategy (0–1.0 *M* potassium chloride gradient) was employed to determine whether immobi-

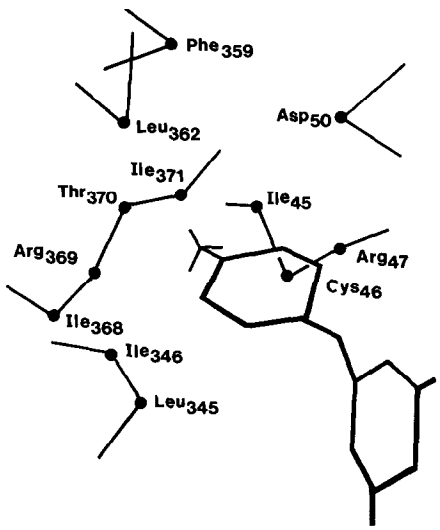


Fig. 6. Structure of the putative C.I. Reactive Blue 2 terminal ring binding site of horse liver alcohol dehydrogenase.

lised dye I displayed a genuinely high affinity for the enzyme or was merely binding non-competitively with respect to NADH. The concentration of potassium chloride required to elute the enzyme from immobilised dye I (0.25 *M*) was notably higher than the concentration required for elution from immobilised dye V (0.16 *M*). This observation suggested that immobilised dye I had a higher affinity for horse liver alcohol dehydrogenase than would have been predicted from the performance of other C.I. Reactive Blue 2 analogues.

DISCUSSION

Crystallographic studies reveal that the terminal phenyl ring of C.I. Reactive Blue 2 is bound by horse liver alcohol dehydrogenase in a region not normally associated with coenzyme binding[14]. From information given by Biellmann *et al.*[14] and deductions made from constructed molecular models, the terminal ring binding site is composed of residues which are located in the catalytic domain of the enzyme. The majority of the residues (Ile_{45}, Leu_{345}, Ile_{346}, Phe_{359}, Leu_{363}, Ile_{368} and Ile_{371}) are apolar. Of the remaining residues, two are cationic (Arg_{47} and Arg_{369}), two are polar (Thr_{370} and Cys_{46}) and one is anionic (Asp_{50}), with the latter being located at the top of the putative binding pocket. The molecular architecture of this site (Fig. 6) can entirely account for the observed affinity of the various C.I. Reactive Blue 2 analogues.

The preponderance of hydrophobic residues in the terminal ring binding site will favour the binding of predominantly hydrophobic species. Thus, dye X was bound more tightly than most C.I. Reactive Blue 2 analogues with hydrophilic groups attached to the terminal phenyl ring (Table II). The two juxtaposed arginine residues (Arg_{47} and Arg_{369}) will encourage the entry of anionic species into the pocket. Thus, dyes bearing acidic groups (I–VII) displayed a much higher affinity for the enzyme compared to dyes with basic groups on the terminal phenyl ring (XI, XII). Dyes bearing terminal rings substituted with carboxylates had higher affinities than dyes possessing sulphonate residues at equivalent positions on the terminal ring. These differences in affinity may be attributed to the smaller size of the carboxyl group relative to the sulphonate group, thereby permitting entry into a relatively narrow pocket, and the smaller hydration shell of a carboxylic acid. A small hydration shell on an anionic terminal ring substituent will promote electrostatic interaction with the cationic arginine residues which line the pocket and will also facilitate hydrophobic interaction by reducing the number of water molecules in the vicinity of the pocket. The tight binding of V is therefore attributable to the optimal hydrophobic and electrostatic interaction between the terminal ring of the dye and its binding site. This conclusion may be significant, since reactive textile dyes invariably rely on sulphonate as opposed to carboxylate groups for solvation. The observation that *para*-orientated terminal ring substituents do not bind as tightly as equivalent *ortho*- and *meta*- substituents may be explained by steric hindrance, since the interchain distance between residues at the bottom of the terminal ring binding pocket (Gly_{44} and Ile_{371}) is only 4.2 Å. *Para*-orientated anionic groups are also unfavourably positioned for interaction with Arg_{47}. The low affinity of XII is thus readily accounted for by unfavourable steric and charge interactions created by the *p*-trimethylammonium cation.

The difference spectra generated on binding C.I. Reactive Blue 2 analogues to horse liver alcohol dehydrogenase suggested that the environment of the anthraquinone chromophore was influenced by the structure of the terminal phenyl ring. The position of anionic groups on the terminal ring may favour interaction with one of the two arginine residues which line the pocket. Such an event may indirectly influence the binding of the anthraquinone moiety of the dye to the adenine binding pocket of the enzyme. Furthermore, by analogy to the binding of NAD^+ to horse liver alcohol dehydrogenase[13], a change in enzyme conformation might also be triggered by the binding of C.I. Reactive Blue 2 analogues, in a manner dependent upon the nature and orientation of the terminal ring substituent. This hypothesis is supported by studies of Cibacron Blue F3G-A binding to glutamine synthetase[28], where the difference spectrum generated upon dye binding was shown to be influenced by the conformational state of the enzyme. The form of the observed difference spectrum may be explained by the bound chromophore interacting with different residues within the adenosine binding crevice of horse liver alcohol dehydrogenase. A hydrophobic type difference spectrum (as observed for II, see Fig. 4) was probably due to strong interaction of the anthraquinone region of the dye with hydrophobic residues such as Phe_{198}. The electrostatic type difference spectrum (as observed for I) is probably the result of a preferred interaction between the anthraquinone-2-sulphonate group and Arg_{271}.

C.I. Reactive Blue 2 analogue I differs from the other analogues in that the terminal ring of the dye is not rotationally mobile due to the presence of an *ortho*-orientated sulphonic acid group. This feature of I may account for the tight binding of the immobilised dye to the enzyme during chromatography. The hindrance to free rotation of the terminal sulphonated phenyl ring of I will be increased by immobilisation of the dye through the triazine ring. Thus, the immobilised dye may be locked into a position which is highly favourable for binding the enzyme. The orientation of the terminal phenyl ring sulphonate group had a marked effect on the affinity of the dye for horse liver alcohol dehydrogenase.

Differences in affinity of C.I. Reactive Blue 2 isomers towards proteins such as dopamine-β-monooxygenase have also been reported[29]. However, on the basis of our work, claims that the *meta*-sulphonate isomer of C.I. Reactive Blue 2 is not an inhibitor of horse liver alcohol dehydrogenase[14] must be discounted. Thus, care must be exercised in selecting affinity adsorbents composed of immobilised C.I. Reactive Blue 2, since commercial dye preparations are known to be heterogeneous[17] and different isomeric forms of the dye may exhibit entirely unexpected chromatographic properties.

Although large differences in affinity towards horse liver alcohol dehydrogenase were displayed by C.I. Reactive Blue 2 analogues in free solution, this was not apparent in the majority of cases when the dyes were immobilised. This observation suggests that the method of immobilisation used does not allow the terminal ring of the dye to interact fully with the enzyme. The weak binding of XIV relative to XIII (Table II) suggests that the triazine ring does not contribute significantly to dye binding. Immobilisation of the dye by the triazine ring may worsen its already weak binding to render this part of the molecule redundant in enzyme binding. This hypothesis is supported by crystallographic data which suggest that the triazinyl chlorine atom of C.I. Reactive Blue 2 bound to horse liver alcohol dehydrogenase is directed towards

the active site of the enzyme[14]. It is highly improbable that the triazine ring adopts a similar orientation when immobilised to a solid support matrix due to severe steric hindrance. Thus, although the anthraquinone and terminal ring regions of the dye contribute to enzyme binding in free solution, it is unlikely that this situation exists when the dye is immobilised. Under the latter circumstances, protein binding is apparently dominated by the anthraquinone ring region.

The effectiveness of the blue chromogen of C.I. Reactive Blue 2 in binding proteins has been amply demonstrated[30–32]. This region of the dye has a particularly high affinity for adenine nucleotide binding proteins, where it frequently mimics the binding of AMP[6,23,33]. The general nature of the hydrophobic/electrostatic interaction of proteins with the anthraquinone region of the dye may account for the wide variety of unrelated proteins which are bound by the immobilised dye. It might be anticipated therefore, that immobilisation of C.I. Reactive Blue 2 analogues by the anthraquinone ring will reduce non-specific dye–protein interaction and allow the terminal ring of the immobilised dye to interact more fully with its complementary binding site. The true effect of modifications to the terminal ring of C.I. Reactive Blue 2 on protein purification by affinity chromatography may then be more readily observed.

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