Mechanism of Activation by Adenosine 3':5'-Cyclic Monophosphate of a Protein Phosphokinase from Rabbit Reticulocytes*

Mariano Tao†, Maria L. Salas‡, and Fritz Lipmann

THE ROCKEFELLER UNIVERSITY, NEW YORK CITY 10021

Communicated June 24, 1970

Abstract. Two protein phosphokinases (EC 2.7.1.37) were found to be present in rabbit reticulocytes. The two enzymes were separated by DEAE-cellulose chromatography and called kinases I and II. Adenosine 3':5'-cyclic monophosphate stimulated the activity of both enzymes. However, the degree of stimulation was different and depended on the protein acceptor used. In the presence of adenosine 3':5'-cyclic monophosphate, protein kinase I dissociated into two subunits: a subunit binding adenosine 3':5'-cyclic monophosphate, and a catalytic subunit. The component binding the cyclic nucleotide appeared to act as an inhibitory protein, regulating the activity of the catalytic subunit. The mechanism of action of the cyclic nucleotide on kinase II appeared to be different from that of kinase I.

A stimulation of amino acid activation and polymerization by adenosine 3':5'-cyclic monophosphate (cAMP) was recently observed in rabbit reticulocyte hemolysates by Malkin and Lipmann.¹ In the hope of finding some clues to explain the mechanism of this effect, we studied the binding of cAMP by the supernatant fraction and subfractions thereof. During this search, a concentrate of the reticulocyte translocation factor T₂ was found to bind cAMP strongly. This, however, proved to be misleading as this fraction was contaminated with protein phosphokinases (ATP:protein phosphotransferase, EC 2.7.1.37), and further separation by DEAE-chromatography indicated that the kinases rather than the translocase were responsible for the cAMP binding. In this paper we describe details of the cAMP binding by kinases present in reticulocyte supernatant fractions.

A binding of cAMP by a protein phosphokinase was discovered by Walsh et al.² in their analysis of cAMP stimulation of phosphorylase b activation. They traced this effect to a cAMP stimulation of a relatively nonspecific protein phosphokinase, a kinase kinase, that activates phosphorylase b kinase. We have now isolated two protein phosphokinases, designated I and II, from reticulocyte hemolysate. Both bind, and are activated by, cAMP. From results obtained in our study, a mechanism for the activation of kinase I by cAMP is proposed involving separation of an inhibitor protein from the catalytically active unit.

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**Methods. Assay for protein kinase:** Protein kinase activity was determined by measuring the amount of \(^{32}\)P incorporated into histone. The reaction mixture contained, in a final volume of 0.2 ml: 25 \(\mu\)mol of Tris-Cl, pH 7.5, 4 \(\mu\)mol of MgCl\(_2\), 0.04 \(\mu\)mol of \(\gamma\)-ATP, \(\pm 0.02 \mu\)mol of cAMP, 0.4 mg of calf thymus histone, and enzyme protein. The mixture was incubated at 37°C for 5 min and the reaction terminated by the addition of 10% trichloroacetic acid (TCA). The protein precipitate was collected on Whatman GF/C glass fiber paper, washed with 10% TCA, and counted in 5 ml of Bray’s solution. Protein kinase activity was determined by the method of Lowry et al. with bovine plasma albumin as standard.

**Enzyme purification:** Rabbit reticulocytes were prepared and lysed according to the procedure described by Allen and Schweet. About 500 ml of blood was collected from five rabbits by heart puncture. The supernatant fluid obtained from lysis of reticulocytes was centrifuged at 100,000 \(\times\) \(g\) for 90 min. The pellet was discarded, and the supernatant was made 1% with streptomycin sulfate. The mixture was allowed to stand for 15 min, and then centrifuged. Solid ammonium sulfate was added slowly with stirring to the supernatant fraction to 50% saturation, and left for 30 min. After centrifugation, the precipitate was dissolved in 0.02 M Tris-HCl, pH 7.5, containing 1 mM dithiothreitol (buffer A), and was dialyzed overnight against this buffer. The enzyme solution was then applied to a DEAE-cellulose column (2 \(\times\) 8 cm) which had been previously equilibrated with buffer A. The column was washed with 100 ml of buffer A, and the enzymes were eluted with a KCl gradient from 0 to 0.2 M using a total volume of 300 ml. Protein concentration was monitored at 280 nm, and kinase activity was determined as described in the previous section. The peak fractions of kinase activity were pooled, concentrated by ammonium sulfate precipitation, dialyzed overnight against buffer A, and then stored in liquid nitrogen until used. The overall purification was about 30-fold. All operations were carried out at 0-4°C.

**Materials.** \(\gamma\)-ATP was purchased from International Chemical and Nuclear Corp. Schwarz BioResearch supplied cAMP and \(\text{[H]}\)cAMP. Calf thymus histone, lysine-rich histone, arginine-rich histone, salmon sperm protamine, cUMP, cGMP, and cCMP were purchased from Sigma Chemical Co. Phosvitin and casein were obtained from Nutritional Biochemicals Corp.

**Results. DEAE-cellulose chromatography:** Fig. 1 shows the elution profile of protein kinases of rabbit reticulocytes from a DEAE-cellulose column. Two major kinase activities were demonstrated, one eluting at about 0.1 M KCl (designated as protein kinase I), and the other eluting at 0.2 M KCl (protein kinase II). No kinase activity was detected beyond 0.2 M KCl up to a KCl concentration of 0.4 M.

**Stimulation by cAMP:** Both protein kinases I and II were found to be stimulated by cAMP. The reaction required ATP as the phosphoryl donor, histone as the protein acceptor, and Mg\(^{++}\). The dependence of kinase activity on cAMP concentration is shown in Fig. 2. The phosphorylation of histone by protein kinase I was stimulated by cAMP about 400%. The increase of

![Fig. 1. DEAE-cellulose chromatography of protein kinases. About 700 mg of the enzyme solution obtained from the ammonium sulfate fraction was applied to a 2 \(\times\) 8 cm column. The column was then washed with 100 ml of buffer A and the enzymes were eluted with a linear 0–0.2 M KCl gradient in a total volume of 300 ml. Kinase activity was determined as described under Methods. Protein concentration was measured at 280 nm.](image-url)
kinase II activity was less, due, in part, to a higher activity in the absence of cAMP. Maximum stimulation was observed just above a cAMP concentration of about 5 \times 10^{-7} M for both kinases. Other cyclic nucleotides, such as cUMP, cGMP, and cCMP, at 10^{-4} M, were found to be equally effective in stimulating the activity of kinases I and II. AMP, at a concentration of 10^{-4} M, was found to have no effect on the activity of kinase I, and slightly inhibited kinase II activity.

Specificities of acceptor proteins: The effect of cAMP on the phosphorylation of different protein acceptors by kinases I and II is presented in Table 1. The degree of stimulation of phosphorylation by cAMP depends on the protein acceptor used. The phosphorylation of casein by both kinases was not affected by the cyclic nucleotide. When protamine was used as the acceptor, kinase I showed a stimulation of about 110% by cAMP, but kinase II activity was not affected. Of all acceptors used, arginine-rich histone appeared to be the best. The degree of stimulation by cAMP of the phosphorylation of lysine-rich histone and arginine-rich histone, was different for kinases I and II. In all cases, cAMP had a greater effect on kinase I.

The histone-\(^{32}\)P linkage was found to be stable when heated at 100° for 15 min at neutral pH. In 1 N KOH, under the same heating conditions, the histone-\(^{32}\)P linkage was completely hydrolyzed. This alkaline lability suggests that the

![Fig. 2. Effect of cAMP concentration on kinase I and II activity. Assay conditions were as described under Methods. The reaction mixture contained either 46 μg of kinase I or 100 μg of kinase II. \([\gamma-^{32}\text{P}]\text{ATP} = 15 \text{cpm/pmole.}

Table 1. Effect of cAMP on \(^{32}\)P incorporation into different protein acceptors by protein kinases I and II.

<table>
<thead>
<tr>
<th>Protein</th>
<th>(^{32})P incorporated (pmol)</th>
<th>Stimulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP</td>
<td>+ cAMP</td>
</tr>
<tr>
<td>Kinase I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histone</td>
<td>137</td>
<td>665</td>
</tr>
<tr>
<td>Lysine-rich</td>
<td>49</td>
<td>318</td>
</tr>
<tr>
<td>Arginine-rich</td>
<td>179</td>
<td>1080</td>
</tr>
<tr>
<td>Protamine</td>
<td>306</td>
<td>643</td>
</tr>
<tr>
<td>Casein</td>
<td>234</td>
<td>234</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>71</td>
<td>98</td>
</tr>
<tr>
<td>Kinase II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histone</td>
<td>198</td>
<td>490</td>
</tr>
<tr>
<td>Lysine-rich</td>
<td>152</td>
<td>206</td>
</tr>
<tr>
<td>Arginine-rich</td>
<td>366</td>
<td>800</td>
</tr>
<tr>
<td>Protamine</td>
<td>298</td>
<td>312</td>
</tr>
<tr>
<td>Casein</td>
<td>97</td>
<td>90</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>37</td>
<td>52</td>
</tr>
</tbody>
</table>

The reaction mixture contained, in a final volume of 0.2 ml: 25 μmol of Tris-HCl, pH 7.5, 4 μmol of MgCl₂, 0.04 μmol of \([\gamma-^{32}\text{P}]\text{ATP} \(12 \text{cpm/pmole, 46 μg of kinase I or 40 μg of kinase II, and 0.4 mg of calf thymus histone, protamine, casein, or phosvitin. In the case of arginine-rich and lysine-rich histone, 0.25 mg was added as acceptor. Incubation was at 37°C for 10 min and the reaction terminated by the addition of 10% TCA. The protein precipitate was collected on glass filters and radioactivity determined as described in Methods.}\\\\

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sites of phosphorylation are likely to be the OH groups of serine and/or threonine residues.

**Binding of cAMP to kinases**: As already shown in Fig. 2, the concentration of cAMP required for maximum stimulation of both kinases I and II was about $5 \times 10^{-7}$ M. It appears that both kinases have a high affinity for the cyclic nucleotide. The binding of cAMP to the enzymes could be demonstrated by Sephadex G-50 gel filtration. Fig. 3 shows that when a mixture of enzyme and

![Fig. 3](image)

**Fig. 3**. Binding of $[^{3}H]$cAMP to protein kinase I as determined by Sephadex G-50 gel filtration. The Sephadex G-50 (coarse) column, 1 x 37 cm, was equilibrated with buffer A. 10 µl of $[^{3}H]$cAMP (0.16 µmol/1 ml; 12.7 Ci/mmol) was added to 0.5 ml of a kinase I (4.6 mg/ml) solution and incubated at 0°C for at least 10 min. The mixture was then applied to the column and elution was carried out with buffer A; 0.9-ml fractions were collected at a flow rate of about 3.6 ml/min, 0.1 ml of each being used for radioactivity determination. From each fraction, 50 µl was withdrawn for assay of kinase activity. Protein concentration was measured at 280 nm. $[^{3}P]$ATP = 17 cpm/pmol.

**Fig. 4**. Binding of cAMP on Millipore filters. The binding mixture contained, in a final volume of 0.11 ml: 2 µmol of Tris-HCl, pH 7.5, 1 µmol of MgCl$_2$, 20 pmol of $[^{3}H]$cAMP (1600 cpm/pmol), and various amounts of kinases I or II. The mixture was incubated at 37°C for 3 min then kept at 0°C. Millipore filters (HA 0.45-µm pore size) were presoaked in cold 0.02 M Tris-HCl, pH 7.5, buffer containing 10 mM MgCl$_2$ (buffer B). After the incubation period, the binding mixture was diluted with cold buffer B and filtered through Millipore filters. The filters were washed with cold buffer B, dissolved in 5 ml of Bray's solution, and counted. To test for the effect of Mg$^{2+}$ on the retention of cAMP-kinase complex on Millipore filters, Mg$^{2+}$ was omitted both in the incubation mixture, and in buffer B, at the points marked -Mg$^{2+}$. ---, kinase I; O---O, kinase II.

$[^{3}H]$cAMP was applied to a Sephadex G-50 column, a portion of the radioactivity was found to co-elute with the enzyme fraction. Similar results were obtained for both kinases I and II. When the radioactive material that eluted with kinase I was chromatographed on a thin-layer of cellulose, all the radioactivity remained with it and corresponded to cAMP. However, in a similar experiment using kinase II, only 60 to 70% of the radioactive cAMP was recovered, perhaps because of contamination with degrading enzyme. This binding of cAMP to both enzymes did not require Mg$^{2+}$.

The binding of cAMP to kinases could also be demonstrated using Millipore membrane filtration. Fig. 4 shows the relationship between the amount of protein added and the amount of $[^{3}H]$cAMP bound on Millipore filters. The binding reaction was very rapid and appeared to reach completion in 30 sec at
37°C. However, the mixture was routinely incubated at 37°C for 3 min. Mg⁺⁺ appeared to promote a better retention on the Millipore filters of the complex between [³H]cAMP and the kinases. This effect was greater for the cAMP-kinase II complex than for the cAMP-kinase I complex. The adsorption of the cAMP-kinase complex on Millipore filters constitutes a useful technique for the binding assay.

**Dissociation of protein kinase I in the presence of cAMP:** The sedimentation behavior of protein kinase I in a 5 to 20% sucrose gradient is depicted in Fig. 5. Sucrose density gradient centrifugation was carried out according to the procedure described by Martin and Ames. Kinase I activity sedimented into two

![Fig. 5](image5.png)

**Fig. 5.** Sucrose density gradient centrifugation of kinase I. 0.12 ml of kinase I (4.6 mg/ml) was layered over 5 ml of a 5–20% sucrose gradient. The sucrose solution was prepared in buffer A. After about 15-hr centrifugation at 49,600 rpm at 0°C in a SW50L rotor, the bottom of the tube was punctured and 2-drop (about 0.1–0.15 ml) fractions were collected. The fractions were assayed for kinase activity in the presence (●●●) and absence (○○○) of cAMP. [γ-³³P]ATP = 13 cpm/pmol.

**Fig. 6.** Sedimentation of kinase I activity and cAMP-binding activity with or without cAMP present in the sucrose gradient. Centrifugations were carried out as described in Fig. 5.

(a) Kinase I was sedimented in a sucrose gradient containing no cAMP. Kinase activity (●●●) was determined in the presence of cAMP. The cAMP-binding activity (○○○) was determined using the Millipore technique described in Fig. 4. (b) Kinase I was sedimented in a sucrose density gradient containing 4 × 10⁻⁴ M [³H]cAMP. 0.15 ml of kinase I (4.6 mg/ml) and 5 µl of [³H]cAMP (0.16 µmol/ml) were incubated at 0°C for at least 10 min before layering over the gradient. Two-drop fractions were collected and the binding of cAMP was determined by taking an aliquot of each fraction, which was made 10 mM in MgCl₂ and then filtered through a Millipore membrane. The filter was washed as described in Fig. 4. Kinase activity was determined in the presence of cAMP. ●●●, kinase activity; ○○○, [³H]cAMP bound.

**Fig. 7.** Sucrose density gradient centrifugation of kinase II in the presence and absence of cAMP. The experimental conditions were similar to those of Fig. 5. (a) Centrifugation was carried out in the absence of cAMP. The kinase activity was measured in the presence (●●●) and absence (○○○) of cAMP. (b) Centrifugation of kinase II in the presence of 0.1 mM cAMP in the sucrose gradient. The kinase activity was measured in the presence of cAMP.
distinct peaks. Using yeast alcohol dehydrogenase (MW 151,000) and horse liver dehydrogenase (MW 84,000) as standards, the molecular weight of the heavy peak was estimated to be about 140,000 and the light peak about 60,000. The heavy peak appeared to be almost completely dependent on cAMP for activity. On the other hand, the activity of the light peak was independent of cAMP. The binding activity for cAMP as measured by the Millipore technique was found to coincide with the dependent peak, as shown in Fig. 6a. The light kinase that was independent of cAMP did not bind cAMP.

When the centrifugation was carried out in the presence of [3H]eAMP, a single peak of protein kinase activity was obtained which sedimented at the same position as the independent fraction (Fig. 6b). This seemed to indicate that, in the presence of cAMP, the heavy, dependent, fraction had dissociated into a light component possessing enzymatic activity. This dissociation must have involved the interaction of this protein with cAMP. Therefore, it was of interest to determine the distribution of the binding activity for cAMP in the sucrose gradient following the dissociation. Since the sucrose gradient contained labeled cAMP, the sedimentation pattern of the binding activity could be determined readily by using the Millipore filtration technique. As shown in Fig. 6b, the cAMP binding activity appeared as a single peak which sedimented slightly ahead of the light, cAMP-independent, enzyme. The molecular weight of the cAMP-binding protein was estimated to be 80,000.

The sedimentation pattern of kinase I was not affected by AMP or ATP. cGMP appeared to be as effective as cAMP in promoting dissociation.

Sucrose density gradient centrifugation of kinase II in the presence and absence of cAMP: The sedimentation profile of kinase II in a sucrose density gradient is shown in Fig. 7. In the absence of cAMP, the kinase activity sedimented as a single symmetrical peak. The molecular weight of this peak was estimated to be 140,000, similar to that of heavy kinase I. The kinase activity of this peak was stimulated by cAMP. When the sedimentation of kinase II was carried out in a sucrose gradient containing 0.1 mM cAMP, the kinase activity was distributed in a broad asymmetrical peak. The cyclic nucleotide also seemed to cause some molecular alteration of kinase II. However, the changes appeared to be different from that observed for kinase I.

Conclusions. Adenosine 3',5'-cyclic monophosphate-dependent protein kinases have been demonstrated in different animal tissues.\textsuperscript{5,8-12} In this communication, we have shown the occurrence of two different protein kinases in rabbit reticulocytes. The two enzymes could be separated by DEAE-cellulose chromatography. Depending on their sequence of elution with a salt gradient from the DEAE-column, the enzymes were identified as kinase I and II. Of these, the kinase I proved to be most interesting.

When kinase I was sedimented in a sucrose density gradient, two peaks possessing enzymatic activities were detected. However, only the heavier sedimenting component was stimulated by cAMP. The activity of the light component was independent of the cyclic nucleotide, and did not bind cAMP. The molecular weight of the heavy fraction was estimated to be about 140,000, and that of the light about 60,000.
With cAMP present in the gradient, a single kinase activity peak sedimented at a position analogous to the light fraction, suggesting that cAMP causes a dissociation. This is supported by finding an inactive component which sediments slightly ahead of the light activity peak and which binds cAMP. The molecular weight of this component was estimated to be 80,000. These results suggest that the heavy protein kinase I fraction is an inactive form and that cAMP binds to and dissociates an inhibitor of a little more than half the molecular weight leaving the light, catalytically active, form behind. We realize that there are limitations to such an interpretation since the experiments were done with an impure system. However, the proposition does constitute an attractive working hypothesis and would present a novel mode of enzyme regulation. Since completion of this work, a paper by Gill and Garren has appeared which reports experiments, with a protein kinase from adrenal cortex, that are interpreted to indicate a similar mechanism of cAMP stimulation.

Kinase II, in a sucrose density gradient, sedimented at a position very similar to the heavy kinase I fraction. Its molecular weight was estimated to be about 140,000. In the presence of cAMP, the sedimentation profile of kinase II became asymmetrical and was different from that observed for kinase I. This indicates that cAMP may also cause structural changes of this molecule. However, since some cAMP-degrading enzyme appears to contaminate kinase II preparations, the interpretation of these results has to await further enzyme purification.

We are grateful to Dr. Jean Lucas-Lenard for her help with the preparation of this manuscript.

Abbreviations used: cAMP, adenosine 3′:5′-cyclic monophosphate; buffer A, 0.02 M Tris-HCl, pH 7.5–1 mM dithiothreitol; TCA, trichloroacetic acid.

* This work was supported by grant GM-13972 from the U. S. Public Health Service.
† Recipient of a fellowship from the American Cancer Society (PF-506). Present address: Department of Biological Chemistry, College of Medicine, University of Illinois at the Medical Center, Chicago.
‡ Recipient of an International Postdoctoral fellowship from the U. S. Public Health Service. Present address: Instituto Maranon, Centro de Investigaciones Biologicas, C.S.I.C., Madrid, Spain.