GENERAL LIGAND AFFINITY CHROMATOGRAPHY: N^6-(6-AMINOHEXYL) 3′,5′-ADP SEPHAROSE AS AN AFFINITY ADSORBENT FOR THE CoA-DEPENDENT ENZYME, SUCCINATE THIOKINASE

Standish BARRY, Peter BRODELIUS* and Klaus MOSBACH
Biochemical Division, Chemical Centre, University of Lund, P.O. Box 740, S-220 07 Lund 7, Sweden

Received 30 August 1976

1. Introduction

General ligand affinity chromatography has been shown in recent years to be an effective technique in the separation and purification of various groups of enzymes in particular the adenine nucleotide dependent enzymes such as the NAD-linked [1–5] and NADP-linked [6–9] dehydrogenases as well as ATP-dependent enzymes [10] and cyclic-AMP enzymes [11,12]. An analogue of AMP (the adenine nucleotide half of NAD substituted at position 6 of the adenine moiety with a hexamethylene diamine spacer-arm has been shown to be an effective general ligand for NAD-linked dehydrogenases [1,3,4]. Similarly the corresponding analogue of 2′,5′-ADP (the adenine nucleotide half of NADP) has been shown to be effective as a general ligand for NADP-linked dehydrogenases [6,8]. We decided to investigate the potential of such a 'half-molecule' of CoA i.e., 3′,5′-ADP, as a general ligand for use in the affinity chromatographic separation of CoA-dependent enzymes. We show here that an analogue of 3′,5′-ADP, i.e. N^6-(6-aminohexyl) 3′,5′-ADP, (which is a byproduct in the syntheses of the above mentioned analogues having the same spacer molecule and point of substitution in the adenine moiety), is an effective affinity chromatographic ligand when coupled to CNBr-activated Sepharose 4 B or to epoxy activated Sepharose 6 B. Succinate thiokinase was chosen as a model enzyme and its binding to the three adenine nucleotide gels compared. Effective biospecific binding was observed only with the 3′,5′-ADP analogue which was in accord with the K_i-values obtained with the corresponding soluble analogues. The enzyme was also purified from crude extracts of pig heart using this affinity chromatographic procedure.

*Present address: Department of Chemistry, University of California, San Diego, La Jolla, Calif., USA

2. Experimental

Coenzyme A (grade I-L from yeast), 2′,5′-ADP, 3′,5′-ADP, GTP (type II-S, sodium salt), ATP (disodium salt), $NADP^+$ (sodium salt) and succinate thiokinase (succinyl-CoA synthase), (EC 6.2.1.4) from pig heart (specific activity = 6.9 U/mg) were all obtained from Sigma Chemical Co., St. Louis. Sepharose 4 B, and Epoxy-activated Sepharose 6 B were supplied by Pharmacia, Uppsala.

Succinate thiokinase was assayed at 30°C by spectrophotometrically monitoring the increase in absorbance at 233 nm due to thioester formation according to the method of Cha [13]. N^6-(6-aminohexyl) 3′,5′-ADP and N^6-(6-aminohexyl) 2′,5′-ADP were synthesized by the method of Brodelius et al. [6] and N^6-(6-aminohexyl) 5′-AMP was synthesized by the method of Guilford et al. [14]. These ligands were coupled to Sepharose 4 B by a modification of the CNBr-activation technique [15], except that the CNBr was dissolved in acetonitrile. (The 2′,5′-ADP Sepharose and 5′-AMP Sepharose are now commercially available from Pharmacia, Uppsala.)

The 3′,5′-ADP analogue was also coupled to epoxy-activated Sepharose 6 B as follows: The 3′,5′-ADP

analogue (0.148 mmol) dissolved in 0.2 M $NaHCO_3$ (3 ml) was added to a suspension of 3 ml (1 g dry weight) epoxy-activated Sepharose 6 B in an equal volume of 0.4 M $NaHCO_3$ which had been previously titrated to pH 11.7 with 6 M NaOH. The gel–nucleotide mixture (pH 10.7) in a sealed container was gently agitated for 20 h at 28°C and then washed extensively with 0.2 M $NaHCO_3$, 0.2 M NaCl and distilled water respectively. The substitution levels of the nucleotide gels (which ranged from 1.8–2 μmol ligand per ml of wet gel – see legends to figs.1 and 2 for exact quantities) were estimated by direct ultraviolet spectroscopy as follows (taking the molar absorption coefficient of the N^6-(6-aminohexyl) 3′(2′),5′-ADP ligands at 267 nm to be 18 500 $M^{-1} \times cm^{-1}$ [6] and that of N^6-(6-aminohexyl) 5′-AMP at 267 nm to be 17 300 $M^{-1} \times cm^{-1}$ [14]); 100 mg of moist nucleotide gel (i.e., gel sucked on a sintered glass funnel until the gel begins to crack) is suspended in 2.9 ml of 0.5% aqueous solution of polyethyleneglycol (Polyox WSR 301) (to prevent rapid setting of gel) and the absorbancy at 267 nm is read against a blank consisting of an equal amount of CNBr- (or epoxy-)-activated Sepharose made up the same way.

Protein concentrations were determined by a modification of the Lowry method as described by Hartee [16]. Samples were first precipitated with 10% TCA to remove interfering nucleotides and Tris. Bovine serum albumin was used as a standard and was treated in the same way.

A crude extract of pig heart was prepared accord-

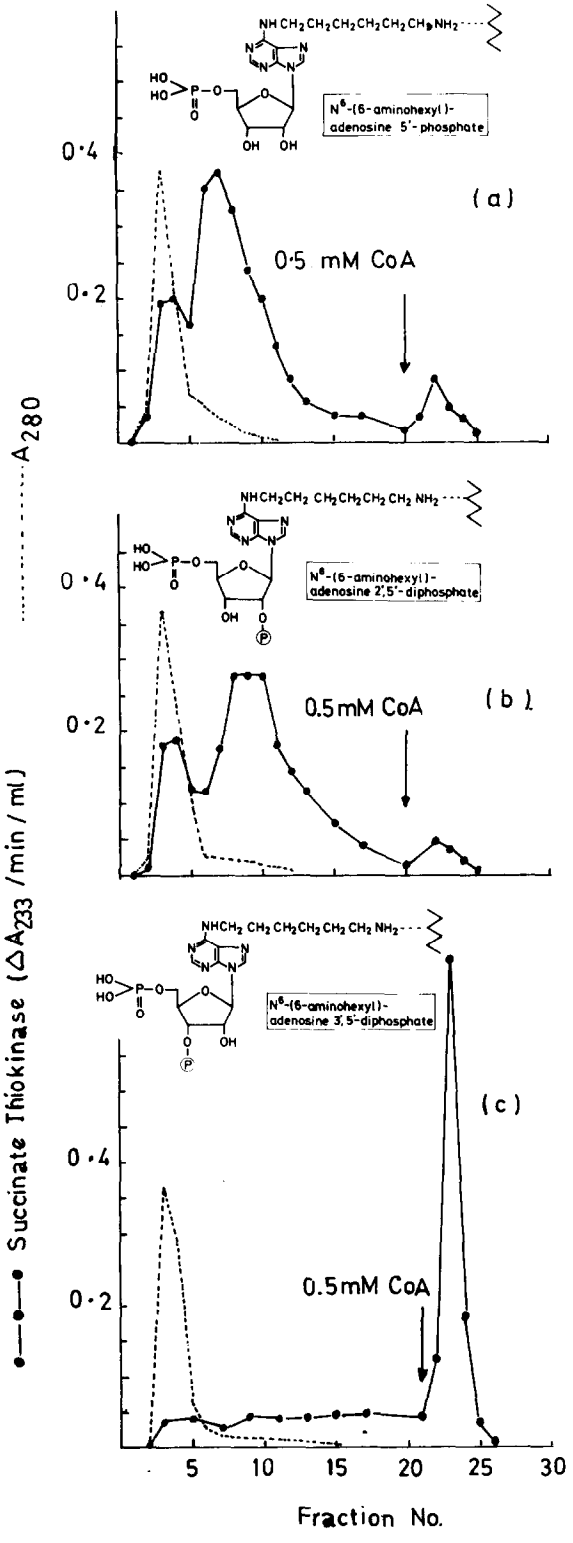

Fig.1. Affinity chromatographic behaviour of succinate thiokinase on three different 6-immobilized adenine nucleotide Sepharose derivatives (see inserts on the individual diagrams): Samples (500 μl) of crude extract of pig heart (prepared as described in Experimental) containing approx. 0.75 units of succinate thiokinase activity were applied to miniature columns containing 1 ml of nucleotide gel (CNBr coupled) which had the following substitution levels: (a) 5′-AMP Sepharose (2.00 μmol immobilized nucleotide per ml wet gel), by 2′,5′-ADP Sepharose (1.89 μmol immobilized nucleotide per ml wet gel), and (c) 3′,5′-ADP Sepharose (1.88 μmol immobilized nucleotide per ml wet gel). The irrigating buffer was 20 mM Tris–HCl, pH 7.2 to which CoA was added (in a total vol. of 2 ml) as indicated by the vertical arrows. 1 ml fractions were collected at a flow rate of 4.2 min per ml.

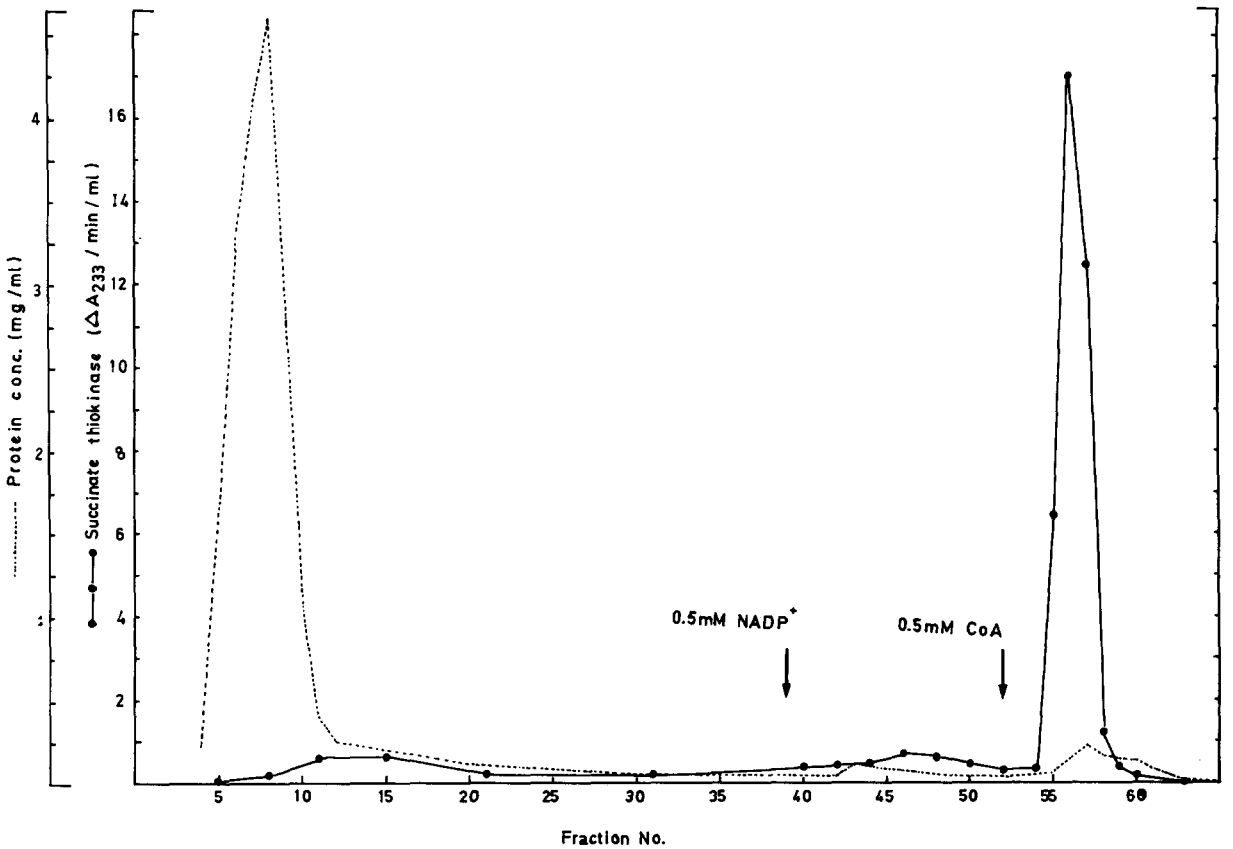

Fig.2. Partial purification of succinate thiokinase from pig heart by affinity chromatography on 3′,5′-ADP Sepharose (CNBr coupled): A column (140 × 10 mm) containing 8 ml of 3′,5′-ADP Sepharose (2.00 μmol immobilized nucleotide per ml of wet gel) was equilibrated with a 20 mM Tris–HCl irrigating buffer pH 7.0. A sample (7 ml) of crude extract of pig heart (prepared as described in Experimental) containing 18 units of succinate thiokinase was applied to the column and 2 ml fractions were collected at a flow rate of 3 ml/min. $NADP^+$ and CoA (dissolved in 8 ml of irrigating buffer each) were applied to the column separately as indicated by the vertical arrows.

ing to Cha [13]. Fresh, non-frozen pig heart with excess fat removed, was minced and washed with cold distilled water and rinsed through a cheese-cloth and excess water squeezed out by hand. The mince was homogenized with 3 vol of ice-cold 10 mM KH_2PO_4 and centrifuged for 30 min at 10 000 × g and the residue discarded. The supernatent was brought to 70% saturation with ammonium sulfate and left stand overnight after which the extract was centrifuged at 26 000 × g for 20 min and the supernatent discarded. The pellet was stored at 3–5°C small portions (approx. 9 g) (when required for affinity experiments) were redissolved in 15 ml of 20 mM Tris–HCl buffer, pH 7.0, and dialysed overnight against 3 litres of 20 mM Tris–HCl, pH 7.0, at 3–5°C. All chromatography experiments were carried out in the cold.

3. Results and discussion

Succinate thiokinase from pig heart was shown to bind strongly to 3′,5′-ADP Sepharose and could be competitively eluted with CoA (fig.1c). The biospecificity of the binding was further evaluated by applying the enzyme to nucleotide–gel columns closely analogous to the 3′,5′-ADP Sepharose derivative. As shown in fig.1b, succinate thiokinase was only

retarded on the corresponding 2,'5'-ADP Sepharose gel and retarded even less on the 5',AMP-Sepharose gel (fig.1a). Care was taken in these comparative studies to use gel-preparations of equal nucleotide concentration to permit proper evaluation.

These results show that there is an essential requirement for the phosphate group on the 3'-position of the ribose moiety for binding of succinate thiokinase to be effective. The stronger affinity of this CoA-dependant enzyme for its 'natural' 3'-phosphate derivative in contrast to both 2',5'-ADP Sepharose and 5'-AMP Sepharose is substantiated by the varying abilities of the soluble analogues to inhibit the thiokinase reaction (see table 1).

Binding of succinate thiokinase to 3',5'-ADP Sepharose considerably weakened at higher buffer strengths (100 mM) the enzyme being only retarded. This would seem to suggest that at the lower buffer strengths, the biospecific binding is reinforced by non-specific components which are abolished at higher buffer strengths leaving just the weak biospecific binding which is only capable of weakly retarding the enzyme. Such 'compound affinity' [2] has been suggested to occur in other affinity systems [17,18] and is probably due to non-specific hydrophobic and ionic forces [19] contributed by the CNBr-coupled spacer-arm matrix assemblies.

The epoxy coupled 3',5'-ADP Sepharose, which had a comparable substitution level (1.8 μmol immobilized nucleotide per ml wet gel) to that of the CNBr coupled 3',5'-ADP Sepharose, also bound succinate thiokinase. The exact chemical nature of this epoxy–Sepharose–nucleotide complex remains to be determined, a negative trinitro benzene sulfonate test for free aminogroups on the gel [20] indicates that the terminal aminogroup of the analogue is involved in the binding.

A 50-fold purification of succinate thiokinase from crude extracts of pig heart was obtained using the 3',5'-ADP Sepharose gel followed by biospecific elution of the enzyme with a pulse of CoA (fig.2 and table 2). It was shown that a pre-wash of the column with a pulse of $NADP^+$ was advantageous (table 2) probably removing NADP-dependent enzymes which may also weakly bind to the gel. We found that succinate thiokinase could also be eluted with GTP (the other nucleotide participating in the enzymic reaction), however, the purity of the recovered enzyme was much lower in comparison to elution with CoA (see table 2).

Preliminary studies with phosphotransacetylase from *Clostridium kluyveri* and carnitine acetyl transferase from pigeon breast muscle have shown that these enzymes also bind to 3',5'-ADP Sepharose (S. Barry

Table 1
Inhibition of succinate thiokinase from pig heart[a] *by AMP and ADP ligands*

Ligands	Ligand concentration (mM)	CoA concentration (mM)	% Inhibition	K[b] (mM)
5'-AMP	0.6	0.1	78	–
N^6-(6-aminohexyl) 5'-AMP	0.6	0.1	32	1.0
2',5'-ADP	0.6	0.1	82	–
N^6-(6-aminohexyl) 2',5'-ADP	0.6	0.1	38	0.9
3',5'-ADP	0.6	0.1	83	–
N^6-(6-aminohexyl) 3',5'-ADP	0.6	0.1	46	0.6

[a]Undialysed commercial preparations of succinate thiokinase were used for these assays.

[b]The inhibition constants were calculated from double reciprocal plots of the initial velocity activities versus variable concentrations of CoA in the presence of 0.2 mM nucleotide analogues. Held constant were succinate (Tris salt, pH 7.4) at 50 mM, GTP at 0.1 mM and $MgCl_2$ at 10 mM. A K_m of 0.0042 mM was calculated for CoA in the absence of inhibitor.

The nucleotide ligands, dissolved in 100 μl of 100 mM Tris–HCl, pH 7.4, were added to the standard assay and the reaction started by the addition of the enzyme.

Table 2
Comparison of the effectiveness of various specific elution methods for succinate thiokinase from 3′,5′-ADP Sepharose

Method of elution	Specific activities[a]		Purification (fold)	Recovery (%)
	crude extract	peak fraction		
8 ml pulse of 0.2 mM CoA[b]	0.24	6.96	30.0	62
'Pre-wash' with 8 ml pulse of 0.5 mM $NADP^+$ followed by elution with 8 ml pulse of 0.5 mM CoA (see fig.2)	0.28	14.17	50.4	54
GTP gradient (0–2 mM) peakfraction (0.7 mM GTP)	0.23	2.84	12.6	50
8 ml pulse of 0.5 mM ATP	0.22	N.D.[c]	N.D.[c]	24

[a] Specific activity is expressed in units of enzyme per mg protein where one unit of enzyme is the amount of enzyme catalyzing 1 μmol of substrate per minute.
[b] Lower specific activities (e.g., specific activity = 2.8) were obtained when the affinity column was irrigated with a 0.2 mM CoA pulse immediately after the emergence of the non-specific protein peak.
[c] N.D. = Not determined.

The pig heart extract was prepared as described in Experimental and the chromatographic conditions were as desribed in legend to fig.2.

and K. Mosbach, unpublished data). However further studies on 3′,5′-ADP Sepharose are needed to evaluate the generality of this system for affinity chromatography of CoA-dependent enzymes and also to determine whether the affinity of these enzymes for the 3′,5′-ADP 'half molecule' of CoA is sufficient for effective binding as has been shown to be the case for the corresponding adenine-containing half molecules of NAD and NADP used in affinity chromatography of NAD(P)-linked enzymes.

Acknowledgement

Part of this investigation has been supported by the Swedish National Science Foundation.

References

[1] Mosbach, K., Guilford, H., Ohlsson, R. and Scott, M. (1972) Biochem. J. 127, 625–631.
[2] Barry, S. and O'Carra, P. (1973) Biochem. J. 135, 595–607.
[3] Craven, D. B., Harvey, M. J., Lowe, C. R. and Dean, P. D. G. (1974) Eur. J. Biochem. 41, 329–333.
[4] Kaplan, N. O., Everse, J., Dixon, J. E., Stolzenbach, F. E., Lee, C-Y., Lee, C-L., Taylor, T. and Mosbach, K. (1974) Proc. Natl. Acad. Sci. USA 71, 3450–3454.
[5] Lee, C-Y., Lappi, D. A., Wermuth, B., Everse, J. and Kaplan, N. O. (1974) Arch. Biochim. Biophys. 163, 561–569.
[6] Brodelius, P., Larsson, P. O. and Mosbach, K. (1974) Eur. J. Biochem. 47, 81–89.
[7] Lowe, C. R. and Mosbach, K. (1974) Eur. J. Biochem. 49, 511–520.
[8] Morelli, A. and Benatti, U. (1974) Ital. J. Biochem. 23, 279–291.
[9] Lee, C-Y. and Kaplan, N. O. (1975) Arch. Biochem. Biophys. 168, 665–676.
[10] Trayer, J. P., Trayer, H. R., Small, D. A. P. and Bottomley, R. C. (1974) Biochem. J. 139, 609–623.
[11] Wilchek, M., Salomon, Y., Lowe, M. and Sellinger, Z. (1971) Biochem. Biophys. Res. commun. 45, 1177–1184.
[12] Jergil, B., Guilford, H. and Mosbach, K. (1974) Biochem. J. 139, 441–448.
[13] Cha, S. (1974) Methods in Enzymol. (Lowenstein, J. M. ed) vol. 13, pp. 62–69, Academic press.
[14] Guilford, H., Larsson, P. O. and Mosbach, K. (1972) Chemica Scripta 2, 165–170.
[15] Porath, J., Aspberg, K., Drevin, H. and Axén, R. (1973) J. Chromatogr. 86, 53–56.
[16] Hartee, H. F. (1972) Analyt. Biochem. 48, 422–427.

[17] O'Carra, P., Barry, S. and Griffin, T. (1974) FEBS Lett. 43, 169–175.
[18] Lowe, C. R. and Mosbach, K. (1975) Eur. J. Biochem. 52, 99–105.
[19] Jost, R., Miron, T. and Wilchek, M. (1974) Biochim. Biophys. Acta 362, 75–82.
[20] Inman, J. K. and Dintzis, H. M. (1969) Biochemistry 8, 4074–4082.