Biospecific Affinity Chromatography of an Adenosine 3':5'-Cyclic Monophosphate-Stimulated Protein Kinase (Protamine Kinase from Trout Testis) by using Immobilized Adenine Nucleotides

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1. Two adenine nucleotides, 8-(6-aminoethyl)aminoadenosine 3':5'-cyclic monophosphate and 8-(6-aminoethyl)amino-AMP, were synthesized. Their structures were established in particular by using mass spectroscopy. 2. Free cyclic AMP and 8-(6-aminoethyl)amino cyclic AMP both stimulate protamine kinase activity at low concentrations, but are inhibitory at concentrations above 0.1 mM. AMP is an inhibitor of enzymic activity, whereas neither 8-(6-aminoethyl)amino-AMP nor the earlier synthesized *N*⁶-(6-aminoethyl)-AMP is inhibitory. 3. The nucleotides were coupled to Sepharose 4B and used for biospecific chromatography of partially purified protamine kinase. Enzyme applied at high buffer concentrations to the cyclic AMP-Sepharose material was retarded and thereby purified tenfold. At low buffer concentrations the enzyme was adsorbed to the affinity material, and was subsequently released by a pulse of the inhibitor AMP, yielding a 50–100-fold purification. Enzyme applied to immobilized 8-(6-aminoethyl)amino-AMP or *N*⁶-(6-aminoethyl)-AMP was eluted together with the main protein peak in the void volume. 4. Protamine kinase eluted from 8-(6-aminoethyl)amino cyclic AMP-Sepharose was no longer activated by cyclic AMP. Results from sucrose gradient centrifugation suggest that a dissociation of the enzyme took place on the immobilized nucleotide. 5. Further information on the mass spectroscopy has been deposited as Supplementary Publication SUP 50026 at the British Library (Lending Division) (formerly the National Lending Library for Science and Technology), Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies may be obtained on the terms given in *Biochem. J.* (1973) 131, 5.

Protein kinases are a group of enzymes involved in the phosphorylation of various proteins. The activity of several of them is stimulated by cyclic AMP (for a review see Krebs, 1972), which generally seems to act by dissociating the enzyme into a regulatory, enzymically inactive, cyclic AMP-binding component and a fully active catalytic component (Brostrom *et al.*, 1970; Gill & Garren, 1970; Kumon *et al.*, 1970; Tao *et al.*, 1970). Attempts have been made to use this property for the separation of the cyclic AMP-binding and catalytic fractions. Rat parotid and rabbit skeletal-muscle protein kinases have been chromatographed on columns containing *N*⁶- ϵ -aminohexanoyl cyclic AMP covalently coupled to Sepharose (Wilchek *et al.*, 1971). The enzymically active fractions were apparently eluted together with the main protein peak. They were no longer stimulated by cyclic AMP, nor did binding of this nucleotide occur.

Protein kinases from rat adipose tissue (Corbin *et al.*, 1972a) and rabbit skeletal muscle (Corbin *et al.*, 1972b) can be adsorbed on to casein- or histone-Sepharose columns. Regulatory components

with negligible enzymic activity are dissociated and are eluted on application of 10 μ M-cyclic AMP. The catalytic components, no longer stimulated by cyclic AMP, can subsequently be released by increasing the ionic strength of the eluting buffer.

Earlier investigations into kinetic parameters of protamine kinase, a protein kinase present in trout testis, have shown that this enzyme is also stimulated by low concentrations of cyclic AMP (Jergil & Dixon, 1970). At nucleotide concentrations above 0.1 mM, however, enzymic activity is inhibited. In addition it has been found that protamine kinase is also inhibited by 5'-AMP. These findings prompted an investigation into the possibility that immobilized derivatives of these nucleotides might be useful as biospecific adsorbents for affinity chromatography of this enzyme.

Experimental

Materials

Cyclic AMP used in the preparation of the 8-(6-aminoethyl)amino derivative was purchased from Serva, Heidelberg, West Germany; that used for enzyme analyses was obtained from Sigma, St. Louis,

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Mo., U.S.A., as were other nucleotides. Immobilized N^6 -(6-aminohexyl)-AMP and 8-(6-aminohexyl)-aminoadenosine 3':5'-cyclic monophosphate were prepared as described elsewhere (Guilford *et al.*, 1972). [γ - 32 P]ATP was prepared essentially by the method of Glynn & Chappell (1964) and protamine as described earlier (Jergil & Dixon, 1970). Other chemicals and chromatographic materials were obtained from standard commercial sources and were used without further purification.

Methods

Protamine kinase was partially purified from rainbow-trout testes (Dan Trout, Brande, Denmark) by hydroxyapatite chromatography (Jergil, 1972). Generally, active fractions were concentrated by ultrafiltration by using collodion membranes. The concentrated enzyme solution was stored at 0°C. The kinase was assayed with protamine as substrate as described by Jergil & Dixon (1970). Protein analyses were performed after precipitation of the protein with 10% (w/v) trichloroacetic acid, by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as standard.

The t.l.c. systems used were: (a) 2-methylbutan-2-ol-formic acid-water (3:2:1, by vol.) (0.1 mm-thick cellulose plates); (b) propan-2-ol-NH₃-water (7:1:2, by vol.) (0.1 mm-thick silica-gel plates). Spots were detected in u.v. light (wavelength 254 nm), then sprayed with ninhydrin reagent [0.2% ninhydrin in butan-1-ol-1 M-acetic acid (19:1, v/v)] and heated for 5 min at 120°C.

High-voltage electrophoresis was done on Whatman 3 MM paper in a Camag 61000 cell, with a 0-5000 V d.c. power supply, at 5000 V and 115 mA for 21 min in a buffer containing acetic acid and water (1:29, v/v) adjusted to pH 3.6 with ammonium acetate. Under these conditions the dye marker Xylene Cyanol FF migrated towards the anode ($m = 7.2$ cm).

8-Bromo-AMP. AMP (0.9 g, 2.9 mmol) was dissolved in 2 M-NaOH (15 ml), and a solution of bromine (0.8 ml, 7.5 mmol) in 1 M-sodium acetate buffer, pH 3.9 (50 ml), was added. The mixture was agitated at ambient temperature for 5 days in the dark. Anisole (10 ml) was added and the shaking continued vigorously for 2 days, after which time the organic layer was discarded. The residue was concentrated to 5 ml and applied to a column (1.6 cm $\times$ 55 cm) of Sephadex G-10 (50 g). Fractions (3 ml) were collected, water being used as eluent. In a typical run, fractions 9-18 contained bromine, 19-24 a mixture of bromine and nucleotides and 25-125 the required product. Rechromatography of the intermediate fractions gave more pure material, bringing the total yield after freeze-drying to 1.13 g (93%). The product was homogeneous in t.l.c. systems *a* ($R_F = 0.36$; AMP

$R_F = 0.27$), *b* ($R_F = 0.70$; AMP $R_F = 0.70$) and *c* ($R_F = 0.40$), and migrated towards the anode with $m = 4.4$ cm in the high-voltage electrophoresis system ($m_{AMP} = 3.3$ cm; $m_{8\text{-bromo-cyclic AMP}} = 3.9$ cm). It had a light-absorption maximum in water of 263 nm. Analysis showed it to contain bromine, monophosphate and ribose in the proportions of approx. 1:1:1 [Found: C, 25.4; H, 2.6 (drying loss 5.8%); C₁₀H₁₁BrN₅Na₂O₇P requires C, 25.5; H, 2.4]. These data confirm the structure as a monobromo-AMP, as do the following mass-spectrometric results: molecular-weight peaks of m/e 785 (^{79}Br) and 787 (^{81}Br) respectively for the trimethylsilylated derivative penta-'Me₃Si'-(8-bromo-AMP). The halogen atom is thought to be at C-8 both on theoretical grounds and by analogy with other reactions (cf. Muneyama *et al.*, 1971).

8-(6-Aminohexyl)amino-AMP. A solution of 8-bromo-AMP(Na⁺)₂ (118 mg, 0.25 mmol) and 1,6-diaminohexane (1.08 g, 10 mmol) in water (10 ml) was incubated at 140°C for 2 h in a sealed tube. After cooling, the mixture, now free of 8-bromo-AMP, was applied to a Dowex 1 column (X8; acetate form) (2 cm $\times$ 20 cm), which was then washed with 500 ml of water to remove the excess of diamine. A gradient of water (500 ml)-1 M-acetic acid (500 ml) was applied and fractions (5 ml) were collected; those with appropriate u.v. spectra and t.l.c. properties and which gave a positive ninhydrin reaction (typically fractions 54-80) were retained. Freeze-drying yielded 45 mg (40%) of a new compound, shown to be 8-(6-aminohexyl)amino-AMP by the following properties and mass-spectrometric analysis: the product is homogeneous in t.l.c. systems *a* ($R_F = 0.48$) and *c* ($R_F = 0.32$) migrates to the anode with $m = 0.5$ cm and gives a pink colour with ninhydrin. The light-absorption maximum in water is at 277 nm, the minimum at 237 nm ($E_{277}/E_{237} = 4.7$). Analysis reveals monophosphate, ribose and primary amine in the proportions 1:1:1. The mass-spectrometric data of the compound are reported in a supplement to this paper (SUP 50026).

Coupling of 8-(6-aminohexyl)amino-AMP to Sepharose 4B. Sepharose 4B (5 ml, about 0.2 g dry wt.) was activated with CNBr (0.25 g) (Axén *et al.*, 1967) and washed with ice-cold 0.1 M-Na₂CO₃. The gel was suspended in a solution of 8-(6-aminohexyl)-amino-AMP (22 mg) in the same buffer (5 ml) and slurried at 4°C overnight. The Sepharose was filtered off and extensively washed on a sinter with, successively, 0.1 M-Na₂CO₃, water, 0.5 M-NaCl and water. Approx. 12 mg of the nucleotide was coupled to the gel (i.e. 60 $\mu\text{mol/g}$ of dry Sepharose).

Chromatography on immobilized nucleotides. The gel was equilibrated with ammonium phosphate buffer, pH 8.0, of the same molarity as the buffer used in the subsequent elution (see the Figure legends). A small column, 0.5 cm in diameter, was packed to a height of 10-12 cm, washed thoroughly with the

buffer (at least 100ml) and connected to a peristaltic pump operating at 1ml/h. The protamine kinase preparation was dialysed against the same buffer before being applied to the column. Fractions (250 μ l) were collected and analysed for protamine kinase activity and protein content. After elution the column was washed with 8–10ml of 8M-urea, re-equilibrated with buffer and was then ready for re-use. All buffers were 1mM in dithiothreitol. Chromatographic procedures were carried out at 4°C.

Sucrose gradient centrifugation. Linear 5–30% (w/v) sucrose gradients were prepared in centrifuge tubes for the Spinco SW 40 rotor. Sucrose solutions were made up in 0.1M-ammonium phosphate buffer, pH 8.0, 1mM in dithiothreitol. The total volume of the gradient was 13.4ml. Samples (200 μ l) were dialysed against the ammonium phosphate buffer, layered on top of the sucrose gradients and centrifuged for 30h at 30°C at 40000rev./min (200000 $g_{av.}$) in a Spinco L2-65B ultracentrifuge. After centrifugation the gradients were collected in 28 fractions and analysed for protein kinase activity with protamine as substrate. In one case protamine kinase was incubated with 0.1mM-cyclic AMP for 15min at room temperature (22°C) before the centrifugation. The sucrose gradient for this sample contained 0.5mM-cyclic AMP.

Results and Discussion

The structures of the adenine nucleotides applied in this study are summarized in Fig. 1. Mass spectrometry was used in their structural determination and is discussed in a supplement to this paper (SUP 50026).

Effect of cyclic nucleotides on protamine kinase activity

Before the chromatographic characteristics of protamine kinase on the immobilized derivatives were examined, the effect of the various nucleotides on enzymic activity was investigated. At concentrations between 0.1 μ M and 0.1mM, cyclic AMP stimulated enzymic activity, maximally by about 60% (Fig. 2a), which is in accord with previous findings (Jergil & Dixon, 1970). The 8-substituted derivative affected the enzyme to the same degree but in the concentration range 1 μ M–0.1mM. The affinity constants of 8-(6-aminohexyl)amino cyclic AMP and cyclic AMP for the enzyme are 8×10^{-7} M and 5×10^{-8} M respectively, as computed from the semi-logarithmic plot in Fig. 2(a). This observation is consistent with the report of Muneyama *et al.* (1971) on the effect of a number of 8-alkylamino cyclic AMP derivatives on the activity of a bovine brain protein kinase. In addition to the stimulation by the cyclic nucleotides, however, inhibition of protamine kinase activity is observed at concentrations in excess of

0.1mM (Fig. 2a). Preliminary investigations have shown that the inhibition is competitive with respect to ATP, with K_i 3.3×10^{-4} M. Thus at higher concentrations the cyclic nucleotides exhibit a dual effect on this enzyme, perhaps by operating at different sites. A biospecific adsorbance caused by either or both of these effects might therefore be expected when protamine kinase is applied to a column of cyclic AMP–Sephacrose.

Chromatography on immobilized 8-(6-aminohexyl)-amino cyclic AMP

Protamine kinase partially purified on hydroxyapatite was chromatographed on 8-(6-aminohexyl)-amino cyclic AMP immobilized on Sepharose (Fig. 3a). When the column was developed with 0.1M-ammonium phosphate buffer a major inactive protein peak was eluted with the void volume. Protamine kinase, appearing in the 'trailing' part of the protein peak, was appreciably retarded under these conditions. The degree of retardation seemed to be independent of buffer concentration within the range of 0.1M to (at least) 0.5M with this nucleotide–Sepharose preparation. When the ammonium phosphate concentration was 0.01M, however, protamine kinase was retained on the column. The enzyme was subsequently released by 0.1M buffer with an elution profile similar to that shown in Fig. 3(a), except that the protein peak was relatively small, since much inactive protein was eluted at the lower buffer concentration. In control experiments chromatography was performed on both Sepharose 4B and CNBr-activated Sepharose lacking nucleotide. The enzyme was eluted from these columns with the void volume (cf. Fig. 3b). An approximately tenfold purification was obtained by using the cyclic AMP–Sepharose column and recovery was 40–50%. After chromatography the enzyme was usually unstable and tended to lose half of its activity within 10h, probably mainly because of the low protein concentration in the eluate. About 50% of the total protein was recovered. Increasing the buffer concentration of the eluent from 0.1M to 0.5M released only a trace of protein and did not release additional enzyme. Urea (8M) released more protein, bringing the total recovery to 75–80%. In the control with unsubstituted Sepharose, recovery of both enzyme and protein was about 80%.

Cyclic AMP–Sepharose columns could be used several times without appreciable change in their elution properties. Gradually, however, the retardation of protamine kinase diminished, and the buffer concentration below which the enzyme was retained on the column decreased. These changes may be due to hydrolysis of the cyclic phosphate ester, either through the action of phosphodiesterase activity present in the applied samples, or, less likely (cf.

Fig. 1. Structures of the three adenine nucleotides applied

(I), 8-(6-Aminoheptyl)amino cyclic AMP; (II), 8-(6-aminoheptyl)amino-AMP; (III), N^6 -(6-aminoheptyl)-AMP. The dashed lines indicate binding to Sepharose (M).

Sutherland & Rall, 1958), through non-enzymic hydrolysis. It is also possible that leakage from the column gradually decreased the amount of bound nucleotide (Tesser *et al.*, 1972).

Different preparations of cyclic AMP-Sepharose exhibited different chromatographic properties in that the buffer concentration below which the enzyme was retained on the column varied. The general pattern of elution of the enzyme, however, was quite consistent.

We next examined whether the protamine kinase was still subject to cyclic AMP activation after passage through a column of cyclic AMP-Sepharose (Table 1). The enzyme preparation used was activated by 60%

by the nucleotide before chromatography, but after elution enzyme activity was independent of cyclic AMP. These data are consistent with the observations by Wilchek *et al.* (1971) on the behaviour of skeletal-muscle protein kinases after chromatography on immobilized N^6 -hexanoyl cyclic AMP. The enzyme eluted from our control column of unsubstituted Sepharose was still activated by cyclic AMP (Table 1). To examine whether the observed cyclic AMP independence could have been caused by leakage of the ligand from its carrier, a sample of unchromatographed protein kinase of known degree of cyclic AMP stimulation was mixed with an enzyme

fraction eluted from the column, and tested for activity in the presence and absence of the nucleotide (Table 1). Since the mixture was activated by cyclic AMP to the same extent as was the unchromatographed enzyme, the conversion of the enzyme into a cyclic AMP-independent form on affinity chromatography is not an apparent effect caused by leakage of the ligand from the column.

Since several cyclic AMP-activated protein kinases have been reported to dissociate in the presence of the cyclic nucleotide into catalytic and cyclic AMP-binding units, there is a possibility that a similar dissociation of protamine kinase will take place on the cyclic AMP-Sepharose column resulting in the elution of the catalytic activity while the cyclic AMP-binding unit remains adsorbed to the immobilized ligand. To test this possibility protamine kinase was subjected to sucrose gradient centrifugation before and after chromatography on 8-(6-aminohexyl)amino cyclic AMP-Sepharose, and also after treatment with free cyclic AMP (Figs. 4a and 4b). Untreated protamine kinase showed, on centrifugation in a linear 5–30% (w/v) sucrose gradient (Fig. 4a), two peaks of enzymic activity, with the major peak, containing approx. 80% of the total activity, having a lower rate of sedimentation than the minor peak. Both peaks were activated by cyclic AMP to approximately the same degree. Treatment of protamine kinase with cyclic AMP before centrifugation converted the enzyme into a single enzymically active component sedimenting

Fig. 2. Effect of nucleotides on protamine kinase activity

Various concentrations of nucleotides were included in a standard reaction mixture containing 3.4 μ g of enzyme protein. Incubation time was 15 min at 20°C. (a) Cyclic AMP (○); 8-(6-aminohexyl)amino cyclic AMP (Δ). (b) AMP (○); 8-(6-aminohexyl)amino-AMP (Δ); N^6 -(6-aminohexyl)-AMP (●).

Fig. 3. Chromatography of protamine kinase on columns containing nucleotides immobilized on Sepharose 4B

Chromatography was performed as described in the Experimental section on columns previously equilibrated with 0.1M-ammonium phosphate buffer, pH8.0, 1mM in dithiothreitol. (a) 8-(6-Aminoethyl)amino cyclic AMP-Sepharose. Enzyme protein (2.5mg) in 0.5ml of 0.1M buffer was applied. Identical separation patterns were obtained when chromatography was performed either with 0.5M-ammonium phosphate buffer or with 0.1M buffer containing 0.1mM-cyclic AMP as eluent. (b) 8-(6-Aminoethyl)amino-AMP-Sepharose. Enzyme protein (1.8 mg) in 0.3 ml of 0.1 M buffer was applied. Chromatography on N^6 -(6-aminoethyl)-AMP-Sepharose, Sepharose 4B, or CNBr-activated Sepharose yielded identical separation patterns. ○, [32 P]P_i incorporated into protamine (nmol/15 min per 10 μ l of sample); Δ, protein concentration (mg/ml).

Table 1. Effect of cyclic AMP on protamine kinase activity before and after chromatography on Sepharose-coupled adenine nucleotides

Enzyme analyses were performed by using 5 μ l of enzyme preparation in the absence (–) or presence (+) of 10 μ M-cyclic AMP. [32 P]P_i incorporated (nmol/15 min)

Nucleotide coupled to Sepharose 4B	Applied enzyme			Eluted enzyme		
	–	+	Ratio +/-	–	+	Ratio +/-
8-(6-Aminoethyl)amino cyclic AMP	0.54	0.85	1.6	0.14	0.14	1.0
8-(6-Aminoethyl)amino-AMP	1.94	3.10	1.6	0.65*	0.98*	1.5*
N ⁶ -(6-Aminoethyl)-AMP	1.94	3.10	1.6	0.45	0.83	1.8
Unsubstituted Sepharose 4B	2.47	3.95	1.6	1.71	2.43	1.4

* Mixture of applied and eluted enzyme, 5 μ l of each.

Fig. 4. Sucrose gradient centrifugation of protamine kinase. Sucrose gradients were prepared and centrifuged as described in the Experimental section. The fractions (0.5 ml) were tested for protamine kinase activity in the presence (●) or in the absence (Δ) of 10 μ M-cyclic AMP. (a) Protamine kinase purified through the hydroxyapatite step. Enzyme protein (0.9 mg) was applied. (b) The same enzyme source (0.9 mg of protein) preincubated in 0.1 mM-cyclic AMP and sedimented in a sucrose gradient, 0.5 mM in cyclic AMP. Protamine kinase eluted from an 8-(6-aminoethyl)amino cyclic AMP-Sepharose column showed an identical sedimentation pattern.

at a slower rate than the two peaks observed with untreated enzyme (Fig. 4b). Sucrose gradient centrifugation of the cyclic AMP-independent enzyme, recovered after chromatography through the cyclic AMP-Sepharose column, also showed a single catalytically active component with the same rate of sedimentation as that found with the cyclic AMP-treated enzyme (Fig. 4b). These data suggest (a) that protamine kinase undergoes a dissociation in the presence of free cyclic AMP which is analogous to that observed for other cyclic AMP-stimulated protein kinases (Brostrom *et al.*, 1970; Gill & Garren, 1970; Kumon *et al.*, 1970; Tao *et al.*, 1970) and (b) that dissociation also takes place with the immobilized cyclic AMP analogue, resulting in an elution of the catalytic component of the enzyme, whereas the cyclic AMP-binding moiety presumably remains adsorbed to the immobilized ligand. We have not been able to recover the cyclic AMP-binding material in the eluate or after treatment of the column with 1 M-ammonium phosphate buffer.

In addition to an apparent dissociation of protamine kinase on the cyclic AMP-Sepharose column, a retardation of the catalytic component was observed, indicating weak interactions with the column material. The retardation might be due to (a) the time required for dissociation of the enzymic components, (b) non-specific interactions between the column material and the enzyme and (c) biospecific interactions. Since the dissociation of protamine kinase in solution on treatment with cyclic AMP is very rapid, the substantial retardation of the enzymically active fractions renders (a) unlikely. The degree of retardation was also independent of buffer concentration within defined limits and was therefore less likely to be due to non-specific ionic interactions between the column material and the enzyme. The possibilities for biospecific interactions are of two kinds, either of reversible binding of catalytic components to cyclic AMP-binding components already adsorbed on the ligands, or of a direct interaction between a 'binding' site on the catalytic component and the cyclic AMP ligand of

Fig. 5. Chromatography of protamine kinase on 8-(6-aminohexyl)amino cyclic AMP-Sepharose; specific elution of the enzyme with AMP

Enzyme protein (3 mg) in 0.5 ml of 0.3 M-ammonium phosphate buffer (pH 8.0), 1 mM in dithiothreitol, was applied to a 0.5 cm $\times$ 12 cm column, equilibrated with the same buffer. The column was washed with the buffer before a pulse of 5'-AMP (5 mM dissolved in the buffer) was applied as indicated by the arrow. In these experiments another batch of immobilized nucleotide retaining the enzyme at a higher buffer concentration than that in Fig. 3(a) was used. O, Incorporation of [32 P]P_i into protamine (μ mol/15 min per 10 μ l of sample); Δ , protein concentration (mg/ml).

the column. To distinguish between these two possibilities we tried to determine whether cyclic AMP-independent enzyme is retarded when chromatographed on a cyclic AMP-Sepharose column. Owing to the instability of the enzyme, however, no enzymic activity was detected either in the eluates or when the column material was suspended in buffer and tested for activity. Evidence that the adsorption involves direct interaction between the ligand and the catalytic component, perhaps at the ATP site, is provided by the results of eluting with AMP, which is described further below.

Effect of AMP and AMP derivatives on protamine kinase activity; chromatography on immobilized AMP derivatives

Since preliminary experiments indicated that AMP is an inhibitor of protamine kinase, we decided to examine the possibility that AMP derivatives immobilized on Sepharose could be used for bio-specific purification of the enzyme. *N*⁶-(6-Amino-hexyl)-AMP and 8-(6-aminohexyl)amino-AMP were first tested for their influence on protamine kinase activity. Whereas AMP itself is an inhibitor acting competitively with respect to ATP (preliminary investigations have shown the *K_i* to be 3×10^{-4} M)

neither of the AMP derivatives inhibited enzyme activity even at a concentration of 1 mM (Fig. 2b). In fact, at this concentration *N*⁶-(6-aminohexyl)-AMP activated the enzyme to a small but significant extent. Despite the lack of inhibitory effect by the AMP derivatives we decided to test whether they would act as ligands for protamine kinase purification when coupled to Sepharose (and hence modified further). Thus the enzyme was applied to columns containing either of the AMP derivatives. In 0.1 M-ammonium phosphate buffer the enzyme was not retained on either column and was eluted together with the protein peak in the void volume (Fig. 3b). Lowering the buffer concentration of the eluent to 0.005 M did not affect the elution pattern. The enzyme chromatographed on these columns was activated by cyclic AMP (Table 1). These experiments clearly show that even a minimum of alteration of the nucleotide can completely change its effect on the enzyme, a fact that must be borne in mind when designing compounds to be used for affinity chromatography (Guilford, 1973). In a number of other cases, however, *N*⁶-(6-aminohexyl)-AMP immobilized on Sepharose has been used successfully as a 'general ligand' in biospecific chromatography of enzymes and isoenzymes (Mosbach *et al.*, 1972; Brodelius & Mosbach, 1973; J. Dixon, J. Everse, L. Y. Lee,

S. S. Taylor, N. O. Kaplan & K. Mosbach, unpublished work). In these cases it is acting similarly to AMP, as an inhibitor for these enzymes.

Specific elution of protamine kinase from 8-(6-aminohexyl)amino cyclic AMP-Sepharose

Since protamine kinase is retained on the cyclic AMP-Sepharose column at low buffer concentrations, it was decided to explore the possibilities for a more extensive purification of the enzyme by using a specific eluent. As the retention of kinase is most likely due to a direct interaction between the cyclic AMP ligand and the catalytic unit, we used AMP, another inhibitor of the enzyme (cf. Fig. 2b). At 5mM, AMP readily eluted adsorbed protamine kinase from the column without also releasing substantial amounts of inactive protein (Fig. 5). The released enzyme was not activated by cyclic AMP. To show that the elution is dependent on the inhibitory property of the added adenine nucleotide and not due to the addition of the nucleoside 5'-monophosphate itself to the eluting buffer, GMP, which is not an inhibitor, was tested. Protamine kinase was not released by this nucleotide but remained on the column. The enzyme could subsequently be eluted by the addition of AMP. The degree of purification obtained when AMP was used was approx. 50–100-fold (Fig. 5), which is substantially higher than that obtained when the enzyme was purified tenfold by retardation on the column (cf. Fig. 3a). An exact value is difficult to assess, since the protein concentration in the eluate is quite low, about 10 µg/ml in the active fractions, and therefore subject to relatively large errors in measurement. Because of the low protein concentration it has not been possible to perform an electrophoretic analysis of the purified enzyme to investigate its degree of purity. There seems to be a limit to the amount of material that can be purified in a comparatively concentrated form on the column, since the enzyme tends to be released as a broad peak if the amount of material applied is increased substantially, when a small column is used.

Thus the chromatography studies carried out with the various adenine nucleotides have established that one compound, 8-(6-aminohexyl)amino cyclic

AMP, is suitable for the purification of protamine kinase, and that a substantial purification of the enzyme can be achieved when a biospecific eluting agent such as AMP is applied.

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