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IMMOBILIZED ANTHRAQUINONE DYES FOR AFFINITY CHROMATOGRAPHY

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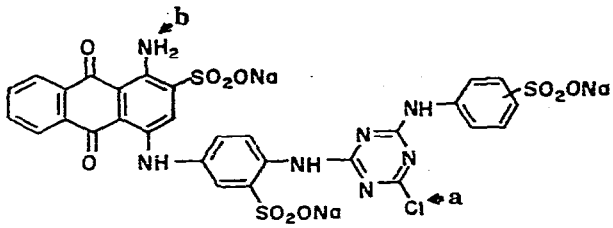
SUMMARY

Cibacron Brilliant Blue BR-P, a structural isomer of Cibacron Blue 3G-A (the chromophore in Blue Dextran), and Procion Brilliant Blue MR, Cibacron Brilliant Blue BR-P without the sulfonated benzyl moiety, were covalently attached to Sepharose CL-6B by the triazine rings. The use of these materials for column affinity chromatography was compared with commercial Blue Sepharose CL-6B, Cibacron Blue 3G-A covalently attached by its triazine ring to Sepharose CL-6B, and with a column of agarose succinyl-polyacrylic hydrazine with Cibacron Blue 3G-A attached on its anthraquinone ring. It was found likely that the 1-amino-anthraquinone-2-sulfonic acid moiety was the critical structure for binding of the selected enzymes used, one kinase and three dehydrogenases. These enzymes, bound to the dyes, attached to the columns by their triazine rings and elution of the enzymes occurred at similar concentrations of eluting buffers indicating that the entire Cibacron Blue 3G-A molecule is not required for biospecific binding and elution. The new columns described in this report may allow for selective enzyme purification due to differences in binding and steric interactions of the proteins with the different dyes.

INTRODUCTION

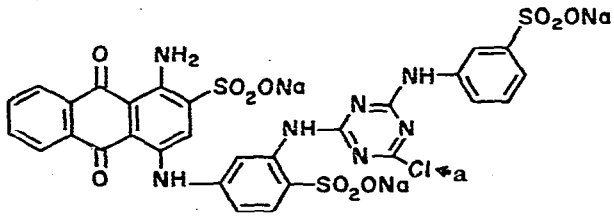
Blue Dextran, a dextran polymer with the blue dye Cibacron Blue 3G-A (CB3G-A, I in Fig. 1) covalently attached, was developed as an exclusion volume marker for gel filtration chromatography. During such use, it was observed to form conjugates with enzymes such as glutathione reductase[1], pyruvate kinase[2–4], phosphofructokinase[5–6], lactate dehydrogenase[7], and others[8–11]. This interaction, which was initially a problem in molecular-weight estimations on columns, has been exploited for purification. A protein sample with added Blue Dextran can be chromatographed on a molecular-sieve column that has been equilibrated in a low-ionic-strength buffer. If a conjugate is formed and the protein is eluted in the void volume, the eluant is then rechromatographed at a high ionic strength[1,4,9–11] or with the addition of a competing ligand[2,3] to separate the protein from the Blue Dextran. This process could potentially be quite effective for purifying some proteins.

To allow more specific elution of the species being isolated, both Blue Dextran



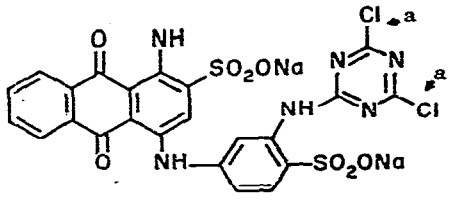
Cibacron Blue 3G-A (CB3G-A)

I



Cibacron Brilliant Blue BR-P (CBB)

II



Procion Brilliant Blue M-R (PBB)

III

Fig. 1. The structures of the dyes used in the study. The dyes were attached to the column supports at the positions indicated (a) by substitution at the triazine ring (b) by carbodiimide activation of the amine.

and its chromophore, CB3G-A, have been immobilized to several supports including polyacrylamide[6,12], agarose[7,13,14] and Sephadex[14–16]. These materials have been used to isolate a wide variety of proteins and extensive lists of such applications have been compiled[14,17–19]. Proteins bound to dye-substituted columns have been eluted by increasing ionic strength or, in several instances, by the addition of specific substrates or effectors, indicating biospecific interactions[14,17–19]. The biospecific cases are effective examples of affinity chromatography.

Experiments by Böhme *et al.*[16] on phosphofructokinase led to the suggestion that the dye molecule, 1-amino-4-(4′-aminophenylamino) anthraquinone-2,3′-disulfonic acid, was a structural analogue of ATP and was the portion of CB3G-A responsible for its interactions with phosphofructokinase. This idea was expanded further by Stellwagen with the suggestion that CB3G-A was a structural analogue of NADH and could be used as a diagnostic probe for the protein super-secondary

structure termed the "dinucleotide fold"[19–21]. Since yeast phosphofructokinase bound to Blue Dextran immobilized in polyacrylamide but not to Cibacron Brilliant Blue BR-P (CBB, II), a structural isomer of CB3G-A, attached to Dextran 2000 (ref. 16), Stellwagen further suggested that the complete structure of CB3G-A was required to produce the precise orientation of the charged groups necessary for binding to occur[21]. Wilson has suggested that an enzyme without a dinucleotide fold may interact biospecifically with CB3G-A, either immobilized or in solution, and that all enzymes that have a binding site for nucleotides, cyclic nucleotides, and dihydrofolate and/or folate may interact with CB3G-A[22]. We have recently shown from inhibition studies that the major interaction of selected nucleotide requiring enzymes with CB3G-A was with the 1-amino-4(4′-aminophenylamino) anthraquinone-2,3′-disulfonic acid portion of the dye[23].

Due to widespread interest in the use of this dye immobilized to column material for enzyme purification, this study was undertaken to determine if the entire CB3G-A molecule is required for the biospecific binding of proteins to dye-substituted columns. CB3G-A, CBB, and a portion of CBB, Procion Brilliant Blue MR (PBB, III), were covalently bound to a Sepharose CL-6B. CB3G-A was also covalently bound to a column support by linkage of its primary amine. Several nucleotide requiring proteins were chromatographed on these materials to evaluate the specificity and potential for general preparative use of such columns.

EXPERIMENTAL

Coenzymes, ATP, ITP, dithiotreitol, glucose-6-phosphate, pig heart malate dehydrogenase, rabbit muscle aldolase, glycerophosphate dehydrogenase/triosephosphate isomerase, and 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide were all purchased from Sigma (St. Louis, Mo., U.S.A.). Yeast glucose-6-phosphate dehydrogenase and oxaloacetate were obtained from Calbiochem (Los Angeles, Calif., U.S.A.). Rabbit muscle lactate dehydrogenase was from P-L Biochemicals (Milwaukee, Wisc., U.S.A.) and pyruvate was from J. T. Baker (Phillipsburgh, N.J., U.S.A.). Boehringer (Mannheim, G.F.R.) supplied the fructose-6-phosphate. Phosphofructokinase was partially purified from yeast using the first six steps of the method of Stellwagen and Wilgus[24]. Procion Brilliant Blue MR (PBB, C.I. reactive blue 4, C.I. constitution number 61205) was purchased from ICN K&K Labs. (Plainview, N.Y., U.S.A.). Blue Sepharose CL-6B (CB3G-A attached to Sepharose CL-6B) and Sepharose CL-6B were obtained from Pharmacia (Uppsala, Sweden). The agarose succinyl-polyacrylic hydrazide was from Miles-Yeda (Elkhart, Ind., U,S,A.). Ciba-Geigy (Basel, Switzerland) kindly provided Cibacron Blue 3G-A (CB3G-A, C.I. reactive blue 2, C.I. constitution number 61211) and Cibacron Brilliant Blue BR-P (CBB, C.I. reactive blue 5, C.I. constitution number 61205:1).

Procion Brilliant Blue Sepharose CL-6B and Cibacron Brilliant Blue Sepharose CL-6B were prepared according to the method used by Böhme *et al.*[16] for preparing Cibacron Blue 3G-A Sephadex with substitution of the appropriate dye and Sepharose CL-6B.

The agarose succinyl-polyacrylic hydrazide-CB3G-A column was prepared by the condensation of agarose succinyl-polyacrylic hydrazide (1 ml wet volume) with CB3G-A (0.25 grams) using the water soluble carbodiimide (1-ethyl-3-(3-dimethyl-

aminopropyl) carbodiimide, 0.38 g). A 13 ml-solution of 0.5 *M* NaCl at pH 4.5 with the above ingredients was gently shaken for 1 h. The gel was then washed on a glass sintered funnel with successive additions of water, 2 *M* KCl, water, 6 mg/ml bovine serum albumin, 2 *M* KCl, 8 *M* urea, and finally water until no chromophore was eluted. The control column made by eliminating the carbodiimide had a small amount of dye adsorbed to the column material (0.015 μmol/ml).

The concentration of the dyes bound to the Sepharose CL-6B was determined as described by Easterday and Easterday[14] by hydrolysis of the column material in 6 *M* HCl at 40° and spectrophotometric measurement of the released dye. Dye substitution on the agarose succinyl-polyacrylic hydrazide was estimated by hydrolysis in 6 *M* HCl for 1 h in a boiling-water bath and spectral measurement of the dye with an unsubstituted gel blank used as a control.

A 1.0-ml portion of each gel was poured into a 0.7 × 4 cm polypropylene column from Bio-Rad Labs. (Richmond, Calif., U.S.A.) and equilibrated with 10 m*M* Tris, 1 m*M* EDTA, and 1 m*M* dithiothreitol at pH 7.5 (standard buffer). They were then loaded with 80–500 μg of protein which was eluted by successive application of five column volumes each of the standard buffer, then 10 m*M* KCl, 100 m*M* KCl, and 1 *M* KCl in standard buffer. The columns were then washed with the standard buffer, reloaded with protein, and eluted with 1 m*M* of the respective ligand in standard buffer. The Cibacron Blue 3G-A Sepharose CL-6B was washed, loaded, and eluted with 50% ethylene glycol and standard buffer. All fractions were collected and enzyme activity in each determined as described below.

All enzymes were dialyzed extensively against the standard buffer except yeast phosphofructokinase which was exchanged into the standard buffer using a Bio-Gel P-2 column equilibrated with the standard buffer. Lactate dehydrogenase[25], glucose-6-phosphate dehydrogenase[22], malate dehydrogenase[26], and phosphofructokinase[24] were all assayed as described previously. The assays were carried out in a Cary Model 118 spectrophotometer at 25° and no activity was observed when one substrate was omitted from the assay mixtures. Protein concentrations were estimated by A_{280}.

RESULTS AND DISCUSSION

The binding characteristics of phosphofructokinase, malate dehydrogenase, lactate dehydrogenase and glucose 6-phosphate dehydrogenase to dye columns where the chromophore is attached to the column support by the triazine ring are listed in Table I. The per cent of the total activity applied that was eluted from the columns under the conditions described in Experimental is listed in the table. The dye substitution on the Sepharose CL-6B was approximately 3 μmol/ml of gel for PBB, 2 μmol/ml of gel for CBB, and 1 μmol/ml of gel for CB3G-A. The enzymes used in this report all bind to the CB3G-A, CBB and PBB columns and elute at somewhat similar concentrations of salt or substrate. These results indicate that even with the enzymes containing a dinucleotide fold, pig heart malate dehydrogenase and rabbit muscle lactate dehydrogenase[27], neither the absolute configurations of CB3G-A nor its entire structure is required for binding or biospecific elution of these enzymes as Stellwagen has suggested[21]. Böhme *et al.* did not observe affinity of yeast phosphofructokinase for the condensation product of CBB with Dextran 2000[16], however,

the dyes used in this work were supported by Sepharose CL-6B and the CBB derivative bound yeast phosphofructokinase quite tightly.

It should also be pointed out from the data in Table I that the biospecific elution of phosphofructokinase and lactate dehydrogenase is more easily carried out with the PBB-Sepharose CL-6B than the other columns. Glucose-6-phosphate dehydrogenase is harder to elute biospecifically from GB3G-A-Sepharose CL-6B than from the other columns. Increases in ionic strength appear to be more effective for the elution of lactate dehydrogenase from PBB-Sepharose CL-6B than from the other derivatives and less effective in the elution of phosphofructokinase from CBB-Sepharose CL-6B than from the other derivatives. These differences in per cent activity eluted are probably due to the different steric interactions between the enzymes and the different dyes and to a small part due to inherent technical problems of quantitative work on such small columns.

CB3G-A was coupled covalently, presumably at the primary amine, to agarose succinyl-polyacrylic hydrazide by a carbodiimide condensation. The degree of substitution was 0.4 μmol/ml of gel. The amount of dye adsorbed to the control column, made as described in Experimental, was 0.015 μmol/ml of gel. In a linkage of this type, the 1-amino-anthraquinone-2-sulfonate group of the dye should be blocked from binding to the enzymes. As can be seen in Table II, where the binding criteria are the same as in Table I, this portion of the dye is important for the binding of these enzymes. Glucose-6-phosphate dehydrogenase and phosphofructokinase do not bind to this column. Lactate dehydrogenase and malate dehydrogenase show approximately the same affinity for the dye-substituted and the control column. These affinities are significantly less than those exhibited when the dye is attached at the triazine ring. Some binding of the enzymes to these columns may occur through dye adsorbed to the columns as observed with the control column. Böhme *et al.*[16] also concluded that this portion of the dye was important for binding from experiments in which CNBr-activated Sepharose 4B had been reacted with the primary amine of CB3G-A in Blue Dextran. Such treatment should not have blocked all the dye molecules present in Blue Dextran; it would block only those molecules linking the Blue Dextran to the Sepharose 4B. Yeast phosphofructokinase did not bind to such a column[16], but the enzyme from *E. coli* does[19].

Hydrophobic binding of proteins to immobilized CB3G-A columns has been observed in several cases. Blue Dextran-Sepharose binds human heart NADP-specific isocitrate dehydrogenase at its $NADP^+$ site. This binding is enhanced by high concentrations of salts in the order of the Hofmeister series, and the protein is eluted by the hydrophobic solvent glycerol and by nicotinamide which is an ineffective inhibitor[28]. Interferon binds to Blue Dextran-Sepharose and can be displaced by polynucleotides or ethylene glycol, but not by 1 *M* NaCl (refs. 29 and 30). The degree of hydrophobicity involved in the binding of the enzymes studied in this paper to CB3G-A Sepharose CL-6B was tested by washing a loaded column with 50% ethylene glycol. Only 37% of the total activity of yeast phosphofructokinase could be eluted while no activity could be eluted using the other three enzymes.

It is apparent that both hydrophobic and electrostatic forces are involved in the binding of proteins to these anionic dyes. Glazer[31] has suggested that the binding of dyes to proteins takes place predominately in areas that overlap the binding sites for protein ligands in preference to other surface areas of the protein. In his examples

TABLE I

ELUTION OF VARIOUS NUCLEOTIDE ENZYMES FROM THE DYE-SUBSTITUTED COLUMNS

Enzyme	*Column*	*Percentage of Total Activity Eluted*					
		Salt Washes				*Ligand Washes*	
		10 mM Tris	*0.01 M KCl*	*0.1 M KCl*	*1 M KCl*		
						1 mM ATP	*10 mM ATP*
Phosphofructokinase (yeast)	PBB-Seph	1	0	1	91	89	76
	CB3G-A-Seph	0	0	0	91	26	69
	CBB-Seph	0	0	0	52	26	76
						10 mM NADH	
Malate dehydrogenase (pig heart)	PBB-Seph	0	0	0	33	0	
	CB3G-A-Seph	0	0	0	32	0	
	CBB-Seph	0	0	0	31	0	
						1 mM NADH	
Lactate dehydrogenase (rabbit muscle)	PBB-Seph	0	0	0	68	76	
	CB3G-A-Seph	0	0	0	30	48	
	CBB-Seph	0	0	0	20	48	
						1 mM $NADP^+$	
Glucose-6-phosphate dehydrogenase (yeast)	PBB-Seph	0	0	0	93	52	
	CB3G-A-Seph	0	0	0	100	27	
	CBB-Seph	0	0	0	92	48	

The columns were prepared and used as described in Experimental. Abbreviations: PBB-Seph = PBB-Sepharose CL-6B; CB3G-A-Seph = CB3G-A-Sepharose CL-6B; CBB-Seph = CBB-Sepharose CL-6B.

TABLE II

ELUTION OF VARIOUS NUCLEOTIDE ENZYMES FROM CB36-A-SEPHAROSE LINKED AT THE ANTHRAQUINONE AMINE

The columns were prepared and used as described in Experimental. The CB3G-A was attached by its amine group to agarose succinyl-polyacrylic hydrazide by a carbodiimide mediated condensation. ND = Not determined.

Enzyme	*Column*	*Percentage of Total Activity Eluted*				
		Salt Washes				*Ligand Washes*
		10 mM Tris	*0.01 M KCl*	*0.1 M KCl*	*1 M KCl*	
						1 mM ATP
Phosphofructokinase (yeast)	CB3G-A	90	3	0	0	ND
	Control	82	3	9	1	ND
						1 mM ATP
Glucose-6-phosphate dehydrogenase (yeast)	CB3G-A	63	9	9	0	ND
	Control	5	1	95	2	ND
						1 mM NADH
Lactate dehydrogenase (rabbit muscle)	CB3G-A	1	1	78	5	90
	Control	4	54	16	1	83
						1 mM NADH
Malate dehydrogenase (pig heart)	CB3G-A	6	6	80	4	68
	Control	5	2	87	5	29

of such binding, there is generally little obvious similarity between the dyes and the protein ligands, but it was proposed that one reason for the binding could be the hydrophobicity of these sites relative to the rest of the protein. In Glazer's work, the interactions of proteins with multiple charged ligands were specifically excluded, but there is no reason to exclude the same type of interaction with the chromophores used in our experiments. The charged groups of the dyes undoubtedly make a large contribution to binding.

Many enzymes containing a dinucleotide fold super-secondary protein structure or very similar folding pattern bind to CB3G-A[19–21]. Rao and Rossman[32] have described the β-pleated sheet of this super-secondary structure in the following manner, "...restrictive in amino acid sequence. For instance, the hydrophobic character of the residues in sheet regions is apparent. The presence of a hydrophobic patch or pocket at the end of the parallel sheet may possibly be required to produce this particular fold." The hydrophobic pocket created by this structure and similar protein structures of a hydrophobic central β-pleated sheet flanked by hydrophilic α-helixes could provide for exactly the type of interaction that Glazer[31] has suggested. The interaction of CB3G-A with enzymes not containing a dinucleotide fold[33] has been shown with yeast hexokinase[22]. Phosphoglyceromutase and aldolase are examples of enzymes that do not use nucleotides or dinucleotides as natural substrates, but do interact with CB3G-A biospecifically at their substrate sites. However, most of the enzymes that bind to CB3G-A columns biospecifically or otherwise either have substrates or effectors that are anionic in nature or are themselves basic[14,17–19]. Therefore, CB3G-A-Sepharose and the other dye columns used in this study will show a propensity to interact with enzymes containing a dinucleotide-fold type folding pattern and with enzymes that bind hydrophobic and/or anionic ligands such as nucleotides.

The column support material for CB3G-A has a great deal of influence on the interaction of the dye with some enzymes. Rat brain hexokinase has been shown to bind to CB3G-A-Sepharose CL-6B but not to Blue Dextran-Sepharose[22]. Pig heart malate dehydrogenase can be eluted from Blue Dextran-Sepharose with 10 m*M* NADH[19] but not from the CB3G-A-Sepharose CL-6B. Different ionic strengths are needed to elute yeast phosphofructokinase from Blue Dextran immobilized in polyacrylamide or CB3G-A-Sephadex[16]. Böhme *et al.*[16] were also unable to elute yeast phosphofructokinase from CB3G-A-Sephadex with ATP or from CB3G-A-Cellulose under any conditions, while it could be eluted from Blue Dextran-polyacrylamide gel with 2 m*M* ATP (ref. 6). We were able to effect elution of a high percentage of yeast phosphofructokinase from CB3G-A-Sepharose CL-6B with 10 m*M* ATP. Also, Wilson has shown differences in the interaction of free CB3G-A and free Blue Dextran in solution with several enzymes[22]. In general, changes in environment of the dye and attachment position can affect the binding.

It is felt that the new columns presented here, PBB and CBB-Sepharose CL-6B offer alternatives to CB3G-A-Sepharose for enzyme purification due to differences in their binding and steric interactions. It should be noted that the optimum conditions for the use of each column in this paper were not determined; instead the procedure was the same for all to allow comparisons in the enzyme-binding properties of the column. The availability of a large number of reactive dyes based on anthraquinone structures and similar molecules may allow development of new, more

specific columns so that selectivity for different nucleotides and substrate sites may be available. This is the direction further research in this area should follow.

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