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DESIGN AND APPLICATION OF BIO-MIMETIC DYES IN BIOTECHNOLOGY

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SUMMARY

The last decade or so has been the introduction of multi-coloured reactive dyes as substitutes for natural biological ligands in the purification of proteins by affinity chromatography. This paper reviews the evidence for the remarkable selectivity of the interaction of reactive dyes with proteins and describes our recent work with dye analogues. Terminal ring, bridging ring and anthraquinone ring analogues of Cibacron Blue F3G-A were synthesised de novo and shown to interact selectively with the NAD^+-binding site of horse liver alcohol dehydrogenase but with affinities differing by several orders of magnitude. It is anticipated that these novel dye ligands will lead to affinity adsorbents with improved affinity, capacity and specificity.

INTRODUCTION

Genetic engineering has provided a tool for the production of large quantities of diagnostic and therapeutic proteins. However, the successful exploitation of such biotechnological processes requires the development of new techniques for the selective purification of these proteins. Techniques such as affinity chromatography harness the natural molecular recognition

encountered between biological macromolecules and complementary ligands such as enzyme substrates, inhibitors and effectors, coenzymes, hormones, immunoligands, nucleic acids and lectins [1, 2]. Affinity technology has been extensively developed over the last few years in response to greater demands being placed on purification technology by the commercial value of therapeutic proteins derived from genetically engineered organisms and the need for more rigorous quality criteria imposed on such products. Not surprisingly, therefore, the more stringent requirements of scale, yield and purity of today's products has led to a critical reappraisal of many of the early ideas of affinity chromatography [3]. Thus, the qualities of the matrix, the activation chemistry, ligand selection and the purification format itself have all been reexamined in the light of these new requirements. For example, whilst agarose has always been regarded as an ideal matrix for laboratory-scale purifications, it exhibits serious shortcomings as a column packing material for large-scale operations. These deficiencies may be circumvented to some extent by substituting alternative solid supports such as cross-linked polysaccharides, silica or the acrylic copolymers Trisacryl® and hydroxyalkyl methacrylate. Similarly, solvolysis of the ligand–matrix bond and thereby contamination of the potential therapeutic product may be reduced by using novel procedures for the activation of the hydroxylic functions on the matrix. For example, the exploitation of sulphonyl chlorides such as tosyl, mesyl and tresyl chlorides [4] and, more recently, the coloured or fluorescent derivatives dipsyl, dansyl and dabsyl chlorides [5] has been described for the activation of matrices and subsequent coupling of proteins and other ligands under mild conditions. These, and other procedures based on 1,1'-carbonyldiimidazole [6], produce very stable, uncharged linkages between the bioligand and the matrix backbone and circumvent many of the problems associated with the widely adopted cyanogen halide activation procedure.

The ligand selected for immobilisation has also been scrutinised more closely in recent years. The high cost, relatively low protein binding capacity and lability to both chemical and biological degradation has seriously undermined the usefulness of affinity adsorbents exploiting natural biological ligands. For this reason, entirely synthetic ligands such as immobilised textile dyes have been investigated as alternatives to conventional biological media [7]. Reactive textile dyes offer significant advantages as ligands for large-scale affinity chromatography. For example, they are available as commodity chemicals in large quantities and at low cost and are easily coupled directly to matrix materials via their reactive functions. The synthetic textile dyes are almost completely resistant to chemical and biological degradation and are immobilised via a stable bond to the matrix. The characteristic spectral properties of the dyes facilitate measurement of immobilised-ligand concentrations and permit easy identification of column materials. However, the most important advantage of reactive textile dyes is their remarkable propensity to bind, selectively and reversibly, a plethora of proteins and enzymes. The immobilised dyes display high capacity for complementary proteins, and adsorbed proteins may be released in good yield and often with significantly enhanced specific activities [7, 8]. For

example, the anthraquinone textile dye, Cibacron Blue F3G-A, is known to interact with representatives from almost every conceivable class of enzyme plus a host of other seemingly unrelated proteins. Over the last few years the specificity of a number of other commercially available textile dyes has been examined systematically. Reactive dyes such as Procion Red HE-3B, Procion Red H-8BN, Procion Yellow MX-8G, Procion Scarlet MX-G, Procion Yellow H-A, Procion Green H-4G and Procion Brown MX-5BR have all been found to be suitable ligands for the purification of selected proteins [9- 11]. In addition to conventional low-pressure affinity chromatography, textile dyes have also been exploited in high-performance liquid affinity chromatography [12, 13], in affinity precipitation [14, 15] and in affinity partition [16]. However, to date, all the previous studies have been executed with a handful of commercially available textile dyes, and most notably, the blue anthraquinone dye, Cibacron Blue F3G-A.

The present paper reports on a systematic study of the effects of defined structural modifications of this dye on its ability to interact with a model protein, horse liver alcohol dehydrogenase. In many cases these dyes have been designed using the principles of computer-aided molecular design and tailor-made to the target protein. We believe that this represents the first recorded case of the directed design and synthesis of artificial affinity ligands for use in affinity purification techniques.

INTERACTION OF REACTIVE DYES WITH PROTEINS

The reactive dyes exploited in the purification of proteins by affinity chromatography were developed originally by ICI in the mid-1950s for staining textiles and paper. They comprise a chromophoric component, often an azo, anthraquinone, phthalocyanine or metal complex, attached to a reactive functional group [7]. The most extensively studied dye, Cibacron Blue F3G-A, comprises four distinct structural units (Fig. 1): (a) the sulphonated anthraquinone moiety; (b) the bridging diaminobenzene sulphonate; (c) the reactive chlorotriazine functional group; (d) the terminal *o*-aminobenzene sulphonate

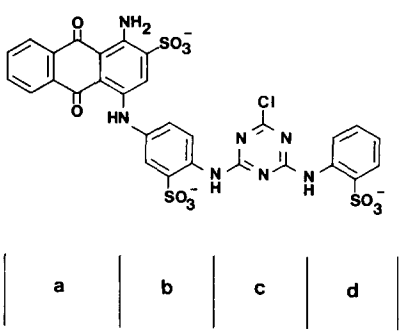


Fig. 1. The principal structural elements of the anthraquinone dye, Cibacron Blue F3G-A. (a) Sulphonated anthraquinone moiety; (b) bridging diaminobenzene sulphonate; (c) reactive chlorotriazine functional group; (d) terminal *o*-aminobenzene sulphonate ring.

ring. The four principal structural features are linked via secondary amine bridges and possess both aromatic and anionic character as well as significant hydrogen bonding potential. Cibacron Blue F3G-A interacts with a variety of proteins including adenine coenzyme-dependent oxidoreductases and phosphokinases, hydrolases, transferases, nucleases, polymerases, synthetases, lyases, decarboxylases and a number of glycolytic enzymes and blood proteins [7]. Difference spectral titrations provide evidence for both hydrophobic and ionic contributions to dye—protein binding. However, in order to interpret spectral changes in the dye in association with different polar or apolar binding sites, the spectral characteristics of Cibacron Blue F3G-A with organic solvents, salts, oligopeptides and model proteins have been examined [17, 18]. The difference spectra of Cibacron Blue F3G-A were significantly affected by the environmental polarity and such differences were used to interpret the spectra generated on binding the dye to dihydrofolate reductase [17]. However, great caution should be exercised in interpreting dye–protein difference spectra since it is known that at concentrations greater than 5 μM Cibacron Blue F3G-A exists partly in a stacked state and that destacking of the dye on binding to proteins is partly responsible for the observed spectral perturbations [19].

More compelling evidence for the detailed nature of the dye–protein interaction has arisen from enzyme inhibition and affinity labelling studies. For example, enzyme inhibition studies with fragments of the Cibacron Blue F3G-A molecule revealed that for several oxidoreductases, phosphokinases and ATPases, the presence of rings a and b (Fig. 1) was critical for strong inhibitory behaviour [20]. This observation was subsequently confirmed by noting that ring a alone was a relatively poor inhibitor, whilst an analogue comprising rings a and b was only marginally less inhibitory than the complete parent dye itself [21]. The conclusions from these studies were confirmed by chromatographing proteins on immobilised analogues of anthraquinone dyes; the anthraquinone moiety (ring a) was again highlighted as a key structural feature essential for tight binding to proteins [21].

Affinity labelling with reactive dyes has yielded more definitive information on dye–protein interactions [22, 23]. However, the ability of monochlorotriazinyl dyes such as Cibacron Blue F3G-A to inactivate enzymes covalently is limited by their reactivity and under the majority of experimental conditions selected, the dye behaves as a competitive, but not an irreversible inhibitor. The use of triazine dyes as irreversible active site-directed affinity labels has therefore centred on the more reactive dichlorotriazinyl dyes. For example, the reactive Cibacron Blue F3G-A analogue, Procion Blue MX-R, inactivates horse liver alcohol dehydrogenase in a manner competitive with the binding of the pyridine nucleotides NAD^+ and NADH [23]. Quantitatively inhibited enzyme incorporated 1 mol dye per mol of subunit of molecular weight 40 000. Chymotryptic digestion of the labelled protein and resolution of the peptides by reversed-phase high-performance liquid chromatography (HPLC) yielded a single blue penta-peptide, in which the affinity label, Procion Blue MX-R, was unambiguously identified as being covalently attached to the thiol side-chain of Cys-174. This amino acid (Cys-174) is a key residue in horse liver alcohol dehydrogenase and lies at the bottom of the active site crevice in the

interdomain region of the subunit within the bounds of the catalytic domain. The side-chain of Cys-174 is one of three residues, including Cys-76 and His-67, which provide ligands to coordinate the catalytic zinc atom. The most likely explanation of the labelling studies is that Procion Blue MX-R is bound to the active site of alcohol dehydrogenase in a manner similar to that in which the natural coenzyme is bound. The anthraquinone moiety of the dye presumably occupies the apolar adenosine binding pocket in such a way that the reactive dichlorotriazinyl ring is positioned close to the thiol side-chain of Cys-174 at the bottom of the active site pocket. Fortunately, the binding of Cibacron Blue F3G-A to orthorhombic crystals of horse liver alcohol dehydrogenase has been studied by X-ray diffraction at 0.37 nm resolution [24]. These studies have defined the binding sites for the different rings of the dye, but not the precise orientations nor conformational details of the rings. The anthraquinone ring a binds in a wide apolar cleft which constitutes, at one end, the adenine binding site. The bridging diaminobenzene sulphonate ring b is positioned such that its sulphonate group could interact with the guanidinium side-chain of Arg-271. Ring c binds close to where the pyrophosphate moiety of the nicotinamide coenzyme binds with the reactive triazinyl chlorine adjacent to the nicotinamide ribose binding site. The terminal ring d is bound in a cleft between the catalytic and coenzyme binding domains with a possible interaction of the anionic sulphonate with the side-chain of Arg-369. The binding of Cibacron Blue F3G-A to horse liver alcohol dehydrogenase thus resembles ADP binding but differs significantly at the nicotinamide end of the coenzyme, with the mid-poing position of ring d differing from the mid-point of the nicotinamide ring of bound NAD^+ by about 10 Å. Thus, modifications of the terminal ring d of Cibacron Blue F3G-A were considered the most likely to produce significant variations in binding affinity to proteins.

TERMINAL RING MODIFICATIONS

Terminal ring analogues of Cibacron Blue F3G-A were synthesised de novo from the key intermediate in the synthesis of sulphonated anthraquinone dyestuffs, bromamine acid (Fig. 2). The bromine is readily displaced by amines in the presence of a copper catalyst to yield compounds with substituted amino groups in the 4-position and displaying the characteristic blue shades of the anthraquinone dyestuffs. Condensation of bromamine acid with the arylamine, 1,4-diaminobenzene-3-sulphonic acid, yields the chromophoric base (1-amino-4,4′-aminophenylamino)anthraquinone-2,3′-disulphonic acid (Fig. 2) used in the production of reactive dyes for cellulosic fibres. The blue base is condensed with 1,3,5-trichloro-*s*-triazine (cyanuric chloride) to yield the corresponding dichloro-*s*-triazine which on substitution with a second base yields monochloro-*s*-triazines (Fig. 2). For example, final substitution with *o*-aminobenzenesulphonic acid produces Cibacron Blue F3G-A (Fig. 1), whereas condensation with a number of other substituted arylamines produced a collection of analogues of the parent dye. In each case, the synthesised dye analogues were purified to > 95% homogeneity by chromatography on Sephadex LH-20, with their purity being monitored by reversed-phase HPLC on a C_{18} μBondapak column support.

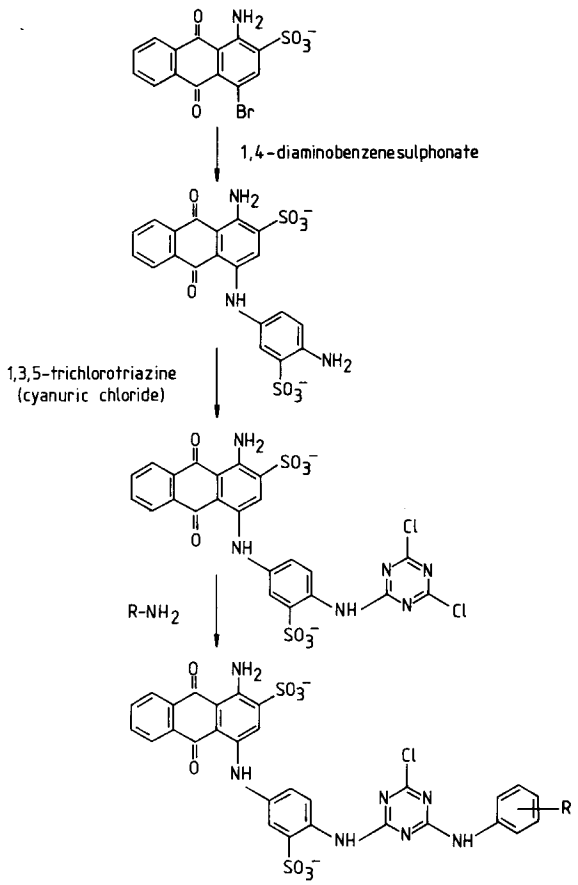
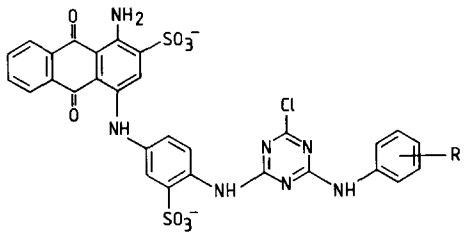


Fig. 2. Synthesis of terminal ring substituents.

The affinities of the purified dye analogues for horse liver alcohol dehydrogenase were assessed by determining the apparent dissociation constants (K_d) by difference spectroscopy at 25°C. Table I lists a number of the terminal ring analogues together with their apparent dissociation constants deduced by difference spectroscopy. However, despite the reservations expressed earlier about the use of spectral perturbations to monitor dye binding to proteins, the apparent K_d values listed in Table I were similar to those determined by enzyme inactivation studies [22, 23]. The data demonstrate that small substituents bind more tightly than more bulky species and especially so if substituted in the *o*- or *m*-positions on the terminal aryl-amine ring with a neutral or anionic group. Substitutions at the *p*-position produce analogues with a more linear structure which do not fit well into the coenzyme binding site of horse liver dehydrogenase. In particular, substitution with a cationic trimethylammonium group [Table I, R = $N^+(CH_3)_3$ produces a dye analogue which displays very low affinity for the enzyme, presumably because not only is it a *p*-substituent but also is cationic and therefore likely to interact with the positively charged side-chain of Arg-369 which is postulated to be in the vicinity and attracts the smaller anionic substituents [24]. Thus, altering the structure of the parent dye, Cibacron Blue F3G-A

TABLE I

APPARENT AFFINITIES OF TERMINAL RING ANALOGUES OF ANTHRAQUINONE DYES FOR HORSE LIVER ALCOHOL DEHYDROGENASE



R	Apparent K_d (μM)
m-COOH	0.06
H	0.2
o-COOH	0.2
o-SO_3H	0.4
m-SO_3H	1.6
m-CH_2OH	4.5
m-$CONH_2$	5.7
p-COOH	5.9
p-SO_3H	9.3
p-PO_3H_2	10.5
p-$N^+(CH_3)_3$	172

(Table I, R = *o*-SO_3H) in a relatively minor way, can lead to very substantial changes in the apparent affinity of the analogues for the enzyme. This affinity can be altered by several orders of magnitude for horse liver alcohol dehydrogenase.

BRIDGING RING MODIFICATIONS

Molecular modelling studies using a computer-aided design package have indicated that the parent dye, Cibacron Blue F3G-A, and the terminal ring analogues described above are too short and rigid to bind to horse liver alcohol dehydrogenase in an identical manner to the natural coenzyme, NAD^+. Thus, two analogues of the parent dye were synthesised which possess central spacer groups in order to lengthen and de-rigidify the structure and make it more comparable to the hooked binding mode of NAD^+ [24]. The first analogue (SJB/25/C19) contained an ethyl spacer molecule inserted between the bridging diaminobenzene sulphonate ring (b) (Fig. 1) and the reactive triazine ring (c) (Fig. 1) whilst the second analogue (SJB/30/C23) comprised a second bridging aminobenzene sulphonate ring in addition to the normal bridging ring (b) (Fig. 1). Difference spectral titrations revealed that the second analogue (SJB/30/C23) displayed an apparent affinity almost ten-fold higher for horse liver dehydrogenase than the former (SJB/25/C19).

ANTHRAQUINONE MODIFICATIONS

It is well known that for immobilised NAD^+ to be effective either as an affinity adsorbent [25] or a catalytically active coenzyme [26] for oxidoreductases, it must be attached to the matrix via a spacer molecule substituted at the exocyclic N^6-amino group on the adenine moiety. The adenosine part of the coenzyme is bound in a shallow hydrophobic crevice with the exocyclic N^6-amino function protruding into solvent and therefore accessible to modification with spacer molecules without impairing the native interaction with the complementary oxidoreductases. By analogous reasoning, in view of the similarity of binding the anthraquinone and the adenine moieties in the same apolar crevice, three Cibacron Blue F3G-A analogues have been synthesised in which a spacer molecule has been substituted on the anthraquinoid amino group. The procedure entails reaction of the methoxylated dye analogue with excess chloroacetyl chloride in dimethyl formamide followed by nucleophilic displacement with 1,2-diaminoethane to yield the substituted analogues illustrated in Fig. 3. Unlike the parent dye analogues which have typical bright blue shades, the new anthraquinoid-substituted analogues are characteristically red.

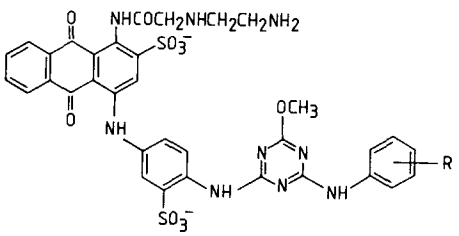


Fig. 3. Anthraquinone-substituted dye analogues for affinity chromatography. R = *o*-, *p*-SO_3^-, *m*-COO^-.

AFFINITY CHROMATOGRAPHY ON IMMOBILISED DYE ANALOGUES

Reactive dye analogues prepared by modification of the terminal ring, bridging ring or anthraquinone moiety were immobilised via an aminoalkyl spacer molecule to Sepharose 4B activated with 1,1′-carbonyldiimidazole. An immobilised-dye concentration of approx. 2 μmol/g moist weight gel was achieved by controlling the extent of activation of the matrix. Preliminary affinity chromatography experiments were performed with purified horse liver alcohol dehydrogenase at 4°C with glass minicolumns containing 1 g moist weight gel equilibrated with 0.02 *M* N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) buffer, pH 7.5. Dialysed enzyme (2—3 U) was applied to the column, washed with equilibrating buffer and eluted with a 0—0.5 m*M* NADH gradient. In general, the concentration of NADH at which peak enzyme activity was eluted on the gradient, correlated well with the observed apparent dissociation constants determined for the dye analogues and the enzyme in free solution. Highest affinity for horse liver alcohol dehydrogenase was displayed for the immobilised *m*-carboxy analogue of Cibacron Blue F3G-A (Table I, R = *m*-COOH) and other analogues with this structural feature, notably the two

bridging ring analogues SJB/25/C19 and SJB/30/C23. The immobilised *p*-substituted terminal ring analogues displayed weaker affinity for the enzyme than the corresponding *o*- and *m*-substituted analogues. The cationic terminal ring analogues, bearing *m*- and *p*-trimethylammonium substituents [Table I, R = *m*-, *p*-$N^+(CH_3)_3$], as anticipated from their apparent K_d values, when immobilised did not adsorb horse liver alcohol dehydrogenase. The mean recovery of enzyme activity in all cases was > 80%.

One dye analogue (SJB/25/C19) apparently resolved the commercial sample of horse liver alcohol dehydrogenase into two active components. The structure of SJB/25/C19 was predicted from molecular design computing to be capable of adopting a similar shape and flexibility to NAD^+ (Fig. 4). Rechromatography of the two resolved components showed identical chromatographic behaviour and electrophoresis revealed them to be distinct enzyme species. Affinity chromatography on the immobilised parent dye, Cibacron Blue F3G-A, under identical conditions failed to resolve the active components of horse liver alcohol dehydrogenase. These observations clearly demonstrate the value in selective design and synthesis of affinity ligands for the chromatographic resolution of proteins.

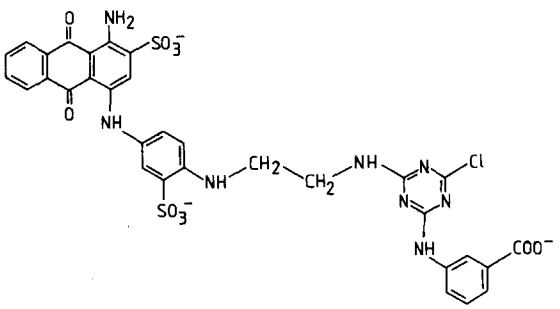


Fig. 4. Structure of the bridging ring analogue, SJB/25/C19.

As predicted by analogy to the efficacy of N^6-adenine-substituted derivatives of NAD^+ in the purification of nicotinamide nucleotide coenzyme-dependent enzymes by affinity chromatography, the anthraquinone-substituted analogues of Cibacron Blue F3G-A proved particularly effective. Horse liver alcohol dehydrogenase was particularly tightly bound to immobilised dyes coupled through the anthraquinonoid moiety and containing a terminal ring substituted with an *o*-sulphonate or *m*-carboxylate (Fig. 3). These observations are in keeping with previous data on the effect of terminal ring substitutions.

CONCLUSIONS

It is well established that immobilised sulphonated aromatic dyes of the chlorotriazine type such as Cibacron Blue F3G-A are effective affinity adsorbents for the isolation and purification of hundreds of enzymes and other proteins. The literature abounds with examples of applications of dye—ligands in solid phase, liquid phase and large-scale processing of diagnostic, therapeutic

and genetically engineered proteins. However, to date, all studies have exploited commercially available textile dyes which were destined for large-scale applications in the printing and textile industries. We now report preliminary data from an extensive and on-going study into the rational design of bio-mimetic dyes specifically targetted at individual proteins. Horse liver alcohol dehydrogenase has been selected as a model system since the interaction of Cibacron Blue F3G-A [24] and Procion Blue MX-R [23] with this enzyme is understood to some extent. Modifications to the terminal ring, bridging ring and anthraquinone moiety dramatically alter the interaction with the complementary protein and change the dye—protein affinity by several orders of magnitude. These structural modifications are in accordance with the anthraquinone moiety occupying the adenosine binding site and the terminal ring residing within the protein at a region adjacent to the active site. It is anticipated that these novel dye ligands will lead to improved affinity, capacity and specificity of the affinity adsorbents and related techniques.

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