Schneider for his helpful discussions during the preparation of this manuscript.

References

Avruch, J., & Fairbanks, G. (1974a) *Biochemistry 13*, 5507.
Avruch, J., & Fairbanks, G. (1974b) *Biochemistry 13*, 5514.
Bodemann, H., & Passow, H. (1972) *J. Membr. Biol. 8*, 1.
Cannan, R. K. (1958) *Am. J. Clin. Pathol. 30*, 211.
Dodge, J. T., Mitchell, C. D., & Hanahan, D. J. (1963) *Arch. Biochem. Biophys. 100*, 119.
Erlichman, J., Hirsch, A. H., & Rosen, O. M. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 731.
Ford, D. L., & Omachi, A. (1972) *Biochim. Biophys. Acta 279*, 587.
Graff, J. C., Wohlhueter, R. M., & Plagemann, P. G. W. (1977) *J. Biol. Chem. 252*, 4185.
Guthrow, C. E., Jr., Allen, J. E., & Rasmussen, H. (1972) *J. Biol. Chem. 247*, 8145.
Hanahan, D. J., & Ekholm, J. E. (1974) *Methods Enzymol. 30* (Part A), 168.
Holman, G. D. (1978) *Biochim. Biophys. Acta 508*, 174.
Kant, J. A., & Steck, T. L. (1973) *Biochem. Biophys. Res. Commun. 54*, 116.
Keely, S. L., Corbin, J. D., & Park, C. R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1501.
Kury, P. G., & McConnell, H. M. (1975) *Biochemistry 14*, 2798.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Plagemann, P. G. W., Graff, J. C., & Wohlhueter, R. M. (1977) *J. Biol. Chem. 252*, 4191.
Plut, D. A., Hosey, M. M., & Tao, M. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 320.
Rasmussen, H., Lake, W., & Allen, J. E. (1975) *Biochim. Biophys. Acta 411*, 63.
Rubin, C. S., & Rosen, O. M. (1973) *Biochem. Biophys. Res. Commun. 50*, 421.
Rubin, C. S., Erlichman, J. S., & Rosen, O. M. (1972) *J. Biol. Chem. 247*, 6135.
Rubin, C. S., Rosenfeld, R. D., & Rosen, O. M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3735.
Ryan, W. L., & Hendrick, M. L. (1974) *Adv. Cyclic Nucleotide Res. 4*, 81.
Sachs, J. R., Knauf, P. A., & Dunham, P. B. (1975) in *The Red Blood Cell* (Surgenor, D. M., Ed.) Vol. II, p 613, Academic Press, New York.
Shwoch, G., & Passow, H. (1973) *Mol. Cell. Biochem. 2*, 197.
Tao, M. (1974) *Methods Enzymol. 38* (Part C), 155.
Taverna, R. D., & Langdon, R. G. (1973) *Biochim. Biophys. Acta 323*, 207.
Thomas, E. L., King, L. E., Jr., & Morrison, M. (1979) *Arch. Biochem. Biophys. 196*, 459.
Zail, S. S., & Van den Hoek, A. K. (1975) *Biochem. Biophys. Res. Commun. 66*, 1078.
Zar, J. H. (1974) in *Biostatistical Analysis*, p 121, Prentice-Hall, Englewood Cliffs, NJ.

Kinetic Studies on the Dissociation of Adenosine Cyclic 3′,5′-Monophosphate from the Regulatory Subunit of Protein Kinase from Rabbit Skeletal Muscle†

Vincent Chau, Laura C. Huang,‡ Guillermo Romero, Rodney L. Biltonen, and Ching-hsien Huang*

ABSTRACT: The exchange rate of unlabeled adenosine 3′,5′-monophosphate (cAMP) with labeled [^{3}H]cAMP in the dimeric regulatory subunit–cAMP complex of cAMP-dependent protein kinase, type I, purified from rabbit skeletal muscle is described by using the equilibrium isotope exchange technique. Results indicate that the rate of exchange carried out in the absence of the catalytic subunit (C) is rather slow with a half-life of ~870 s. This slow exchange rate is not affected by the presence of MgATP (50 μM). However, when both MgATP (50 μM) and C (1–13 nM) are present, the rate of isotope exchange is observed to increase markedly. Furthermore, less than stoichiometric amounts of C are required for the increase in the rate of cAMP exchange, indicating that the effect of C on the rate enhancement is a catalytic process. These results indicate that in the presence of MgATP, a ternary complex between C and regulatory subunit–cAMP complex must be formed, and a dynamic equilibrium between the ternary complex and its dissociable species must be reached within seconds. On the basis of our kinetic data, it is proposed that the formation of this ternary complex intermediate allows the rapid activation or the inactivation of cAMP-dependent protein kinase following changes in the cellular cAMP levels.

Adenosine 3′,5′-monophosphate (cAMP)[1] dependent protein kinase from mammalian skeletal muscle is a relatively well understood tetrameric enzyme composed of two pairs of dissimilar subunits, R_2C_2. Its function is to serve as a receptor for cAMP and to phosphorylate one or more specific serine or threonine residues in a number of protein or enzyme substrates (Krebs, 1972; Rubin & Rosen, 1975; Cohen, 1978). Since cAMP is a general and common "second messenger" for many hormones and since phosphorylation of enzymes often results in the alteration of many enzymatic activities, cAMP-dependent protein kinase thus plays an important role in the hormonal regulation of glycogen metabolism in mam-

† From the Departments of Biochemistry and Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22908. *Received July 10, 1979.* Portions of this paper are derived from the dissertation of V.C. presented in partial fulfillment of the requirements for a Ph.D. degree in Biophysics, University of Virginia (1979). A preliminary account of this work has been presented at the ASBC/AAI Meeting, June 4–8, 1978, Atlanta, GA. This research was supported in part by U.S. Public Health Service Grants GM-22430, GM-20637, and GM-17452.

‡ The recipient of Career Development Award 1K04-AM-00212 from the U.S. Public Health Service.

[1] Abbreviations used: cAMP, adenosine cyclic 3′,5′-monophosphate; R_2, dimeric regulatory subunit of cAMP-dependent protein kinase; C, catalytic subunit monomer of cAMP-dependent protein kinase.

0006-2960/80/0419-0924$01.00/0 © 1980 American Chemical Society

malian skeletal muscle (Cohen, 1978). The mode of action of cAMP on the protein kinase is well understood in a phenomenological way as described by the dissociation model (Beavo et al., 1975). In this model, two molecules of cAMP bind to one molecule of the type I cAMP-dependent protein kinase and promote the dissociation of the inactive holoenzyme (R_2C_2) into a dimeric regulatory subunit–cAMP complex $(R{\cdot}cAMP)_2$ and two molecules of active catalytic subunits (2C). Although the dissociation of the holoenzyme in the presence of cAMP can be attributed to the high affinity of the regulatory subunit for cAMP, the detailed mechanism of this dissociation reaction remains to be established.

Since protein kinase has a multisubunit structure, a large number of reaction mechanisms for the mode of activation of the enzyme by cAMP is theoretically possible (Ogez & Segel, 1976; Boeynaemes & Dumont, 1977). Two major models have dominated the discussion of the protein kinase activation reaction (Walsh et al., 1972; Walsh & Krebs, 1973). In the first model, it is assumed that the holoenzyme in the absence of cAMP is in equilibrium with its subunits, $R_2C_2 \rightleftharpoons R_2 + 2C$, and that cAMP perturbs the preexisting equilibrium toward the dissociated species by binding to R_2 with high affinity (Ogez & Segel, 1976; Schwechheimer & Hofmann, 1977; Hoppe et al., 1978a). In the second model, it is assumed that cAMP first binds to the holoenzyme, thereby eliciting a conformational change in the regulatory subunit. As a result of this conformational change, the interaction between the regulatory and catalytic subunits is weakened and, consequently, the inactive holoenzyme readily dissociates to form the active C and the regulatory subunit–cAMP complex (Huang & Huang, 1975). In this second model, the preequilibrium between the holoenzyme and its subunits, which may or may not exist appreciably at equilibrium, is not of primary concern; emphasis is placed on the change in the subunit–subunit interactions upon cAMP binding.

In this communication, we report a kinetic study on the dissociation of cAMP from the regulatory subunit of cAMP-dependent protein kinase, type I, isolated from rabbit skeletal muscle by using equilibrium isotope exchange techniques. Our results indicate that the dissociation of cAMP from the regulatory subunit–cAMP complex in the presence of MgATP is greatly accelerated by the addition of the catalytic subunit, which implies that the rapid dissociation must proceed via an intermediate; this intermediate is a ternary complex of cAMP, the dimeric regulatory subunit, and the catalytic subunit. These data argue in favor that the protein–ligand interaction in regulatory subunit–cAMP is markedly weakened by a conformation change of the regulatory subunit elicited by binding of C. The converse situation that a conformation change of R_2C_2 elicited by the binding of cAMP promotes the dissociation of the holoenzyme is thus strongly implied.

Experimental Section

Materials

[γ-^{32}P]ATP was prepared according to the method of Glynn & Chappell (1964), utilizing $^{32}P_i$ from New England Nuclear. Histones type IIA and cAMP were obtained from Sigma Chemical Co. Nitrocellulose filter disks (Schleier and Schuler BA85, 25 mm, 0.45-μm pore size) were obtained from Arthur H. Thomas Chemical Co. All other chemicals used are standard reagent grade.

Methods

Preparation of Protein Kinase and Its Subunits. cAMP-dependent protein kinase, type I, from rabbit skeletal muscle was purified according to the procedure of Huang & Huang (1975), and the enzymatic activity was assayed as described previously (Huang, 1974). The catalytic subunit was prepared according to the procedure of Matsuo et al. (1978).

The regulatory subunit–cAMP complex was prepared according to the following procedure. A sample of the purified holoenzyme was preincubated with 1 mM cAMP at 4 °C for 1 h in 50 mM Tris-HCl buffer (pH 7.3), 2 mM EDTA, and 10 mM 2-mercaptoethanol (buffer A). The protein sample was then applied to a DE-52 column (2.5 × 20 cm) previously equilibrated with buffer A, followed by successive washing with 2 bed volumes of buffer A. The regulatory subunit–cAMP complex was then eluted from the column with buffer A containing, in addition, 0.2 M NaCl.

The purified holoenzyme, the cAMP–regulatory subunit complex, and the catalytic subunit were dialyzed separately against 0.02 M phosphate buffer (pH 7.0) containing 0.1 M NaCl and 1 mM dithiothreitol (DTT) at 4 °C. Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

The purified enzyme and its subunits were found to be highly homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Huang & Huang, 1975). Moreover, no kinase activity was detectable with the regulatory subunit–cAMP complex, indicating that the isolated complex was not contaminated by C.

Preparation of $R_2{\cdot}([^3H]cAMP)_2$ Complex. The $R_2{\cdot}([^3H]cAMP)_2$ complex was prepared by equilibration of the purified $R_2{\cdot}cAMP_2$ complex with 1×10^{-7} M [^{3}H]cAMP at 30 °C in buffer C (0.02 M phosphate, pH 7.0, containing 0.1 M NaCl, 10 mM $MgCl_2$, and 1 mM DTT). The amount of radioactivity incorporated into the complex was determined by the filtration procedure of Gilman (1970). The nitrocellulose filter disk was washed 3 times with 2 mL of ice-cold buffer C, and the washed filter disk was then dissolved in 1 mL of 2-methoxyethanol in a 7-mL counting vial. Subsequently, a 5-mL aliquot of PPO–POPOP–toluene scintillation counting solution was added to the vial, and the radioactivity was determined in a liquid scintillation counter.

Equilibrium Isotope Exchange. Unless noted otherwise, all equilibrium isotope exchange reactions were carried out in buffer C at 30 °C. The reaction was initiated by mixing a 3-mL buffer solution containing the $R_2{\cdot}([^3H]cAMP)_2$ complex, having a radioactivity of ~30 000 cpm/mL, with a buffer solution containing unlabeled cAMP. The reaction mixture was then incubated in a constant temperature bath. At specified times, 500-μL aliquots were withdrawn and immediately passed through a nitrocellulose filter disk. The filter disk was then washed, and the radioactivity remaining bound to the filter disk was counted as described previously.

The isotope exchange reaction in the presence of the catalytic subunit, C, was carried out in a rapid filtration apparatus which was constructed by connecting a rapid mixing device to a filtration unit. A detailed description of the apparatus was described elsewhere (Chau, 1979). The apparatus was thermostated at 30 °C. At zero time, 0.5 mL of a buffer solution containing the $R_2{\cdot}([^3H]cAMP)_2$ complex (20 000 cpm/mL) was rapidly mixed with 0.5 mL of a buffer solution containing unlabeled cAMP and C. The reaction mixture was aged on top of a nitrocellulose filter disk in the filtration unit and filtered after a predetermined time interval at a rate of ~5 mL/s. The uncertainty of each time point was ±0.2 s.

Analysis of the Kinetic Data. The first-order rate constant of the isotope exchange, k_{obsd}, was obtained by a nonlinear least-squares fitting of the data to the equation

$$\mathrm{cpm}_t = \mathrm{cpm}_0 \exp[-(k_{obsd}t)] + \mathrm{cpm}_\infty \qquad (1)$$

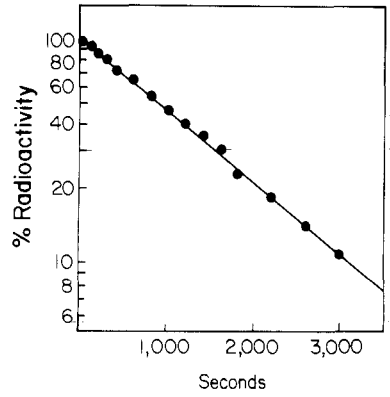

FIGURE 1: Semilogarithmic plot of the time course of the exchange of the [^{3}H]cAMP in the absence of the catalytic subunit. The reaction mixture at time zero contained 10 nM labeled cAMP–regulatory subunit complex and 1 μM cAMP. The solid line is a calculated curve obtained by fitting the data according to eq 1. The first-order rate constant (k_{obsd}) obtained from the nonlinear least-squares fitting was $7.9 \times 10^{-4} \pm 0.00004$ s^{-1}.

where cpm_t, cpm_0, and cpm_∞ are the radioactivity in counts per minute retained by the filter disk at reaction time t, zero, and infinity, respectively.

Results

Equilibrium Isotope Exchange. When a solution containing the R_2·([^{3}H]cAMP)$_2$ complex is mixed with excess unlabeled cAMP, the amount of [^{3}H]cAMP in the complex is observed to decrease with increasing time. This decrease is a result of the exchange of bound [^{3}H]cAMP in the complex with unlabeled cAMP in the reaction mixture. Since the amount of unlabeled cAMP is always in large excess over that of [^{3}H]-cAMP (greater than 50-fold), the exchange of labeled [^{3}H]-cAMP into the unlabeled R_2·cAMP$_2$ complex is negligible at all times. If the two cAMP binding sites are equivalent with respect to the dissociation rate constant of cAMP, the rate equation for the exchange is given by

$$-d(R\cdot[^3H]cAMP)/dt = k_{obsd}(R\cdot[^3H]cAMP) \quad (2a)$$

or

$$(R\cdot[^3H]cAMP) = (R\cdot[^3H]cAMP)_0 \exp(-k_{obsd}t) \quad (2b)$$

where (R·[^{3}H]cAMP) and (R·[^{3}H]cAMP)$_0$ are the monomeric concentrations of the complex containing [^{3}H]cAMP at time t and time zero, respectively, and k_{obsd} is, in this case, the dissociation rate constant of cAMP. The amount of [^{3}H]-cAMP in the complex at a given reaction time interval is measured by counting the radioactivity retained on the nitrocellulose filter disks as described under Experimental Section. Since only the bound cAMP in the complex is retained, the measured radioactivity can be used to calculate the amount of [^{3}H]cAMP in the complex. A semilogarithmic plot of the data, given in Figure 1, clearly shows that the exchange follows a single-exponential decay. An average value of k_{obsd} obtained from three separate exchange experiments is estimated to be $(8 \pm 0.4) \times 10^{-4}$ s^{-1}. As expected, the value of k_{obsd} is found to be independent of the concentrations of the reactants (10–500 μM cAMP; 5–10 nM R_2·cAMP$_2$) under the condition that the amount of unlabeled cAMP is always in excess over that of [^{3}H]cAMP. The addition of 50 μM ATP or the removal of Mg^{2+} from the reaction mixture did not affect the value of k_{obsd}.

Effect of the Catalytic Subunit on the Exchange Rate. Addition of small amounts of the catalytic subunit, C, together with 50 μM MgATP to the exchange reaction mixture markedly accelerates the rate of the exchange. It can be seen from Figure 2 that the exchange in the presence of C again follows first-order kinetics. A value of 0.17 ± 0.01 s^{-1} is calculated for k_{obsd}, the first-order rate constant for this exchange. It should be emphasized that all [^{3}H]cAMP initially present in the R_2·([^{3}H]cAMP)$_2$ complex is exchanged at this accelerated rate. Since the molar ratio of C to R_2·([^{3}H]-cAMP)$_2$ at time zero is ~1:12, the observed single-exponential decay in Figure 2 means that 1 mol of the catalytic subunit is able to facilitate the exchange of cAMP for several moles of R_2·([^{3}H]cAMP)$_2$ during the time course of the experiment. Therefore, C behaves as a catalyst in the accelerated exchange. Figure 3 further shows that k_{obsd} is linearly dependent upon the concentration of C over a concentration range of 1.6–12.8 nM. Because the exchange is carried out with the reaction mixture incubated in the presence of the filter disk, it is necessary to rule out the possibility that the observed increase in the exchange rate may be due to the presence of the filter disk. For this purpose, the exchange in the absence of C has been carried out by using the filtration apparatus. The exchange over a time interval of 2 min can be qualitatively described by a k_{obsd} of 8×10^{-4} s^{-1} as measured for the previous exchange (data not shown). Moreover, the dependence of k_{obsd} on the concentration of C indicates that the accelerated exchange is mediated by C rather than an artifact.

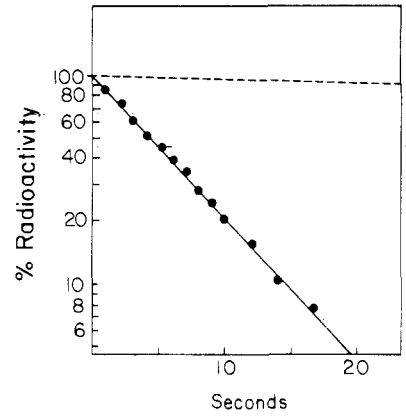

FIGURE 2: Semilogarithmic plot of the time course of the exchange of the [^{3}H]cAMP in the presence of the catalytic subunit. The reaction mixture contained 0.15 μM labeled cAMP–regulatory subunit complex, 50 μM cAMP, and 12.8 nM C. The solid line is a calculated curve obtained by fitting the data according to eq 1. The value of k_{obsd} obtained from the fitting was 0.17 ± 0.01 s^{-1}. The dashed line is the calculated one for the decay curve based on the exchange data given in Figure 1.

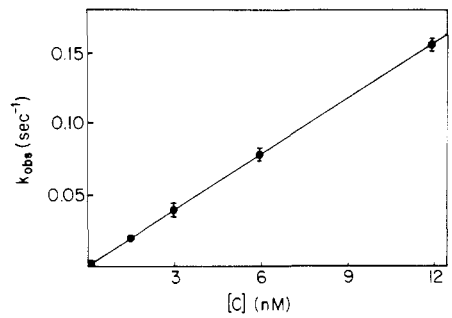

FIGURE 3: Dependence of k_{obsd} on the concentration of the catalytic subunit. The concentrations of C are 1.6, 3.2, 6.4, and 12.8 nM, and the corresponding values of k_{obsd} are 0.022 ± 0.002, 0.040 ± 0.003, 0.081 ± 0.01, and 0.17 ± 0.01 s^{-1}, respectively. The solid line is a calculated curve using parameters obtained from a least-squares fit of the data to a straight line.

The effect of the catalytic subunit on the exchange rate is found to be dependent on the presence of MgATP. Removal of either Mg^{2+} or ATP from the reaction mixture abolishes

the observed increase in the exchange rate (data not shown). In agreement with this observation, we have found that the exchange carried out with a reaction mixture containing 0.1 μM holoenzyme which has been previously equilibrated with [^{3}H]cAMP for 5 min at 30 °C has a rate similar to that observed for purified regulatory subunit in the absence of C.

Discussion

In this paper, we present results of our kinetic studies on the dissociation of cAMP from the regulatory subunit–cAMP complex of cAMP-dependent protein kinase (type I) purified from rabbit muscle. The polypeptide chain of the regulatory subunit–cAMP complex is highly homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The dissociation reaction is followed by the exchange of bound [^{3}H]cAMP in the regulatory subunit–cAMP complex with unlabeled cAMP in solution.

The data obtained from equilibrium isotope exchange experiments indicate that the rate of exchange of the [^{3}H]cAMP of regulatory subunit–[^{3}H]cAMP complexes with excess unlabeled cAMP in solution containing MgATP is relatively slow with a half-life of ~870 s (Figure 1). This slow exchange is not unexpected since the regulatory subunit of cAMP-dependent protein kinase binds cAMP rather tightly with an intrinsic dissociation constant of ~3 nM (Schwechheimer & Hofmann, 1977). It should be noted that the exchange reaction follows a single exponential (Figure 1), indicating that the two cAMP binding sites in the regulatory subunit are equivalent in terms of their rates of dissociation; otherwise, a biphasic kinetic would be observed. In contrast to the present result, Døskeland (1978) reported biphasic kinetics for the dissociation of cAMP from bound protein kinase at 37 °C over a time range of 2400 s. Our measured value of k_{obsd} corresponds to the slow phase observed by Døskeland (1978). It is not clear at the present time what may have caused the discrepancy. We do, however, want to emphasize that the single-exponential decay shown in Figure 1 is present in all of our studies.

The rate of the isotope exchange is observed to increase markedly by the addition of a catalytic amount of C in the presence of MgATP as indicated in Figure 2. This stimulating effect can best be explained by postulating (1) the formation of a ternary complex among C, R_2, and cAMP and (2) a rapid equilibrium between the ternary complex and its dissociable species.

It has been reported by Brostrom et al. (1971) that [^{3}H]-cAMP can be partially released from the R_2·([^{3}H]cAMP)$_2$ complex in the presence of C. Moreover, if MgATP is present, the amount of [^{3}H]cAMP released from the complex is further increased by C. These results are taken to imply the existence of a dynamic equilibrium among the holoenzyme, cAMP, R_2·cAMP$_2$, and C (Brostrom et al., 1971). Wilchek et al. (1971) have reported that MgATP stimulates the rate of the exchange of cAMP in solution with bound [^{3}H]cAMP in the R_2·([^{3}H]cAMP)$_2$ complex prepared by incubating the crude holoenzyme with [^{3}H]cAMP. It is thus proposed that the stimulating effect of MgATP may be mediated by C (Wilchek et al., 1971). Finally, it should be mentioned that the formation of a short-lived ternary complex containing R, C, and cAMP has also been suggested independently by Armstrong & Kaiser (1978) and Tsuzuki & Kiger (1978) using protein kinase from bovine heart and *Drosophila melanogaster* embryos, respectively.

In the present study the isotope exchange reactions were carried out with highly purified regulatory subunit–[^{3}H]cAMP complex and excess unlabeled cAMP in the presence of various amounts of C. It is apparent from the linear dependence of k_{obsd}, the observed first-order rate constant of the exchange, on the concentration of C (Figure 3) that the accelerated exchange rate must be mediated by C. Furthermore, in these studies the initial concentration of R_2·([^{3}H]cAMP)$_2$ complex is always kept in excess of C (12–100-fold excess) and the observed exchange is invariably found to follow a single-exponential decay, indicating that the effect of C on the rate enhancement is a catalytic process. Since the observed accelerated process is mediated by C, a ternary complex must be formed between C and R_2·cAMP$_2$. Also, the enhanced rate of the exchange by C means that the rate of dissociation of cAMP from the bound cyclic nucleotide–regulatory subunit complex in the presence of C is considerably faster than that in the absence of C. Furthermore, since the C-mediated exchange is a catalytic process, the ternary complex must have turned over during the time course of the exchange reactions; consequently, the rate of dissociation of either C or cAMP or both C and cAMP from the ternary complex must proceed with a rate equal to or faster than the observed rate of the isotope exchange.

The catalytic effect of C on the dissociation rate of cAMP from R_2·cAMP$_2$ complex is shown to depend on the presence of MgATP. From binding studies, Beavo et al. (1975) have shown that MgATP binds very strongly to the holoenzyme with a K_d of ~50 nM. Also, binding of MgATP leads to a shift in the K_a, the concentration of cAMP required for half-maximal binding to the holoenzyme, to a higher value, indicating that the binding of MgATP stabilizes the holoenzyme (Beavo et al., 1975). Their results obtained from the equilibrium studies cannot be used directly to interpret the effect of MgATP on the rate of cAMP dissociation observed in the study. However, since the catalytic effect of C is dependent on MgATP, it is reasonable to assume that MgATP is also present in the ternary complex, although the role of MgATP is not immediately apparent. One possibility is that MgATP may have stabilized the ternary complex to such an extent that the concentration of the ternary complex has increased appreciably. Since the value of k_{obsd} depends on the concentration of the ternary intermediate (Chau, 1979), an increase in k_{obsd} is thus expected when the ternary complex is stabilized by MgATP. An alternative possibility is that the rapid dissociation of either C or cAMP from the ternary complex can only occur upon MgATP binding. These possibilities remain to be investigated. Taking both the effect of MgATP on the rate of cAMP dissociation and the maximum number of MgATP binding sites per holoenzyme to be two (Hoppe et al., 1978b) into consideration, the rapid equilibrium between the ternary complex and its dissociated species can be represented as

$$R_2C_2MgATP_2 + 2cAMP \rightleftharpoons \text{(ternary complex)}MgATP_2 \rightleftharpoons R_2\cdot cAMP_2 + 2C + 2MgATP$$

The activation of cAMP-dependent protein kinase by cAMP has been proposed by several investigators to follow the "sequential mechanism" (Ogez & Segel, 1976; Schweichheimer & Hofmann, 1977; Hoppe et al., 1978a):

$$\begin{array}{ccc} R_2C_2 & \rightleftharpoons & R_2 + 2C \\ 2cAMP \downarrow\uparrow & & \uparrow\downarrow 2cAMP \\ & R_2\cdot cAMP_2 & \end{array}$$

In this mechanism, the dissociation of R_2 and 2C from the inactive holoenzyme precedes the binding of cAMP by R_2 in the activation process. Likewise, in the reversed inactivation

process, dissociation of cAMP from R_2·cAMP$_2$ precedes the association of the dissimilar subunits into the holoenzyme. From our isotope exchange experiments (Figure 1), it is evident that the rate of dissociation of cAMP from R_2·cAMP$_2$ in the absence of C is rather slow with a half-life of ~870 s. According to the sequential mechanism, the inactivation process must, therefore, also be slow with a half-life equal to or slower than ~870 s, depending on whether the dissociation of cAMP from R_2·cAMP$_2$ is a rate-limiting step or not. The rate of the dissociation of the type I holoenzyme into its dissimilar subunits has recently been reported by Builder et al. (1979) to be rather slow with a rate constant of 2.4×10^{-3} s^{-1}. Consequently, the activation process according to the sequential mechanism is also slow. The point to be made is that the activation and inactivation of cAMP-dependent protein kinase must lag behind the rise and fall of cellular concentration of cAMP in a time coordinate if the activation and inactivation of the enzyme indeed follow the sequential mechanism.

Based on our kinetic data which indicate clearly the existence of a ternary complex formed between C and R_2·cAMP$_2$, we suggest that the conversion of cAMP-dependent protein kinase from an active form to an inactive form proceeds in the presence of excess MgATP via a mechanism involving the ternary complex as follows:

$$R_2C_2 \text{ (inactive)} + 2\text{cAMP} \rightleftharpoons \text{(ternary complex)} \rightleftharpoons R_2\text{·cAMP}_2 + 2\text{C (active)}$$

The fast dissociation of cAMP and C from the ternary complex intermediate as shown in this study (Figures 2 and 3) allows the interconversion of protein kinase between an active and an inactive form to proceed much more rapidly. One major difference between the proposed mechanism and the sequential mechanism is, therefore, the response time of the enzyme in terms of activities toward the changes in cellular concentration of cAMP. A similar conclusion has also been drawn by Builder et al. (1979) and Krebs & Beavo (1979).

It is well-known now that the cellular level of cAMP increases immediately following hormonal stimuli. It is also known that cAMP can be converted to 5′-AMP by the catalytic action of phosphodiesterase, but the conversion does not occur when cAMP is bound to protein kinase (Brostrom et al., 1971). An important metabolic question is how the cellular level of cAMP falls back rapidly to the basal level after hormonal activation is over. The response of rapid dissociation of cAMP from R_2·cAMP$_2$ complex upon C binding observed in this study may offer a means of controlling the cellular cAMP level. We suggest that immediately after the dissociation of holoenzyme caused by the high level of cellular cAMP, the active C exists mainly as an enzyme–substrate complex to catalyze the phosphorylation of its protein substrate. When the phosphorylation has stopped, the catalytic subunit will be free, which, in turn, interacts with R_2·cAMP$_2$ to promote the rapid dissociation of cAMP. It is through this mechanism that phosphodiesterase can reduce the level of cellular cAMP rapidly, and at the same time inactive holoenzyme is regenerated.

After completion of this paper, Weber & Hilz (1979) reported that the stoichiometry of cAMP binding to R_2 isolated from type I protein kinase is four instead of two. However, this change in stoichiometry will not affect our conclusions of the existence of the ternary complex and the rapid equilibrium between the ternary complex and its dissociated species.

Acknowledgments

We thank B. Turner for his assistance in constructing the rapid filtration apparatus.

References

Armstrong, R. N., & Kaiser, E. T. (1978) *Biochemistry 17*, 2840–2845.

Beavo, J. A., Bechtel, P. J., & Krebs, E. G. (1975) *Adv. Cyclic Nucleotide Res. 5*, 241–251.

Boeynaemes, J. M., & Dumont, J. E. (1977) *Mol. Cell. Endocrinol. 7*, 275–295.

Brostrom, C. O., Corbin, J. D., King, C. A., & Krebs, E. G. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2444–2447.

Builder, S. E., Beavo, J. A., & Krebs, E. G. (1979) *J. Supramol. Struct., Suppl. 3*, 26.

Chau, V. (1979) Ph.D. Dissertation, University of Virginia.

Cohen, P. (1978) *Curr. Top. Cell. Regul. 14*, 117–196.

Døskeland, S. O. (1978) *Biochem. Biophys. Res. Commun. 83*, 542–549.

Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 305.

Glynn, I. M., & Chappell, J. B. (1964) *Biochem. J. 90*, 147–149.

Hoppe, J., Rieke, E., & Wagner, K. G. (1978a) *Eur. J. Biochem. 83*, 411–417.

Hoppe, J., Lawaczeck, R., Rieke, E., & Wagner, K. G. (1978b) *Eur. J. Biochem. 90*, 585–593.

Huang, L. C. (1974) *Biochim. Biophys. Acta 358*, 281–288.

Huang, L. C., & Huang, C. (1975) *Biochemistry 14*, 18–24.

Krebs, E. G. (1972) *Curr. Top. Cell. Regul. 5*, 99–133.

Krebs, E. G., & Beavo, J. A. (1979) *Annu. Rev. Biochem. 48*, 923–959.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Matsuo, M., Huang, C., & Huang, L. C. (1978) *Biochem. J. 173*, 441–447.

Ogez, J. R., & Segel, I. H. (1976) *J. Biol. Chem. 251*, 4551–4556.

Rubin, C. S., & Rosen, O. M. (1975) *Annu. Rev. Biochem. 44*, 831–887.

Schwechheimer, K., & Hofmann, F. (1977) *J. Biol. Chem. 252*, 7690–7696.

Tsuzuki, J., & Kiger, J. A. (1978) *Biochemistry 17*, 2961–2970.

Walsh, D. A., & Krebs, E. G. (1973) *Enzymes, 3rd Ed. 8*, 55–581.

Walsh, D. A., Brostrom, C. O., Brostrom, M. A., Chen, L., Corbin, J. D., Reimann, E., Soderling, T. R., & Krebs, E. G. (1972) *Adv. Cyclic Nucleotide Res. 1*, 33–45.

Weber, W., & Hilz, H. (1979) *Biochem. Biophys. Res. Commun. 90*, 1073–1081.

Wilchek, M., Salomen, Y., Lowe, M., & Selinger, Z. (1971) *Biochem. Biophys. Res. Commun. 45*, 1177–1184.