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# Binding Activity Regulation of Rabbit Skeletal Muscle Adenosine 3′-5′-Monophosphate-Dependent Protein Kinases

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A cAMP-dependent protein kinase has been isolated from rabbit muscle and purified. The affinity constant of the enzyme for the nucleotide is $K_a = 9.3 \times 10^{-9}$ M, with a $V_{max} = 0.013 \times 10^{12}$ moles bound cAMP/1 $\mu$g protein. The influence exerted by different factors is studied: *a)* Inhibitor (I) of kinase activity: increases the binding capacity for cAMP, by percentages which depend on the amount of I. In the presence of inhibitor (120 $\mu$g/100 $\mu$l) the affinity constant is $K_a = 4.1 \times 10^{-9}$ M, without change in $V_{max}$. *b)* Effect of pH: it has a complex influence over binding, being also regulated by cAMP concentration. The positive effect on binding of ionic and bovine serum albumin concentrations, and the negative effect of enzyme preincubation before additions of ($H^3$) cAMP, have also been studied. The importance of these effectors to obtain a high degree of sensitivity in the binding protein method has been assertained.

Some of the biological functions of cyclic 3′-5′Adenosine Monophosphate (cAMP) are performed through the activation of some protein kinase (ATP: protein phosphotranspherase EC 2.7.1.37), which have been identified in a wide variety of sources (10, 19). As originally proposed by BROSTROM *et al.* (4), cAMP-dependent protein kinase are composed of two dissimilar subunits, which dissociate in the presence of cAMP, according to the following equation: RC + cAMP $\rightleftharpoons$ R-cAMP + C. Cyclic AMP activates protein kinase *in vitro* by binding to its regulatory (R) subunit, effecting the release of the active, catalytic subunit (C).

This mechanism has been utilized by GILMAN (8) and others (6, 17, 21) as a basis for the radioanalytic determination of cAMP. It has also been reported (2, 3, 18) that the presence of an inhibitor-protein molecule (I) of the subunit (C) stabilizes the R-cAMP complex. In the present study it has been evaluated the influence exerted over the dissociation of RC, in the presence of cAMP, and subse-

quent stabilization of the R-cAMP complex, by the following factors: the concentration of the inhibitory molecule I, pH of medium, preincubation of protein kinase before addition of cAMP, and saline and bovine serum albumin (BSA) concentration of incubation medium. It has been tried to establish the optimal conditions for binding of protein kinase to cAMP, in order to obtain an improvement in the technique of the protein binding of the cyclic nucleotide.

## Materials and Methods

cAMP-dependent protein kinase has been prepared essentially by the method of WALSH *et al.* (20), with some modifications, starting from rabbit skeletal muscle. Two peaks of maximum binding activity are obtained in DEAE-cellulose chromatography (DE-11 Whatman, 2.5 × 30 cm): DEAE-I and DEAE-II. Automatic recording of the O.D. of the eluate provide two peaks which are correlated with the fractions showing maximum binding activity. A higher purification has also been achieved by chromatography in Sephadex G-200.

Preparation of inhibitor was carried out according to APPLEMAN *et al.* (1), from rabbit muscle and rat liver (Wistar albino male). The concentration of the cyclic AMP (Boehringer Mannheim) was determined by spectrophotometry at 259 nm (15). The protein concentration was determined following LOWRY *et al.* (11). The pH in the range 4-5.5 were obtained with an acetic acid/sodium acetate and those in the range pH 6-7 with potassium phosphate buffer solution respectively.

*Binding activity of protein kinase.* The binding activity of the cAMP-dependent protein kinase was assayed at pH 6.5 in an incubation volume of 0.1 ml at 4° C. The assay mixture contained the following: 200 mM potassium phosphate buffer, 8 mM theophylline, 6 mM 2-mercaptoethanol, 5 mM EDTA, 2 % bovine serum albumin (BSA) (Behringwerke, Marburg-Lahn), $1 \times 10^{-9}$ mol ($H^3$)cAMP (38 Ci/mmol. The Radiochemical Centre, Amersham, Bucks.), 0.02 ml (52 $\mu$g) of the protein kinase preparation (or 0.05 ml of the eluated fraction in DEAE cellulose chromatography). In the standard curves cAMP ($0.05 - 4 \times 10^{-9}$ mol/tubo) is added, before additions of ($H^3$)cAMP, during 90 min at 4° C. The R-cAMP complex is separated through filtration in cellulose acetate membrane filters (HAWP, 0.45 $\mu$m. Millipore) or by activated charcoal (Sigma) (2.6 %, BSA 2 %) (17). The filters are processed according to GILMAN (8). The charcoal suspension (0.1 ml) were added to each tube. After centrifugation (7.500 × *g* for 10 min at 4° C) 0.1 ml sample of supernatans (bound fraction) were removed and their radioactivity determined by liquid scintillation counting in PCS (10 ml). The chromatographic fractions which yield some binding activity peaks (DEAE-I and DEAE-II) were pooled and dialized against Tris-HCl 5 mM, pH 7.5, 2 mM EDTA. They are frozen at —20° C in aliquots (1 ml).

## Results

The binding relation of protein kinase to cAMP is of hyperbolic pattern, with an affinity constant $K_a = 9.3 \times 10^{-9}$ Molar, with respect to the cAMP, and a $V_{max} = 0.013 \times 10^{-12}$ moles bound cAMP/1 $\mu$g protein. Reaction equilibrium is achieved in 60-70 min, but the reaction is maintained systematically up to 90 min.

*Effect of inhibitor (I) on binding activity.* The formation of R-cAMP complex is increased in the presence of I by a percentage which oscillates between 35 and 55 % in experiences with a constant amount of enzyme (26 $\mu$g/0.1 ml), and variable amounts of I (0, 24, 48 $\mu$g/0.1 ml). The absence of specificity of I is significant since the one obtained from rat liver

increases by 45 % the binding capacity of rabbit muscle protein kinase for cAMP.

In other experiencies it has been evaluated the effect exerted by two concentrations of I (48-120 $\mu$g/0.1 ml) throughout a series of dilutions of protein kinase, noticing an increase of 18-25 % of binding for the largest concentrations of I.

The affinity constant ($K_a$ in the presence of I (120 $\mu$g/0.1 ml) of the R-cAMP complex, determined with variable concentrations of ($H^3$) cAMP (0.2-1.5 $\times$ $10^{-9}$ M), is $K_a = 4.1 \times 10^{-9}$ M and $V_{max} = 0.011 \times 10^{-12}$ moles bound cAMP/1 $\mu$g protein kinase (fig. 1).

*Effect of pH on fractions of protein kinase.* The binding activity of protein kinase as a function of medium pH is a relationship which is also influenced by cAMP concentration (fig. 2). The critical points are between pH 4.5 and 6.5 (both for DEAE-I and DEAE-II). In the process of change of pH (from 4.5 to 6.5), there is a high increase in enzyme binding activity for concentrations greater than $5 \times 10^{-9}$ M, and a decrease for lower concentrations of cAMP.

*Effect of saline and albumin concentrations on binding activity.* Acetate ion concentrations of 0.02, 0.05 and 0.2 M at pH 4 yield, for DEAE-I fraction, percentages of binding of protein kinase with cAMP of 10, 12 and 24 %, respectively. Similar results are obtained for phosphate ion, in both DEAE-I and DEAE-II peaks.

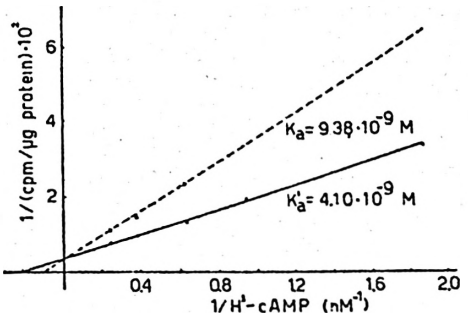


Fig. 1. *Effect to inhibitor on binding affinity constant for cyclic AMP at pH 6.5.*
●—● with Inhibitor protein (120 $\mu$g/100 $\mu$l);
●·····● without Inhibitor; binding protein concentration 52 $\mu$g/100 $\mu$l.

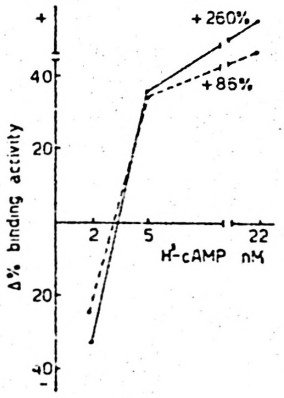


Fig. 2. *Effect of pH on cAMP binding activity in the increase pH from 4.5 to 6.5.*
Binding protein concentration: 120 $\mu$g/100 $\mu$l. % Binding activity = % Binding pH 6.5/% Binding activity pH 4.5. Buffers were: 0.2 M acetate-acetic acid pH 4.5; 0.2 M phosphate pH 6.5 (both plus additions).

Bovine serum albumin yields increases of binding activity of 28-37 % at pH 4.5. This influence is not observed for the fractions obtained from Sephadex G-200 chromatography at pH 6.5.

*Effect of preincubation of protein kinase. Sensitivity of radioassay.* In the quantitative determination of cAMP by protein binding, before the additions of ($H^3$) cAMP, the protein kinase is preincubated with cAMP during 60 min at 4° C. A higher sensitivity of the method is obtained, established as an increase in the slope of the asymptote at the zero point.

In previous experiences, in order to evaluate the effect that this preincubation could exert over the enzyme activity, the protein kinase is diluted in 0.05 ml phosphate buffer 0.2 M pH 6.5 incubation medium (plus additions) for 60 min at 0-4° C. Afther this, the ($H^3$) cAMP is

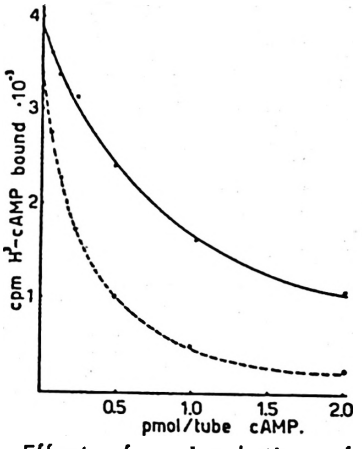


Fig. 3. *Effect of preincubation of protein kinase with unlabelled cAMP.*
Two standard curves, obtained under different conditions: ●——● unlabelled cAMP, ($H^3$) cAMP (1 pmol) protein kinase (26 μg) and inhibitor (24 μg) were incubated for 90 min. ●—● Protein kinase and Inhibitor were preincubated with unlabelled cAMP for 60 min. Later on ($H^3$) cAMP (1 pmol) is added and incubated for 90 min. The separation is carried with charcoal.

added. A very significant loss of enzyme binding capacity is observed: 35 % for DEAE-I and 27 % for DEAE-II at the two pH's.

## Discussion

The standardization of the protein binding method (6, 8, 13) for the determination of cAMP presents special problems because o high degree of sensitivity is needed, due to its low concentrations in tissues (7).

There is no agreement about the optimal pH to achieve maximum binding activity of the protein kinase. Several pH values between 4 and 7.5 have been reported for proteins extracted from bovine and rabbit muscle and from adrenal glands (6, 8, 12, 17). These results are in perfect agreement with those described by GILMAN (8) about the influence which also exerts the cAMP concentration, even through there are differences about the absolute values of such a concentration. The results obtained with the inhibitor suggest that it is a stability factor of the R-cAMP complex. It has been postulated (2) that the inhibitor is a modified form of R, which keeps a binding site with C, with which it interacts directly. It has been found the positive influence exerted by BSA over the protein kinase binding capacity. It has been indicated (17) that the addition of BSA increases the affinity in the same way as the inhibitor does. Similar results have been described for other proteins (14, 16) which suggests that, in a general way, they serve to stabilize the protein binding or to increase its affinity for cAMP (14). The increase in saline concentration of the incubation medium increases the binding of protein kinase and cAMP. BROSTROM *et al.* (5) has studied the influence of NaCl on binding, with complex results, due to its interrelation with the medium pH and with the method of separation for the free and bound fractions. The exposure of the enzyme to cAMP solution or the samples, before addition of ($H^3$) cAMP was already used by HALES *et al.* (9) to sensitize the radioimmunoassay, and COOPER *et al.* (7) for determination of cAMP, obtaining a higher sensitization on the protein binding method. In these studies it has been found, besides an improvement in sensitivity, a loss of protein kinase binding capacity due to the effect of this preincubation.

Even though the internal mechanism of the influence exerted by these factors remains obscure, the quantitative effect must be determined correctly to obtain: (i) a high degree, of binding between RC and (ii) stability of R-cAMP complex in its manipulations. Both of these conditions are necessary to obtain a good protein binding technique.

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## Resumen

De músculo de conejo se ha aislado y purificado una proteína quinasa, dependiente del cAMP. La constante de afinidad del enzima para el nucleótido es $K_a = 9{,}3 \times 10^{-9}$ M, con una $V_{max} = 0{,}013 \times 10^{-12}$ moles cAMP enlazado/1 $\mu$gr proteína. Se estudia la influencia ejercida por diferentes factores: *a*) Inhibidor (I) de la actividad quinasa: incrementa la capacidad de unión para el cAMP, en valores que dependen de la cantidad de I. En presencia del inhibidor (120 $\mu$g/100 $\mu$l) la constante de afinidad es $K_a = 4{,}1 \times 10^{-9}$ M, sin variación en la $V_{max}$. *b*) Efecto del pH. Tiene una influencia compleja sobre el enlace, estando también regulada por la concentración de cAMP. También se ha estudiado el efecto positivo sobre el enlace de la concentración iónica y de la albúmina sérica bovina; y el negativo de la preincubación del enzima, antes de añadir ($H^3$) cAMP. Se constata la importancia de esos efectores para obtener un alto grado de sensibilidad en la técnica de binding protein.

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