KINETIC STUDIES ON THE INTERACTION OF cAMP WITH BOVINE CARDIAC PROTEIN KINASE

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1. Introduction

The activation of protein kinases by cAMP involves the dissociation of inactive holoenzyme (R_2C_2) into a regulatory subunit dimer (R_2) and active catalytic subunits (C) [1,2], as shown in eq. (1).

$$R_2C_2 + n\text{cAMP} \rightleftharpoons R_2(\text{cAMP})_n + 2\,C \quad (1)$$

Recent studies [3,4] on bovine skeletal muscle protein kinase types I and II indicate that 2 mol cAMP are bound/regulatory subunit monomer. Whether or not total occupancy of the cAMP-binding sites is required for activation has not been determined. Disregarding the possibility of cooperative interactions, the activation may proceed through two mechanistically distinct routes [5]:

(i) Cyclic AMP may exert its effect by binding to free R_2 and displacing a pre-existing equilibrium between holoenzyme and its subunits towards active C.
(ii) Cyclic AMP may bind to holoenzyme, followed by dissociation of the ternary complex into C and $R_2(\text{cAMP})_n$.

Evidence in favour of the existence of R · C · cAMP complexes [4,6,7] suggests that the latter mechanism must be contributing at least to the activation process.

This study deals with the kinetics of association of cAMP with bovine cardiac protein kinase type II and its release, under different conditions, from the resultant cAMP · kinase complex(es), with the aim of identifying the significant route in the activation of this enzyme.

2. Materials and methods

2.1. *The enzyme preparation*

Bovine cardiac protein kinase type II was partially purified to spec. act. 52.6 nmol phosphate transferred to protamine . min^{-1} . mg $protein^{-1}$ (kinase activity) and 173 pmol cAMP bound/mg protein (binding activity). Kinase activity was measured at 30°C; the assay system contained 100 mM potassium phosphate buffer (pH 7.0), 10 mM mercaptoethanol, 10 mM $MgSO_4$, 2 μM cAMP, 100 μM [U-^{14}C]ATP (The Radiochemical Centre, Amersham) at spec. act. 28 000 cpm/nmol and ~20 μg enzyme/ml. The reaction was monitored by following the formation [^{14}C]-ADP by thin-layer chromatography on PEI-Cellulose F (Merck, FRG). After the initial phases of purification, the production of ADP was found to be strictly dependent on the inclusion of protamine in the assay medium. Cyclic AMP binding activity was assayed according to [8] as modified [9]. The initial purification steps were essentially as in [9]; DEAE–Sephadex A-50 chromatography was substituted for batch elution, followed by gel filtration on Sephadex G-200 (Pharmacia, Sweden), which yielded coincident and symmetrical peaks of protein kinase and cAMP binding activity.

2.2. *Kinetics of cAMP binding and release*

The time course of cAMP binding to and release from protein kinase was followed by the filter method [8]. Binding was initiated by the addition of c[8-^{3}H]AMP (The Radiochemical Centre, Amersham); the final composition of the assay medium was 40 mM potassium phosphate (pH 7.0), 4 mM mercaptoethanol, 10 mM $MgSO_4$, 40 nM cAMP (spec. act. 15 000 cpm/pmol) and 15 μg enzyme/ml. Incubation

was carried out at 20°C. Protein-bound radioactivity was determined by filtration of 100 μl aliquots on Sartorius (FRG) SM 11306 filters at 0°C and counting the filters following solubilization. After binding equilibrium was reached, a 2500-fold excess of non-radioactive cAMP was added to the remaining incubation mixture, in such a way that the concentrations of the remaining components did not change significantly. Protein-bound radioactivity was monitored as above.

3. Results

3.1. *Cyclic AMP binding and release in the absence of substrate*

Semilogarithmic plots of the approach to binding equilibrium and of isotope exchange versus time showed multiphasic character (fig.1). In a series of experiments done at 30°C instead of 20°C, the plots had more biphasic character (not shown) and could be analyzed in terms of a fast and a slow component, the contribution of the slow component decreasing with increasing age of the enzyme preparation.

3.2. *The effects of protamine sulfate and Mg · ATP*

The inclusion of 0.73 mg protamine sulfate/ml or of 5 mM Mg · ATP in the assay medium was found to increase equilibrium binding capacity by >100%, at the same time reducing the kinetics of cAMP binding and release essentially to that of a single-component system (fig.2,3). The rate constants for the approach

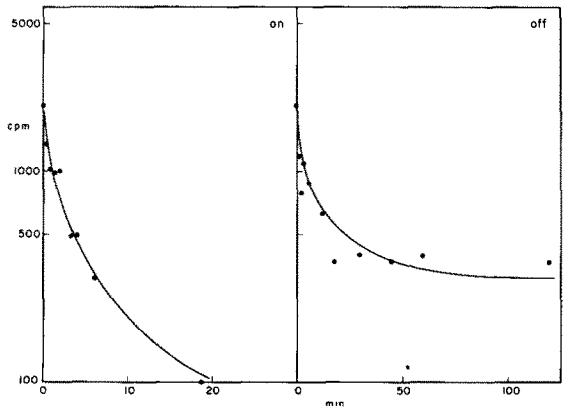

Fig.1. Semilogarithmic plots of protein-bound radioactivity *vs* time: 'on', the approach to c[^{3}H]AMP binding equilibrium (cpm ≡ $cpm_{eq} - cpm_t$); 'off', c[^{3}H]AMP release upon addition of 100 μM cAMP (cpm ≡ cpm).

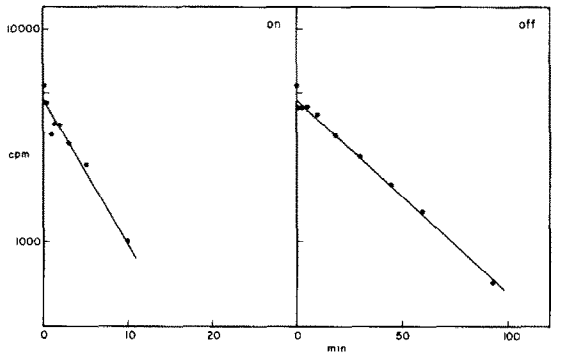

Fig.2. The effect of protamine sulfate (0.73 mg/ml) on c[^{3}H]-AMP binding and release.

to binding equilibrium (k_{on}) and for release (k_{off}) were 0.12 ± 0.02 min^{-1} and 0.023 ± 0.002 min^{-1}, respectively, regardless of whether protamine sulfate of Mg · ATP was the added effector.

4. Discussion

The multiphasic character of cAMP binding and release in the absence of substrate could arise from:

(i) Exclusive binding to a single species (most reasonably R_2) with non-equivalent binding sites; or

(ii) Simultaneous binding to more than one species (R_2C_2, R_2C, R_2), the binding sites of which may or may not be interacting. Here the component phases may reflect the species composition of the enzyme preparation or may in part be due to rate-limiting transitions between kinetically significant binding and releasing species. Recent studies on

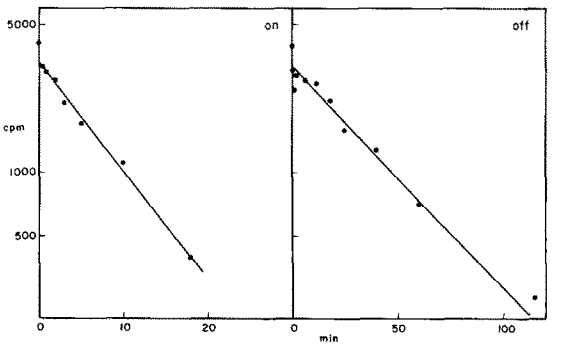

Fig.3. The effect of 5 mM Mg · ATP on c[^{3}H]AMP binding and release.

the stoichiometry of cAMP binding to R_2 [3] and of cAMP release from $R_2(cAMP)_2$ [10] indicate that the two cAMP binding sites on R_2 are kinetically distinct, and that the membrane filtration technique detects only the slow-dissociating $R_2 \cdot cAMP$ complex. This study (fig.1) then provides additional evidence for the existence [4,6,7] of ternary $R \cdot C \cdot cAMP$ complexes.

The 'off' phase in fig.2 must reflect the dissociation of cAMP from R_2, since the only conceivable effect of protamine sulfate is to displace the holoenzyme $\rightleftharpoons$ subunit equilibrium in favour of $R_2(cAMP)_n$ and C. The half-life for isotope exchange (29 ± 2 min) is in close agreement with that obtained for rabbit muscle R_2 in the absence of C [11]. Regarding the linearity of the 'on' phase in the presence of protamine sulfate, it has been shown [12] that in the absence of cAMP, the protein substrate does not bring about appreciable dissociation of the type II enzyme. Therefore the principal event under observation must be the binding of cAMP to holoenzyme, followed by fast dissociation of the resultant complex. These results indicate that significant activation (dissociation) of protein kinase takes place only when both cAMP and substrate are available.

The identity of the binding and release rates in the presence of protamine sulfate and of ATP suggests that autophosphorylation [13–15] affects only subunit dissociation in the holoenzyme as in [14], and that the basic R_2–cAMP interaction is not affected. In conclusion, all forms of bovine cardiac protein kinase type II are able to bind and to release cAMP. Occupancy of the cAMP binding sites increases the rate of holoenzyme dissociation, the extent of which, however, depends on the presence of the protein substrate and on the state of phosphorylation of the regulatory subunits.

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