RELATIONSHIP BETWEEN CYCLIC AMP DEPENDENT PROTEIN KINASE(S) AND CYCLIC AMP BINDING PROTEIN(S) IN RAT LIVER

A.M. CHAMBAUT*, F. LERAY and J. HANOUNE**

Institut de Pathologie Moléculaire†, 24 rue du Faubourg Saint Jacques, Paris 14e, France

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1. Introduction

It is now widely recognized that adenosine-3′, 5′-cyclic monophosphate (cAMP) acts essentially within the cell by the activation of one or several more or less specific protein kinases. Such cAMP sensitive kinases have been described in a variety of tissues and throughout the animal kingdom [1–2]. More specifically, these protein kinases have been shown to catalyse the phosphorylation and activation of muscle phosphorylase kinase [3] and lipase from adipose tissue [4] as well as phosphorylation and inactivation of glycogen synthetase [5]. The mechanism by which cAMP stimulates the various protein kinases is not as yet understood. Direct and indirect evidence from studies on muscle, adrenal gland and in reticulocytes has lead to the hypothesis that cAMP can dissociate an inactive (or weakly active) kinase into a fully active form by binding to an inhibitory subunit, thus releasing the free, active catalytic subunit [6–8].

From the sucrose gradient centrifugation data reported here, it appears that the same mechanism applies to liver cytoplasmic enzymes. Moreover, this separation procedure enabled the isolation of a cAMP binding protein, heavier than the protein kinase fraction and devoid of any kinase activity. The commonly proposed model for the physiological activation of cAMP dependent protein kinase is discussed.

* Chargée de Recherches au Centre National de la Recherche Scientifique, on leave from the Institut de Biochimie (432), Faculté des Sciences, 91-Orsay, France.

** Chargé de Recherches à l'Institut National de la Santé et de la Recherche Médicale.

† Université de Paris, Groupe U.15 de l'Institut National de la Santé et de la Recherche Médicale, Laboratoire associé au Centre National de la Recherche Scientifique.

2. Materials and methods

The partially purified enzyme was prepared according to the procedure of Kuo and Greengard [1] modified as follows: livers from 3–4 male, Wistar rats were pooled, homogenized in 3 volumes of neutralized 4 mM EDTA. The homogenate was centrifuged at 20,000 *g* for 20 min, then at 10,000 *g* for 45 min. The pH of the supernatant was adjusted to 4.9 with 1 N acetic acid. The precipitate was removed by centrifugation and the pH of the supernatant readjusted to 6.9 with 1 M potassium phosphate buffer (pH 7.2). Solid ammonium sulfate (0.34 g/ml) was slowly added and the solution stirred for 30 min at 4°. After removal of the precipitate by centrifugation, the solution was extensively dialyzed against 50 mM potassium phosphate buffer (pH 7.2) containing 2 mM EDTA. After a last centrifugation at 75,000 *g* for 30 min, the enzyme preparation was kept in several small tubes, at -70°, without any loss of neither activity nor cAMP sensitivity for weeks.

2.1. *Standard assay for protein kinase*

As standard conditions, the activity of the cAMP dependent protein kinase was assayed in an incubation volume of 0.2 ml containing 50 mM tris-HCl

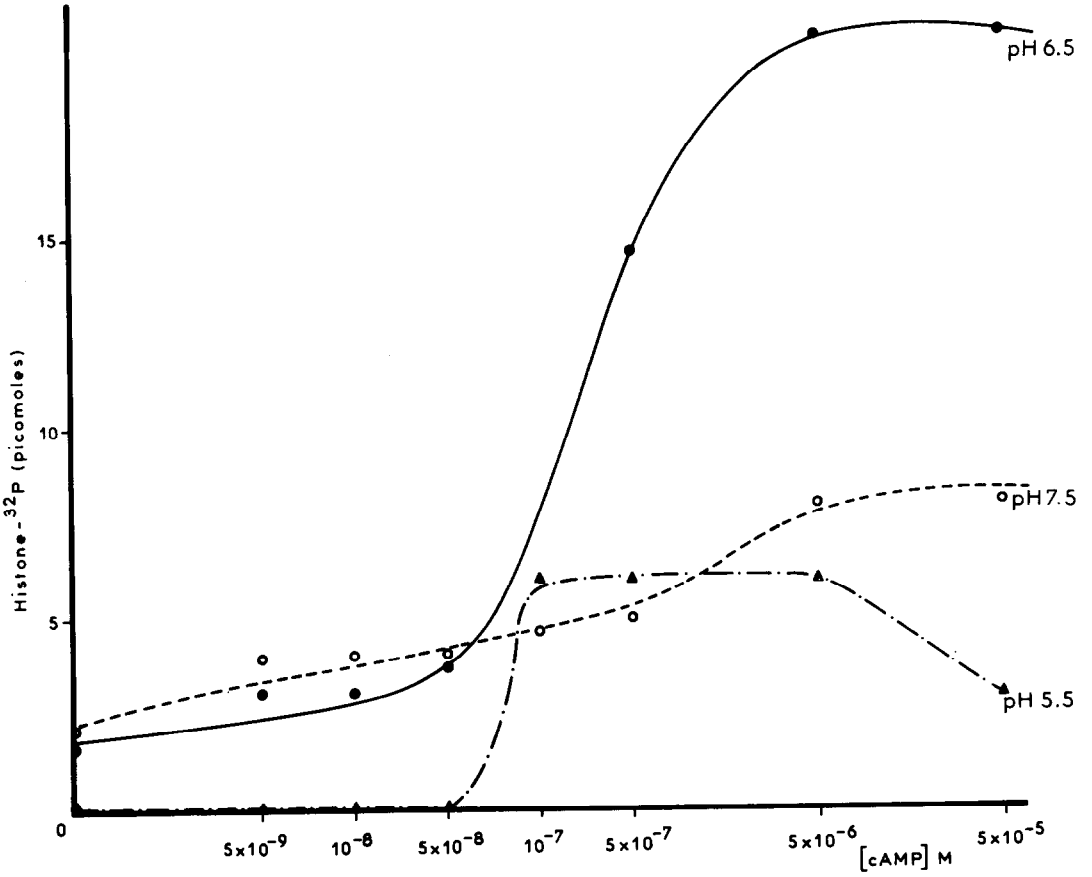

Fig. 1. pH dependence of the kinase activity: kinase activity was determined in duplicate for each point either in acetate buffer 50 mM, pH 5.5 or in tris-HCl buffer 50 mM pH 6.5, or in potassium phosphate buffer 50 mM pH 7.5 in the presence of 5 mM Mg^{2+} and of increasing concentrations of cAMP. The specific activity of the γ-^{32}P-ATP was 600 cpm/pmole.

buffer (pH 6.5); 5 mM $MgCl_2$; γ-^{32}P-ATP, 12.5 μM, about 1.5 X 10^6 cpm per assay; various amounts of enzyme preparation (up to 50 μg); 40 μg of total thymus histones and, when indicated, 5 X 10^{-6} M cAMP. The reaction was always started by the addition of the enzyme and the mixture was incubated at 30° for 5 min, in a shaking bath. The reaction was terminated by the addition of 2 ml of 25% cold trichloroacetic acid containing 0.1 mM potassium monophosphate and the solution was filtered on a nitrocellulose filter (Schleicher and Schüll 0.45 μm pore size). The filter was washed with 50 ml of 5% cold trichloroacetic acid, dissolved in 10 ml of Instagel (Packard Inst. Co) and counted in a Nuclear Chicago Liquid Scintillation Counter Mark I. Each experimental series included an assay without histone. Results are expressed in pmoles of ^{32}P from γ-^{32}P-ATP recovered in protein in the standard assay system.

2.2. *Determination of cyclic AMP binding activity*

Duplicate or triplicate reaction mixtures were prepared by adding 150 μg of protein in 0.2 ml reaction mixture containing ^{3}H-cAMP at various concentrations as indicated in the legends to the figures. Buffers were either potassium phosphate 50 mM or tris-HCl 50 mM, pH 5.5, 6.5 or 7.5 with or without 10 mM $MgCl_2$. Theophylline 8 mM was added when pH 7.5 buffer was used. The mixture was incubated for 90 min at 0°. A Millipore

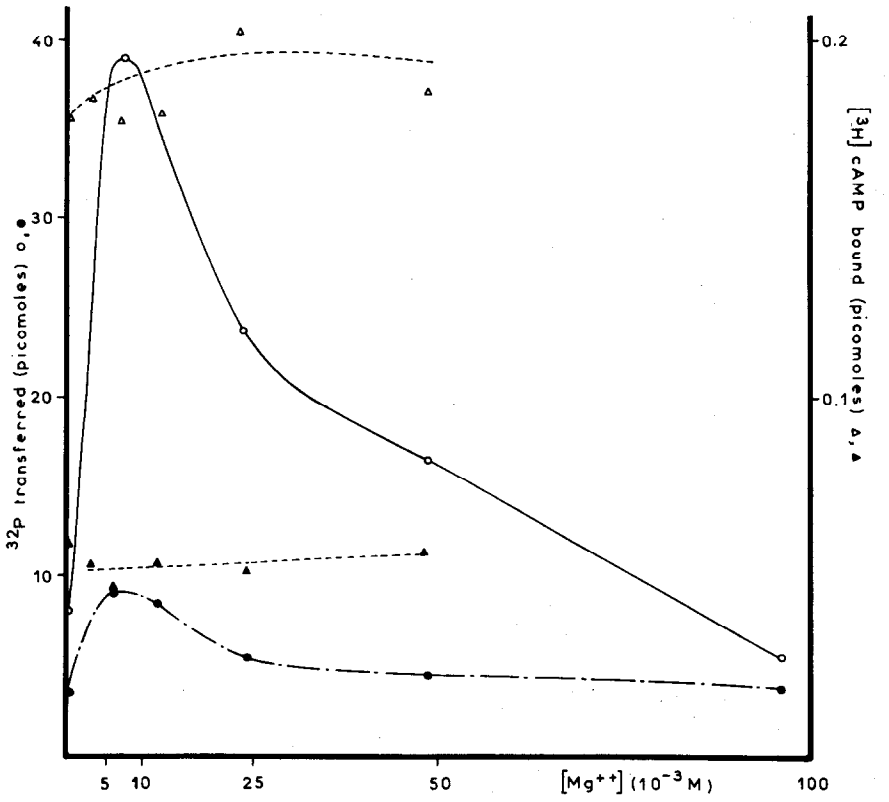

Fig. 2a. Influence of cations upon kinase and cAMP binding activities: effect of increasing concentration of $MgCl_2$. The kinase and the cAMP binding activities were assayed as described in Materials and methods. ●, kinase activity without cAMP; ○, kinase activity in the presence of 5×10^{-6} M cAMP. Binding activities were tested with two concentrations of ^{3}H-cAMP: ▲, 2.2×10^{-9} M; △, 5.6×10^{-8} M.

filter apparatus was used to separate the free from protein-bound cyclic AMP. The membrane filter (Schleicher and Schüll, 0.45 μm pore size) was initially immersed in the same buffer used for incubation to which $MgCl_2$ 10 mM was eventually added. The reaction mixture was diluted with the above buffer and quantitatively transferred onto the filter. The filter was washed with 12 ml of cold buffer and then dissolved in 10 ml of Instagel and counted in a Liquid Scintillation Counter. Results are expressed in cpm or in pmoles of cAMP bound/min per reaction mixture.

Table 1
pH dependence of cAMP binding activity.

pH	^{3}H-cAMP bound (cpm)
5.5	2,943 ± 75
6.5	3,278 ± 243
7.5	2,684 ± 43

Binding activity was determined in the same buffers as in the legend to fig. 1 and in the presence of 5.6×10^{-8} M ^{3}H-cAMP. Protein kinase preparation: 120 μg per assay. Values are given as means ± standard error for 4 assays.

2.3. *Materials*

Total calf thymus histone and cold adenosine-3′,5′-cyclic monophosphate were obtained from Calbiochem; γ-^{32}P-ATP (about 2 Ci/mmole) and 8-^{3}H-adenosine 3′,5′-cyclic monophosphate (19 Ci/mmole) were purchased from C.E.A. Saclay, France. Purity of both products was checked by thin layer chromatography. Theophylline was from Merck (Germany).

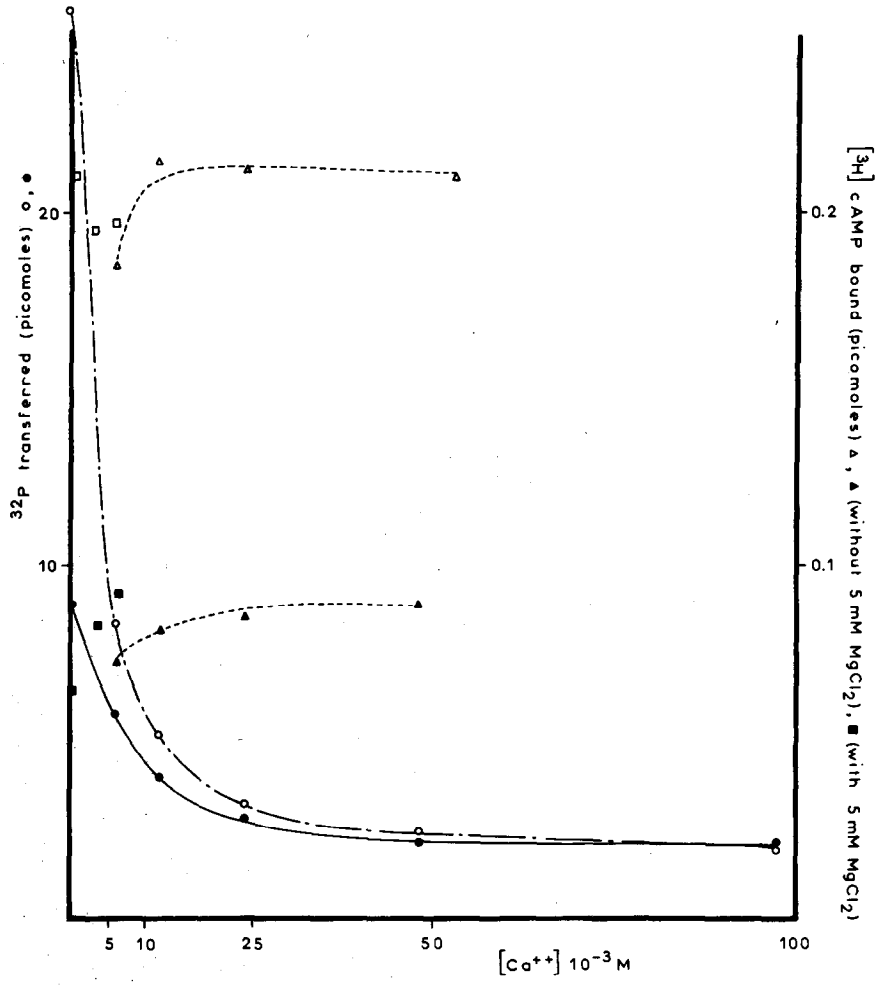

Fig. 2b. Influence of cations upon kinase and cAMP binding activities: effect of increasing amount of $CaCl_2$. Kinase activity was tested in the presence of $MgCl_2$ 5 mM, with (○) or without (●) 5×10^{-6} M cAMP. Binding activity was tested without $MgCl_2$ (■, □) and with 5 mM $MgCl_2$ (▲, △); closed symbols refer to 2.2 ± 10^{-9} M ^{3}H-cAMP and open ones to 5.6×10^{-8} M ^{3}H-cAMP.

3. Results

We first tried to compare the optimal biochemical parameters for both the kinase and binding properties of our preparation.

Fig. 1 shows the effect of pH on both the basal and the cAMP dependent activities of the protein kinase preparation. At pH 5.5 no basal activity could be detected and the enzyme was completely dependent upon the presence of cAMP. At pH 7.5, the activating effect of cAMP was rather weak. The optimal pH for the cAMP activation was found to be 6.5.

Similar studies were conducted with regards to the cAMP binding properties of this preparation. As has been shown in other systems [9, 10], cAMP binding was much less dependent on pH between 5.5 and 7.5 (table 1).

Fig. 2 reveals some other discrepancies between the cAMP sensitive kinase activity and the cAMP binding properties of the preparation with regards to the cation concentration of the assay mixture.

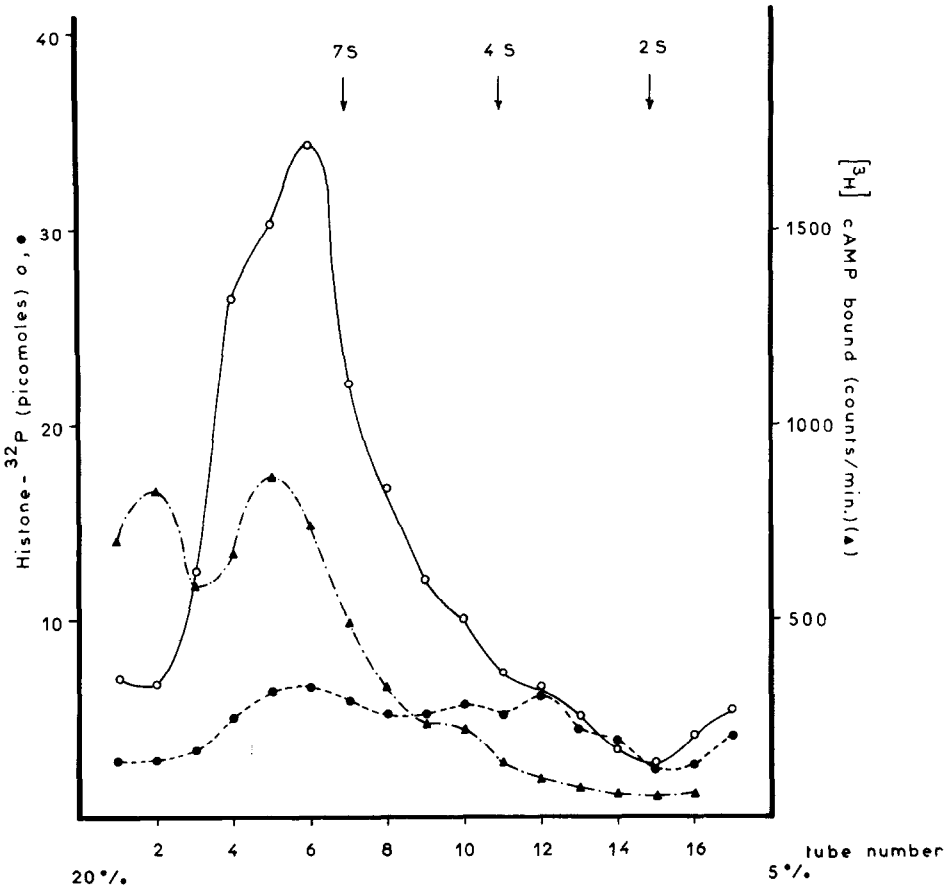

Fig. 3a. Sucrose density gradient centrifugation. 0.2 ml (6.25 mg/ml) of protein kinase preparation was layered over 4.8 ml of a 5–20% linear sucrose gradient. The sucrose solution was prepared in tris-HCl buffer 20 mM, pH 7. The protein was sedimented without added cAMP. After 18 hr centrifugation at 38,000 rpm at 0° in a SW 39 rotor of a L Spinco Centrifuge, the bottom of the tube was punctured and 4-drop fractions were collected (about 0.25 ml). The fractions were assayed for kinase activity in the presence (o——o) and absence (•——•) of 5 × 10^{-6} M cAMP and for cAMP binding activity (▲ – · – ▲) (^{3}H-cAMP 5 × 10^{-9} M; 10,500 cpm/pmole). Approximate S values were calculated according to the procedure of Martin and Ames [14], using myoglobin, serum albumin and lacticodeshydrogenase as standards.

It is clear that maximal kinase activity and cAMP sensitivity occur at 5 mM Mg^{2+} (fig. 2a). Both are strongly inhibited by Ca^{2+} (fig. 2b). On the contrary the binding properties are not altered by modifications of the cation concentration.

The sedimentation behaviour of protein kinase in a 5 to 20% linear sucrose gradient is shown in fig. 3a. Kinase activity sedimented into two distinct fractions: the heavy one (approximately 8 S) appeared to be almost completely dependent on cAMP for activity. The light one (about 4 S) was independent of cAMP. The binding activity for cAMP was found to sediment also in two peaks, one with an S value > 8, the other coinciding with the cAMP sensitive kinase peak. The light kinase that was independent of cAMP did not bind cAMP. Practically no kinase activity was found associated with the heavier binding peak.

After preincubation for 100 min at 0° with 5.6 × 10^{-6} M ^{3}H-cAMP (2,250 cpm/pmole) the same preparation was centrifuged in a sucrose gradient containing 5.1 × 10^{-6} M ^{3}H-cAMP (230 cpm/pmole). In each elution fraction, total kinase activity and ^{3}H-cAMP bound were determined. It

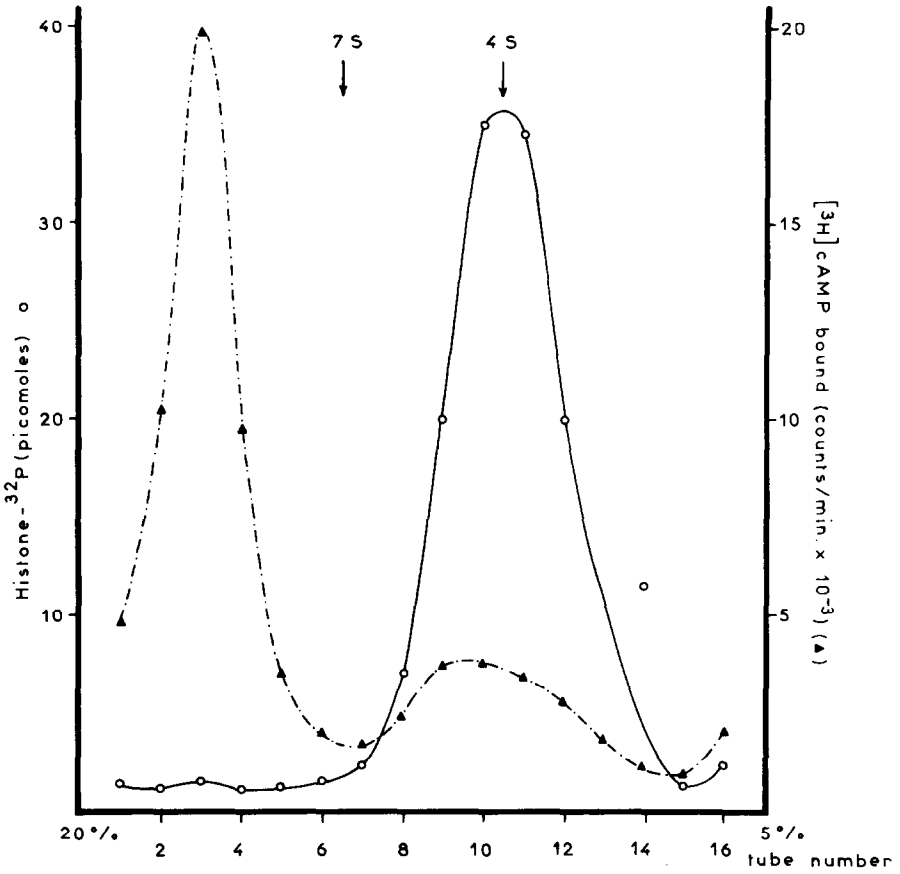

Fig. 3b. Sucrose density gradient centrifugation. The sucrose solution was prepared in tris-HCl buffer 20 mM, pH 7. The protein was sedimented in a sucrose gradient containing ^{3}H-cAMP (5.1×10^{-6} M; 230 cpm/pmole). The protein kinase preparation was incubated at 0° for 100 min with ^{3}H-cAMP (5.6×10^{-6} M; 2,250 cpm/pmole) in tris buffer (20 mM, pH 7.0), theophylline 8 mM, before layering onto the gradient. The fractions were assayed for kinase activity in the presence of 5×10^{-6} M cAMP (o——o) and for binding activity by taking an aliquot of each fraction which was made 5 mM in $MgCl_2$ and filtered through a membrane (▲ –·–▲). Approximate S values were calculated according to the procedure of Martin and Ames [14], using myoglobin, serum albumin and lacticodeshydrogenase as standards. All other assay conditions were as in fig. 3a.

is clear in fig. 3b that, in this condition, the heavier kinase peak is shifted to the same position as the cAMP-dependent fraction (4 S). The cAMP binding activity, previously associated with the heavy kinase, now appeared at a position analogous to the light kinase, sedimenting slightly ahead. The heavier binding peak, devoid of any kinase activity, was not shifted and remained at the same place.

4. Discussion

These data suggest that in liver as well as in other organs, cAMP acts on protein kinase by virtue of its binding to an 'inhibitory subunit', thus leading to a dissociation of the enzyme into an active, lighter catalytic subunit and a regulatory component.

Discrepancies in the pH dependence and cations sensitivity of both cAMP binding properties and cAMP sensitive kinase activity can be explained in terms of different physiochemical conditions necessary for the expression of the two phenomena and are still compatible with the above hypothesis. Nevertheless, we wish to emphasize that, in our conditions, sucrose gradient centrifugation revealed the existence of an heavy cAMP binding fraction, now under study, characterized by a probably high affinity

for cAMP but without any kinase activity. Such a fraction was not described in previous work on muscle and adrenal systems, may be due to a purification procedure focussed on the enzymatic activity.

Although the interpretation of the above data must await purification of further enzyme and cAMP binding protein fractions, it is already clear that in physiological conditions the regulation of the kinase(s) activity by cAMP may be dependent on the presence of some unknown binding fractions.

As already emphasized by several teams, cAMP level in liver [11, 12] as well as in muscle [13] is quite high and would constantly keep all kinases fully activated. As a working hypothesis, we suggest that binding of cAMP with other sites could modulate the amount of the free nucleotide necessary for kinase activation.

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