An Examination of Potential Matrices for the Affinity Chromatography of NADP⁺-Linked Dehydrogenases with Special Reference to 6-Phosphogluconate Dehydrogenase

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1. 6-phosphogluconate dehydrogenase from sheep liver has been purified 350-fold by affinity chromatography with a final specific activity of 18 μmol of NADP⁺/reduced min per mg of protein and an overall yield of greater than 40%. 2. A systematic investigation of potential ligands has been carried out; these included 6-phosphogluconate and NADP⁺, pyridoxal phosphate and several immobilized nucleotides. The results indicate that NADP⁺ is the most suitable ligand for the purification of 6-phosphogluconate dehydrogenase. 3. The effects of pH and alternative eluents have been examined in relation to the parameters known to affect the desorption phase of affinity chromatography; careful manipulation of the elution conditions permitted the separation of glucose 6-phosphate dehydrogenase, glutathione reductase and 6-phosphogluconate dehydrogenase from sheep liver on NADP⁺-Sephacrose 4B. 4. A large-scale purification scheme for 6-phosphogluconate dehydrogenase is presented that uses the competitive inhibitors inorganic pyrophosphate and citrate as specific eluents.

Mammalian 6-phosphogluconate dehydrogenase (EC 1.1.1.44) has been extensively studied by Villet & Dalziel (1972*a,b*), Dyson & D'Orazio (1973), and Pearse & Rosemeyer (1974). These workers (Villet & Dalziel, 1969; Silverberg & Dalziel, 1973; Dyson *et al.*, 1973; Pearse & Rosemeyer, 1974) have also described purification schemes for the enzyme which use conventional procedures; however, these methods are invariably time-consuming and the yield is low. Kinetic data and inhibition studies for 6-phosphogluconate dehydrogenase indicate that this enzyme interacts with a wide range of nucleotides and thus might be purified on group-specific affinity matrices (Villet & Dalziel, 1972*a,b*; Dyson & D'Orazio, 1973; Dyson *et al.*, 1973).

Recent advances in the chemistry of specific immobilization of nucleotides have led to a spate of publications on the interaction of NADP⁺-dependent dehydrogenases with group-specific matrices (Dean & Harvey, 1975); conversely, however, few NADP⁺-linked dehydrogenases have been studied in detail on such materials (Lowe *et al.*, 1973*b*; Brodelius *et al.*, 1974; Godinot *et al.*, 1974; Lowe & Mosbach, 1974). Consideration of the enzyme's reaction immediately suggests two potential ligands, namely NADP(H) and 6-phosphogluconate; further, alternative ligands such as pyridoxal phosphate are suggested by the literature (Rippa & Pontremoli, 1969). We hoped that a study of relevant affinity matrices would lead first

to a rapid and simple method for purification of the enzyme and secondly to some insight into the interaction of the enzyme with these matrices.

A preliminary account of this work has already been published (Dean & Harvey, 1974). By using this procedure, Betts & Mayer (1975) have successfully purified 6-phosphogluconate dehydrogenase from rabbit mammary gland.

Experimental

Materials

5'-AMP and NADP⁺ were obtained from Boehringer Corp. (London) Ltd., (London, W.5, U.K.). 5-Amino-1-hexanol, 5,5'-carbonyl di-imidazole and *p*-nitrobenzoyl azide sulphamic acid were purchased from R. N. Emanuel Ltd. (Wembley, Middx., U.K.). HBr was supplied by Kodak (Liverpool, U.K.). Hydrazine hydrate, sodium metaperiodate, pyridoxal phosphate and adipic acid diethyl ester and all other chemicals were of the highest purity available from BDH Chemicals Ltd. (Poole, Dorset, U.K.).

Methods

Assay of 6-phosphogluconate dehydrogenase. The enzyme was assayed spectrophotometrically by using a Gilford 2000 spectrophotometer and follow-

ing the A_{340} in a 1 cm light-path. The reaction mixture of 0.75 ml consisted of 0.15 M-glycine buffer, pH 8.9, 5 mM-MgCl₂, 0.4 mM-NADP⁺ and 2 mM-6-phosphogluconate; the reaction was started by the addition of a portion (20 μ l) of the enzyme preparation. A unit of enzyme activity was defined as 1 μ mol of NADP⁺ reduced per min at 30°C, and the specific activity expressed as units/mg of protein. Protein was determined from the A_{280} assuming $A_{280}^{1\%} = 10$.

Purification of 6-phosphogluconate dehydrogenase. Each step was performed at 0–4°C, and all buffer solutions contained 1 mM-EDTA and 1 mM-2-mercaptoethanol; when used in conjunction with the affinity matrices the buffers were supplemented with 0.02% NaN₃. Steps 1 and 2 (see Table 1) of the purification procedure are equivalent to those of Silverberg & Dalziel (1973), except that the (NH₄)₂SO₄ fractionation was between 0.45 and 0.65 saturation. Details of subsequent steps are given below.

Affinity adsorbents. The matrix used in the present study was Sepharose 4B activated by the CNBr method of Axen *et al.* (1967); C₆ methylene spacer arms were used where appropriate. NADP⁺ was coupled to Sepharose by using three different procedures (Lamed *et al.*, 1973; Larsson & Mosbach, 1971; Hocking & Harris, 1973); the latter method was also used to immobilize pyridoxal phosphate and 5'-AMP. The bishydrazide procedure of Lamed *et al.* (1973) was used to couple ATP, pyridoxal phosphate and pyridine 3-aldehyde-adenine dinucleotide; in the preparation of the last two adsorbents the periodate-oxidation step was omitted. 2'-AMP was immobilized by using the procedure of Larsson & Mosbach (1971): N⁶-5'-AMP and N⁶-NAD⁺ were prepared and coupled by using previously described methods (Craven *et al.*, 1974a,b). The method outlined by Trayer *et al.* (1974) was used, with some modifications, to immobilize 5'-AMP at C-8, the reaction mixture, containing 8-bromo-AMP and hexane diamine (1:20, w/w), being heated overnight at 80°C. P¹-(6-Aminohexyl) P² - (5' - adenosyl) pyrophosphate -

Sepharose was prepared by the method of Harvey *et al.* (1974).

Preparation of P²-(6-aminohexyl) P²-(6-gluconate) pyrophosphate-Sepharose 4B. The methyltri-*n*-octylammonium salt of 6-phosphogluconate (1 mmol) was dissolved in -dimethylformamide (1.6 ml) and treated with a solution of 6-trifluoroacetylaminohexanol phosphate imidazolid (2 mmol) (Barker *et al.*, 1972) in dimethylformamide (2.8 ml). The reaction was carried out over 3 days in a stoppered flask at room temperature (22°C). After dilution with 50% (v/v) methanol (40 ml) the resultant mixture was applied to a column (2.5 cm $\times$ 25 cm) of Dowex 1-X8 (Cl⁻ form, 200–400 mesh). The column was washed with 50% (v/v) methanol (250 ml) and then a linear gradient of LiCl (0–0.4 M; total volume 1 litre) in 10 mM-HCl. Fractions (10 ml) were collected, and analysed for phosphate (Hanes & Isherwood, 1949) and unchanged 6-phosphogluconate. Fractions 41–140 were pooled, adjusted to pH 11 (with LiOH) and freeze-dried. Excess LiCl was removed by extraction (Barker *et al.*, 1972) and the product coupled to Sepharose.

The ligand concentration of the affinity adsorbents was maintained at 2.0 μ mol/g moist wt. of Sepharose 4B with two exceptions; first, those adsorbents prepared by the method of Lamed *et al.* (1973) had a ligand concentration of 3.5 μ mol/g, and secondly, a variable ligand concentration was used in the study of ribose-immobilized NADP⁺ adsorbents, and these values are recorded in the text.

Results and Discussion

In a preliminary investigation based on the kinetic data of Dyson & D'Orazio (1973) we chromatographed partially purified enzyme extracts (in 10 mM-KH₂PO₄/KOH, pH 7.5) on the following group-specific adsorbents: NADP⁺: ribose-linked, carbodi-imide-linked, and diazo-linked; NAD⁺: N⁶-linked and 3-aldehyde-linked; 2'-AMP: carbodi-imide-

Table 1. Purification of 6-phosphogluconate dehydrogenase from 1.63 kg of sheep liver

The initial extract was taken through to Step 2; thereafter the results are an average of three individual purifications (for details see the text). The protein concentration was determined from A_{280} and an assumed value for $A_{280}^{1\%}$ of 10.0.

Step	Volume (ml)	Protein (g)	Total activity (μ mol/min)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
1. Initial extract (supernatant)	3350	181.9	3750	0.0206	100	1.0
2. 0.45–0.65 satd. (NH ₄) ₂ SO ₄	265	37.6	3140	0.0832	83.4	4.06
3. NADP ⁺ -Sepharose, citrate	610	1.528	2380	1.55	63.5	75.3
4. 0.80 satd. (NH ₄) ₂ SO ₄	46	1.072	2100	1.96	56.1	95.4
5. NADP ⁺ -Sepharose, pyrophosphate	675	0.224	1650	7.33	44.0	356.0

linked; 5'-AMP: N⁶-linked, diazo-linked and C-8-linked; ADP: P₂-linked; and ATP: ribose-linked. Significant binding of the enzyme to these group-specific adsorbents was only observed in two instances: ribose-linked and carbodi-imide-linked

Fig. 1. Binding of 6-phosphogluconate dehydrogenase to heptanoyl-NADP⁺-Sephacrose as a function of pH

Partially purified extracts of sheep liver 6-phosphogluconate dehydrogenase were dialysed against equilibration buffers (a-e) and samples (1 ml) were chromatographed on 1 g columns (5 mm × 40 mm) of NADP⁺-Sephacrose (2 μmol/g moist wt.) (Larsson & Mosbach, 1971). The sample was washed into the column with a small volume of equilibration buffer before elution of the non-adsorbed protein with the same buffer (9.5 ml). The columns were developed with a linear gradient of KCl (0–1.0 M; 20 ml total volume) in the appropriate equilibration buffer. The flow rate was maintained between 8 and 10 ml/h, and fractions (1.5 ml) were collected and assayed for protein (....), KCl (---, by conductivity) and enzyme activity (—). The sample applied to columns (a-d) was purified by DEAE-cellulose chromatography (Silverberg & Dalziel, 1973), and had a specific activity of 1.0; after elution the specific activity values were (a) 1.05, (b) 1.56, (c) 1.98 (for the enzyme eluted in the gradient) and (d) 5.5. The equilibration buffer for columns (a-d) was 10 mM-KH₂PO₄, adjusted with KOH to pH values (a) 6.0, (b) 7.0, (c) 7.5 and (d) 8.0. The sample (Table 1, Step 2) applied to column (e) had a specific activity of 0.085 unit/mg which was increased to 1.13 after chromatography; the equilibration buffer was 10 mM-borate/HCl, pH 8.5.

NADP⁺. These adsorbents were examined in greater detail along with the substrate, column P¹-(6-amino-hexyl) P²-(6-gluconate) pyrophosphate-Sephacrose 4B and a variety of immobilized pyridoxal phosphate adsorbents.

Fig. 1 demonstrates that considerable selective binding of 6-phosphogluconate dehydrogenase could be achieved by using the undefined carbodi-imide-linked NADP⁺ (Larsson & Mosbach, 1971). These results were dependent on the pH of the irrigating buffer. A KCl gradient at pH 6.0 eluted a high proportion of the non-specific protein along with the enzyme; however, as the buffer pH was increased to 8.0 there was an increase in the apparent binding (β) (Craven *et al.*, 1974a) for 6-phosphogluconate dehydrogenase, together with a concomitant decrease in the retention of non-specific protein. The application of a dialysed crude extract (Step 2, Table 1) at pH 8.5 and subsequent elution by a KCl gradient resulted in a 13-fold purification of the enzyme (Fig. 1e).

Similar pH effects were observed in the interaction of the enzyme with P¹-(6-amino-hexyl) P²-(6-gluconate) pyrophosphate Sepharose. In this instance binding was favoured at pH 6.2, rather than 8.5. The interaction of the enzyme with this affinity adsorbent was only apparent with a partially purified extract (sp. activity = 1.0), the application of the crude extract at pH 6.2, resulting in little or no specific retention of the enzyme. These results conflict with the free-solution kinetic data, where a plot of *K_m* for 6-phosphogluconate against pH showed an inflection point at pH 8.3 (Dyson *et al.*, 1973) with the conclusion that a group with a p*K* of 8.3 is involved in binding substrate to enzyme; likewise, the possibility of ordered substrate addition has been demonstrated for this enzyme (Villet & Dalziel, 1972a). Bearing these results in mind the observed binding was probably non-specific; further, incorporating either 100 μM-NADP(H) or 1 mM-2'-AMP in the irrigating buffer failed to promote retention of the enzyme. This would suggest that the phosphate group of 6-phosphogluconate is essential for the substrate-enzyme interaction in the presence of co-factor.

The purification of 6-phosphogluconate dehydrogenase on pyridoxal phosphate-Sephacrose could be increased from four- to 13-fold by incorporating a DEAE-cellulose DE-52 column before the affinity-matrix step. Unfortunately, the diazo coupling of pyridoxal phosphate to Sepharose (Hocking & Harris, 1973) was very unstable, and examination of the column eluant showed that pyridoxal phosphate was eluted together with the enzyme. There was also a temperature-dependent ligand leakage associated with the stored polymer, resulting in a decreased capacity for enzyme. The same problem of ligand leakage was apparent when pyridoxal

phosphate was immobilized through the aldehyde group (Lamed *et al.*, 1973).

Studies with ribose-linked NADP⁺-Sephacrose

As a result of the above studies, all subsequent work was carried out with the defined NADP⁺-Sephacrose prepared as described by Lamed *et al.* (1973). Initial experiments with this polymer were designed to measure the apparent binding (β) of 6-phosphogluconate dehydrogenase with several eluents. Fig. 2 shows the elution profiles obtained by using KCl, NADP⁺ and NADPH gradients, the respective values for β being 510, 20 and 3mm. Each of these eluents has disadvantages when applied to a large-scale preparation. The use of nucleotide gradients on a large scale is prohibitively expensive, and KCl, although yielding 60–70-fold purification, resulted on occasions in extremely low recoveries. Alternative methods of elution were examined; development of

the column with a pH gradient (pH 6–11) yielded an enzyme fraction at pH 10.0 with a 45-fold purification, but again instability of the enzyme prevented any further application of this method.

Elution with competitive inhibitors

Pyrophosphate, a competitive inhibitor of both 6-phosphogluconate and NADP⁺ (Dyson & D'Orazio, 1973), was examined as a potential eluent and had an apparent binding (β) of 10mm at pH 9.0. The purification factor was dependent on the dialysis/equilibration buffer, i.e. phosphate > Tricine* Tris/acetate > triethanolamine at pH 7.5, and could be enhanced by the addition of low concentrations of pyrophosphate. This concentration of pyrophosphate was dependent on the immobilized ligand concentration, e.g. at a NADP⁺ concentration of 1.9 μ mol/g moist wt. of Sepharose, the pyrophosphate concentration of the dialysis/equilibration buffer was 1.0mm. When the pyrophosphate concentration was increased, a percentage of the enzymic activity was located in the void-volume fractions. Increased ligand concentrations allowed higher concentrations of pyrophosphate to be included in the irrigant buffers; this resulted in purer enzyme fractions on elution, since more non-specific protein passed unretarded through the affinity adsorbent. A similar control over ligand and eluant concentrations is required with respect to P_i, since this also inhibits the enzyme in a competitive manner (Dyson & D'Orazio, 1973).

The application of the enzyme preparation at pH 7.5 and subsequent chromatographic development by a pyrophosphate gradient at pH 9.0 necessitates a large pH change through the affinity matrix. Previous work (Lowe *et al.*, 1974a) has shown that pH itself can be used to elute enzymes from affinity adsorbents, and consequently attempts were made to introduce pH 'jumps' before the pyrophosphate-induced enzyme elution, and thereby achieve a further purification. Fig. 3 shows that a considerable amount of protein is eluted by 0.05M-glycine/KOH, pH 9.0, before development with a pyrophosphate gradient. Unfortunately, exposure to glycine, Bicine and borate at pH 8.5 and 9.0 resulted in the loss of enzyme activity. This is a complicated effect dependent on buffer molarity, the presence of salt or dithiothreitol, and time. Under the experimental conditions reported here, the enzyme activity remaining after 0.25, 2.5, 4.5 and 24h was 100, 53, 26 and 3% respectively. Nevertheless, in Fig. 3 it is noteworthy that of the enzymes examined, only those requiring NADP⁺ as a cofactor were retained by the affinity adsorbent. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was eluted by an NADP⁺ pulse (Lamed *et al.*, 1973) and gluta-

*Abbreviations: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Bicine, *NN*-bis-(2-hydroxyethyl)-glycine.

Fig. 2. Chromatography of 6-phosphogluconate dehydrogenase on ribose-immobilized NADP⁺-Sephacrose. Partially purified enzyme (Table 1, Step 2) was dialysed against equilibration buffer, and samples (2ml) were chromatographed on 1 g columns (5 mm × 40 mm) of NADP⁺-Sephacrose (3 μ mol/g moist wt.) (Lamed *et al.*, 1973). Elution was affected with linear gradients of (a) NADP⁺ (0–20mM; 10ml total volume), (b) NADPH (0–5mM; 10ml total volume) and (c) KCl (0–1.0M; 20ml total volume) prepared in equilibration buffer [10mM-KH₂PO₄/5mM-EDTA/0.02% Na₂S₂O₅/KOH (pH 7.5)]. Fractions [0.75 ml (a and b) and 1.5 ml (c)] were collected at a flow rate of 10ml/h and assayed for protein (·····), enzyme activity (—) and eluent concentration (----).

Fig. 3. Sequential elution of 6-phosphogluconate dehydrogenase, glutathione reductase and glucose 6-phosphate dehydrogenase from ribose-immobilized NADP⁺-Sephacrose

Partially purified enzyme (Table 1, Step 2) was dialysed against equilibration buffer and a sample (32 ml) was chromatographed on a column (2.2 cm × 10 cm) containing 50 g of NADP⁺-Sephacrose (1.9 μ mol/g moist wt.) (Lamed *et al.*, 1973), equilibrated in the same buffer. The column was developed with the following buffers: (a) equilibration buffer; 50 mM-KH₂PO₄/1 mM-MgCl₂/0.5 mM-Na₄P₂O₇/KOH (pH 7.5, 350 ml); (b) 50 mM-glycine/1 mM-MgCl₂/0.5 mM-Na₄P₂O₇/KOH (pH 9.0, 210 ml); (c) 50 mM-Na₄P₂O₇/1 mM-MgCl₂/HCl (pH 9.0, 210 ml); (d) 50 mM-Na₄P₂O₇/1 mM-MgCl₂/1 M-KCl (pH 9.0, 110 ml); (e) 50 mM-NADP⁺ (5 ml pulse) in buffer (d). Fractions (10 ml) were collected and assayed for phosphoglucose mutase (●), phosphoglucose isomerase (■), glutathione reductase (□) and glucose 6-phosphate dehydrogenase (Δ) by using the methods cited by Barman (1969); assays for 6-phosphogluconate dehydrogenase (○) and protein (····) are described under 'Methods'.

thione reductase (EC 1.6.4.2) by 1.0 M-KCl; at this stage the latter enzyme had specific activity of 4.5 units/mg of protein, and had been purified 280-fold. Phosphoglucose mutase (EC 2.7.5.1), phosphoglucose isomerase (EC 5.3.1.9) and hexokinase (EC 2.7.1.1) (not shown in the Figure) were located in the void volume. Thus the usual five enzyme contaminants of commercially available 6-phosphogluconate dehydrogenase can be separated cleanly from the desired enzyme by using this affinity adsorbent. A 52-fold purification of 6-phosphogluconate dehydrogenase was obtained by using these conditions; however, as mentioned earlier, this was limited by the lack of reproducibility in enzyme recovery. Similar stability problems were observed when the column was washed with 0.1 M-KCl to elute non-specific protein before development in a pyrophosphate gradient.

Knowing that the kinetic coefficients of sheep liver 6-phosphogluconate dehydrogenase are sensitive to pH values and the buffer used (Villet & Dalziel,

1972b), we examined the effect of pH on enzyme recovery by using two competitive inhibitors as eluents. With both pyrophosphate and citrate the percentage recovery decreased as the pH value was increased above 7.0. However, whereas the apparent binding constant (β) for pyrophosphate decreased from 44 to 19 mM with increasing pH values (6.0–8.5), the β value for citrate increased from 48 mM to more than 100 mM over the same pH range. These factors, although assisting in the derivation of a suitable purification scheme for this enzyme also reiterate our previous work, and give further emphasis to the need for maximum control over variables such as pH and ligand concentration in assessing the performance of an affinity adsorbent (Lowe *et al.*, 1974a; Harvey *et al.*, 1974). The capacity of the NADP⁺ column determined by frontal-analysis chromatography was only 0.2% of the total 'available' ligand concentration. This is consistent with previous observations (Lowe *et al.*, 1973a, 1974b).

Fig. 4. *Partial purification of 6-phosphogluconate dehydrogenase on NADP⁺-Sephacrose by using citrate elution*. The enzyme sample (100 ml; Table 1, Step 2) was diluted to 1200 ml with 10 mM-KH₂PO₄/0.5 mM-Na₄P₂O₇/KOH (pH 6.0) and chromatographed on a 200 g column of NADP⁺-Sephacrose (1.2 μmol/g moist wt.) (Lamed *et al.*, 1973); see the text for conditions. (a) 10 mM-KH₂PO₄/0.5 mM-Na₄P₂O₇/KOH, pH 6.0; (b) 50 mM-KH₂PO₄/0.5 mM-Na₄P₂O₇/KOH, pH 6.0; (c) 0.25 M-citrate; pH 6.0. Protein (—) and enzyme (▲) were assayed for in the eluate volume.

Fig. 5. *Chromatography of 6-phosphogluconate dehydrogenase on NADP⁺-Sephacrose by using a pyrophosphate gradient*. The (NH₄)₂SO₄ precipitate (Table 1, Step 4) was dialysed against 10 mM-KH₂PO₄/0.5 mM-Na₄P₂O₇/KOH, pH 7.0; a sample (46 ml, 150 mg of protein) was chromatographed under the conditions described in the text. (a) 10 mM-KH₂PO₄/0.5 mM-Na₄P₂O₇/KOH, pH 7.0; (b) 75 mM-KH₂PO₄/0.5 mM-Na₄P₂O₇/KOH, pH 7.0; (c) Na₄P₂O₇ gradient (0.5–100 mM) in 75 mM-KH₂PO₄/KOH, pH 7.0. Fractions (20 ml) were collected and assayed for 6-phosphogluconate dehydrogenase (▲) and protein (—).

Large-scale purification procedure

The purification scheme adopted as a result of the above studies is summarized in Table 1.

Steps 1 and 2. The initial extraction and $(\text{NH}_4)_2\text{SO}_4$ fractionation (pH 6.0) was performed as described by Silverberg & Dalziel (1973) (see under 'Methods'). The heat treatment (50°C) used by Dyson *et al.* (1973) could be incorporated at this stage with a twofold increase in specific activity; however, this had no apparent advantages during the subsequent affinity-chromatography steps.

Step 3. Affinity chromatography on NADP^+ -Sephacrose: citrate elution. The affinity adsorbent was equilibrated with 10 mM- KH_2PO_4 /0.5 mM- $\text{Na}_4\text{P}_2\text{O}_7$ /KOH, pH 6.0, at a flow rate of 1 litre/h. The enzyme containing $(\text{NH}_4)_2\text{SO}_4$ precipitate was diluted with the equilibration buffer to a conductivity value of 10 mmho. This solution was applied to an NADP^+ -Sephacrose 4B column (6.5 cm $\times$ 6.5 cm, 1.2 μmol NADP^+ /g moist wt. of Sephacrose) and allowed to pass in by gravity flow. The sample was washed into the column with a further 500 ml of 10 mM- KH_2PO_4 /0.5 mM- $\text{Na}_4\text{P}_2\text{O}_7$ /KOH, pH 6.0, and subsequently with 2 litres of 50 mM- KH_2PO_4 /0.5 mM- $\text{Na}_4\text{P}_2\text{O}_7$ /KOH, pH 6.0, to wash through non-adsorbed protein. Enzyme elution was effected with 0.25 M-citrate, pH 6.0, 20 ml fractions being collected. Total recoveries at this stage ranged from 56 to 77%. A typical elution profile is shown in Fig. 4.

Step 4. $(\text{NH}_4)_2\text{SO}_4$ precipitation, pH 6.0. To the pooled enzyme-containing fractions from the previous step $(\text{NH}_4)_2\text{SO}_4$ was added to 80% saturation. The suspension was left overnight, centrifuged, and the precipitate obtained dissolved in 10 mM- KH_2PO_4 /0.5 mM- $\text{Na}_4\text{P}_2\text{O}_7$ /KOH (pH 7.0). This solution was dialysed over a period of 6 h against three changes of the same buffer. After this step the total purification ranged from 87- to 103-fold.

Step 5. Affinity chromatography on NADP^+ -Sephacrose: pyrophosphate elution. The affinity matrix from Step 3 was washed with 500 ml of 6 M-urea and then equilibrated against 10 mM- KH_2PO_4 /0.5 mM- $\text{Na}_4\text{P}_2\text{O}_7$ /KOH, pH 7.0. The dialysed enzyme solution was applied to an NADP^+ -Sephacrose column (40 cm $\times$ 2.6 cm; 1.2 μmol of NADP^+ /g moist wt. of Sephacrose) and washed into the matrix with an equal volume of 10 mM- KH_2PO_4 /0.5 mM- $\text{Na}_4\text{P}_2\text{O}_7$ /KOH, pH 7.0, followed by approx. 2 litres of 75 mM- KH_2PO_4 /0.5 mM- $\text{Na}_4\text{P}_2\text{O}_7$ /KOH, pH 7.0. The column was then developed with a 2-litre linear gradient of pyrophosphate (0.5–100 mM; in 75 mM- KH_2PO_4 /KOH, pH 7.0). Enzymically active fractions (20 ml) were collected between 10- and 25 mM-pyrophosphate at a flow rate of 250 ml/h. An enzyme-elution profile from this column is shown in Fig. 5. Total recoveries after this step ranged from 34 to 50%. Peak specific activities ranged from 12.0 to 16.6 units/mg; approx. 70% of the applied enzyme was recovered, with a

specific activity in the order of 11 units/mg, and the total active fractions when combined had a specific activity of 7.3 units/mg (Table 1). Re-chromatography of the active fractions by the procedures outlined in Step 5 resulted in almost quantitative recovery of an enzyme having a specific activity of 18 units/mg. Samples of the enzyme preparation were analysed by 7.5% (w/v) polyacrylamide-gel electrophoresis, and gave a single band when stained for protein or enzyme activity. The procedure applied in Step 5 has also been successfully used in the purification of 6-phosphogluconate dehydrogenase from rabbit mammary gland (Betts & Mayer, 1975).

These results demonstrate the usefulness of a comparatively simply prepared affinity adsorbent, namely ribose-immobilized NADP^+ -Sephacrose 4B. It should be noted that ligand leakage from the adsorbent has been observed (9% in 14 days), but this does not preclude its use at cold-room temperatures (0–4°C). Provided that the adsorbent is washed with 6 M-urea between runs it may be used repeatedly and reproducibly with crude extracts. We have shown that several eluents may be used to effect the desorption of 6-phosphogluconate dehydrogenase from this adsorbent, and, as with selection of suitable ligands, this is best achieved by a systematic approach based on the free-solution kinetics of the enzyme under consideration.

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