The use of various immobilized-triazine affinity dyes for the purification of 6-phosphogluconate dehydrogenase from *Bacillus stearothermophilus*

Firdausi QADRI and Peter D. G. DEAN

Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

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1. 6-Phosphogluconate dehydrogenase from *Bacillus stearothermophilus* was purified approximately 260-fold on triazine-immobilized dye columns to a final specific activity of 54 μmol of NADP⁺ reduced/min per mg of protein and an overall yield of 62%. 2. An investigation of the capacities of different triazine dyes that inhibit 6-phosphogluconate dehydrogenase was carried out. Cibacron Blue F3G-A and Procion Red HE-3B strongly inhibited the enzyme in free solution and were therefore chosen as the ligands in the purification scheme. 3. KCl was found to be the most suitable agent for eluting 6-phosphogluconate dehydrogenase from Procion Red HE-3B–Sephacrose 6B. NADP⁺ could specifically elute 6-phosphogluconate dehydrogenase from Cibacron Blue F3G-A–Sephacrose 6B. 4. A study of the effect of temperature on the binding of pure 6-phosphogluconate dehydrogenase to both Cibacron Blue–Sephacrose and Procion Red–Sephacrose showed that the binding increased with an increase in temperature.

6-Phosphogluconate dehydrogenase catalyses the following reaction:

6-Phospho-D-gluconate–NADP⁺ oxidoreductase (decarboxylating) (EC 1.1.1.44) has been extensively studied from a wide range of sources (Villet & Dalziel, 1969). These studies have generally employed conventional procedures, and only in a few cases have affinity (nucleotide) matrices been used (Griffiths *et al.*, 1977). Economic considerations, however, limit the preparation of sufficient immobilized-nucleotide supports for the purification of large quantities of enzyme. The usefulness of polyaromatic sulphonated textile dyes in protein purification (examples of which are Cibacron Blue and Procion dyes; Figs. 5, 6 and 7) lies in their value as high-capacity stable alternatives to nucleotide ‘group-specific’ adsorbents.

In a recent review (Dean & Watson, 1979) it was shown that the enzymes that bind to polyaromatic dyes include not only dehydrogenases and kinases but also many other classes of proteins, e.g. albumin. Suggestions that the dehydrogenases and other enzymes that bind to these dyes might also contain the dinucleotide-binding fold has prompted many workers to explore the selectivity of individual dyes. Although Procion Red HE 3B appears in some

studies to be NADP⁺-selective (Watson *et al.*, 1978), this specificity is probably not as marked as one would prefer in a group-specific adsorbent. Nevertheless, the binding of at least one dye, Cibacron Blue F3GA, has been established beyond all reasonable doubt to be at the nucleotide-binding site for alcohol dehydrogenase (Biellmann *et al.*, 1979). This last-mentioned result underlines the importance of a screening programme at present being carried out in several laboratories to find specific applications for individual affinity dye matrices. Among several dozens of dye–enzyme combinations examined in our own laboratory, 6-phosphogluconate dehydrogenase has shown some interesting properties that may tell us something of the mechanism of interaction of proteins with these dyes.

The aim of the present study was (i) to examine the interaction of 6-phosphogluconate dehydrogenase from *Bacillus stearothermophilus* with triazine ‘affinity’ dyes and investigate their mechanism of binding, and (ii) to obtain pure enzyme in high yield suitable for X-ray crystallography.

Materials and methods

Materials

NADP⁺ was obtained from P-L Biochemicals, Milwaukee, WI, U.S.A. 6-Phosphogluconate was

obtained from Boehringer, Mannheim, Germany. Sepharose-6B was purchased from Pharmacia, London W.5, U.K. Cibacron Blue F3G-A was a gift from CIBA-GEIGY, Manchester, U.K. Procion dyes were gifts from ICI Organics Division, Blakely, Manchester 9, U.K. Frozen cells of *Bacillus stearothermophilus* were obtained from the Microbiological Research Establishment, Porton, Wilts., U.K.

General methods

6-Phosphogluconate dehydrogenase was assayed routinely at 30°C in semi-microcuvettes in a total volume of 1.0 ml. The reaction mixture consisted of 6-phosphogluconic acid (0.5 mM), NADP⁺ (0.6 mM) and MgCl₂ (10 mM) in 0.1 M-Tris/HCl buffer, pH 7.5. The reaction was initiated by the addition of enzyme solution and was followed by measuring the rate of NADPH formation at 340 nm with a Gilford 250 spectrometer. One unit of enzyme activity catalyses the reduction of 1 μmol of NADP⁺/min. Specific activity is expressed as units/mg of protein. Protein was determined from the absorbance at 280 nm assuming $A_{280}^{1\%, 1\text{ cm}} = 10$ and by the tannin micro method (Katzenellenbogen & Dobryzycka, 1959). Cibacron Blue and Procion dye derivatives of Sepharose and Sephadex were prepared by the method of Heyns & DeMoor (1974). Cibacron Blue-Sepharoses of different ligand concentrations were prepared by varying the dye concentration during the coupling reaction. Ligand concentrations were determined by using 'wet gel' as well as 'dry gel' weight. The dyed matrices were dried by washing on a sintered-glass funnel with methanol, followed by diethyl ether and then in air. Samples of all Sepharose derivatives were hydrolysed by boiling for 10 min in 50% (v/v) acetic acid or 3 M-HCl. The Sephadex derivatives were hydrolysed in 3 M-HCl. Cibacron Blue concentrations were determined at 610 nm ($\epsilon = 9 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 7) and Procion Red HE-3B at 536 nm ($\epsilon = 2 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ pH 7) with a Gilford 250 spectrophotometer and were based on dilutions that fell within the linear range of absorption.

Disc gel electrophoresis

Analytical polyacrylamide-gel electrophoresis was performed with 7.5% (acrylamide) gels in Tris/glycine buffer, pH 8.5, at 3 mA per tube (Ornstein, 1964). 6-Phosphogluconate dehydrogenase activity was detected in polyacrylamide gels by tetrazolium reduction (Veronese *et al.*, 1974). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Weber & Osborn (1969).

Enzyme purification

The purification procedure adopted for the isolation of 6-phosphogluconate dehydrogenase is summarized in Table 1.

Step 1. Disruption of cells. Frozen cells (100 g) of *Bacillus stearothermophilus* were suspended in 300 ml of equilibration buffer (50 mM-Tris/HCl buffer, pH 7.2, containing 1 mM-EDTA and 1 mM-2-mercaptoethanol) and allowed to thaw overnight at 4°C. The cells were disrupted in an MSE Ultrasonic disintegrator for 2 min (ten times) and then centrifuged at 10000g for 30 min in a Sorvall RC2-B centrifuge. The supernatant was collected. The debris was resuspended in equilibration buffer (200 ml), sonicated and centrifuged. Both supernatants were pooled and enzyme activity as well as protein concentration were measured.

Step 2. (NH₄)₂SO₄ fractionation. The precipitate after 35% saturation with (NH₄)₂SO₄ was removed by centrifugation at 5000g for 15 min. The solution was adjusted to 75% saturation with (NH₄)₂SO₄ and re-centrifuged. The precipitate was dissolved in equilibration buffer (164 ml) and dialysed exhaustively until the conductivity of the enzyme solution decreased to 3.4 mΩ⁻¹ (being that of the equilibration buffer).

Step 3. Chromatography on Procion Red HE-3B-Sepharose 6b. A column (18.5 cm × 4.2 cm) of Procion Red HE-3B-Sepharose 6B (ligand concentration 40.5 μmol/g dry wt. of gel) was pre-washed with equilibration buffer. The dialysed (NH₄)₂SO₄ extract (240 ml) was applied to the column. The column was then washed with equilibration buffer (850 ml) until the absorbance at 280 nm decreased

Table 1. Purification of 6-phosphogluconate dehydrogenase from *B. stearothermophilus*
Experimental details are given in the text.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification factor (fold)	Yield (%)
Crude extract	5514	1143	0.21	1	100
(NH ₄) ₂ SO ₄ fractionation	2624	1002	0.381	0.84	87.6
Procion Red HE-3B-agarose chromatography	390	940	2.52	12.1	82.2
Cibacron Blue F3G-A-agarose chromatography	19.5	897	46	222	78.4
Sephadex G-100	13	707	54.2	261	61.9

to less than 0.1. A linear (0–0.8M) KCl gradient (2 litres) of equilibration buffer was applied. The column flow rate was controlled at 60ml/h using a peristaltic pump. Fractions (12ml) were assayed for enzyme activity, conductivity and protein at 280nm (Fig. 1). Fractions containing enzyme activity (nos. 143–169) (325 ml) were pooled, concentrated and then dialysed overnight against equilibration buffer.

Step 4. Chromatography on Cibacron Blue F3G-A–Sephacrose. The dialysed enzyme solution (150ml) from step 3 was applied to a Cibacron Blue F3G-A–Sephacrose 6B column (18 cm × 2.5 cm) (ligand concentration 31 μ mol/g dry wt. of gel). The flow rate was maintained at 60ml/h. The column was washed with equilibration buffer containing 80mM-KCl (200ml). 6-Phosphogluconate dehydrogenase was eluted with a pulse of 0.5mM-NADP⁺ (250ml) (Fig. 2). Fractions containing enzyme activity were pooled and dialysed (130ml).

Step 5. Sephadex G-100. The pooled enzyme fractions from step 4 were concentrated by ultra-filtration to 1.5 ml and then applied to a Sephadex G-100 column (125 cm × 2.1 cm). The column was pre-washed and eluted with equilibration buffer, and 3.28 ml fractions were collected (Fig. 3).

Fig. 1. Chromatography of 6-phosphogluconate dehydrogenase from *B. stearothermophilus* on Procione Red HE-3B–Sephacrose 6B

The dialysed enzyme preparation (240 ml, 1002 units) obtained from the (NH₄)₂SO₄ fractionation (see the Materials and methods section) was applied to a Procione Red HE-3B–Sephacrose-6B column (232 ml bed volume, 18.5 cm × 4 cm) that had been previously equilibrated with 50mM-Tris/HCl buffer, pH 7.2 (equilibration buffer). The column was then washed with equilibration buffer (850 ml). A linear gradient of KCl (0–0.8M) in equilibration buffer was applied to the column. Fractions (12 ml) were collected and assayed for 6-phosphogluconate dehydrogenase activity (●), conductivity (----) and absorbance at 280nm (○). The flow rate of the column was maintained at 60 ml/h. Fractions 143–169 were pooled.

Fig. 2. Chromatography of 6-phosphogluconate dehydrogenase from *B. stearothermophilus* on Cibacron Blue F3G-A–Sephacrose 6B

The dialysed enzyme preparation (150 ml) from the Procione Red HE-3B–Sephacrose 6B column (see Fig. 1) was applied to a Cibacron Blue F3G-A–Sephacrose 6B column (89 ml bed volume, 18 cm × 2.5 cm) that had been pre-equilibrated with 50mM-Tris/HCl buffer, pH 7.2 (equilibration buffer). The same buffer containing (in addition) 80mM-KCl was used to wash the column (200 ml). Then 0.5mM-NADP⁺ in equilibration buffer (250 ml) was applied to the column. Fractions (13 ml) were collected and assayed for enzyme activity (●) and protein by the tannin procedure (○). The flow rate of the column was maintained at 60 ml/h. Fractions 35–44 were pooled.

Fig. 3. Gel filtration of 6-phosphogluconate dehydrogenase on Sephadex G-100

The enzyme sample (130 ml) from the previous step (Fig. 2) was concentrated to 1.5 ml and applied to a Sephadex G-100 column (125 cm × 2.1 cm). The column was eluted with 50mM-Tris/HCl buffer, pH 7.2. The arrow indicates the void volume (151 ml) determined separately with bovine thyroglobulin solutions. Fractions (3.28 ml) were assayed for enzyme activity (●) and absorbance at 280nm (○). Fractions 51–61 were pooled.

Fig. 4. Polyacrylamide-gel electrophoresis of 6-phosphogluconate dehydrogenase samples obtained during enzyme purification

(a) After Procion Red HE-3B–Sephadex 6B chromatography (Amido Schwartz); (b) after Cibacron Blue F3G-A–Sephadex 6B chromatography (Amido Schwartz) [and (b') (specific 6-phosphogluconate dehydrogenase stain)]; (c) after Sephadex G-100 chromatography (Amido Schwartz); (d) after Sephadex G-100 chromatography (specific 6-phosphogluconate dehydrogenase stain); (e) after Sephadex G-100 chromatography after treatment with sodium dodecyl sulphate (Amido Schwartz). Experimental details are given in the text.

Criteria of purity

Samples of enzyme from each step were analysed by disc gel electrophoresis (Fig. 4). The samples from the Cibacron Blue–Sephadex 6B column occasionally showed multiple bands when stained for protein and for enzyme activity. However, pure protein could be routinely prepared by this procedure, which when dissociated by sodium dodecyl sulphate and run on sodium dodecyl sulphate/polyacrylamide gels gave only one band of protein.

Results

Figs. 1 and 2 show the purification of 6-phosphogluconate dehydrogenase with the use of immobilized triazine dyes. The interaction between various polyaromatic sulphonated dyes and 6-phosphogluconate dehydrogenase was examined by: (i) chromatography of the purified enzyme on columns of the Sephadex 6B-immobilized dyes, namely Procion Red HE-3B, Procion Red H-3B, Procion Red MX-2B, Procion Scarlet MX-G, Cibacron Blue F3G-A, Procion Blue MX-3G, Procion Blue MX-R,

Procion Brown H-2G and Procion Yellow MX-R; (ii) inhibition with the use of unhydrolysed dyes in free solution. The results are presented in Table 2. The structures of the relevant dyes are given in Figs. 5, 6 and 7. The dyes that showed the greatest degree of inhibition, namely Procion Red HE-3B and Cibacron Blue F3G-A, were used in the purification process (Table 1).

KCl, citrate and pyrophosphate were compared for their selectivity in eluting 6-phosphogluconate dehydrogenase from the Procion Red HE-3B–Sephadex 6B column. KCl gradients gave not only higher purification but also higher recoveries. 6-Phosphogluconate and NADP^+ were investigated as specific eluents for the enzyme; both elution methods met with little success on the Procion Red HE-3B–Sephadex 6B columns. A KCl gradient satisfactorily eluted 6-phosphogluconate dehydrogenase from the Cibacron Blue F3G-A–Sephadex 6B column (ligand concentration $70 \mu\text{mol/g}$ dry wt. of gel). Biospecific elution was attempted by using 6-phosphogluconate and NADP^+ ; 6-phosphogluconate (10–100 mM) failed to elute 6-phospho-

Table 2. Binding of *Bacillus stearothermophilus* 6-phosphogluconate dehydrogenase to free and immobilized triazine dyes

Experimental details are given in the text.			
	Free solution [concn. required to give 50% inhibition (μM)]	Column	Concn. of KCl coincident with peak of enzyme activity (M)
Procion Red HE-3B	3	Binds	See the text
Procion Red H-3B	222	Binds	0.22
Procion Red MX-2B	260	—	—
Procion Scarlet MX-G	453	—	—
Cibacron Blue F3G-A	27	Binds	0.23
Procion Blue MX-3G	168	Binds weakly (leaks)	0.12
Procion Blue MX-R	265	—	—
Procion Brown H-2G	150	Binds	0.21
Procion Yellow MX-R	220	—	—

Fig. 5. Structure of some red triazine dyes and their interactions with *B. stearothermophilus* 6-phosphogluconate dehydrogenase
Abbreviation used: S, SO₃H or SO₃Na.

gluconate dehydrogenase. When NADP⁺ (10mM) was used, the polyacrylamide gels showed numerous protein bands. However, when a concentration of NADP⁺ lower than 10mM was used, no enzyme was

eluted. Lowering the ligand concentration of Cibacron Blue on Sepharose 6B (to 31μmol/g dry wt. of gel) enabled a pulse of 0.5mM-NADP⁺ to elute the enzyme specifically. The binding capacity of pure

Fig. 6. Structures of some blue triazine dyes and their interactions with *B. stearothermophilus* 6-phosphogluconate dehydrogenase
Abbreviation used: S, SO₃H or SO₃Na.

Fig. 7. Structures of brown and yellow triazine dyes and their interactions with *B. stearothermophilus* 6-phosphogluconate dehydrogenase
Abbreviation used: S, SO₃H or SO₃Na.

Table 3. Capacity of immobilized Procion Red HE-3B for 6-phosphogluconate dehydrogenase from *B. stearothermophilus*. Experimental details are given in the text.

Support matrix	Concn. of ligand (mg/g wet wt.)	Capacity for 6-phosphogluconate dehydrogenase (units/g wet wt.)
Sephadex G-15	0.13	0
Sephadex G-25	0.26	0
Sephadex G-50	0.48	0
Sephadex G-75	0.50	4.2
Sephadex G-100	0.55	14.5
Sephadex G-200	0.81	20.0
Sepharose 6B	1.14	15.6

Fig. 8. Effect of variation of column temperature on the salt-dependent elution of *B. stearothermophilus* 6-phosphogluconate dehydrogenase from triazine dye-agarose columns

Dialysed enzyme samples (10 units in 0.2 ml) were applied to pre-equilibrated columns of immobilized dye (1 ml). After further washing of the column with equilibration buffer (10 ml), linear gradients (0–1.0 M) of KCl in equilibration buffer were applied (20 ml). Fractions (1 ml) were collected and assayed for enzyme activity. Salt concentrations were measured at room temperature and are the values determined at the peaks of emerging activity. Chromatography was on Procion Red HE-3B-agarose (■) and on Cibacron Blue F3G-A-agarose (▲).

Fig. 9. Effect of variation of column temperature on the salt-dependent elution of yeast 6-phosphogluconate dehydrogenase from Cibacron Blue F3G-A-agarose. The experimental conditions were the same as those described in Fig. 8.

6-phosphogluconate dehydrogenase for Procion Red HE-3B as a ligand was compared by using the support matrices shown in Table 3. This Table shows that the ligand capacity for the enzyme with Sephadex G-100 and G-200 is high and comparable with that of Sepharose 6B. However, ligand leakage from the immobilized dye-Sephadex columns made it difficult to measure the enzyme activity because the free dye strongly inhibited 6-phosphogluconate dehydrogenase. The Sephadex columns also had very poor flow rates when compared with agarose gels.

The effect of temperature on the binding of 6-phosphogluconate dehydrogenase to Cibacron

Blue F3G-A- and Procion Red HE-3B-Sephacrose 6B were studied. When the column temperature was increased from 0 to 55°C, binding to the immobilized dye increased (Fig. 8). Binding (β) was measured at the concentration of KCl (mM) at the centre of the enzyme peak when the enzyme was eluted with a linear gradient. A similar experiment was carried out with yeast 6-phosphogluconate dehydrogenase on Cibacron Blue F3G-A-Sephacrose 6B (Fig. 9). Higher temperatures increased the binding, although the recovery of enzyme decreased.

Discussion

Use of triazine dyes to purify 6-phosphogluconate dehydrogenase

The purification of 6-phosphogluconate dehydrogenase is described that uses a combination of $(\text{NH}_4)_2\text{SO}_4$ fractionation and triazine dye adsorption chromatography. The enzyme from *B. stearothermophilus*, in common with the same enzyme from yeast and from *Acer pseudoplatanus* (W. Jessup & P. D. G. Dean, unpublished work), is easily separated from glucose 6-phosphate dehydrogenase by using Procion Red HE-3B-agarose columns. This purification procedure demonstrates the 'in tandem' use of Cibacron Blue F3G-A and Procion Red HE-3B as 'affinity' ligands.

The specific activity of the purified protein was 52 units/mg (pH 7.5, 30°C), which compares with reported values of 20 units/mg (pH 9.0, 25°C) (Pearse & Harris, 1973) and 87 units/mg (pH 8.0, 43°C) (Veronese *et al.*, 1974). Under the latter conditions of pH and temperature our purified protein had a specific activity of 91.6 units/mg.

We have examined the interaction of a variety of Procion dyes (for a discussion of Procion dyes see Beech, 1970) for their ability to bind 6-phosphogluconate dehydrogenase selectively, and from these studies and free-solution inhibition data we chose Procion Red HE-3B and Cibacron Blue F3G-A as ligands for adsorption chromatography.

Dependence of binding of 6-phosphogluconate dehydrogenase on the structure of various dyes

The free-solution inhibition by various dyes correlates with the chromatographic behaviour of the enzyme on the immobilized dyes. A 50% inhibition constant above about 200 μM measured under the conditions described in the Materials and methods section is associated with an immobilized-dye column that fails to bind enzyme at the ligand concentration used. However, higher ligand concentrations could promote enzyme binding (Dean & Watson, 1979).

The dimeric structure Procion Red HE-3B has been shown to bind a wide variety of enzymes, particularly NAD(P)^+ -dependent dehydrogenases.

(Dean & Watson, 1978, 1979; Watson *et al.*, 1978). The univalent analogue Procion Red H-3B shows similar characteristics, but the free-solution 50% inhibition constant is almost two orders higher (222 μM). Nevertheless, immobilized Procion Red H-3B binds 6-phosphogluconate dehydrogenase effectively.

On the other hand, removal of the benzene ring linked to the triazine moiety (Procion Red MX-2B) results in abolishment of the binding and an increase of the free-solution inhibition constant to 290 μM . Furthermore, alteration of the point of attachment of the triazine ring to the naphthylamine moiety and removal of one sulphonyl residue raises the free-solution inhibition constant still further (Procion Scarlet MX-G). Some dye structures (Procion Brown H-2G) seem to be able to bind 6-phosphogluconate dehydrogenase despite two major structural changes away from the structure of Procion Red HE-3B.

The structure-binding relationship for the anthroquinone dyes shows that the tightest binding (Cibacron Blue F3G-A) is severely weakened by eliminating the sulphanilic acid moiety attached to the triazine ring, and is abolished when a further change is made to alter the position of substitution to the central phenylenediamine ring.

The above structure-inhibition-binding studies should enable one to define the shape and charge distribution of polyaromatic dye ligands that bind to this enzyme.

Specific elution

Considerable attention has been paid to the selective binding of various groups of enzymes (dehydrogenases and kinases) to Cibacron Blue and other polyaromatic triazine dyes (Stellwagen, 1977; Dean & Watson, 1979), and structural similarity between these dyes and nucleotides has been claimed (Wilson, 1976). Recent work has attempted to clarify the molecular basis of this selectivity. Stellwagen and his group (Stellwagen, 1979) have questioned the original claims that allow one to predict the presence of nucleotide-binding sites in a protein on the basis of whether or not a protein binds to immobilized Cibacron Blue. Furthermore, Leatherbarrow & Dean (1979) have shown by inhibition studies and n.m.r. that the specific binding of human serum albumin to Cibacron Blue F3G-A is competitive with bilirubin, indicating that binding sites of molecules that are structurally dissimilar to nucleotides can be occupied by this type of dye.

Many workers have used biospecific elution to deduce the nature of the mechanism of adsorption of a protein to an affinity matrix. Data from the present and other studies (Dean & Watson, 1978) have indicated that the phenomena of adsorption

and desorption are not necessarily related and that biospecific elution (with nucleotides, for example) is not always as effective as ionic elution (KCl). Biospecific elution, however, was effective with the Cibacron Blue column in the present study, but its success was very dependent on the immobilized ligand concentration used.

Overall capacity

It has been shown that the operational capacities of two triazine dye columns are considerably higher than those of many immobilized nucleotides [e.g. AMP, NAD⁺ (Watson & Dean, 1977; Dean & Watson, 1978)].

Matrices

Group-specific affinity chromatography can be usefully combined with gel filtration (Lowe & Dean, 1971). We have extended this work in the study of triazine dyes, which are readily coupled to Sephadex gels. The resulting matrices are undeformed by this treatment, which overcomes the bead damage associated with CNBr activation (cf. Neame *et al.*, 1975). As observed by other workers (Baird *et al.*, 1976), triazine dye–Sephadex columns are often more effective (in terms of ligand utilization) than their agarose counterparts. These observations are confirmed in the present study: G-100 and G-200 Sephadex-based Procion Red HE-3B have the same effective capacity (26.2 units/mg of dye), whereas the agarose equivalent has only half the latter value (13.6 units/mg), despite the higher ligand concentration.

However, we find not only that the agarose-based dyes have superior flow properties but also that ligand leakage is less pronounced than with the Sephadex series.

Temperature studies

Temperature effects in affinity chromatography have been studied with immobilized nucleotide columns: mesophilic dehydrogenases and kinases (four examples are given by Harvey *et al.*, 1974) showed decreased binding in response to increased temperatures, whereas three thermophilic enzymes show the opposite effect (Comer *et al.*, 1975). The present study suggests that ionic as well as hydrophobic interactions play an important part in the chromatography of at least two enzymes on both immobilized triazine dyes studied, and this observation is in agreement with conclusions reached by Stellwagen (1979).

General conclusions

The successful use of both Cibacron Blue F3G-A and Procion Red HE-3B–agarose in protein puri-

fication is extended in the present work to a thermophilic protein. The binding of these dyes is highly structure-dependent, and dyes can therefore be selected that give greater or lesser binding.

When selecting dyes for affinity applications, 50% inhibition constants give useful indications of how individual dyes are likely to behave under column conditions. The free-solution binding data for Cibacron Blue F3G-A and Procion Red HE-3B confirm that the red dye binds 6-phosphogluconate dehydrogenase more tightly, and reinforce the conclusions drawn from previous observations (Watson *et al.*, 1978) that the latter dye has demonstrably higher capacities for NADP⁺-dependent dehydrogenases. It is also clear that several dyes are able to bind both classes of dehydrogenase as well as many other proteins.

The mechanism of binding of the triazine dyes has an important hydrophobic component and dye conformation need not necessarily mimic nucleotides alone.

A method for the rapid purification of 6-phosphogluconate dehydrogenase from *B. stearrowthermophilus* is described that gives homogeneous enzyme in high yield and specific activity. The method illustrated here has encouraged us to believe that the triazine dyes are excellent group-specific ligands and that screening studies for other applications will be fruitful.

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