THE KINETICS OF ASSOCIATION OF CYCLIC AMP TO THE TWO TYPES OF BINDING SITES ASSOCIATED WITH PROTEIN KINASE II FROM BOVINE MYOCARDIUM

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1. Introduction

Cyclic AMP-dependent protein kinase type I (cAKI) and type II (cAKII) are tetramers composed of 2 regulatory (R) and 2 catalytic (C) subunits. R has 2 types of binding sites for cyclic AMP, one of which (A) exchanges its bound cyclic AMP more rapidly than the other one (B) [1–3]. Cyclic AMP activates the enzyme by promoting its dissociation into a regulatory subunit dimer and 2 catalytically active subunits (reviews [4,5]):

$$R_2C_2 + 4 \text{ cyclic AMP} \rightleftarrows R_2 \text{ cyclic AMP}_4 + 2\ C$$

The details of the above reaction are unknown, and only recently has enough evidence accumulated to decide that cyclic AMP may activate the enzyme by binding directly to R in the holoenzyme rather than to free R only [5–8].

This study was undertaken to find if the 2 types of binding sites (A en B) that in free RII show different kinetics in their interaction with cyclic AMP [3,9] also differ functionally in the cAKII holoenzyme. For this, the time course of the association between cyclic [^{3}H]AMP and site A and B of cAKII holoenzyme was monitored. The rate of association was measured in the physiologically relevant range (20 nM–2 μM) of cyclic AMP concentrations.

These data suggest that the site of type A can interact with cyclic AMP only after the B-site has been occupied by the nucleotide.

2. Experimental

Adenosine 5′-[γ-^{32}P]triphosphate (~2 Ci/mmol) was from the Radiochemical Centre, Amersham. Urea (analytical grade) was from Merck, and methylamine from Sigma. Other reagents were from the sources in [3,9]. Buffer A is 15 mM Hepes–NaOH (pH 7.0) containing 0.3 mM EGTA, 0.1 mM EDTA and 20 mM 2-mercaptoethanol.

Buffer B is 62.5 mM Hepes–NaOH (pH 7.0) containing 4 M NaCl, 37.5 mM EDTA, 0.5 mM dithiothreitol, 20 mM 2-mercaptoethanol, 0.5 mg bovine serum albumin/ml and 0.2 mg heat-stable protein fraction/ml [9].

2.1. *Preparation of cAKII and RII*

This was as described in [3]. RII was eluted from the affinity column with urea.

2.2. *Preparation of C*

The catalytic subunit (C) was prepared from bovine heart cAKII purified through the carboxymethylcellulose step [3]: 20 ml of cAKII (0.2 μM with respect to cyclic AMP binding capacity) was dissociated by incubation with 0.1 mM cyclic AMP and 0.2 mM 3-isobutyl-1-methylxanthine for 30 min at 0°C, passed through a carboxymethylcellulose column (0.9 × 2 cm) equilibrated with buffer A and the column washed with 10 ml buffer A. The catalytic subunit was then eluted with 4 ml buffer A containing 250 mM KCl into a tube containing 2 mg albumin in 0.1 ml. The concentration of C-subunit was determined by comparing its catalytic efficiency in the phosphotransferase assay [10] with that of preparations of cAKI and cAKII of known concentrations.

2.3. *cAKII autophosphorylation*

cAKII (0.2 μM) was incubated in buffer B with 0.5 mg bovine serum albumin/ml, 10 mM Mg $(CH_3OO)_2$ and 10 μM [γ-^{32}P]ATP (1 μCi/ml) for 30 min at 0°C. About 1.4 mol ^{32}P was incorporated into trichloroacetic acid-precipitable or ammonium

sulphate-precipitable protein under those conditions. No loss of acid-precipitable or $(NH_4)_2SO_4$-precipitable ^{32}P occurred under the conditions of the kinetic experiments in which the autophosphorylated enzyme was used.

2.4. *Association rate studies: Differentiation of cyclic [^{3}H]AMP bound to site A and B*

Samples (0.1 ml) of cAKII in buffer A with 0.15 M KCl were placed in the bottom of plastic tubes. The reaction was started by squirting 0.1 ml cyclic [^{3}H]AMP in the same buffer into the tube. 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 quench-solution, and the amount of bound isotope determined in the precipitate formed by mixing the aliquot with 4 ml 90% satd. ammonium sulphate [3]. Since the cyclic [^{3}H]AMP associated with site A of RII exchanges with a half-time of 30 min under these conditions <2% of the labelled nucleotide initially associated with this site remains bound after 3.5 h in the quenchsolution [3]. 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.

2.5. *Plotting of kinetic data*

The association rate constant (k_a) for the interaction between cyclic AMP and site A and B was calculated essentially as in [11]. For site A:

$$k_a \cdot t = \frac{1}{[A]_o - ([L]_o - [B \cdot L]_t)}$$

$$\ln \frac{([L]_o - [B \cdot L]_t) \cdot ([A]_o - [A \cdot L]_t)}{[A]_o \cdot ([L]_o - [B \cdot L]_t - [A \cdot L]_t)}$$

For the calculation of k_a for site B, A and B should be interchanged in all the terms above: $[A]_o$, total concentration of cyclic AMP binding sites of type A; $[L]_o$, total concentration of cyclic AMP; $[B \cdot L]_t$ and $[A \cdot L]_t$, concentration of cyclic AMP complexed to site B and A, respectively, after a certain time of reaction.

3. Results

3.1. *On the reliability of the methods used to study the rate of association to site A and B of cAKII*

To have an idea of the precision obtained with the manual mixing procedure six parallel determinations of bound cyclic AMP were performed 'immediately' after the association reaction was stated, after ~0.5 s and after 1, 2 and 3 s of reaction. When the concentrations of cyclic [^{3}H]AMP and binding protein (RII or cAKII) were adjusted so that the amount bound after 1 s of reaction was at least twice the blank values of the assay, the range of values for bound nucleotide determined were within ±40% of the mean for 0.5 s, ±25% for 1 s and ±15% for 2 s and 3 s of reaction. The mean value for the amount of nucleotide 'immediately' bound was 1/10th that after 1 s of reaction, suggesting that the lag involved in quenching the reaction was <0.1 s.

To ensure that the exchange reaction used to differentiate cyclic [^{3}H]AMP bound to site A and B was unaffected by the various conditions used during the association reaction, detailed exchange rate studies were performed at 0°C and 30°C for various concentrations (0.4–30 nM) of autophosphorylated and non-phosphorylated cAKII saturated to various degrees with cyclic [^{3}H]AMP. The slopes of neither the rapid nor the slow phase of exchange were affected by the concentrations or degree of saturation of cAKII at either temperature. The presence of 20 nM of the catalytic subunit of protein kinase did not alter the exchange rates.

It should be noted that the excess unlabelled cyclic AMP in the quench solution served not only to quench the association rate by isotope dilution, but also to dissociate cAKII into subunits. The RII cyclic [^{3}H]-AMP in the quench-solution existed thus in the free form. A final point of concern was, however, that cyclic [^{3}H]AMP bound in a putative ternary complex with C and R might be perturbed in the interval between the addition of quench-solution and the complete dissociation of cAKII into subunits. It was reasoned that this interval would be shorter at high concentrations of cyclic AMP, and that any perturbation would be more pronounced at low than at high concentrations of cyclic AMP in the quench-solution. In an experiment like that of fig.1a the reactions were quenched by the addition of 0.2 ml of 20 μM or 20 mM cyclic AMP in buffer A with 0.15 M KCl, and then 1.6 ml buffer B with 0.13 mM cyclic AMP added.

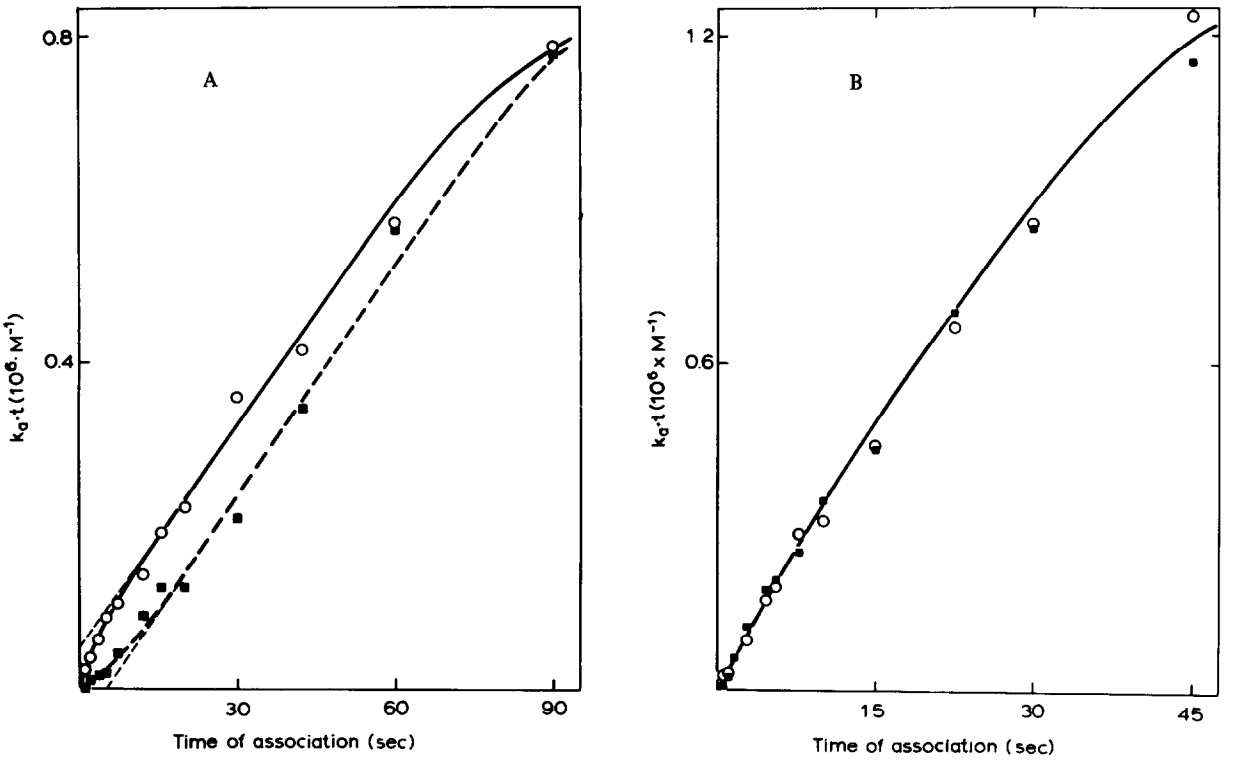

Fig.1. Association of cyclic [^{3}H]AMP and cAKII. Cyclic AMP and cAKII were reacted for the periods of time indicated on the abscissa, and the amount of nucleotide bound to site A (■) and site B (○) determined. $k_a \cdot t$ was calculated as explained in section 2: (a) cyclic [^{3}H]AMP was 30 nM, cAKII binding sites 6 nM; (b) cyclic [^{3}H]AMP was 0.6 μM, cAKII binding sites 20 nM.

The results were the same within experimental error whether the reactions were quenched with 20 μM or 20 mM cyclic AMP in 0.15 M KCl or as in section 2 (0.13 mM cyclic AMP in buffer B). It seems thus that the method used gives a true estimate of the amount of cyclic [^{3}H]AMP bound to cAKII at the moment of quenching.

3.2. *Kinetics of the initial phase of association of cyclic AMP and cAKII*

The time course of association at 0°C in 0.15 M KCl is shown in fig.1,2. An initial burst was observed for the rate of occupancy of site B by cyclic AMP and a corresponding lag for site A. There was no significant difference in association rate between autophosphorylated and nonphosphorylated cAKII (fig.2). Furthermore, cAKII preincubated at 37°C for 10 min with 10 mM Mg-acetate/5 mM ATP, and incubated with 2 mM Mg-acetate/0.1 mM ATP showed kinetics similar to that of enzyme treated similarly, but not exposed to ATP during preincubation and incubation.

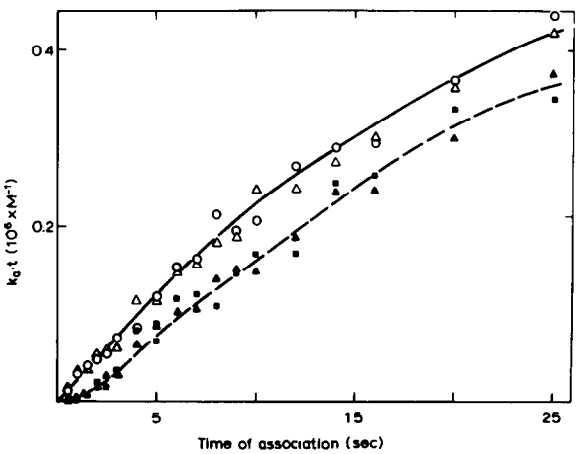

Fig.2. Comparison of association rate of cyclic [^{3}H]AMP to untreated and autophosphorylated cAKII. The time course of binding (expressed as $k_a \cdot t$ to site A (■) and site B (○) of untreated, and to site A (▲) and site B (△) of autophosphorylated cAKII is shown: cyclic [^{3}H]AMP was 0.1 μM, cAKII binding sites 10 nM.

The initial nonlinear kinetics of binding was more easily studied at low concentrations of cyclic AMP (fig.1a), the reaction proceeding too fast at higher concentrations (fig.1b) to be monitored with manual mixing. The pattern was independent of enzyme concentration from 2–20 nM, indicating that liberation of free C was not responsible for the burst and lag for binding to site B and A, respectively.

3.3. *Kinetics of the second phase of association of cyclic AMP and cAKII*

After the initial nonlinear phase of binding, most evident at the lower concentrations of cyclic AMP tested (fig.1a,2), the binding to site A and B proceeded at a near linear rate for some time. The apparent association rate constant (k_a) of this phase was higher at high than at low concentrations of cyclic AMP. The k_a thus increased from $\sim 10^4$ $M^{-1} . s^{-1}$ at 20 nM cyclic AMP to $\sim 8 \times 10^4$ $M^{-1} . s^{-1}$ at 2 μM cyclic AMP when incubations were at 0°C in 0.15 M KCl.

To find if k_a varied also at a physiological temperature and in the physiologically relevant range of cyclic AMP concentrations, association rate studies were performed at 37°C in 0.15 M KCl with 0.5 μM and 2 μM cyclic AMP. The linear phase of cyclic AMP accumulation was shorter than at 0°C, presumably due to a substantial C-induced dissociation of the RII–cyclic AMP complex at 37°C. The k_a at 2 μM cyclic AMP ($\sim 3.5 \times 10^5$ $M^{-1} . s^{-1}$) and at 0.5 μM cyclic AMP ($\sim 2 \times 10^5$ $M^{-1} . s^{-1}$) was nevertheless determined with sufficient precision to conclude that k_a increased with cyclic AMP concentration also at 37°C.

4. Discussion

Some aspects of the interaction between cyclic AMP and site A and B of the free regulatory moiety (RII) of protein kinase II (cAKII) were presented in [9]. The nucleotide bound more rapidly to site A than to site B of the free RII, the initial rate of accumulation to either site was linear, and the apparent association rate constant (k_a) was independent of the contration of cyclic AMP [9].

The initial phase of cyclic AMP binding to the cAKII holoenzyme was more complex (fig.1,2). Thus, there was a burst of cyclic AMP binding to site B and a corresponding lag for the binding to site A. This suggests that in the ground state of cAKII only site B is free to react with cyclic AMP, and that binding to site B alters the conformation of cAKII so that also site A may react with cyclic AMP.

In [11,12] the time course of the accumulation of cyclic AMP occurring after $\geqslant$1 min incubation with endometrial cAK was shown. These data strongly suggest that an initial burst of binding must have occurred during the first 1 min incubation. It is of special interest that the millipore filtration method used in [11,12] seems to detect only binding to site B of cAK [3], which is the site showing a burst of accumulation here (fig.1,2).

After the initial nonlinear phase the accumulation of cyclic AMP to site A and B of cAKII proceeded near linearly and at a similar rate for the 2 sites (fig. 1,2). The apparent k_a calculated for this phase of the association increased as a function of the cyclic AMP concentration. These observations suggest that the complex between the first molecule of cyclic AMP bound and site B of cAKII is unstable, being stabilized by the binding of further molecules of cyclic AMP to cAKII. The stabilization of the binding of cyclic AMP may correspond to the dissociation of one or both of the C-subunits from the cAKII–cyclic AMP complex. The kinetic data obtained for the interaction between cyclic AMP and an insect protein kinase of composition CR was found to fit the model:

$$\text{CR} + \text{cyclic AMP} \rightleftarrows \text{CR–cyclic AMP} \rightleftarrows \text{R–cyclic AMP} + \text{C}$$

where the cyclic AMP dissociates much more rapidly from the ternary complex than from the R–cyclic AMP complex [7]. For the mammalian tetrameric kinase with 4 binding sites the kinetic analysis is more complex. Thus, after one of the B-sites of cAKII has been occupied there are 6 possible sequences for binding to the 3 remaining sites (fig.3). To find which of the sequences is the preferred one(s), and at what points in the sequence the catalytic subunits become active will require more detailed kinetic studies preferably using cyclic AMP analogues with a high degree of preference for site A or B. To establish the true sequence and determine to what degree the intermediary complexes accumulate may be of more than theoretical interest, since it has been suggested that some intermediary complex of cAKII and cyclic AMP rather than the ground state of the holoenzyme or the fully saturated RII is transported across the nuclear membrane [13,14].

One of the important features of the cyclic AMP-

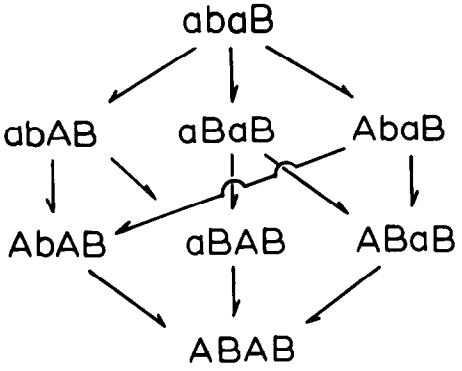

Fig.3. The possible sequences of cyclic AMP binding to cAKII with one B-site occupied are shown. Occupied sites are indicated with capital letters. That only the forward reaction has been indicated (→) does not imply that the steps are irreversible.

dependent protein kinases is their ability to be significantly activated in vivo in response to a very slight increase of the intracellular concentration of cyclic AMP, the in vivo binding of cyclic AMP showing apparent positive cooperativity (discussed in [5,15]).

One mechanism responsible for this apparent positive cooperativity is presumably the increasing retardation of the reassociation of R and C subunits when the concentration of cyclic AMP is increased within the physiological range [5]. Another mechanism may be the increase of the apparent k_a for the association between cyclic AMP and cAKII noted here when the concentration of cyclic AMP was increased. This increase was evident at a physiological temperature, pH and ionic strength and in the physiologically relevant range of cyclic AMP concentrations.

There is evidence that cAKII exists mainly in a phosphorylated form in freshly prepared extracts of bovine cardiac muscle. By exposing the enzyme to phosphatases (deliberately or during lengthy purification procedures) it is converted to a form that readily accepts the terminal phosphate of ATP in an autophosphorylation reaction [16].

The unusual kinetics of the association of cyclic AMP and cAKII noted here may be due to heterogeneity in the content of endogenous phosphate at the autophosphorylable site(s). The fully autophosphorylated cAKII, presumed to be the predominating form in vivo [16], might show kinetics different from those here. However, the similar rate of cyclic AMP accumulation to untreated cAKII and cAKII exposed to conditions favourable for autophosphorylation (fig.2), rendered those possibilities unlikely.

According to [17] each RII subunit contains ⩾2 mol of phosphate. About 1.4 mol phosphate was transferred to the RII subunit during the autophosphorylation reaction (see section 2). It is therefore possible that our preparation of cAKII contained endogenous phosphate, and that the completely dephosphorylated cAKII might have kinetic properties distinct from those described here.

The interaction with cyclic AMP is different for site A and B when in the cAKII holoenzyme than when in free RII. The contention that cyclic AMP may interact directly with cAK holoenzyme and not only with free R is thus supported. The initial burst of cyclic AMP binding to site B and lag in binding to site A suggest that only site B is free to react with cyclic AMP in the cAKII holoenzyme, and that binding to site A occurs after site B has been occupied. The finding that the rate of cyclic AMP binding to cAKII increased more than proportionally to the cyclic AMP concentration may be of relevance to explain the apparent positive cooperativity observed for the binding of cyclic AMP to cAK in vivo [5,15].

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