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Binding Proteins for Adenosine 3′:5′-Cyclic Monophosphate in Bovine Adrenal Cortex

By STEIN OVE DØSKELAND and PER MAGNE UELAND
Cell Biology Research Group, The Preclinical Institutes, University of Bergen, N-5000 Bergen, Norway

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Five peaks of cyclic AMP-binding activity could be resolved by DEAE-cellulose chromatography of bovine adrenal-cortex cytosol. Two of the binding peaks co-chromatographed with the catalytic activities of cyclic AMP-dependent protein kinases (ATP–protein phosphotransferase, EC 2.7.1.37) of type I or type II respectively. A third binding protein was eluted between the two kinases, and appeared to be the free regulatory moiety of protein kinase I. Two of the binding proteins for cyclic AMP, sedimenting at 9S in sucrose gradients, could also bind adenosine. They bound cyclic AMP with an apparent equilibrium dissociation constant (K_d) of about 0.1 μM, and showed an increased binding capacity for cyclic AMP after preincubation in the presence of K^+, Mg^{2+} and ATP. The two binding proteins differed in their apparent affinities for adenosine. The isolated regulatory moiety of protein kinase I had a very high affinity for cyclic AMP (K_d < 0.1 nM). At low ionic strength or in the presence of MgATP, the high-affinity binding of cyclic AMP to the regulatory subunit of protein kinase I was decreased by the catalytic subunit. At high ionic strength and in the absence of MgATP the high-affinity binding to the regulatory subunit was not affected by the presence of catalytic subunit. Under all experimental conditions tested, dissociation of protein kinase I was accompanied by an increased affinity for cyclic AMP. To gain some insight into the mechanism by which cyclic AMP activates protein kinase, the interaction between basic proteins, salt and the cyclic nucleotide in activating the kinase was studied.

Specific binding proteins for cyclic AMP were first discovered in bovine adrenal-cortex extract (Gill & Garren, 1969). The binding proteins appeared to be the regulatory moieties of cyclic AMP-dependent protein kinases (Gill & Garren, 1970, 1971), earlier shown to exist in muscle extracts (Walsh *et al.*, 1968). It is well established that cyclic AMP-dependent protein kinase holoenzymes from mammalian tissues consist of two regulatory (R) subunits and two catalytic (C) subunits, which, in the presence of cyclic AMP, can dissociate into a regulatory-subunit dimer (R_2) and two free active catalytic subunits according to the scheme (Rosen *et al.*, 1975; Hofmann *et al.*, 1975):

$$R_2C_2 = R_2 + 2C$$

The detailed mechanism for the activation of protein kinase is not yet known. Several models are compatible with the experimental data presently available (Ogez & Segel, 1976).

We have presented kinetic evidence that binding sites with different affinities for cyclic AMP exist in adrenal-cortex extract. The high-affinity binding in the extract was enhanced in the presence of KCl or NaCl (Døskeland *et al.*, 1977). In the present study the protein kinase carrying the high-affinity site for cyclic AMP was separated from the other binding proteins by DEAE-cellulose chromatography, and the conditions governing its affinity were investigated. It was also decided to study the effect of the catalytic subunit on the cyclic AMP-binding properties of the regulatory moiety of the protein kinase.

Cyclic AMP/adenosine-binding proteins of the type found in rabbit erythrocytes (Yuh & Tao, 1974) and mouse liver (Døskeland & Ueland, 1975*a*; Ueland & Døskeland, 1977) were sought. Such proteins may possibly function to modulate the cyclic AMP response of the cell. Their presence in adrenal cortex could explain some of the problems encountered when cyclic AMP is measured by competitive binding to minimally purified adrenal extract (Albano *et al.*, 1974; Døskeland *et al.*, 1977).

Experimental

Materials

Crystallized bovine serum albumin, calf thymus histone (type II), ATP (disodium salt), cyclic AMP (free acid) and other purine derivatives were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Cyclic [8-^{3}H]AMP (27 Ci/mmol) and [2-^{3}H]adenosine (21 Ci/mmol) of more than 95% purity, as judged by

t.l.c. (Ueland & Døskeland, 1977), were from The Radiochemical Centre, Amersham, Bucks., U.K. [γ-^{32}P]ATP was prepared by the method of Glynn & Chappell (1964). DEAE-cellulose (DE-52) and phosphocellulose (P-11) were from Whatman Biochemicals, Maidstone, Kent, U.K. Sephadex G-25 (medium) and Sephadex G-150 (fine) were from Pharmacia, Uppsala, Sweden. Membrane filters (HAWP, 0.45μm pore size) were from Millipore Corp., Bedford, MA, U.S.A.

Buffers

Buffer A is 50mM-Hepes*/30mM-EDTA/800mM-NaCl/10mM-2-mercaptoethanol, adjusted to pH7.2 at 0°C with NaOH. Buffer B is 15mM-Hepes/1mM-EDTA/10mM-2-mercaptoethanol, adjusted to pH7.0 at 0°C with NaOH.

Assay of binding of cyclic [^{3}H]AMP and [^{3}H]adenosine

Except when otherwise indicated the incubations were carried out at 0°C. The binding reaction was stopped by mixing a portion of the mixture with 10vol. of ice-cold 80%-satd. $(NH_4)_2SO_4$, containing 0.1mM-unlabelled ligand to prevent binding of labelled ligand during the precipitation (Døskeland *et al.*, 1977). Free ligand was separated from bound by suction through membrane filters, which were processed for liquid-scintillation counting as described by Døskeland & Ueland (1975*a*). In the absence of binding protein 0.1–0.3% of the added radioactivity was retained by the filters. As a routine cyclic [^{3}H]AMP and [^{3}H]adenosine were diluted with unlabelled ligands to specific radioactivities of 2.7Ci/mmol and 2.1Ci/mmol respectively, for the studies of cyclic AMP/adenosine-binding proteins, whereas cyclic [^{3}H]AMP of the original specific radioactivity (27Ci/mmol) was used for studies of binding to protein kinase. Variations in the degree of isotopic dilution of either of the ligands did not affect the amount of ligand bound.

Protein kinase activity

This was measured essentially as described by Ueland & Døskeland (1976). Except when otherwise indicated the incubations were carried out in a volume of 150μl for 10min at 30°C in 15mM-Hepes/NaOH, pH7.0, containing 0.3mM-EGTA, 0.1mM-EDTA, 10mM-magnesium acetate, 30μM-[γ-^{32}P]-ATP (1μCi/ml) and histone (0.67mg/ml). One unit of enzyme activity is the amount of activity incorporating 1pmol of phosphate into histone/min at 30°C. The protein kinase activity ratio is defined as the activity obtained in the absence of added cyclic AMP divided by the activity in the presence of 2μM-cyclic AMP.

* Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Cyclic AMP phosphodiesterase (EC 3.1.4.17) activity

This was measured as described by Ueland & Døskeland (1977).

Adenosine deaminase (EC 3.5.4.4) activity

This was determined by a spectrophotometric method, essentially as described by Kalckar (1947).

Protein

This was measured by the method of Klungsøyr (1969), with bovine serum albumin as the standard.

Preparation of adrenal-cortex cytosol

Adrenal glands were obtained within a few minutes after slaughter of the animals and brought to the laboratory on ice; adrenal cortex and medulla were separated, and the cortical tissue was put into ice-cold homogenization buffer [15mM-Tris/HCl (pH7.6)/4mM-EDTA/10mM-2-mercaptoethanol/0.25M-sucrose]. Homogenization (1:10, w/v) was performed by six strokes in a glass/Teflon homogenizer (Thomas, type C) with a pestle speed of 750rev./min. The high-speed (20000$g_{av.}$, 20min) supernatant of this homogenate was centrifuged for 1h at 100000$g_{av.}$, and the resulting cytosol was desalted by passage through a column (2.6cm×40cm) of Sephadex G-25 equilibrated with 10mM-Tris/HCl (pH7.5)/1mM-EDTA/10mM-2-mercaptoethanol.

DEAE-cellulose chromatography of cytosol

The desalted cytosol was applied to a column (2.6cm×36cm) of DEAE-cellulose equilibrated with 10mM-Tris/HCl (pH7.5)/1mM-EDTA/10mM-2-mercaptoethanol. After washing with 1.2 litres of this buffer a linear gradient (total volume 1 litre) of 0–0.35M-NaCl in the same buffer was set up. Fractions (18ml) were collected and samples (500μl) desalted by passage through Sephadex G-25 columns equilibrated with buffer B, as described by Døskeland & Ueland (1975*b*).

Sucrose-density-gradient centrifugation

Linear density gradients (10ml) of 5–20% (w/v) sucrose in buffer B were overlayered with 250μl of sample and spun for 22h (19h for rat liver binding proteins) at 40000rev./min in the 488 rotor of an International B60 ultracentrifuge. Fractions (0.65ml) were collected. For determinations of sedimentation velocities, bovine haemoglobin (4.5S) was added to the samples as an internal marker. Cyclic AMP/adenosine-binding protein from mouse liver (8.8S; Ueland & Døskeland, 1977), run in parallel tubes, served as an external marker.

Polyacrylamide-gel electrophoresis

Gels (0.7cm×7cm) of 9% (w/v) polyacrylamide with 3.3% cross-linking were prepared with *NNN'N'*-

tetramethylethylenediamine (7mM) and ammonium peroxydisulphate (5mM) as catalysts. The gels were pre-electrophoresed at constant voltage until the current had declined to a constant value. After the sample (50μg of protein in 50μl) of buffer B with about 15%, w/v, sucrose had been applied, electrophoresis was conducted at 2°C for 5h at 1.5mA/tube. The same buffer (20mM-Tris/glycine, pH8.8) was used in the gels and the electrode compartments. The gels were either stained for protein with Coomassie Blue or cut into slices of 1.2mm thickness with a gel cutter. The gel slices were finely divided with scissors and extracted overnight at 2°C with 350μl of buffer B. For analytical purposes 50μl portions of the extracts were incubated for 2h at 0°C in the presence of 0.5μM-[^{3}H]adenosine in buffer B; other samples (100μl) were incubated for 2h at 30°C in buffer B containing 2μM-cyclic [^{3}H]-AMP, 10mM-magnesium acetate and 150mM-KCl, and other samples (100μl) were assayed for adenosine deaminase activity. When polyacrylamide-gel electrophoresis was used as a preparative step, the cyclic AMP/adenosine-binding proteins were assayed by incubating 25μl portions in the presence of 0.5μM-[^{3}H]adenosine. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed in 7.5% gels as described by Weber *et al.* (1972).

Preparation of cyclic AMP/adenosine-binding proteins

The cytosol fraction precipitated between 12 and 31g of poly(ethylene glycol) (mean mol.wt. 6000) per 100ml of cytosol was dissolved in 10mM-Tris/HCl (pH7.5)/1mM-EDTA/10mM-2-mercaptoethanol and chromatographed on DEAE-cellulose under the same conditions as used for the chromatography of cytosol. The two peaks of cyclic AMP/adenosine-binding activity separated (Fig. 1*c*) were termed cyclic AMP/adenosine-binding protein I and cyclic AMP/adenosine-binding protein II according to the order of elution from the column. The peak fractions were pooled separately, adjusted to pH8.8 by the addition of 0.5M-Tris base, and applied to another column (2.6cm×10cm) of DEAE-cellulose, equilibrated with 10mM-Tris/HCl (pH8.8)/0.5mM-EDTA/10mM-2-mercaptoethanol. The binding activity was eluted with a linear gradient (0–200mM) of NaCl in the equilibration buffer and the peak fractions were pooled, precipitated with 65%-satd. $(NH_4)_2SO_4$, desalted by Sephadex G-25 chromatography, and subjected to sucrose-density-gradient centrifugation. Cyclic AMP/adenosine-binding protein I was obtained devoid of cyclic AMP phosphodiesterase or adenosine deaminase activities by this procedure, but contained several protein contaminants, as judged by polyacrylamide-gel electrophoresis in the absence or presence of sodium dodecyl sulphate. Cyclic AMP/adenosine-binding protein II so prepared contained adenosine deaminase, and was further purified by polyacrylamide-gel electrophoresis. The migration of the cyclic AMP/adenosine-binding activity and the adenosine deaminase activity was compared with the protein bands in a gel run in parallel. The major protein band corresponded to the cyclic AMP/adenosine-binding activity. The adenosine deaminase activity was associated with a minor band which had migrated about half as far as the major band. A minor rapidly moving band and a band with a relative mobility about 0.75 that of the major band were devoid of both adenosine deaminase and binding activities.

To obtain cyclic AMP/adenosine-binding protein II free of adenosine deaminase, the peak fractions of binding activity from 12 separate gels were pooled. This preparation gave only one band on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate and was used for the binding studies.

Preparation of the regulatory and catalytic moieties of protein kinase I

Protein kinase I was obtained by DEAE-cellulose chromatography of cytosol as described above, except that a more shallow (0–150mM) NaCl gradient was used. The early fractions of protein kinase I (up to and including the peak fraction) were pooled, precipitated with 45%-satd. $(NH_4)_2SO_4$ and dissolved in a minimal volume of buffer A containing 1mg of bovine serum albumin/ml. A sample (0.4ml) was applied to a column (0.9cm×40cm) of Sephadex G-150 equilibrated with 15mM-Hepes/NaOH (pH 7.2)/1mM-EDTA/800mM-NaCl/10mM-2-mercaptoethanol/1mg of albumin/ml. Fractions (0.6ml) were collected. The fractions containing cyclic AMP-binding activity without phosphotransferase activity (R_2) were pooled and desalted by passage through a column (0.9cm×9cm) of Sephadex G-25 equilibrated with buffer B containing 1mg of albumin/ml. The fractions containing catalytic activity devoid of binding activity (C) were pooled separately. This preparation had a kinase activity of 950 units/ml when assayed in the presence of 160mM-NaCl.

Miscellaneous

Except when otherwise indicated all handling of tissue extracts, binding protein or enzymes was in the cold (2°C). All data given are the means of duplicate determinations. In cases where the same experiments were performed several times, the data are those of a typical experiment.

Results

Fractionation of binding proteins for cyclic [^{3}H]AMP and [^{3}H]adenosine by DEAE-cellulose chromatography

Two peaks of highly cyclic AMP-dependent protein kinase activity were eluted from DEAE-cellulose by the gradient. The protein kinase eluted at low ionic strength was activated by salt (Figs. 3 and 6*c*) and histone (Fig. 6*a*) and could thus be classified as type I (Corbin *et al.*, 1975*a*), whereas the protein kinase eluted at high ionic strength was classified as type II on the basis of its resistance to activation by salt (Fig. 3). Sometimes species of protein kinase, less dependent on cyclic AMP for activity under standard assay conditions, were eluted slightly after protein kinase I. Most of the kinase activity in those fractions sedimented at 5–6S on sucrose-gradient centrifugation, and probably represented protein kinase that had undergone partial proteolysis.

High-affinity binding sites for cyclic AMP were sought (Fig. 1*a*) by incubating desalted portions of

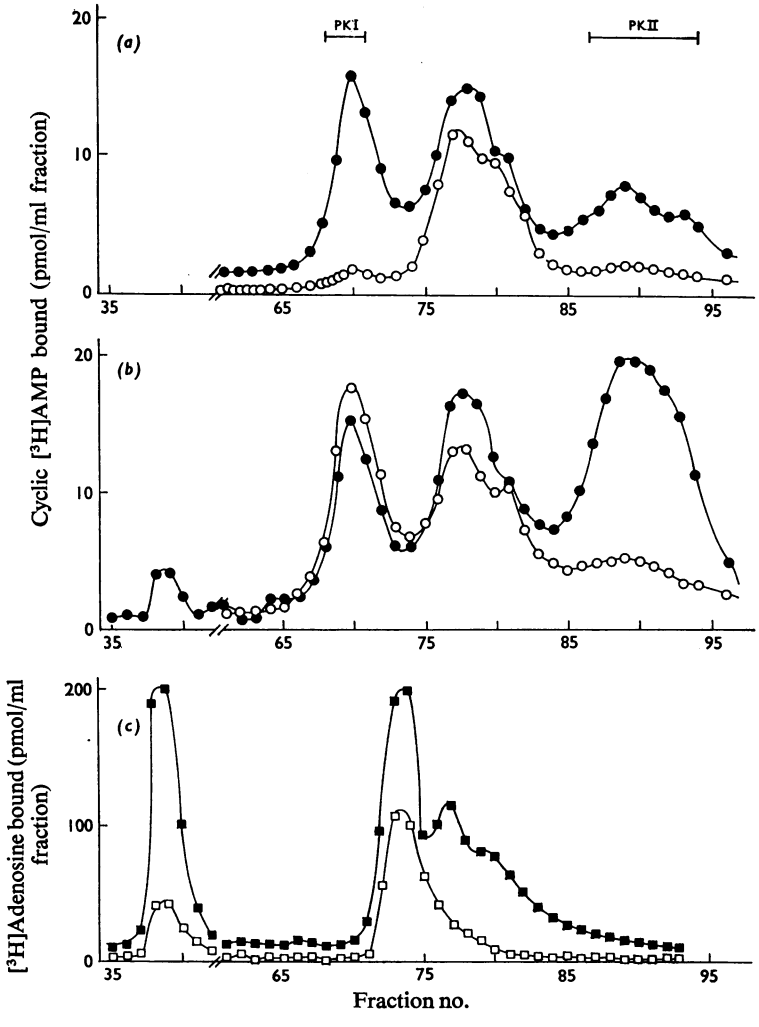

Fig. 1. *DEAE-cellulose chromatography of adrenal-cortex cytosol*

Details of the chromatographic procedure are given in the Experimental section. Desalted portions of the fractions were incubated for 2½h in the presence of cyclic [^{3}H]AMP or [^{3}H]adenosine. (*a*) Amount of nucleotide bound (pmol/ml) at 0.5nM-cyclic [^{3}H]AMP in buffer B (○) or in buffer A containing 0.2mg of histone/ml (●). (*b*) Nucleotide bound at 50nM-cyclic [^{3}H]AMP in buffer B (○) or buffer A containing histone (●). (*c*) Amount of [^{3}H]adenosine bound at 0.5μM- (□) or 10μM- (■) labelled nucleoside in buffer B. The salt gradient was started at fraction 60. The positions of protein kinase I (PK I) and protein kinase II (PK II) are indicated.

the DEAE-cellulose fractions with 0.5 nM-cyclic [^{3}H]AMP. At low ionic strength, only one major peak without associated kinase activity showed high-affinity binding. In the presence of a salt/histone mixture, high-affinity binding of cyclic AMP appeared also to be associated with protein kinase I. The fractions corresponding to protein kinase II required a high concentration of cyclic AMP (50 nM) and the presence of the salt/histone mixture to exhibit maximal binding (Fig. 1*b*).

Adenosine-binding activity was assayed at 0.5 μM- and 10 μM-adenosine. Two peaks were apparent at the lower concentration (Fig. 1*c*). As judged by the ratio of binding at 0.5 μM- and 10 μM-adenosine, the second peak had the higher affinity for adenosine.

Sucrose-density-gradient centrifugation of adrenal cytosol and cyclic AMP/adenosine-binding protein: comparison with rat liver cyclic AMP/adenosine-binding proteins

Protein kinase, highly dependent on cyclic AMP for activity, sedimented in the 7S region of a sucrose gradient loaded with cytosol (Fig. 2). A shoulder of kinase activity, less dependent on cyclic AMP, sedimented in the 5–6S region. A single sharp 9S peak was found for the binding of adenosine. The cyclic AMP-binding activity had a maximum at 7S, with one shoulder at about 9S, and another at about 4.5S. The latter probably corresponded to the high-affinity binding protein eluted between protein kinases I and II (Fig. 1*a*), since the main binding activity of that part of the DEAE-cellulose eluate also sedimented at 4–5S (results not shown). Each of the two peaks of cyclic AMP/adenosine-binding activity from the DEAE-cellulose column (Fig. 1*c*) sedimented at 9S. Two binding proteins for adenine analogues, one (14.2S) possibly the dimer of the other one (10.1S), have been described in rat liver (Sugden & Corbin, 1976). The relation between the two forms of rat liver and adrenal binding proteins was studied by subjecting rat liver cytosol to DEAE-cellulose chromatography and the peaks of cyclic AMP/adenosine-binding activity to sucrose-gradient centrifugation under the same conditions as had been used for adrenal cytosol and binding proteins. DEAE-cellulose chromatography of rat liver cytosol revealed a major peak of cyclic AMP/adenosine-binding activity sedimenting at 9S, which was eluted like the cyclic AMP/adenosine-binding protein I from adrenal cortex. A second minor peak was eluted from the column at a slightly higher ionic strength than was required to elute adrenal binding protein II from a similar column. It could be distinguished from the adrenal binding proteins by Sephadex G-200 chromatography and gradient centrifugation, by virtue of its larger molecular size. Mouse liver cytosol was found to contain the same types of cyclic AMP/adenosine-binding proteins as rat liver cytosol. The

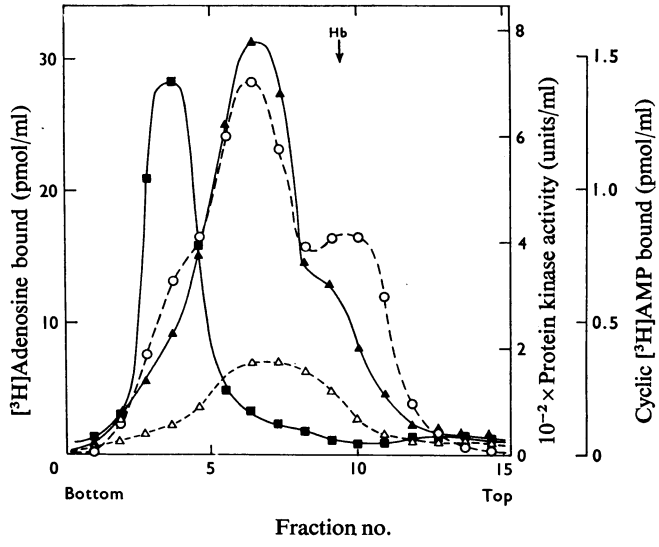

Fig. 2. *Sucrose-density-gradient centrifugation of adrenal-cortex cytosol*

Details of the procedure are given in the Experimental section. Samples (100 μl) of each fraction were assayed for binding activity in the presence of 0.5 μM-[^{3}H]adenosine in buffer B (■) and 10 nM-cyclic [^{3}H]AMP in buffer A containing 1 mg of albumin/ml (○). Portions (25 μl) were assayed for protein kinase activity in the absence (△) or presence (▲) of 2 μM-cyclic AMP. The position of haemoglobin (Hb) is indicated by the vertical arrow.

Table 1. *Binding of [^{3}H]adenosine and cyclic [^{3}H]AMP to cyclic AMP/adenosine-binding proteins at various concentrations of the radioactively labelled ligands, and in the presence of potential inhibitors*

The preparations of cyclic AMP/adenosine-binding proteins I and II from adrenal cortex (obtained as described in the Experimental section) were passed through columns of Sephadex G-25 equilibrated with 50mM-Hepes/NaOH (pH7.2)/1mM-EDTA/10mM-2-mercaptoethanol/20% (v/v) glycerol, and further diluted in the same buffer. Samples (120μl) of binding protein were added to 60μl of a mixture of radioactive ligand and inhibitor. After 18h of incubation at 0°C a sample (150μl) was withdrawn and mixed with 2ml of 80%-satd. $(NH_4)_2SO_4$ for determination of the amount of bound ligand. The concentration of cyclic AMP/adenosine-binding protein was four times as high during incubations with cyclic [^{3}H]AMP as in incubations with [^{3}H]adenosine.

	Amount of cyclic [^{3}H]AMP or [^{3}H]adenosine bound (pmol/ml)										
Total concns. (final) of potential binding inhibitors	—	—	3μM-cyclic AMP	150μM-cyclic AMP	6μM-adenosine	6μM-AMP	6μM-ADP	12μM-ATP	12μM-adenine	12μM-cyclic GMP	150μM-inosine
Total concns.' (final) of the labelled ligands (μM)	Cyclic AMP/adenosine-binding protein I	Cyclic AMP/adenosine-binding protein II									
Cyclic [^{3}H]AMP											
0.1	11.2	29	1.1	—	1.5	4.8	8.9	14	22	31	18
0.3	19.2	49	5.7	—	10	18	22	25	34	51	35
0.9	22.0	54	15	—	18	35	39	43	46	56	55
2.7	23.8	62	30	—	45	49	48	56	57	59	61
10	24.3	59	52	—	54	52	58	61	60	61	62
[^{3}H]Adenosine											
0.15	6.5	39	—	—	—	—	—	—	—	—	—
0.3	13	59	61	56	9.4	44	46	53	43	62	55
0.6	27	98	86	92	28	75	80	93	67	102	92
1.2	51	143	141	144	35	116	118	132	102	155	128
4.5	82	222	202	238	104	213	216	242	212	230	196
12	121	241	—	—	—	—	—	—	—	—	—
60	213	228	—	—	—	—	—	—	—	—	—

adrenal cyclic AMP/adenosine-binding protein II could thus be distinguished from the binding proteins of liver.

Some properties of the adrenal cyclic AMP/adenosine-binding proteins

Both of these proteins showed an increased binding capacity for cyclic AMP after preincubation in the presence of K^+, Mg^{2+} and ATP at 30°C, as described for binding protein from mouse liver (Døskeland & Ueland, 1975*a*). When either of them was incubated at 30°C in the presence of K^+, Mg^{2+} and cyclic AMP, the cyclic nucleotide was rapidly bound during the first minutes of incubation, followed for several hours by a slower but progressive increase in binding. The slowly progressing binding could be explained if cyclic AMP, like ATP, could activate binding sites for the cyclic nucleotide. In fact, binding protein preincubated in the presence of 10 μM-cyclic AMP, followed by removal of excess of cyclic AMP by Sephadex G-25 chromatography, could bind more cyclic [^{3}H]AMP in the subsequent assay than binding protein preincubated in the absence of cyclic AMP. Lowering the temperature, inclusion of 20% (v/v) glycerol or the omission of K^+ and Mg^{2+} markedly decreased the slowly progressing binding, the first rapid phase of binding being less affected. When the binding protein was incubated at 0°C in the presence of 20% glycerol (and in the absence of K^+ and Mg^{2+}) the ability of cyclic AMP to activate its own binding sites was nearly abolished. Under such conditions the substances that decrease the cyclic [^{3}H]AMP binding (Table 1) would not be expected to do so by interfering with the activation process. Table 1 shows that adrenal binding proteins I and II were half-maximally saturated at about 0.1 μM-cyclic [^{3}H]AMP. The most potent inhibitors of the binding of cyclic [^{3}H]AMP to binding protein II were cyclic AMP, adenosine, AMP, ADP and ATP, in descending order. Adenosine was an inefficient inhibitor when binding protein II had not been freed of contaminating adenosine deaminase by polyacrylamide-gel electrophoresis (results not shown). The adrenal binding protein II apparently bound [^{3}H]adenosine with higher affinity than did binding protein I (Table 1). Of several substances tested, only adenosine itself was a strong inhibitor of [^{3}H]adenosine binding to binding protein II (Table 1).

Effect of ionic strength on the high-affinity cyclic AMP binding to and the degree of activation of the protein kinase isoenzymes

Only a small fraction of the binding sites for cyclic AMP were occupied when protein kinase I or II was incubated with 1.5 nM-cyclic [^{3}H]AMP in the absence of added salt. Whereas the binding to protein kinase II was little enhanced by salt at such a low concentration of nucleotide, the binding to protein kinase I dramatically increased as a function of the concentration of KCl (Fig. 3).

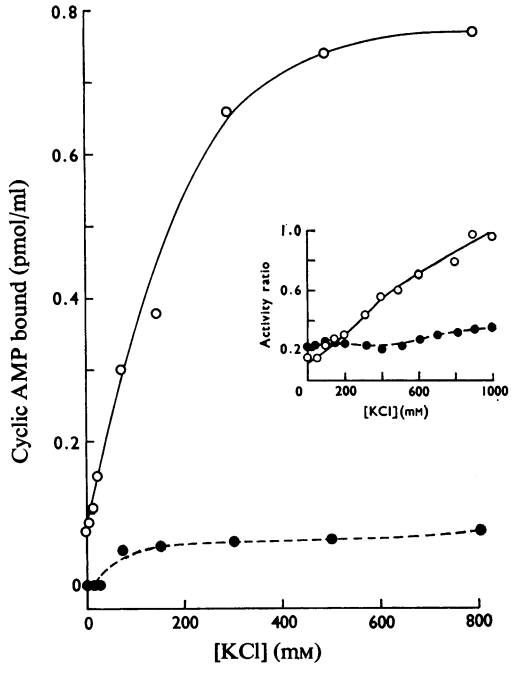

Fig. 3. *Effect of increasing ionic strength on cyclic AMP binding to and on activity ratio of protein kinases I and II*
Fraction 68 (protein kinase I) from the DEAE-cellulose column (Fig. 1) was precipitated with poly(ethylene glycol) (18 g/100 ml of fraction) and redissolved in buffer B. Fraction 88 (protein kinase II) from DEAE-cellulose (Fig. 1) was passed through a column (0.2 cm × 2 cm) of phosphocellulose equilibrated with buffer B to remove the cyclic AMP-independent protein kinases that preferentially phosphorylate casein (Ueland & Døskeland, 1976). The amount of kinase preparation added was adjusted so that the catalytic activity and binding capacity were the same for the incubations containing protein kinase I (○) and protein kinase II (●). The incubations were carried out for 2 h in buffer B with 1.5 nM-cyclic [^{3}H]AMP and various concentrations of KCl. The inset shows the effect of KCl on the activity ratio of protein kinase I (○) and protein kinase II (●) after incubation for 30 min in the absence of cyclic AMP. For measurement of the activity ratio, portions (30 μl) were taken for assays of protein kinase activity in the absence and presence of 2 μM-cyclic AMP.

In confirmation of the results of Corbin *et al.* (1975*a*), protein kinase I but not II was progressively activated at increasing concentrations of salt (Fig. 3, inset). NaCl had the same effect on the binding and activity ratio as KCl. Since NaF (Table 2), as well as trichloroacetate and perchlorate at low concen-

Table 2. *Effect on cyclic [^{3}H]AMP binding of agents activating protein kinase I*

Protein kinase I (prepared as explained in the legend to Fig. 3) was incubated for 2h in 1ml of buffer B in the presence of 1nM-cyclic [^{3}H]AMP and the agents listed in the Table. Two samples (400μl) were mixed with $(NH_4)_2SO_4$ for measurement of cyclic [^{3}H]-AMP bound, and two samples (30μl) were taken for measurement of protein kinase activity, in the absence and presence of 2μM-cyclic AMP respectively. The concentration of magnesium acetate during the kinase assay was 15mM instead of 10mM, to minimize the likelihood of Mg depletion due to formation of MgF_2.

Concentrations (final) of agents present during the binding reaction	Cyclic [^{3}H]AMP bound (pmol/ml of incubation)	Protein kinase activity ratio
—	0.023	0.18
2mM-NaF	0.025	0.20
4mM-NaF	0.022	0.25
8mM-NaF	0.030	0.19
20mM-NaF	0.045	0.37
40mM-NaF	0.073	0.58
80mM-NaF	0.109	0.68
200mM-NaF	0.141	0.78
400mM-NaF	0.149	0.87
800mM-NaCl	0.157	1.07
0.2mg of protamine sulphate/ml	0.163	0.98
200mM-NaF+800mM-NaCl	0.151	0.94
0.2mg of protamine sulphate/ml+800mM-NaCl	0.153	1.04
800mM-NaCl+10mM-magnesium actate+30μM-ATP	0.024	0.19
1mg of albumin/ml	0.024	0.12

trations (Døskeland *et al.*, 1977), also increased the high-affinity binding to and activity ratio of protein kinase I, the effect of salts seems to be non-specific, possibly according to their chaotropic potencies, as suggested by Huang & Huang (1975).

To obtain the data of Table 2, samples from the same preincubations were taken for determination of bound ligand and measurement of the protein kinase activity ratio. It has been shown that no loss of bound cyclic [^{3}H]AMP nor any binding of labelled ligand occurred under the conditions used for $(NH_4)_2SO_4$ precipitation of protein kinase I (Døskeland *et al.*, 1977). To ascertain that reassociation of the protein kinase subunits to inactive holoenzyme did not occur during the phosphotransferase assay, protein kinase I was activated to various degrees by preincubation with different concentrations of cyclic AMP. The preincubations were rapidly run through columns of Sephadex G-25, portions of the excluded fraction added to protein kinase assay mixtures (with and without added cyclic AMP) and the phosphotransferase activity was determined after various periods of time (2–20min). The activity ratio was constant during the periods of incubation tested, except for a slight increase with time of the cyclic AMP-independent activity of the samples not completely activated by the preincubation with cyclic AMP. Such an increase in the basal activity was expected from our previous findings with such dilute preparations of protein kinase (Ueland & Døskeland, 1976). Although changes in activity ratio occurring immediately after addition of the protein kinase to the reaction mixture could not be ruled out, the experiment indicated that continuous reassociation of protein kinase subunits during the assay was not a problem. A close correlation was found

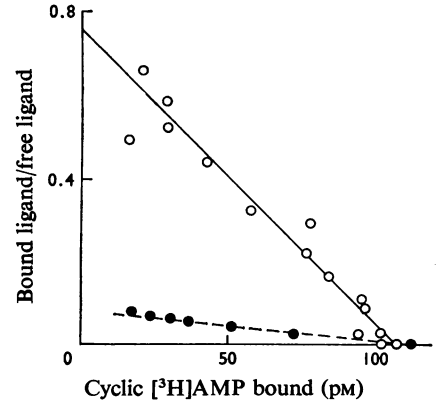

Fig. 4. *Scatchard plot for the interaction between cyclic [^{3}H]AMP and protein kinase I at high ionic strength in the absence and presence of MgATP*

Protein kinase I was incubated in a volume of 4.4ml for 4h in the presence of various concentrations of cyclic [^{3}H]AMP and 1mg of albumin/ml in either buffer A (○) or buffer A containing 1mM- (instead of 30mM-) EDTA, 5mM-magnesium acetate and 0.1mM-ATP (●). At the end of the incubation 1ml samples were mixed with $(NH_4)_2SO_4$ solution to determine the amount of bound ligand. Other 1ml samples were mixed with 0.5ml of buffer A containing 30nM-cyclic [^{3}H]AMP, 120mM-EDTA and 1mg of albumin/ml and incubated for another 30min before being precipitated with $(NH_4)_2SO_4$. Under the latter conditions protein kinase I is saturated with cyclic [^{3}H]AMP and maximally stabilized (Døskeland *et al.*, 1977), so the number of cyclic AMP-binding sites remaining after the first incubation could be estimated. The slightly (2–10%) lower recovery of binding sites for samples from the incubations which contained the lowest concentrations of cyclic [^{3}H]AMP was not corrected for when the plots were constructed.

(Table 2) between the protein kinase activity ratio and the amount of cyclic [^{3}H]AMP bound. The plots according to Scatchard (1949) constructed for the interaction between cyclic [^{3}H]AMP and protein kinase I at high ionic strength (Fig. 4) were consistent with an apparent equilibrium dissociation constant (K_d) of 0.14nm in the absence and of 1.5nM in the presence of MgATP. The calculated K_d for the interaction between cyclic [^{3}H]AMP and protein kinase I (in the absence of MgATP) ranged between 0.09 and 0.24nM (mean 0.15nM) for experiments with six different batches of protein kinase assayed at concentrations of binding sites for cyclic AMP between 0.08 and 0.3nM.

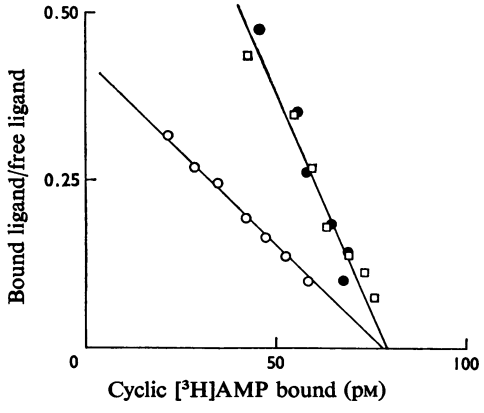

Fig. 5. *Scatchard plot for the binding of cyclic [^{3}H]AMP to the isolated regulatory subunit of protein kinase I*
The regulatory moiety of protein kinase I, obtained as described in the Experimental section, was incubated in the presence of 1mg of albumin/ml in buffer A (○), buffer B (□) or buffer B containing 5mM-magnesium acetate and 0.1mM-ATP (●). The conditions were otherwise as described in the legend to Fig. 4, except that 1.6ml portions were removed for determination of the amount of cyclic [^{3}H]AMP bound. The difference in recoveries of cyclic AMP-binding sites between samples from incubations containing low and high concentrations of the cyclic nucleotide was less than 10%.

Binding of cyclic [^{3}H]AMP to the free regulatory moiety of protein kinase I

The protein kinase subunits were dissociated by high ionic strength instead of by cyclic nucleotides, to avoid interference with the subsequent studies of the binding of cyclic [^{3}H]AMP. The data of Fig. 5 showed that the free regulatory subunit of protein kinase I had a very high affinity for cyclic [^{3}H]AMP at low ionic strength, apparent K_d about 0.08nM, whether MgATP was present or not. The apparent K_d (0.2nM) at high ionic strength (Fig. 5) was within the range found for the interaction between cyclic [^{3}H]AMP and preparations of protein kinase I holoenzyme under similar conditions (see the preceding section; Fig. 4). Whereas the addition of catalytic subunit was without effect on the high-affinity binding to the regulatory subunit at high ionic strength, the binding was decreased at low ionic strength, especially in the presence of MgATP (Table 3).

A factor whose high-affinity binding of cyclic AMP was only moderately enhanced by salt was eluted between protein kinase I and protein kinase II on DEAE-cellulose chromatography (fractions 76–79 in Fig. 1*a*). When the desalted fractions corresponding to that activity were briefly incubated in the presence of MgATP and added catalytic subunit, and then rechromatographed on DEAE-cellulose, a salt-stimulated high-affinity cyclic AMP-binding activity was eluted at a position corresponding to protein

Table 3. *Effect of catalytic subunit on the high-affinity cyclic [^{3}H]AMP binding to the regulatory subunit of protein kinase I*
The conditions for binding of cyclic [^{3}H]AMP to the isolated regulatory subunit were exactlyas described in the legend to Fig. 5, except that catalytic subunit was present in some of the incubations. The preparation of catalytic subunit (see the Experimental section) was used directly, being diluted 16-fold in the incubation mixture. When the remaining cyclic AMP-binding capacity was measured as described in the legend to Fig. 4, no difference was noted between the samples incubated in the absence or presence of catalytic subunit, indicating that the effect of the catalytic subunit was not to destroy the binding sites. Bovine serum albumin (1mg/ml) was present in all the incubations. The incubations were started by the addition of regulatory subunit.

Agents present during the incubation	Cyclic [^{3}H]AMP bound (pmol/ml)		
Total concn. of cyclic [^{3}H]AMP (nM) ...	0.1	0.2	0.8
Buffer B (low-salt)	30.7	42.8	54.5
Buffer B+catalytic subunit	18.6	32.1	49.9
Buffer B+10mM-magnesium acetate+0.2mM-ATP	32.7	41.9	48.6
Buffer B+10mM-magnesium acetate+0.2mM-ATP+catalytic subunit	3.41	5.76	13.5
Buffer A (high-salt)	18.7	27.4	44.2
Buffer A+catalytic subunit	20.8	29.5	44.0

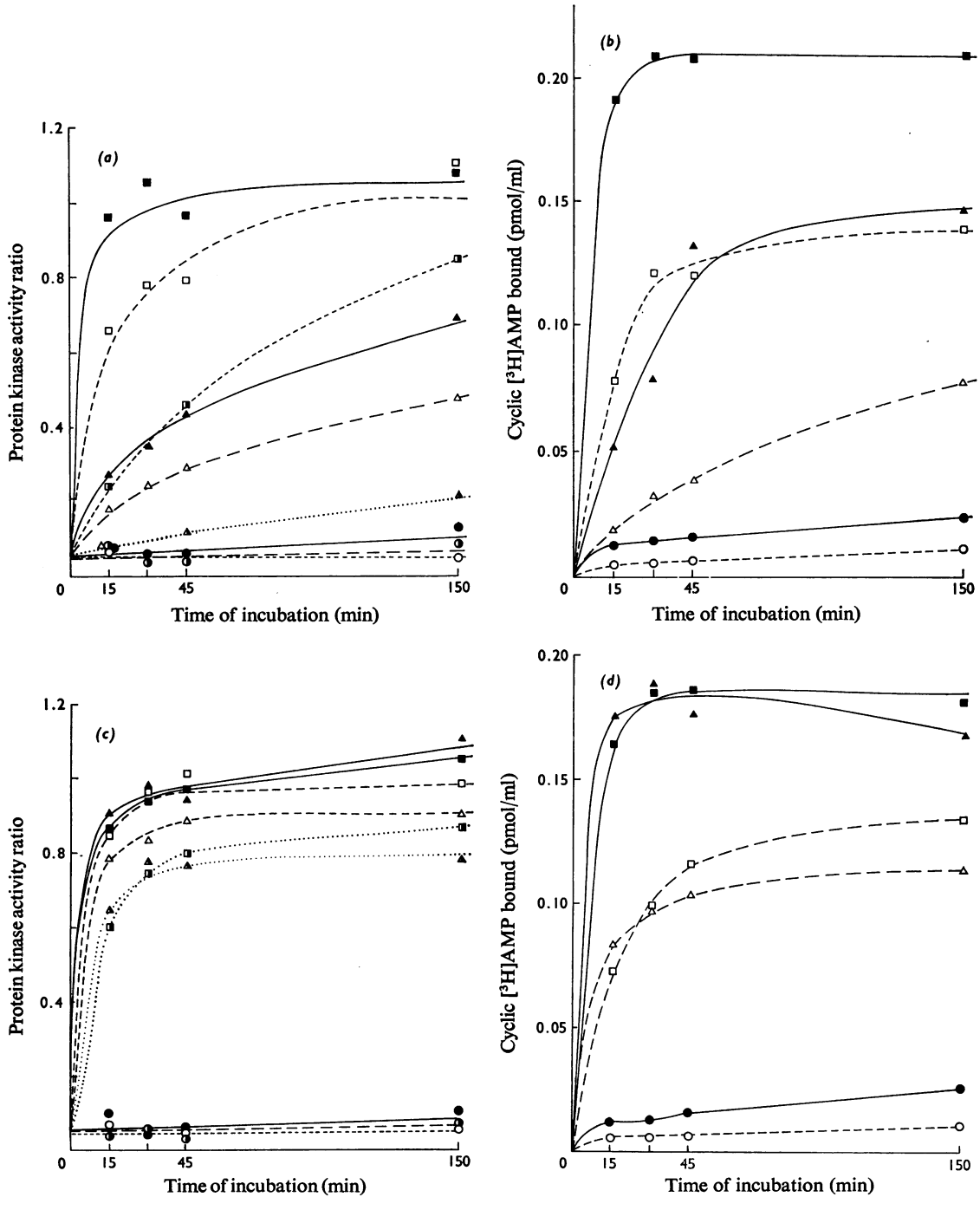

Fig. 6. *Binding of cyclic [^{3}H]AMP and degree of activation of protein kinase as a function of time and incubation conditions*
Protein kinase I was incubated in buffer B variously supplemented with exogenous proteins and/or KCl in either the absence or the presence of cyclic [^{3}H]AMP. Portions (30μl) were added to 120μl of assay mixture for determination of the protein kinase activity ratio. The contents of the assay mixture were adjusted to give final concentrations of 0.2mg of albumin/ml and 0.67mg of histone/ml for all the incubations. From the incubations containing cyclic

kinase I. As the freshly obtained high-affinity binding protein sedimented in sucrose gradients and chromatographed on Sephadex G-200 as expected for a dimer of the regulatory subunits of protein kinases (Rosen *et al.*, 1975; Hofmann *et al.*, 1975; Sugden & Corbin, 1976), it probably represented the dimer of the regulatory subunits of protein kinase I. The preparation was not found useful for binding studies, as it contained cyclic AMP phosphodiesterase, some phosphotransferase, and probably proteolytic activity (as judged by a slight decrease in the sedimentation velocity and a marked and progressive decrease in the Stokes radius on storage).

Correlation between cyclic [^{3}H]AMP binding to and activation of protein kinase

The time course of protein kinase I activation by cyclic [^{3}H]AMP, salt and basic proteins, alone or in combination, is shown in Figs. 6(*a*) and 6(*c*). The use of labelled nucleotide allowed the activating effect of cyclic [^{3}H]AMP to be correlated with the degree of its occupancy of binding sites (Figs. 6*b* and 6*d*). Preliminary experiments showed that the amount of cyclic [^{3}H]AMP carried over from the primary incubation to the phosphotransferase assay was too low to affect the protein kinase activity ratio. Under the conditions of the assay the KCl carried over inhibited the phosphotransferase activity about equally in the absence and presence of cyclic AMP. Bovine serum albumin (0.2mg/ml) slightly enhanced the activity in the presence of cyclic AMP, and partly prevented the progressive increase of basal activity noted on prolonged incubations of highly diluted protein kinase (Ueland & Døskeland, 1976). The assay mixtures were therefore prepared so that the concentration of albumin was the same (0.2mg/ml) in all the phosphotransferase assays.

Fig. 6(*a*) shows that the protein kinase was progressively activated in the presence of histone. The presence of 1.5nM-cyclic [^{3}H]AMP alone had little effect on the protein kinase activity, but was able to activate the kinase completely within 30min in the presence of histone. Histone and cyclic AMP thus activated the kinase synergistically, the synergism being most evident at the early time points. The activation in the presence of 800mM-KCl (Fig. 6*c*) proceeded more rapidly than in the presence of histone, no increase in degree of activation being noted from 45 to 150min of incubation. Salt and cyclic AMP activated the kinase synergistically (Fig. 6*c*), but the synergism was less obvious than for the nucleotide and histone. The presence of histone only slightly enhanced the activating effect of salt (Fig. 6*c*). The inclusion of albumin partly prevented the activation of protein kinase by histone (Fig. 6*a*), and also decreased the high-affinity binding of cyclic [^{3}H]AMP in the presence of histone (Fig. 6*b*).

Discussion

In agreement with the results of Gill & Garren (1970, 1971) two peaks of cyclic AMP-dependent protein kinase activity were resolved by DEAE-cellulose chromatography of bovine adrenal-cortex cytosol. A high-affinity binding protein for cyclic AMP eluted between the protein kinases (Fig. 1*a*) was probably the free regulatory moiety of protein kinase I, on the basis of rechromatography after its recombination with catalytic subunit of protein kinase. A similar binding protein was found by Gill & Garren (1970) to inhibit the cyclic AMP-independent activity of protein kinase II.

Two additional cyclic AMP-binding proteins, not previously described in adrenal gland, were most conveniently detected in the DEAE-cellulose fractions by their ability also to bind adenosine (Fig. 1*c*). They were termed cyclic AMP/adenosine-binding proteins I and II according to their order of elution from DEAE-cellulose. The binding activities for cyclic AMP and adenosine co-sedimented on sucrose-density-gradient centrifugation (Fig. 2) and co-migrated on polyacrylamide-gel electrophoresis Both activities were present in a preparation of cyclic AMP/adenosine-binding protein II, which gave a single band on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The adrenal binding proteins differed in their apparent affinities for adenosine (Fig. 1*c*; Table 1).

The results of the present study, as well as our recent experiments on a cyclic AMP/adenosine-

[^{3}H]AMP, portions (0.8ml) were also removed for determination of the amount of bound nucleotide. (*a*) Protein kinase activity: one series of incubations was carried out in the presence of 1mg of albumin/ml in the absence (◑), or in the presence of 0.5nM- (○) or 1.5nM- (●) cyclic [^{3}H]AMP. The second series of incubations contained 1mg of albumin/ml, 0.2mg of histone/ml and no (▲), 0.5nM- (△) or 1.5nM- (▲) cyclic [^{3}H]AMP. The third series contained 0.2mg of histone/ml and no (◨), 0.5nM- (□) or 1.5nM- (■) cyclic [^{3}H]AMP. The activity ratio of protein kinase is given as a function of the incubation time. (*b*) The amount of cyclic [^{3}H]AMP bound in those of the incubations above containing labelled nucleotide is given as a function of incubation time. The symbols are as in (*a*). (*c*) Protein kinase activity: one series of incubations was in buffer B without cyclic AMP (◑) or in the presence of 0.5nM- (○) or 1.5nM- (●) cyclic [^{3}H]AMP. The second series contained 800mM-KCl, no (▲), 0.5nm- (△) or 1.5nM- (▲) cyclic [^{3}H]AMP. In the third series 800mM-KCl, 0.2mg of histone/ml and no (◨), 0.5mM- (□) or 1.5nM- (■) cyclic [^{3}H]AMP were present. (*d*) Cyclic [^{3}H]AMP binding; the incubation conditions and symbols correspond to those in (*c*).

binding protein from mouse liver, indicate that cyclic AMP, like ATP, may increase the cyclic AMP-binding capacity of such proteins, especially at high temperature and in the presence of K^+ and Mg^{2+}. Some discrepancies in the apparent affinities of such proteins for cyclic AMP may thus be explained. In the present study and in an investigation of a binding protein from mouse liver (Ueland & Døskeland, 1977), half-maximal binding was obtained at 0.1 μM-cyclic AMP when the incubations were performed at 0°C in the presence of glycerol and in the absence of K^+ or Mg^{2+}. On the other hand, half-maximal binding was at 2 μM- and 1 μM-cyclic AMP respectively for binding protein from mouse liver (Døskeland & Ueland, 1975*a*) and bovine liver (Sugden & Corbin, 1976), incubated at 30°C in the presence of K^+ ions.

The data on the competition for the cyclic AMP-binding site of adrenal cyclic AMP/adenosine-binding protein II (Table 2) indicated that cyclic AMP itself had the highest affinity, followed by adenosine, AMP, ADP and ATP. Cyclic GMP and inosine were inefficient competitors. In the system of Sugden & Corbin (1976) adenosine inhibited the binding of cyclic [^{3}H]AMP more efficiently than did unlabelled cyclic AMP, and inosine produced a substantial inhibition. Either the difference in species or in incubation conditions may be responsible for the discrepancy. The present study indicated that the adrenal cyclic AMP/adenosine-binding proteins are not associated with histone kinase, cyclic AMP phosphodiesterase or adenosine deaminase activities. The question remains whether this class of binding protein is associated with other enzyme activities or acts merely to control the effective intracellular concentrations of its ligands.

A correlation between cyclic AMP binding to and activation of protein kinase has been found under phosphotransferase assay conditions (Ueland & Døskeland, 1976), and in the presence of various concentrations of trichloroacetate (Døskeland *et al.*, 1977). In the present study such a correlation was found when protein kinase I was activated by increasing concentrations of NaF (Table 2), NaCl or KCl (Fig. 3), and as a function of time when the kinase was activated at a fixed concentration of histone or KCl (Fig. 6). Furthermore the decreased ability of histone to activate the kinase in the presence of albumin (Fig. 6*a*) was accompanied by a correspondingly diminished high-affinity binding of cyclic [^{3}H]AMP (Fig. 6*c*).

There is uncertainty whether cyclic AMP binds to holoenzyme before dissociation of C or whether cyclic AMP binds only to R_2, thereby shifting a pre-existing equilibrium between holoenzyme and subunits in favour of the free subunits (Ogez & Segel, 1976). The tight coupling observed between binding of cyclic AMP and activation of protein kinase would be expected if (1) cyclic AMP only could bind to free R_2 or (2) cyclic AMP after dissociating the kinase remained bound to free R_2, owing to a tight binding of cyclic AMP to free R_2. The data of Fig. 5 and Table 3 demonstrate that free R_2 and cyclic AMP do interact with such a high affinity that mechanism (2) is also compatible with the observed tight coupling between binding of cyclic AMP and activation of protein kinase by the nucleotide.

The activation of protein kinase by histone occurred at a low rate (Fig. 6*a*), as would be expected if histone shifted the slow equilibrium $R_2C_2 = R_2+2C$ to the right by interaction with the free subunits. The marked suppression of histone activation by albumin (Fig. 6*a*), the presence of which did not impair the ability of histone to serve as a substrate for C (see the Results section), does not support the idea that enzyme–substrate interaction between C and histone is a major mechanism of activation. On the basis of earlier results (Ueland & Døskeland, 1976), we have suggested that the activation occurred by complex-formation between histone and R_2, which are oppositely charged at neutral pH. The synergistic activation of the protein kinase by histone and low concentrations of cyclic AMP (Fig. 6*a*) would not be expected if cyclic AMP acted only by the same mechanism (decreasing the probability of recombination of R_2 and C by its binding to R_2), as has been outlined above for histone. The simplest explanation of the synergism would be that cyclic AMP at low concentrations binds to and dissociates only a small fraction of the holoenzyme molecules, the dissociated subunits rapidly reassociating (at low ionic strength in the absence of histone). In that way the equilibrium $R_2C_2 = R_2+2C$, slightly shifted to the right, would be converted from a slow into a rapid equilibrium, favouring more rapid complex-formation between R_2 and histone. The data obtained thus suggest that interaction between cyclic AMP and free R_2 is an insufficient explanation of the activation of protein kinase I by cyclic AMP. Further studies, preferably conducted under near-physiological conditions, are required to conclude by what mechanism cyclic AMP activates the protein kinase intracellularly. Such studies could give clues to the 'control efficiency' (Swillens & Dumont, 1976) of protein kinase activation by cyclic AMP. It would be of interest to know if the significant activation of intracellular protein kinase occurring in the absence of a detectably raised intracellular concentration of cyclic AMP (Corbin *et al.*, 1975*b*; Moyle *et al.*, 1976; Cooke *et al.*, 1976) could result from a control mechanism for protein kinase promoting a high responsiveness to the appearance of cyclic AMP (Swillens & Dumont, 1976), or if liberation of cyclic AMP from intracellular binding sites must be postulated to account for those findings. One of the functions of the cyclic AMP/adenosine-binding

proteins reported in the present work might be to regulate the intracellular concentration of free nucleotide.

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