ISOLATION AND PROPERTIES OF THE CATALYTIC SUBUNIT OF CYCLIC AMP-DEPENDENT PROTEIN KINASE FROM RABBIT SMALL INTESTINAL MUCOSA

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The protein kinases constitute a large class of enzymes catalyzing the phosphorylation of protein substrates and performing important regulatory functions in the cell. There are several different types of protein kinases, differing in their method of regulation and in their substrate specificity. There are cyclic AMP- and cyclic GMP-dependent protein kinases, and also protein kinases independent of the action of cyclic nucleotides.

The problem of the specificity of many protein kinases to endogenous cell proteins and of the functions of the different types of protein kinases in the cell still remains unexplained. Of the whole wide class of protein kinases those which have been studied the most are the cyclic AMP-dependent protein kinases, which have been found in and purified from many animal tissues. Cyclic AMP-dependent protein kinases are located mainly in the cell cytosol [3, 11]. The molecule of a cyclic AMP-dependent protein kinase consists of two types of subunits: regulatory and catalytic [9, 14]. The free catalytic subunit, dissociating from its complex with the regulatory subunit under the influence of cyclic AMP, is the active form of the enzyme [6].

The object of this investigation was to study the protein kinase composition of the cytosol of the mucosa of the rabbit small intestine and also the properties of the catalytic subunit of the cyclic AMP-dependent protein kinase isolated from this source.

EXPERIMENTAL METHOD

Protein kinase activity was determined from the incorporation of radioactive phosphate from [γ-^{32}P]-ATP into protein substrate. The incubation medium in a final volume of 70 µl contained 50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 5 mM glycerol, 3 mM $MgCl_2$, 1 mM dithiothreitol, and 50 µM [γ-^{32}P]-ATP (1.5×10^5-3×10^5 cpm). Incubation was carried out at 30°C for 5-15 min. After the end of incubation aliquot samples of 50 µl were taken and applied to filters (dimension 18 × 18 mm, Whatman 3MM paper). The filters were washed twice in 10% TCA containing 10 mM Na_2HPO_4, and then 4 or 5 times in 5% TCA with the same additive. The filters were dehydrated in an acetone–ethanol (1:1) mixture and dried at 100°C. Radioactivity was counted in ZHS-106 toluene scintillator on a type SL 4000 liquid radiospectrometer (from Intertechnique, France).

Cyclic AMP binding was determined [4] in 100 µl of incubation medium containing 50 mM potassium phosphate, 2 M NaCl, and 5 mM theophylline in the presence of 400 nM ^{3}H-cyclic AMP (3×10^5-4×10^5 cpm).

Electrophoresis in polyacrylamide gel in the presence of sodium dodecylsulfate was carried out in cylindrical (measuring 5 × 70 mm) gels with a current 3 mA applied to the tube [10]. The protein concentration was determined by the method [16] and histones were isolated as in [8].

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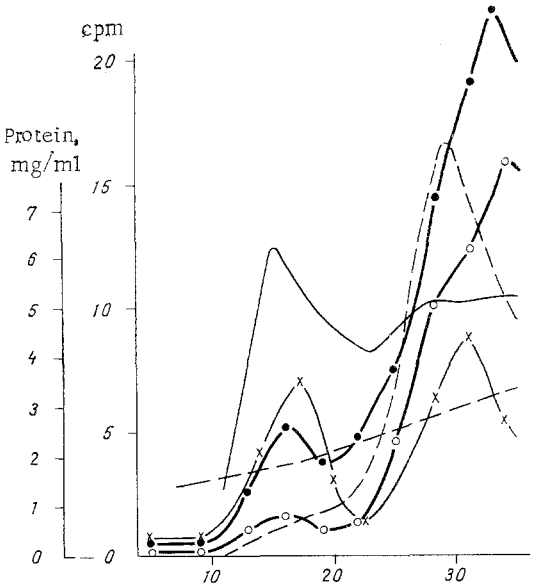

Fig. 1. Ion-exchange chromatography of protein kinases in cytosol from intestinal mucosa on DEAE-cellulose. Cytosol applied to column (1.2 × 15 cm) with DEAE-cellulose equilibrated with buffer A. Protein kinases eluted by NaCl gradient from 0 to 500 mM (V = 130 ml) at the rate of 25 ml/h. Fractions of 2 ml collected. Protein kinase activity determined in 5-µl aliquots with histone as substrate for 5 min and with casein for 15 min; binding with cyclic AMP determined in 20-µl aliquots for 1 h. 1) Protein; 2) electrical conductance; 3) phosphorylation of histone H_1 in presence of cyclic AMP; 5) phosphorylation of casein; 6) binding of ^{3}H-cyclic AMP. Abscissa, fractions; ordinate: on left — protein concentration (in mg/ml), on right — velocity (in cpm).

The rabbit's small intestine was removed and washed with cold physiological saline. The mucosa was curetted and homogenized in three volumes of buffer containing 4 mM EDTA, pH 7.0, 10 mM mercaptoethanol, 0.2 mM CMPP, 0.2 mM benzamidine, and 20 mM NaF (buffer A), and centrifuged for 30 min at 20,000g. The resulting cytosol was used for the later work.

EXPERIMENTAL RESULTS

The effect of cyclic nucleotides on protein kinase activity in the cytosol was studied by the use of histone H_1 and casein as substrates. Histone H_1 is a good substrate for cyclic AMP- and cyclic GMP-dependent protein kinases [17] and casein for some cyclic nucleotide-independent protein kinases [7]. Cyclic AMP stimulated phosphorylation of histone H_1 by 2-3 times, evidence that the cytosol from the small intestinal mucosa contains cyclic AMP-dependent protein kinase. Cyclic GMP, in a concentration of 1 µM, did not stimulate phosphorylation of histone H_1. This can be explained either by the low activity of the cyclic GMP-dependent protein kinase in the cytosol or by its insensitivity to cyclic GMP.

Phosphorylation of casein by the cytosol protein kinases was independent of the presence of cyclic AMP and cyclic GMP, and was evidently effected by a specific cyclic nucleotide-independent protein kinase.

The elution profile of the cytosol protein kinases from DEAE-cellulose is shown in Fig. 1. Clearly cyclic AMP-dependent protein kinase is represented in the mucosa by two types of enzyme, I and II, which were eluted from the ion-exchange resin by 50-60 mM (type I) and 130-150 mM (type II) NaCl, respectively. Measurements of activity of the protein kinases of types I and II in the presence of cyclic AMP showed it to be 10 and 90%, respectively.

TABLE 1. Phosphorylation of Different Substrates by Catalytic Subunit of Cyclic AMP-Dependent Protein Kinase from Intestinal Mucosa

Peptide substrate	Specific activity*		Specific activity †	
	nmoles · mg^{-1} · min^{-1}	%	nmoles · mg^{-1} · min^{-1}	%
Histones				
H_1	186	32	253	43
H_{2a}	71	12	81	14
H_2	580	100	580	100
H_3	6,9	1,2	—	—
H_4	0	0	0	0
Casein (3 mg/ml)	1,9	0,33	—	—
Protamine (1.6 mg/ml)	—	—	63	11

*Concentration of histones 100 μM.
†Histone concentration 200 μM.
Legend. Histone H_3 only sparingly soluble in incubation medium.

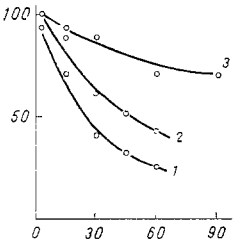

Fig. 2. The study of the protective action of ATP and Mg-ATP on catalytic subunit against inhibitory action of adenosine p-fluorosulfobenzoate (AFSB). Catalytic subunit incubated in presence of: 1) 0.05 mM AFSB, 2) 0.05 mM AFSB + 5 mM ATP, 3) 0.2 mM AFSB + 0.5 mM ATP + 10 mM $MgCl_2$. Abscissa, time (in min); ordinate, activity (in %).

It will also be clear from Fig. 1 that casein protein kinase is represented in the rabbit small intestinal mucosa by only one form, eluted by 110-120 mM NaCl. Cyclic GMP did not activate phosphorylation of histone H_1 in any of the fractions obtained after ion-exchange chromatography.

As was shown above, the type II cyclic AMP-dependent protein kinase is predominant in the cytosol from the rabbit small intestinal mucosa. To study the properties of this enzyme the catalytic subunit of the type II protein kinase was isolated in a homogeneous state. Cytosol obtained as described above from 200 g of mucosa was mixed with 200 ml DEAE-cellulose, equilibrated with 10 mM K-phosphate buffer, pH 6.7, containing 1 mM EDTA, 50 mM NaCl, and 10 mM mercaptoechanol (buffer B). A column (7 × 50 cm) was washed with 5 volumes of K-phosphate buffer (10 mM, pH 6.7) containing 1 mM EDTA, 80 mM NaCl, and 10 mM mercaptoethanol, and then with 15 volumes of buffer B. The catalytic subunit was eluted with buffer B containing 0.1 mM cyclic AMP. The eluate was the applied to a column (1 × 0.5 cm) with hydroxyapatite. The catalytic subunit was eluted from the hydroxyapatite with K-phosphate buffer (50 mM, pH

6.7) containing 500 mM NaCl, 1 mM EDTA, and 10 mM mercaptoethanol. The resulting enzyme was kept at 4°C in the same buffer.

The catalytic subunit II preparation obtained after specific elution from DEAE-cellulose and concentration on hydroxyapatite contained two protein fractions with mol. wt. of 40-42 and 78-82 kilodaltons, in the ratios of 40 to 60%, respectively. Gel-filtration of this preparation on a column with Sephacryl S-300 yielded a homogeneous catalytic subunit with mol. wt. of 39-40 kilodaltons. The substrate specificity of the catalytic subunits of the cyclic AMP-dependent protein kinase from the rabbit intestinal mucosa was studied with the aid of a number of known substrates of this enzyme (Table 1). As Table 1 shows, in its ability to phosphorylate the different substrates, the low-molecular-weight catalytic subunit which we isolated differed only a little from that of pig brain [15] and the high-molecular-weight subunit from rabbit skeletal muscles [13]. Values of K_m determined for histone H_1 (65 μM) and ATP (12 μM) also are close to those given in the literature [12].

Some analogs of ATP containing reactable groups in the triphosphate part of the molecule are known to block irreversibly the active center of the catalytic subunit of cyclic AMP-dependent protein kinases from different sources [1]. The use of specific protein kinase inhibitors is of great importance both when studying the structure of the active center of the enzyme and when developing preparations for clinical use based on substrate analogs as inhibitors. We studied the action of two ATP analogs containing reactable groups: chloromethylpyrophosphonate and adenosine p-fluorosulfobenzoate (AFSB). In a concentration of 0.25 mM AFSB reduced protein kinase activity by 80-90% during preincubation of the analog with the enzyme for 20-30 min. p-Fluorosulfobenzoic acid in the same concentration had only a very slight effect on enzyme activity, thus ruling out the possibility of any nonspecific action of the analog. The specificity of action of p-fluorosulfobenzoate (PFSB) with respect to the active center of the catalytic subunit is also confirmed by data on protection of the enzyme against inactivation by Mg-ATP, the substrate of the protein kinase reaction. As Fig. 2 shows, ATP itself had only a negligible protective action on inactivation of the catalytic subunit by PFSB. To define the inhibitory action of PFSB on the catalytic subunit of cyclic AMP-dependent protein kinase from intestinal mucosa, the inhibition constants and inactivation velocities of the enzyme through the action of the inhibitor were determined. The value of k_2, calculated by extrapolation to zero value of the reciprocal of inhibitor concentration was 0.051 min^{-1}, and the value of K_i was 0.1 mM. These values of the inhibition constants for PFSB are close to the values of the constants for this analog given by other workers [5, 18] for the catalytic subunit obtained from other sources.

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