THE KINETICS OF THE INTERACTION BETWEEN CYCLIC AMP AND THE REGULATORY MOIETY OF PROTEIN KINASE II

Evidence for interaction between the binding sites for cyclic AMP

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1. Introduction

The regulatory moiety (RII) of cyclic AMP-dependent protein kinase II (cAKII) has 2 binding sites for cyclic AMP/subunit. One of the binding sites (A) exchanges its bound cyclic AMP more rapidly than the other one (B) [1,2]. By following the time course of exchange of cyclic [^{3}H]AMP from the RII–cyclic [^{3}H]AMP complex the relative distribution of bound cyclic [^{3}H]AMP between site A and B can be determined. This technique has allowed us to show that cyclic [^{3}H]AMP binds in an ordered sequence, i.e., binding to site B preceeds binding to site A, to cAKII holoenzyme [3].

Here, this technique was used to determine separately the time course of cyclic AMP accumulation to site A and B of free RII. In addition the rate of dissociation of the RII–cyclic [^{3}H]AMP complex has been determined under exchange (excess unlabelled cyclic AMP) and non-exchange conditions. The results to be presented strongly indicate that binding of cyclic AMP to site A of RII affects both the rate of cyclic AMP association to and the rate of dissociation from site B of RII.

2. Experimental

Cyclic [5′,8-^{3}H]AMP (45 Ci/mmol) was from The Radiochemical Centre, Amersham. Active charcoal was from Merck, Damstadt. The charcoal was treated as in [4], washed repeatedly in 15 mM Hepes–NaOH (pH 7.0), 0.3 mM EGTA, 0.1 mM EDTA, 0.5 mM dithiothreitol, 20 mM 2-mercaptoethanol, 0.5 mg bovine serum albumin/ml, 0.3 mg heat-stable protein/ml (buffer A), and finally in 50 mM Hepes–NaOH (pH 7.0), 3.2 M NaCl, 30 mM EDTA, 20 mM 2-mercaptoethanol (buffer B) containing 10 mg albumin/ml and 10 mg heat-stable protein/ml. The latter was prepared from muscle (bovine heart or rabbit skeketal muscle) as in [5], and serves to protect RI against inactivation [5]. Other materials were from sources described in [2,5].

RII was prepared by cyclic AMP–affinity chromatography of partially purified cAKII using elution with urea [2]. The procedures for autophosphorylation of cAKII and preparation of the catalytic subunit of cAKII are described in [3].

2.1. *Association rate studies*

For these RII in 0.1 ml buffer A was mixed with cyclic [^{3}H]AMP (in 0.1 ml buffer A which sometimes contained 0.3 or 1.6 M of KCl or NaCl, or 0.8 M Na_2SO_4 or $(NH_4)_2SO_4$). The reaction was quenched by the addition of 0.8 ml ice-cold buffer B with 0.13 mM unlabelled cyclic AMP. Aliquots (0.17 ml) were removed 0, 3.5, 6 and 16 h after the addition of the quench-solution, mixed with 4 ml 90% satd. ammonium-sulphate and bound cyclic [^{3}H]AMP measured [2,5]. As explained in [3] cyclic [^{3}H]AMP associated with site A of RII exchanges more rapidly ($t_{1/2}$ = 0.5 h) than that associated with site B ($t_{1/2}$ = 11 h) in the quench-solution at 0°C. Thus, <2% of the cyclic [^{3}H]AMP initially bound to site A remains

Abbreviations: cAKII, cyclic AMP-dependent protein kinase, type II; RII, the regulatory moiety of cAKII; k_a, the association rate constant for the interaction between cyclic AMP and RII; k_d, the dissociation rate constant for the dissociation of the complex of cyclic AMP and RII

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bound after 3.5 h in the quench-solution.

By plotting the logarithm of the values found for bound cyclic [^{3}H]AMP after 3.5, 6 and 16 h of exchange *vs* time, and extrapolating to zero time, the amount of cyclic [^{3}H]AMP bound to site B at the moment of quenching could be determined. The amount of nucleotide associated with site A was calculated as total nucleotide bound (site A + site B) minus nucleotide bound to site B.

2.2. *Dissociation rate studies*

For these studies RII saturated with cyclic [^{3}H]-AMP (by prior incubation of 0.1 μM RII binding sites with 0.2 μM cyclic [^{3}H]AMP) was incubated in buffer B. Rebinding of isotope dissociated from the RII–cyclic [^{3}H]AMP complex was prevented by conducting the experiments at very low concentration of RII (0.03 nM) and cyclic [^{3}H]AMP (0.06 nM) or by including active charcoal (10 mg/ml) or unlabelled cyclic AMP (0.1 mM) in the incubations. In the presence of charcoal and especially at >30°C, RII was unstable unless serum albumin (5 mg/ml) and heat-stable protein (2 mg/ml) were present. These proteins were therefore included in all dissociation rate experiments.

The amount of cyclic [^{3}H]AMP bound to site A and B was determined in samples removed after various periods of dissociation of the RII–cyclic [^{3}H]-AMP complex. For the incubations without charcoal the discrimination between cyclic [^{3}H]AMP bound to site A and B was as described above for the association rate experiments, except that the samples were mixed with 9 vol. quench-solution precooled to –5°C. For the samples containing charcoal aliquots could be removed for determination of bound cyclic [^{3}H]-AMP only after the charcoal had been sedimented (13 000 × *g* for 3 min at 0°C).

3. Results

3.1. *The association of cyclic [^{3}H]AMP and free RII*

In the initial phase the cyclic AMP accumulation to site A and site B of RII proceeded linearly (fig.1). The rate of association in the initial phase was the same for all concentrations of RII (1–100 nM) or cyclic [^{3}H]AMP (6–600 nM) tested. The association rate constant (k_a), calculated as explained in [3], was 4×10^4 $M^{-1} . s^{-1}$ for the initial binding to site A and 1.5×10^4 $M^{-1} . s^{-1}$ for site B.

The k_a for the association of cyclic AMP to site A decreased to a similar extent in the presence of SO_4^{2-} (0.4 M Na_2SO_4 or 0.4 M $(NH_4)_2SO_4$) and Cl^- (0.8 M NaCl or 0.8 M KCl). The k_a for the binding to site B, however, decreased much more in the presence of Cl^- than SO_4^{2-} (not shown).

In all experiments the accumulation of cyclic AMP to site B showed a transition to a slower rate (fig.1). In the presence of 0.4 M SO_4^{2-} this transition occurred at a higher degree of occupancy of site B than in the

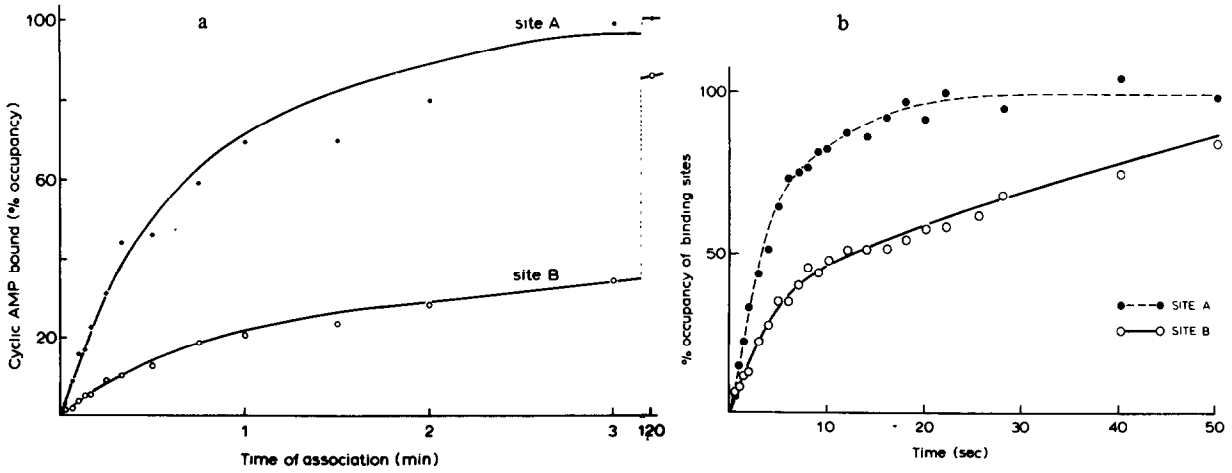

Fig.1. Rate of association of cyclic [^{3}H]AMP and RII. Cyclic [^{3}H]AMP and RII were allowed to react at 0°C in 0.15 M KCl for the periods of time indicated on the abscissa, and the amount of nucleotide bound to site A (•) and site B (○) determined as in section 2: (a) cyclic [^{3}H]AMP was 30 nM and RII 2 nM; (b) cyclic [^{3}H]AMP was 0.6 μM and RII 40 nM.

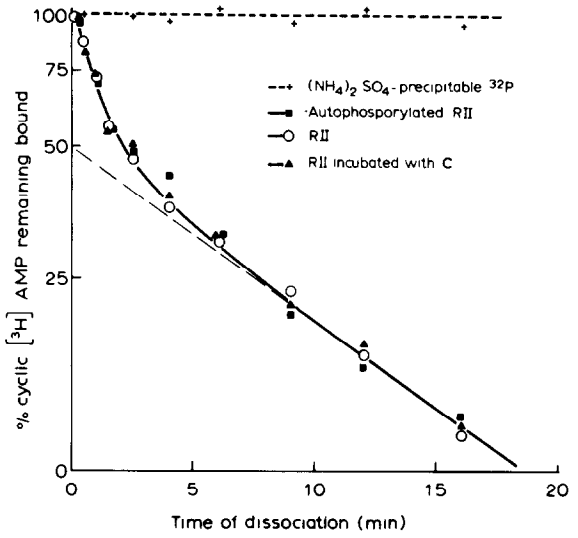

Fig.2. Rate of exchange of cyclic [^{3}H]AMP bound to RII. The cyclic [^{3}H]AMP–RII complex (10 nM) was incubated at 30°C in buffer B containing 0.1 mM unlabelled cyclic AMP, albumin and heat-stable protein. Samples were removed after various time periods and precipitated in $(NH_4)_2SO_4$ for the determination of the amount of cyclic [^{3}H]AMP remaining bound. In one case the source of RII was cAKII that had been autophosphorylated with [γ-^{32}P]ATP [3]. In that case the amount of ammonium-sulphate precipitable ^{32}P as well as of cyclic [^{3}H]AMP was determined. In another case 20 nM of the catalytic subunit of cAKII was present.

experiment of fig.1, and in 0.8 M Cl^- the transition occurred at a lower degree of occupancy of site B. In all cases, however, the transition occurred when site A was about half-saturated with cyclic AMP.

3.2. *Kinetics of the dissociation of the RII–cyclic [^{3}H]AMP complex*

The time course of dissociation of the complex was studied in the presence of excess unlabelled cyclic AMP in buffer B at 30°C (fig.2). The rapid phase of exchange (from site A) had a dissociation rate constant (k_d) = 13 × 10^{-3} s^{-1} and the slow phase (from site B) had a k_d = 1.6 × 10^{-3} s^{-1}. An Arrhenius-plot of these data as well as those obtained at 0°C and 37°C [2] allowed the activation energies for the dissociation of the complex of cyclic AMP and site A (77 kJ/mol, 1 J = 0.24 cal) and site B (98 kJ/mol) to be estimated. The dissociation of cyclic [^{3}H]AMP from either site was unaffected by the presence of the catalytic subunit of cAKII, and was the same whether RII had been autophosphorylated or not (fig.2).

Under conditions where rebinding of dissociated cyclic [^{3}H]AMP was prevented by working with very low concentrations of RII and cyclic [^{3}H]AMP or in the presence of charcoal, only ~10% of the cyclic [^{3}H]AMP initially associated with site A remained bound after 2 min at 30°C (not shown). The dissociation from site B proceeded with a lag of ~2 min under these conditions and thereafter showed first order kinetics, the rate being ~5-times higher than when site A was saturated (excess unlabelled cyclic AMP present) (fig.3).

The difference in apparent dissociation rate in the absence and presence of unlabelled cyclic AMP might be due to a lower stability of RII when not saturated [6,7]. This was not the case, however, since >80% of the cyclic AMP binding capacity was recovered after 20 min incubation at 30°C in the absence or presence of charcoal under the conditions of the experiment of fig.3. To ensure that the measures taken to prevent rebinding of cyclic [^{3}H]AMP were adequate, 0.6 nM free RII was added to the incubations in experiments like those shown in fig.3. For incubations containing charcoal or unlabelled cyclic AMP the dissociation curves were identical whether RII had been added or not. The additional binding observed in the absence of those agents was too slight to affect the estimation of k_d. The time course of dissociation was the same when the concentration of charcoal was increased to 20 mg/ml.

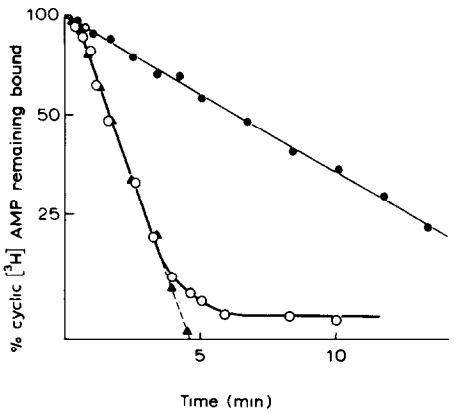

Fig.3. The rate of dissociation of cyclic [^{3}H]AMP from its complex with site B of RII. The cyclic [^{3}H]AMP–site B complex (0.6 nM) was allowed to dissociate in the presence of 0.1 mM unlabelled cyclic AMP (•), 10 mg active charcoal/ml (▲) or (0.015 nM complex) in the absence of those agents (○).

4. Discussion

The regulatory moiety (RII) of cyclic AMP-dependent protein kinase II (cAKII) has 2 types of binding sites (A and B). Cyclic [^{3}H]AMP exchanges more rapidly with unlabelled nucleotide when bound to site A than when bound to site B [1,2], fig.2).

This study has shown that cyclic AMP associates more rapidly to site A than to site B of RII (fig.1). Thus, site A differs from site B by having higher rate constants for the association with cyclic AMP as well as for the dissociation of bound nucleotide. The pattern of cyclic AMP association to cAKII [3] was very different from that to free RII. The method used to discriminate binding to site A and B (rate of exchange of bound cyclic [^{3}H]AMP with excess unlabelled cyclic AMP in 3.2 M NaCl at 0°C, conditions that ensure complete dissociation of cAKII into free RII and C) was identical for the experiments with RII and cAKII. The observed difference must thus mean that the cyclic AMP binding domains have different configurations in RII and cAKII. This difference is especially marked for site A whose rate of cyclic AMP binding in cAKII is much slower than in free RII. This lowering of the k_a for cyclic AMP binding to site A of cAKII may be explained if the area of contact between RII and C overlaps with the A-site of RII, or if the interaction of RII and C at a site distant from site A alters the conformation of RII at site A.

The initial rate of accumulation of cyclic AMP to site A or site B of RII was independent of the concentration of cyclic AMP. A sharply reduced rate of accumulation to site B occurred, however, when ~50% of site A had been saturated with cyclic AMP (fig.1). This rate transition was observed under several experimental conditions and makes us suggest that site B exists in a state with a higher k_a when site A of the subunit is vacant than when site A is occupied by cyclic AMP.

An inverse relationship between the k_a and the concentration of cyclic AMP was noted in [7]. The discrepancy between [7] and this study is presumably due to their k_a-values being based on the accumulation of cyclic AMP occurring after incubating for $\geq$1 min and longer, and that their method (membrane filtration) allowed only cyclic AMP bound to site B to be detected [2]. Thus, as the concentration of cyclic AMP was raised more of site A became occupied and more of site B was converted from the 'high k_a state' to the 'low k_a state'.

As discussed in [8] information about site–site interaction in multisite complexes can be gained by comparing the rate of dissociation of bound labelled ligand under conditions when a site that has lost its ligand remains empty (no rebinding) and when the site is rapidly filled by unlabelled ligand (exchange conditions). The rate of dissociation of cyclic [^{3}H]-AMP from the two B-sites of RII was several times more rapid under conditions of no rebinding than under exchange conditions (fig.3). For a protein with ligand bound at 2 similar sites the overall dissociation rate can at most be 2 times higher under conditions of no rebinding than under exchange conditions. This follows from the fact that the first molecule of ligand leaving the protein will always leave a saturated complex and therefore will dissociate at the same rate whether unlabelled ligand is present or not. Since the preparation of RII used in the experiment of fig.3 was saturated with cyclic [^{3}H]AMP at the onset of the dissociation, and the nucleotide dissociated 5-times quicker from site B under conditions of no rebinding than under exchange conditions (fig.3), it follows from the above that site B–B interactions alone are insufficient to explain the data. We therefore suggest that mainly site A–B interactions are responsible for the observed data, i.e., the cyclic AMP–site B complex exists in a 'low k_d state' when site A is occupied by cyclic AMP and in a 'high k_d state' when site A is vacant. This interpretation is supported by the observation that the dissociation from site B under conditions of no rebinding proceeded with a lag (fig.3) corresponding to the time needed for cyclic AMP to dissociate from site A.

In contrast to these results in [9] it was reported that the cyclic [^{3}H]AMP–RII complex dissociated with the same rate whether unlabelled cyclic AMP was present or not. This discrepancy is not easily explained. However, additional evidence that the binding sites of RII do indeed interact comes from recently conducted unpublished experiments, in which the rate of dissociation of the cyclic [^{3}H]-AMP–RII complex was found to depend on the type of the unlabelled ligand present. Thus, the rate of dissociation of the complex was more rapid in the presence of cyclic GMP or cyclic IMP than in the presence of cyclic AMP itself.

This study has shown that cyclic AMP binds more rapidly to site A than site B of free RII. Interactions between site A and site B have been revealed. Thus, binding of cyclic AMP to site A affects the vacant

site B (lowered k_a) as well as the cyclic AMP–site B complex (lowered k_d).

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