THE ISOLATION AND CHARACTERISATION OF A DIMERIC CYCLIC AMP-DEPENDENT PROTEIN KINASE AND OF THE CORRESPONDING R-SUBUNIT

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1. Introduction

The cyclic AMP-dependent protein kinase are composed of 4 subunits, 2 monomeric catalytic ones (C) responsible for the phosphorylation of proteins and one dimeric regulatory (R) responsible for the cyclic AMP binding [1]. It is further demonstrated that two isoenzymes exist, named protein kinase I and II, each containing identical C-subunits (M_r 40 000) and different R-subunits, R_I (M_r 49 000) and R_{II} (M_r 54 000–56 000), respectively [2]. Besides the 2 R-subunits, digestion products of these regulatory proteins which also bind cyclic AMP have been detected [3–8].

There is also evidence that cyclic AMP-dependent protein kinases containing <4 subunits may be present in animal tissues or are formed during the isolation procedure of the tetrameric enzyme by proteolytic cleavage especially of the R-subunit [9,10].

During the isolation of a tetrameric protein kinase from bovine liver we found a cyclic AMP-dependent protein kinase which seems to be dimeric containing 1 C-subunit and 1 R-subunit with M_r 41 000 (R-41).

2. Materials and methods

[γ-^{32}P]ATP and cyclic [8-^{3}H]AMP were purchased from Amersham Buchler GmbH and CNBr-activated Sepharose 4B from Pharmacia. Ultrogel AcA 34 was obtained from LKB.

Protein kinase activity was determined as in [9] and the cyclic AMP binding capacity according to [3] at 0°C. The 8-(6-aminohexyl)amino-cyclic AMP was prepared according to [11] and coupled to the CNBr-activated Sepharose 4B as in [12]. The procedure in [13] was applied for the polyacrylamide gel electrophoresis and in the presence of SDS that in [14]. Protein concentrations were determined according to [15], with bovine serum albumin as a standard. The Stokes radius (r_S) and frictional ratio was calculated according to [16]. Sucrose density centrifugation was performed according to [17].

3. Results

3.1. *Isolation of the dimeric protein kinase*

For the isolation of the dimeric protein kinase (R-41–C) from bovine liver the procedure in [18] for the tetrameric cyclic AMP-dependent protein kinase was used until step 5. At step 6, when the protein kinases were adsorbed to hydroxylapatite the R-41–C protein kinase was eluted with 160 mM Na_2HPO_4 (pH 7.0) containing 15 mM 2-mercaptoethanol (a 250 mM Na_2HPO_4 buffer (pH 7.0) was necessary for the tetrameric enzyme). The protein kinase containing fractions were collected and the proteins precipitated by the addition of $(NH_4)_2SO_4$ (70% saturation). The precipitate was collected by centrifugation and dissolved in a minimum of 30 mM Na_2HPO_4 (pH 7.0) containing 15 mM 2-mercaptoethanol (buffer B). After dialysis the solution was applied to molecular sieve column (Ultrogel AcA 34, 3 × 140 cm), equilibrated with buffer B containing 100 mM NaCl. The elution profile showed 2 zones with protein kinase activity: (1) due to traces of the tetrameric protein kinase; (2) corresponding to R-41–C. The fractions of the zone (2) were collected and used for characterisation of R-41–C.

3.2. *Isolation of the R-41 subunit*

For the isolation of the R-41 subunit the R-41–C containing fractions were combined and applied onto

a small column (1 ml), containing 8-(6-aminohexyl)-amino-cyclic AMP–Sepharose 4B (5 μmol cyclic AMP/ml matrix). The column was washed with buffer B containing 2 M NaCl. After incubation of the matrix with buffer B containing 10 mM cyclic AMP at 20°C for 2 h the R-41 subunit was eluted. R-41 subunit (~0.5 mg) was obtained from 2.5 kg bovine liver.

3.3. *Properties of the R-41–C protein kinase*

In the presence of 1 μM cyclic AMP the phosphotransferase activity of the R-41–C protein kinase could be stimulated 5-fold. By molecular sieve chromatography r_S 40 Å (fig. 1) was determined and by sucrose density centrifugation a $s_{20,w}$ 4.9 S. By using these data M_r 86 000 could be calculated. With regard to M_r 40 000 for the C and 41 000 for the R-41 subunit, a dimeric structure had to be postulated.

To guarantee that the R-41 subunit is really present in the R-41–C protein the enzyme solution was incubated in the presence of Mg-[γ-^{32}P]-ATP. Under these conditions the R-subunit of the

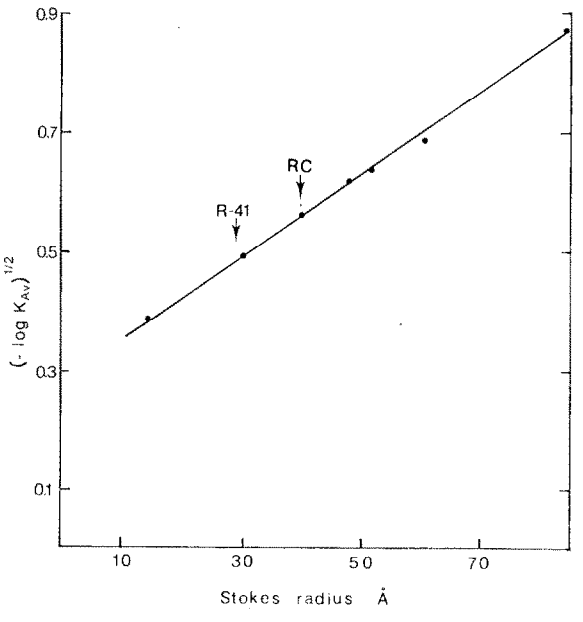

Fig.1. Determination of the Stokes radii on an Ultrogel AcA 34 column (1.5 × 120 cm), equilibrated with buffer B containing 100 mM NaCl. The flow rate was 8 ml/h and 2 ml fractions were collected. R-41–C, dimeric protein kinase; R-41, cyclic AMP binding protein. Calibration proteins (Stokes radius): myoglobin (14 Å), ovalbumin (29.5 Å), glyceraldehyde 3-phosphate dehydrogenase (40.5 Å), aldolase (48 Å), catalase (52 Å), urease (61.5 Å) and thyroglobulin (85 Å).

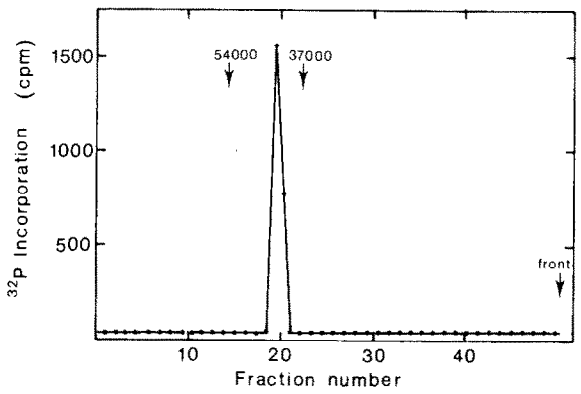

Fig.2. Incubation of the R-41–C protein kinase with Mg-[γ-^{32}P]ATP. R-41–C (50 μg) incubated for 10 min at 0°C with 10 nmol [γ-^{32}P]ATP (100 000 cpm/nmol) and Mg^{2+}. The reaction was stopped by addition of SDS and 2-mercaptoethanol (final conc. 1% each). The solution was incubated for 3 min at 95°C and then a SDS gel electrophoresis was performed. After staining and destaining the gel was cut into 2 mm slices and the radioactivity was determined.

tetrameric protein kinase was phosphorylated autocatalytically [19,20]. After SDS gel electrophoresis of the incubation mixture radioactivity could only be detected in the zone corresponding to M_r 41 000 (fig.2).

3.4. *Properties of the R-41 subunit*

3.4.1. Purity

By polyacrylamide gel electrophoresis (7.5%) and SDS gel electrophoresis only 1 protein zone could be detected (fig.3).

3.4.2. Molecular parameters

By SDS gel electrophoresis M_r 41 000 was estimated. A r_S = 29 Å was determined by molecular sieve chromatography (fig.1) and $s_{20,w}$ = 2.8 S by sucrose density centrifugation. Assuming the same partial specific volume ($\bar{\nu}$) of 0.724 (cm^3/g) as determined for the R-subunit [18] M_r 34 000 was obtained. This value was lower than those found by SDS gel electrophoresis. Inaccuracy of the method and a too low $\bar{\nu}$ might explain the discrepancy. According to [21] an axial ratio of 1:7 was determined with a frictional ratio (f/f_o) of 1.36.

3.4.3. Cyclic AMP binding sites

The R-41 subunit was incubated with an excess of

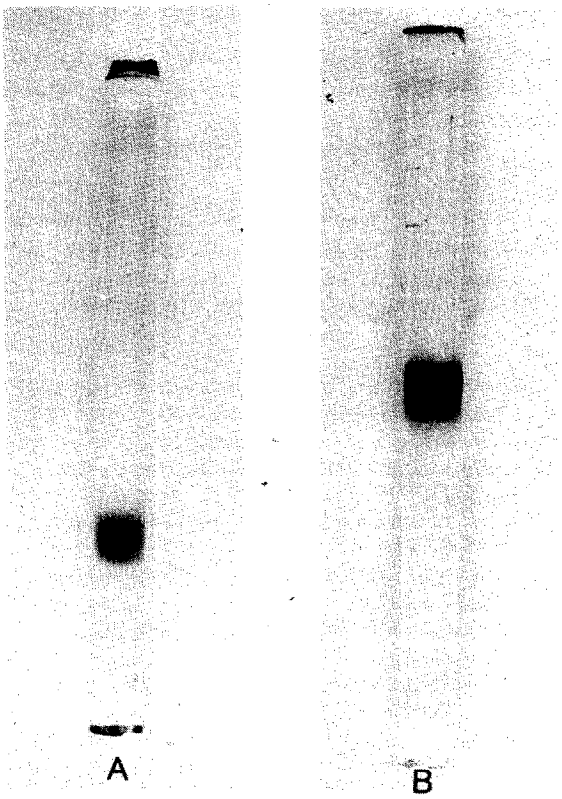

Fig.3. Polyacrylamide gel electrophoresis of the R-41 subunit: (A) 12 μg protein in the absence; (B) 7 μg protein in the presence of SDS.

cyclic [^{3}H]AMP then the solution was exhaustively dialysed. Radioactivity and protein concentration were determined. Under these conditions 2 cyclic AMP binding sites could be detected for the R-41 subunit.

3.4.4. Inhibition of the C-subunit

As shown for the dimeric R-subunit the catalytic activity of the C-subunit was depressed by the R-subunit due to association forming the tetrameric protein kinase [8,12]. Similar results were determined for the R-41 subunit (fig.4). Inhibition of >50% could be observed. However, from the data cited above a dimeric structure must be formed.

3.4.5. Phosphorylation of the R-41 subunit

If the R-41 subunit was incubated with small amounts of the C-subunit in the presence of Mg-[γ-^{32}P]ATP the protein was phosphorylated (see also section 3.3 and fig.2).

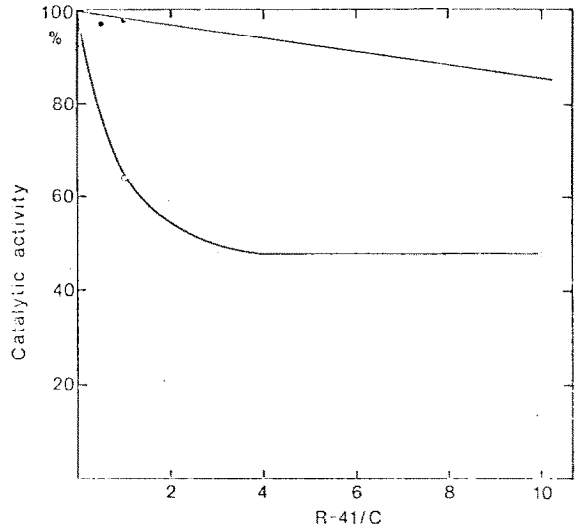

Fig.4. Inhibition of the catalytic activity by the R-41 subunit. A purified C-subunit (bovine liver, 1.5 pmol) was incubated for 30 min with increasing amounts of R-41 subunit at 4°C. Finally the phosphotransferase activity was tested in the presence (•) and in the absence (○) of cyclic AMP (1 μM). The activity of the C-subunit alone was specified as 100%.

4. Discussion

The new protein kinase which was isolated from bovine liver is composed of 1 C-subunit (M_r 40 000) and 1 R-subunit with M_r 41 000 determined by SDS gel electrophoresis. Like the tetrameric enzyme the activity could be stimulated by the addition of cyclic AMP. Whether the new protein kinase is present in bovine liver as a native enzyme with special functions different from those of the other protein kinases, or whether it is formed from a tetrameric protein kinase by proteolysis, could not be established.

If we compare the data of the R-41 subunit with those obtained for the R_{II}-subunit the following similarities are evident:

(1) Both subunits possess 2 binding sites for cyclic AMP.
(2) They are Mg-ATP-dependent phosphorylated in the presence of C-subunit.
(3) The phosphotransferase activity of the C-subunit can be inhibited by both R-proteins.

However, the R-subunit is isolated as a dimeric form in contrast to the monomeric R-41 subunit. This may be a hint that the amino acid sequence necessary for recognition and binding of the homologous R-subunit is missing in the R-41 subunit or may have been

removed by proteolysis of the dimeric R-subunit. It is also possible that the removal of ~110–120 amino acids changed the conformation drastically, making the association impossible. Compared with the tryptic derivate of the R_{II}-subunit showing M_r 37 000 by SDS gel electrophoresis the R-41 subunit contains the same number of cyclic AMP binding sites contains phosphate and is a monomer [22]. However, in contrast to the tryptic digestion product the R-41 subunit can be further phosphorylated and can associate with the C-subunit. This means that the R-41 protein contains the amino sequence or the correct conformation necessary for binding C-subunit and for phosphorylation.

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