THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 253, No. 5, Issue of March 10, pp. 1667-1676, 1978
Printed in U.S.A.

An Adenosine 3′:5′-Monophosphate-Adenosine Binding Protein from Mouse Liver

A STUDY ON ITS INTERACTION WITH ADENOSINE 3′:5′-MONOPHOSPHATE AND ADENOSINE*

(Received for publication, February 17, 1977, and in revised form, August 30, 1977)

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The binding of cyclic [^{3}H]AMP to the untreated cyclic AMP-adenosine binding protein increases slowly in a time-dependent manner when incubated at 30° in the presence of radioactive cyclic AMP (0.05 to 10 μM). Data are presented suggesting that cyclic AMP activates its own sites (homologous activation). The homologous activation is promoted by low pH, Mg^{2+}, and K^+; Na^+ is inefficient.

Binding of cyclic AMP at 30° to the binding protein activated by treatment with ATP (Ueland, P. M., and Døskeland, S. O. (1977) *J. Biol. Chem.* 252, 677-686), showed a progressive increase after the initial equilibrium phase at high concentrations (>1 μM) of cyclic AMP, whereas a decrease was observed at low concentrations of cyclic AMP. This is explained by activation or deactivation. These processes caused interference with the equilibrium binding studies when performed at 30°. At 0°, only minor changes in activation occurred. Under this condition (0°) the binding assay used could be validated by the equilibrium binding method of Hummel and Dreyer (Hummel, J. P., and Dreyer, W. J. (1962) *Biochim. Biophys. Acta* 63, 530-532).

The dissociation rate for both cyclic AMP and adenosine bound to the activated binding protein was determined under conditions of sufficient dilution to prevent rebinding. The dissociation showed first order kinetics for cyclic AMP. The dissociation rate constant was 0.14 min^{-1}. The log bound *versus* time graph for the dissociation of adenosine was hyperbolic indicating heterogeneity of the adenosine binding sites. The dissociation rate for neither cyclic AMP nor adenosine was affected by the presence of the homologous or heterologous ligand, suggesting absence of heterotrop or homotrop cooperative effects.

Adenosine was a potent inhibitor of the homologous activation. The inhibition could be differentiated from the competitive inhibition of cyclic AMP binding by adenosine.

The cyclic AMP binding activity co-sedimenting in a sucrose gradient with adenosine binding activity was compared to the cyclic AMP binding activity co-sedimenting with phosphotransferase activity. In the presence of assumedly physiological concentrations of adenine nucleotides and 2 μM of cyclic [^{3}H]AMP, the amount of cyclic AMP bound to the cyclic AMP-dependent protein kinase seemed to be of the same order of magnitude as the amount bound to the cyclic AMP-adenosine binding protein.

A cyclic AMP-adenosine binding protein not associated with cyclic AMP-dependent protein kinase has been purified and partly characterized from mouse liver (1). Several properties serve to distinguish this protein from the cyclic AMP binding component of cyclic AMP-dependent protein kinase. The cyclic AMP-adenosine binding protein does not inhibit the phosphotransferase activity of the free catalytic subunit of the cyclic AMP-dependent protein kinase (1). Furthermore, cyclic AMP binding to the cyclic AMP-adenosine binding protein could be inhibited by other adenine derivatives but not by cyclic GMP (1) whereas the reverse is true for cyclic AMP binding to the regulatory subunit of protein kinase (2-4). Finally, the cyclic AMP binding moiety of protein kinase (5) has lower molecular weight than the cyclic AMP-adenosine binding protein (1).

The binding protein is isolated in an inactive form which has low binding capacity for cyclic AMP compared to the amount of adenosine that binds at saturating concentration of this adenine derivative (1). Conditions favoring its conversion from the inactive to the active form, the latter characterized by high binding capacity for cyclic AMP and high affinity for adenosine relative to the inactive protein, have been presented (6). No enzymatic activity has hitherto been ascribed to this binding protein (1) or a similiar protein from rabbit erythrocytes (7).

The possibility exists that the cyclic AMP-adenosine binding protein has allosteric properties (8), *i.e.* binding of one ligand to a site affects the binding of the same or another ligand to another site. The aim of this work was to investigate the possibility of mutual interactions among binding sites.

* This work was supported by grants from the Norwegian Research Council for Science and the Humanities. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The binding of cyclic AMP and adenosine were studied because among the low molecular substances tested, these ligands seem to have the highest affinity toward the binding protein as judged by competition studies (1). With respect to cyclic AMP, the following possibilities were investigated. Cyclic AMP binding to one site could make available another site for cyclic AMP (here termed homologous activation) or binding to one site could alter the affinity for cyclic AMP to another site (homotrop cooperative effect (8)). The possible effect of adenosine on cyclic AMP binding was also investigated together with some studies on possible effects of cyclic AMP or adenosine on the binding of adenosine. The amount of cyclic AMP and adenosine bound in the presence of competing adenine nucleotides, was determined. On the basis of these results, the possible functional role of this class of protein will be discussed.

MATERIALS AND METHODS

The sources of the reagents used have been given previously (1). The binding protein was prepared as described earlier (1). 3-Isobutyl-1-methylxanthine was obtained from Sigma Chemical Co., St. Louis.

Measurement of Cyclic [^{3}H]AMP and [^{14}C]Adenosine Binding – Binding protein was incubated in the presence of cyclic [^{3}H]AMP (0.54 Ci/mmol) or [^{14}C]adenosine (0.12 Ci/mmol) or both at 30° in the buffer given in the legend to the separate figures. Aliquots of 100 μl were added to ice-cold 80% saturated ammonium sulfate containing unlabeled cyclic AMP and adenosine (0.1 mM), the protein was allowed to precipitate and collected on Millipore filters, washed, and radioactivity determined as described (1, 6).

Equilibrium Binding Studies – Binding protein (5 mg/ml) was activated by ATP by preincubating in the presence of 10 mM magnesium acetate, 150 mM KCl, 6 mM ATP at 30° for 30 min. Samples, containing 450 μg of protein, were passed through Sephadex G-25 columns (0.45 × 6 cm) equilibrated with 30 mM Hepes,[1] pH 8.0, containing 20% glycerol and eluted in the same buffer. Temperature was 0–2°. The effluent (250 μl) was mixed with equal volume (250 μl) of distilled water containing cyclic [^{3}H]AMP.

The equilibrium binding was performed by the gel filtration method of Hummel and Dreyer (9). Columns (0.7 × 29 cm) were packed with Sephadex G-25 fine and equilibrated with 15 mM Hepes, pH 8.0, containing cyclic [^{3}H]AMP (54 mCi/mmol) at concentrations indicated in legend to Fig. 5. The gel filtration experiments were performed either at 0° or at room temperature (25°) as follows:

1. Gel filtration at 0° was conducted by immersion of the column into ice water. The protein (450 μg) was preincubated for 2 h in the equilibration solution at 0° and then applied to the column in a volume of 500 μl. The column was eluted with the same solution and fractions of 330 μl were collected. The flow rate was 1 ml/h.
2. The experiments conducted at room temperature (25°) were performed as above with the following modifications. The column was placed in room temperature and the activated binding protein was preincubated for 5 min at 25°. The flow rate was 14 ml/h.

Aliquots of 100 μl were taken from the fractions and mixed with 1 ml of 0.2% sodium dodecyl sulfate in scintillation vials. After 45 min, 8 ml of Diluene (Packard) was added.

The samples from the fractions were mixed with sodium dodecyl sulfate to denature the protein and thus avoid co-precipitation of bound cyclic [^{3}H]AMP and protein which probably could explain the low counting efficiency observed when samples containing protein were added directly to the scintillation fluid (10).

Determination of Dissociation Rate. Theoretical Basis and Experimental Design – The relation between the association rate constant (k_1) and the dissociation rate constant (k_{-1}) and the equilibrium dissociation constant (K_d) is given by the equation

$$K_d = \frac{k_{-1}}{k_1} \quad (11)$$

The determination of the dissociation rate constant as an experimental approach to investigate cooperative kinetics has been proposed by De Meyts *et al.* (12). In case of homotrop cooperative effects, the dissociation rate of ^{3}H-labeled ligand is affected by the presence of excess unlabeled ligand (12). Furthermore, if the binding of one ligand (A) to a site increases or decreases the affinity for the other ligand (B) to another site, the dissociation rate for ligand B would be expected to decrease or increase, respectively, in the presence of ligand A.

The dissociation rate experiments were conducted as follows. Binding protein (4 mg/ml) was activated by preincubation in the presence of 6 mM ATP, 10 mM Mg^{2+}-acetate, and 150 mM KCl in 20 mM Mes buffer, pH 6.0. The preincubation was allowed to proceed for 30 min at 30°. Samples of 30 μl were applied to a Sephadex G-25 column (0.45 × 6 cm) equilibrated with 20 mM Hepes, pH 7.0. The protein was eluted in 200 μl of the same buffer and an aliquot of 60 μl incubated either in the presence of 10 μM cyclic [^{3}H]AMP (20 Ci/mmol) or 10 μM [^{3}H]adenosine (21 Ci/mmol) for 30 min at 30° in a total volume of 90 μl. The incubation mixture was cooled and applied to a Sephadex G-25 column (0.45 × 6 cm) equilibrated with the buffer in which the eluate was diluted (see legend to Figs. 6 and 7) and eluted in 250 μl of the same buffer. The temperature was kept at 0–2°. The protein excluded from the column was immediately diluted in the appropriate buffer containing 0.2% bovine serum albumin, to a final concentration of 0.5 μg of binding protein/ml. The temperature was 30°. At times, indicated on the separate figures, samples of 500 μl were added to and mixed with 4 ml of ice-cold 90% saturated ammonium sulfate containing 0.1 mM unlabeled adenosine and cyclic AMP. The filtration through the Millipore filters and the determination of radioactivity were performed as described (1).

The following experiments were conducted to reassure that rebinding did not interfere with the determination of the dissociation rate. The dissociation rate was determined at progressively higher dilutions until the slope of the log bound *versus* time graph was independent on the dilution. The dissociation rate was routinely measured at 0.5 μg of binding protein/ml. A 10-fold increase in the concentration of the ligand·protein complex or a 4-fold dilution did not affect the apparent dissociation rate for either cyclic AMP or adenosine. (Further dilution was restricted by the specific radioactivity of the ligand.) Activated (as described above) binding protein not incubated in the presence of ^{3}H-labeled ligand was added to the incubation mixture to a final concentration of 5 μg/ml without affecting the apparent dissociation rate measured at a dilution corresponding to 0.5 μg of binding protein/ml. Thus, a 10-fold increase in the number of binding sites did not result in a detectable increase in the amount of radioactive ligand bound during the dissociation rate experiments.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis – Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Weber *et al.* (13) in tubes (75 × 5 mm) containing 7.5% gel. The electrophoresis was run for 4 h using 6 mA/gel. The gels were stained in Coomassie blue and destained in 7% acetic acid containing 40% methanol.

Sucrose Gradient Centrifugation – Samples of 300 μl were loaded on the top of 10 ml (5 to 20% w/v) sucrose gradients. Centrifugation was performed at 40,000 rpm for 24 h at 4° in an International B-60 ultracentrifuge. The tubes were punctured, and fractions of 0.7 ml were collected.

Thin Layer Chromatography – This was carried out at room temperature on polyethyleneimine-impregnated cellulose sheets. Cyclic AMP was separated from its decomposition products (AMP and adenosine) by developing the chromatograms in 0.5 M ammonium acetate:96% ethanol (5:2, v/v).

Determination of Protein – This was performed by the method of Klungsöyr (14) using bovine serum albumin as standard.

RESULTS

Homologous Activation of Cyclic AMP Site – Nonactivated binding protein and activated binding protein (treated with Mg^{2+}-ATP as described in legend to Fig. 1) were incubated with cyclic [^{3}H]AMP (5 μM) at 30° in the absence and presence of KCl and Mg^{2+} (which promote the activation of the binding protein by ATP (6) (Fig. 1). The binding of cyclic [^{3}H]AMP to the nonactivated binding protein showed a slow time-dependent increase which was accelerated under activation conditions (*i.e.* in the presence of KCl and Mg^{2+}). The time course

[1] The abbreviations used are: Hepes, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

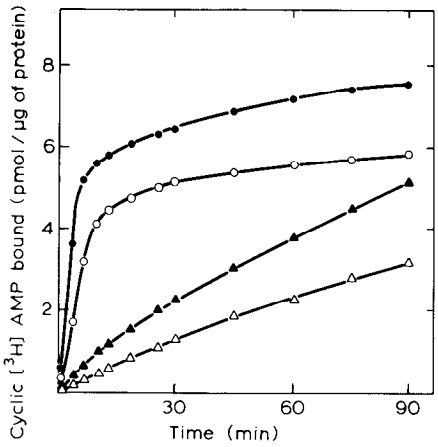

FIG. 1. Time course of cyclic [^{3}H]AMP binding to nonactivated and activated binding protein in the absence and presence of Mg^{2+} and KCl. Binding protein (4 mg/ml) was preincubated with 6 mM ATP, 10 mM Mg^{2+}, and 150 mM KCl in 20 mM Hepes buffer, pH 7.0. The preincubation was allowed to proceed for 30 min at 30° and was terminated by applying a sample of 60 µl to a Sephadex G-25 column (0.45 × 6 cm) equilibrated with 20 mM Hepes, pH 7.0, and eluted in the same buffer. Binding protein treated in this way (activated) and untreated protein (nonactivated) were incubated at 30° for the time indicated with 5 µM cyclic [^{3}H]AMP in the absence and presence of 10 mM Mg^{2+}-acetate and 150 mM KCl. The incubation buffer was 15 mM Hepes, pH 7.0, and the concentration of binding protein 160 µg/ml. The binding of cyclic [^{3}H]AMP to the activated binding protein in the absence (○——○) and presence (●——●) of Mg^{2+} and KCl, and to the nonactivated protein in the absence (△——△) and presence (▲——▲) of Mg^{2+} and KCl are plotted against time of incubation.

of the cyclic [^{3}H]AMP binding to the activated binding protein was biphasic. There was a rapid increase for the first 10 min followed by a slow increase. In the presence of Mg^{2+} and KCl principally the same time course was observed, but the binding during both phases was increased. These results could be explained by a time-dependent activation of cyclic AMP binding site by cyclic AMP (homologous activation). Equilibrium of cyclic AMP binding to the activated protein was obtained within 10 min under the conditions used (30°).

To further investigate the possibility of homologous activation, the following experiment was conducted. Binding protein (1 mg/ml) was preincubated in the presence of increasing concentration of either ^{3}H-labeled cyclic AMP or unlabeled cyclic AMP for 1 h at 30° (Table I). After gel filtration to remove unbound cyclic AMP, the protein was incubated with either 10 µM cyclic [^{3}H]AMP or 10 µM cyclic AMP for 0, 15, 30, and 60 min at 30°. Panel A in Table I (preincubation with labeled cyclic AMP and incubation with labeled cyclic AMP) shows the sum of the binding during the preincubation and incubation. Panel B (preincubation in the presence of labeled cyclic AMP and incubation with unlabeled nucleotide) shows the amount of cyclic [^{3}H]AMP bound during the preincubation and the subsequent dissociation during the incubation. Panel C (preincubation in the presence of unlabeled cyclic AMP and incubation with labeled cyclic AMP) shows the amount of cyclic [^{3}H]AMP bound during the incubation. From the data presented (Table I) the following statements can be made. Binding protein preincubated in the presence of unlabeled cyclic AMP bound more cyclic [^{3}H]AMP than the protein preincubated in the absence of the nucleotide (Panel C) even though cyclic AMP was bound to the protein during the preincubation and carried over into the incubation mixture

TABLE I

Binding protein (1 mg/ml) was preincubated for 60 min at 30° in the presence of cyclic [^{3}H]AMP or unlabeled cyclic AMP at the concentrations indicated. The incubation buffer was 15 mM Hepes, pH 7.0, containing 10 mM Mg^{2+}-acetate and 150 mM KCl. Samples of 60 µl were applied to Sephadex G-25 columns (0.45 × 6 cm) equilibrated with 15 mM Hepes, pH 7.0, containing 20% glycerol; and eluted in the same buffer. Binding protein (250 µg/ml) was incubated for the time indicated in the presence of 10 µM cyclic [^{3}H]AMP or 10 µM unlabeled cyclic AMP. Samples of 50 µl were taken for the determination of cyclic [^{3}H]AMP bound (see "Materials and Methods").

Preincubation in presence of	Incubated in presence of	Cyclic [^{3}H]AMP bound in incubation time (in min)			
		0	15	30	60
µM		*pmol/ml*			
A. Cyclic [^{3}H]AMP					
0	Cyclic [^{3}H]AMP (10 µM)		121	146	263
0.5	Cyclic [^{3}H]AMP (10 µM)	113	177	326	356
1.5	Cyclic [^{3}H]AMP (10 µM)	196	282	386	441
2.5	Cyclic [^{3}H]AMP (10 µM)	327	425	463	505
5.0	Cyclic [^{3}H]AMP (10 µM)	401	508	552	568
10.0	Cyclic [^{3}H]AMP (10 µM)	538	640	736	750
B. Cyclic [^{3}H]AMP					
0.5	Cyclic AMP (10 µM)	120	83	80	47
1.0	Cyclic AMP (10 µM)	186	148	128	86
2.5	Cyclic AMP (10 µM)	340	241	199	136
5.0	Cyclic AMP (10 µM)	410	293	259	196
10.0	Cyclic AMP (10 µM)	541	400	307	251
C. Cyclic AMP (unlabeled)					
0.5	Cyclic [^{3}H]AMP (10 µM)		157	203	242
1.0	Cyclic [^{3}H]AMP (10 µM)		133	257	291
2.5	Cyclic [^{3}H]AMP (10 µM)		183	237	339
5	Cyclic [^{3}H]AMP (10 µM)		204	306	311
10	Cyclic [^{3}H]AMP (10 µM)		209	344	439

(Panel B). This indicates an activation of cyclic AMP binding sites by the homologous ligand.

Concentration of Cyclic AMP—The gel filtration technique is not useful for the measurement of a small degree of homologous activation. Results presented in the preceding paragraph indicate that cyclic AMP binding to the activated binding protein reaches equilibrium within 10 min of incubation at 30°. The activation process is thus the time-limiting step in the binding of cyclic [^{3}H]AMP to the nonactivated protein. Therefore, the binding of cyclic AMP as a function of time is a measurement of the velocity of activation.

Nonactivated binding protein was incubated in the presence of various concentrations of cyclic [^{3}H]AMP (Fig. 2*A*). At low concentrations of cyclic [^{3}H]AMP (0.05 and 0.1 µM) maximal

binding was reached within 40 min, whereas at high concentrations (5 and 10 μM) the binding progressively increased with time for at least 90 min under the conditions used (Fig. 2*A*).

The same experiment was conducted using binding protein activated by treatment with Mg^{2+}-ATP (Fig. 2*B*). At all concentrations of cyclic AMP tested (0.05 to 10 μM) binding of cyclic [^{3}H]AMP displayed a rapid time course within the first 10 min. The further binding depended upon the concentration of cyclic AMP. The binding at 0.05 and 0.1 μM cyclic [^{3}H]AMP showed a small but reproducible decrease after a plateau was reached. The degree of activation obtained by the Mg^{2+}-ATP treatment could possibly not be sustained by the low concentration of cyclic AMP. At high concentrations of the nucleotide (5 and 10 μM), a progressive increase in cyclic [^{3}H]AMP binding was observed after the initial period (Fig. 2*B*). This could be explained by further activation of the protein by cyclic AMP.

Cation Requirement—The homologous activation as determined by the time course of cyclic [^{3}H]AMP binding to the nonactivated protein, increases in the presence of KCl (Fig. 3*A*). NH_4Cl could replace KCl whereas NaCl was inefficient (data not shown). Magnesium also stimulates the activation in a concentration-dependent manner (Fig. 3*B*).

Effect of pH—The homologous activation was measured at pH 6, 7, and 8. The results presented in Fig. 3*C* shows that the activation was more pronounced at low pH.

Equilibrium Binding Studies under Activation Conditions—When the affinity for cyclic AMP to the activated binding protein is measured, a constant amount of protein is incubated in the presence of various concentrations of cyclic [^{3}H]AMP. If the incubations are performed under conditions (30°) where changes in the degree of activation occur, the cyclic AMP concentration of half-maximal binding would appear to be higher than when the incubation is performed under conditions preserving the activation. These principles are illustrated in Fig. 4 showing equilibrium binding at 0° (18 h) and 30° (15 min). This may explain the high cyclic AMP concentration of half-maximal binding and thus the low apparent affinity for cyclic AMP reported in a preceding communication (15).

Equilibrium Binding Studies by Gel Filtration—To validate the binding assay involving membrane filtration of precipitated protein (1), the binding of cyclic [^{3}H]AMP was measured by the gel filtration method of Hummel and Dreyer (9).

The results obtained when the gel filtration was performed at 0° indicate that an equilibrium between free and bound ligand exists as the base-line was reached after the protein-bound nucleotide was eluted (*left panel*, Fig. 5). Under these conditions, about the same affinity toward cyclic AMP was obtained as that published previously (1). The binding capacity (6 pmol/μg of protein) was identical with that obtained

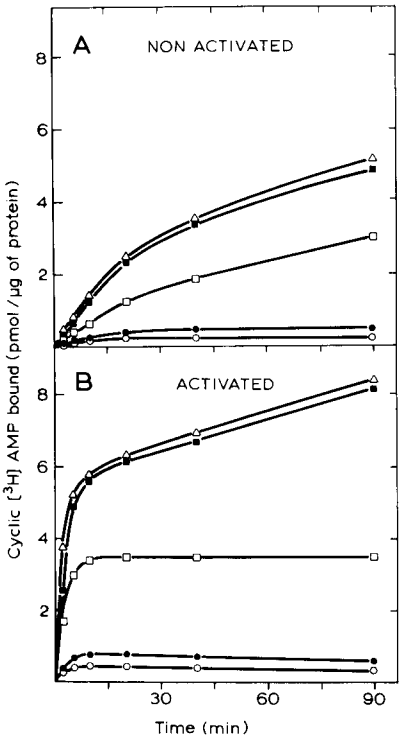

FIG. 2. Time course of cyclic [^{3}H]AMP binding to activated and nonactivated binding protein at various concentrations of cyclic [^{3}H]AMP. Activation of the protein and gel filtration were performed as described in legend to Fig. 1. *A*, binding of cyclic [^{3}H]AMP to the nonactivated binding protein (160 μg/ml) at 0.05 μM (○——○), 0.1 μM (●——●), 1.0 μM (□——□), 5 μM (■——■), or 10 μM (△——△) in 15 mM Hepes, pH 7.0, containing 10 mM Mg^{2+}-acetate and 150 mM KCl, is plotted against time of incubation. *B*, binding of cyclic [^{3}H]AMP to the activated protein (160 μg/ml) at the same concentrations as given above. The same symbols are used as in *A*.

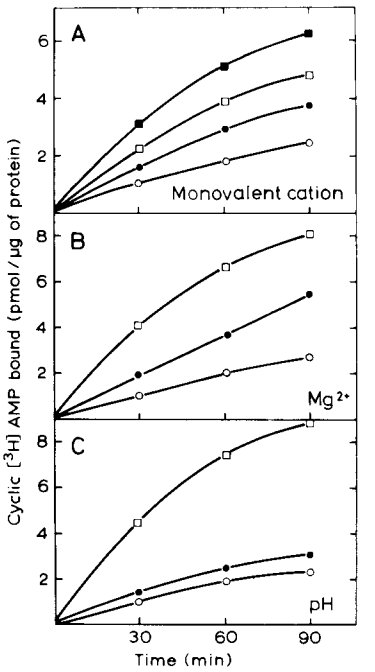

FIG. 3. *A*, requirement for monovalent cations. Binding protein (160 μg/ml) was incubated in the presence of 5 μM cyclic [^{3}H]AMP, 10 mM Mg^{2+}-acetate in 20 mM Hepes, pH 7.0, containing no salt (○——○), 50 mM KCl (●——●), 150 mM KCl (□——□), 450 mM KCl (■——■). The incubations were run at 30° for the periods of time indicated. *B*, effect of magnesium ion on the homologous activation. Binding protein (160 μg/ml) was incubated in the presence of 5 μM cyclic [^{3}H]AMP, 150 mM KCl in 20 mM Hepes, pH 7.0, containing the following concentrations of Mg^{2+}-acetate, 0 mM (○——○), 10 mM (●——●), or 40 mM (□——□). Incubations were run at 30° for the periods of time indicated. *C*, effect of pH on the homologous activation. Binding protein (160 μg/ml) was incubated in the presence of 5 μM cyclic [^{3}H]AMP, 75 mM KCl, 5 mM Mg^{2+}-acetate in 20 mM Hepes buffer, pH 8.0 (○——○); 20 mM Hepes, pH 7.0 (●——●); or 20 mM Mes buffer, pH 6.0 (□——□). Incubation was run at 30° for the time indicated on the figure.

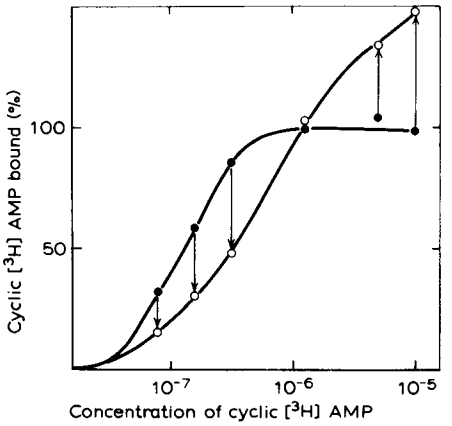

FIG. 4. Effect of incubation temperature on the apparent cyclic AMP concentration of half-maximal binding. Binding protein, activated as described in the legend to Fig. 1, was incubated in the presence of a 100-fold concentration range (0.08 to 10 μM) of cyclic [^{3}H]AMP. The binding protein was incubated either at 0° for 18 h in 15 mM Hepes buffer, pH 7.5, containing 20% glycerol (●——●) or at 30° for 15 min in the same buffer (○——○). The binding capacity for cyclic AMP at 0° is taken as 100%. The assumed inactivation or activation during the incubation at 30° is indicated by *arrows*.

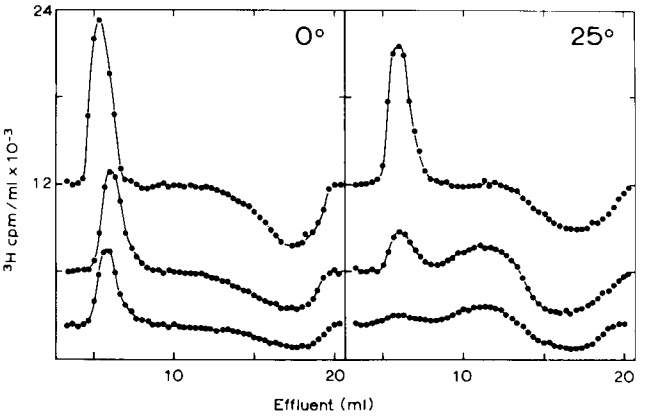

FIG. 5. Equilibrium binding studies by gel filtration at 0° and 25°. This was performed as described under "Materials and Methods" using 450 μg of binding protein. *Left panel* shows the elution profile at 0°. The *upper curve*, the *middle curve*, and the *lower curve* represent the profile obtained at 1.0, 0.5, and 0.2 μM of cyclic [^{3}H]AMP, respectively. *Right panel* shows the elution profile at 25° using the same concentrations of cyclic [^{3}H]AMP.

using the ammonium sulfate precipitation membrane filtration method in parallel experiments. A 10- to 15-fold increase in the binding capacity for cyclic AMP was obtained by treatment with ATP (data not shown). Adenosine as an inhibitor of the activation by ATP (6) was also confirmed by this method.

The elution profile obtained at 25° (Fig. 5) showed a peak of radioactivity which appeared in the void volume. This probably represents protein-bound ligand. The first peak was followed by a second increase in radioactivity (between 8 and 13 ml of effluent) after which a trough appeared. The concentration of cyclic [^{3}H]AMP was 0.2, 0.5, or 1 μM. The second increase in radioactivity was relatively more pronounced at low concentrations of cyclic AMP. This probably represents release of cyclic [^{3}H]AMP caused by deactivation at low concentrations of the nucleotide. These results are in agreement with those reported in Fig. 4 showing that changes in the degree of activation cause interference with equilibrium binding studies conducted at high temperature (30°).

The fact that binding protein, left at 25° in the equilibration buffer for the time needed to perform the gel filtration experiment, could be activated by both cyclic AMP and ATP, argues against the possibility of denaturation of the protein during the experiment.

Test for Proteolysis during Activation by Cyclic AMP or ATP—Binding protein was activated by preincubation for 30 min at 30° in the presence of cyclic AMP (50 μM) or ATP (6 mM) in 15 mM Hepes, pH 6.0, containing 150 mM KCl and 10 mM magnesium acetate. After dialysis for 6 h against the gel buffer containing 1% sodium dodecyl sulfate and 100 mM 2-mercaptoethanol, the protein (10 to 50 μg) was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis as described under "Materials and Methods." No additional band appeared upon activation indicating that activation is not accompanied by proteolysis.

Dissociation Rate for Cyclic [^{3}H]AMP—A linear Scatchard plot was obtained for the binding of cyclic [^{3}H]AMP to the activated binding protein (1). This indicates homogeneity and noncooperativity among the cyclic AMP binding sites (11). Furthermore, data have been presented suggesting that adenosine competitively inhibits the binding of cyclic [^{3}H]AMP (1). An alternative interpretation of the experimental data is that the binding of adenosine reduces the apparent affinity for cyclic AMP (1) by inducing structural changes in the protein molecule. Another question to be settled is whether pH affects the affinity for cyclic AMP to the activated protein or if the apparent low affinity at low pH (6) could be explained by incubation under conditions (low pH) not preserving the degree of activation (as shown for high temperature in Fig. 3).

The dissociation rate was measured by diluting the cyclic [^{3}H]AMP·protein complex (see "Materials and Methods") in the following buffers: 30 mM Mes buffer, pH 6.0, 30 mM Hepes buffer, pH 7.0, and 30 mM Hepes, pH 8.0, all of which contained 0.2% bovine serum albumin. In some experiments the buffer contained 5 μM cyclic AMP or 5 μM adenosine. The data were plotted as log bound *versus* time of dissociation. pH homologous or heterologous ligand did not affect the dissociation rate. The log bound *versus* time graph was linear (Fig. 6). The dissociation rate constant (k_{-1}) was 0.14 min^{-1}. The dissociation displayed first order kinetics which further suggests that cyclic AMP binds to a homogeneous population of noncooperative binding sites. Cyclic AMP (5 μM) did not affect the log bound *versus* time graph at pH to 6, 7, and 8. This observation is also in favor of noncooperativity, but conveys additional information. From the results presented in Fig. 1 it is obvious that 5 μM of cyclic AMP can maintain or increase the degree of activation of the binding protein (at pH 7.0). The fact that the dissociation rate curve was the same in the absence and presence of 5 μM cyclic AMP indicates that inactivation of the binding protein during the dissociation rate experiment was not a source to erratic results. The lack of effect of adenosine (5 μM) suggests the absence of heterotrop cooperative effects.

Low pH decreases the apparent affinity for cyclic AMP (6). The observation that the pH does not affect the dissociation rate for cyclic AMP is in favor of the interpretation that the low apparent affinity at low pH could be explained by incubation under conditions not preserving the degree of activation.

Dissociation Rate for [^{3}H]Adenosine—The nonlinear Scat-

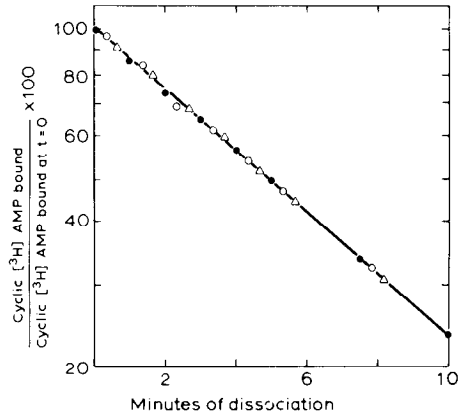

FIG. 6. Dissociation rate for cyclic [^{3}H]AMP. This was determined as described under "Materials and Methods." The binding protein was diluted to 0.5 μg/ml in 30 mM Hepes buffer, pH 7.0, containing 0.2% bovine serum albumin, no adenine derivative (●), 5 μM cyclic AMP (○), or 5 μM adenosine (△). The results are plotted as log bound *versus* time of dissociation.

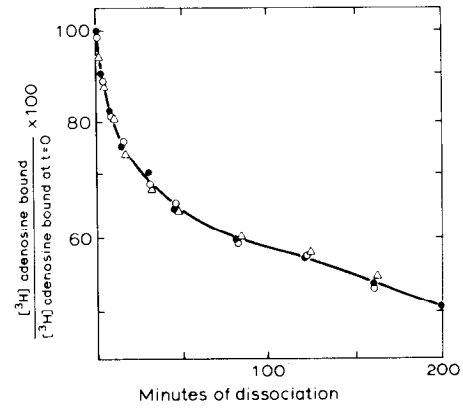

FIG. 7. Dissociation rate for [^{3}H]adenosine. This was determined as described under "Materials and Methods." The binding protein was diluted to 0.5 μg/ml in the buffer given in legend to Fig. 5 either containing no adenine derivative (●), 5 μM cyclic AMP (○), or 5 μM adenosine (△). The results are plotted as log bound *versus* time of dissociation.

chard plot for the binding of [^{3}H]adenosine to the activated binding protein has been explained by heterogeneity of binding sites (1). An alternative interpretation is negative cooperativity among the binding sites for adenosine. To investigate the possibility of site to site interactions on the activated binding protein, the dissociation rate for adenosine was measured by diluting the [^{3}H]adenosine protein complex (see "Materials and Methods") in the same buffers (containing 0.2% bovine serum albumin) as those used in the dissociation rate experiment for cyclic AMP (see preceding paragraph) which in some experiments contained 5 μM of cyclic AMP or adenosine. The log bound *versus* time graph was unaffected by the presence of 5 μM adenosine or 5 μM cyclic AMP and pH did not increase or decrease the dissociation rate for [^{3}H]adenosine. The dissociation rate was lower for adenosine than for cyclic AMP and the log bound *versus* time graph was nonlinear indicating the presence of more than one site binding adenosine (Fig. 7). No effect of adenosine or cyclic AMP on the dissociation rate curve suggests the absence of cooperative effects.

Competition studies indicate that adenosine binds to the cyclic AMP site (1). To investigate whether part of the dissociation rate curve could be explained by liberation of adenosine from the cyclic AMP site, the dissociation rate experiment was performed as described under "Materials and Methods" except that 100 μM unlabeled cyclic AMP was included in the incubation mixture to inhibit the binding of adenosine to the cyclic AMP binding site. No detectable change in the dissociation rate curve was obtained under the conditions used. These results together with the observation that the Scatchard plot for [^{3}H]adenosine binding in the presence of 100 μM unlabeled cyclic AMP was hyperbolic (1), are compatible with adenosine binding to at least two sites apart from the cyclic AMP site.

Effect of Adenosine on Homologous Activation—Adenosine has been shown to inhibit the activation of the binding protein by Mg^{2+}-ATP (6). It was therefore of interest to investigate whether the homologous activation was affected by the presence of this adenine derivative. Adenosine probably interacts with the cyclic AMP binding site (1 and a preceding paragraph). Thus, when measuring the possible effect of adenosine on the homologous activation, the concentrations of cyclic AMP and adenosine must be chosen so that only minor and predictable inhibition of the cyclic AMP binding by adenosine occurs. The inhibition can be calculated by the following rearrangement of the Michaelis-Menten equation:

$$\% \text{ inhibition} = 100 - 100\,\frac{K_i\,(\text{cyclic AMP})}{K_d\,(\text{I}) + [(\text{cyclic AMP}) + K_d]K_i}$$

where K_i (8×10^{-7} M) is the inhibition constant for adenosine (1), K_d (1.5×10^{-7} M) the equilibrium dissociation constant for cyclic AMP (1) and (I) the concentration of inhibitor (adenosine). If the concentrations of cyclic AMP and adenosine are chosen to be 5 μM and 2.5 μM, respectively, adenosine would inhibit the cyclic AMP binding by 11%.

The homologous activation of the cyclic AMP binding site determined as cyclic [^{3}H]AMP binding as a function of time, was measured at 5 μM cyclic [^{3}H]AMP in the absence and presence of 2.5 μM of [^{14}C]adenosine (Fig. 8). After 15 min of incubation a part of the incubation mixture not containing adenosine was made 2.5 μM in [^{14}C]adenosine (arrow) and the incubation was run for further 30 min. As pH affects the activation process (Fig. 4*C*), the experiment was conducted at pH 6, 7, and 8. Fig. 8 shows the results obtained at pH 6 and pH 8.

Adenosine (2.5 μM) added at zero time, inhibited the binding of cyclic [^{3}H]AMP by 70 to 80%. By addition of adenosine after 15 min of incubation (arrow), the graph for cyclic [^{3}H]AMP binding *versus* time displayed a small downward curvature immediately after the incubation mixture was supplemented with adenosine. This probably represents the competitive inhibition of cyclic AMP binding by adenosine. This small decrease in the binding of cyclic [^{3}H]AMP was in the order of magnitude calculated above (11%). After the addition of adenosine the curve ran parallel with that obtained for cyclic [^{3}H]AMP binding in the presence of 2.5 μM adenosine from time zero, whereas the binding in the absence of adenosine continued to increase at nearly the same velocity as before. The binding of [^{14}C]adenosine was determined simultaneously and reached a plateau within 10 to 12 min of incubation (Fig. 7, *A* and *B*).

After 45 min of incubation, sufficient time had elapsed for re-equilibration of cyclic AMP binding after the addition (at 15 min) of adenosine, as judged from the dissociation kinetics

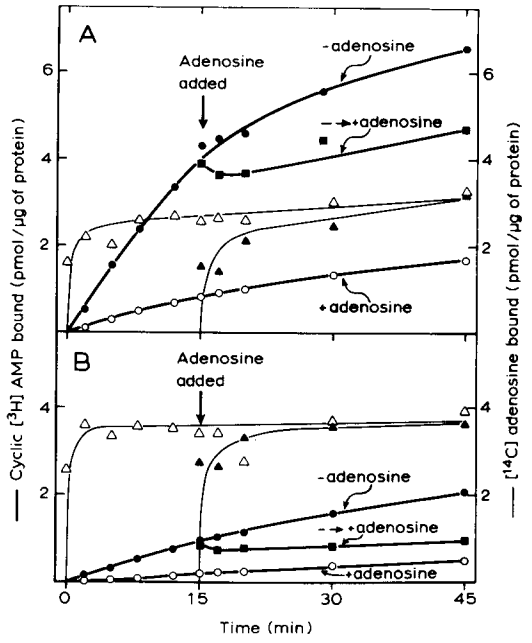

FIG. 8. Inhibition of homologous activation by adenosine. Binding protein (160 µg/ml) was incubated at 30° in the presence of 5 µM cyclic [^{3}H]AMP with and without the addition of [^{14}C]adenosine (2.5 µM). After 15 min of incubation, part of the incubate not containing adenosine was made 2.5 µM in [^{14}C]adenosine (*arrow*). Samples (100 µl) were taken for determination of cyclic [^{3}H]AMP and [^{14}C]adenosine bound at time indicated on the figure. ●——●, cyclic [^{3}H]AMP bound in the absence of adenosine; ○——○, cyclic [^{3}H]-AMP bound in the continuous presence of [^{14}C]adenosine; ■——■, cyclic [^{3}H]AMP bound in the fraction made 2.5 µM in [^{14}C]adenosine at time 15 min; △——△, [^{14}C]adenosine bound in the incubate containing adenosine from time zero minutes; ▲——▲, [^{14}C]-adenosine bound in the incubate made 2.5 µM in [^{14}C]adenosine at time 15 min. *Panel A* shows the results obtained at pH 6.0 (20 mM Mes buffer containing 5 mM Mg^{2+}-acetate and 75 mM KCl); *Panel B*, the results obtained at pH 8.0 (20 mM Hepes buffer containing 5 mM Mg^{2+}-acetate and 75 mM KCl).

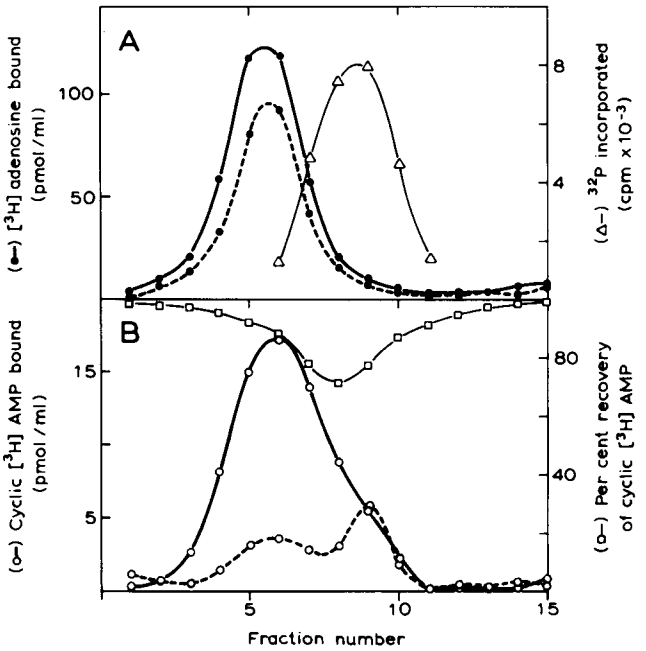

FIG. 9. Sucrose gradient centrifugation of mouse liver extract and measurement of cyclic [^{3}H]AMP and [^{3}H]adenosine binding in the absence and presence of competing adenine nucleotides. Livers were rapidly removed and put in ice-cold 30 mM Tris/HCl buffer, pH 7.6, containing 10 mM EDTA and homogenized (1:2, w/v) in a Thomas type C homogenizer with four strokes at 465 rpm. After centrifugation at 20,000 *g* for 30 min, samples (300 µl) from the supernatant were immediately subjected to sucrose gradient centrifugation as described under "Materials and Methods." *A*, aliquots of 20µl from the fractions were assayed for adenosine binding activity by incubating in the presence of 2 µM [^{3}H]adenosine, 10 mM magnesium acetate, 150 mM KCl, 1 mM methylisobutylxanthine in 15 mM Hepes, pH 7.0, in the absence (●——●) and presence (●- - -●) of 0.1 mM AMP, 0.5 mM ADP, and 2 mM ATP. The incubation was run for 30 min at 37° in a total volume of 120 µl. The protein kinase activity (△——△) was determined in samples of 20 µl as described previously (13). *B*, cyclic [^{3}H]AMP binding was assayed exactly as above except that aliquots (20 µl) were incubated in the presence of cyclic [^{3}H]AMP (2 µM) instead of [^{3}H]adenosine. The binding was determined in the absence (○——○) and presence (○- - -○) of competing adenine nucleotides. The recovery of cyclic [^{3}H]AMP (□——□) after 30 min of incubation, was determined by thin layer chromatography as described under "Materials and Methods."

for cyclic AMP (Fig. 6) and the time course of binding of cyclic AMP to the activated binding protein (Figs. 1 and 2*B*). At this time (45 min of incubation) the amount of [^{14}C]adenosine bound was the same whether adenosine was added at time zero (incubation mixture A) or after 15 min (incubation mixture B) whereas the amount of cyclic [^{3}H]AMP bound in incubate A was one-half to one-third of the cyclic [^{3}H]AMP bound in incubate B. These data suggest that after 45 min of incubation A contained one-half (pH 8.0) to one-third (pH 6.0) of the binding sites for cyclic AMP present in B, but the amount of binding protein was the same. Thus, the binding protein in incubate B had acquired a higher degree of activation than in A during 15 min of activation in the absence of adenosine. The amount of cyclic [^{3}H]AMP bound to the binding protein incubated in the absence of adenosine for 45 min, was severalfold higher than for the protein incubated in the presence of adenosine (Fig. 7, *A* and *B*). Only a part (11%) of the reduction could be explained by competitive inhibition.

Binding of Cyclic [^{3}H]AMP and [^{3}H]Adenosine to Liver Proteins in Absence and Presence of Physiological Concentrations of Other Adenine Derivatives—To approach the biological function of the cyclic AMP-adenosine binding protein, the amount of cyclic AMP and adenosine that might be bound *in vivo* by this protein was determined. This was performed by subjecting mouse liver extract to sucrose gradient centrifugation and measuring the amount of cyclic [^{3}H]AMP binding activity that co-sedimented with adenosine binding activity and protein kinase activity, respectively (Fig. 9). Cyclic [^{3}H]AMP binding was measured at 2 µM in the absence and presence of assumedly physiological concentrations (16) of AMP (0.1 mM), ADP (0.5 mM), and ATP (2 mM). The experiment was conducted in the presence of 1-methyl-3-isobutylxanthine to inhibit the hydrolysis of cyclic AMP which occurs under activation conditions. The recovery of cyclic [^{3}H]AMP was higher than 70% after 30 min of incubation (Fig. 9*B*).

The incubations were run for 30 and 60 min at 37°. The results obtained after 30 min of incubation are shown in Fig. 9. In the presence of AMP, ADP, and ATP no increase in the amount of cyclic [^{3}H]AMP bound to the protein co-sedimenting with adenosine binding activity and phosphotransferase activ-

ity was obtained by increasing the incubation time from 30 to 60 min (data not shown). In contrast, a slight increase (40%) in the binding of cyclic [^{3}H]AMP to protein co-sedimenting with the adenosine binding activity was observed in the absence of AMP, ADP, and ATP. This could be explained as follows: In the presence of ATP (AMP and ADP), no further activation was obtained by increasing the incubation time from 30 to 60 min and equilibrium was obtained. In the absence of these nucleotides, the homologous activation proceeded after 30 min of incubation.

Adenosine binding was only slightly inhibited by AMP, ADP, and ATP whereas cyclic AMP binding under this condition was about 20% of the binding observed in the absence of competing nucleotides[2] (Fig. 9*B*). These results indicate that about equal amounts of cyclic AMP binding activity as determined in the presence of physiological concentrations of AMP, ADP and ATP, are associated with the cyclic AMP-adenosine binding protein and cyclic AMP-dependent protein kinase.

DISCUSSION

The conclusions that can be made from the experimental data presented in this paper depend on the degree of purity of the cyclic AMP-adenosine binding protein. The protein appears to be homogenous as judged by ultracentrifugation and polyacrylamide gel electrophoresis in the absence and presence of sodium dodecyl sulfate (1). The impression of a high degree of purity has recently been supported by NH_2-terminal amino acid analysis with the dansyl method.[3] However, the possibility exists that minor impurities not detectable by the methods used are present and that these could affect the binding characteristics. The existence of impurities in sufficient concentrations to account for the binding of either cyclic AMP or adenosine is unlikely. Co-migration of the cyclic AMP and adenosine binding activities in polyacrylamide gels under various conditions[4] argues against that these binding activities reside on different molecules.

A property of the cyclic AMP-adenosine binding protein is its ability to undergo activation when incubated under certain defined conditions. Two types of activation can be distinguished, (*a*) Heterologous activation, *i.e.* activation of the cyclic AMP and adenosine sites by ATP (6), (*b*) homologous activation, *i.e.* activation of the cyclic AMP binding site by cyclic AMP. The concentrations of ATP or cyclic AMP required for activation were within the physiological range (16, 18). The activation process was promoted by cations (Mg^{2+} and K^+) present in the intracellular sap. It is possible that the cyclic AMP-adenosine binding protein in the cell may be subjected to similar changes characterized by severalfold increase in the number of binding sites for cyclic AMP.

In addition to an increase in the number of binding sites, the binding properties of the protein could be modulated by an increase or a decrease in the affinity for cyclic AMP or adenosine. The dissociation rate experiments presented (Figs. 6 and 7) were in favor of the absence of cooperative phenomena on the activated binding protein. The studies confirmed the assumption based on equilibrium binding studies published previously (1). Cyclic AMP binds to a homogeneous population of sites whereas adenosine seems to interact with at least two sites in addition to the cyclic AMP binding site. The low dissociation rate for adenosine compared to that for cyclic AMP further points to the difference between adenosine and cyclic AMP binding sites.

There is a theoretical objection to the conclusions based on the dissociation rate experiments. A factor could influence the association rate without affecting the dissociation rate. Thus, changes in the equilibrium dissociation constant could be brought about which would not be detected by measuring the dissociation rate. However, determination of the association rate constant for cyclic AMP (at 30° at least) would be obscured by the homologous activation. The determination of the association rate constant has another disadvantage compared to the measurement of the dissociation rate. The former cannot be determined in the presence of excess of a competitive inhibitor. On this basis the dissociation rate was chosen as an experimental approach to further studies on the binding properties of the cyclic AMP-adenosine binding protein.

Adenosine (10 μM) inhibits the activation of the binding protein by ATP (6) and also inhibits the homologous activation of the cyclic AMP binding site (Fig. 8, *A* and *B*). This type of experiment illustrates the problems encountered when determining the specificity of the cyclic AMP-adenosine binding protein by competition studies under activation conditions. An inhibitor of activation will appear to be an efficient inhibitor of cyclic [^{3}H]AMP binding.

At the present stage of knowledge one may speculate whether adenosine exerts some of its biological effects by increasing the amount of free cyclic AMP available to the cyclic AMP-dependent protein kinase by two synergistic mechanisms. By inhibiting the activation of cyclic AMP binding sites and, secondly, by competitive inhibition of cyclic AMP binding. The inhibitory effects of adenosine on the activation was obtained at concentrations within the physiological range (19).

The results presented in Fig. 9 do not argue against the concept that the cyclic AMP-adenosine binding protein may function to sequestrate cyclic AMP in mouse liver. A significant portion of cyclic AMP was bound to the cyclic AMP-adenosine binding protein relative to the binding activity that co-sedimented with the phosphotransferase activity when the cyclic AMP binding was measured at 2 μM and in the presence of other adenine nucleotides. The concentration of cyclic AMP used is within the order of magnitude reported for half-maximal activation of cyclic AMP-dependent protein kinase in brown adipose tissue (20) and rat heart (21).

The necessary requirement for the validity of this experiment is that cyclic [^{3}H]AMP exchanges with cyclic AMP that might be bound to the proteins *in vivo*. Evidence has been presented suggesting that in the presence of MgATP, incubation with cyclic [^{3}H]AMP allows the determination of the number of cyclic AMP binding sites in rat diaphragm extract irrespectively of the amount of cyclic AMP bound *in vivo* (22). Addition of MgATP to crude extracts from various tissues has been used for the detection of the amount of regulatory

[2] The number of binding sites for cyclic AMP (as measured in the absence of other nucleotides) generated in the presence of cyclic AMP, AMP, ADP, and ATP (Fig. 9) is probably higher than the amount of cyclic AMP bound to the cyclic AMP-adenosine binding protein in the absence of AMP, ADP, and ATP. The reason for this is the higher degree of activation obtained in the presence of ATP. Therefore, the displacement is probably underestimated by comparing the binding of cyclic AMP in the absence and presence of other adenine nucleotides.

[3] P. M. Ueland, T. Skotland, S. O. Døskeland, and T. Flatmark, manuscript in preparation.

[4] J. Saebø and P. M. Ueland, unpublished results.

subunit of protein kinase already existing as cyclic AMP complex (23). This is based on the observation that MgATP decreases the affinity of the cyclic AMP-dependent protein kinase for cyclic AMP (24, 25) at least with one isoenzyme form of the enzyme. As judged from the high dissociation rate constant for cyclic [^{3}H]AMP from the cyclic AMP-adenosine binding protein (Fig. 6), cyclic AMP bound to this protein *in vivo* is probably not a source to erratic results. Furthermore, no increase in the binding of cyclic [^{3}H]AMP from 30 to 60 min of incubation, indicates that equilibrium has been obtained.

In the presence of assumedly physiological concentrations of AMP, ADP, and ATP, and under the experimental conditions presented in Fig. 9, the amount of cyclic AMP bound to the cyclic AMP-adenosine binding protein was about the same as the binding capacity of the cyclic AMP-dependent protein kinase. In addition, the data presented in the figure also give clues to the molar ratio that may exist in the cell between the cyclic AMP binding sites residing on these two classes of proteins. The adenosine binding (Fig. 9*A*) is about 20-fold higher than the cyclic AMP binding associated with the cyclic AMP-dependent protein kinase (Fig. 9*B*). Furthermore, the purified cyclic AMP-adenosine binding protein could be activated to the extent that the cyclic AMP binding capacity equals the adenosine binding capacity (1). Assuming that the same degree of activation could be obtained *in vivo*, these data indicate that the number of cyclic AMP sites (as measured in the absence of other adenine nucleotides) on the cyclic AMP-adenosine binding protein could under certain circumstances be about 20-fold higher than the number of sites associated with the cyclic AMP-dependent protein kinase. The cyclic AMP binding to the cyclic AMP-adenosine binding protein is partly displaced by other adenine nucleotides, but a substantial part of cyclic AMP may be bound to this protein assuming that the same binding properties exist *in vivo* as those found under the experimental conditions used. However, in bovine adrenal cortex, the concentration of this class of protein is about 10 times lower than in mouse liver.[5] This argues against the concept that cyclic AMP sequestration by this protein could take place to the same extent in all mammalian cells.

Based on measurement of cyclic AMP binding in the presence of competing adenine nucleotides, Sudgen and Corbin (26) state that cyclic AMP does probably not bind to similar proteins in rat liver *in vivo*. Our results do not exclude that significant binding of cyclic AMP may occur intracellularly. The discrepancy may be explained by different incubation conditions used and by the fact that these workers measured cyclic AMP binding by a method involving nitrocellulose membrane filtration of protein without prior precipitation of the protein. This assay, in our hands, does not give quantitative estimate of the cyclic AMP·protein complex as the cyclic AMP-adenosine binding protein is poorly retained by the filters (1). The ammonium sulfate precipitation Millipore filtration technique (1) seems to give a true estimate of the cyclic AMP bound both to this class of protein (1) and to the cyclic AMP-dependent protein kinase (27).

Recently, reports have appeared dealing with cyclic AMP binding to proteins in perfused rat liver (28) and to protein kinase in rat liver *in vivo* (29). Castagna *et al.* (28) using perfusion with ^{3}H-labeled dibutyryl cyclic AMP and subsequent sucrose gradient centrifugation, suggest that the only binding species detected in cytosol could be attributed to the regulatory subunit of the cyclic AMP-dependent protein kinase. Schwoch and Hilz (29) found a close correlation at low concentrations of cyclic AMP between activation of protein kinase and the amount of cyclic AMP bound to proteins in rat liver extract. These workers, separating free and bound nucleotide by charcoal treatment of cytosol, suggest that binding to low affinity binding proteins would not have been detected by this method. Because of the high dissociation rate for cyclic AMP (Fig. 6) the binding of this nucleotide to the cyclic AMP-adenosine binding protein could probably not have been detected by either method. In contrast, Skare *et al.* (30) observed at least 10 sites for cyclic AMP of apparent different molecular weights in whole sarcoma 37 cells using 8-azido-adenosine-3',5'-monophosphate as a photoaffinity probe.

Another interesting hypothesis is based on the assumption that the protein has enzyme activity. Cyclic AMP may interact with a regulatory site whose effects are antagonized by binding of adenosine to the site specific for this adenine derivative. However, apart from phosphofructokinase (10, 31) and cyclic AMP-dependent protein kinase (24), no reports have appeared yet on the interaction between an enzyme and cyclic AMP or adenosine characterized by the same or higher affinity for these adenine derivatives other than that reported for the cyclic AMP-adenosine binding protein.

Acknowledgment—The technical assistance of T. Ellingsen is highly appreciated.

[5] S. O. Döskeland, unpublished results.

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