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ISOLATION AND STUDY OF THE PROPERTIES OF THE REGULATORY SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE

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Cyclic AMP (cAMP) exerts its activating action on cAMP-dependent protein kinase through its regulatory subunit (R). Under the influence of cAMP the holoenzyme protein kinase dissociates according to the scheme:

$$R_2C_2 \xrightarrow{2cAMP} (R\cdot cAMP)_2 + 2C,$$

which leads to the appearance of the active catalytic subunit (C) [4].

It is also known that the regulatory subunit of type II cAMP-dependent protein kinase (R-II) contains four functional regions responsible for binding cAMP, for contact with C, and for forming the R=R bond in the holoenzyme, and an autophosphorylation site [6, 14, 15]. According to some workers, R is a single polypeptide chain, readily broken down by proteolytic enzymes into fragments, one of which preserves the cAMP binding center [13].

The investigation described below was a continuation of the study of protein kinases in the mucosa of the rabbit's small intestine [3]. To study the cAMP binding site the method of inhibitor analysis was used, with analogs of cAMP containing substitutents in different parts of the molecule.

EXPERIMENTAL METHOD

Binding of [^{3}H]-cAMP with the regulatory subunit was determined by the method in [7] in 100 µl of incubation medium containing 50 mM potassium phosphate buffer, pH 6.5, 2 mM NaCl, 5 mM theophylline, bovine serum albumin in a concentration of 1 mg/ml, and 4-7 µM of [^{3}H]-cAMP (2×10^5 to 4×10^5 cpm).

Electrophoresis in 12.5% polyacrylamide gel in the presence of sodium dodecylsulfate was carried out [9] in cylindrical (5 × 70 mm) gels with a g-250 and scanned at 600 nm in a Carl Zeiss (East Germany) densitometer.

The protein concentration was determined by the method in [12]. Protein solutions were concentrated by dialysis against 20% polyethyleneglycol solution (mol. wt. 40,000 daltons).

The 8-(2-hydroxyethylthio)-cAMP was immobilized on epoxy-activated sepharose by the method in [16]. The cAMP analogs were generously provided by N. N. Gulyaev.

EXPERIMENTAL RESULTS

The small intestine of a rabbit was removed and washed with cold physiological saline. The mucosa was curetted, frozen, and kept at −70°C. To obtain a cytosol, the frozen tissue

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TABLE 1. Isolation of Regulatory Subunit of Type II Protein Kinase from Mucosa of Rabbit's Small Intestine

Stage of purification	Protein, mg/ml	Binding activity, pmoles ^{3}H-cAMP/mg protein	Yield, %	Purification, number of times
105,000g supernatant	7800	2,8*	100	1
Treatment with CM-Sephadex	5600	3,6*	92	1,3
Chromatography on DEAE cellulose and affinity chromatography on hydroxyethylthio-cAMP-sepharose 6B	0,65	15 000†	45	5400

*Binding of cAMP determined with [^{3}H]-cAMP in a concentration of 0.25 μM for 1 h at 4°C.
†Binding of cAMP determined with [^{3}H]-cAMP in a concentration of 2 μM for 1 h at 20°C.

(200 g) was passed twice through a mincer, homogenized in 2.5 volumes of buffer containing 4mM EDTA, pH 7.0, and 10 mM mercaptoethanol, and centrifuged, first for 30 min at 20,000g, then for 90 min at 105,000g (all procedures were carried out at 4°C).

The resulting supernatant (volume 450 ml), whose electrical conductance was first adjusted to 5.5 mC with saturated KCl solution, was treated with CM-Sephadex, equilibrated with 10 mM potassium phosphate buffer, pH 6.7, containing 1 mM EDTA, 30 mM KCl, and 10 mM mercaptoethanol (buffer A) in the proportion of one volume of thick Sephadex suspension to six volumes of protein solution. The mixture was stirred on a magnetic mixer for 20 min and quickly freed from Sephadex by filtration on a Büchner funnel. The procedure was repeated five times with fresh portions of CM-Sephadex.

The protein solution obtained after treatment with CM-Sephadex (volume 800 ml) was treated with 150 ml of a dense suspension of DEAE-cellulose, equilibrated with buffer A. The mixture was stirred for 30 min, the resin was then allowed to settle, and the supernatant was poured off and the cellulose transferred to a 7 × 4 cm column. The column was washed with 10 mM potassium phosphate buffer, pH 6.7, containing 1 mM EDTA, 70 mM KCl, and 10 mM mercaptoethanol. Protein kinase II was eluted with 10 mM potassium phosphate buffer, pH 6.7, containing 1 mM EDTA, 400 mM KCl, and 10 mM mercaptoethanol, at the rate of 40 ml/h. The eluate was immediately applied to a 1.2 × 4 cm affinity column with immobilized cAMP. The affinity column was washed first with 10 mM potassium phosphate buffer, pH 6.7, containing 50 mM NaCl and 1 mM EDTA (buffer B), then with buffer B containing 2 M NaCl until zero absorption at 280 nm, and again with 50 ml of buffer B containing 10 mM AMP and 0.2 mM cGMP. The regulatory subunit was eluted at 20°C as follows: the column was filled with buffer B containing 30 mM cAMP, the affinity adsorbent was mixed, and left to incubate for 1 h at 20°C. The eluate was collected in 1-ml fractions. Fractions containing protein were pooled and concentrated to a volume of 2-3 ml. The results of purification of the R-II are given in Table 1.

To verify the purity of the preparation of regulatory subunit and to determine its molecular weight the method of disc electrophoresis in polyacrylamide gel in the presence of sodium dodecylsulfate was used. The preparation of R-II contained three protein fractions with mol. wt. of 36,000, 24,500, and 13,500 daltons respectively. Protein fractions with mol. wt. of 24,500 and 13,500 daltons, which were present in small quantities, evidently did not contain a cAMP-binding site, and for that reason most of them was lost during washing of the affinity column. The fraction with the highest molecular weight (36,000 daltons) accounted for about 70% of the total protein of the preparation and was evidently the cAMP-binding fragment of R-II (R-II').

It can be suggested that R-II', which is sensitive to the action of proteases, was degraded both during ion-exchange chromatography and during elution from the affinity column.

To study the cAMP-binding properties the R-II preparation obtained by affinity chromatography was freed from excess of cAMP. For this purpose the protein was applied to a 1.5 × 50 cm column with Sephadex G-25 equilibrated with buffer B. Gel filtration was carried out at 20°C at the rate of 20 ml/h. Fractions containing protein were collected and concentrated.

The cAMP-binding activity of R-II' was determined by the method in [7]. Maximal binding was achieved after incubation of the R-II for 2 h at 20°C in the presence of 4-7 μM [^{3}H]-cAMP and amounted to 17 nmoles cAMP/mg protein.

The apparent dissociation constant (K'_d) for cAMP was determined by the method in [10]. The character of the relationship obtained suggests the presence of two types of cAMP binding sites in the preparation with K'_d values of 8.8×10^{-8} and 9.1×10^{-7} M respectively. The number of sites with high affinity for cAMP was 40%. The presence of two types of cAMP binding sites can evidently be explained by the presence of phospho- and dephospho-forms of R-II' in the preparation. Such forms of R-II have been described in the literature both for native R-II and for its proteolytic fragment containing a cAMP binding site [5, 8].

The competitive action of cAMP analogs on binding of [^{3}H]-cAMP was studied with the cAMP-binding fragment of the regulatory subunit [11]. Analogs containing substituents in the 8- and N^6-positions of the adenine base [2] — the compounds 8-Br-cAMP, 8-(N-acetylaminoethylamino)-cAMP, and N^6-acetylaminoethyoxy-cAMP — had a fairly high affinity for the cAMP-binding site. Substitutions in the cyclophosphate part of the molecule, associated with removal of the negative charge from the oxygen atom [1], lowered the affinity of the analog for R-II' (8-Br-ethyl ester of cAMP) catastrophically. If, however, the negative charge was preserved, as in the case of the carboxymethyl ester of cAMP, affinity of the analog for R-II' remained quite high. Substitutions in the N-1 region of the purine ring (the compound N-1-methyoxy-cAMP) and of the 2'-hydroxyl group of the ribose (the compound 2'-deoxy-2'-amino-8-hydroxy-cAMP) substantially reduced the competitive power of the analog.

This investigation showed that there are a number of common features both in physicochemical properties and in structure of the cAMP-binding site of regulatory subunits of different cAMP-dependent protein kinases.

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