The Selective Retardation of NADP⁺-Dependent Dehydrogenases by Immobilized Procion Red HE-3B

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The capacities of Procion Red HE-3B and Cibacron Blue F3G-A immobilized to Sepharose CL-4B and Matrex 201R for NAD⁺-, NADP⁺- and NAD(P)⁺-dependent dehydrogenases were measured. Procion Red HE-3B columns retarded NADP⁺-dependent dehydrogenases more effectively than NAD⁺-dependent dehydrogenases, whilst immobilized Cibacron Blue F3G-A retarded NAD⁺-dependent dehydrogenases more effectively than NADP⁺-dependent dehydrogenases. The capacity of Procion Red HE-3B–Sepharose CL-4B for five dehydrogenases was highest in the region of 70 nmol of immobilized ligand/ml of settled gel. The effects of using poly(ethyleneimine) as a spacer for both porous and pellicular supports were also examined. Four NADP⁺-dependent dehydrogenases were purified from yeast extract by using Procion Red HE-3B–Sepharose CL-4B. Two NAD⁺-dependent dehydrogenases were purified from the same source using Cibacron Blue F3G-A–Sepharose CL-4B. These results are discussed in relation to the use of immobilized Procion Red HE-3B to purify dehydrogenases. This immobilized dye's chromatographic behaviour is compared with that of immobilized nucleotides. The most important feature of immobilized triazine dyes seems to be their high operational capacities when compared with group-specific nucleotide adsorbents.

The purification of dehydrogenases on group-specific affinity-chromatographic adsorbents is now commonplace. It has been noted that, at least for immobilized nucleotides, such group-specific ligands possess low capacities (Griffiths *et al.*, 1977; Watson & Dean, 1977). Furthermore, the preparation of this type of ligand often requires considerable synthetic effort involving several stages to produce a chemically defined adsorbent. Chemically ill-defined nucleotide adsorbents are less satisfactory as affinity matrices. On the other hand, immobilized Cibacron Blue F3G-A columns are easily prepared and have demonstrably high capacities (Travis *et al.*, 1976). This type of adsorbent has been used for the purification of dehydrogenases, kinases (Easterday & Easterday, 1974) and other proteins (Travis & Pannell, 1973) as well as to resolve isoenzymes (Nadal-Ginard & Markert, 1975). It has been claimed that the dye binds to the dinucleotide fold of proteins (Thompson *et al.*, 1975).

We have investigated the chromatography of NAD⁺- and NADP⁺-dependent dehydrogenases on immobilized Cibacron Blue and another immobilized triazine dye, Procion Red HE-3B. The latter has

previously been used in the purification of carboxypeptidase G from *Pseudomonas* A.T.C.C. 25301 (Baird *et al.*, 1976).

The results presented in this paper suggest that the above two dyes can be used in a complementary fashion in that Cibacron Blue is NAD⁺-specific, whereas Procion Red is NADP⁺-specific.

Materials and Methods

Materials

Procion Red HE-3B was a gift from Dr. C. V. Stead, I.C.I. Organics Division, Manchester, U.K. Cibacron Blue F3G-A was from Ciba-Geigy (U.K.), Manchester, U.K. Poly(ethyleneimine) (Polymin 'P') was from BDH Chemicals, Poole, Dorset, U.K. Sepharose 4B was from Pharmacia Fine Chemicals (G.B.), London, U.K. Matrex 201R was a gift from Dr. W. Bowers, Amicon Corp., Lexington, MA, U.S.A. All enzymes, except *Lactobacillus casei* dihydrofolate reductase and *Neurospora crassa* L-glutamate dehydrogenase, were from the Boehringer Corp., Lewes, Sussex, U.K. Yeast enzymes concentrate (type II) was from Sigma (London) Chemical Co., Kingston-upon-Thames, U.K. *L. casei* was a gift from Dr. A. Atkinson, M.R.E., Porton Down, Wilts., U.K.

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Methods

Dihydrofolate reductase was purified from *L. casei* by affinity chromatography on NADP⁺-adipic acid dihydrazide-Sepharose 4B with NaCl as eluent (Dean & Watson, 1978). *N. crassa* L-glutamate dehydrogenase, *am*¹, *am*³ and wild-type forms, were purified by the method of Ashby *et al.* (1974).

Triazine-dye immobilization

The Procion Red HE-3B and Cibacron Blue F3G-A were immobilized to cross-linked Sepharose 4B by the method of Baird *et al.* (1976). Sepharose 4B was cross-linked by the method of Porath *et al.* (1971). Dye-poly(ethyleneimine) derivatives were synthesized as follows: poly(ethyleneimine) (2g, 0.4nmol) was mixed with 40 μ mol of triazine dye in 0.1% Na₂CO₃ solution (5ml). The mixture was incubated at room temperature for 7 days on a Coulter mixer. Unchanged dye and poly(ethyleneimine) were then resolved from the dye-poly(ethyleneimine) product by a modification of the method of Zapelli *et al.* (1975). The reaction mixture was diluted with 20ml of 0.5M-potassium phosphate buffer, pH 6.0, and further diluted to a volume of 200ml with deionized water. The required insoluble dye-poly(ethyleneimine) product [which was probably composed of dye molecules coupled to poly(ethyleneimine) groups via the triazine moiety of the dye] was precipitated by centrifugation in a bench centrifuge, resuspended in deionized water (5ml) and reprecipitated. This procedure was repeated five times. Unchanged dye and poly(ethyleneimine) were retained in the supernatant. Poly(ethyleneimine)-Procion Red HE-3B (1.32g, 65% yield) and poly(ethyleneimine)-Cibacron Blue (1.65g, 81% yield) were immobilized to cross-linked Sepharose 4B and Matrex 201R by the CNBr-activation method of Axen *et al.* (1967). Immobilized-ligand concentrations were measured by direct spectral analysis of the adsorbents suspended in 10% glycerol against the appropriate matrix blanks at 550 and 610nm respectively.

Affinity chromatography

Purified enzyme was applied at 10nmol/ml in 0.1M-potassium phosphate buffer, pH 7.4, containing 1mM-EDTA (disodium salt) to a column (5mm $\times$ 50mm) containing 1g of adsorbent equilibrated in the same buffer at 6°C, at a flow rate of 0.7ml/h. In certain experiments (see the Results section) enzyme was applied at a variety of concentrations. Yeast enzyme concentrate was applied at 5mg/ml. Enzyme application was continued until the concentration of protein and enzyme in the eluate and the applied sample were the same. Unretarded enzyme was then eluted with the above buffer (10ml). Retarded enzyme

was eluted by a linear gradient of 0–1M-NaCl in buffer (0–1.0M, 16ml of total volume). The column was then washed with 8M-urea in buffer and re-equilibrated with buffer. The amount of retarded enzyme was measured by inspection of the frontal elution pattern and compared with that for enzyme applied to a column of unsubstituted support under the above experimental conditions. Over a period of 3 months, following the stated experimental conditions, ligand leakage was observed to be less than 0.1%.

Enzyme and protein assay

Enzyme activities were measured as described by Barman (1969) or for *N. crassa* L-glutamate dehydrogenase by the methods of Watson & Wootton (1977). Protein was measured by *A*₂₈₀ (Warburg & Christian, 1931).

Results

Fig. 1 shows that immobilized Cibacron Blue F3G-A was able to interact effectively with NAD⁺-dependent dehydrogenases. On the other hand, NADP⁺-dependent dehydrogenases were more effectively retarded by Procion Red HE-3B-Sepharose CL-4B.

Table 1 compares the capacities of triazine-dye adsorbents with the capacities of two nucleotide adsorbents, *N*⁶-5'-AMP and *N*⁶-NADP⁺, having defined affinities for NAD⁺- and NADP⁺-dependent dehydrogenases respectively, for six nucleotide-linked enzymes in a yeast extract. The capacity of Cibacron Blue F3G-A-Sepharose for alcohol dehydrogenase and malate dehydrogenase was 4–20 times greater than that observed for *N*⁶-5'-AMP-Sepharose, whereas the capacity of Procion Red HE-3B-Sepharose for the NADP⁺-linked enzymes glutathione reductase, glutamate dehydrogenase, 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase was 50–100 times greater than that observed for *N*⁶-NADP⁺-Sepharose. The variation in the capacity of Procion Red HE-3B-Sepharose for these four enzymes was related to the concentration of each enzyme in the yeast extract. A correlation existed between the applied enzymes concentration and the capacity of the adsorbent (at 3 μ M-glucose 6-phosphate dehydrogenase the capacity was 6% at a ligand concentration of 0.17 μ mol of Procion Red HE-3B/ml of Sepharose). Table 1 also compares the capacities of the immobilized triazine dyes for the same enzymes in yeast extract with the capacities of two nucleotide adsorbents for the same enzymes. The higher capacities observed for the adsorption of purified enzymes probably reflects the lack of competition from other enzyme species, as seen with the yeast extract.

Fig. 1. Comparison of the capacities for the retardation of NADP⁺- and NAD⁺-dependent dehydrogenase by immobilized Procion Red HE-3B and Cibacron Blue F3G-A. Capacities of Procion Red HE-3B and Cibacron Blue F3G-A immobilized to Sepharose CL-4B for several dehydrogenases (A to L) were measured as described in the Materials and Methods section and are expressed as mol of ligand used per mol of immobilized ligand $\times 100\%$. The immobilized ligand concentrations of these adsorbents were the same as those given in Table 2. Capacities were measured by frontal analysis and confirmed by NaCl elution. Key to enzymes: L. casei NADP⁺-dependent dihydrofolate reductase (A); pig heart NADP⁺-dependent isocitrate dehydrogenase (B); ox liver NAD(P)⁺-dependent L-glutamate dehydrogenase (C); pig heart NAD⁺-dependent lactate dehydrogenase (D); yeast NAD⁺-dependent malate dehydrogenase (E); yeast NADP⁺-dependent 6-phosphogluconate dehydrogenase (F); yeast NADP⁺-dependent glucose 6-phosphate dehydrogenase (G); wild-type *N. crassa* NADP⁺-dependent L-glutamate dehydrogenase (H); *am*¹ *N. crassa* NADP⁺-dependent L-glutamate dehydrogenase [this mutant glutamate dehydrogenase does not interact with NADPH (Watson & Wooton, 1977)]. (J); *am*³ *N. crassa* NADP⁺-dependent L-glutamate dehydrogenase (K); yeast NAD⁺-dependent alcohol dehydrogenase (L).

The effect of varying the ligand concentration on the capacity of Procion Red HE-3B–Sepharose CL-4B for three enzymes is shown in Fig. 2, where it can be seen that the capacity reached an optimum between 30 and 80nmol of dye/ml of Sepharose. Similar experiments with *N. crassa* L-glutamate dehydrogenase and pig heart isocitrate dehydrogenase gave almost identical results to those obtained with 6-phosphogluconate dehydrogenase.

To optimize the adsorption of dehydrogenases to immobilized Procion Red HE-3B, we examined the effect on capacity and selectivity of (i) incorporating a spacer, and (ii) immobilizing the ligand to the surface of the pellicular support, Matrex 201R. The capacities of the adsorbents for five dehydrogenases are compared with appropriate controls in Table 2.

Comparing the capacities of immobilized Cibacron Blue for NAD⁺-specific enzymes (D and E) and of immobilized Procion Red HE-3B for NADP⁺-specific enzymes (A and B), it can be seen that the highest capacities were observed, except for dihydrofolate reductase, when the dye was coupled to the pellicular support, Matrex 201R, via a poly(ethyleneimine) spacer arm.

The selectivity of these adsorbents also altered (Table 2) in that the capacities of Procion Red for NAD⁺-specific enzymes (D and E) and of Cibacron Blue for NADP⁺-specific enzymes (A and B) decreased with the incorporation of the spacer. Furthermore, the additional effect of the pellicular support further narrowed the specificity in all cases but dihydrofolate reductase.

The capacity of both the Procion Red HE-3B and Cibacron Blue F3G-A adsorbents for the NADP⁺-linked enzyme glutamate dehydrogenase was improved both by the incorporation of a spacer [except in the case of Cibacron Blue–poly(ethyleneimine)–Sepharose CL-4B] and by the use of the pellicular support.

Table 1. Comparative capacities of triazine-dye and nucleotide adsorbents for six dehydrogenases. Adsorbent capacities (%) were measured by frontal enzyme application (see the Materials and Methods section) for purified enzymes (all from yeast, except glutamate dehydrogenase from *Neurospora crassa*) and for the same enzymes in yeast enzyme concentrate. Key to adsorbents: Red and Blue, Procion Red HE-3B– and Cibacron Blue F3G-A–Sepharose CL-4B respectively; N⁶-NADP⁺ and N⁶-5'-AMP, N⁶-(6-aminohexylcarbamoymethyl)-nucleotide–Sepharose 4B. Abbreviation: n.m., not measured.

	Adsorbent capacity (%)						Enzyme concentration in yeast concentrate (nmol/ml)
	Purified enzymes		Yeast enzyme concentrate				
	Red	Blue	Red	Blue	<i>N</i> ⁶ -NADP	<i>N</i> ⁶ -5'-AMP	
Alcohol dehydrogenase	9.7	9.7	1.9	1.2	0.0079	0.31	3.6
Malate dehydrogenase	6.5	8.9	1.4	2.0	0	0.11	1.8
Glutathione reductase	n.m.	n.m.	11.2	0.32	0.119	0.0014	1.95
Glutamate dehydrogenase	13.0	1.6	0.5	0.05	0.0045	0	0.2
6-Phosphogluconate dehydrogenase	13.6	2.2	5.8	0.24	0.099	0.023	1.4
Glucose-6-phosphate dehydrogenase	10.6	1.9	0.22	0.04	0.0041	0.006	0.08

Enzyme desorption from dye-poly(ethyleneimine)-Matrex adsorbents could not be successfully achieved by using 1 M-salt or 4 mM-nucleotide (oxidized). How-

Fig. 2. Variation of capacity with immobilized Procion Red HE-3B concentration

The capacity for the retardation of *L. casei* dihydrofolate reductase (a), yeast 6-phosphogluconate dehydrogenase (b) and yeast glucose 6-phosphate dehydrogenase (c) by Procion Red HE-3B-Sepharose CL-4B at each immobilized ligand concentration was measured by frontal analysis (see the Materials and Methods section).

ever, quantitative recovery was achieved by using electrophoretic desorption (Dean *et al.*, 1977).

Using ionic-strength gradients (Fig. 3), NADP⁺-dependent dehydrogenases could be selectively eluted from Procion Red HE-3B-Sepharose CL-4B and likewise NADP⁺-dependent dehydrogenases from Cibacron Blue F3G-A-Sepharose CL-4B with purification factors between 60- and 1000-fold. On the other hand by using similar gradients to elute NADP⁺-dependent dehydrogenases from Procion Red columns as well as NADP⁺-dependent dehydrogenases from Cibacron Blue columns, purification factors of only 2–10-fold were observed. Although NADP⁺-linked enzymes have an affinity for Procion Red HE-3B-Sepharose they are eluted at very low ionic strengths and do not interfere with the resolution of NADP⁺-linked enzymes.

The Procion Red column was incorporated into the specific purification (Table 3) of NADP⁺-specific *Neurospora crassa* glutamate dehydrogenase and was able to replace two ion-exchange columns (DEAE-Sephadex, A50) and a gel-filtration step (Sephadex G-200) in the scheme described by Ashby *et al.* (1974), and yield a protein preparation of equivalent homogeneity and specific activity.

Discussion

The results demonstrate the more effective resolution of NADP⁺-dependent dehydrogenases by

Table 2. Effect of support porosity and the presence of a spacer arm on the capacities of immobilized Procion Red HE-3B and Cibacron Blue F3G-A

Absorbent capacities (%) were measured by frontal analysis as described in the Materials and Methods section. The enzymes studied were: *L. casei* dihydrofolate reductase (A); pig heart isocitrate dehydrogenase (B); ox liver L-glutamate dehydrogenase (C); pig heart lactate dehydrogenase (D); yeast malate dehydrogenase (E). The adsorbents' immobilized ligand concentrations (μmol/ml of gel) were as follows: Procion Red HE-3B-Sepharose-CL-4B (0.170); Procion Red HE-3B-poly(ethyleneimine)-Sepharose CL-4B (0.173); Procion Red HE-3B-poly(ethyleneimine)-Matrex 201R (0.150); Cibacron Blue F3G-A-Sepharose-CL-4B (0.173); Cibacron Blue F3G-A-poly(ethyleneimine)-Sepharose CL-4B (0.175); Cibacron Blue F3G-A-poly(ethyleneimine)-Matrex 201R (0.150).

(a) Immobilized Procion Red HE-3B

Enzyme	Dye-Sepharose CL-4B	Dye-poly(ethyleneimine)-Sepharose CL-4B	Dye-poly(ethyleneimine)-Matrex 201R
A	30.6	14.3	19.2
B	12.4	9.3	17.0
C	7.5	10.6	15.0
D	5.6	1.0	0.5
E	6.5	1.0	0.6

(b) Immobilized Cibacron Blue F3G-A

Enzyme	Dye-Sepharose CL-4B	Dye-poly(ethyleneimine)-Sepharose CL-4B	Dye-poly(ethyleneimine)-Matrex 201R
A	3.8	0.2	2.4
B	1.2	0.6	0.3
C	8.3	6.4	9.9
D	10.4	5.4	14
E	8.9	4.6	10

Fig. 3. Partial resolution of NAD^+ - and NADP^+ -dependent dehydrogenases by salt-gradient elution from Procion Red HE-3B and Cibacron Blue F3G-A-Sepharose CL-4B

Yeast enzyme concentrate was applied frontally to Procion Red HE-3B (a) and Cibacron Blue F3G-A-Sepharose CL-4B (b) until each adsorbent was saturated with each enzyme (not shown) as described in the Materials and Methods section. Both adsorbent columns were washed with buffer until no further enzyme activity or protein was eluted. A linear gradient of 0–1 M-NaCl in buffer (—), was then applied (at the arrow). Protein concentration (A_{280}/ml) (∇) and the following enzyme activities were measured in the eluted fractions: alcohol dehydrogenase ($\circ$); malate dehydrogenase ($\bullet$); glucose 6-phosphate dehydrogenase (Δ); NADP^+ -dependent L-glutamate dehydrogenase ($\square$); 6-phosphogluconate dehydrogenase ($\blacksquare$); glutathione reductase ($\blacktriangle$).

Table 3. *N. crassa* glutamate dehydrogenase purification

Stage	Volume (ml)	Total protein (A_{280})	Total glutamate dehydrogenase (units)	Recovery (%)	Specific activity (units/ A_{280})	Purification factor
5 g of freeze-crude extract of dried mycelium	250	—	872	100	—	—
30000g Supernatant	214	2049	656	75	0.3	—
Heat step 30000g supernatant	208	1042	450	52	0.4	1.4
0–35%–Satd.-(NH_4) $_2$ SO $_4$	224	119	428	49	3.6	11.3
30000g supernatant						
35–60%–Satd.-(NH_4) $_2$ SO $_4$	3.6	24	406	47	17	53
30000g pellet*						
Procion Red HE-3B–Sepharose column	3.0	0.9	73	(92)†	79	245

* Dialysed overnight against 0.1 M- $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer, pH 7.4 (1 mM-EDTA).

† Enzyme (406 units) applied frontally to a 1 ml column; 73 units were bound and were recovered after NADPH elution.

immobilized Procion Red HE-3B when compared with immobilized Cibacron Blue. In contrast, the latter was more effective than Procion Red in resolving NAD^+ -dependent dehydrogenases. This is not to say that Cibacron Blue columns cannot be used to purify NADP^+ -dependent enzymes (Kawai & Eguchi, 1976a,b; Easterday & Easterday, 1974; Heyns & de Moor, 1974).

Several advantages of using immobilized triazine bound dyes over the corresponding specific nucleotide affinity adsorbents are apparent: (i) higher capacities (by an order of magnitude or more); (ii) comparative ease of immobilization, with or without spacers; (iii) economy; (iv) availability; (v) lack of difficult organic syntheses.

Some disadvantages also exist, such as the broader

specificity of the triazine dyes, although this can be narrowed by using various spacer molecules, as shown here with poly(ethyleneimine), or by changing the support from agarose to a pellicular support, such as Matrex 201R, or agarose-polyacrylamide copolymers (Doley *et al.*, 1976). These effects, especially those associated with the introduction of a polyethylene spacer molecule, may be related to the observations of Wilson (1976) on the interaction of nucleotide-binding enzymes with Cibacron Blue F3G-A and the conjugate 'Blue Dextran'.

Regarding the nature of the interaction of dehydrogenases with triazine dyes, both muscle and heart lactate dehydrogenases are retarded under similar conditions, whereas negatively charged ion exchange columns would retard only the heart enzyme below pH 8. Similarly *am*¹ glutamate dehydrogenase is not retarded as well as either the wild type or *am*³ enzyme by the immobilized dye column; but ion-exchange columns (DEAE) retarded *am*¹ and wild-type enzymes equally well, whilst the *am*³ enzyme was poorly retarded. Therefore some degree of bio-specificity probably exists between the dye column and dehydrogenases, the nature of which has yet to be elucidated. Whilst the idea of an interaction with the enzyme-dinucleotide fold is attractive, it is unlikely to be applicable to the observed adsorption of cytochrome *c* and ovalbumin by both immobilized Procion Red HE-3B and Cibacron Blue F3G-A.

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