Journal of Chromatography, 586 (1991) 51–59
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 582

High-performance affinity chromatography of $NADP^+$ dehydrogenases from cell-free extracts using a nucleotide analogue as general ligand

J. Alhama, J. López-Barea and F. Toribio*

Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Córdoba, Avda. Medina Azahara s/n., 14071 Córdoba (Spain)

(First received March 8th, 1991; revised manuscript received June 10th, 1991)

ABSTRACT

An epoxy-activated silica column (50 cm × 0.45 cm I.D.) was derivatized with 8-[(6-aminohexyl)amino]-2′-phosphoadenosine-5′-diphosphoribose; the bound ligand concentration was 11.4 μmol/g of dry silica, and the useful loading capacity was 2.3 mg of glutathione reductase. The new high-performance liquid chromatographic column specifically retained $NADP^+$-dependent enzymes, which were quantitatively eluted specifically by $NADP^+$ or, with better resolution, by potassium chloride. The new high-performance liquid chromatographic support was applied to the purification of glutathione reductase and glucose-6-phosphate dehydrogenase from cell-free extracts of baker's yeast, fish liver and rabbit hemolysates, with high recoveries and excellent purification factors.

INTRODUCTION

High-performance liquid affinity chromatography (HPLAC) combines the biospecificity of conventional affinity chromatography with the speed of operation, resolution and sensitive detection of high-performance liquid chromatography (HPLC) [1]. Adenine nucleotide derivatives have been used for many years as general ligands for conventional affinity chromatography [2,3], covalently attached to insoluble supports through either the N-6 [4] or the C-8 [5] adenine ring position, the hydroxyl groups of the ribose ring [6] or the phosphate groups [7]. Their ability to retain a certain enzyme depends on whether the geometrical orientations of the modified nucleotide properly fit the enzyme-binding site [8,9]. The type of immobilized adenine nucleotide also selects the kind of enzymes retained: ATP, ADP and AMP bind mainly kinases [10]; NAD^+, $NADP^+$, 2′-phosphoadenosine, 5′-diphosphoribose (ATPR) and 2′,5′-ADP retain $NAD(P)^+$-dependent dehydrogenases [11,12]; and 3′,5′-ADP is specific for coenzyme A-related enzymes [13].

Epoxy-silica has been successfully used in the field of HPLAC [14–16] as a carrier for biological ligands, since its mechanical rigidity and porosity allows high pressures and flow-rates, resulting in a fast technique for purifying biologically active molecules. Epoxy groups on the support react in the pH range 3–7.5 with nucleophiles such as primary amine, sulfhydryl or hydroxyl. The presence of such groups in a wide range of biomolecules allows their binding to the epoxy-activated silica. Other alternative chemical modifications of silica-based supports, with reactive groups such as tresyl [17], isothiocyanate [18], or primary hydroxyl [19], have also been used to link covalently several ligands containing primary or secondary amino groups.

Alcohol dehydrogenase [17] and lactate dehydrogenase [20,21] have been purified using AMP and NAD^+ bound to silica-based supports. Dyes structurally related to pyridine nucleotides have also been used for purification by HPLAC of several enzymes [2,22–25]. In this paper, we report the development of a new support with an $NADP^+$ analogue, 8-[(6-aminohexyl)amino]-2′-phosphoadeno-

0021-9673/91/$03.50

sine-5′-diphosphoribose (AHC8-ATPR), attached through the C-8 position of the adenine ring to epoxy-activated silica, as a specific group ligand for purification by HPLAC of several $NADP^+$-dependent dehydrogenases. The new support shows very high specificity, excellent yields of the retained enzymes and short operation times, and has been used with several cell-free extracts, from which glutathione reductase and glucose-6-phosphate dehydrogenase have been highly purified.

EXPERIMENTAL

Chemicals

Bromine, Dowex 1-X8 resin, bovine serum albumin, ovalbumin, baker's yeast alcohol: NAD^+ dehydrogenase (EC 1.1.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glutathione reductase (EC 1.6.4.2) and hexokinase (EC 2.7.1.1), *Thermoanarobium brockii* alcohol: $NADP^+$ dehydrogenase (EC 1.1.1.2), rabbit muscle lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.1.40), pig heart isocitrate dehydrogenase (EC 1.1.1.42) and citrate synthase (EC 4.1.3.7), and $NAD(P)^+$, NAD(P)H and 3-[N-morpholine]propane sulfonic acid (MOPS) were purchased from Sigma. Bacto peptone was from Difco. Salts for the mobile phases were from Merck. All other chemicals used were of the highest purity.

Equipment

HPLC was performed using a Beckman apparatus equipped with two 110B pumps, an Altex 210A injection valve, a 163 UV detector and a 406 analog interface module. Integration was performed by an AT computer with the System Gold software from Beckman. Affinity chromatography was carried out with Beckman Ultraffinity-EP (5.0 cm × 0.45 cm I.D.) columns, packed with epoxy-activated silica. An LKB 2112 Redirac collector was used to fractionate the column eluate for monitoring enzymatic activities. A Spherisorb amino-propyl silica column (5 μm, 25 cm × 0.4 cm I.D.) from Tracer Analitica was used for chromatographic control throughout the synthesis of the nucleotide analogue. Cell-free extracts were filtered through 0.45-μm filters before application to the HPLC affinity columns.

Centrifugations were carried out on J2-21 and L8-80M Beckman centrifuges. High-purity water was obtained from a Milli-Q water purifier (Millipore). The eluting solvents were degassed under vacuum for 30 min in an ultrasonic bath and filtered through 0.2-μm membrane filters. Spectrophotometric measurements and enzyme kinetics were carried out in a Beckman DU-7 spectrophotometer. A Braun Labsonic apparatus was used for sonic disintegration of yeast cells, and an IKA Ultraturrax T-25 homogenizer for preparation of fish-liver extracts.

Enzyme assays

The enzymatic activities were assayed as previously published at the temperatures indicated: alcohol: NAD^+ dehydrogenase [26] 25°C, alcohol: $NADP^+$ dehydrogenase [27] 40°C, lactate dehydrogenase [28] 37°C, isocitrate dehydrogenase [29] 37°C, glucose-6-phosphate dehydrogenase [30] 25°C, glutathione reductase [12] 30°C, hexokinase [31] 25°C, pyruvate kinase [32] 37°C and citrate synthase [33] 37°C. One unit of activity is defined as the amount of enzyme catalyzing the transformation of 1 μmol of substrate into product per minute.

Cell-free extract preparations

Baker's yeast. Cells grown with 1% bacto peptone and 2% glucose as carbon source were harvested by centrifugation at 15 000 *g* for 10 min, washed with 250 m*M* MOPS buffer, pH 7.5, containing 1 m*M* EDTA and centrifuged at 48 400 *g* for 10 min. The pellet was resuspended with four volumes of buffer and sonicated four times for 15 s at 1-min intervals. Cellular debris was removed by centrifugation at 48 400 *g* for 10 min, and the supernatant was dialyzed overnight against extraction buffer. The cell-free extract contained 0.62 U/ml glutathione reductase (0.09 U/mg specific activity) and 0.42 U/ml glucose-6-phosphate dehydrogenase (0.06 U/mg specific activity).

Rabbit hemolysate. Cells from freshly drawn rabbit arterial blood were collected by centrifugation and lysed by osmotic shock with 5 m*M* MOPS buffer, pH 7.0, containing 1 m*M* EDTA (2 ml per ml of packed cells). Cellular debris was removed by centrifugation at 48 400 *g* for 10 min and the supernatant was again centrifuged at 184 000 *g* for 1.5 h; the hemolysate was dialyzed overnight against a suitable MOPS buffer (25–250 m*M*, pH 7.0–7.5). The extract contained 0.31 U/ml glutathione reduc-

tase (0.003 U/mg) and 0.61 U/ml glucose-6-phosphate dehydrogenase (0.005 U/mg).

Fish liver. Frozen *Mugil cephalus* liver was minced and ground in liquid nitrogen. The powder was extracted with a similar volume of 5 m*M* Tris–HCl buffer, pH 7.4, containing 250 m*M* sucrose and 10 m*M* reduced glutathione (GSH). The slurry was diluted three-fold with 1.15% (w/v) potassium chloride solution and centrifuged at 9000 *g* for 10 min. The supernatant was ultracentrifuged at 105 000 *g* for 1 h, and dialyzed overnight against an appropriate MOPS buffer (25–250 m*M*, pH 7.0–7.5). The glutathione reductase activity in this extract was 0.28 U/ml (0.052 U/mg) and that of glucose-6-phosphate dehydrogenase 0.96 U/ml (0.158 U/mg).

Protein determination. Protein concentrations were estimated by the bicinchoninic acid protein assay [34] using bovine serum albumin as standard.

Synthesis of the ligand

The synthesis of 8-[(6-aminohexyl)amino]-2′-phosphoadenosine-5′-diphosphoribose (AHC8-ATPR, Fig. 1) was carried out as a modification of the procedure described by López-Barea and Lee [12]: NADP$^+$ (4 g, 3.945 mmol), was dissolved in 15 ml of 1 *M* sodium acetate buffer, pH 4.5. Liquid bromine (2.5 ml, 48.56 mmol) was then added dropwise to the solution with vigorous stirring; during this process the pH was maintained between 3.9 and 4.5 by adding 1 *M* sodium hydroxide. After 70 min at room temperature the unreacted bromine was extracted seven times, with 30 ml of carbon tetrachloride each time. To the aqueous phase which contained 8-bromo-NADP$^+$ (C8-BrNADP$^+$), 350 ml of cold acetone (−80°C) were added and stored overnight at −80°C. The yellow precipitate was washed with 60 ml of cold acetone and redissolved in 20 ml of water. Then, 1,6-diaminohexane (12.0 g, 103 mmol) in 5.0 ml of water was added to the C8-BrNADP$^+$ solution and heated at 60°C for 3 h. The coupling of the spacer arm was monitored spectrophotometrically by following the shift in absorbance maximum from 267 nm (C8-BrNADP$^+$, ε = 20.7 mM^{-1} cm^{-1}) to 279 nm (AHC8-ATPR, ε = 18.0 mM^{-1} cm^{-1}): the A_{279}/A_{267} ratio changed from 0.71 to 1.11 within 4.5 h of coupling. The reaction mixture was diluted to 2 l with HPLC-grade water and loaded at a flow-rate of 100 ml/h into a Dowex 1-X8 column (180-ml bed), previously equilibrated with 1 *M* ammonium carbonate and extensively washed with water. The elution was performed by a linear ammonium carbonate gradient (0–1.5 *M*). The fractions (15 ml) with an absorbance maximum at 279 nm were pooled and lyophilized. Ion-exchange HPLC was performed to follow the synthesis of the ligand (Fig. 1).

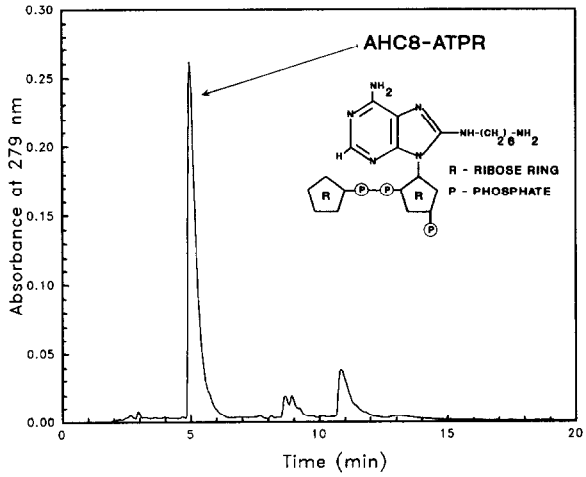

Fig. 1. Monitoring of AHC8-ATPR synthesis. Column, Spherisorb APS (25 × 0.4 cm I.D.); back-pressure, 135 bar; flow-rate, 1.0 ml/min; temperature, ambient; irrigant buffer, 0.04 *M* phosphate, pH 2.8; applied sample, 20 µl of a 0.75 mg/ml solution of AHC8-ATPR lyophilized after Dowex 1-X8 chromatography dissolved in irrigant buffer. Elution was effected with a linear gradient of potassium chloride (0–0.8 *M* in irrigant buffer, pH 2.7) lasting 15 min after sample injection.

Coupling of the ligand to the epoxy silica column

Optimization of the coupling reaction was performed in a batchwise mode using the Beckman Ultraffinity-EP column capacity kit, which contains 100 mg of the same support as the prepacked column. For coupling to the column, the purified AHC8-ATPR (0.813 g) was dissolved in 10 ml of 0.85 *M* potassium phosphate buffer, pH 8.4, containing 0.3 *M* sodium hydrogencarbonate. The epoxy-silica prepacked column was attached to an HPLC pump primed with the same loading buffer; column flow was adjusted to 1 ml/min until liquid eluted from column end, then it was shifted to 0.2 ml/min and recycled overnight at 45°C. To remove ionically bound ligand, the column was washed with 1 *M* potassium chloride for 30 min at 1 ml/min. Hydrophobically bound ligand was removed

by washing the column with 20 m*M* potassium chloride under identical conditions. The unreacted epoxy groups were converted into the corresponding diol structures by a mild acid hydrolysis with 10 m*M* hydrochloric acid, pH 2.2, pumped at 0.5 ml/min for 2 h at 50°C. After washing with equilibration buffer the column was ready for use.

Ligand density was determined as follows: ligand-substituted epoxy silica (100 mg) was heated at 60°C for 30 min in 1 ml of 1 *M* sodium hydroxide. To the solubilized support 4 ml of 100 m*M* sodium phosphate buffer, pH 7.0, 1 ml of 1 *M* hydrochloric acid and distilled water were added to 10 ml final volume. A reference solution was prepared similarly with underivatized epoxy-silica. Ligand concentration was calculated from the molar absorptivity of the AHC8-ATPR at 279 nm.

The breakthrough capacity of the affinity column was determined as follows: a solution of glutathione reductase (0.182 mg/ml) in 100 m*M* MOPS buffer, pH 7.0, containing 1 m*M* EDTA was passed through the column at a flow-rate of 1 ml/min until the detector indicated an increasing baseline at 280 nm. At that point the enzyme was just beginning to break through, and if the loading process continued the curve would reach a maximum; the amount of enzyme retained up to this point represented the maximum dynamic capacity [35].

Chromatographic procedures

Ion-exchange high-performance liquid chromatography. The Spherisorb column was equilibrated with 40 m*M* potassium phosphate, pH 2.8 (buffer A), at a flow-rate of 1 ml/min. After sample injection (20 μl), elution was carried out with a linear gradient of buffer B (buffer A + 0.8 *M* potassium chloride, pH 2.7) lasting 15 min. Detection was performed in each case at the maximum absorption wavelength.

Affinity chromatography. Unless otherwise stated the Ultraffinity column derivatized with AHC8-ATPR was equilibrated with loading buffer C (250 m*M* MOPS buffer, pH 7.5, containing 1 m*M* EDTA) at 1 ml/min. Samples of 500 μl containing a mixture of nine commercial enzymes shown in Table III (0.1–0.2 mg of each enzyme per ml of buffer C) were injected into the column. The column was extensively washed with buffer C until the A_{280} returned to zero. The retained enzymes were eluted either non-specifically by a linear potassium chloride gradient (0–1.25 *M* in buffer C) lasting 10 min, or specifically with a linear $NADP^+$ gradient (0–1.5 m*M* in buffer C) lasting 15 min. Fractions of 400 μl were collected and assayed for enzymatic acitivities. All chromatographic procedures were performed at room temperature.

RESULTS AND DISCUSSION

The nucleotide analogue AHC8-ATPR bound to Sepharose 4B has been used previously to purify mouse liver glutathione reductase [12]: the enzyme was purified 35-fold in 36 h with 86% overall yield. The use of agarose for preparative affinity chromatography is limited by its low flow-rates, diminished peak resolution and bacterial degradation, resulting in ligand leakage. The HPLC methodology would be essentially free of such problems and highly valuable for unstable proteins requiring rapid isolation procedures. For this reason we decided to use the same ligand to purify by HPLAC several $NADP^+$-dependent enzymes.

The different steps throughout the synthesis of AHC8-ATPR were controlled by ion-exchange HPLC. Fig. 1 shows the chromatogram obtained with a sample of the Dowex 1-X8 column eluate: the main peak, corresponding to AHC8-ATPR (retention time 5.0 min), represented at least 82% of the total peaks area.

The ligand concentration on the support was 11.4 μmol/g of dry silica, corresponding to 16% of the initial epoxy groups of the underivatized silica, a value higher than the usual 10% previously described for epoxy-activated silica [20]. A standard measurement of the useful loading capacity of the columns is the 1% breakthrough capacity [35]: using glutathione reductase as the reference $NADP^+$ dehydrogenase, this was determined to be 2.3 mg of enzyme for a 5.0 cm x 0,45 cm I.D. analytical column.

The suitability of AHC8-ATPR for affinity chromatography was studied by measuring the inhibition constants [36] of the soluble uncoupled ligand against different enzymes. Table I summarizes the results obtained: with the exception of isocitrate dehydrogenase, the $NADP^+$-dependent enzymes showed low inhibition constant (K_i) values for AHC8-ATPR. The other enzymes tested, NAD^+-,

TABLE I

INHIBITION CONSTANTS OF DIFFERENT ENZYMES TOWARD AHC8-ATPR IN SOLUTION

Enzyme	Specificity	Inhibition constant[a] (K_i in μM)
Glucose-6-phosphate dehydrogenase	$NADP^+$	7
Glutathione reductase	$NADP^+$	58
Alcohol dehydrogenase	$NADP^+$	83
Isocitrate dehydrogenase	$NADP^+$	–[b]
Alcohol dehydrogenase	NAD^+	–[b]
Lactate dehydrogenase	NAD^+	639
Citrate synthase	Coenzyme A	409
Pyruvate kinase	ATP	2614
Hexokinase	ATP	7615

[a] Determined by Dixon plot [36] using at least two substrate and six AHC8-ATPR concentrations.
[b] No inhibition observed under our experimental conditions.

ATP- and coenzyme A-dependent enzymes, displayed much higher K_i values or were not inhibited. Thus, as expected, AHC8-ATPR could be selectively used for affinity chromatography of $NADP^+$-dependent dehydrogenases.

The effect of different factors, namely ionic strength, type of buffer and pH, on the binding of different classes of enzymes was studied in a batchwise mode using the Ultraffinity-EP column capacity kits. The kit was derivatized under the conditions stated above, although conveniently scaled down. The results obtained with four commercial enzymes are shown in Table II. MOPS buffer was the most effective for specific binding of $NADP^+$-dependent dehydrogenases: ionic strengths higher than 100 mM quantitatively retained glutathione reductase and glucose-6-phosphate dehydrogenase, which were eluted by potassium chloride with recoveries close to 100%; the other two enzymes were not retained under identical conditions. The bind-

TABLE II

EFFECT OF TYPE OF BUFFER, IONIC STRENGTH AND pH ON THE BINDING OF DIFFERENT ENZYMES TO THE AFFINITY SUPPORT

	Phosphate buffer, pH 7.0				MOPS buffer, pH 7.0				100 mM MOPS buffer		
	15[a]	30	60	150	20[a]	50	100	150	6.5[b]	7.0	7.5
Glutathione reductase											
Loaded[c]	8.9	9.2	8.9	8.9	11.9	10.6	10.5	11.5	11.0	10.5	10.0
Washed[d]	0.4	0.7	1.5	4.1	0.8	0.2	0.7	2.0	2.5	0.7	2.1
Eluted[e]	7.9	8.2	6.9	4.0	10.9	8.9	8.6	9.4	7.9	8.6	6.6
Glucose-6-phosphate dehydrogenase											
Loaded[c]	8.7	11.9	8.8	8.9	9.9	9.9	9.9	11.1	11.3	9.9	11.3
Washed[d]	1.0	3.2	5.3	6.3	0.8	0.0	0.2	0.1	0.7	0.2	0.1
Eluted[e]	5.9	5.9	2.6	0.4	8.3	9.1	9.9	11.6	12.0	10.0	10.6
Lactate dehydrogenase											
Loaded[c]	10.1	9.3	9.1	9.3	12.2	9.2	11.9	10.9	11.8	11.9	11.5
Washed[d]	0.5	3.6	7.5	7.2	0.2	1.9	9.3	8.5	0.4	9.3	8.3
Eluted[e]	3.6	2.1	0.2	0.0	7.9	3.2	0.1	0.1	6.1	0.0	0.0
Pyruvate kinase											
Loaded[c]	3.7	3.5	4.5	4.2	8.5	13.8	6.0	8.9	8.5	6.0	10.2
Washed[d]	1.4	1.4	2.6	3.7	0.5	4.3	5.1	5.5	0.1	5.1	9.8
Eluted[e]	0.9	1.5	0.6	0.2	8.6	10.8	1.1	0.4	6.9	1.2	0.7

[a] Ionic strength of loading buffer expressed in mM.
[b] pH value of loading buffer.
[c] Total units present in 1.0 ml of loading buffer.
[d] Total units washed with 6.0 ml of each loading buffer.
[e] Total units eluted with 6.0 ml of 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 0.5 M potassium chloride.

ing of the model enzymes was less effective and specific using phosphate buffer: while at low ionic strength glutathione reductase and glucose-6-phosphate dehydrogenase were mostly bound to the support, an increase in ionic strength significantly lowered their binding, especially that of the second enzyme. With both buffers, at low ionic strengths, the support indiscriminately retained all four enzymes tested, probably because of unspecific ionic interactions with the immobilized ligand, avoided at higher ionic strengths [20]. Even at low ionic strengths, the recoveries obtained using MOPS buffer were much higher than with phosphate buffer. The poorer results with phosphate buffer could be due to competition of free phosphate ions and the 2′-phosphate of C8-ATPR for the phosphate-binding site present in $NADP^+$-dependent dehydrogenases [37]. The effect of different pH values on the binding of the model enzymes was studied with 100 m*M* MOPS buffer. At pH 7.0 and 7.5 the support almost completely retained both $NADP^+$-dependent enzymes, while mostly excluding the other two enzymes. Consequently, MOPS buffer of at least 100 m*M* and pH 7.0–7.5 was selected for the standard operation with the new affinity support.

The chromatographic behavior of the new affinity column was studied using a mixture of nine different commercially available enzymes. Fig. 2A and B shows the chromatograms obtained with two mixtures of enzymes using non-specific potassium chloride elution and specific elution by $NADP^+$, respectively, while Table III shows quantitative data about the total units loaded, washed and eluted. Glutathione reductase and glucose-6-phosphate dehydrogenase were fully retained and eluted out of the column with recoveries close to 100%. Of the $NADP^+$-dependent alcohol dehydrogenase loaded, 60–75% was also retained and recovered from the column, as well as a small amount of pyruvate kinase, in agreement with the previous results shown in Table II. All other enzymes tested were completely unretained by the column under such conditions. The $NADP^+$-dependent isocitrate dehydrogenase was not retained at all by the affinity column, in agreement with the lack of inhibition shown by AHC8-ATPR in Table I, and its lack of binding to a $NADP^+$-type support in conventional affinity chromatography [11,13]. As shown in Fig. 2A, elution with potassium chloride yielded a better resolution

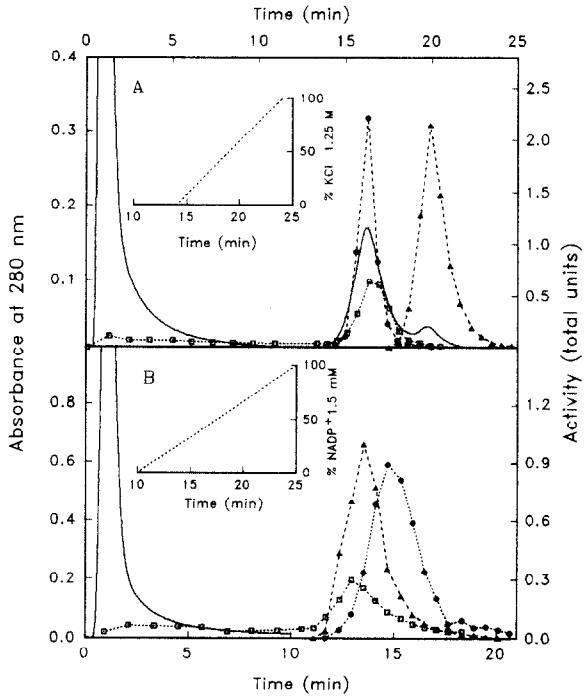

Fig. 2. HPLAC of mixtures of commercially available enzymes. Conditions were as described in the *Chromatographic procedures* section and sample compositions are shown in Table III. Temperature, ambient; back-pressure, 28 bar; flow-rate, 1.0 ml/min. (A) Unspecific elution by potassium chloride gradient: ——— = absorbance at 280 nm; ● = glutathione reductase activity; ▲ = glucose-6-phosphate dehydrogenase activity; □ = $NADP^+$-dependent alcohol dehydrogenase (U × 4). (B) Specific elution by $NADP^+$ gradient: —— = absorbance at 280 nm; ● = glutathione reductase activity; ▲ = glucose-6-phosphate dehydrogenase activity (U × 0.5); □ = $NADP^+$-dependent alcohol dehydrogenase (U × 2).

of glutathione reductase and glucose-6-phosphate dehydrogenase, while with $NADP^+$ (Fig. 2B) glucose-6-phosphate dehydrogenase was eluted before glutathione reductase, in agreement with their K_i values for AHC8-ATPR (Table I). In addition, $NADP^+$ elution did not improve the peak shapes, thus leading to significant overlapping.

The real test of excellent performance of an HPLAC support should be the efficient purification of enzymes from cell-free extracts. Thus after the results obtained with model mixtures of enzymes, we initiated the purification of glutathione reductase and glucose-6-phosphate dehydrogenase from three different cell-free extracts. The following

TABLE III

CHROMATOGRAPHIC BEHAVIOR OF THE AFFINITY COLUMN TOWARDS A MIXTURE OF MODEL ENZYMES

Enzyme	Unspecific elution			Specific elution		
	Loaded[a]	Washed[b]	Eluted[c]	Loaded[a]	Washed[b]	Eluted[c]
Hexokinase	2.6	2.3	0.0	4.5	4.0	0.0
Pyruvate kinase	3.4	2.8	0.1	3.4	2.7	0.0
Citrate synthase	3.5	3.5	0.0	3.3	3.5	0.0
Lactate dehydrogenase	7.5	7.8	0.0	6.4	6.7	0.0
Isocitrate hydrogenase	3.5	3.5	0.0	2.2	2.2	0.0
Alcohol: NAD$^+$ dehydrogenase	6.3	6.7	0.0	5.3	5.7	0.0
Alcohol: NADP$^+$ dehydrogenase	1.0	0.3	0.6	1.2	0.3	0.9
Glutathione reductase	5.0	0.0	4.7	4.3	0.0	4.4
Glucose-6-phosphate dehydrogenase	7.3	0.0	7.3	6.4	0.0	7.6

[a] Total units of each enzyme contained in 500 μl of a mixture injected into the column.
[b] Non-retained enzymatic activities expressed as total units.
[c] Activity of the enzymes retained by the column, expressed as total units.

chromatographic conditions were used: 2.0 ml of each cell-free extract were loaded into the column at a flow-rate of 1 ml/min; an extensive washing with the loading buffer was carried out until the A_{280} returned to a stable baseline; elution of the bound enzymes was carried out with a linear potassium chloride gradient (0–1.25 *M*) in loading buffer lasting 10 min. Fractions (0.4 ml) were collected and analyzed for enzymatic activities and protein concentrations.

Table IV summarizes the results obtained with several extracts under different conditions. In yeast cell-free extract, both enzymes were retained and recovered from the column with yields near to 100% and high purification factors. With fish liver extracts, glutathione reductase was not bound in 250 m*M* MOPS buffer, although glucose-6-phosphate dehydrogenase was partially retained and recovered with a 57-fold purification factor, probably because of the lower amount of proteins retained under such conditions. As previously shown in Table II, a lowered ionic strength improved the binding and recovery of both enzymes, although the purification factors decreased in parallel as more proteins were retained. With the rabbit hemolysate, glutathione reductase was not retained at 250 m*M* MOPS buffer, but it did bind at 25 m*M* loading buffer, with a 77% recovery and a 142-fold purification factor. On the other hand, glucose-6-phosphate dehydrogenase was retained under both conditions with similar recoveries and very high purification factors, 502- and 440-fold, respectively.

Fig. 3 shows the chromatogram obtained with the yeast cell-free extract: an excellent resolution was observed between both enzymes with the potassium chloride gradient, in close analogy with the reconstruction experiment of Fig. 2A. These results were obtained with an analytical column at a flow-rate of only 1 ml/min; nevertheless even under such limited conditions the chromatographic procedure was completed in less than 45 min, with quite good purification factors (Table IV).

Modified nucleotides have been employed to purify NAD$^+$-dependent dehydrogenases using HPLAC [17,21]. Nilsson and Mosbach [17] reported an 80% recovery after chromatography of pure lactate and alcohol dehydrogenases using N6-(6-aminohexyl)-NAD$^+$ coupled to tresyl-activated porous silica. Wikström and Larsson [21] purified lactate dehydrogenase from ox heart cell-free extract using an NAD$^+$ analogue coupled to dextran-coated porous quartz fibres with a 90% recovery and a 35-fold purification factor. As far as we know, our work reports for the first time the purification of NADP$^+$-dependent dehydrogenases by HPLAC, using an NADP$^+$ analogue as specific group ligand, coupled through the C-8 position of the adenine ring to as epoxy-activated porous silica matrix.

TABLE IV

PURIFICATION OF GLUTATHIONE REDUCTASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM DIFFERENT CELL-FREE EXTRACTS[a]

Cell-free extracts (loading buffer)	Glutathione reductase		Glucose-6-phosphate dehydrogenase	
	Recovery (%)	Purification (fold)	Recovery (%)	Purification (fold)
Baker's yeast				
MOPS 250 m*M*, pH 7.5	90	104	118	35
Fish liver				
MOPS 250 m*M*, pH 7.5	Unbound	Unbound	59	57
MOPS 50 m*M*, pH 7.5	62	28	63	22
MOPS 25 m*M*, pH 7.0	70	19	69	19
Rabbit hemolysate				
MOPS 250 m*M*, pH 7.5	Unbound	Unbound	66	502
MOPS 25 m*M*, pH 7.0	77	142	67	440

[a] A sample of 2.0 ml of each cell-free extract was loaded into the column. The recovery is expressed as a percentage of the initial activity loaded. The purification is expressed as the ratio of specific activity of the peak fraction to specific activity of the cell-free extract.

The results summarized in Table IV and Fig. 3 show that AHC8-ATPR is an excellent specific group ligand for purification of $NADP^+$-related enzymes by HPLAC in crude extracts from different sources. Three arguments validate such an statement:

(1) Experiments with model enzymes and those carried out with cell-free extracts show that the new affinity support is highly specific for $NADP^+$-dehydrogenases.

(2) The recoveries of the retained enzymatic activities, at least 70%, are similar to those previously obtained in the purification of NAD^+-dependent dehydrogenases by HPLAC [17,21–24].

(3) The specificity and resolving power of our support yield higher purification factors (19- to 502-fold) than those previously reported for NAD^+-dependent enzymes [17,23–25].

Our results also significantly improve those obtained using the same ligand, AHC8-ATPR, in conventional affinity chromatography, with a 50-fold decrease in chromatographic run time.

In conclusion, we propose the new support, with AHC8-ATPR covalently attached to epoxy-activated porous silica, as a very convenient affinity matrix for separation of $NADP^+$-dependent enzymes. The chromatographic procedure described in this work can also be easily scaled up for laboratory semipreparative purposes, by using a commercially available prepacked column instead of an analytical one.

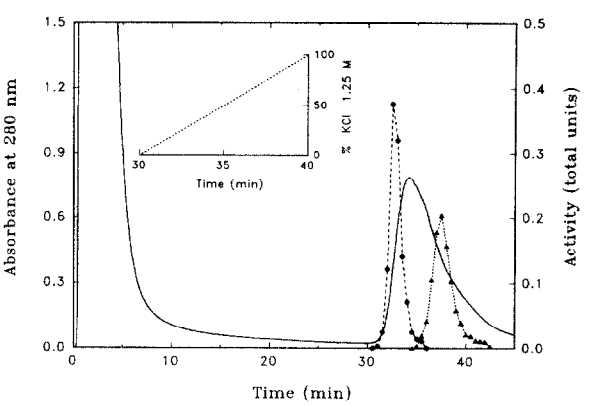

Fig. 3. HPLAC of a cell-free extract of baker's yeast. A 2-ml sample of baker's yeast cel-free extract was injected into the column at a flow-rate of 1 ml/min. Starting solvent: 250 m*M* MOPS buffer, pH 7.5, 1 m*M* EDTA. Elution: linear potassium chloride gradient (0–1.25 *M*) in starting solvent. —— = Absorbance at 280 nm; ● = glutathione reductase activity; ▲ = glucose-6-phosphate dehydrogenase activity.

REFERENCES

1 S. Ohlson, L. Hansson, P. O. Larsson and K. Mosbach, *FEBS Lett.*, 93 (1978) 5.
2 C. R. Lowe, *An Introduction to Affinity Chromatography*, Elsevier Biomedical, Amsterdam, 1985, p. 430.
3 P. P. G. Dean, W. S. Johnson and F. A. Middle, *Affinity Chromatography: A Practical Approach*, IRL Press, Oxford, 1985, p. 136.
4 S. Barry and P. O'Carra, *FEBS Lett.*, 37 (1973) 134.
5 C. Y. Lee, D. A. Lappi, B. Wermuth, J. Everse and N. O. Kaplan, *Arch. Biochem. Biophys.*, 163 (1974) 561.
6 R. Lamed, Y. Levin and M. Wilcheck, *Biochim. Biophys. Acta*, 304 (1973) 231.
7 M. J. Harvey, C. R. Lowe, D. B. Graven and D. G. Dean, *Eur. J. Biochem.*, 41 (1974) 335.
8 I. M. Chaiken, *J. Chromatogr.*, 376 (1986) 11.
9 P. Mohr and K. Pommerening, *Affinity Chromatography: Practical and Theoretical Aspects (Chromatographic Science Series*, Vol. 33), Marcel Dekker, New York, 1986 p. 67.
10 C. Y. Lee, L. H. Lazarus, D. S. Kabakoff, P. J. Russel, M. Laver and N. O. Kaplan, *Arch. Biochem. Biophys.*, 178 (1977) 8.
11 C. Y. Lee and N. O. Kaplan, *Arch. Biochem. Biophys.*, 168 (1975) 665.
12 J. Lopez-Barea and C. Y. Lee, *Eur. J. Biochem.*, 98 (1979) 487.
13 P. Brodelius, P. O. Larsson and K. Mosbach, *Eur. J. Biochem.*, 47 (1974) 81.
14 Y. D. Clonis, in R. W. A. Oliver (Editor), *HPLC of Macromolecules: A Practical Approach*, IRL Press, Oxford, 1989 p. 157.
15 P. O. Larsson, *Methods Enzymol.*, 104 (1984) 212.
16 A. Fallon, R. F. G. Booth and L. D. Bell, in R. H. Burdon and P. H. Knippenberg (Editors), *Applications of HPLC in Biochemistry. Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier, Amsterdam, 1987 p. 106.
17 K. Nilsson and K. Mosbach, *Biochem. Biophys. Res. Commun.*, 102 (1981) 449.
18 F. B. Anspach, H. J. Wirth, K. K. Unger, P. Stanton and J. R. Davies, *Anal. Biochem.*, 179 (1989) 171.
19 K. Ernst-Cabrera and M. Wilchek, *Anal. Biochem.*, 159 (1986) 267.
20 P. O. Larsson, M. Glad, L. Hansson, M. O. Mansson, S. Ohlsson and K. Mosbach, *Adv. Chromatogr.*, 21 (1983) 41.
21 P. Wikström and P. O. Larsson, *J. Chromatogr.*, 388 (1987) 123.
22 B. Anspack, K. K. Unger, J. Davies and M. T. W. Hearn, *J. Chromatogr.*, 450 (1988) 195.
23 Y. D. Clonis, *J. Chromatogr.*, 407 (1987) 179.
24 D. A. P. Small, T. Atkinson and C. R. Lowe, *J. Chromatogr.*, 266 (1983) 151.
25 Y. D. Clonis, K. Jones and C. R. Lowe, *J. Chromatogr.*, 363 (1986) 31.
26 L. A. Decker (Editor), *Worthington Enzyme Manual*, Worthington Biochemical, Freehold, NJ, 1977 p. 9.
27 R. J. Lamed and J. G. Zeicus, *Biochem. J.*, 195 (1981) 183.
28 H. U. Bergmeyer, *Methods of Enzymatic Analysis*, Vol. 2, Academic Press, New York, 1974 p. 575.
29 H. U. Bergmeyer, *Methods of Enzymatic Analysis*, Vol. 2, Academic Press, New York, 1974 p. 624.
30 L. A. Decker, (Editor), *Worthington Enzyme Manual*, Worthington Biochemical, Freehold, NJ, 1977 p. 27.
31 W. Gruber, H. Höllering and H. U. Bergmeyer, *Enzymol. Biol. Clin.*, 7 (1966) 115.
32 H. U. Bergmeyer, *Methods of Enzymatic Analysis*, Vol. 1, Academic Press, New York, 1974 p. 510.
33 H. U. Bergmeyer, *Methods of Enzymatic Analysis*, Vol. 1, Academic Press, New York, 1974 p. 443.
34 P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson and D. C. Klenk, *Anal. Biochem.*, 150 (1985) 76.
35 D. F. Hollis, S. Ralston, E. Suen, N. Cooke and R. G. Shorr, *J. Liq. Chromatogr.*, 10 (1987) 2349.
36 M. Dixon, *Biochem. J.*, 55 (1953) 170.
37 N. S. Scrutton, A. Berry and R. N. Perham, *Nature*, 343 (1990) 38.