ISOLATION OF A CYCLIC AMP-ADENOSINE BINDING PROTEIN FROM RAT HEART

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1. Introduction

Adenosine has been shown to have a number of actions on the cardiovascular system [1–4]. It relaxes smooth muscles resulting in dilation of coronary arteries and exercises a local control of blood flow in relation to myocardial oxygen needs [5,6].

It is not known how adenosine causes vasodilation and how it exercises its effects on vascular smooth muscle. However, adenosine has been shown to augment the intracellular cAMP level in the ventricular myocardium [6]; moreover, in a number of tissues it has been possible to demonstrate direct stimulation of adenylate cyclase by adenosine [7].

On the other hand, cAMP and adenosine appear to be interrelated also at the level of recently discovered cAMP-adenosine binding factors [8–10], among which the binding activity of the mouse liver protein, that depends on a process of 'activation' which has already been described in some detail [10]. Therefore we searched directly for a protein capable of binding cAMP and adenosine in the heart, a tissue in which adenosine is thought to act as a physiological regulator.

Here we report the purification by affinity chromatography to apparent homogeneity of a cAMP-adenosine binding protein from the soluble fraction of rat heart. Recently, data have also been reported suggesting that the cAMP-adenosine binding protein from human placenta [10] and mouse liver [11] is associated with SAH hydrolase activity. The protein from rat heart, purified by affinity chromatography, was devoid of SAH hydrolase activity.

Abbreviations: SAH, *S*-adenosyl-L-homocysteine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

2. Experimental

2.1. *Materials*

[2-^{3}H]Adenosine (22 Ci/mmol), [8-^{3}H]adenosine 3′,5′cyclic phosphate (30 Ci/mmol) and adenosine 5′-[γ-^{32}P]triphosphate (13.8 Ci/mmol) were obtained from the Radiochemical Centre. Millipore filters (HAWP 0.45 μm) were obtained from Millipore S. A. All other chemicals were of analytical grade.

2.2. *Thin-layer chromatography*

Thin-layer chromatography was carried out on Cellulose F glass plates (Merck) (20 × 20). The chromatographic system used was: *n*-butanol–methanol–water–NH_3 (60:20:19:1). Adenosine (R_F 0.6); SAH (R_F 0.15).

2.3. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis was carried out in the absence or presence of sodium dodecyl sulphate according to [12,13] on 5% polyacrylamide gels.

2.4. *Sucrose density gradient*

The sucrose density gradient centrifugation was performed according to [14].

2.5. *Assay for SAH hydrolase activity*

The enzyme activity was assayed in the direction of synthesis according to [10]. The reaction mixtures contained in 0.05 ml: 100 μM [^{3}H]adenosine; 5 mM L-homocysteine (prepared immediately before use by mild alkaline hydrolysis of the thiolactone) and 25 mM potassium phosphate buffer (pH 7.0); containing 1 mM EDTA and 2 mM 2-mercaptoethanol. The reaction was started by addition of the protein. Incubations were performed for 10 min at 37°C and

stopped by addition of 5 μl 8 M formic acid. A 20 μl portion was then applied to a Cellulose thin-layer chromatographic plate along with an unlabeled SAH marker and developed as described above. The SAH spots were visualized under ultraviolet light, scraped off and counted by liquid scintillation.

2.6. *Measurement of cyclic [^{3}H]AMP and [^{3}H]adenosine binding*

Cyclic AMP and adenosine binding activity was measured essentially according to [9] as follows. Samples (20 μl) of the solutions to be assayed were incubated in presence of 10 μM cyclic [2-^{3}H]AMP (0.6 Ci/mmol); 150 mM KCl and 10 mM magnesium acetate in 15 mM Hepes buffer (pH 7.0) or 10 μM [8-^{3}H]adenosine (1.48 Ci/mmol) and 100 μM unlabeled cAMP in 15 mM Hepes buffer (pH 7.0). In the inhibition assays, samples were incubated also in the presence of 50 μM 9-(*p*-aminobenzyl) or 9-(*p*-acetamidobenzyl)adenine in 10% DMSO (final). When the first steps of purification were assayed, 1 mM theophylline was added to inhibit phosphodiesterase activity. Incubations were performed at 30°C for 60 min in 120 μl. All incubations were stopped by adding 2 ml ice-cold 80% saturated ammonium sulphate containing 100 μM unlabeled cAMP or adenosine. After blending in a Vortex mixer, the protein was allowed to precipitate at 0°C for 1 h, then collected by suction through Millipore filters. The filters were washed 3 times with 2 ml 60% saturated ammonium sulphate and put into scintillation vials. The precipitate was dissolved in 1 ml 1% SDS and 10 ml Insta-gel were added.

2.7. *Protein assay*

The estimation of protein was based on the method in [15]. Bovine serum albumine was utilized as standard.

2.8. *Assay for enzyme activity*

Protein kinase activity was assayed essentially as in [16] by determining the amount of phosphate incorporated into histone (2.5 mg/ml) or casein (1.5 mg/ml) from [γ-^{32}]ATP (60 μM). Adenosine deaminase activity and nucleoside phosphorylase activity were assayed according to [17]. Phosphodiesterase activity was assayed according to [18].

2.9. *Synthesis of the specific adsorbent*

The adsorbent was synthesized by coupling the amino group of 9-(*p*-aminobenzyl)adenine to the carboxilate groups of succinylaminopropylaminopropyl–Sepharose in presence of the water-soluble carbodiimide: cyclohexyl-3-(2-morpholinoethyl)carbodiimide methyl-*p*-toluensulfonate, as described [19,20].

3. Results and discussion

The preliminary steps of purification of the binding protein were carried out according to [9] until DEAE-cellulose chromatography as follows: hearts (46 g) were frozen into liquid N_2 then homogenized in a Waring blendor in 15 mM Tris–HCl (pH 7.6) containing 4 mM EDTA and 5 mM 2-mercaptoethanol (1/4, w/v). The homogenate was centrifuged for 40 min at 20 000 × *g*. The supernatant solution was precipitated with polyethylene glycol 6000 (11 g/100 ml), centrifuged, the pellet discarded and polyethylene glycol 6000 (13 g/100 ml) was again added to the supernatant.

After centrifugation (20 000 × *g* for 40 min), the pellet so obtained was dissolved in 10 mM Tris–HCl (pH 7.5), 4 mM EDTA and 5 mM 2-mercaptoethanol. The solution was then applied to a DEAE-cellulose column (1.6 × 12 cm), equilibrated and eluted with the same buffer. The fractions containing the binding activity were pooled and exhaustively dialyzed against 10 mM Tris–HCl (pH 7.2), 4 mM EDTA and 5 mM 2-mercaptoethanol. Most of the SAH hydrolase activity of the crude extract was present in this pool. On the contrary, adenosine deaminase activity was absent in the pooled fractions after DEAE-cellulose.

Because 9-(*p*-aminobenzyl) and 9-(*p*-acetamidobenzyl)adenine were powerful inhibitors of adenosine binding activity (50 μM caused an inhibition of 70% in the standard assay (section 2), we used the resin synthesized as above described for purifying the binding protein by affinity chromatography. The dialyzed solution from DEAE was then applied to the affinity column (1.6 × 13 cm) equilibrated with 10 mM Tris–HCl (pH 7.2), 4 mM EDTA and 5 mM 2-mercaptoethanol and exhaustively washed with the same buffer. In the washings no binding activity was recovered. The protein was then eluted with 4 mM guanyl–urea in 10 mM Tris–HCl (pH 8.0), 4 mM

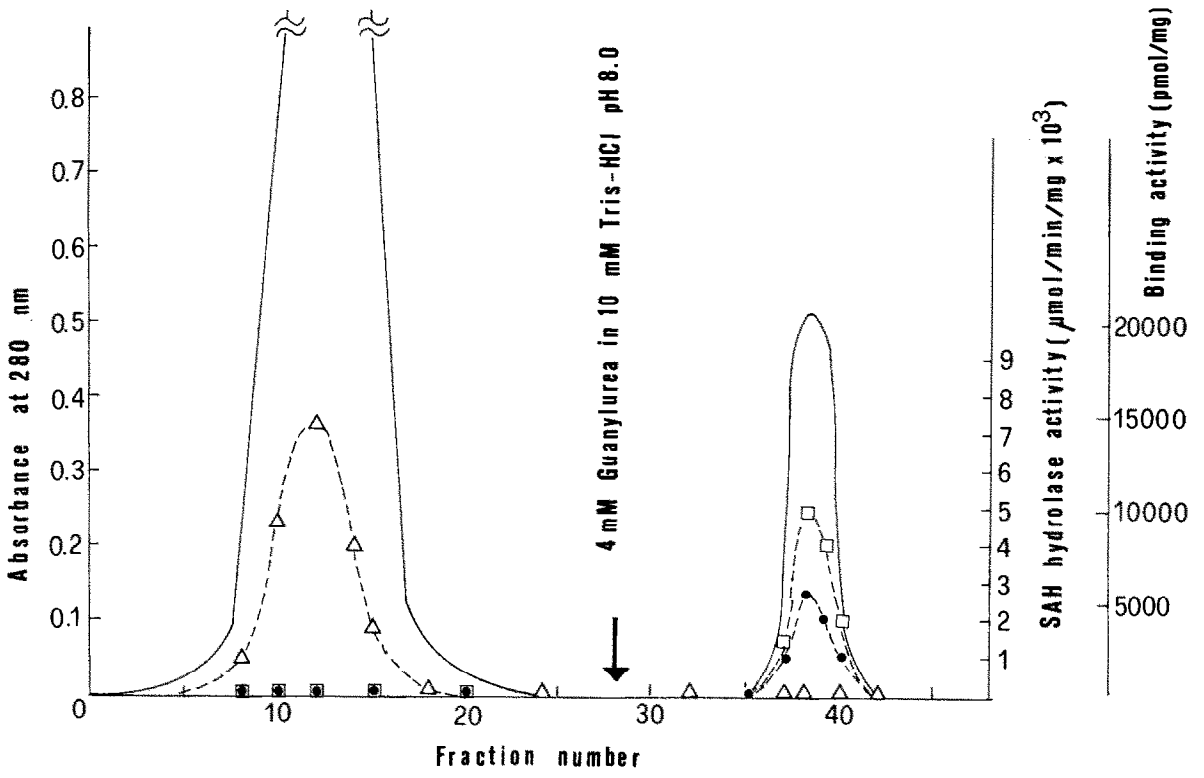

Fig.1. Affinity chromatography elution profile for rat heart cAMP-adenosine binding protein. The column (1.6 × 13 cm) was equilibrated with 10 mM Tris–HCl (pH 7.2) containing 4 mM EDTA and 5 mM 2-mercaptoethanol. Elution was performed using 4 mM guanyl-urea in 10 mM Tris–HCl (pH 8.0) 4 mM EDTA and 5 mM 2-mercaptoethanol. A_{280} (——). SAH hydrolase activity μmol . min^{-1} . mg^{-1} . 10^3 (△——△). cAMP binding activity (pmol bound/mg protein •——•). Adenosine binding activity (pmol bound/mg protein (□——□).

EDTA and 5 mM 2-mercaptoethanol with a recovery of nearly 100%. Guanyl–urea is a powerful inhibitor of adenosine binding to the protein (guanyl–urea) (at 50 μM caused 77% inhibition of adenosine binding in the conditions of section 2). In fig.1 the elution pattern for the cAMP-adenosine binding protein was reported. No SAH hydrolase activity was found in the elution peak; all the enzymatic activity present in the dialyzed fraction from DEAE was recovered in the washings, as shown in fig.1. The fractions containing the binding protein eluted with 4 mM guanyl–urea were pooled and dialyzed against 10 mM Tris–HCl (pH 7.2), 4 mM EDTA and 5 mM 2-mercaptoethanol.

The washings from the affinity column containing SAH hydrolase were incubated in the presence of 5 mM L-homocysteine and [^{3}H]adenosine under the above conditions, except that adenosine was varied from 0.3–100 μM. The double reciprocal plot for SAH hydrolase activity versus the concentration of adenosine was linear and consistent with K_m 6.5 × 10^{-7} M (fig.2), which is in agreement with the value reported in [10]. When assayed in the presence of 9-(*p*-aminobenzyl)adenine (50 μM), the K_m value for adenosine was the same indicating that there is no competition for the binding of adenosine to the enzyme and that there is no inhibition of SAH hydrolase activity (fig.2). Therefore SAH hydrolase was not retained on the affinity column.

The cAMP-adenosine binding protein from rat heart was devoid of adenosine deaminase, nucleoside phosphorylase, phosphodiesterase and protein kinase activity and shows spec. act. 8800 pmol adenosine

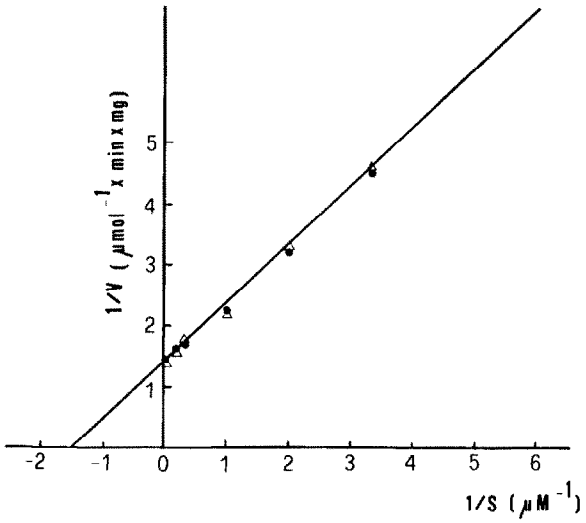

Fig.2. Double reciprocal plot for SAH hydrolase activity versus the concentration of adenosine. The enzyme activity was measured under the conditions in section 2.5; adenosine was varied from 0.3–100 μM in the absence (●——●) and presence (△——△) of 50 μM 9-(*p*-aminobenzyl) adenine.

bound/mg protein and 5120 pmol cAMP bound/mg protein. The binding protein was purified to apparent homogeneity as judged by polyacrylamide gel electrophoresis in the absence and presence of sodium dodecyl sulphate. A sharp single band was observed. The purified protein sedimented as a sharp single peak in a sucrose density gradient. The native protein was mol. wt 176 000 (data not shown). Judged by SDS–polyacrylamide gel electrophoresis the subunit was mol. wt 43 000 (data not shown). These data are in agreement with the tetrameric structure previously suggested for the mouse liver protein.

However, it should be noted that the cAMP-adenosine binding protein and SAH hydrolase from rat heart show different structural requirements; in fact, the absence of inhibition of SAH hydrolase activity by 9-(*p*-aminobenzyl)adenine and, on the contrary, the high inhibitory effectiveness of the same compound towards adenosine binding to the cAMP-adenosine binding protein, strongly indicates that the adenosine binding site of the two proteins differs for the purine and 'not purine' moieties of the adenine nucleoside or its analogues.

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