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3′:5′-Cyclic AMP Binding Proteins

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The binding of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) to various cellular components has been studied using a Millipore filtration method. The activity to bind cyclic AMP is distributed among various subcellular fractions of rat liver. From the soluble fraction apparently four binding proteins have been partially purified and their properties investigated. The binding is specific for cyclic AMP, although it is partially inhibited by other adenine derivatives. Kinetic studies have revealed that there are two discrete binding states which are characterized by different binding velocities and affinities. The slowly developing state which corresponds to the low affinity binding ($Kd=8.8\times10^{-6}$ M) is markedly affected by ionic strength, indicating that the linkage is of ionic nature. The rapidly equilibrating state, by contrast, which is assigned to the high affinity binding ($Kd=1.1\times10^{-8}$ M) is insensitive to the alteration in ionic milieu. Enzymological studies have suggested that two of the four binding proteins are associated with protein kinase which is dependent on cyclic AMP and the other two have an activity to suppress the protein kinase.

INTRODUCTION

Since the discovery of cyclic AMP** as a thermostable hyperglycemic factor by Sutherland and Rall in 1957,[1] substantial evidence has been accumulated that cyclic AMP is an intracellular mediator of various hormonal responses.[2] The mechanism of action of this nucleotide, however, had been totally unknown until recently. The first clue to this problem was obtained in 1968 by Walsh, Perkins and Krebs,[3] who showed that a protein kinase partially purified from rabbit skeletal muscle was activated specifically by cyclic AMP. Similar observations were subsequently reported by others with various protein kinase preparations.[4~7] Another clue to the problem was obtained from the study of interaction between the nucleotide and cellular components. Gill and Garren first reported in 1969[8] that there was a protein in adrenocortical microsomes and soluble fraction which specifically bound cyclic AMP. The subsequent analysis of the protein has suggested that it is closely related to protein kinase which is dependent on cyclic AMP.[9]

The present study has been undertaken in order to clarify the specificity of binding with respect to both nucleotide and protein and to characterize the binding physicochemically and enzymologically.

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** The abbreviations used are: cyclic AMP, adenosine 3′:5′-cyclic monophosphate; cyclic GMP, guanosine 3′:5′-cyclic monophosphate; cyclic IMP, inosine 3′:5′-cyclic monophosphate; cyclic CMP, cytidine 3′:5′-cyclic monophosphate; cyclic UMP, uridine 3′:5′-cyclic monophosphate; cyclic TMP, thymidine 3′:5′-cyclic monophosphate; DTT, dithiothreitol.

A preliminary report of this work was presented at the 43rd general meeting of Japanese Biochemical Society.[10]

MATERIALS AND METHODS

Preparation of Subcellular Fractions——Wistar rat livers were homogenized 1 : 3 (w/v) in Littlefield's medium (0.25 *M* sucrose-0.025 *M* KCl-0.005 *M* $MgCl_2$-0.05 *M* Tris-Cl, pH 7.6)[11] by means of a Potter-Elvehjem type tissue grinder. The homogenate strained through four layers of gauze was centrifuged for 10 min at $700\times g$. Nuclei were prepared from this precipitate by the method of Chauveau, Moulé and Rouiller.[12] The $700\times g$ supernatant fraction was further centrifuged for 20 min at $10{,}000\times g$. The mitochondrial pellet obtained was washed by suspending in Littlefield's medium and spinning down at $8{,}000\times g$. The soluble and microsomal fractions were prepared from the postmitochondrial supernatant by centrifugation at $75{,}000\times g$ for 60 min. All above particulates (nuclei, mitochondria and microsomes) were finally suspended in a medium containing 0.35 *M* sucrose, 0.07 *M* KCl, 0.004 *M* $MgCl_2$, 0.05 *M* Tris-Cl, pH 7.8 and 0.06 *M* 2-mercaptoethanol.[7]

Assay of Cyclic AMP Binding——The standard reaction mixture contained 20 μmoles of Tris-Cl, pH 8,0, 5 μmoles of $MgCl_2$, 132 pmoles of cyclic AMP-^{3}H(G) (1,490 cpm/pmole) and a protein sample in a total volume of 0.2 ml. The incubation (10 min, 30°C) was terminated with 5 ml of ice-cold 0.01 *M* Tris-Cl, pH 8.0, and the mixture was immediately passed through a Millipore filter (HA type; pore size 0.45 μ). The filter was washed with five aliquots of the same buffer and dried. The radioactivity retained on the filter was determined in a toluene based scintillator using a Beckman *DPM 100* liquid scintillation spectrometer.

Assay of Protein Kinase——The standard reaction mixture (0.25 ml) contained 5 μmoles each of potassium phosphate buffer, pH 7.2 and $MgCl_2$, 3 nmoles of ATP-γ-^{32}P (50 to 100 cpm/pmole), 120 μg of calf thymus histone (type III, Sigma), 0.6 nmole of cyclic AMP, if added, and an enzyme preparation. The reaction was carried out for 8 min at 30°C and stopped by the addition of 20% trichloroacetic acid. The mixture was passed through a Millipore filter and the filter washed five times with 5% trichloroacetic acid. The radioactivity on the filter was measured with a Nuclear-Chicago gas flow counter.

Other Assays——Aminoacyl transferase II was assayed according to the method of Honjo *et al.*[13] employing diphtheria toxin and NAD-adenosine-^{14}C.

Protein was assayed after Lowry *et al.*[14] using bovine serum albumin as standard.

Labeled Compounds——ATP-γ-^{32}P was prepared enzymatically from inorganic phosphate-^{32}P[15] or obtained from Radiochemical Centre. NAD-*abenosine*-^{14}C was prepared from ATP-^{14}C(U) as described previously.[16] ATP-^{14}C(U), cyclic AMP-^{3}H(G) and other tritiated nucleotides were obtained from Schwarz BioResearch or from Radiochemical Centre.

RESULTS

Distribution of Cyclic AMP Binding Activities——The activity to bind cyclic AMP was found in all subcellular fractions. As shown in Table I, the highest activity

Table 1. Distribution of Cyclic AMP and 5′-AMP Binding Activities among Subcellular Fractions.

Fraction	Cyclic AMP Binding	5′-AMP Binding
	pmoles/mg protein	pmoles/mg protein
Homogenate	0.80	0.43
Soluble	1.22	0.55
Microsomal	2.87	2.78
Mitochondrial	1.14	0.88
Nuclear	0.27	0.18

The assay of cyclic AMP binding was carried out under the standard condition except for a concentration of cyclic AMP (2 μM) and the addition of 10 mM theophylline. The activity of 5′-AMP binding was assayed under the similar condition except that cyclic AMP-^{3}H was substituted for 5′-AMP-^{3}H.

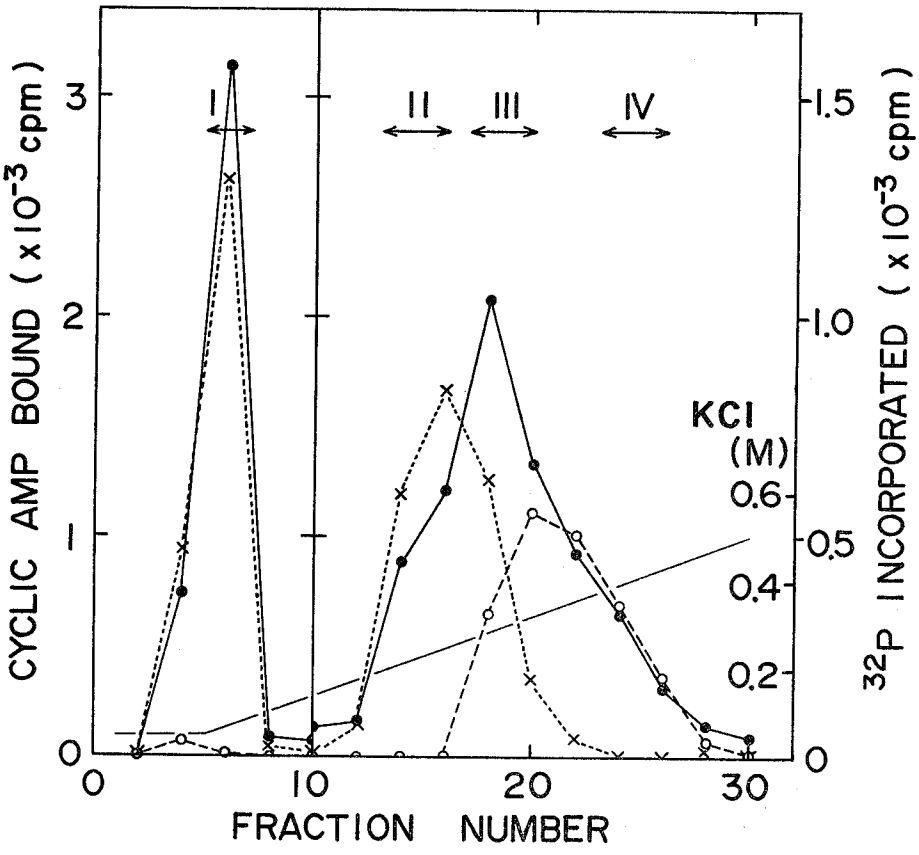

Fig. 1. DEAE-cellulose column chromatography of cyclic AMP binding proteins.

The calcium phosphate gel extract (114 mg of protein) was applied on a DEAE-cellulose column (2×18 cm). The column was eluted with a gradient of KCl (——). Aliquots from each fraction (6 ml) was assayed for cyclic AMP binding (×---×), protein kinase with (●—●) or without cyclic AMP (○--○). For the sake of clarity, the doubled values are plotted for the lattr three activities in fractions 10 to 30. Arrows specify the combined fractions I, II, III and IV.

was located in the microsomal fraction and the nuclei were relatively scant of this activity. The situation was also true with the capacity to bind 5′-AMP (Table I). However, the specificity for binding cyclic AMP, as judged by the ratio of ability to bind cyclic AMP to that of binding 5′-AMP, appeared to be highest in the soluble fraction. For this reason and because of the greater total activity, the soluble fraction was employed as the source material for purification of cyclic AMP binding proteins.

The binding activities in all fractions were almost totally lost by boiling and by trypsin treatment.

Purification of Cyclic AMP Binding Proteins——Wister rat livers were homogenized with 2.5 volumes of 0.25 *M* sucrose containing 3.3 m*M* $CaCl_2$ and centrifuged for 60 min at 105,000×*g*. To the supernatant or *soluble fraction* solid $(NH_4)_2SO_4$ was added to obtain 0.5 saturation. After stirring for 15 min, the solution was centrifuged for 15 min at 7,000×*g* and the precipitate dissolved in 0.2 *M* potassium phosphate buffer, pH 7.0, containing 0.5 m*M* DTT (*0.5 $(NH_4)_2SO_4$ precipitate*). About

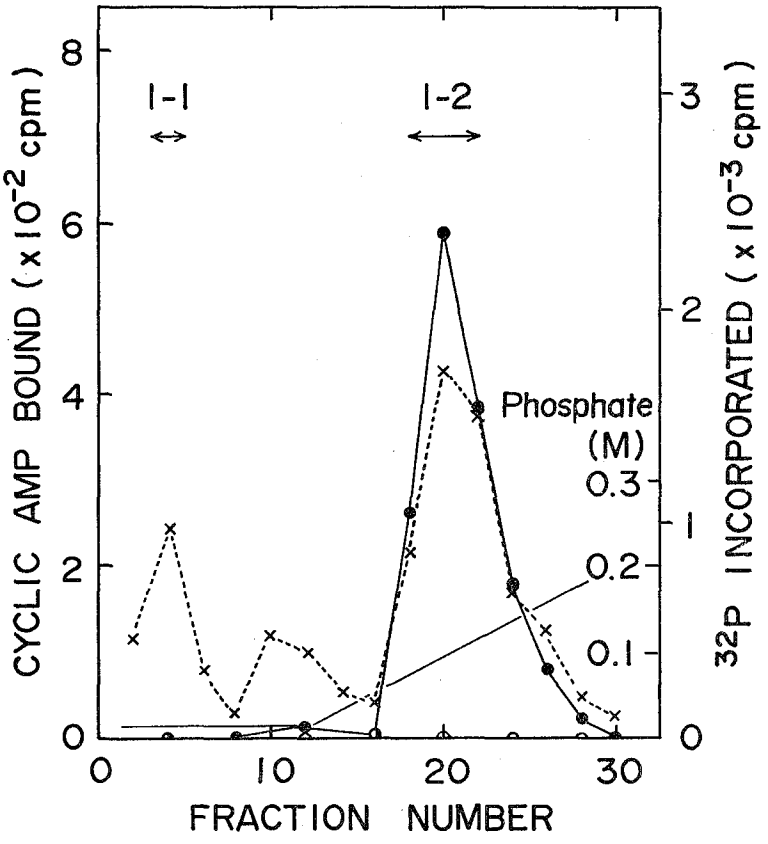

Fig. 2. Hydroxylapatite column chromatography of cyclic AMP binding proteins (I).

The DEAE-cellulose fraction I (approximately 25 mg of protein) was applied on a hydroxylapatite column (0.8×8 cm) and the column eluted with a gradient of phosphate buffer (——). 2 ml fractions were collected and assayed for cyclic AMP binding (×----×), protein kinase in the presence (●—●) and absence of cyclic AMP (○--○). Arrows specify the combined fractions I-1 and I-2.

0.2 volume of. 1 *M* $CaCl_2$ was added to this solution, and the mixture stirred for 30 min. Resulting calcium phosphate gel was separated by centrifugation, washed once with water, and treated with 0.2 *M* potassium phosphate, pH 7.0, containing 0.5 m*M* DTT.

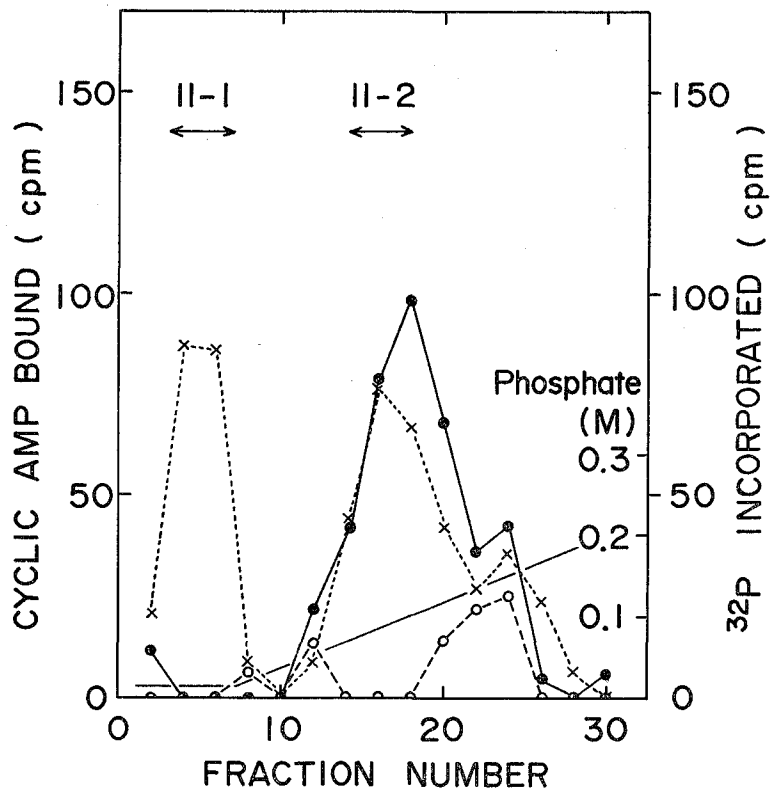

Fig. 3. Hydroxylapatite column chromatography of cyclic AMP binding proteins (II).

The DEAE-cellulose fraction II (approximately 7 mg of protein) was applied on the column (0.8×10 cm). Simbols are the same as in Fig. 2. Fractions specified by arrows were combined and designated as II-1 and II-2.

Table 2. Purification of Cyclic AMP Binding Proteins.

Step	Specific Activity
	pmoles/mg protein
Soluble fraction	7.57
0.50 $(NH_4)_2SO_4$ precipitate	18.1
Calcium phosphate gel extract	20.5
DEAE-cellulose fraction I	26.0
// // II	36.8
Hydroxylapatite fraction I-1	37.8
// // I-2	35.3
// // II-1	121.3
// // II-2	51.3

The *gel extract* obtained by centrifugation (7,000 × *g*, 15 min) was 10-fold diluted with water, concentrated to a suitable volume by the use of a Diaflo ultrafiltration apparatus (Amicon, membrane type UM-2) and applied on a DEAE-cellulose column which was equilibrated with 0.02 *M* potassium phosphate, pH 7.0 and 0.5 m*M* DTT. The column was eluted with 0.05 *M* KCl-0.02 *M* potassium phosphate, pH 7.0–0.5 m*M* DTT, and then with a linear gradient of KCl from 0.05 *M* to 0.5 *M* containing the same buffer and DTT. The elution pattern from the DEAE-cellulose column is shown in Fig. 1. The fractions with cyclic AMP binding activities were combined as indicated and dialyzed overnight against 0.02 *M* potassium phosphate, pH 7.0–0.5 m*M* DTT (*DEAE-cellulose fractions I and II*). The dialyzed material was applied on a hydroxyl-

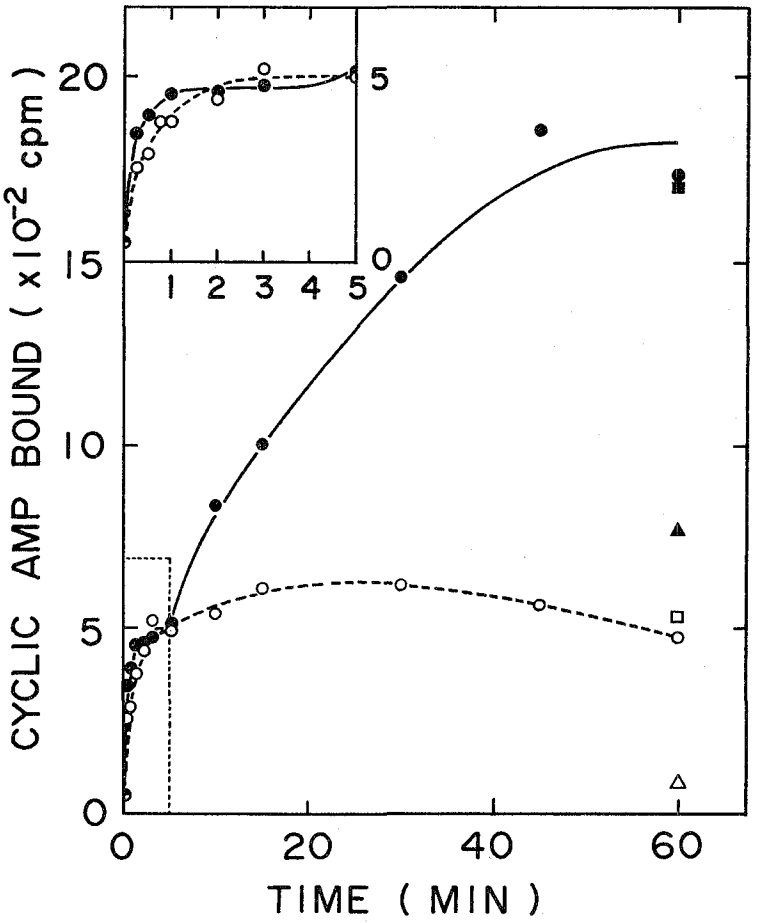

Fig. 4. Time course of cyclic AMP binding.

The binding reaction was carried out for the time period indicated under the standard condition in the presence (○- -○) or absence of 1 *M* NaCl (●—●). DEAE-cellulose fraction III was employed. ▲ and ■ represent the bindings of the salt-less mixture which was diluted at 30 min with 5 ml of 0.01 *M* Tris-Cl, pH 8.0, with or without 50 μM cyclic AMP, respectively, and incubated for additional 30 min. △ and □ represent the bindings of the salted mixture similarly treated. The early stages of the reaction is replotted with a different time scale in the accompanying figure.

apatite column equilibrated with the same buffer. Elution was performed first with the equilibrating buffer and then by introducing the phosphtae buffer, pH 7.0, of a linearly increasing concentration from 0.02 to 0.3 *M*, supplemented with 0.5 m*M* DTT. The hydroxylapatite column chromatograms obtained for DEAE-cellulose fractions I and II are shown in Figs. 2 and 3, respectively. The fractions with cyclic AMP binding activity from each column were combined as specified and designated as *hydroxylapatite fractions I-1, I-2 and II-1, II-2.*

All above manipulations were carried out at 0 to 4°C.

Table II gives the summary of purification procedures and specific activities at each step. It is evident that approximately 5- to 15-fold purification of cyclic AMP binding proteins was achieved by these procedures.

Kinetics of Cyclic AMP Binding——Binding of cyclic AMP to proteins appeared to precede in two steps. The typical time courses of binding in the absence and presence of concentrated salt are shown in Fig. 4. Under the condition of low ionic strength an obvious inflection point was observed at around 5 min of incubation, and it seemed that there were two separate states of binding, *i.e.* the rapidly developing and time-consuming ones. The former was achieved in 1 or 2 min and the latter took about one hour to reach completion. It is notable that the latter or a slowly developing binding state was highly sensitive to the alteration in ionic milieu and disappeared almost completely under the condition of high ionic strength, *e.g.* 1 *M* NaCl. The rapid binding was, by contrast, insensitive to the change in the ionic environment. When the concentration of cyclic AMP was lowered as low as 10^{-8} *M*,

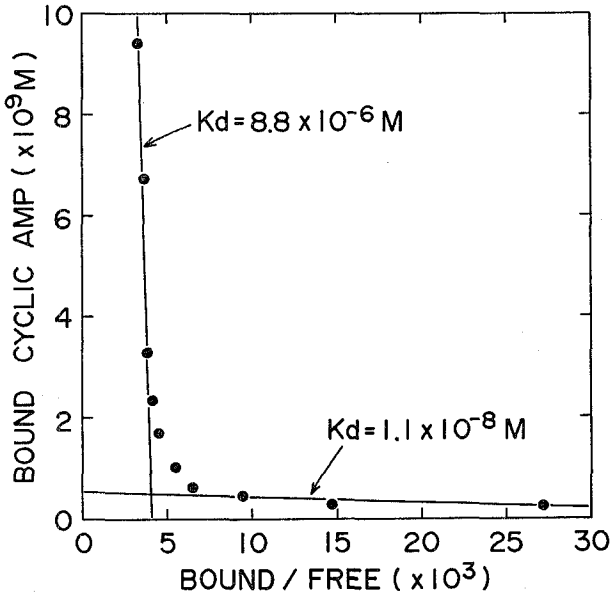

Fig. 5. Scatchard plot of binding of cyclic AMP to protein.

Conditions were as described under "Materials and Methods" except that the concentration of the nucleotide was varied. A hydroxylapatite fraction II-1, purified by a modified method, was employed as the binding protein.

the slowly developing binding phase became much less apparent. These facts may indicate that there are two classes of linkages between cyclic AMP and its binding proteins and the binding observed at a later incubation stage under the condition of a low ionic strength and of a high nucleotide concentration is of ionic nature. The activity as assayed by the conventional method (10 min incubation) includes both phases of cyclic AMP binding. These two states of binding were observed with all binding proteins tested.

The binding reaction appeared to be reversible. As shown in Fig. 4, the dilution of the reaction mixture with buffer at 30 min of incubation seemed to give no effect on the binding thereafter for at least 30 min under the present condition, but the addition of 100 times concentration of nonradioactive cyclic AMP to the diluting buffer effected a marked loss of bound radioactivity, irrespective of the ionic strength. This implies that radioactive cyclic AMP once bound was exchanged with nonradioactive one and both bindings described above were reversible.

Mg^{2+} at a concentration of 0.025 to 0.05 *M* had an apparent activating effect when added in the reaction mixture. Since the ion added solely in the diluting buffer gave a less, although significant, effect, the activation appeared to be caused partly by a better retention of proteins on filters and partly by a promotion of closer interaction between the protein and nucleotide. The optimal pH for binding was around 6 to 8.

The affinity of cyclic AMP for the binding protein was determined by the Scatchard method.[17] As shown in Fig. 2, apparently two discrete bindings were observed; one had a dissociation constant (Kd) of 1.1×10^{-8} *M* and the other that of 8.8×10^{-6} *M*. The former or a high affinity binding was not significantly affected by ionic strength, but the latter or a low affinity binding was markedly influenced by the salt concentration. In the presence of 1 *M* NaCl, only the high affinity binding with a slightly lower Kd value was observed and the Kd for the low affinity binding could not be determined. The Kd values as well as salt effects were similar among all four binding proteins.

On reference to the effects of salt and nucleotide concentration on the time course described above, these bindings with different affinities appear to be ascribable to the discrete binding states discriminated by velocity, *i.e.* the high affinity binding to the rapid phase and the lower one to the slowly developing phase. The observation that the binding of 5'AMP gave the Kd values of 7.0×10^{-8} *M* and 8.3×10^{-6} *M* under the same condition as in Fig. 5 may imply that the binding of high affinity is specific for cyclic AMP.

Linkage between Cyclic AMP and Protein——The linkage between cyclic AMP and its binding proteins did not appear to be covalent. A brief treatment (10 min at 0°C) of the protein-cyclic AMP complex with either mild acid (0.01 *N* HCl) or alkali (0.01 *N* NaOH) resulted in the loss of the bulk of bound nucleotide. Boiling for a minute also effected the almost complete unbinding of cyclic AMP. No appreciable incorporation into 5% trichloroacetic acid-insoluble fraction was thus far detectable. The material which was recovered in the Millipore filtrate after the acid treatment was totally cyclic AMP, indicating no molecular alteration of the nucleotide upon the binding and unbinding.

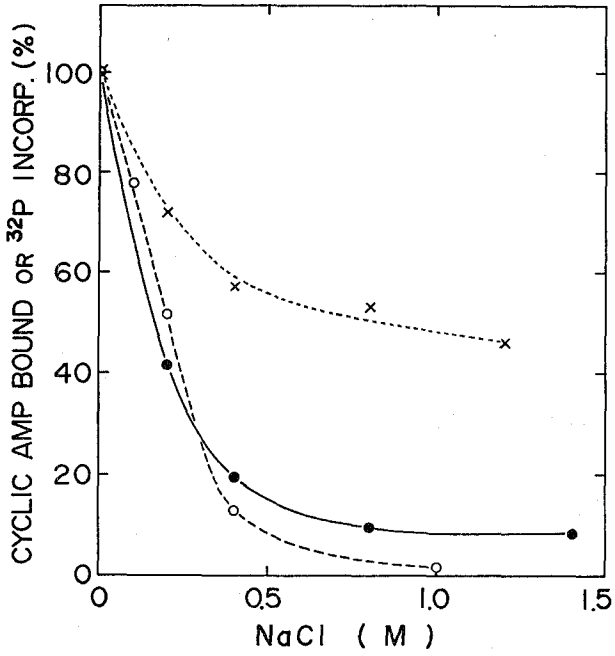

Fig. 6. Effect of ionic strength on cyclic AMP binding and protein kinases.

Assays of protein kinases were conducted under the standard condition except that various concentrations of NaCl were included. DEAE-cellulose fractions III and IV were employed for cyclic AMP dependent and independent kinases, respectively. The mixture for cyclic AMP binding was modified to contain 20 m*M* Tris-Cl buffer, pH 8.0, 20 m*M* of $MgCl_2$, 2.4 μM cyclic AMP-^{3}H and the protein (DEAE-cellulose fraction III) in a total volume of 0.25 ml. This mixture was comparable with that for protein kinase assay. The results were expressed in terms of percent as compared with each value at an NaCl-free state. ×----×, cyclic AMP binding; ●—● and ○--○, protein kinase in the presence and absence of cyclic AMP, respectively.

The observation suggesting that a part of the linkage is of ionic nature have already been described. The inhibitory effect of elevated ionic strengths on the binding was evident upon the titration, as shown in Fig. 6.

Specificity of Binding Nucleotide——The specificity of nucleotide for binding was examined by the competition with cyclic AMP binding and a direct measurement of binding of other nucleotides. Table III shows the competitive effect of various nucleotides and nucleosides on the binding of cyclic AMP-^{3}H. As obvious from the incomparably marked effect of unlabeled cyclic AMP, the binding to the protein was highly specific for this nucleotide. Among other substances studied, adenosine and cyclic IMP were potent competitors. They brought about approximately 75% decrease in binding of cyclic AMP at a concentration of 400 times that of the latter. Other adenine derivatives and cyclic nucleotides were also effective to varying degrees. These results may suggest that both structures of adenosine and cyclic phosphate esters were important in this binding.

The high specificity of cyclic AMP is also evident in Table IV which shows the

Table 3. Competitive Effect of Various Nucleotides and Nucleosides upon Cyclic AMP-^{3}H Binding.

Addition	Bound Cyclic AMP-^{3}H
	cpm
None	1,343
Cyclic AMP	120
Cyclic GMP	745
Cyclic IMP	463
Cyclic CMP	631
Cyclic UMP	714
Cyclic TMP	1,149
3′-AMP	645
5′-AMP	843
5′-GMP	1,055
5′-CMP	1,364
5′-UMP	1,355
5′-ADP	756
3′,5′-ADP	1,092
ATP	818
GTP	1,357
Adenosine	432
Guanosine	910

The reaction mixture (0.2 ml) contained 20 μmoles of Tris-Cl, pH 7.4, 5 μmoles of $MgCl_2$, 25 pmoles of cyclic AMP-^{3}H (7,130 cpm/pmoles), 10 nmoles of additional substance as indicated and a DEAE-cellulose fraction I protein. The incubation and measurement of bound radioactivity were performed under the standard conditions. The values listed were corrected for a radioactivity observed in the absence of protein.

Table 4. Binding of Various ^{3}H-Labeled Nucleotides.

Nucleotide	Binding
	pmole
Cyclic AMP	0.321
Cyclic GMP	0.044
5′-AMP	0.030
5′-GMP	0.000
ATP	0.078
GTP	0.000
CTP	0.000
UTP	0.000

The reaction was carried out under the standard condition with a DEAE-cellulose fraction I except that cyclic AMP-^{3}H was substituted for other ^{3}H-labeled nucleotide as indicated. The concentration of all nucleotides was 2.0 $\pm$0.1 μM. The values were corrected for the binding in the absence of protein.

Table 5. Inhibition of Protein Kinase by Cyclic AMP Binding Proteins.

Expt	Proteins	^{32}P Incorporated	
		−Cyclic AMP	+Cyclic AMP
		cpm	
1.	DE IV (14 μg)	353	343
	HA I-1 (42 μg)	0	1
	DE IV (14 μg)+HA I-1 (21 μg)	318	338
	DE IV (14 μg)+HA I-1 (42 μg)	284	314
2.	DE IV (14 μg)	582	634
	HA II-1 (16 μg)	0	0
	DE IV (14 μg)+HA II-1 (16 μg)	395	393
	DE IV (14 μg)+HA II-1 (32 μg)	242	283

Protein kinase activity was assayed under the standard condition with or without cyclic AMP. Amounts of proteins employed were specified in the brackets. DE IV, HA I-1 and HA II-1 represent DEAE-cellulose fraction IV, hydroxylapatite fractions I-1 and II-1, respectively.

result of binding of various labeled nucleotides. At the identical concentration (2 μM), ATP and cyclic GMP bound to the protein to less than a quarter of cyclic AMP.

Relationship to Protein Kinases——As already mentioned, a close relation between the cyclic AMP binding protein and protein kinase has been suggested by Gill and Garren.[9] This relation was also suggested in the present study. As shown in Figs. 1 to 3, the activity of protein kinase which was dependent on cyclic AMP accompanied that of cyclic AMP binding. Two of the four proteins purified were, in fact, associated with the protein kinase. The kinase which was independent of this nucleotide did not contain the binding activity. Other two binding proteins free of protein kinase had an activity to inhibit the latter enzyme. As shown in Table V, both proteins (hydroxylapatite fractions I-1 and II-1) caused a significant suppression of the kinase independent of cyclic AMP. It may be noted that the suppression was partially relieved by the addition of cyclic AMP. These facts, altogether, appears to be compatible with the view that the binding of cyclic AMP is closely related to the activation of protein kinase.

The attempt to determine which of the two binding states mentioned earlier was responsible for this kinase activation from the view point of salt effect was unsuccessful, since both dependent and independent activities of kinase were markedly affected by the increase in ionic strength (Fig. 6). As judged by the rapidity of kinase activation and a low activation constant ($Ka=2\times10^{-8}\ M$), however, it seems that the rapid and high affinity binding of cyclic AMP is accountable for the activation of kinase.

No relation to Aminoacyl Transferase II——Based on a report by Kuwano and Schlessinger[18] that the bacterial G-factor specifically binds cyclic AMP in the presence of GTP, the relation of binding proteins to the functionally corresponding factor in mammals, aminoacyl transferase II, was investigated. The experiment with a hydroxylapatite column chromatography suggested that aminoacyl transferase II did not associate with any activity to bind cyclic AMP. No binding of the nucleotide to the transferase II was further confirmed using a homogeneous preparation (a kind gift from Dr. T. Honjo).[19]

DISCUSSION

The interaction of cyclic AMP with cellular components has been noted as the link between the hormonal stimulation of adenyl cyclase and the modification of cell function. The resolution of this interaction at a molecular level appears to be just beginning. The present study was undertaken in an attempt to approach this problem through the analysis of binding reaction.

The binding of cyclic AMP to biomacromolecules, as assayed by the Millipore filtration method, appears to precede in two steps. Kinetic studies have suggested that the rapid phase corresponds to the high affinity binding and the slower one to the low affinity binding. These two classes of binding are clearly distinguished by their different sensitivities to ionic strength. Although some indication is available which suggests the high affinity binding to be crucial for the activation of protein kinase, which of the two bindings is involved in the regulatory action of cyclic AMP must be determined carefully with a more purified preparation. This will concern with the assay condition of cyclic AMP action of more physiological significance.

The relation of cyclic AMP binding proteins to the protein kinase has been another central subject of this study. As shown above, two of the four isolated binding proteins are associated with protein kinase which is dependent on cyclic AMP. Further, the other two binding proteins have an activity to suppress the protein kinase. This effect appears to be partially counteracted by cyclic AMP. These observations are in accord with the recent reports[20~25] that the protein kinase dependent on cyclic AMP is composed of two subunits and that the attachment of cyclic AMP to the "regulatory" or "inhibitory subunit" causes the dissociation of these two and makes the "catalytic subunit" fully active. A preliminary experiment has revealed that there are a variety of kinases and cyclic AMP binding proteins besides those reported here. To which combination of those kinases and binding proteins the above sort of mechanism is applicable is currently under investigation.

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