CONVERSION OF PROTEIN KINASE TO A CYCLIC AMP INDEPENDENT FORM BY AFFINITY CHROMATOGRAPHY ON N⁶-CAPROYL 3',5'-CYCLIC ADENOSINE MONOPHOSPHATE-SEPHAROSE

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SUMMARY

A method was developed to couple 3',5'-cyclic adenosine monophosphate (c-AMP) to Sepharose. Chromatography of protein kinases from rat parotid, and rabbit skeletal muscle on c-AMP-Sepharose, resulted in preparations which were fully active in the absence of c-AMP. The c-AMP independent kinases thus obtained no longer bind c-AMP. It is suggested that c-AMP-Sepharose activates the protein kinase by removal of a regulatory unit that binds c-AMP. So far no method has been found to recover the c-AMP binding unit from the c-AMP-Sepharose column.

Cyclic 3',5'-adenosine monophosphate has been shown to exert its effect by activation of a c-AMP dependent protein kinase (1-3). It is now generally accepted that activation of the protein kinase by c-AMP involves binding of the nucleotide to a regulatory subunit thus causing a dissociation of the enzyme into regulatory and catalytic subunits (4-10). In bacterial systems in which c-AMP regulates the level of several catabolic enzymes, the nucleotide was shown to be bound by a specific protein which modulates gene transcription (11, 12). The potential use of affinity chromatography in studies of c-AMP-binding proteins prompted the preparation of a c-AMP derivative covalently linked to Sepharose. The present report describes the use of the c-AMP-Sepharose column for affinity chromatography of two protein kinase preparations.
MATERIALS AND METHODS

Preparation of c-AMP-Sepharose. The N°-ε-aminocaproyl-c-AMP derivative was covalently linked to Sepharose by the cyanogen bromide method of Porath et al. (13).

Carbobenzoxy-ε-aminocaproic acid anhydride: CBZ-ε-aminocaproic acid (5.3 g) and Dicyclohexylcarbodiimide (2.9 g) were dissolved in 40 ml of absolute ethylacetate. After 1 h incubation at room temperature the dicyclohexyurea was removed by filtration and the anhydride was precipitated by addition of petroleum ether. The yield was quantitative. m.p. 75-77°.

N°-carbobenzoxy-ε-aminocaproyl-c-AMP: c-AMP (250 mg) was dissolved in a minimal volume of cold aqueous 0.4 M triethylamine. The solution was evaporated to dryness under reduced pressure, taken up in an anhydrous pyridine, reevaporated and dried for 5 hr in a vacuum desiccator. The dried material was dissolved in 9 ml of hot anhydrous pyridine, to which 4 g of CBZ-ε-aminocaproic anhydride were added. This mixture was boiled for 3-4 minutes and was left at room temperature for 8 days. At the end of this incubation period the mixture was evaporated to dryness, the residue was dissolved in ethyl acetate and was extracted with cold water containing sodium bicarbonate in an amount of two equivalent of the anhydride. The aqueous solution was cooled in ice and the pH adjusted to 13. After 5 min the alkaline solution was acidified to pH 2 with 2 M HCl and extracted with ether. The aqueous solution was brought to pH 6.2 with 2 N NaOH and evaporated to dryness. The dried residue was extracted several times with boiling absolute ethanol until the last ethanol extract showed negligible absorption at 272 nm. The combined ethanol extracts were concentrated by evaporation and the N°-CBZ-ε-aminocaproyl-c-AMP was precipitated with
dry ether. The yield was 180 mg. The material showed λmax at 272 and
gave one spot on thin layer chromatography in a solvent system of ethanol
(5 vol), 0.5 M ammonium acetate (2 vol) Rf 0.77.

**N⁶-ε-aminocaproyl-c-AMP:** Palladium on charcoal (10% w/w) was added to
a methanolic solution of N⁶-CBZ-ε-aminocaproyl-c-AMP (0.5 g/30 ml).

Reduction was carried out in a Parr hydrogenation apparatus for 1.5 hr at
room temperature. After hydrogenation the palladium charcoal was re-
moved by filtration and the solution was evaporated to dryness. The residue
was redissolved in a small volume of methanol and was precipitated by
addition of ether. The yield was quantitative. The product showed one spot
of ultraviolet absorption with an Rf of 0.38 by thin layer chromatography
using the same solvent as above. The λmax of this compound was 272 nm
and \( EM^{-1} \text{cm}^{-1} \) 14,500.

**Sepharose-ε-aminocaproyl-c-AMP** was prepared by the cyanogen bromide
method as described previously (13,14). Sepharose containing up to 5μmolec
c-AMP per ml of Sepharose bed volume was prepared.

3',5'-adenosine monophosphoric acid was a product of Boehringer, [8-³H]
adenosine 3'-5' monophosphate with a specific activity of 16.3 C/mmole was
obtained from Schwarz Bioresearch and Sepharose 4B was purchased from
Pharmacia.

**RESULTS**

Binding of c-AMP to Sepharose by the cyanogen bromide activation
method was unsuccessful, probably because the N⁶-amino group of c-AMP
was relatively unreactive. Since binding studies showed that N⁶-butyryl
c-AMP efficiently binds to parotid membranes (15), N⁶-aminocaproyl c-AMP
was prepared. Such an introduction of a hydrocarbon chain with a free
amino group facilitated the binding of c-AMP to Sepharose and caused the
purine nucleotide to extend out of the gel matrix. Chromatography of crude rat parotid protein kinase and partially purified protein kinase from rabbit skeletal muscle on the c-AMP-Sepharose column resulted in preparations which were fully active in the absence of c-AMP (Table I). It is also shown that the column eluates containing protein kinase activity lost their ability to bind c-AMP. Attempts to recover the c-AMP binding component from the column by elution with solutions containing high salt concentration, urea, buffers at alkaline pH, or 1 mM c-AMP were unsuccessful. It was particularly surprising that c-AMP was unable to elute the c-AMP binding component since an exchange between c-AMP-Sepharose bound to the regulatory unit and free c-AMP in solution should have taken place. With the hope of understanding this lack of exchange experiments were carried out to test the exchange reaction

| Table I
| Chromatography of Protein Kinase on Cyclic AMP-Sepharose Columns |
|-----------------|-----------------|-----------------|-----------------|
| Fraction        | Protein kinase activity | Recovery of protein kinase activity | c-AMP binding |
|                 | µmol incorporated | %               | pmoles/mg protein |
|                 | pmol/mg protein/min |                 | protein |
| with c-AMP      | without c-AMP     |                 |                 |
| I. Parotid supernatant | 240              | 100 (100)       | 3.6             |
| Column eluate   | 210              | 200             | 50              |
| II. Muscle kinase | 1470             | 70 (100)        | 12              |
| Column eluate   | 2000             | 2000            | 70              |

Purification of muscle protein kinase (purified to the first DEAE cellulose column step) and determination of protein kinase activity were carried out as described by Walsh et al. (17). c-AMP binding was determined on millipore filters which serve to isolate the protein-c-AMP complex. The assay system in a final volume of 100 µl contained glycylglycine buffer pH 7.5, 50 mM, MgCl₂ 5 mM and [³H]-c-AMP 0.2 µM. The reaction was started by the addition of enzyme and incubation was carried out for 5 min at 30°. The reaction was terminated by the addition of 3 ml magnesium glycylglycine buffer and filtration through a 24 mm millipore filter (0.45 µ). The filter was washed 3 times with 3 ml of magnesium glycylglycine buffer, dried and counted in a toluene scintillation solution.
between \([^{3}H]c-AMP\) bound to the protein kinase and excess of unlabeled free c-AMP. This exchange reaction was previously studied by Gilman (16) who reported that at pH 4.0 and 0°C \([^{3}H]c-AMP\) bound to the enzyme was exchanged with a half life of 2 hr in the presence of 10 mM MgCl\(_2\) and 7 hr in the absence of magnesium. In the present work the exchange reaction was found to be very slow also at pH 7.5 and 30°C (Fig. 1). Since under physiological conditions the cell responds to changes in the level of c-AMP quite rapidly, it was reasoned that some compound normally present within the cell must increase the rate of dissociation between c-AMP and the protein kinase. It was found that ATP greatly enhanced the rate of the exchange reaction (Fig. 1). Although the parotid and muscle protein kinases behave very similarly in the exchange reaction, the parotid preparation which was only partially dependent on c-AMP (cf. Table 1) retained about 25% of the bound \([^{3}H]c-AMP\) in a non-exchangeable form even in the presence of ATP (Fig. 1). Enhancement
of the exchange reaction was maximal at ATP concentration of 10 μM and about half maximal at 2 μM ATP. The effect of ATP was dependent on the presence of magnesium and was completely abolished by the addition of EDTA. GTP or 5' AMP at 100 μM concentration could not replace ATP in promoting the exchange reaction. In spite of the effect of Mg-ATP on the c-AMP exchange reaction, addition of 0.1 mM ATP and 5 mM MgCl₂ to an elution solution containing 1 mM c-AMP did not result in a recovery of the c-AMP binding component from the c-AMP Sepharose column.

**DISCUSSION**

The present report demonstrates that c-AMP-Sepharose can be used to prepare protein kinases which are fully active in the absence of c-AMP and no longer bind this nucleotide. Taking into account the reports on a c-AMP-induced dissociation of the protein kinases into catalytic and regulatory subunits (4-10) it is likely that the dependence on c-AMP was abolished by absorption of the regulatory unit to the c-AMP-Sepharose column. The failure to recover the c-AMP binding component from the c-AMP-Sepharose deserves some comments. It is possible that the c-AMP binding unit was not recovered from the column due to its lability upon dissociation from the catalytic subunit. Such an inactivation was recently reported in the case of the protein kinase from bovine brain (10). Another possibility, however, is that the c-AMP binding unit adsorbed to c-AMP-Sepharose did not in fact undergo exchange with free c-AMP and was therefore not eluted. Certainly the binding of c-AMP to the protein kinase is not a simple reaction. The affinity of the enzyme for c-AMP was shown to be increased five fold by a protein kinase inhibitor (17) and the rate of association and dissociation of c-AMP and the protein kinase was increased by magnesium (16). In the present work it was found
that Mg-ATP markedly enhanced the exchange between [H³] c-AMP bound to the enzyme and an excess of unlabeled c-AMP. The effect of ATP on the exchange reaction was specific for this nucleotide neither GTP or 5' AMP could replace ATP and enhance the exchange reaction. Since the exchange reaction required the presence of both ATP and magnesium and showed the same specificity for ATP as the catalytic reaction it is conceivable that Mg-ATP influenced the exchange reaction through an interaction with the catalytic subunit. In line with this hypothesis was the finding that the parotid preparation which was only partially dependent on c-AMP and might have undergone some dissociation of the catalytic and the regulatory subunits retained about 25% of the bound c-AMP in a non-exchangeable form. The above hypothesis might also explain why an addition of Mg-ATP to the c-AMP solution did not lead to elution of the c-AMP binding component from the c-AMP-Sepharose column. Probably the fact that the catalytic subunit was already dissociated and eluted from the column prevented the exchange reaction from taking place. We are now preparing a derivative containing c-AMP from which the nucleotide can be split off by mild chemical reaction and this might make possible the isolation of the regulatory subunit. Since the present preparation of c-AMP-Sepharose retains the regulatory subunit it can serve as an excellent agent for isolation of the free catalytic unit.

REFERENCES