The Specific Interaction of Cibacron and Related Dyes with Cyclic Nucleotide Phosphodiesterase and Lactate Dehydrogenase

By ANTHONY R. ASHTON* and GIDEON M. POLYA

Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia

(Received 13 March 1978)

1. Reactive Blue 2 (Cibacron Blue 3G-A) is a competitive inhibitor of bovine heart cyclic nucleotide phosphodiesterase (K_i 0.3 μM). The K_i increases with increasing temperature, suggesting that hydrophobic interactions are not largely responsible for the binding of the dye. Another 25 sulphonated aromatic dyes are also competitive inhibitors of the cyclic nucleotide phosphodiesterase (K_i values in the range of 0.06–13.6 μM). 2. These dyes (covalently linked to Dextran 40) inhibit bovine heart cyclic nucleotide phosphodiesterase. Reactive Blue 2 (covalently linked to Dextran 40) is a competitive inhibitor of the phosphodiesterase (K_i 0.4 μM). 3. Bovine heart cyclic nucleotide phosphodiesterase is retained on a column of Reactive Blue 2–Sephacryl S-200 and can be eluted from the column by 3':5'-cyclic AMP. 4. A variety of the dyes (either free or covalently linked to Dextran 40) are competitive inhibitors of rabbit muscle lactate dehydrogenase. 5. The effectiveness of a wide range of structurally dissimilar dyes as competitive inhibitors of lactate dehydrogenase and cyclic nucleotide phosphodiesterase compromises proposals for the use of Reactive Blue 2 as a specific probe for the dinucleotide-binding structural domain present in many dehydrogenases and kinases. Detailed information of the various dyes used has been deposited as Supplementary Publication SUP 50089 (7 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1978) 169, 5.

Blue Dextran is a dextran with an average mol.wt. of 2000000 to which the dye Cibacron Blue 3G-A (Colour Index name: Reactive Blue 2) has been covalently coupled. Based on their studies of the interaction of Blue Dextran with phosphofructokinase, Bohme *et al.* (1972) proposed that Reactive Blue 2 had sufficient structural similarities with ATP to enable ATP-reversible binding of the enzyme to Blue Dextran. Similarly Ryan & Vestling (1974) observed NADH-reversible binding of lactate dehydrogenase to Blue Dextran.

The 'dinucleotide fold' is an evolutionarily conserved region of secondary structure that forms the nucleotide-binding site for various dehydrogenases and kinases (Rossmann *et al.*, 1974). Thompson & Stellwagen (1976) examined a range of enzymes for specific (substrate-reversible) binding to Blue Dextran. Enzymes possessing the 'dinucleotide-fold' were bound specifically to Blue Dextran, whereas proteins known not to possess this structural domain did not bind the Blue Dextran in this substrate-reversible fashion. It has been proposed that Reactive Blue 2 specifically interacts with the 'dinucleotide-fold' structural domain and that this

interaction was due to structural similarities between this dye and NAD (Thompson & Stellwagen, 1976; Stellwagen, 1977).

The present study describes the specific interaction of Reactive Blue 2 and a wide range of structurally dissimilar sulphonated aromatic dyes with a cyclic nucleotide phosphodiesterase. The structural requirements for interaction of these dyes with lactate dehydrogenase (an enzyme possessing the 'dinucleotide-fold' structure) are also described.

Experimental

Materials

Coomassie Brilliant Blue R (Acid Blue 83) and Coomassie Brilliant Blue G (Acid Blue 90) were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Trypan Blue (Direct Blue 14) was obtained from George T. Gurr, London S.W.6, U.K. Remazol Brilliant Blue R (Reactive Blue 19) was provided by Hoechst Australia, Melbourne, Australia. Cibacron dyes were provided by Mr. F. Colaci of CIBA-GEIGY (Australia), Melbourne, Australia, and Procion dyes, Nylomine dyes and Lissamine Blue 2R (Acid Blue 47) were provided by Mr. K. Brown of I.C.I. (Australia), Melbourne,

* Present address: Department of Biological Sciences, University of Illinois at Chicago Circle, Chicago, IL 60680, U.S.A.

Australia. All dyes were used without further purification. For Reactive Blue 13 and Cibacron Blue TRE (structures of both unknown) a mol.wt. of 1000 was assumed for calculation of ϵ_{mM} values. Chromatography of these dyes on cellulose thin-layer sheets developed with butan-1-ol/pyridine/water (1:1:1, by vol.) revealed the presence of minor dye species in addition to the major species. No chromatographic species common to all the dye samples was detectable in either visible or u.v. light. The commercial name(s), generic name, Colour Index (1971) constitution number, extinction coefficient and structural formula for each of the dyes used in this study are deposited with the British Library Lending Division, Boston Spa, Wetherby, W. Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1978) **169**, 5. The dyes are referred to in this paper by their Colour Index generic names (Colour Index, 1971).

Bovine heart cyclic nucleotide phosphodiesterase (0.2 unit/mg) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The phosphodiesterase solution (1 mg/ml) was stored frozen at -20°C and before each series of assays a sample was thawed and diluted to the appropriate concentration (usually $20\text{ }\mu\text{g/ml}$) with water. Lactate dehydrogenase, purified from rabbit skeletal muscle by affinity elution chromatography (Scopes, 1977), was provided by Dr. R. K. Scopes and Mrs. A. Stoter. Dextran 40 was from Watson Victor, Melbourne, Australia. Polyox WSR 301 was provided by Union Carbide, Melbourne, Australia. 3':5'-Cyclic AMP, bovine serum albumin, pyruvate and NADH were from Sigma. 3':5'-Cyclic [2,8- ^3H]AMP (32 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Sephacryl S-200 was from Pharmacia Fine Chemicals, AB, Uppsala, Sweden. All other chemicals were of analytical reagent grade.

Enzyme assays

Lactate dehydrogenase (EC 1.1.1.27) was measured spectrophotometrically at 30°C as described by Scopes (1977), but with the omission of bovine serum albumin (which binds the dyes used). NADH (0.1 mM) and 2 mM-pyruvate were present in the standard assay.

Cyclic AMP phosphodiesterase (EC 3.1.4.17) was measured at 30°C in an assay reaction medium (30 μl) containing 50 mM-Tris/HCl, pH 7.5, 5 mM-MgCl₂, 1 μM -cyclic AMP and 30 nCi of cyclic [2,8- ^3H]AMP. The reaction was initiated by addition of heart cyclic nucleotide phosphodiesterase (0.2 μg). The reaction was stopped by spotting 25 μl of the reaction mixture on to a poly(ethyleneimine)-cellulose thin-layer plate containing 0.1 μmol each of cyclic AMP, 5'-AMP and adenosine. The product of the reaction (5'-[^3H]AMP) was purified and measured as described previously (Ashton & Polya, 1977).

Synthesis of substituted dextrans

Dextran (0.5 g) was dissolved in water (18 ml) containing 12.5% (w/v) NaCl at 60°C . A freshly prepared solution of dye [3 ml containing 3.3% (w/v) dye] was added to the dextran solution and the mixture incubated for 30 min at 60°C . Na₂CO₃ (0.2 g) was then added to the dextran solution, which was kept at 80°C for 90–180 min, then cooled and free dye separated from the covalently coupled dye. The substituted dextran was precipitated directly by addition of 4 vol. of ethanol; however, repeated precipitations by this procedure were found to be necessary to remove all non-covalently bound dye. Substituted dextrans were purified before use by passage through a column of Sephadex G-25 equilibrated with water. The degree of substitution of the dextrans was determined spectrophotometrically assuming invariance of the extinction coefficient of the free and covalently attached dye and a dextran monomer mol.wt. of 162. The degree of substitution of various dextrans ranged from 0.3 to 1.0 residues/100 residues (commercial Blue Dextran contains 1.1 residue/100 residues).

Reactive Blue 2 was coupled to Sephacryl S-200 (added to the reaction mixture as a slurry in water) as described above. The degree of substitution was determined from the absorbance of substituted gel particles suspended in either glycerol or 0.5% (w/v) Polyox. The substituted Sephacryl S-200 contained 2.5–3.0 μmol of Reactive Blue 2/ml of settled beads.

Results and Discussion

Competitive inhibition of cyclic nucleotide phosphodiesterase by dyes

The rate of hydrolysis of cyclic AMP in the presence and absence of Reactive Blue 2 was a linear function of phosphodiesterase concentration (Fig. 1). Irreversible inhibition of the phosphodiesterase would be indicated by deviations from linearity (Ackermann & Potter, 1949; Cha, 1975). Bovine serum albumin (5 μM) substantially overcomes the inhibition due to the presence of 1 μM -Reactive Blue 2 in the standard phosphodiesterase assay. Bovine serum albumin has been previously shown to bind Reactive Blue 2 (Travis & Pannell, 1973). The inhibition of the phosphodiesterase by Reactive Blue 2 is competitive (Fig. 2; Table 1). A large number of other sulphonated dyes, which differ in chromophore structure or lack of a chlorotriazine residue (e.g. Acid Blue 47), are also competitive inhibitors of the phosphodiesterase (Table 1). With the exception of Reactive Red 8 (K_i 14 μM), all of these dyes are more effective inhibitors than the known inhibitor 3-isobutyl-1-methylxanthine (K_i 10 μM) (Fig. 2; Table 1) and they represent the most potent

Fig. 1. Ackermann-Potter plot for cyclic nucleotide phosphodiesterase preincubated with Reactive Blue 2. Bovine heart cyclic nucleotide phosphodiesterase was preincubated in the absence or presence of Reactive Blue 2 for 10 min at 22°C and then incubated in the presence of substrate in the standard assay conditions (see the Experimental section) for 20 min at 30°C. ○, No dye present; ●, 0.3 μM-dye present; ■, 1.3 μM-dye present in the final assay mixture.

Fig. 2. Competitive inhibition of cyclic nucleotide phosphodiesterase by sulphonated aromatic dyes. Initial rates of cyclic AMP hydrolysis were determined at 30°C as described in the Experimental section and the data are presented as double-reciprocal plots. The initial concentration of cyclic AMP was varied in the presence or absence of various inhibitors. ○, No inhibitor present; ●, plus 1.0 μM-Reactive Blue 2 (linked to Dextran 40); □, plus 0.68 μM-Reactive Blue 2; ■, plus 20 μM-3-isobutyl-1-methylxanthine.

inhibitors of mammalian cyclic nucleotide phosphodiesterase yet described (cf. Amer & Kreighbaum, 1975; Chasin & Harris, 1976).

Table 1. Inhibition of cyclic nucleotide phosphodiesterase by various dyes and dextran-linked dyes

K_i values of the cyclic nucleotide phosphodiesterase for the free dyes were determined as described in the legend to Fig. 2; all dyes thus examined were competitive inhibitors of the enzyme. The inhibition of the phosphodiesterase by inclusion of dextran-linked dyes (at 10 or 1 μM) in the standard assay was determined in duplicate as described in the Experimental section. The control assays (no added dye) were performed with an eightfold replication (s.d. approx. 8% of mean) and duplicate estimates of the percentage inhibition were within 10% of the mean value.

Dye	K_i for free dye (μM)	Inhibition of phosphodiesterase by dextran-linked dyes (%)	
		10 μM	1 μM
Direct Blue 14	0.7		
Acid Blue 25	2.0		
Acid Blue 40	5.5		
Acid Blue 47	1.2		
Acid Blue 83	1.2		
Acid Blue 90	1.3		
Reactive Black 1	1.0	88	61
Reactive Brown 1	1.1	94	38
Reactive Red 1	5.5		
Reactive Red 3	0.7	28	12
Reactive Red 4	0.9	99	90
Reactive Red 6	2.7		
Reactive Red 7	0.2	92	68
Reactive Red 8	13.6	53	32
Reactive Red 12		65	7
Reactive Orange 1	3.3	21	7
Reactive Orange 2	1.4	51	7
Reactive Yellow 1	4.7	81	3
Reactive Yellow 2	0.7	55	24
Reactive Yellow 3	0.7	100	69
Reactive Yellow 4	0.7	26	20
Reactive Blue 2	0.3	92	80
Reactive Blue 4	1.4	93	72
Reactive Blue 5	0.06	97	94
Reactive Blue 7		86	52
Reactive Blue 13		90	19
Reactive Blue 19	0.6	99	77
Cibacron Blue TRE	1.3	68	37
Reactive Violet 2	2.1	87	11

Interaction of dextran-linked dyes with the phosphodiesterase

Reactive Blue 2 coupled to Dextran 40 is a competitive inhibitor (K_i 0.4 μM) of the cyclic nucleotide phosphodiesterase (Fig. 2). The phosphodiesterase is also inhibited by low concentrations of all other dextran-linked dyes examined (Table 1). Cyclic nucleotide phosphodiesterase has been partially purified by conventional means from a variety of tissues (Goren & Rosen, 1972; Cheung & Lin, 1974), but the only affinity purification procedures used

have made use of the reversible interaction of the Ca^{2+} -binding modulator protein and cyclic nucleotide phosphodiesterase (Ho *et al.*, 1977; Watterson & Vanaman, 1976). The interaction of the phosphodiesterase with Reactive Blue 2 coupled to Sephacryl S-200 can be used for affinity chromatography of the enzyme. The cyclic nucleotide phosphodiesterase was retained by such an affinity column and was eluted by 10mM-cyclic AMP (Fig. 3a) or at high ionic strength (Fig. 3b). Elution with 1M-KCl yielded a phosphodiesterase elution profile with an extended trailing edge; replacement of 1M-KCl with 5mM-cyclic AMP/0.5M-KCl as the eluting solution resulted in displacement of the remaining phosphodiesterase from the affinity column (Fig. 3b). The phosphodiesterase does not interact with unmodified Sephacryl S-200 in the conditions used here.

Fig. 3. Affinity chromatography of cyclic nucleotide phosphodiesterase on Reactive Blue 2-Sephacryl S-200. Bovine heart phosphodiesterase (1 mg, partially purified as described by Butcher & Sutherland, 1962) dissolved in 0.5 ml of buffer [containing 50mM-Tris/HCl, 5mM-MgCl₂, 10mM-2-mercaptoethanol, 1mM-EGTA and 10% (v/v) glycerol (pH 7.5)], was applied to columns (0.24 cm² × 2.5 cm) of Reactive Blue 2-Sephacryl S-200 and eluted with 4 ml of the same buffer. The column was eluted as indicated with (a) buffer containing 10mM-cyclic AMP, (b) buffer containing 1M-KCl and then buffer containing 5mM-cyclic AMP and 0.5M-KCl. Phosphodiesterase was assayed as described in the Experimental section.

Temperature-dependence of the phosphodiesterase-Reactive Blue 2 interaction

The K_m of the bovine heart phosphodiesterase for cyclic AMP increases with temperature: from 11 μM at 0°C to 46 μM at 40°C. Similarly, the K_i of the phosphodiesterase for Reactive Blue 2 increases from 0.08 μM at 0°C to 0.33 μM at 30°C, Reactive Blue 2 acting as a competitive inhibitor at all temperatures examined in this range. Because hydrophobic interactions are favoured at high temperatures (Nemethy, 1967), this result suggests that other forces are responsible for binding of the dye to the enzyme. Estimates of ΔG° , ΔH° and ΔS° for the binding of cyclic AMP and Reactive Blue 2 to the phosphodiesterase and ΔE_{act} for the phosphodiesterase-catalysed reaction are given in Table 2. The energy of activation for the bovine heart phosphodiesterase-catalysed reaction (93.5 kJ·mol⁻¹) is similar to the value of 79 kJ·mol⁻¹ for dog heart phosphodiesterase (Nair, 1966), but much higher than the value for brain cyclic nucleotide phosphodiesterase (31 kJ·mol⁻¹) (Cheung, 1967).

Interaction of the dyes with lactate dehydrogenase

The dyes, both free and covalently linked to Dextran 40, also inhibit rabbit muscle lactate dehydrogenase competitively with respect to NADH (Table 3, Fig. 4). Seven of the dyes were more effective inhibitors than Reactive Blue 2, which has previously been reported to inhibit lactate dehydrogenase (Thompson *et al.*, 1975).

Stellwagen (1977) proposed that Reactive Blue 2 is structurally similar to NAD and thus can specifically bind to the nucleotide-binding domain of many dehydrogenases and kinases. However, the present study shows that a wide range of otherwise structurally dissimilar aromatic sulphonated dyes are potent competitive inhibitors of both cyclic

Table 2. Thermodynamic parameters for the interaction of bovine heart phosphodiesterase with Reactive Blue 2 and cyclic AMP

	Reactive Blue 2	Cyclic AMP
ΔH° (kJ·mol ⁻¹)	-31.6	-24.6
ΔG° (kJ·mol ⁻¹)	-37.7	-25.7
ΔS° (J·mol ⁻¹ ·K ⁻¹)	+20	+ 3.7
ΔE_{act} (kJ·mol ⁻¹)		93.5

Table 3. *Inhibition of lactate dehydrogenase by various dyes and dextran-linked dyes*

K_i values of lactate dehydrogenase for free dyes and dyes covalently linked to dextran 40 were determined as described in the legend to Fig. 4; the dyes and dextran-linked dyes thus examined were all competitive inhibitors of the enzyme. The inhibition of lactate dehydrogenase by inclusion of dextran-linked dyes at $10\mu\text{M}$ in the standard assay was determined in duplicate as described in the Experimental section. The control assays (no added dye) were conducted in quadruplicate (s.d. approx. 5% of mean) and duplicate estimates of percentage inhibition were within 10% of the mean value.

Dye	Inhibition of lactate dehydrogenase by dextran-linked dyes (%)	K_i for free dye (μM)	K_i for dextran-linked dye (μM)
Direct Blue 14		0.6	
Acid Blue 25		12.6	
Acid Blue 83		9.3	
Reactive Black 1	32	0.9	
Reactive Brown 1	12	17.8	
Reactive Red 1	12		
Reactive Red 3	27		
Reactive Red 4	40	0.5	0.9
Reactive Red 7	10	9.1	
Reactive Red 8	1		
Reactive Red 12	9	17.8	
Reactive Orange 1	3		
Reactive Orange 2	26		
Reactive Yellow 1	9		
Reactive Yellow 2	5	6.1	
Reactive Yellow 3	66	2.7	1.2
Reactive Yellow 4	15		
Reactive Blue 2	22	2.5	6.7
Reactive Blue 4	30	5.4	
Reactive Blue 5	28	3.8	
Reactive Blue 7	31		
Reactive Blue 13	10		
Reactive Blue 19	13	27.0	
Cibacron Blue TRE	80		
Reactive Violet 2	29	7.5	33.0

nucleotide phosphodiesterase and lactate dehydrogenase (Tables 1 and 3). It is therefore difficult to believe that the specific interactions of Reactive Blue 2 and related dyes with these proteins are due to structural similarities between these dyes and the nucleotide substrates of the proteins. Other non-sulphonated dyes have also been shown to bind at or near the nucleotide-binding sites of various enzymes (Wolff & Wolff, 1957; Brand *et al.*, 1967; Bond *et al.*, 1970; Rippa *et al.*, 1970; Wassarman & Lentz, 1971). These specific dye-protein interactions could possibly derive from flexibility of the nucleotide-binding domain enabling accommodation of the multiplicity of potential binding determinants on these structurally complex dye molecules.

Fig. 4. *Competitive inhibition of lactate dehydrogenase by sulphonated aromatic dyes*

Kinetic parameters for each dye were determined from a single reaction progress curve as described by Yun & Suelter (1977). The initial reaction mixture was the same as described in the Experimental section, but contained 5 mM-pyruvate. (a) $\square$, No inhibitor; $\circ$, $5\mu\text{M}$ -Reactive Red 4; $\bullet$, $5\mu\text{M}$ -Reactive Black 1; $\triangle$, $5\mu\text{M}$ -Reactive Yellow 3. (b) $\square$, No inhibitor; $\circ$, $5\mu\text{M}$ -Reactive Red 4 (linked to Dextran); $\bullet$, $5\mu\text{M}$ -Reactive Yellow 3 (linked to Dextran).

Since a variety of sulphonated aromatic dyes are competitive inhibitors of both cyclic nucleotide phosphodiesterase and lactate dehydrogenase, we suggest that a broad range of such dyes can be used as general ligands for affinity chromatography of nucleotide-binding enzymes such as those that bind Reactive Blue 2.

This study was supported by a grant from the Australian Research Grants Committee. A. R. A. was supported by a Commonwealth Postgraduate Research Award.

References

- Ackermann, W. W. & Potter, V. R. (1949) *Proc. Soc. Exp. Biol. Med.* **72**, 1-9
- Amer, M. S. & Kreighbaum, W. E. (1975) *J. Pharm. Sci.* **64**, 1-37
- Ashton, A. R. & Polya, G. M. (1977) *Biochem. J.* **165**, 27-32

- Bohme, H. J., Kopperschlager, G., Schultz, J. & Hofmann, E. (1972) *J. Chromatogr.* **69**, 209–214
- Bond, J. S., Francis, S. H. & Park, J. H. (1970) *J. Biol. Chem.* **245**, 1041–1053
- Brand, L., Gohlke, J. R. & Rao, D. S. (1967) *Biochemistry* **6**, 3510–3518
- Butcher, R. W. & Sutherland, E. W. (1962) *J. Biol. Chem.* **237**, 1244–1250
- Cha, S. (1975) *Biochem. Pharmacol.* **24**, 2177–2185
- Chasin, M. & Harris, D. N. (1976) *Adv. Cyclic Nucleotide Res.* **7**, 225–264
- Cheung, W. Y. (1967) *Biochemistry* **6**, 1079–1087
- Cheung, W. Y. & Lin, Y. M. (1974) *Methods Enzymol.* **38**, 223–239
- Colour Index (1971) 3rd edn., The Society of Dyers and Colourists, Bradford
- Goren, E. N. & Rosen, O. M. (1972) *Arch. Biochem. Biophys.* **153**, 384–397
- Ho, H. C., Wirch, E., Stevens, F. C. & Wang, J. H. (1977) *J. Biol. Chem.* **252**, 43–50
- Nair, K. G. (1966) *Biochemistry* **5**, 150–157
- Nemethy, G. (1967) *Angew. Chem. Int. Ed.* **6**, 195–206
- Rippa, M., Picco, C. & Pontremoli, S. (1970) *J. Biol. Chem.* **245**, 4977–4981
- Rossmann, M. G., Moras, D. & Olsen, K. W. (1974) *Nature (London)* **250**, 194–199
- Ryan, L. D. & Vestling, C. S. (1974) *Arch. Biochem. Biophys.* **160**, 279–284
- Scopes, R. K. (1977) *Biochem. J.* **161**, 253–263
- Stellwagen, E. (1977) *Acc. Chem. Res.* **10**, 92–98
- Thompson, S. T. & Stellwagen, E. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 361–365
- Thompson, S. T., Cass, K. H. & Stellwagen, E. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 669–672
- Travis, J. & Pannell, R. (1973) *Clin. Chim. Acta* **49**, 49–52
- Wassarman, P. M. & Lentz, P. J. (1971) *J. Mol. Biol.* **60**, 509–522
- Watterson, D. M. & Vanaman, T. C. (1976) *Biochem. Biophys. Res. Commun.* **73**, 40–46
- Wolff, J. & Wolff, E. C. (1957) *Biochim. Biophys. Acta* **26**, 387–396
- Yun, S. L. & Suelter, C. H. (1977) *Biochim. Biophys. Acta* **480**, 1–13