SEPARATION OF TWO NONIDENTICAL cAMP BINDING SITES IN THE cAMP-DEPENDENT PROTEIN KINASE TYPE I

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1. Introduction

Two forms of cytosolic cAMP-dependent protein kinases exist in mammalian tissues [1,2], type I and type II, according to their order of elution during chromatography on DEAE-cellulose. Both forms are tetramers. The stoichiometry of the activation by cAMP has been revised to be [3–6]:

$$R_2C_2 + 4\ cAMP \rightleftharpoons R_2(cAMP)_4 + 2\ C$$

cAMP dissociates the tetrameric complex into the dimeric cAMP binding regulatory subunit (R) and the free active catalytic subunit C. This equation implies that the monomeric R-subunit binds 2 molecules of cAMP. In [7–9] both sites had similar affinities to cAMP but differed in their dissociation rate. Using nitrocellulose filtration for the determination of bound cAMP a stoichiometry of only 1 mol bound cAMP/mol monomeric R-subunit is obtained. This discrepancy has been assigned to a specific conformational effect of the nitrocellulose filter on the R-subunit [6]. We are now engaged in elucidating the activation process of the kinase by means of derivatives of cAMP [10–12]. One prerequisite of these studies is a detailed description of the cAMP binding sites and the conformational change they undergo in the activation process. It was therefore necessary to ascertain that 1 homogeneous population of sites is recovered by the filtration technique. Furthermore, conditions were found (a 6-fold excess of catalytic subunit) where 2 cAMP sites/R-subunit monomer could be measured in the filter assay. In the presence of an excess of catalytic subunit and ATP, these 2 sites showed different sensitivity towards the ionic strength of the buffer.

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2. Materials and methods

c[8-^{3}H]AMP was purchased from Amersham Buchler. ATP, cAMP were from Boehringer (Mannheim). 2-(*N*-morpholino)ethane sulfonic acid (MES); ethyleneglycol-bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) were from Sigma. Nitrocellulose filters (25 mm diam.) were from Sartorius. All other chemicals were of highest available purity. Protein kinase type I from rabbit skeletal muscle was prepared as in [13]. Free regulatory subunit and catalytic subunit were prepared as in [14]. Enzymes were stored at 0–4°C in 5 mM MES (pH 6.7), 0.1 mM EDTA, 0.1 mM phenylmethanesulfonylfluoride, 0.1 mM benzamidine and 15 mM mercaptoethanol. Protein content was determined as in [15] using bovine serum albumin as a standard.

Bound c[^{3}H]AMP was determined as in [16]. c[^{3}H]AMP had spec. act. 2000 cpm/pmol. Incubations were done in 100 μl total vol. at 25°C. The incubation buffer (pH 6.9) contained 25 mM MES, 4 mM Mg^{2+}-acetate, 0.125 mM EGTA.

3. Results

3.1. *Stoichiometry and stability of cAMP bound to the regulatory subunit*

Fig.1 shows that the recovery of bound c[^{3}H]AMP on the nitrocellulose filter is dependent on the amount of catalytic subunit present in the incubation mixture. Under the conditions employed (R-subunit, 7.5 nM) a

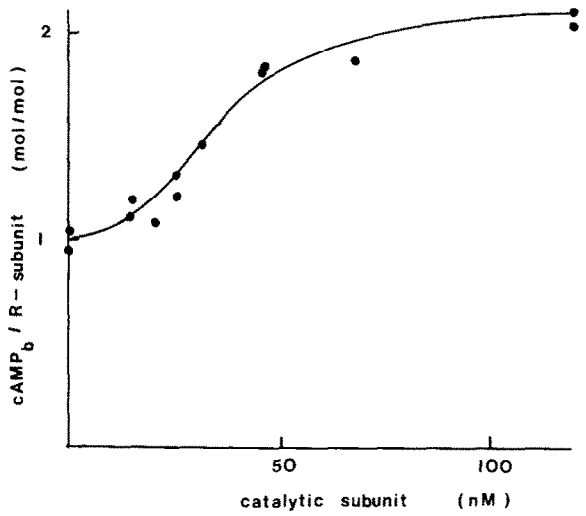

Fig.1. Stoichiometry of the cAMP binding to the regulatory subunit in dependency of the concentration of the catalytic subunit. Regulatory subunit (7.5 nM) was incubated with various concentrations of the catalytic subunit for 30 min at room temperature in the presence of 1 μM c[^{3}H]AMP.

6-fold excess of the catalytic subunit (50 nM) yielded a stoichiometry of 2 mol cAMP bound/mol monomeric R-subunit. In a control experiment the catalytic subunit did not bind detectable amounts of c[^{3}H]-AMP. Furthermore, the effect of excess catalytic subunit is independent of whether isolated R-subunit or holoenzyme is used.

As shown in table 1 the presence of excess catalytic subunit stabilizes the R-subunit. After 3.5 h incubation the recovery of bound c[^{3}H]AMP remained constant whereas a considerable loss was observed in the absence of catalytic subunit.

Table 1
Binding stoichiometry of c[^{3}H]AMP to the regulatory subunit under saturation conditions

Incubation time (h)	Additions	cAMP bound to R (mol/mol)
0.25	–	0.98
3.5	–	0.15
3.5	PMSF (0.1 mM); benzamidine (0.1 mM)	0.17
3.5	Mercaptoethanol (15 mM)	0.17
3.5	BSA (1 mg/ml)	1.95
3.5	Catalytic subunit (105 mM)	2.05
2.25	Catalytic subunit (105 mM) added after 2 h, second incubation for 15 min	1.90

Regulatory subunit (5.5 nM) was incubated with 2 μM c[^{3}H]AMP at room temperature

This loss is probably due to dissociation of the dimeric subunit into its monomers [17]. Proteolytic degradation or oxidation is unlikely since the addition of protease inhibitors or mercaptoethanol did not prevent the loss of binding sites (table 1). Addition of catalytic subunit after 2 h incubation can reverse the loss of bound c[^{3}H]AMP. BSA affords the same protection but a higher concentration (1 mg/ml) has to be used compared with the catalytic subunit (5 μg/ml). This indicates a rather specific effect of the catalytic subunit preventing structural changes in the regulatory subunit leading to a loss of bound cAMP.

3.2. *Dissociation of bound c[^{3}H]AMP on the nitrocellulose filter*

It was suggested [6] that nitrocellulose filters induce a conformational change in the regulatory subunit leading to a loss of bound cAMP. It was therefore interesting to investigate directly the influence of the nitrocellulose on the recovery of bound c[^{3}H]-AMP. Filters loaded with the complex R_2cAMP_2 or R_2cAMP_4 (fig.2) were incubated at 20°C for various times.

This incubation generally leads to a release of bound c[^{3}H]AMP. A slow drop in recovery is found at conditions where 1 site/monomer R-subunit is recovered. Under conditions where 2 sites/monomer are recovered (presence of 105 nM catalytic subunit) a rapid initial loss is observed. After 1 min both curves coincide indicating that all the bound cAMP of 1 site was released. About 50% of cAMP bound to the slow releasing site is recovered at that time. These results strongly suggest that upon contact with nitrocellulose, 1 specific site is affected; i.e., that under normal conditions (without stabilizing agents) 1 homogeneous population of sites is observed.

3.3. *Salt effect on the binding of c[^{3}H]AMP*

Titration of the cAMP binding site on the R-subunit in the presence of excess catalytic subunit results in a straight line in the Hill plot (fig.3). Both sites appear to have the same binding constant and the same cooperativity (K_D 45 nM, $n = 1.62$). The K_D is ~2-fold higher than that observed without excess C-subunit (K_D 20 nM). This effect is explained by a higher fraction of undissociated enzyme. The reduced affinity for

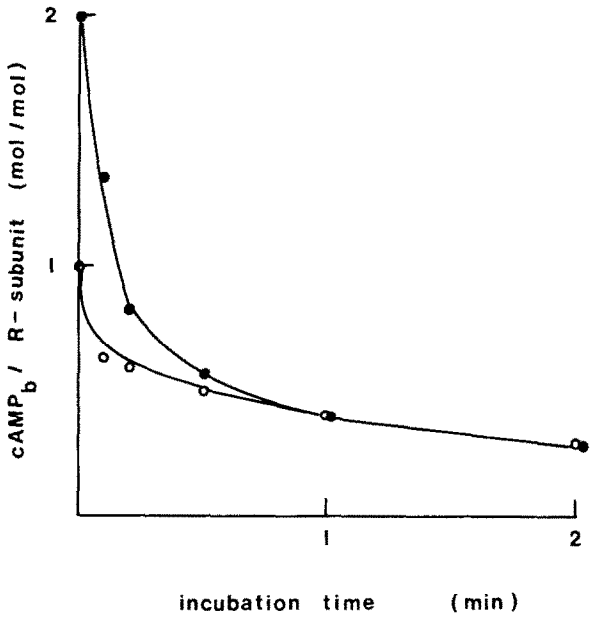

Fig.2. Loss of c[^{3}H]AMP recovery caused by raising the temperature on the nitrocellulose filter. Samples of holoenzyme (7.5 nM) were incubated with 2 μM c[^{3}H]AMP in the presence (•) and the absence (○) of 105 nM catalytic subunit. After 30 min incubation at room temperature the samples were cooled on ice for 30 min and subjected to nitrocellulose filtration. The filters were then incubated with 10 ml assay buffer at 20°C for different time periods. Incubation was stopped by quickly removing the buffer by vacuum filtration. Zero values were obtained without incubation at 20°C of the nitrocellulose filter.

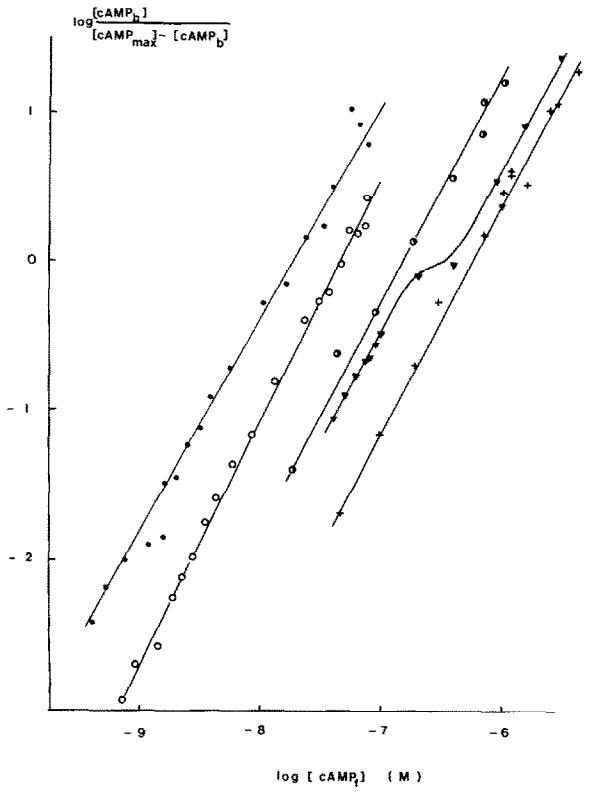

Fig.3. Binding of cAMP to the regulatory subunit in the presence of an excess of catalytic subunit and ATP. Data are presented in the form of Hill-plots. Holoenzyme in all the experiments was 7.5 nM: (•) holoenzyme; (○) holoenzyme, C-subunit (105 nM); (+) holoenzyme, C-subunit (105 nM), ATP (0.2 mM); (▾) holoenzyme, C-subunit (105 nM), ATP (0.2 mM), potassium phosphate (12 mM); (◑) holoenzyme, C-subunit (105 nM), ATP (0.2 mM), potassium phosphate (40 mM); incubation was for 4.5 h.

cAMP is more pronounced in the presence of ATP which is known to shift the equilibrium towards association to the tetramer [18]. In the presence of 105 nM catalytic subunit and ATP a straight line in the Hill plot was observed with K_D 550 nM and a Hill-coefficient of $n = 1.56$, indicating that under these conditions the 2 sites still cannot be distinguished. In contrast, in the presence of 10 mM potassium phosphate a clear biphasic binding is observed (K_{D1} 150 nM, K_{D2} 550 nM). The cooperativity apparently is not changed. Increasing the potassium phosphate concentration results again in a monophasic plot with K_D 145 nM. KCl had a similar effect, though a less clear biphasic character was observed and higher concentration (40 mM) had to be applied (not shown).

To decide which of the 2 sites is more sensitive towards potassium phosphate concentrations the following experiment was performed. cAMP sites were titrated in the holoenzyme in the presence of excess catalytic subunit, ATP, and 10 mM potassium phosphate as in fig.3. Parallel to the usual filtration at 0°C the filters loaded with the enzyme were incubated at 20°C for 40 s using the method in fig.2. This releases all c[^{3}H]AMP bound at the 'labile' site. 50% of the 'stable' sites are recovered under these conditions (see fig.2). The data plotted in fig.4 are corrected for this 50% loss. Fig.4 clearly shows that the 'stable' sites are first to be occupied. Saturation is achieved at ~1 μM c[^{3}H]AMP whereas >2 μM c[^{3}H]AMP is needed to saturate the 'labile' sites. These results suggest that the 'stable' sites are more sensitive to low potassium phosphate concentrations.

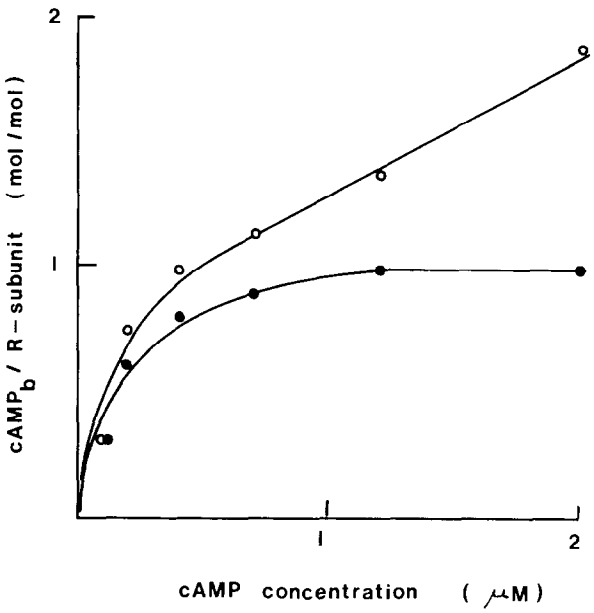

Fig.4. Different occupation of the 2 sites by c[^{3}H]AMP. Holoenzyme (7.5 nM) was incubated with catalytic subunit (105 nM), ATP (0.2 mM) and potassium phosphate (10 mM) at various concentrations of c[^{3}H]AMP: (○) total amount of bound c[^{3}H]AMP; (●) amount of bound c[^{3}H]AMP after 40 s incubation of enzyme on the nitrocellulose filter at 20°C. Data were corrected for a 50% loss of the 'stable' sites during this procedure.

4. Discussion

It was suggested [6] that nitrocellulose filters have a conformational effect on the regulatory subunit leading to a release of bound cAMP from one site. Modifications of the filter assay [3,7–9] using either addition of histone and high [NaCl] or precipitation by ammonium sulfate. Here, we describe the protection of bound cAMP by low concentrations of the catalytic subunit. This method allowed the investigation of the salt dependency of the 2 different binding sites. The direct effect of the nitrocellulose filter on the dissociation of the cAMP from the regulatory subunit was investigated by incubation at 20°C of the loaded filter. One site releases bound c[^{3}H]AMP rapidly. In [8] different exchange rates of bound c[^{3}H]AMP were demonstrated in the 2 different sites of type II regulatory subunit. The fast exchanging site was not observed using the unmodified filterassay, whereas the c[^{3}H]AMP bound to the slow exchanging site was completely recovered [8]. Together with our experiments it is now evident that using the unmodified assay 1 homogeneous population of cAMP binding sites is observed. This would allow us to map and identify this site specifically. Maps with analogues of cAMP have been obtained by the filterassay [12]. These studies have been performed using the unmodified filter assay. The results thus reflect the structural requirements of the 'stable' cAMP binding site. In [9], differences were described of the 2 sites regarding their nucleotide specificity. By comparing the maps of the binding sites with the requirements for the activation a possible role of the 2 distinct sites could be evaluated (R. J. W. de W. et al., unpublished).

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