Biochimica et Biophysica Acta, 585 (1979) 512—526

BBA 28939

CYCLIC AMP-ADENOSINE BINDING PROTEIN/*S*-ADENOSYLHOMOCYSTEINASE FROM MOUSE LIVER

A FRACTION OF ADENOSINE BOUND IS CONVERTED TO ADENINE

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(Received November 3rd, 1978)

Key words: Adenosine hydrolysis; S-Adenosylhomocysteinase; Cyclic AMP-adenosine binding protein

Summary

1. Adenosine bound to the cyclic AMP-adenosine binding protein/*S*-adenosylhomocysteinase from mouse liver was partly converted to a product which was identified as adenine in four chromatographic systems. Ribose was formed in equivalent amounts.

2. The time course of the reaction was characterized by an initial burst phase lasting for less than one second followed by a slow progressive phase. The reaction was partly reversed by prolonged incubation, slow denaturation of the protein, dilution of the incubation mixture and removal of adenosine by converting it to inosine by the enzyme adenosine deaminase.

3. Both the ATP-treated (Ueland, P.M. and Døskeland, S.O. (1978) Arch. Biochem. Biophys. 185, 195—203) and the non-treated protein were subjected to polyacrylamide gel electrophoresis at pH 8.8. The adenosine-adenine, the cyclic AMP binding activities and the conversion activity comigrated with the main protein band, indicating that these properties reside on the same protein molecule.

4. Adenine generated by hydrolysis of adenosine was mainly bound to the protein as judged by nearly complete reversion of the conversion upon dilution in the presence of excess unlabelled adenine and by Sephadex G-25 chromatography.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid.

5. The conversion of adenosine to inosine by the enzyme adenosine deaminase was decreased in the presence of the binding protein.

6. Adenine formation could also be demonstrated under condition of enzymic formation of S-adenosylhomocysteine, i.e. in the presence of homocysteine.

Introduction

A class of proteins not associated with protein kinase activity has been identified in various tissues because of their ability to bind cyclic AMP [1—7]. Some reports have appeared showing that these cyclic AMP binding proteins bind adenosine with high specificity [3,6,8]. The cyclic AMP-adenosine binding protein(s) also interact(s) with a wide variety of adenine derivatives [4,6,8, 9] although cyclic AMP and adenosine seem to have the highest affinity towards the protein [4,8,9].

We have purified to apparent homogeneity a cyclic AMP-adenosine binding protein from mouse liver [8] and a similar protein from bovine adrenal cortex [10]. A rather detailed picture of the molecular properties of the cyclic AMP-adenosine binding protein from mouse liver was emerged [8,11]. Data on the interaction between this protein and cyclic AMP, adenosine, adenine and adenine nucleotides have been presented [8,9,12,13]. The protein could be differentiated from the cyclic AMP binding moiety of the cyclic AMP dependent protein kinase and from phosphofructokinase [8]. Recently, it has been shown that the binding protein is associated with *S*-adenosylhomocysteinase activity [14].

In the present paper data are presented suggesting that a fraction of adenosine bound is hydrolyzed to adenine and ribose. Adenine formation could also be demonstrated under the condition where *S*-adenosylhomocysteine is formed from adenosine and homocysteine.

Materials and Methods

Chemicals. Hepes, AMP, ADP, ATP, cyclic AMP, hypoxanthine, inosine, *S*-adenosyl-L-homocysteine, DL-homocysteine, ribose 1-phosphate (dicyclohexylammonium salt) and ribose were obtained from Sigma Chemical Co., St. Louis, U.S.A. Polyethyleneimine-impregnated cellulose thin-layer sheets (Polygram Cel 300 PEI, 0.1 mm) were from Macherey Nagel and Co., F.R.G. and TLC plastic sheets coated with silica gel 60 F_{254} (0.25 mm) from Merck, Darmstadt. [2-^{3}H]Adenine (20 Ci/mmol), [2-^{3}H]adenosine (27 Ci/mmol), [2-^{3}H]-adenosine 5′-monophosphate (21 Ci/mmol), [2-^{3}H]adenosine 5′-diphosphate (20 Ci/mmol), cyclic [8-^{3}H]AMP (21 Ci/mmol) and [U-^{14}C]adenosine (0.5 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, and the purity was checked by thin-layer chromatography.

Thin-layer chromatography. Five chromatographic systems (A—E) were used. Systems A, B and C are defined as chromatography on PEI-cellulose thin-layer sheets developed in 1.2 M LiCl (system A), 0.5 M ammonium acetate/96% ethanol (5 : 2, v/v) (system B) or distilled water (system C). System D refers to

chromatography on silica gel thin-layer sheets using 0.5 M ammonium acetate/96% ethanol (5 : 2, v/v) as solvent. Adenine (R_F = 0.61), adenosine (R_F = 0.52), ribose (R_F = 0.41) and ribose 1-phosphate (R_F = 0.10) were separated on PEI-cellulose plates developed in isobutanol/ethanol/water (2 : 1 : 1, v/v) (system E). Reducing sugars were identified by spraying the dried chromatogram with anilinephthalate and heating for 10 min at 110°C.

Chromatography was carried out at room temperature.

Purification of cyclic AMP-adenosine binding protein/S-adenosylhomocysteinase. This was carried out by a slight modification [9] of a procedure described previously [8]. The protein was homogeneous as judged by polyacrylamide gel electrophoresis in the absence and presence of sodium dodecyl sulfate [8].

Purification of nucleotides. AMP, ADP and ATP were purified as described [9].

Measurement of the conversion of adenosine to adenine. Binding protein was incubated in the presence of [^{3}H]adenosine under conditions described in the legends to the separate figures. Aliquots of 25 μl from the incubation mixture were mixed with 25 μl of 0.8 M perchloric acid containing 1 mM adenine, adenosine and inosine. After 5 min at 0°C, the acid was neutralized by adding 50 μl of ice-cold 0.36 M KOH containing 0.30 M $KHCO_3$ and the mixture was left at 0°C for 30 min. The precipitate was removed by centrifugation and samples (20 μl) from the supernatant (pH 7.5) were subjected to thin-layer chromatography in system A. The chromatographic spots were visualized by absorption in ultraviolet light. The spots corresponding to adenine, adenosine and inosine were cut out and put in scintillation vials containing 1 ml of 1 M HCl. After 60 min, 7 ml Dilusolve (Packard) were added. The radioactivity was recorded in a Packard scintillation counter.

The recovery of [^{3}H]adenosine and [^{3}H]adenine eluted from the thin-layer cellulose sheets developed in system A, was 95% and 93%, respectively.

Assay for enzyme activities. Adenosine phosphorylase (EC 2.4.2.-) activity was tested for as follows. Binding protein (100 μg/ml) was incubated in the presence of 1 mM ribose 1-phosphate, 0.1 or 1 or 10 μM [^{3}H]adenine (21 Ci/mmol) in 15 mM Hepes, pH 7.0, containing 10 mM magnesium acetate and 150 mM KCl. The incubations were run for 5, 30 and 60 min at 30°C. Adenosine and adenine were separated by thin-layer chromatography in system A and B.

Adenosine deaminase activity was determined by a radioisotope technique which measures the conversion of [^{3}H]adenosine to [^{3}H]inosine which were separated by thin-layer chromatography in system A. *S*-Adenosylhomocysteinase (EC 3.3.1.1) activity was assayed as described previously [14].

Polyacrylamide gel electrophoresis. This was performed at pH 8.8 (15 mM Tris/glycine) and at 6, 7.5 and 9% gel. The protein (96 μg) in 20% sucrose was applied to the top of the gel in 0.7 × 8.5 cm tubes. The electrophoresis proceeded at 4°C for 1—4 h at 3 mA per gel. The gels were either stained in Coomassie blue and destained in 7% acetic acid containing 40% methanol or cut into slices (1.7 mm) with a gel cutter. The slices were homogenized in 150 μl of 15 mM Hepes, pH 7.0, containing 0.4% bovine serum albumin and eluted for 36 h at 0°C.

Determination of protein. This was performed by the method of Klungsøyr [15] using bovine serum albumin as standards.

Results

Conversion of adenosine to adenine. Cyclic [^{3}H]AMP (2 μM), [^{3}H]AMP (100 μM), [^{3}H]ADP (100 μM) or [^{3}H]adenosine (1 μM) were incubated for 30 min at 30°C in the presence of binding protein (100 μg/ml). The incubation buffer was 15 mM Hepes, pH 7.0, containing 5 mM magnesium acetate and 150 mM KCl. The incubations were terminated as described under Materials and Methods and analyzed by chromatography in systems A and B. Adenosine alone was metabolized. [^{3}H]Adenosine (40%) was converted to a radioactive substance which was identified as adenine in four chromatographic systems (Table I). The conversion was not affected by the presence of 50 mM sodium phosphate (data not shown).

Identification of ribose and stoichiometry of the reaction. [U-^{14}C]Adenosine was hydrolysed by heating (100°C) for 15 min in the presence of 1 M HCl and the radioactivity residing in the ribose and adenine residue was determined by separating the reaction products by chromatography in system E. [U-^{14}C]Adenosine (1 μM) was incubated in the presence of the binding protein under the conditions given in the preceeding paragraph. Ribose and adenine were demonstrated to be formed in equivalent amounts (data not shown).

Evaluation of the protein denaturation procedures. The results presented above were seemingly in conflict with data given previously. No conversion of [^{3}H]adenosine to [^{3}H]adenine could be detected when the bound and free radioactivity were separated by Sephadex G-25 chromatography and the bound

TABLE I

COMPARISON OF R_F VALUES OF THE PRODUCT OF THE METABOLISM OF [^{3}H]ADENOSINE WITH THE R_F VALUES OF VARIOUS ADENOSINE DERIVATIVES IN FOUR CHROMATOGRAPHIC SYSTEMS

[^{3}H]Adenosine (1 μM) was incubated for 15 min at 30°C in the presence of binding protein (150 μg/ml) and the reaction was terminated using percholoric acid as described under Materials and Methods. The mobility of adenosine derivatives chromatographed in systems A, B, C and D (see Materials and Methods were determined by absorption at 254 nm, whereas the mobility of the radioactive product was determined by scintillation counting.

	Chromatographic systems			
	A	B	C	D
Cyclic AMP	0.55	0.51		
AMP	0.48	0.09	0.04	0.78
ADP	0.28	0	0	0.57
ATP	0.11	0	0	0.66
Adenosine	0.52	0.67	0.69	0.89
Inosine	0.73	0.78	0.74	0.87
Hypoxanthine	0.55	0.67	0.57	0.85
Adenine	0.33	0.59	0.55	0.65
Product	0.32	0.59	0.56	0.65

TABLE II

EFFECT OF VARIOUS PROTEIN DENATURATION PROCEDURES ON THE DETECTION OF THE CONVERSION OF [^{3}H]ADENOSINE TO [^{3}H]ADENINE BY THE CYCLIC AMP-ADENOSINE BINDING PROTEIN/*S*-ADENOSYLHOMOCYSTEINASE

Binding protein (100 μg/ml) was incubated in the presence of 1 μM [^{3}H]adenosine in 15 mM Hepes, pH 7.0, containing 5 mM magnesium acetate and 150 mM KCl. The incubation was allowed to proceed for 15 min and was terminated by one of the following procedures: A. 25 μl of the incubation mixture was added to and mixed with 25 μl 0.8 M perchloric acid and cooled (0°C) for 5 min. The precipitate removed by centrifugation. The supernatant was neutralized by the addition of 50 μl of alkali as described under Materials and Methods and the precipitated perchloric acid was removed by centrifugation. B. 25 μl of the incubate was mixed with 50 μl of 0.36 M KOH/0.30 M $KHCO_3$ and neutralized by the addition of 25 μl of 0.8 M perchoric acid. The precipitate was removed by centrifugation. C. 25 μl of the incubation mixture was mixed with 25 μl of 2% sodium dodecyl sulfate containing 1 mM adenine and 1 mM adenosine. After 15 min at 25°C the mixture was cooled (0°C) and the precipitated sodium dodecyl sulfate removed by centrifugation. D. The reaction tube was immersed into boiling water for 5 min, cooled (0°C) and centrifuged. E. 25 μl from the incubation mixture was added to 975 μl of boiling water. After 5 min the mixture was cooled (0°C) and centrifuged. Five replicates were processed by each procedure and aliquots of 20 μl were subjected to thin-layer chromatography in system A. The results are shown in the left row of the table. The column to the right shows the percentage conversion of [^{3}H]adenosine measured under the following conditions: Perchloric acid (A), alkali (B) or sodium dodecyl sulfate (C) was mixed with [^{3}H]-adenosine prior to the addition of the native binding protein. In the experiment corresponding to D and E, binding protein denatured by heat treatment was used instead of native binding protein.

Protein denaturation by	Conversion of [^{3}H]adenosine to [^{3}H]adenine detected (% ± SD)	Conversion of [^{3}H]adenosine to [^{3}H]-adenine caused by the protein denaturation procedure (%)
A Perchloric acid	49 ± 1.26	0.02
B Alkali ($KOH/KHCO_3$)	47.1 ± 3.12	0.05
C Sodium dodecyl sulfate	6.9 ± 0.12	0.00
D Heating of reaction tube	1.39 ± 0.49	0.02
E Addition of sample to boiling water	16.3 ± 2.5	0.02

fraction analyzed by thin-layer chromatography after being denatured by heating (100°C) [18]. In the experiment presented in the preceding paragraphs, perchloric acid was used as a protein denaturant.

These observations led us to evaluate the following procedures used for the termination of the incubation: Addition of perchloric acid or alkali or sodium dodecyl sulfate to the incubation mixture, or heating the incubation mixture (Table II). No one of these procedures (see legend to Table II for details) caused cleavage of the nucleoside linkage in adenosine under the conditions used (left row of Table II). Sodium dodecyl sulfate did not alter the chromatographic migration of adenine and adenosine under the condition used.

A high percentage conversion (about 50%) was detected by terminating the reaction by addition of strong alkali or acid, whereas a low value (7%) was obtained using sodium dodecyl sulfate. Almost no conversion (1—2%) was detected when the incubation was terminated by immersion of the reaction tubes in boiling water bath. To investigate whether these findings could be explained by reversion of the reaction converting adenosine to adenine during a slow denaturation process, this value was compared to the percentage conversion measured (16%) when adding 25 μl from the same incubation mixture to

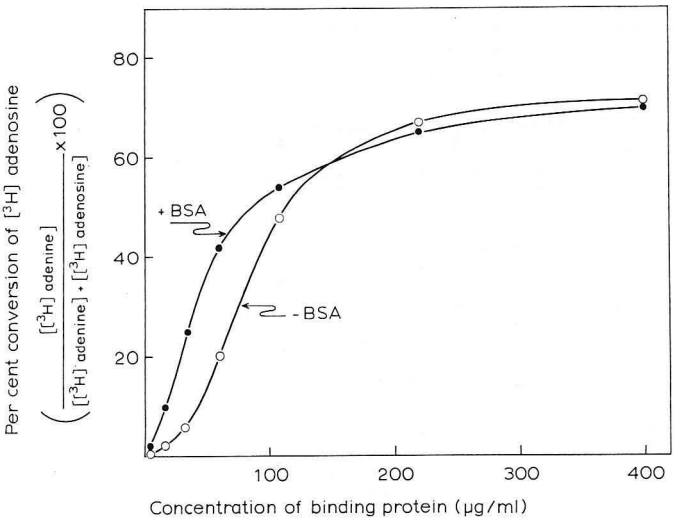

Fig. 1. Conversion of [^{3}H]adenosine to [^{3}H]adenine in the presence of various concentrations of cyclic AMP-adenosine binding protein/S-adenosylhomocysteine. [^{3}H]Adenosine (1 µM) was incubated for 15 min at 30°C in the presence of binding protein at concentrations indicated on the figure. The incubation buffer was 15 mM Hepes, pH 7.0, containing 5 mM Mg^{2+}, 150 mM KCl and 0.2% bovine serum albumin (BSA) (●——●) or no serum albumin (○——○). The reaction was stopped by mixing samples from the incubation mixture with perchloric acid as described under Materials and Methods.

975 µl of boiling water. The possibility also existed that reversion could take place during denaturation of the protein by addition of perchloric acid. However, the observation that under certain conditions (low concentration of adenosine relative to the amount of binding protein, high pH) nearly complete conversion of adenosine to adenine was detected using perchloric acid as protein denaturant (data not shown), does not support this possibility.

Concentration of protein. The conversion of [^{3}H]adenosine to [^{3}H]adenine was measured at various concentrations of binding protein and in the absence and presence of bovine serum albumin (Fig. 1). Under the conditions used (1 µM of [^{3}H]adenosine), the conversion was almost linear with respect to the concentration of protein up to about 100 µg of binding protein per ml in the presence of bovine serum albumin. In the absence of albumin, the conversion deviated from linearity at a low concentration of binding protein (Fig. 1). No conversion was detected in the presence of bovine serum albumin (0.2%) when the binding protein was omitted from the reaction mixture.

Time course. The time course of the conversion (Fig. 2) was characterized by an initial burst phase lasting for less than 1 s (Fig. 2, inset). The initial burst was followed by a slow progressive phase lasting for 5—15 min. A reversal of the reaction was observed by prolonged incubation.

Concentration of [^{3}H]adenosine. The time course of the formation of [^{3}H]-adenine from [^{3}H]adenosine was determined at increasing concentrations (0.1—10 µM) of [^{3}H]adenosine (Fig. 2). The percentage conversion was low and the burst phase less pronounced at high concentrations of the nucleoside. Bovine serum albumin stimulated the conversion at high concentrations of [^{3}H]adenosine (Fig. 2).

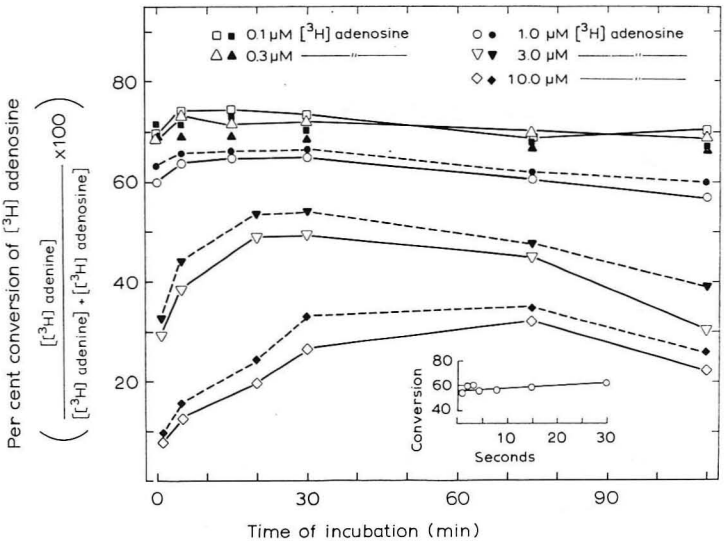

Fig. 2. The formation of [^{3}H]adenine from [^{3}H]adenosine in the presence of binding protein as a function of time, at various concentrations of [^{3}H]adenosine. Binding protein (150 μg/ml) was incubated at 30° C in the presence of [^{3}H]adenosine at concentrations indicated on the figure. The incubation buffer was 15 mM Hepes, pH 7.0, containing 5 mM Mg^{2+}, 150 mM KCl and 0.2% bovine serum albumin (filled symbols) or no serum albumin (open symbols). Inset shows the time course of the conversion of adenosine determined at 1 μM of adenosine and up to 30 s of incubation.

Reversion of the formation of [^{3}H]adenine from [^{3}H]adenosine. The reversion observed upon prolonged incubation (Fig. 2) could not be inhibited by making the incubation mixture 0.1 mM in adenine after 30 min of incubation (data not shown). Binding protein (100 μg/ml) was incubated in the presence of unlabelled adenosine (1 μM) and the incubation mixture was made 0.1, 1 or 10 μM in [^{3}H]adenine (20 Ci/mmol) at 15 min of incubation. No radioactivity corresponding to [^{3}H]adenosine could be detected after 90 min of incubation (data not shown).

Reversion was brought about by dilution of the incubation mixture (Fig. 3). The reversion was increased in the presence of excess unlabelled adenine (Fig. 3).

Separation of free and bound [^{3}H]adenine and [^{3}H]adenosine. Samples from an incubation mixture containing [^{3}H]adenosine (1 μM) and either non-treated binding protein (Fig. 4A) or protein preincubated in the presence of ATP (Fig. 4B) were subjected to Sephadex G-25 chromatography. The amount of [^{3}H]-adenine and [^{3}H]adenosine in the separate fractions of the effluent was determined. The sharp peak of radioactivity excluded from the gel represents the protein bound fraction. 62% of the [^{3}H]adenine formed in the presence of the non-treated protein and 35% in the presence of protein treated with ATP, is protein bound. [^{3}H]Adenine liberated from the protein prior to or during the gel filtration appears in the void volume. By comparison of the percentage conversion prior to gel filtration (54% in the presence of non-treated protein, 15% in the presence of protein treated with ATP) with the percentage [^{3}H]-adenine of the total radioactivity eluted from the column (30% using non-treated protein, 10% in the presence of protein preincubated with ATP), it is

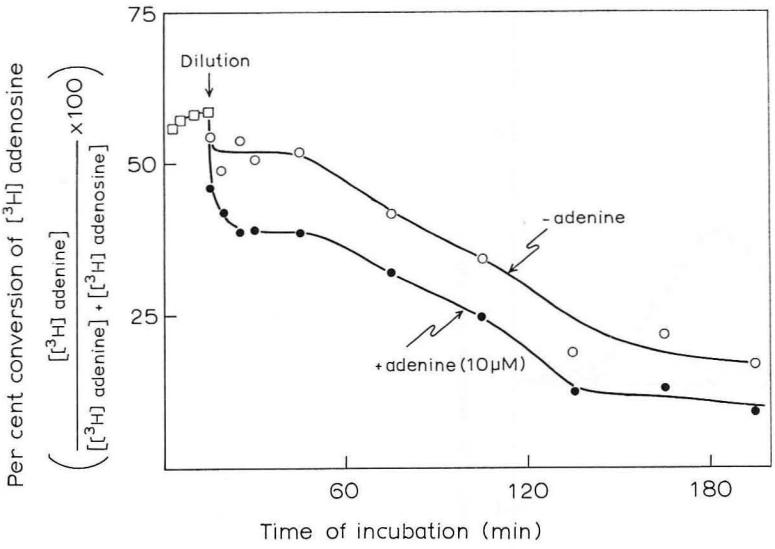

Fig. 3. The reversion of the formation of [^{3}H]adenine from [^{3}H]adenosine upon dilution in the absence and presence of excess unlabelled adenine. Binding protein (150 μg/ml) was incubated at 30°C in the presence of 1 μM [^{3}H]adenosine in 15 mM Hepes, pH 7.0, plus 5 mM Mg^{2+}, 150 mM KCl and 0.2% bovine serum albumin. After 15 min of incubation a sample from the incubation mixture was diluted 100-fold (arrow) in the same buffer containing either 10 μM or no adenine. The percentage [^{3}H]adenine is plotted against time of incubation prior to dilution (□———□), after dilution in the absence (○———○) and presence (●———●) of 10 μM adenine.

apparent that a partial reversion of the reaction has taken place during the chromatographic run. This could perhaps be explained by dilution (see Fig. 3). Cooling (0°C) of the incubation mixture for 15 min did not reverse the conversion of adenosine. Because of the reversion, the ratio of [^{3}H]adenine bound relative to free prior to chromatography is probably higher than the ratio obtained by integrating the elution profiles presented in Figs. 4A and B.

The same results were obtained when the incubation time prior to gel filtration was reduced to 1 min, except that the protein bound radioactivity was reduced by about 50%. Under these conditions, no denaturation or decomposition of the protein could be detected by polyacrylamide gel electrophoresis (data not shown).

The possibility existed that the elution profiles obtained from the experiments presented in Fig. 4 were the result of repeated dissociation and rebinding of [^{3}H]adenosine during the gel filtration. This possibility was excluded by performing the gel filtration on Sephadex G-25 columns equilibrated with sufficiently high concentration of adenine (1 mM) to inhibit almost completely the binding of [^{3}H]adenosine [9]. Under these conditions, the protein bound fraction was slightly (25%) reduced, but the results were essentially the same as in the absence of adenine (data not shown).

Polyacrylamide gel electrophoresis. Binding protein was preincubated for 0, 5 and 30 min in the presence of ATP as described in legend to Fig. 5 and then subjected to polyacrylamide gel electrophoresis. One band was observed using the non-treated protein [8]. A somewhat diffuse band with higher mobil-

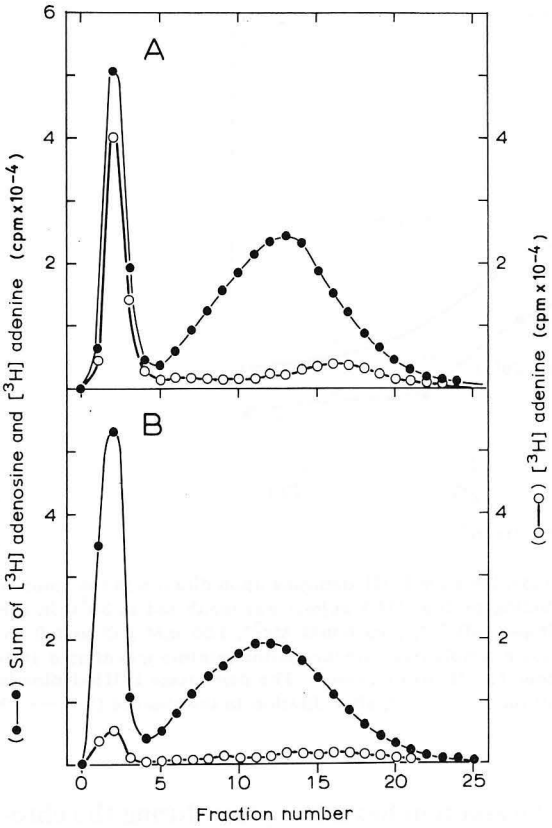

Fig. 4. Separation of bound and free [^{3}H]adenine and [^{3}H]adenosine by Sephadex G-25 chromatography. A. Binding protein (125 μg/ml) was incubated for 15 min at 30°C in the presence of [^{3}H]adenosine (1 μM) in 15 mM Hepes, pH 7.0, plus 5 mM Mg^{2+}, 150 mM KCl and 0.2% bovine serum albumin. Samples of 60 μl from the incubate were applied to a Sephadex G-25 column (0.45 × 6 cm), equilibrated with 15 mM Hepes and eluted in the same buffer (0°C). Fractions of 100 μl were collected and immediately mixed with 100 μl 0.8 M perchloric acid. The mixture was neutralized as described under Materials and Methods by addition of alkali (200 μl). Aliquots of 50 μl were counted directly and 20 μl were subjected to thin-layer chromatography in system A to separate [^{3}H]adenine from [^{3}H]adenosine. B. Binding protein (3 mg/ml) was preincubated at 30°C for 30 min in the presence of 6 mM ATP, 10 mM Mg^{2+} and 150 mM KCl in 15 mM Mes buffer, pH 6.0 [12]. Samples (30 μl) from the incubate were desalted by filtration through a Sephadex G-25 column (0.45 × 6 cm) equilibrated with 15 mM Hepes, pH 7.0. The ATP-treated binding protein was diluted to 125 μg/ml, incubated in the presence of 1 μM [^{3}H]adenosine, subjected to Sephadex G-25 chromatography, fraction collected and analyzed as described under A.

ity appeared upon prolonged preincubation (Figs. 5B and C). This probably represents a decomposition product. Parallel gels or half of a gel cut longitudinally were sliced and the protein was eluted as described under Materials and Methods. The non-treated protein displayed high conversion activity and relatively low cyclic AMP binding activity, whereas the cyclic AMP binding capacity increased and the conversion activity decreased upon treatment with ATP (Fig. 5). These activities comigrated exactly with the main protein band. The mobility of the non-treated protein and the protein treated with ATP was the same (Fig. 5).

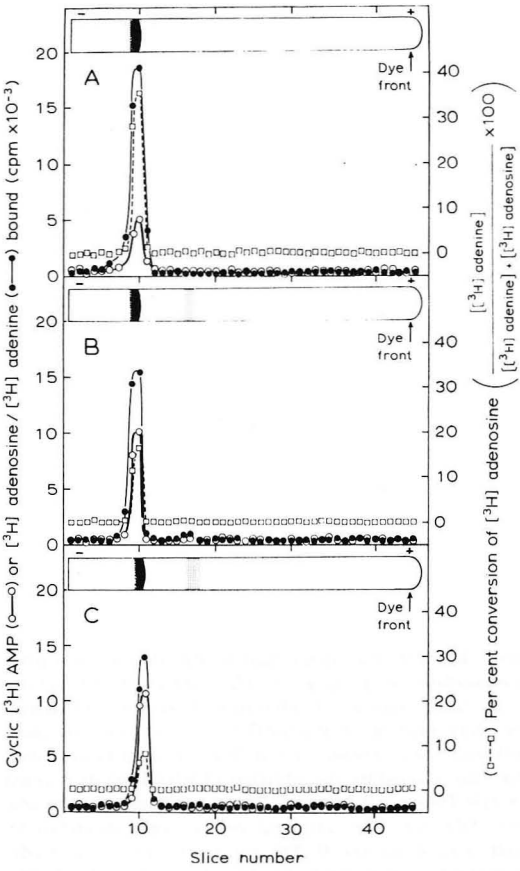

Fig. 5. Polyacrylamide gel electrophoresis of the binding protein preincubated in the presence of ATP for 0 min (A), 5 min (B) and 30 min (C). Binding protein (5 mg/ml) was preincubated at 30°C in the presence of 6 mM ATP, 10 mM Mg^{2+} and 150 mM KCl in 15 mM Mes buffer pH 6.0 for 0, 5 or 30 min [12]. Samples from the incubate (40 µl) were subjected to gel filtration on a Sephadex G-25 column (0.45 × 6 cm) equilibrated with 15 mM Tris/glycine, pH 8.8 and the protein eluted in the same buffer. The protein (96 µg/ml) was applied to polyacrylamide gel (9%) in 0.8 × 8.5 cm tubes. The gel buffer was 15 mM Tris/glycine, pH 8.8. The gels were either stained in Coomassie blue and destained or sliced as described under Materials and Methods. The gel slices were eluted in 15 mM Hepes, pH 7.0, plus 0.4% bovine serum albumin. Binding of [^{3}H]adenosine-adenine and cyclic [^{3}H]AMP was measured by adding 50 µl from the eluate to 50 µl of 15 mM Hepes buffer, pH 7.0, containing either 2 µM [^{3}H]adenosine or 2 µM cyclic [^{3}H]AMP. The incubation was allowed to proceed for 15 min at 30°C. The separation of free and bound ligand and the measurement of the protein bound fraction were performed as described [8,13]. Aliquots of 25 µl were added to 25 µl of perchloric acid, neutralized and analyzed by thin-layer chromatography in system A as described under Materials and Methods.

Effect of non-treated binding protein and binding protein treated with ATP on adenosine deaminase activity. The results presented in Fig. 6A show that deamination of adenosine by the enzyme adenosine deaminase was inhibited in the presence of non-treated binding protein and to a lesser degree in the presence of protein treated with ATP. The concentration of [^{3}H]adenine and [^{3}H]-adenosine during the amino hydrolysis of [^{3}H]adenosine in the presence of the non-treated protein (Fig. 6B) and protein preincubated with ATP (Fig. 6C) was determined. Both [^{3}H]adenine and [^{3}H]adenosine decreased as a function of

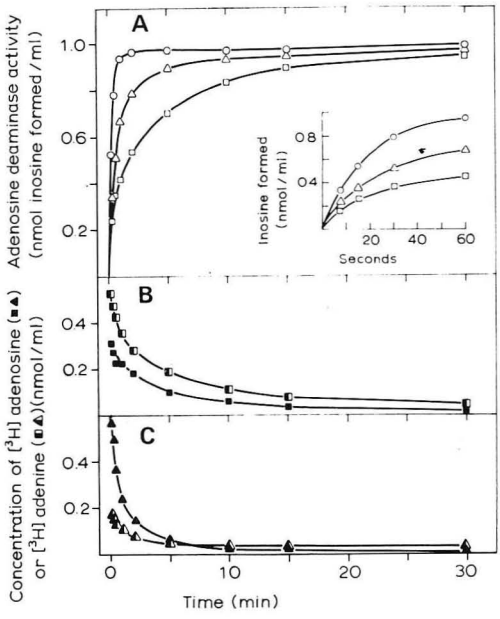

Fig. 6. Deamination of [^{3}H]adenosine to [^{3}H]inosine by adenosine deaminase in the absence and presence of cyclic AMP-adenosine binding protein/*S*-adenosylhomocysteinase. A. The formation of [^{3}H]inosine from [^{3}H]adenosine (1 μM) was measured in the presence of adenosine deaminase (1 μg/ml) (○———○), adenosine deaminase (1 μg/ml) and binding protein (150 μg/ml) (□———□) or adenosine deaminase (1 μg/ml) and binding protein (150 μg/ml) which was treated with ATP for 15 min as described in the legend of Fig. 5 (△———△). The incubations were started by the addition of adenosine deaminase and binding protein. The incubation buffer was 15 mM Hepes, pH 7.0, containing 5 mM magnesium acetate, 150 mM KCl and 0.2% bovine serum albumin. Adenine, adenosine and inosine were separated by thin-layer chromatography in system A (see Materials and Methods). B. The total concentration of adenine (□) and adenosine (■) during the deamination in the presence of non-treated binding protein. C. The toal concentration of adenine (△) and adenosine (▲) during the deamination in the presence of binding protein treated with ATP.

time. Formation of adenosine from adenine must have taken place as adenine is a poor substrate for the enzyme adenosine deaminase [17]. A smaller fraction of adenosine was converted to adenine in the presence of binding protein treated with ATP compared to in the presence of non-treated protein (Figs. 6B and C).

To exclude the possible interference from adenine deaminase activity in the preparation of adenosine deaminase used, the following experiment was conducted. The conversion of [^{3}H]adenine (1 μM) to [^{3}H]hypoxanthine was measured using a concentration of adenosine deaminase 30 times higher than that used in the experiments presented in Fig. 6. Only 1.5% of [^{3}H]adenine was converted to [^{3}H]hypoxanthine after 90 min of incubation.

Test for adenosine phosphorylase activity. Adenosine phosphorylase from rat liver has been shown to catalyze the following reversible reaction [16]:

Ribose 1-phosphate + adenine ⇌ adenosine + P_i.

This activity could not be detected in the preparation of the binding protein.

TABLE III

ADENINE FORMATION AT VARIOUS CONCENTRATIONS OF BINDING PROTEIN UNDER CONDITION OF ENZYMIC INCORPORATION OF ADENOSINE INTO *S*-ADENOSYLHOMOCYSTEINE

[^{14}C]Adenosine (2 μM) was incubated at 37°C in the presence of binding protein at concentrations indicated in the table. The incubation buffer was 15 mM Hepes, pH 7.0, containing 5 mM magnesium acetate, 150 mM KCl, 3 mM DL-homocysteine, 2 mM 2-mercaptoethanol and 0.2% bovine serum albumin. The reaction products were separated by thin-layer chromatography (system E) [14]. SAH, *S*-adenosylhomocysteine.

Concentration of binding protein (μg/ml):	0.16		250		1500	
Time of incubation (min):	0.5	15	0.5	15	0.5	15
Concentration (μM) of						
adenine	0.01	0.01	0.12	0.13	0.27	0.29
adenosine	1.79	0.04	0.02	0.03	0.03	0.02
SAH	0.19	1.94	1.86	1.84	1.70	1.69

Formation of adenine under condition of enzymic formation of S-adenosylhomocysteine. The experiment presented in Table III shows that at high concentration of binding protein, *S*-adenosyl-L-homocysteine is rapidly formed but a fraction of adenosine is not incorporated into *S*-adenosyl-L-homocysteine. This fraction is identified as mainly adenine. At low concentration of protein, suitable for the determination of the time course of *S*-adenosyl-L-homocysteine formation [14], no adenine could be detected.

Discussion

The conversion of adenosine to adenine and ribose by the cyclic AMP-adenosine binding protein/*S*-adenosylhomocysteinase is characterized by an initial burst-phase, slight liberation of the products of the reaction and reversibility. These properties, which serve to distinguish this reaction from enzyme catalysis, will be discussed in the following paragraphs.

A fraction of adenosine is converted to adenine and ribose in the presence of the binding protein in less than 1 s (Fig. 2). This is in agreement with the observation that the binding of [^{14}C]adenosine to the binding protein shows a rapid initial phase [13]. The determination of the initial burst phase is not obscured by reaction blank, which is zero (Table II). No conversion was detected when bovine serum albumin was assayed alone.

The conversion of adenosine is reversed upon prolonged incubation (Fig. 2), by diluting the incubation mixture (Fig. 3), and by removal of adenosine deaminase (Fig. 6). The observation that the reversion effected by prolonged incubation was not inhibited by the addition of excess unlabelled adenine, suggests that adenine formed through hydrolysis of adenosine is not, or only slightly, dissociated from the protein and mixed with adenine added to the incubation mixture. This suggestion is supported by the absence of any formation of [^{3}H]adenosine from [^{3}H]adenine added to the incubation mixture upon initiation of the reversal phase.

The experiment depicted in Fig. 3 was conducted to make available further

evidence that adenine formed from adenosine is mainly bound to the protein and that this adenine only is used for resynthesis of adenosine during the reversion phase. Under the condition of the experiment, 50% of adenosine present (1 μM) is converted to adenine corresponding to the formation of 0.5 μM ribose. The concentration of ribose is 0.005 μM after a 100-fold dilution of the incubation mixture. Assuming that [^{3}H]adenine formed is dissociated from the protein and mixed with unlabelled adenine (10 μM), there would be enough ribose to convert only 0.05% of the adenine present to adenosine. In contrast, a nearly complete reconversion occurred (Fig. 3). The reversion was increased rather than inhibited in the presence of unlabelled adenine (Fig. 3). However, the mechanism of the adenine effect is difficult to suggest, as precaution has not been taken in this experiment to prevent rebinding of adenosine after dilution [13].

The gel filtration experiments shown in Fig. 4 are in agreement with the concept that adenine formed through cleavage of the nucleoside linkage in adenosine is bound mainly to the protein. Similar experiments on ribose would require radioactive labelling of high specific activity in the ribose residue of adenosine, which is not available. Almost complete reversion of the conversion after 100-fold dilution indicates that bound ribose rather than free is used for the reversion to adenosine.

The possibility exists that the slow reversion observed during prolonged incubation (Fig. 3) is caused by denaturation of the protein. The rapid reversion brought about by removal of adenosine by deamination (Fig. 6) shows that this phenomenon occurs under non-denaturating conditions.

The slight liberation of adenine after dilution, addition of unlabelled adenine and incubation for 3 h (Fig. 3) indicates a tight binding of a possibly covalent nature of adenine formed from adenosine. The binding protein may act as an acceptor for autoribosylation or transfer of the adenine moiety.

The results presented in this paper are based on the conversion values obtained by denaturation of the protein by perchloric acid. The conversion of adenosine may be due to an effect of a group at the binding site on acid-(or base)-catalyzed hydrolysis. The observation that the conversion detected when denaturating the protein by rapid heating was about one third of the value obtained using perchloric acid as a protein denaturant (Table II), is not in agreement with this explanation. Adenosine bound to the protein may be hydrolysed during the inactivation of the protein. This explanation seems attractive in the light of the results presented in Table II showing that the conversion detected depends on the denaturation procedure used. The experiments presented in Fig. 4 show that a fraction of the adenine formed from adenosine was liberated from the protein and could be separated from the bound fraction by Sephadex G-25 chromatography. Formation of free adenine was also observed under mild, apparently non-denaturating, conditions (1 min of incubation at 30°C). These data do not support the suggestion that protein denaturation is a prerequisite for adenine formation.

The interpretation of the experimental data presented in this paper is based on the following hypothesis. The rapid denaturation caused by strong acid or alkali preserves the conversion of adenosine to adenine, whereas reversion takes place during a slow denaturation process. The high and low conversion detected

when the protein was denatured by rapid and slow heating, respectively, support this suggestion. The relatively low conversion observed using sodium dodecyl sulfate (Table II) may be related to the fact that denaturation of the protein by 2% sodium dodecyl sulfate is a time-dependent process reaching completion within about 1 min as judged by the reaction of the protein with 5,5′-dithio-bis-(2-nitrobenzoic acid) [11].

The conversion is stimulated by bovine serum albumin at low concentrations of binding protein (Fig. 1). This could be explained by protection of the protein against denaturation or prevention of adsorption to vessel walls. The effect of preincubation in the presence of ATP seems to be a slow transition of the protein from a form where a large fraction of adenosine bound is converted to adenine and which possesses low binding capacity for cyclic AMP to a form characterized by low conversion activity and high cyclic binding AMP capacity (Fig. 5). The data in Fig. 5 confirm the previous observation that this transition is not accompanied by dissociation or aggregation of the protein [8].

The experiments presented in Fig. 6 show that adenosine is protected by the binding protein against hydrolysis by the enzyme adenosine deaminase. This observation points to the possibility that the protein may function to compartmentalize adenosine. The hydrolysis of the nucleoside linkage in adenosine may play a role in the cellular sequestration of the nucleoside. Two observations support this possibility. The protein treated with ATP converts a small fraction of adenosine bound to adenine and is less efficient in protecting adenosine against deamination (Figs. 4, 5, 6). Secondly, the fraction of adenosine not available for *S*-adenosyl-L-homocysteine synthesis at high concentration of binding protein is converted to adenine (Table III). The biological relevance of the latter observation is indicated by the finding that the cellular level of the cyclic AMP-adenosine binding protein/*S*-adenosylhomocysteinase in mouse liver is high and this protein accounts for at least 1.8% of the soluble protein (Ueland and Saebø, unpublished results).

This is the first report indicating that adenine may be formed by hydrolysis of adenosine in mammalian cells. Adenine has been detected in normal serum [18] and in tissues in vivo [19,20]. The enzyme adenine phosphoribosyl transferase has been described in a wide variety of tissues [21] suggesting that the substrate, adenine, is a tissue constituent. There are only a few previous reports providing information on adenine formation in mammalian cells. Adenosine was metabolized to adenine by a crude preparation of a similar protein from bovine liver [6]. Evidence has been presented that adenine is formed from deoxyadenosine in whole tissues [22]. Purine nucleoside phosphorylase (EC 2.4.2.1) is generally believed to be inactive towards adenosine [21,23,24]. Recently, adenosine phosphorylase activity distinct from inosine-guanosine phosphorylase has been demonstrated in crude extract from sarcoma 180 cells and rat liver [16]. The formation of ribose and the independence of phosphate of the conversion of adenosine described here, indicate that the reaction is a hydrolysis and serve to differentiate it from adenosine phosphorylase.

Note added in proof (Received April 2nd, 1979)

Since the submission of this paper we have observed that adenine is a potent inhibitor of both the synthesis and hydrolysis of *S*-adenosylhomocysteine catalyzed by *S*-adenosylhomocysteinase from mouse liver. Thus, the possibility exists that adenine formation is an autoregulatory phenomenon.

Acknowledgements

This work was supported by the Norwegian Research Council for Science and the Humanities and by Nordisk insulinfond. The technical assistance of T. Ellingsen and H. Bergesen is highly appreciated.

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