ISOLATION OF THE REGULATORY SUBUNIT OF PIG-BRAIN HISTONE KINASE BY AFFINITY CHROMATOGRAPHY ON CYCLIC-AMP-CONTAINING ADSORBENT

E. S. SEVERIN, S. N. KOCHETKOV, M. V. NESTEROVA and N. N. GULYAEV

Institute of Molecular Biology, Academy of Sciences of the USSR and Department of Animal Biochemistry, M. V. Lomonosov Moscow State University, Moscow, USSR

Received 11 October 1974

1. Introduction

In recent years much attention was been given to investigation of the mechanism of action of cyclic-AMP*-dependent protein kinases catalysing phosphorylation of different protein substrates [1–3]. Previously we described a purification procedure for pig brain histone kinase which shows high substrate specificity in respect to lysine rich histones [4]. This histone kinase is stimulated by cyclic-AMP and consists of two subunits, a catalytic one with mol. wt. 40 000 and a regulatory subunit with mol. wt. about 90 000. Addition of cyclic-AMP induces dissociation of the enzyme to the subunits. The regulatory subunit binds the cyclic nucleotide, whereas the catalytic one is released and catalyses phosphate transfer from ATP to histone [4]. In the course of these studies we used chromatography on DEAE-cellulose for separation of the subunits. However, by this procedure only the catalytic subunit could be obtained in homogeneous state from partially purified enzyme. Therefore, our efforts were directed to isolation of the regulatory subunit of histone kinase, which belongs to the important class of cyclic-AMP-binding proteins involved in processes of chromatin activation [5–7].

It seemed promising to use for this purpose affinity chromatography on an adsorbent containing an immobilized cyclic-AMP analogue. Specific adsorbents proposed previously were found to be inefficient since binding of a ligand via the exocyclic amino group of cyclic-AMP results in considerable decrease of affinity for the regulatory subunit [8]. Another essential shortcoming of the described adsorbents consisted in gradual elution of the nucleotide from the column owing to hydrolysis of the bonds binding the ligand to the matrix [9].

In this paper we describe the preparation of an adsorbent exhibiting high affinity for the regulatory subunit of histone kinase and stability under the conditions of affinity chromatography. Using this sorbent, it was possible to isolate homogeneous regulatory subunit from partially purified histone kinase.

2. Materials and methods

Sepharose 4B was purchased from Pharmacia, DCC from Fluka, [γ-^{32}P] ATP and cyclic-[^{3}H] AMP from Amersham. Histone F_1 was obtained by the procedure described by Jones [10] and purified on carboxymethyl cellulose [11]. The analogue of cyclic-AMP, 8-(γ-carboxypropylthio)-cyclic-AMP was synthesized as described in our previous publication [12]. MDCC and polylysine were obtained according to [13] and [14], respectively.

Crude histone kinase preparations were obtained from pig brain [4]. Phosphotransferase and cyclic-AMP-binding activities were measured by the assays described in [4].

2.1. *Preparation of the specific adsorbent*

Polylysyl-Sepharose was prepared by the method

* Abbreviations used: cyclic-AMP, adenosine-3′,5′-cyclic phosphate; ATP, adenosine triphosphate; DCC, dicyclohexylcarbodiimide; MDCC, 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine.

of Sica et al. [15]; it contained about 0.7 μmole of free amino groups per 1 ml of sedimented gel, as estimated by conductometric titration.

The general scheme of synthesis of the specific adsorbent is shown in fig. 1.

Equimolecular amounts of 8-(γ-carboxypropylthio)-cyclic-AMP (30 mg) and MDCC (20 mg) were dissolved in dioxane–water mixture (1:10) and evaporated to dryness. The residue was dissolved in 20 ml absolute dioxane and DCC (30 mg) was added. Polylysyl-Sepharose (10 ml of packed gel) was washed with 200 ml dioxane and added to the dioxane solution of the nucleotide in an equal volume. The mixture was shaken for 15 hr at room temperature, every 5 hr a new portion of DCC was added (2 × 30 mg). The gel was washed in sequence with 500 ml dioxane, 2 litres methanol–water mixture (8:2) and 500 ml ice water.

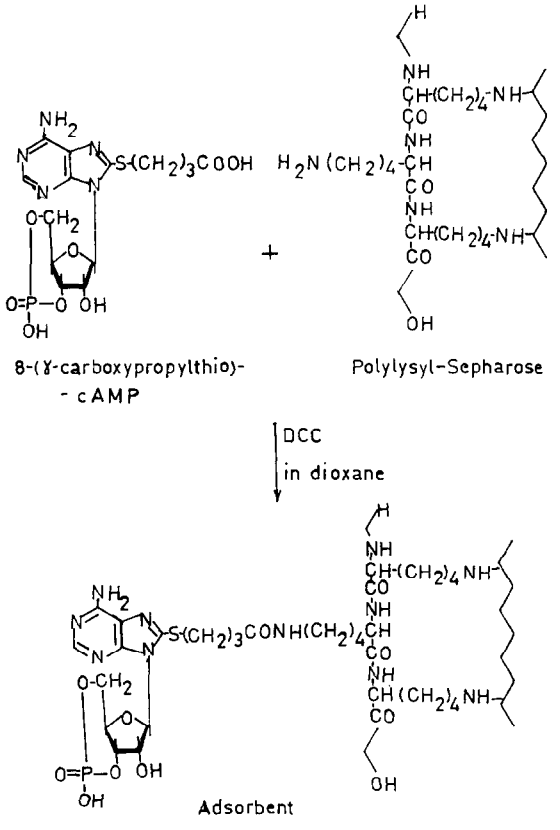

Fig. 1. Scheme of reaction between 8-(γ-carboxypropylthio)-cyclic-AMP and polylysyl-Sepharose.

The reaction was controlled by measuring spectrophotometrically the amount of unreacted nucleotide in washes. The content of analogue was about 5.5 μmoles per 1 ml gel.

2.2. *Affinity chromatography of the regulatory subunit of histone kinase*

Chromatography was carried out at +5°C. A column (3.5 × 0.5 cm) was equilibrated with 10 mM sodium phosphate buffer pH 6.0. The enzyme sample after step 4 ([4]; 30 mg of protein) was applied to the column. Washing was achieved by passing the same buffer at the rate 7.2 ml/hr (fraction volume, 1.8 ml). The protein was eluted with this buffer containing 200 mM NaCl, and thereafter with a NaCl concentration gradient (200–400 mM). Protein elution was monitored by the optical density at 280 nm; phosphotransferase (^{32}P incorporation into histone F_1) and cyclic-AMP-binding activaties were measured in the fractions.

3. Results and discussion

For the efficiency of affinity chromatography, two conditions should be met: stability of the bonds in binding of the immobilized ligand to the matrix, and high affinity of the ligand for the protein to be isolated. The use of polylysyl-Sepharose as a matrix [16,17] results in a considerable rise of stability of the adsorbent.

Position 8 of the purine ring was chosen as the site for modification, since substitution at this position did not result in loss of affinity for protein kinases [12, 18]. To be used as ligand, such an analogue should contain a sufficiently long aliphatic radical (an 'arm'), and a carboxyl group able to undergo condensation reaction with the ε-amino groups of polylysyl-Sepharose. 8-(γ-Carboxypropylthio)-cyclic-AMP previously obtained in our laboratory, satisfied these requirements [12].

Condensation of the analogue with polylysyl-Sepharose was carried out in absolute dioxane; DCC was used as the condensing agent. Choice of these conditions was determined by necessity to reach the highest possible degree of substitution in the ε-amino groups of polylysyl-Sepharose [16]. High degree of substitution made it possible considerably to increase capacity of the adsorbent obtained, and to minimise

ion exchange effects during chromatography. To increase the solubility of 8-(γ-carboxypropylthio)-cyclic-AMP in dioxane we added its MDCC salt to the incubation medium. The techniques suggested made it possible to achieve 80% completion of the condensation reaction, and to obtain a gel containing 5.5 μmoles nucleotide per ml. The gel was stable in sodium phosphate buffer, pH 6.0, for at least one week.

For the affinity chromatography we used a preparation of cyclic-AMP-dependent pig-brain histone kinase obtained after fractionation with ammonium sulphate; it contained about 3% of the enzyme [4]. The elution pattern is shown in fig. 2. One can see that on washing the column with sodium phosphate buffer, pH 6.0, the protein eluted was devoid of phosphotransferase and cyclic-AMP-binding activity. Elution with a buffer of increased ionic strength (200 mM NaCl) made it possible to remove the major part of accompanying proteins. This fact indicates that non-specific binding of some proteins takes place, apparently due to the charges on free ε-amino groups of polylysine. Upon passage of 200 mM NaCl, the elution of proteins occurred possessing almost all of the phosphotransferase activity which was cyclic-AMP-independent. This indicates dissociation of the histone kinase to subunits in the course of chromatography. Elution of the protein exhibiting cyclic-AMP-binding activity took place at higher NaCl concentrations (280 mM). After this the eluate did not contain any protein material. This proves the specific character of binding of the regulatory subunit. The yield was about 70–80% of the calculated amount.

Polyacrylamide-gel electrophoresis of the protein fractions showing cyclic-AMP-binding activity provided evidence for homogeneity of the protein obtained. The mol. wt. of the regulatory subunit, as determined by sodium dodecylsulphate-polyacrylamide-gel electrophoresis, was found to be 90 000, in agreement with our previous results obtained by gel filtration [4].

The data presented show that the cyclic-AMP-containing adsorbent used in this work is extremely efficient for affinity chromatography; its application resulted in isolation of homogeneous regulatory subunit of pig-brain histone kinase. In conclusion, it should be pointed out that this adsorbent may possibly be applicable for the isolation of other cyclic-AMP-binding proteins.

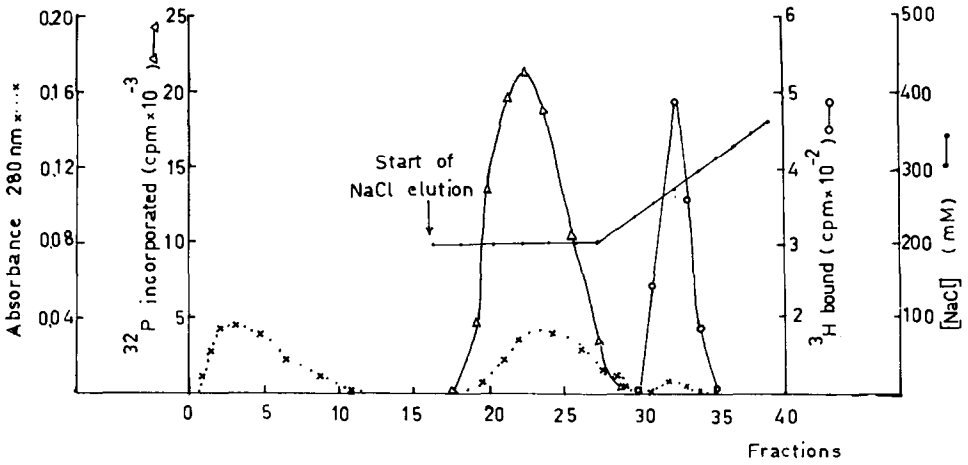

Fig. 2. Elution pattern of the preparation of the pig-brain histone kinase upon chromatography on cyclic-AMP-containing adsorbent. 30 mg protein were applied to the column, and eluted with sodium phosphate buffer, pH 6.0 (fractions 1–15), 200 mM NaCl (fractions 15–26) and NaCl concentration gradient (200–400 mM) (fractions 27–40). (X··X··X), optical density at 280 nm; (△–△–△) ^{32}P incorporation into histone F_1, cpm; (○–○–○), ^{3}H, incorporation into the protein, cpm; (–·–·–), NaCl concentration.

References

[1] Tao, M. and Hackett, P. (1973) J. Biol. Chem. 248, 5324–5332.
[2] Miyamoto, E., Kuo, J. F. and Greengard, P. (1969) J. Biol. Chem. 244, 6325–6402.
[3] Rubin, C. S., Erlichman, J. and Rosen, O. M. (1972) J. Biol. Chem. 247, 36–44.
[4] Nesterova, M. V., Sashchenko, L. P., Vasiliev, V. Yu. and Severin, E. S., Biochim. Biophys. Acta, in press.
[5] Kleinsmith, L. J., Allfrey, V. Y. and Mirsky, A. E. (1966) Science 154, 780–781.
[6] Balhorn, R., Bordwell, J., Sellers, L., Grammer, D. and Chalkley, R. (1972) Biochem. Biophys. Res. Commun. 49, 1326–1333.
[7] Lake, R. S. (1973) J. Cell. Biol. 58, 317–331.
[8] Wilchek, M., Solomon, J., Love, M. and Selinger, Z. (1971) Biochem. Biophys. Res. Commun. 45, 1177–1184.
[9] Tesser, G. J., Fisch, H. U. and Schwyzer, R. (1972) FEBS Lett. 23, 56–58.
[10] Johns, E. W. and Butler, J. A. V. (1962) Biochem. J., 82, 15–18.
[11] Johns, E. W. (1964) Biochem. J. 92, 55–59.
[12] Severin, E. S., Nesterova, M. V., Sashchenko, L. P., Rasumova, V. V., Tunitskaya, V. L., Kochetkov, S. N. and Gulyaev, N. N. Biochim. Biophys. Acta, in press.
[13] Moffat, J. G. and Khorana, H. G. (1961) J. Am. Chem. Soc. 83, 649–658.
[14] Fasman, G. D., Idelson, M. and Blout, E. R. (1961) J. Am. Chem. Soc. 83, 709–712.
[15] Sica, V., Parikh, I., Nola, E., Puca, G. A. and Cuatrecasas, P. S. (1973) J. Biol. Chem. 248, 6543–6558.
[16] Wilchek, M. (1973) FEBS Lett. 33, 70–72.
[17] Cuatrecasas, P., Parikh, I. and Hollenberg, M. D. (1973) Biochemistry 12, 4253–4264.
[18] Muneyama, K., Bauer, R. J., Shuman, D. A., Robins, R. K. and Simon, L. N. (1971) Biochemistry 10, 2390–2395.