PROTEIN KINASE II HAS TWO DISTINCT BINDING SITES FOR CYCLIC AMP, ONLY ONE OF WHICH IS DETECTABLE BY THE CONVENTIONAL MEMBRANE-FILTRATION METHOD

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1. Introduction

Cyclic AMP-dependent protein kinase (ATP: protein phosphotransferase, EC 2.7.1.37) is the only known mediator of cyclic AMP effects (key enzymes subject to phosphorylation by the kinase are reviewed in [1]). The enzyme exists in two forms (cAKI, cAKII) according to their order of elution on DEAE-cellulose [2]. Both forms are tetramers which upon activation by cyclic AMP dissociate to form one regulatory subunit dimer (R_2) and two free catalytic (C) subunits:

$$R_2C_2 \rightleftharpoons R_2 + 2C \qquad [3]$$

The regulatory moiety (RI) of cAKI differs from that (RII) of cAKII in M_r [4,5], primary structure [5] and in immunological properties [6]. RI has two kinetically distinct binding sites for cyclic AMP, present in the proportion 1:1 [7]. With the ammonium sulphate precipitation method [8,9] used here 2 of the 4 cyclic AMP molecules bound/RII dimer were found to exchange more rapidly. This study gives kinetic evidence that RII also has 2 distinct binding sites for cyclic AMP.

With the membrane filtration method [10–14] only 1 of the 2 types of binding sites of RII was detected. This suggests that reports of only 1 type of binding site associated with RII [13] or RI [14] may be due to the selective loss of cyclic AMP bound to one of the binding sites during membrane filtration.

2. Materials and methods

Cyclic [^{3}H]AMP (45 Ci/mmol) was from the Radiochemical Centre, Amersham. Adenosine 3′:5′-cyclic monophosphate agarose (cyclic AMP attached through the N^6-amino group by an 8C-spacer; coupling density 2.6 μmol cyclic AMP/ml gel) was from Sigma. Other reagents were from the sources in [15].

Buffer A is 15 mM Hepes–NaOH (pH 7.0) 0.3 mM EGTA, 0.1 mM EDTA, 0.5 mM dithiothreitol and 20 mM 2-mercaptoethanol.

Buffer B is 50 mM Hepes–NaOH (pH 7.0), 3.2 M NaCl, 30 mM EDTA containing the following factors known to stabilize the binding protein [9,15]: 20 mM 2-mercaptoethanol, 0.5 mg/ml of bovine serum albumin and 0.15 mg/ml of a heat-stable protein fraction.

2.1. *Preparation of cAKII*

Bovine cardiac muscle was homogenized, subjected to precipitation with polyethylene glycol (PEG) and applied to DEAE-cellulose as described for the preparation of cAKI [15]. After DEAE-cellulose chromatography the peak fractions of cAKII were pooled and passed through a column of carboxymethyl cellulose (CM-52) equilibrated with buffer A. The preparation so obtained is referred to as partially-purified cAKII. Partially-purified cAKII was rechromatographed on DEAE-cellulose at pH 8.5, the peak fractions precipitated with 1.9 M (50% satd.) $(NH_4)_2\ SO_4$, the precipitate resuspended in 4 ml buffer A and the resulting slurry briefly dialyzed against the same buffer to completely dissolve the precipitate. The preparation was next chromatographed on a column (2.6 × 90 cm) packed with Ultrogel ACA-34 (LKB) equilibrated with buffer A. The first of the peak fractions of cAKII emerging were concentrated by precipitation with 22% (w/v) PEG 6000 and subjected to glycerol (10–20%, w/v) gradient centrifugation. The final prepara-

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tion was >85% pure when tested by polyacrylamide gel electrophoresis under non-denaturing conditions [16] whether the acrylamide concentration was 5%, 7% or 9%. When 10 μM cyclic AMP was included in the electrophoresis buffer no band was visible at the position corresponding to the major band observed in the absence of this nucleotide.

2.2. *Preparation of RII by affinity chromatography*

This was essentially as in [17]. 35 ml of partially purified cAKII (~40 mg protein) in buffer A was applied (8 ml/h) to the cyclic AMP affinity column (0.7 × 4 cm) at 4°C. After adsorbtion of RII the column was washed with 4 vol. buffer A, and then with 4 vol. same buffer containing 2 M NaCl and 0.5 mM AMP. RII was eluted at 23°C with either buffer A containing 10 M urea and 25 mM methylamine or buffer A containing 20 mM cyclic AMP. SDS gel electrophoresis according to [18] of the protein eluted with 20 mM cyclic AMP revealed one major Coomassie blue-stained band with a mobility corresponding to M_r 55 000. The gels (5–25 μg protein applied) were scanned at 595 nm and the areas under the peaks determined with a MOP-AMO3 Kontron morphometric analyzer. The main band (RII) was in this manner estimated to be ~90% pure. In both preparations (elution with urea or cyclic AMP) RII existed as a dimer since it eluted as a single sharp peak with an estimated Stoke's radius = 5.2 nm by high-performance gel permeation chromatography on a column (0.75 × 60 cm × 63 000 SW from Toyo Soda) equilibrated with buffer A containing 150 mM KCl.

2.3. *Preparation of RI*

cAKI holoenzyme (judged to be 80–90% pure by the same criteria as used for cAKII) was prepared as in [7]. RI was separated from the catalytic subunit and other proteins contaminating cAKI by DEAE-cellulose chromatography in the presence of 0.1 μM cyclic [^{3}H]AMP [7].

2.4. *Estimation of protein-bound cyclic [^{3}H]AMP*

2.4.1. By ammonium-sulphate precipitation

This was essentially as in [9,15]. Aliquots were removed after different periods and precipitated in 10–30 vol. ice-cold 80% satd. ammonium sulphate. The bound cyclic [^{3}H]AMP was collected by membrane filtration, eluted from the filters with aq. 2% (w/v) SDS and scintillation fluid added.

2.4.2. By conventional membrane-filtration

This was essentially as in [12]; 50 μl aliquots were mixed with 6 ml ice-cold buffer (25 mM Tris–HCl (pH 7.0) with 10 mM $MgCl_2$ and 20 mM 2-mercaptoethanol). The sample was then immediately passed through membrane-filters (HAWP; 0.45 μm pore size, 25 mm diam. from Millipore Corp.) and washed with 3 × 6 ml of ice-cold Tris-buffer. Elution of retained cyclic [^{3}H]AMP and scintillation counting were as for the ammonium sulphate precipitation method.

2.5. *Determination of the stoichiometry of cyclic AMP binding to RI and RII*

The amount of cyclic [^{3}H]AMP in the RI nucleotide complex was determined by the ammonium sulphate precipitation method, and the amount of RI by amino acid analysis [19]. The preparation of RII eluted from the cyclic AMP affinity column with cyclic AMP was quickly desalted on a Sephadex G-25 column equilibrated with 15 mM Hepes–NaOH (pH 7.0). The amount of bound cyclic AMP was determined by precipitating aliquots in ammonium sulphate, collecting the precipitates on membrane filters, eluting the bound cyclic AMP by boiling the filters in 1 ml buffer A, and measuring released cyclic AMP by a competitive protein binding assay [15]. The protein content of the desalted RII was determined by the method in [20] with bovine serum albumin as the standard. The protein content of the standard had been pre-checked by amino acid analysis and was a gift from Professor T. Flatmark. For the estimation of the stoichiometry of cyclic AMP binding, the preparation of RI was considered 100% pure and that of RII 90% pure. Subunit M_r = 55 000 and 49 000 were assumed for RII and RI, respectively [3–5].

3. Results

3.1. *On the reliability of measurement of cyclic AMP bound to RI or RII by ammonium sulphate precipitation*

The stoichiometry of cyclic AMP binding to RI or RII was calculated to be 2.0 mol cyclic AMP/subunit using the ammonium sulphate precipitation method. This stoichiometry agrees with results from methods other than membrane filtration (equilibrium dialysis and fluorescence quenching of 1,N^6-etheno-cyclic AMP) [21–23]. This result was obtained in the absence of high concentrations of NaCl or basic proteins

confirming our finding that the ammonium sulphate precipitation method allows the quantitative recovery of cyclic AMP bound to RI independently of the presence of added proteins or salts [9]. Furthermore, 90–100% of the cyclic [^{3}H]AMP incubated with an excess of RI or RII was recovered in the ammonium sulphate precipitate, whether this was collected by membrane filtration or centrifugation. This latter observation supports our proposal that the high degree of retention of bound cyclic AMP on membrane filters with ammonium sulphate is due to formation of precipitates [9], rather than to an altered interaction of R with the filter matrix as proposed in [23].

3.2. *Dissociation rate experiments*

The RII eluted from the affinity column with urea was desalted by Sephadex G-25 chromatography and saturated with cyclic [^{3}H]AMP (0.15 μM for 2 h at 4°C). The exchange reaction was started by adding unlabelled cyclic AMP to 0.1 mM final conc. The experiments were carried out at 0°C (fig.1) and 37°C (fig.2) in buffer B. With the ammonium sulphate precipitation method the rate of dissociation of the cyclic [^{3}H]AMP–RII complex was biphasic at either 0°C (fig.1) or 37°C (fig.2). Half of the bound nucleotide showed a rapid dissociation (A) and the other half a slower dissociation (B). With the membrane-filtration method only one phase of dissociation showing first-order kinetics was observed (fig.2). This phase corresponded to the slowest phase (B) observed with the ammonium-sulphate precipitation method. The k_d for phase A was 0.4×10^{-3} s^{-1} at 0°C and 17×10^{-3} s^{-1} at 37°C. For phase B it was 0.02×10^{-3} s^{-1} at 0°C and 3.4×10^{-3} s^{-1} at 37°C. The main difference from the data presented for RI (7) is that dissociation from site B is more rapid from RII than from RI.

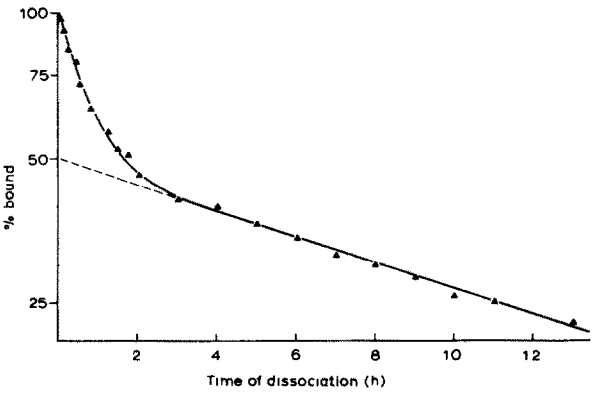

Fig.1. Dissociation rate at 0°C. RII (10 nM) saturated with cyclic [^{3}H]AMP was incubated in buffer B with 0.1 mM unlabelled cyclic AMP. Aliquots of 50 μl were removed at the time points indicated and the amount of bound cyclic AMP determined by the ammonium sulphate precipitation method.

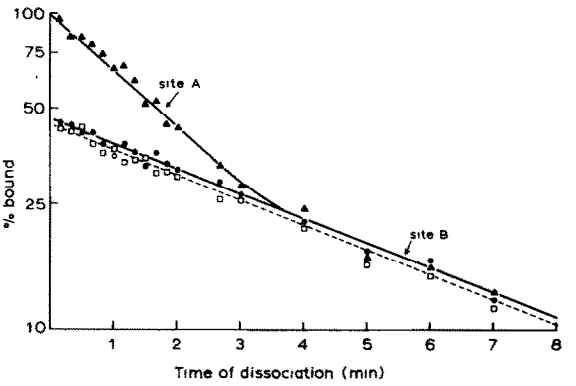

Fig.2. Dissociation rate at 37°C. Comparison of the membrane filtration and ammonium sulphate precipitation methods. RII (10 nM) was incubated in buffer B with 0.1 mM unlabelled cyclic AMP. Aliquots of 100 μl and 50 μl were removed at the time points indicated on the abscissa and mixed with 4 vol. ice-cold Tris-buffer, respectively. The sample in Tris-buffer was immediately passed through Millipore-filters (□). One portion of the sample in buffer B was immediately precipitated with 80% satd. ammonium sulphate (△) to determine bound nucleotide, whereas the remaining portion was left at 0°C and aliquots removed 3.5 h, 6 h and 16 h later for precipitation with ammonium sulphate. As seen in fig.1 essentially no cyclic [^{3}H]AMP remains bound to site A after 3.5–16 h in buffer B at 0°C. Extrapolation of the amount of cyclic [^{3}H]AMP bound (to site B) after 3.5 h, 6 h and 16 h at 0°C to $t = 0$ gives a selective measure of the amount of cyclic [^{3}H]AMP bound to site B (○).

Results similar to those in fig.1,2 were observed whether the binding protein was:

(i) RII eluted from the cyclic AMP affinity column with cyclic AMP and whose bound cyclic AMP had been exchanged with cyclic [^{3}H]AMP;
(ii) Highly purified cAKII;
(iii) Highly purified cAKII in its autophosphorylated form.

The completeness of the autophosphorylation was checked by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP [24]. The dissociation of cyclic AMP from RII derived from autophosphorylated cAKII was not accompanied by any loss of the ^{32}P associated with RII. Two phases of exchange of bound cyclic [^{3}H]AMP were observed also in buffer A in the absence of added salt and with 150 mM KCl. Under

those conditions a retardation of the dissociation in phase A and an acceleration of the dissociation in phase B was observed, underscoring the homology between the binding sites in RII and RI [7].

4. Discussion

Relatively few studies exist on the interaction between cyclic AMP and the regulatory moieties (RI, RII) of cyclic AMP-dependent protein kinases (cAKI, cAKII). In most of those studies protein-bound cyclic AMP has been estimated by relying on quantitative retention of bound nucleotide on membrane filters [11,13,14]. This method has been shown to underestimate the amount of cyclic AMP bound to cAK [9,21–23].

Here, using an ammonium sulphate precipitation assay, a biphasic curve for the dissociation of cyclic AMP bound to RII was observed (fig.1,2) suggesting that RII, like RI [7], has two populations (A,B) of binding sites for cyclic AMP present in the proportion 1:1. With the membrane-filtration method binding to only one of the two populations of binding sites (B) was detected (fig.2).

Using the membrane-filtration assay [13] a monophasic dissociation rate was found for the complex of RII and cyclic [^{3}H]AMP with k_d of 2.4×10^{-3} s^{-1} at 33°C which compares with the value (3.4×10^{-3} s^{-1} at 37°C) found in the present study for site B. About 1 molecule of cyclic AMP bound/RII subunit was found. The apparent discrepancies between [13] and this study may thus be due to the selective loss of cyclic AMP bound to site A during membrane-filtration in that study. We believe that interaction between RII and the matrix of the membrane filter results in the liberation of cyclic AMP bound to site A. Interaction of R with the filter may alter the conformation of R leading to release of cyclic AMP bound to site A. High concentrations of NaCl ([7] and section 3) and chaotropic agents (unpublished) accelerate the dissociation of cyclic AMP preferentially from site A suggesting that R can take on conformations that lead to preferential loss of cyclic AMP from one of the binding sites. This interpretation is supported by the demonstration that membrane filters can remove half of the cyclic AMP bound to R [22].

The estimation of bound cyclic AMP by the ammonium sulphate precipitation method is unaffected by the presence of a number of proteins [9]. Using membrane filtration [25] it was noted, however, that protamine apparently doubled the amount of cyclic AMP bound to cAK. It was unclear whether protamine increased the binding capacity of cAK or enhanced the retention of bound cyclic AMP on the filters. The latter mechanism is more likely since protamine failed to enhance cyclic AMP binding to dissociated cAKI when the ammonium sulphate precipitation assay was used [26]. In [21] 2 mol rather than 1 mol cyclic AMP/RII subunit could be recovered on membrane filters when histone (and NaCl) had been included in the binding assay [21]. Also cyclic AMP bound to cyclic AMP/adenosine-binding proteins from erythrocytes [27], and plant tissue [28] is more efficiently retained on membrane filters when basic proteins are present. This suggests that the enhanced retention observed with basic proteins is non-specific and possibly related to the formation of aggregates between the acidic R and basic proteins. Conditions or agents that destabilize such aggregates (low pH, high ionic strength, chaotropic agents, urea, detergents, high concentrations of acidic proteins competing with R for the formation of aggregates with basic proteins) may interfere with membrane filtration methods using basic proteins. The modified membrane filtration method in [21], using histone and NaCl, does not allow the study of the interaction between cyclic AMP and R at physiological ionic strength or the interaction between the nucleotide and cAK holoenzymes, since the binding of cyclic AMP to the latter is strongly affected by NaCl and histone [9,24,26]. Another advantage of the ammonium sulphate precipitation method as compared to the modified membrane filtration method is that the cyclic AMP–R complex is stable for several hours in ammonium sulphate [9]. We have investigated this phenomenon further and found that the antichaotropic sulphate ion, even at concentrations (0.5–1 M) too low to precipitate R, retards the rate of dissociation of the cyclic AMP–R complex by a factor of ~10.

The finding of two populations of binding sites (A,B) associated with RII here did not seem to result from various degrees of autophosphorylation of RII (see section 3). The fact that both nearly homogeneous RII (isolated by affinity chromatography) and cAKII (isolated by conventional procedures for protein purification) had site A and B in the proportion 1:1 makes it highly unlikely that any one of the binding sites might be associated with a protein contaminating the preparation of RII or cAKII. Furthermore,

experiments on the interaction between cyclic AMP and free RII or cAKII holoenzyme have shown site A–B interactions, suggesting that both binding sites reside in the same macromolecular complex (unpublished). These data are compatible with either a symmetry model of the RII dimer where each subunit has 1 A site and 1 B site, or an asymmetric model in which 1 subunit has 2 A sites and the other 2 B sites.

Each RI subunit may have 1 A and 1 B site based on the fact that interaction between A and B sites is tighter than between similar sites [29]. Experiments in which the monomeric form of RI (isolated by gel chromatography after limited proteolysis of the dimer with trypsin) showed interaction between site A and B, suggest that, at least for RI, the 2 types of sites are located on the same subunit (in preparation).

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Addendum

After this paper was submitted Rannels and Corbin (J. Biol. Chem. (1980) 255, 7085) using a modified membrane filtration assay, have reported a biphasic rate of exchange of bound cyclic AMP from RI and RII. In their hands, however, cyclic AMP dissociated more rapidly from site B of RII than site B of RI. The authors did not address the question of whether any of the two binding sites preferentially escapes detection by conventional membrane filtration.

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