Tandem dye–ligand chromatography and biospecific elution applied to the purification of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*

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1. A total of 65 immobilized triazine dyes were screened for their ability to purify the dual-nucleotide-specific glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*. From this screen a 'negative' (Matrex Gel Purple A) and a 'positive' (Matrex Gel Orange B) adsorbent were found to be the best in terms of overall purification and yield and were therefore combined to give the best purification. 2. Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* was purified approx. 56-fold in a two-step tandem chromatographic system using Matrex Gel Purple A followed by Matrex Gel Orange B chromatography to a specific activity of 228 units/mg of protein in a final yield of 73%. 3. A study of the elution characteristics of glucose-6-phosphate dehydrogenase bound to Matrex Gel Orange B by KCl (pulse and gradient) and biospecific eluents (pulse) was carried out. NADP⁺, NADPH and adenosine 2',5'-bisphosphate were found to be the only effective biospecific eluents. A pulse of 50 μ M-NADP⁺ ($\frac{1}{4}$ column vol.) was found to give a better purification than a 0–1 M-KCl gradient and therefore was the preferred method of elution. 4. Presaturation of the enzyme with various nucleotides was carried out to determine the effect on the subsequent binding of glucose-6-phosphate dehydrogenase to Matrex Gel Orange B. The results of these and biospecific-elution studies lead us to propose two possible schemes to explain the mechanism of the dye–protein interaction. 5. Reusability, capacity of the adsorbent and effect of varying the ligand concentration were also studied in the purification of glucose-6-phosphate dehydrogenase on Matrex Gel Orange B.

Glucose-6-phosphate dehydrogenase [D-glucose-6-phosphate:NAD(P)⁺ 1-oxidoreductase, EC 1.1.1.49] catalyses the first committed step in the pentose phosphate pathway, oxidizing glucose 6-phosphate to 6-phosphogluconate with a subsequent reduction of NADP⁺ (or NAD⁺). The enzyme was initially discovered by Warburg & Christian (1931) in horse erythrocytes, and has since been studied from many varied sources (for a review, see Levy, 1979). Most glucose-6-phosphate dehydrogenases utilize NADP⁺ as the sole coenzyme. However, the enzyme isolated from several microorganisms, including *Leuconostoc mesenteroides*, has the ability to use NAD⁺ as well as NADP⁺. *L. mesenteroides* glucose-6-phosphate dehydrogenase was the first shown to exhibit this dual nucleotide specificity by DeMoss (1955). Levy and co-workers have extensively studied the *Leuconostoc* enzyme, reporting on its physical (Olive & Levy, 1971) and

kinetic (Olive *et al.*, 1971) properties. They have studied the amino acid content and found a complete absence of cysteine (Ishaque *et al.*, 1974), which is unusual for dehydrogenases. It was suggested by these workers that the latter property was responsible for the enzyme's excellent stability. The above resistance to inactivation under a variety of conditions and the dual nucleotide specificity has led to a widespread use of the enzyme in various clinical test kits, e.g. for determining glucose levels in serum (Bondar & Mead, 1974). To date, *L. mesenteroides* glucose-6-phosphate dehydrogenase has been purified by conventional protein purification procedures (Olive & Levy, 1967) and by Choi *et al.* (1978) with a combination of Cibacron Blue 3G-A–Sephacrose 4B chromatography and hydroxyapatite chromatography.

Immobilized triazine dyes provide a well-recognized alternative to the more sophisticated

affinity ligands such as NAD⁺ (Hey & Dean, 1981). Uncertainties regarding the mechanism of at least one dye-protein interaction have been resolved by Biellmann *et al.* (1979), who have clearly established that Cibacron Blue 3G-A binds at the nucleotide-binding site of alcohol dehydrogenase. However, this definitive work does not apply to all interactions involving immobilized Cibacron Blue 3G-A, since many proteins that bind to the dye do not possess a nucleotide-binding site, e.g. albumin (Angal & Dean, 1977) and troponin (Reisler *et al.*, 1980). Conversely, many proteins possessing a nucleotide-binding site show no affinity for the dye. Therefore, in view of the uncertainty surrounding the mechanism, we believe that it is important to approach dye-ligand chromatography empirically: screening a large number of immobilized dyes rather than choosing the most commonly used triazine dye, Cibacron Blue 3G-A. There also remains the often-overlooked possibility of developing a tandem system from dye-screen data. Scawen *et al.* (1982) describe two consecutive dye-ligand chromatography steps to purify two dehydrogenases. However, both steps involve 'positive' chromatography and this work represents a different approach to 'tandem' chromatography. Scheme 1 illustrates our rationale for tandem chromatography. 'Negative' columns are those which bind proteins other than the one of interest giving a purification in the unbound fraction. 'Positive' columns bind the protein of interest selectively which can then be eluted to yield

highly purified enzyme. We have preferred to precede 'positive' adsorbents with a 'negative' column when designing tandem dye-ligand chromatography, since in this way the 'negative' step may protect the 'positive' column against fouling.

The aim of the present study was (i) to purify glucose-6-phosphate dehydrogenase from *L. mesenteroides* by using dye-ligand chromatography as the only protein purification step, (ii) to develop a process suitable for industrial use, and (iii) to study the binding of this dual-nucleotide-specific enzyme to relevant triazine dyes to gain some insight into the mechanism of the protein-dye interaction.

Materials and methods

Materials

NADP⁺, NADPH, NADH, cytochrome *c*, creatine kinase, and glucose 6-phosphate were obtained from Boehringer, Mannheim, Germany. NAD⁺ was obtained from P-L Biochemicals, Milwaukee, WI, U.S.A.; 6-phosphogluconate, 2',5'-ADP, bovine serum albumin, ovalbumin, Nitroblue Tetrazolium, and phenazine methosulphate were from Sigma Chemical Company, Poole, Dorset, U.K. Tannic acid was from BDH Chemicals Ltd., Poole, Dorset, U.K. Matrex Gel Orange B, Purple A and frozen cell extracts of *L. mesenteroides* A.T.C.C. 12291 were supplied by Mr. S. Fulton of the Amicon Corporation, Danvers, MA, U.S.A. A

Scheme 1. Use of dye-screening in the design of tandem systems

60–90%-satd.-(NH₄)₂SO₄ paste of *L. mesenteroides* was supplied by Dr. A. Roeder of Boehringer Mannheim, Tutzing, Germany. Cibacron dyes were a gift from CIBA-Geigy, Manchester, U.K. Procion dyes were a gift from ICI Organics Division, Blackley, Manchester, U.K. All other chemicals used were from BDH Chemicals Ltd. or Fisons Scientific Apparatus, Loughborough, Leics., U.K.

General methods

Glucose-6-phosphate dehydrogenase was assayed routinely at 30°C in semi-micro cuvettes in a total volume of 1 ml. The reaction mixture contained 0.36 mM-glucose 6-phosphate, 0.074 mM-NADP⁺ and 10 mM-MgCl₂ in 45 mM-Tris/HCl buffer, pH 7.8. The reaction was initiated by addition of enzyme and monitored by recording the rate of formation of NADPH at 340 nm with a Cecil CE 292 spectrophotometer fitted with a Servoscribe 1S recorder. One unit of enzyme activity catalyses the reduction of 1 μmol of NADP⁺/min. Specific activity is expressed as units of enzyme per mg of protein.

Protein concentrations were estimated from *A*₂₈₀ and determined accurately by the tannin micro-method of Mejbaum-Katzenellenbogen & Dobrzycka (1959).

Dyes were coupled to Matrex Gel by the method of Heyns & De Moor (1974). Matrices of different ligand concentrations were obtained by varying the concentration of dyes during coupling. The immobilized dye concentration, expressed as mg/g wet weight of gel (referred to as mg/g of gel), was determined by the method of Angal & Dean (1977).

All columns were run at 4°C and after use were regenerated with 1 column-vol. of 4 M-urea in 0.5 M-NaOH before re-equilibration with the appropriate buffer.

Disc-gel electrophoresis

Polyacrylamide-gel electrophoresis was performed on 7.5% (w/v) gels in Tris/glycine buffer, pH 8.5, as described by Davis (1964). Gels were stained for protein with 1% (w/v) Naphthalene Black in 7% (v/v) acetic acid and destained in 7% (v/v) acetic acid and for enzyme activity by Tetrazolium reduction. The reaction mixture contained 0.074 mM-NADP⁺, 0.13 mM-phenazine methosulphate, 0.49 mM-Nitroblue Tetrazolium and 0.36 mM-glucose 6-phosphate in 50 mM-Tris/HCl buffer, pH 7.8. The gels were immersed in staining solution until a purple band formed (5–10 min), then removed, washed in 7% (v/v) acetic acid and stored in distilled water. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Weber & Osborn (1969), with bovine serum albumin, ovalbumin, creatine kinase and cytochrome *c* as molecular-weight markers.

Dye-screening procedure

In all, 65 immobilized dyes were packed into columns (2.3 cm × 0.7 cm) and screened as reported previously (Hey & Dean, 1981), with 50 mM-Tris/HCl buffer, pH 7.8. A solution of the 60–90%-satd.-(NH₄)₂SO₄ *L. mesenteroides* paste was dialysed overnight at 4°C and used in the screening study. A sample of the dialysed solution (50 μl, 40 units, 1.5 mg of protein) was applied to each column. The unbound and bound fractions were assayed for enzyme activity and protein. The purification and yield of enzyme was determined.

'Positive'-column studies

In further studies on some of the 'positive' columns, KCl gradients were applied to 1 ml columns and 1 ml fractions were collected. The flow rates were controlled at 4–6 ml/h. The applied protein was a dialysed solution of a 60–90%-satd.-(NH₄)₂SO₄ precipitate. Protein emerging from the column was estimated by *A*₂₈₀ measurements. More accurate determinations by the tannin micro-method were carried out on the final pooled fractions. All salt gradients were 0–1 M-KCl (30 ml) in equilibration buffer. In the biospecific-elution studies, protein from the unbound fraction of a Matrex Gel Purple A column was applied to a 2 ml column of Matrex Gel Orange B (ligand concentration 17.2 mg/g of gel). Elution was attempted with a pulse of the appropriate eluant.

Purification scheme

Cell extract was dialysed against 50 mM-Tris/HCl buffer, pH 7.8, at 4°C overnight.

Step 1: chromatography on Matrex Gel Purple A. A column (9 cm × 2 cm) of Matrex Gel Purple A (15 ml) (ligand concentration 5 mg/g of gel) was pre-equilibrated with 50 mM-Tris/HCl buffer, pH 7.8. The dialysed cell extract (42 ml, 557 mg of protein) was applied to the column and the column washed with equilibration buffer to remove unbound proteins. The column flow rate was controlled at 9 ml/h using an LKB Varioperpex peristaltic pump. Fractions (4.5 ml) were collected and assayed for enzyme activity and for protein by *A*₂₈₀. Fractions containing enzyme activity were pooled (58 ml).

Step 2: chromatography on Matrex Gel Orange B. The pooled fraction (58 ml, 348 mg of protein) from Step 1 was applied to a Matrex Gel Orange B column (8.5 cm × 1.2 cm) (10 ml of gel; ligand concentration 17.2 mg/g of gel) equilibrated with 50 mM-Tris/HCl buffer, pH 7.8. The column flow rate was maintained at 4.4 ml/h and fractions (2.2 ml) were collected. The column was washed with equilibration buffer to remove the unbound *A*₂₈₀-absorbing material. Glucose-6-phosphate dehydrogenase was eluted with a pulse (5 ml) of

50 μ M-NADP⁺. Fractions containing enzyme activity were pooled (9.1 ml).

Results

Screening of 65 immobilized triazine dyes for their ability to bind glucose-6-phosphate dehydrogenase from *L. mesenteroides* gave four 'negative' columns, ten 'positive' columns and 51 columns exhibiting intermediate behaviour. Table 1 shows the four 'negative' columns obtained from the screen. Matrex Gel Purple A was then chosen as the best of the four adsorbents because it gave the highest purification. Loading 30 mg of protein per ml of gel to this 'negative' adsorbent gives typically a 1.5–2.0-fold purification in the unbound fraction. Fig. 1 shows the polyacrylamide gels of the unbound and KCl eluted proteins from this column. Five of the ten 'positive' columns were studied further by controlling the flow rate and applying gradients of KCl. A comparison between a salt (KCl) pulse and gradient elution for these columns is given in Table 2. Gradient salt elution of enzyme bound to Matrex Gel Orange B gives a typical broad profile with peak enzyme activity at 200 mM-KCl. Immobilized dye Orange B was chosen for the 'positive' column since it gave the best purification and yield.

Table 1. Dye-screen data for the 'negative' columns
Experimental details are given in the Materials and methods section.

Adsorbent	Purification (fold)	Yield (%)
Matrex Gel Purple A	2.7	95
Matrex Gel Procion Navy MX-RB	1.9	90
Matrex Gel Procion Navy H-4R	1.8	72
Matrex Gel Procion Brown MX-GRN	1.6	73

Biospecific elution of the bound enzyme from Matrex Gel Orange B was attempted by using the enzyme's cofactors and substrates and some related

Fig. 1. Polyacrylamide-gel electrophoresis of glucose-6-phosphate dehydrogenase samples obtained during enzyme purification on (a) Matrex Gel Purple A and (b) Matrex Gel Orange B

(a) 1, Dialysed cell extract (100 μ g) (stained with Naphthalene Black); 2, unbound fraction from Matrex Gel Purple A (100 μ g) (Naphthalene Black); 3, bound fraction eluted from Matrex Gel Purple A with 1 M-KCl (75 μ g) (Naphthalene Black). (b) 1, Unbound fraction from Matrex Gel Purple A applied to Matrex Gel Orange B (400 μ g) (stained with Naphthalene Black); 2, protein eluted from Matrex Gel Orange B by 50 μ M-NADP⁺ (40 μ g) (Naphthalene Black); 3, protein eluted from Matrex Gel Orange B by 50 μ M-NADP⁺ (40 μ g) (specific glucose-6-phosphate dehydrogenase stain). Experimental details are given in the Materials and methods section. The arrows indicate the position of glucose-6-phosphate dehydrogenase.

Table 2. A comparison between a salt (KCl) pulse and gradient elution of *L. mesenteroides* glucose-6-phosphate dehydrogenase from immobilized triazine dyes

A solution of a 60–90%-satd.-(NH₄)₂SO₄ *L. mesenteroides* paste dialysed overnight at 4°C against 50 mM-Tris/HCl buffer, pH 7.8, was used. The results of pulsed elution studies were obtained by the method described for the dye-screening procedure (see the Materials and methods section). The gradient results were obtained by applying 0.1 ml (40 units, 2 mg of protein) to a Matrex Gel Orange B column (1 ml bed volume, 2.3 cm $\times$ 0.6 cm) pre-equilibrated with 50 mM-Tris/HCl buffer, pH 7.8. The column was washed with equilibration buffer (10 ml) to remove unbound proteins. A 0–1 M-KCl gradient was then applied (30 ml). Fractions (1 ml) were collected and assayed for glucose-6-phosphate dehydrogenase activity (○) and *A*₂₈₀ (●). The flow rate of the column was maintained at 4 ml/h.

Adsorbent	Pulse elution		Gradient elution	
	Purification (fold)	Yield (%)	Purification (fold)	Yield (%)
Matrex Gel Procion Yellow HE-3G	5.6	100	6.8	77
Matrex Gel Orange B	9.7	91	11.8	100
Matrex Gel Procion Yellow HE-4R	7.0	100	5.7	61
Matrex Gel Procion Orange HER	4.4	100	5.0	77
Matrex Gel Cibacron Black BGA	6.1	88	6.9	77

compounds. The results are shown in Table 3. It can be seen that the only effective biospecific eluents are NADP⁺, NADPH and adenosine 2',5'-bisphosphate. The minimum concentration at which NADP⁺ elutes the enzyme is 50 μ M. Fig. 2 shows that a solution of 42 mM-NAD⁺ causes 8% of the applied enzyme to appear in the eluate. Pulses of 50 μ M-NADP⁺ (as shown) and 1 mM-NADP⁺ (not shown) cause subsequent desorption of a large amount, but not all, of the bound enzyme. This slight release of enzyme by 42 mM-NAD⁺ could be due to the ionic strength of

the NAD⁺ solution, since 1 mM-NAD⁺ desorbs only 3% of the enzyme (Table 3). The results of these studies on Matrex Gel Purple A and Matrex Gel Orange B led to a purification scheme for the enzyme using a tandem system for dye–ligand chromatography as the sole purification method. The scheme involves 'negative' chromatography of the dialysed cell extract on a Matrex Gel Purple A column (15 ml) followed by 'positive' chromatography of the unbound fraction from this column on a Matrex Gel Orange B column (10 ml). Biospecific elution of pure enzyme from the latter column was obtained with a pulse of 50 μ M-NADP⁺ (5 ml) (Fig. 3). Table 4 gives the purification data and Fig. 1 shows the polyacrylamide-disc-gel electrophoretograms of the purified enzyme, showing one band that stains both for protein and enzyme. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis gives a subunit molecular weight of 55 600.

The Matrex Gel Orange B adsorbent was studied further to assess its reusability, capacity and the effect of ligand concentration on its ability to purify the enzyme. A column (1 ml) of the matrix (ligand concentration 4.2 mg/g of gel) was used repeatedly for purifying the enzyme from the same cell extract with no change in the columns performance after 11 repeated purifications.

Matrex Gel Orange B was prepared at four ligand concentrations (3.3, 5.7, 9.8 and 23.5 mg/g) and each was tested in the purification scheme outlined above. The degree of purity of eluted enzyme from each column was assessed by polyacrylamide-gel electrophoresis of the eluates. The results showed no difference over the range tested, with enzyme of specific activity 190 units/mg being eluted in each case. To determine the capacity of Matrex Gel Orange B, increasing amounts of protein were offered to a 2 ml column (ligand concentration 17.2 mg/g of gel). The purification and yield obtained on biospecific elution of bound enzyme was determined in each case. It was observed that the capacity of this adsorbent was 35 mg of applied protein/ml of gel, homogeneous enzyme (0.7 mg/ml of gel) being eluted in 77% yield. A study was carried out on the purified enzyme to determine the effect of the presence of bound nucleotides NAD⁺ and NADP⁺ on its ability to bind to Matrex Gel Orange B, since Metcalf *et al.* (1981) found that endogenous ligands have a dramatic effect on the binding of various albumins to immobilized Cibacron Blue 3G-A. The pure enzyme was treated with activated charcoal (Eby & Kirtley, 1971) before use to remove any bound nucleotides. The treated enzyme was applied to the column and was found to bind. Elution was quantitative with 50 μ M-NADP⁺. Preincubation of the charcoal-treated enzyme for 1 h with 50 μ M-NADP⁺ before ap-

Table 3. Effect of different biospecific eluents to desorb *L. mesenteroides* glucose-6-phosphate dehydrogenase bound to Matrex Gel Orange B

A solution of enzyme (1 ml, 45 units, 10 mg of protein) partially purified on Matrex Gel Purple A was applied to a column of Matrex Gel Orange B (2 ml bed volume, 3.0 cm $\times$ 0.8 cm, dye–ligand concentration 17.2 mg per g of gel) equilibrated with 50 mM-Tris/HCl, pH 7.8. The column was washed with equilibration buffer (7 ml) before attempting elution of the enzyme by applying a solution of the eluent in the volumes and concentrations indicated. The column was then washed again with equilibration buffer (5 ml). Fractions (1 ml) were collected and assayed for glucose-6-phosphate dehydrogenase activity and absorbance at 280 nm. The flow rate of the column was maintained at 4 ml/h.

Biospecific eluent	Pulse volume (ml)	Concn. (mM)	Amount of enzyme eluted (% of that applied)
NADP ⁺	10	1	91
	1	1	85
	1	0.1	80
	1	0.05	78
	1	0.01	13
NADPH	10	1	83
NAD ⁺	1	1	3
	1	42	8
NADH	1	1	4
Glucose 6-phosphate	1	1	7
Glucose 6-phosphate + NADH	1	1	8
Pyrophosphate	1	1	0
	1	10	0
6-Phosphogluconate	1	1	0
6-Phosphogluconate + NADH	1	1	0
Pyrophosphate + NADH	1	1	0
2',5'-Adenosine bisphosphate	1	1	95
	1	0.05	6
3',5'-Adenosine bisphosphate	1	1	0
2'-Adenosine monophosphate	1	1	0
3'-Adenosine monophosphate	1	1	0
5'-Adenosine monophosphate	1	1	0
5'-Adenosine diphosphate	1	1	0
5'-Adenosine triphosphate	1	1	3

Fig. 2. Attempted biospecific elution of *L. mesenteroides* glucose-6-phosphate dehydrogenase from Matrex Gel Orange B. Pooled enzyme fractions from a Matrex Gel Purple A column were used (see the Materials and methods section). A 1 ml portion (48 units, 8.7 mg of protein) was applied directly to a Matrex Gel Orange B column (2 ml bed volume, 3 cm $\times$ 0.8 cm) pre-equilibrated with 50 mM-Tris/HCl buffer, pH 7.8. The column was washed with equilibration buffer (7 ml) to remove unbound proteins. A pulse of 42 mM-NAD⁺ (1 ml) was applied and the column was washed again with equilibration buffer (5 ml). A pulse of 50 μ M-NADP⁺ (1 ml) was then applied, followed by a further wash with equilibration buffer (5 ml). Fractions (1 ml) were collected and assayed for glucose-6-phosphate dehydrogenase activity (○) and absorbance at 280 nm (●). The flow rate of the column was maintained at 4 ml/h.

Fig. 3. Chromatography of glucose-6-phosphate dehydrogenase from *L. mesenteroides* on Matrex Gel Orange B. Pooled enzyme-containing fractions (58 ml, 2172 units, 348 mg of protein) from a Matrex Gel Purple A column (see the Materials and methods section) were applied directly to a Matrex Gel Orange B column (10 ml bed volume, 7.4 cm $\times$ 1.5 cm) that had previously been equilibrated with 50 mM-Tris/HCl buffer, pH 7.8. The column was washed with equilibration buffer to remove unbound proteins before applying a pulse of 50 μ M-NADP⁺ in equilibration buffer (5 ml). Fractions (2.2 ml) were collected and assayed for glucose-6-phosphate dehydrogenase activity (○) and absorbance at 280 nm (●). The flow rate of the column was maintained at 4.4 ml/h.

plication to the column prevented enzyme binding (Fig. 4a). On the other hand, preincubation with 42 mM-NAD⁺ under the same conditions did not have this effect (Fig. 4b). Pre-equilibration of the column with 50 μ M-NADP⁺ did not prevent the enzyme from binding, but pre-equilibration with

1 mM-NADP⁺ prevented 50% of the enzyme binding (Fig. 4c).

Discussion

A purification procedure has been described for glucose-6-phosphate dehydrogenase from *L.*

Table 4. Purification of glucose-6-phosphate dehydrogenase from *L. mesenteroides*
Experimental details are given in the Materials and methods section.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Dialysis of cell extract	2286	557	4.1	1.0	100
Matrex Gel Purple A chromatography	2172	348	6.2	1.5	95
Matrex Gel Orange B chromatography	1664	7.3	228	55.6	73

Fig. 4. Effect of preincubation of *L. mesenteroides* glucose-6-phosphate dehydrogenase with nucleotides before chromatography on Matrex Gel Orange B

Pure enzyme (10 μ l, 4.8 units) that had been charcoal-treated (Eby & Kirtley, 1971) was used. Columns of Matrex Gel Orange B (2 ml bed volume, 3 cm $\times$ 0.8 cm) were pre-equilibrated with 50 mM-Tris/HCl buffer, pH 7.8. After sample application the column was washed with equilibration buffer to remove unbound protein before applying a pulse of 50 μ M-NADP⁺ (1 ml). (a) Enzyme was preincubated with 50 μ M-NADP⁺ (1 ml) for 1 h before applying to the column. (b) Enzyme was preincubated with 42 mM-NAD⁺ (1 ml) for 1 h before applying to the column. (c) The column was pre-equilibrated with equilibration buffer containing 1 mM-NADP⁺ (20 ml) before applying the enzyme in 50 mM-Tris/HCl buffer, pH 7.8 (1 ml). Fractions (1 ml) were collected and assayed for glucose-6-phosphate dehydrogenase activity (○) and A_{280} (●). The flow rate of the columns was maintained at 4 ml/h.

mesenteroides which uses tandem dye–ligand chromatography. The use of immobilized triazine dyes is now established in the purification schemes for a host of widely different proteins. Most workers use immobilized Cibacron Blue 3G-A unquestioningly. The conditions of adsorption and elution are adapted to maximize the purification. Few workers have screened more than five immobilized triazine dyes for their ability to bind individual proteins. Baird *et al.* (1976) screened 11 dye–Sephacrose conjugates for the purification of carboxypeptidase G and reported Procion Red HE-3B to be the most suitable in terms of capacity, specificity of elution and enzyme recovery. Johnson *et al.* (1980) screened 16 dye–agarose conjugates at various ligand concentrations for their ability to bind rat dihydrofolate reductase and found that Procion Blue MX-4GD, Procion Blue HE-RD and Procion Red H3-BN were just as effective at adsorbing and subsequently releasing the enzyme as Cibacron Blue 3G-A. Bruton & Atkinson (1979) examined 32 Sepharose-immobilized triazine dyes including Cibacron Blue 3G-A and screened them for the purification of 13 different amino-acyl-tRNA synthetases. Further investigations of the most interesting dye columns lead to purification schemes for several tRNA synthetases, e.g. tryptophanyl-tRNA synthetase on Procion Brown MX-5BR, and methionyl-tRNA synthetase using Procion Green HE-4BD. The unpredictable nature of dye–protein interactions led Bruton & Atkinson (1979) to suggest screening of as many immobilized dyes as possible, since in their hands they found Cibacron Blue 3G-A Sepharose not to be remarkable in either its binding or elution characteristics.

In our laboratory we routinely screen up to 65 triazine dye columns (Qadri, 1980) and analyse the results to search for both 'negative' and 'positive' supports. The use of a 'negative' column for our purposes eliminates the need for an $(\text{NH}_4)_2\text{SO}_4$ -precipitation step. Crude cell extracts of *L. mesenteroides* can be applied directly to a column of Matrex Gel Purple A at levels of 30 mg/ml of gel. A purification is obtained in the unbound fraction. Furthermore, this column can be used four or five times before fouling necessitates a change of gel. The latter column has a protective effect on the subsequent 'positive' column (Matrex Gel Orange B) since in a re-usability study this column was used 11 times with no change in its chromatographic properties.

The nature of dye–protein interactions has been studied by many workers using a wide variety of methods, including spectral-shift analysis (Thompson & Stellwagen, 1976), X-ray crystallography (Biellmann *et al.*, 1979) and analysis of partial dye structure–binding relationships (Clonis, 1982; Leatherbarrow & Dean, 1980). Biospecific

elution of enzymes bound to dye columns has also been claimed to give some insight into the sites at which binding occurs (Kawai & Eguchi, 1980) and, in addition, may prove invaluable for producing homogeneous protein.

In the present study we have examined the elution of glucose 6-phosphate dehydrogenase bound to Matrex Gel Orange B with a range of relevant cofactors, substrates and related molecules. The only effective eluents were NADP^+ , NADPH and adenosine 2',5'-bisphosphate. The minimum concentration of NADP^+ which quantitatively eluted the enzyme from the gel (ligand concentration 17.2 mg/g) was $50 \mu\text{M}$. Olive & Levy (1967) reported the K_m values for this enzyme to be $7.4 \times 10^{-6} \text{ M}$ for NADP^+ and $1.15 \times 10^{-4} \text{ M}$ for NAD^+ . The K_d values were determined by Grove *et al.* (1976) to be $3 \times 10^{-6} \text{ M}$ for NADP^+ and $2.5 \times 10^{-3} \text{ M}$ for NAD^+ . The minimum concentration of $50 \mu\text{M}$ - NADP^+ required to elute bound enzyme is seven times the K_m and 17 times the K_d for the enzyme (with NADP^+). NAD^+ , however, will not elute the enzyme from the same column when applied at a concentration of 0.8 mM (seven times the K_m for NAD^+) or even at 42 mM, which is 17 times the K_d for NAD^+ . However, it is noteworthy that NADP^+ is not as effective an eluent when applied after 42 mM- NAD^+ (Fig. 2), since even 1 mM- NADP^+ does not elute all the enzyme. Olive & Levy (1967) stated that the binding and catalytic reduction of NAD^+ and NADP^+ by this glucose-6-phosphate dehydrogenase took place at the same site. Later kinetic studies showed that NAD^+ and NADP^+ interact with different conformational enzyme forms (Olive *et al.*, 1971). When 42 mM- NAD^+ is used as an eluent, the nucleotide may bind tightly to the enzyme adsorbed on to the column and induce an NAD^+ -specific conformation. As a result, biospecific elution by NADP^+ becomes more difficult, since NADP^+ requires a different conformational form for effective binding. The only other successful biospecific eluent was adenosine 2',5'-bisphosphate, which was found by Grove *et al.* (1976), who used fluorescent studies, to be strongly inhibitory: competitive with NADP^+ and non-competitive with NAD^+ . Pre-saturating the enzyme or the Matrex Gel Orange B column with NADP^+ before chromatography prevents the enzyme from binding, whereas the same treatment with NAD^+ does not have this effect. We would like to propose that there are two possible mechanisms to explain these observations: (i) dye binding takes place at, or very close to, the nucleotide-binding site and that the binding to ligands is solely determined by the relative K_a values, the K_a for the dye being between that of NAD^+ and NADP^+ . Hence only NADP^+ (or adenosine 2',5'-bisphosphate) can elute the protein from the dye column; (ii) the dye binds to the enzyme at a site remote from the nucleotide-binding site.

Conformational changes induced on NADP⁺ (but not NAD⁺) binding result in weakening of the dye-protein interaction and hence desorption occurs. The dye-protein interaction is clearly not entirely specific, but also ionic in nature, since non-specific eluents such as KCl effectively desorb the enzyme.

In the present work, glucose-6-phosphate dehydrogenase from *L. mesenteroides* has been purified to homogeneity using tandem dye-ligand chromatography involving biospecific elution of pure enzyme from Matrex Gel Orange B using $\frac{1}{2}$ column vol. of 50 μ M-NADP⁺. Olive & Levy (1967) purified this enzyme by a procedure involving precipitation with (NH₄)₂SO₄, treatment with protamine, (NH₄)₂SO₄ fractionation, chromatography on hydroxyapatite followed by two crystallizations. They obtained 22mg of homogeneous enzyme of specific activity 250 units/mg (NADP⁺ as co-enzyme, pH 7.8, 25°C) in 18% yield. More recently Choi *et al.* (1978) reported a procedure for an 80-fold purification of this enzyme by Cibacron Blue 3G-A-Sepharese and hydroxyapatite chromatography, but no further experimental details were published. Our procedure involves two dye-column steps to yield homogeneous enzyme of 228 units/mg (NADP⁺ as coenzyme, pH 7.8, 30°C) in 73% yield. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of our preparation gives subunit mol.wt. of 55600, which compares favourably with the reported values of 54800 by the same method (Ishaque *et al.*, 1974). The latter authors report that the enzyme consists of two identical subunits, since Olive & Levy (1971) determined the molecular weight to be 103700 by sedimentation-equilibrium techniques.

The purification scheme outlined in the present study for glucose-6-phosphate dehydrogenase from *L. mesenteroides* provides a rapid two-step method of obtaining pure enzyme in high yields by dye-ligand chromatography. The advantages of using dye matrices are well documented (Lowe *et al.*, 1981; Hey & Dean, 1981). These results show that these advantages, coupled with the high capacity of Matrex Gel Orange B (35mg/ml of gel) and low concentrations of NADP⁺ required for complete elution of the enzyme, the present two-step procedure constitutes an economically viable system for a large-scale application.

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