A study of the interaction between bovine cardiac-muscle cyclic AMP-dependent protein kinase and cyclic AMP using fluorescence-polarization spectroscopy

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1. The C-subunit of type II cyclic AMP-dependent protein kinase from bovine heart was labelled with the fluorophore fluorecamine (FAM). 2. The association of the dye-labelled subunit (C_{FAM}) with the R-subunit isolated from the same source was monitored by fluorescence polarization spectroscopy. The stoichiometry of C to R in the final complex was close to 1 : 1. The affinity of the two subunits could be described by a dissociation constant in the nanomolar range. 3. Holoenzyme (formed from C_{FAM} and R) was titrated with cyclic AMP, and the changes in fluorescence anisotropy, due to dissociation of the holoenzyme, recorded. The titration curves were analysed in terms of a model which required computer simulation. 4. Cyclic AMP-induced dissociation proceeds via one or more ternary complexes, and all four cyclic AMP-binding sites on the R-dimer are accessible in the holoenzyme. 5. The dissociation constants describing the release of the C-subunits from the two ternary complexes containing four cyclic AMP molecules were both approx. $9\mu M$. 6. The binding of two cyclic AMP molecules to protein kinase is necessary and sufficient to cause the dissociation of both C-subunits. 7. The state of association at 'in vivo' concentrations of protein and cyclic AMP is discussed.

The principal target for cyclic AMP (adenosine 3':5'-cyclic monophosphate), which is generated in the cell after the hormonal activation of adenylate cyclase is cyclic AMP-dependent protein kinase (Walsh *et al.*, 1968). This enzyme has been found to play a key role in the regulation of several metabolic pathways, and a detailed knowledge of how fluctuating cyclic AMP levels control the activity of protein kinase is important to an overall understanding of metabolic regulation.

Brostrom *et al.* (1970) proposed that protein kinase activation was a consequence of cyclic AMP binding to the regulatory (R) subunit and release of the active catalytic (C) subunit from the inactive holoenzyme (R_2C_2). Recent refinements to this model have included the findings that the R-dimer

binds four cyclic AMP molecules (Corbin *et al.*, 1978) and that the reaction proceeds via some form of ternary complex (defined as a species containing R, C and cyclic AMP) (Chau *et al.*, 1980). The mechanism as currently accepted is often written as:

Such a reaction scheme (where cAMP = cyclic AMP) is an oversimplification which ignores a potentially large number of intermediate ternary complexes (containing R-dimer, one or two C-subunits and between one and four cyclic AMP molecules). An understanding of the mechanism by which cyclic AMP mediates the dissociation of the enzyme requires information on the nature of such species.

Progress in understanding the interaction between the protein and cyclic nucleotide has been hampered by the practical difficulties of detecting any intermediate species formed during the dissociation. In an attempt to overcome this problem we have used fluorescence polarization spectroscopy to probe the system. The advantage of this tech-

Abbreviations used: C and R, the catalytic and regulatory subunits of protein kinase; FAM, fluorecamine; C_{FAM} , C-subunit labelled with FAM; ΔA , the overall change in anisotropy; LPD ratio, the ligand-protein dissociation ratio.

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nique is that it is capable of monitoring changes in molecular volume (Weber, 1952), over a wide range of species concentrations. Therefore, if a fluorescent label is attached to the protein, this technique can be used to measure the changing state of aggregation of the component subunits during the interaction of protein kinase with cyclic AMP.

The analysis of any results is theoretically difficult because a potentially large number of intermediates have to be taken into consideration. Analysis of our data was helped by use of the chemistry simulation program FACSIMILE, which is capable of handling multiple-intermediate reactions.

Experimental

Materials

Cyclic AMP was from Boehringer Ltd. Lysine-rich histone (type IIIS) was from Sigma Ltd. [γ - ^{32}P]ATP was from The Radiochemical Centre, Amersham, Bucks., U.K. Fluorescamine was from Hoffmann-La Roche, Basel, Switzerland. All other chemicals were obtained from BDH Ltd.

Protein preparations

Type II cyclic AMP-dependent protein kinase from ox heart was purified by a method modified from that described by Rubin *et al.* (1972). The C- and R-subunits were separated by a non-denaturing method which also removed cyclic AMP from the R-subunit. This depends on dissociating the C- and R-subunits with cyclic AMP, binding both subunits to DEAE-Sepharose CL-6B and eluting the C-subunit with low salt. The cyclic AMP bound to the R-subunit is then exchanged with excess cyclic GMP while the R-subunit is still attached to the DEAE-Sepharose, the R-cyclic GMP complex is eluted with high salt, and the weakly bound cyclic GMP removed from the R-subunit by dialysis. The phosphorylated form of the R-subunit was used throughout the experiments and was prepared by the method of Erlichman *et al.* (1975).

Concentration determinations

The concentrations of the R- and the C-subunits were routinely determined by absorbance measurements at 280 nm using a value of $A_{1\text{cm}}^{1\%} = 14.9$ and an M_r of 39000 for the C-subunit (Peters *et al.*, 1977) and $A_{1\text{cm}}^{1\%} = 6.0$ and an M_r of 45000 for the R-monomer (Corbin *et al.*, 1978; Takio *et al.*, 1982). Concentrations of the R-monomer determined from this factor agreed with those determined independently by amino acid analysis. The concentration of FAM was determined by using a molar absorption coefficient of $6500\text{M}^{-1}\cdot\text{cm}^{-1}$ at 390 nm

(Chen, 1974). The contribution of the dye label to the absorbance of the dye-C-subunit (C_{FAM}) conjugate at 280 nm was accounted for using the molar absorption coefficient we determined for FAM-lysine ($16000\text{M}^{-1}\cdot\text{cm}^{-1}$).

Measurement of enzyme activity

The catalytic activity of the C-subunit was measured by a modification of the filter-paper method (Corbin & Reimann, 1974) using 50 mM-sodium phosphate, pH 7.0, 120 mM-NaCl, 4 mM-mercaptoethanol, 5 mM-magnesium acetate, 0.33 mM-ATP (containing sufficient [γ - ^{32}P]ATP to give about 1.7 Bq/pmol) and 10 mg of histone/ml as the reaction medium. The reaction was initiated by the addition of a 20 μl portion of the sample to 50 μl of assay medium.

Dye-labelling procedure

The C-subunits were labelled with fluorescamine by addition of either a 5-fold or a 15-fold molar excess of the dye dissolved in acetone to a solution of the C-subunits (at about 10 μM) in 50 mM-sodium phosphate (pH 7.0)/120 mM-NaCl/4 mM-mercaptoethanol. The final acetone concentration was less than 1%. The dye-labelled conjugates were then dialysed overnight before use. The number of dye labels per C-subunit were approx. 1.0 and 2.5 for the two conjugates respectively.

Physical properties of C_{FAM}

The effect of the FAM on the properties of the C-subunit was checked by comparing (a) the catalytic activity in the presence of various amounts of R-subunit (no cyclic AMP present) and (b) the gel-filtration behaviour on Sephacryl S-300 of C_{FAM} with that of unlabelled C-subunit. No significant differences between either conjugate and unlabelled C-subunit were detected, indicating that the dye label did not affect the properties of the C-subunit. This conclusion was supported by the observation that changes in the fluorescence anisotropy of C_{FAM} , when titrated with R-subunit and subsequently cyclic AMP, were indistinguishable from those of a solution containing the same total concentration of C-subunit but with unlabelled C-subunit in 5-fold excess over the dye-labelled species.

The species containing 2.5 dye labels per C-subunit was used in experiments where a concentration of less than 0.3 μM was required.

Computing facilities

FACSIMILE is a large computer program for the automatic solution of initial-value problems arising as systems of differential equations (Chance *et al.*, 1977). Its high-level language

facility makes it particularly useful for applications to chemical problems. FACSIMILE was mounted on an ICL 2980 computer.

Results

Fluorescence-anisotropy measurements

The instrument used was an improved version of that described previously by Freyssinet *et al.* (1978). All experiments were carried out at 25°C. The excitation wavelength was 390nm and Wratten number-3 filters were positioned in front of the photomultiplier tubes to select emission wavelengths greater than 450nm.

The buffer used throughout the experiments was 50mM-sodium phosphate, pH7.0, containing 4mM-mercaptoethanol, 2mM-EDTA and 120mM-NaCl.

(1) *The association of C_{FAM} and R-subunit.* The fluorescence anisotropy of the isolated C_{FAM} -subunit was measured at C-subunit concentrations between 0.1 and 3 μ M. There was no discernible variation in the average value obtained (0.20) over this concentration range.

R-subunit was added in small amounts to C_{FAM} (at several concentrations between 0.1 and 3.0 μ M) until no further change in anisotropy was observed. One example is given in Fig. 1. The rate of reaction was too rapid to monitor with the available instrumentation. There was no significant change in total fluorescence during the titration. This indicated that there was no appreciable change in the life-time of the fluorescent label. Therefore the increase in anisotropy could be directly related to an increase in the molecular size of the C-subunits, i.e. to an association with R. The titration-curve shapes indicated that the association was tight. Sufficiently low protein concentrations to allow an accurate determination of the affinity of the two subunits were below the detection limits of the instrumentation. However, from the amount of C-subunit remaining uncomplexed at the breakpoints of the titration curves a dissociation constant in the nanomolar range could be estimated. This value is comparable with the previously determined value of 0.6nM (Builder *et al.*, 1980) and 0.3nM (Hofmann, 1980), which were derived from measurements of enzyme activity. From the break-points of the titration curves a stoichiometry of C-monomer to R-monomer in the complex of 1:1.1 was measured. This is compatible with the previously determined stoichiometry for the complex (R_2C_2) (Erlichman *et al.*, 1973).

The anisotropy of the holoenzyme containing C_{FAM} was measured between 0.05 μ M and 20 μ M, and no discernible trend in the average value (0.23) was observed, indicating that the aggregation state of the enzyme is fixed over this concentration

Fig. 1. Change in anisotropy of C_{FAM} (0.30 μ M) as a function of R-subunit concentration

range. Having determined that the extent of R-C dissociation is negligible over this concentration range (see the above paragraph) this result indicates that the system is uncomplicated by an R dimer-monomer equilibrium and therefore species such as R_1C_1 need not be considered.

(2) *Titration of protein kinase containing C_{FAM} with cyclic AMP.* This section briefly describes the experiments from which the bulk of the results were obtained. C_{FAM} and R-subunit were mixed to a ratio of 1:1 over a range of concentrations of R_2C_2 varying from 0.05 to 20 μ M and were then titrated with cyclic AMP and the changes in anisotropy recorded. Two examples of individual titration curves are given in Fig. 2. The highest protein concentrations were achieved by adding unlabelled protein kinase to the cuvette along with holoenzyme containing C_{FAM} .

Analysis of the binding curves

The titration curves were characterized by two measurable parameters (as shown in Fig. 2): (a) the overall change in anisotropy, ΔA , and (b) the concentration of cyclic AMP at the intercept of the initial slope of the titration curve and the final value of the anisotropy of the titration curve divided by the enzyme concentration. This ratio was called the 'ligand protein dissociation (LPD) ratio'.

Fig. 2. Change in anisotropy of holoenzyme formed from equimolar amounts of C_{FAM} and R-subunit as a function of cyclic AMP concentration

(a) Protein kinase concentration = $0.34 \mu\text{M}$; (b) protein kinase concentration = $7 \mu\text{M}$. Superimposed on each plot are the constructions used to determine the parameters ΔA (}), and the LPD ratio (----).

Fig. 3. The general model

The free C-subunit and cyclic AMP species have been omitted for greater clarity. Bound cyclic AMP is represented by 'a'. The numbers 1–14 identify the equilibrium constants referred to in the text. The equilibrium constants for the unnumbered reactions were calculated from the standard mass-action relationships.

The binding curves, such as those shown in Fig. 2, were analysed by a model of the form depicted in Fig. 3. This does not have a useful analytical solution and its behaviour during titration with cyclic AMP was therefore simulated with the FACSIMILE program.

It was assumed that the observed anisotropy change was due only to the formation of monomeric C-subunit. The implicit assumptions are (a) the binding of cyclic AMP to R-subunit did not produce a change in the lifetime of the FAM bound to the C-subunit, and (b) the anisotropy of tetrameric forms ($R_2C_2cAMP_n$, where $n = 0, 1, 2, 3$ or 4 and $cAMP$ = cyclic AMP) and trimeric forms (R_2CcAMP_n) were indistinguishable. Assumption (a) was supported by the fact that there was no significant change in the total fluorescence on addi-

tion of cyclic AMP to the system. Assumption (b) was supported by the observation that the anisotropy of C_{FAM} in the presence of a 2.5-fold excess of R-subunit (conditions under which one would predict the major species present to be R_2C) was the same as that obtained when equimolar concentrations of C_{FAM} and R-subunit were present (major species present R_2C_2). For the purpose of the simulations a value for the dissociation constant of tetramer in the absence of cyclic AMP (K_1 and K_2 in Fig. 3) was taken from Builder *et al.* (1980) and was 0.6 nM .

(1) ΔA plotted against protein kinase holoenzyme concentration (Fig. 4). Only at the lowest protein kinase concentrations was saturating cyclic AMP capable of generating an overall decrease in anisotropy corresponding to complete dissociation of

Fig. 4. Change in ΔA (at saturating cyclic AMP concentrations) as a function of protein kinase holoenzyme concentration

The broken line was generated by using the fragment of the general model described by K_9 and K_{10} , where $K_9 = 4 \mu\text{M}$ and $K_{10} = 16 \mu\text{M}$. The continuous line was generated by using values for K_9 and K_{10} of $9 \mu\text{M}$.

the holoenzyme. As protein concentration increased, the observed overall change in anisotropy decreased. That is to say, the average molecular weight of C-containing species increased. To explain this behaviour it was necessary to postulate a final step in the overall reaction which involved C-subunit dissociation, the extent of which was protein-concentration-dependent. Any such mechanisms must necessarily involve cyclic AMP binding as a first step and must therefore proceed via some form of ternary complex.

The decrease in the fractional ΔA to values below 0.5 with increasing protein concentration indicates that all four potential cyclic AMP sites must be accessible to cyclic nucleotide, even in the tetramer. If only R_2C and R_2 could bind four cyclic AMP molecules, then the fractional ΔA would reach a limiting value of 0.5, because even at very high protein concentrations, no more than 50% of the C-subunit could be associated with R_2cAMP_4 (to form R_2CcAMP_4) and the remainder would be uncomplexed. Therefore ternary complexes containing four cyclic AMP molecules must be included in the model.

In a quantitative analysis of the data we used

Fig. 5. Change in LPD ratio as a function of protein kinase concentration

The four curves were generated by using models in which the dissociation constants describing the release of C-subunit were either 0.6 nM or $9 \mu\text{M}$. The equilibrium constants having values of $9 \mu\text{M}$ were: model (a), K_5 , K_7 , K_9 and K_{10} ; model (b), K_5 – K_{10} inclusive; model (c), K_3 in addition to K_5 – K_{10} inclusive; model (d), K_3 – K_{10} inclusive. The values given to K_{11} and K_{12} , and K_{13} and K_{14} , in the simulations depicted above, were $0.1 \mu\text{M}$ and $0.7 \mu\text{M}$ respectively, though for each model a wide range of values gave very similar curves.

FACSIMILE to simulate the fragment of the model which comprises those reactions described by K_9 and K_{10} (see Fig. 3). Good fits to the experimental data were obtained with models in which both dissociation constants were around $9 \mu\text{M}$.

(2) LPD ratio plotted against protein kinase holoenzyme concentration (Fig. 5). At the lower protein concentrations the value of the LPD ratio was close to 2.0. The potential information content of the LPD ratio was investigated by simulating plots of the LPD ratio against protein kinase concentration for a series of models inherent in the general model, in which the order of cyclic AMP binding and C-subunit dissociation was varied. This involved assigning values to the dissociation constants describing the release of C-subunit from the various ternary complexes of either $9 \mu\text{M}$ (i.e. significant dissociation) or 0.6 nM (i.e. insignificant dissociation).

The model in which four cyclic AMP molecules had to bind before C-subunit dissociation could occur generated markedly sigmoidal titration curves, unlike the experimental curves, and could be discounted on this basis alone. The models

referred to in Fig. 5 as (a), (c) and (d) (see the legend to Fig. 5 for details of the values used for the dissociation constants) attained LPD ratios of 3.0, 1.7 and 1.0 respectively and could also be discounted. Model (b) attained a value of 2.0, in agreement with the experimental data. This is the model in which both C-subunits only dissociate after the second cyclic AMP molecule has bound to the tetramer. The binding of the third and fourth cyclic AMP molecules does not result in any further decrease of the affinity of C for R.

The use of different values for the various dissociation constants describing the affinity of cyclic AMP for holoenzyme (K_{11} – K_{14}) resulted in only relatively slight differences in the simulated titration curves. This was especially true of K_{13} and K_{14} . Any conclusions about these two equilibrium constants would have required more detailed information at the higher protein concentrations at which the species $R_2C_2cAMP_3$ and $R_2C_2cAMP_4$ predominate.

In the case of model (b) the use of values for both K_{11} and K_{12} of less than $2\mu M$ in the simulations was compatible with the experimental data. (Because of the need to specify values for K_{13} and K_{14} in the simulations, their values were set to be seven times weaker to approximate non-co-operative binding.)

The value for the apparent K_d of $0.5\mu M$ previously reported by Hofmann *et al.* (1975) (obtained by using holoenzyme concentrations at which $R_2C_2cAMP_3$ and $R_2C_2cAMP_4$ concentrations would be negligible) lies within the range that we delineated for K_{11} and K_{12} .

Discussion

The lack of information about the intermediates in the cyclic AMP-induced dissociation of protein kinase has reflected the lack of suitable tools to detect these intermediates. The majority of investigations into the subunit–subunit interactions have relied upon assuming that the catalytic activity of the C-subunit is related to the state of aggregation of the protein. In the present study we have used fluorescence anisotropy to monitor directly changes in the molecular size of the C-subunit (i.e. its association and dissociation from the R-dimer), so the need for such assumptions does not arise. Furthermore, there is no need to have substrates present which could affect the subunit–subunit equilibria. Our data provided both qualitative and quantitative information about some of the intermediate steps in the cyclic AMP-induced dissociation of protein kinase and in particular those involving C-subunit dissociation.

The crucial interrelationship between cyclic AMP binding and C-subunit dissociation occurs

after the second cyclic AMP molecule has bound to the holoenzyme to form the ternary complex $R_2C_2cAMP_2$. The presence of the two cyclic AMP molecules is sufficient to weaken the affinity of the R-dimer for both C-subunits to an extent which is not significantly changed by the binding of the third and fourth cyclic AMP molecules. Thus the dissociation of the C-subunits with respect to cyclic AMP binding appears to be a highly co-operative process. The role of the third and fourth cyclic AMP molecules remains unclear.

To present those equilibrium constants calculated using the standard mass-action relationships (see Fig. 3) is not particularly enlightening because the model does not specify two classes of cyclic AMP-binding site. Use of a model which does explicitly describe different classes of binding sites is of little value because the information content of experiments using C_{FAM} is mainly concerned with C-subunit dissociation as a function of the total cyclic AMP added to the system. The model used in the present study is adequate for this.

Which species are important in the pathway of cyclic AMP-mediated dissociation *in vivo* is determined by the cellular concentration of protein kinase. By using an estimate of $0.7\mu M$ (Hofmann *et al.*, 1977) for the cytosolic enzyme concentration in heart, it can be predicted that about 90% of the available C-subunit will be liberated from the ternary complex of the form $R_2C_2cAMP_2$ if the cyclic AMP concentration is sufficiently high. By using an estimate of $1\mu M$ for the basal cytosolic level of cyclic AMP (Corbin *et al.*, 1977) it can be predicted, using our model and the equilibrium constants that we determined, that approx. 50% of the C-subunit is already dissociated. The experimentally determined level is about 25% (Corbin *et al.*, 1975). Considering that all our measurements were made on a system devoid of normal cellular components, e.g. membranes, protein substrates and MgATP, the difference of a factor of 2 is unsurprising. However, it is clear that the state of aggregation is delicately balanced and very susceptible to changes in cyclic AMP levels.

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