Chemical cross-linking of cyclic AMP-dependent protein kinase and its dissimilar subunits

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Previous kinetic studies have demonstrated that the activation of cyclic AMP-dependent protein kinase by cyclic AMP involves the formation of a ternary complex of cyclic AMP, the regulatory subunit (R) and the catalytic subunit (C). It is suggested that only this ternary complex breaks down to liberate the enzymically active catalytic subunit. We have performed cross-linking experiments with the holoenzyme and its dissimilar subunits in the presence of MgATP and various concentrations of cyclic AMP. Results from these cross-linking studies indicate that regulatory subunits exist as dimers in the native form. Moreover, dissociation of the holoenzyme or the reconstituted enzyme is promoted by cyclic AMP, and the effect of MgATP is to stabilize the enzyme in the tetrameric form. The success in cross-linking the regulatory and the catalytic subunits of protein kinase with the lysine-specific bifunctional cross-linking reagent dimethyl suberimide may be attributed to the presence of a large number of lysine residues in the enzyme.

Cyclic AMP-dependent protein kinase plays a central role in the hormonal regulation of cellular metabolism; its function is to serve as a receptor for the second messenger cyclic AMP and, on the binding of cyclic AMP, to catalyse the phosphorylation of a number of protein or enzyme substrates (Cohen, 1978; Larner *et al.*, 1978; Krebs & Beavo, 1979). Several investigators have proposed, on the basis of their kinetic data, that the activation of the holoenzyme (R_2C_2) by cyclic AMP involves the formation of a ternary complex of cyclic AMP, the regulatory subunit (R) and the catalytic subunit (C), which then breaks down to liberate the enzymically active C subunit (Armstrong & Kaiser, 1978; Tsuzuki & Kiger, 1978; Chau *et al.*, 1980; Builder *et al.*, 1980). The structural evidence for the existence of the ternary complex, however, is still lacking. As a first step to demonstrate structurally the possible existence of the ternary complex, we designed experiments to study the chemical cross-linking of the enzyme.

In the present study we investigated the structural features of cyclic AMP-dependent protein kinase (type I) from rabbit skeletal muscle by using the lysine-specific bifunctional cross-linking reagent

dimethyl suberimide. It is shown that high-molecular-weight polypeptide chains corresponding to R_2C and R_2C_2 can be identified by SDS/polyacrylamide-gel electrophoresis after the holoenzyme has been incubated with cyclic AMP. The amount of these high-molecular-weight species, however, decreases significantly with increasing concentration of cyclic AMP, confirming that cyclic AMP promotes the dissociation of the holoenzyme. Cross-linking of the reconstituted enzyme, made from highly purified dissimilar subunits, in the presence of various concentrations of cyclic AMP and MgATP was also investigated; our results suggest that MgATP favours the association of dissimilar subunits into the tetrameric holoenzyme and that cyclic AMP inhibits the association.

Materials and methods

Procedures for the isolation and purification of cyclic AMP-dependent protein kinase (type I) and its catalytic subunit from rabbit skeletal muscle are described elsewhere (Huang & Huang, 1975; Matsuo *et al.*, 1980). The regulatory subunit of type I protein kinase was purified by using cyclic AMP-Sepharose affinity matrix as described by Weber *et al.* (1979). The regulatory subunit was washed off the cyclic AMP-Sepharose affinity column with the

Abbreviation used: SDS, sodium dodecyl sulphate.

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fluorescent cyclic AMP analogue 1,*N*⁶-ethano-adenosine cyclic 3':5'-monophosphate (approx. 5 mM), and then subjected to gel-filtration chromatography. SDS/polyacrylamide-gel electrophoresis was performed in 0.2 M-Tris/0.2 M-acetate buffer, pH 6.4, containing 0.1% SDS on 5.3%-polyacrylamide gel. Calibration marker proteins for SDS/polyacrylamide-gel electrophoresis were obtained from Boehringer Mannheim Biochemicals, including *Escherichia coli* RNA polymerase, bovine serum albumin and soya-bean trypsin inhibitor. In addition to the general procedure in which enzyme or its subunits were incubated with 2-mercaptoethanol (1.7%, v/v) in the presence of 1.7% (w/v) SDS at 100°C for 10 min before electrophoresis on SDS-containing polyacrylamide gels, samples containing the holoenzyme were preincubated with 1.7% SDS in the absence or in the presence of various concentrations of thiol reducing agents at room temperature for 5 min to examine the effect of 2-mercaptoethanol on the electrophoretic mobility of protein kinase.

The bifunctional cross-linking reagent dimethyl suberimide was obtained from Pierce Chemical Co. The cross-linking of the enzyme or its subunits with dimethyl suberimide was performed in 0.2 M-triethanolamine/HCl buffer, pH 8.35, as described by Davies & Stark (1970). Before the cross-linking with dimethyl suberimide, the holoenzyme or its subunits was washed over an Amicon ultrafilter disc with triethanolamine/HCl buffer. An appropriate concentration of cyclic AMP was added to the buffered enzyme solution. After 10 min incubation at 37°C, MgATP or buffer was added, and the mixture was again incubated at 37°C for an additional 20 min. Finally, dimethyl suberimide was added and the reaction mixture was incubated at 4°C overnight. For instance, the results shown in Fig. 2 were obtained as follows. The enzyme solution (18 µl; 0.5 mg/ml) containing various amounts of

cyclic AMP with or without MgATP (1 mM) was incubated with 2 µl of dimethyl suberimide solution (59 mg/ml) in 0.2 M-triethanolamine/HCl buffer, pH 8.5. The cross-linking reaction was stopped by adding 4 µl of 10% SDS solution containing 10% 2-mercaptoethanol. After being heated at 100°C for 10 min, the sample was then subjected to SDS/polyacrylamide-gel electrophoresis. After staining with 0.05% Coomassie Blue and destaining in 25% methanol/7.5% acetic acid containing 0.0004% Coomassie Blue, gels were scanned at 550 nm with a Gilford gel scanner. When both R and C subunits were subjected to cross-linking experiments. The R subunits were first incubated at 37°C with cyclic AMP and MgATP as described above. Before the final incubation with dimethyl suberimide at 4°C overnight, C subunits were added to the reaction mixture of R subunits, cyclic AMP and MgATP in 0.2 M-triethanolamine/HCl buffer, pH 8.35, and incubated for 30 min at 37°C.

Results

Holoenzyme

The SDS/polyacrylamide-gel-electrophoretic pattern of the cyclic AMP-dependent protein kinase preincubated with 1.7% SDS solution in the presence of an excess of thiol reducing agent, 2-mercaptoethanol (1.29 M), before the electrophoresis shows two distinct polypeptide bands with equal intensities. The electrophoretic mobilities of these two bands correspond to polypeptide chains with apparent *M_r* 48 000 and 42 000; they are in accordance with those reported for the monomeric forms of the regulatory subunit and the catalytic subunit respectively of cyclic AMP-dependent protein kinase (type I) from rabbit skeletal muscle (Dills *et al.*, 1975). The SDS/polyacrylamide-gel-electrophoretic pattern of the holoenzyme can be modified by omitting the thiol reducing agent. In this case the

Fig. 1. Scanning profiles of SDS/polyacrylamide-gel electrophoretograms of the regulatory subunit (R), the catalytic subunit (C), the reconstituted enzyme and the holoenzyme of cyclic AMP-dependent protein kinase

(a) shows scanning profiles 1 and 2 resulting from SDS/polyacrylamide-gel electrophoresis of R subunit (9.4 µg) and of C subunit (5.3 µg) respectively. (b) shows gel scans of electrophoretograms of R subunit (curve 1) and C subunit (curve 2) after individual subunits were incubated with MgATP (—) or buffer (----) followed by cross-linking with dimethyl suberimide. The protein concentrations were identical with those used in (a). (c) shows gel scans of reconstituted protein kinase obtained under various conditions. The regulatory subunit (6 µl; 1.56 mg/ml) was preincubated with 2 µl of 40 µM-cyclic AMP (.....) or 2 µl of 0.2 M-triethanolamine buffer (— and ----) for 10 min at 37°C and then mixed with 2 µl of 10 mM-MgATP (..... and —) or 2 µl of 0.2 M-triethanolamine buffer (----). After incubation for 20 min at 37°C, 6 µl of C subunit (0.88 mg/ml) was added, followed by cross-linking with 2 µl of dimethyl suberimide (53 mg/ml). (d) shows the gel scans of native holoenzyme treated with dimethyl suberimide. Before the cross-linking, the holoenzyme (0.73 mg/ml) was incubated with zero (—) or 76.9 µM-cyclic AMP (----). The final concentration of dimethyl suberimide in the reaction mixture is 5.9 mg/ml, and each gel contains 14.6 µg of protein. The molecular weight of each peak was determined from the plot of log *M_r* versus relative mobility of protein markers in the SDS/polyacrylamide gel after electrophoresis. The protein markers employed are α-, β- and β'-subunits of *E. coli* RNA polymerase (α, β and β' respectively), bovine serum albumin (BSA) and soya-bean trypsin inhibitor (TI), as indicated at the top of the Figure.

band corresponding to the monomeric form of the regulatory subunit disappears, and instead a band corresponding to R_2 dimer with apparent M_r 98 000 is clearly observed.

Regulatory and catalytic subunits

Homogeneous regulatory subunits of the type-I rabbit skeletal-muscle protein kinase, prepared by the method of Weber *et al.* (1979), migrate electrophoretically as a sharp band in the presence of 1.7% SDS and 1.7% 2-mercaptoethanol, and has an apparent molecular weight identical with that of monomeric R subunit of pure cyclic AMP-dependent protein kinase [Fig. 1(a), curve 1]. In addition, a small faint band, corresponding to apparent M_r 98 000, as the R_2 dimer, is barely detectable, as indicated by an arrow in Fig. 1(a) (curve 1). However, if the sample has not been reduced with thiol reagents, the electrophoretogram clearly shows two bands, with apparent M_r 98 000 and 48 000. The catalytic subunit migrates primarily as an intense band of M_r 42 000 [Fig. 1(a), curve 2], with identical mobility in the presence or in the absence of the thiol reducing agent 2-mercaptoethanol.

Cross-linked regulatory subunit

After the regulatory subunit is cross-linked by dimethyl suberimide in 0.2 M-triethanolamine buffer, three additional bands can be seen on SDS/polyacrylamide-gel electrophoresis [Fig. 1(b), curve 1]. One band with relatively high intensity corresponds to the position of R_2 dimer with apparent M_r approx. 98 000. It should be mentioned that before SDS/polyacrylamide-gel electrophoresis, but after the cross-linking, the protein has been reduced by 2-mercaptoethanol in 1.7% SDS. Under the same conditions, the regulatory subunit that has not been exposed to dimethyl suberimide shows no intense band at this position corresponding to the R_2 dimer. Furthermore, two faint trace bands corresponding to R_3 trimer and R_4 tetramer are detectable. These small bands are more pronounced when the regulatory subunits are incubated with MgATP before their cross-linking with dimethyl suberimide [Fig. 1(b), curve 1].

Cross-linked catalytic subunit

In contrast with the results obtained with the regulatory subunit, SDS/polyacrylamide-gel electrophoresis of the purified catalytic subunit pre-incubated with the lysine-specific bifunctional reagent dimethyl suberimide under similar experimental conditions shows no detectable cross-linked polypeptide band of higher molecular weight [Fig. 1(b), curve 2]. However, the electrophoretic band recorded in Fig. 1(b) (curve 2) appears to be more broad and less intense than the corresponding band

obtained without preincubation with dimethyl suberimide [Fig. 1(a), curve 2]. The relatively diffuse broad feature appears to result from an overlap of two bands (M_r 42 000 and 39 000), which may be regarded as intrachain cross-linked isomers.

Cross-linking of reconstituted enzyme

The results of cross-linking of reconstituted cyclic AMP-dependent protein kinase by dimethyl suberimide in the presence of MgATP are shown in Fig. 1(c) (— curve). In contrast with the gel scanning patterns of cross-linked regulatory and catalytic subunits respectively, obtained under similar conditions [Fig. 1(b), curves 1 and 2], the patterns of regulatory subunits in the presence of catalytic subunits in approximately equal molar proportions after cross-linking are noticeably changed by the presence of two additional polypeptide bands, as shown by the arrows in Fig. 1(c), with apparent M_r $186\,000 \pm 1000$ and $145\,000 \pm 1000$. The electrophoretic positions of these bands, as shown by the arrows in Fig. 1(b) (curve 1), are distinctively different from those higher-molecular-weight (R_4 tetramer and R_3 trimer) species present in trace amounts in preparations of cross-linked regulatory subunits [Fig. 1(b), curve 1]; hence the two bands can be reasonably assigned to hybrid species of R_2C_2 and R_2C . The apparent molecular weights of the cross-linked R_2C_2 and R_2C hybrids estimated from their electrophoretic mobilities on SDS/polyacrylamide gels are expected to be higher than the values calculated from the apparent molecular weights of R_2 dimer (M_r 98 000) and C subunit (M_r 42 000). This is due to the fact that the introduction of chemical cross-links will restrict the extensive SDS binding and hence lower the electrophoretic mobilities (Davies & Stark, 1970). Our data indeed bear out the expectation. The extent of cross-linking between the dissimilar subunits can be affected by the presence of cyclic AMP, as shown in Fig. 1(c) (..... curve). Moreover, the generation of cross-linked hybrid R_2C_2 and R_2C species by dimethyl suberimide is found to depend on the presence of MgATP. Removal of either Mg^{2+} or ATP or both from the mixture before the cross-linking reaction abolishes the observed SDS/polyacrylamide-gel-electrophoretic polypeptide bands of higher molecular weight corresponding to R_2C_2 and R_2C hybrid species. The result obtained when MgATP has been omitted in the reaction mixture is shown in Fig. 1(c) (---- curve).

Cross-linking of the holoenzyme

Fig. 1(d) shows scanning profiles of typical SDS/polyacrylamide-gel-electrophoretic patterns of the holoenzyme after cross-linking with dimethyl suberimide. Similarly to the profile for the reconstituted enzyme (Fig. 1c), it is observed that two

Fig. 2. Intensity of R_2C_2 and R_2C hybrid species observed in SDS/polyacrylamide-gel electrophoretograms after the holoenzyme was incubated with various concentrations of cyclic AMP in the absence or in the presence of MgATP (1 mM) followed by cross-linking with dimethyl suberimidate

See the Materials and methods section for experimental details. The intensity was taken directly from the total area under the curve corresponding to the R_2C_2 or R_2C species in the gel scan and was expressed in arbitrary units. Symbols: O, results obtained with the holoenzyme preincubated with various concentrations of cyclic AMP in the presence of MgATP; Δ , results obtained in the absence of MgATP.

heavy bands with apparent M_r 186 000 and 145 000 are generated after the holoenzyme is cross-linked with dimethyl suberimidate, and the intensity of these bands decreases in the presence of cyclic AMP. Since the individual isolated subunits at the corresponding concentration fail to generate marked cross-linked polypeptide bands with apparent M_r higher than 98 000, the two heavy bands with apparent M_r 186 000 and 145 000 observed in Fig. 1(d) can be reasonably taken as cross-linked hybrid species R_2C_2 and R_2C respectively. However, it should be pointed out that, unlike the results obtained with reconstituted enzymes obtained with purified R_2 dimer and C subunit [Fig. 1(b), ——— curve], higher-molecular-weight cross-linked species (186 000- M_r and 145 000- M_r bands) are detectable even if the holoenzyme has not been preincubated with MgATP.

A representative example of the extent of cross-linking of the dissimilar subunits in the holoenzyme

as a function of cyclic AMP is presented in Fig. 2. In this series of studies, the concentration of the holoenzyme is kept constant but the concentration of cyclic AMP is varied in the absence and in the presence of MgATP. It is apparent that the intensity of the 186 000- M_r band (R_2C_2 hybrid) remains relatively constant at low concentration of cyclic AMP and then declines progressively to zero at high concentration (Fig. 2a). Moreover, the presence of MgATP enhances the concentration of the cross-linked R_2C_2 hybrid. It is also observed that the transition depends on the enzyme concentration: the transition curve shifts toward higher concentration of cyclic AMP with increasing concentrations of protein kinase (results not shown). Fig. 2(b) shows the extent of cross-linking of R_2C species as a function of cyclic AMP concentration. The effects of MgATP on the cross-linking and on the transition of the intensity of the R_2C -hybrid band as a function of cyclic AMP concentration are similar to those observed for the holoenzyme shown in Fig. 2(a).

Discussion

The SDS/polyacrylamide-gel-electrophoretic pattern of the purified regulatory subunit after cross-linking with dimethyl suberimidate run under reducing conditions shows a major polypeptide band with apparent M_r 98 000 [Fig. 1(b), curve 1]. Since it is demonstrated that the purified regulatory subunit migrated primarily as a single polypeptide band of apparent M_r 48 000 in SDS/polyacrylamide-gel electrophoresis under reducing conditions [Fig. 1(a), curve 1], the 98 000- M_r band detected after cross-linking with dimethyl suberimidate must be attributed to a dimeric species of the regulatory subunit. The facts that the 98 000- M_r band is a major feature and the intensities of other bands with M_r higher than 98 000 are relatively minute suggest that the native regulatory subunit of type-1 cyclic AMP-dependent protein kinase from rabbit muscle exists as a dimeric form, R_2 . Potter & Taylor (1980) arrived at the same conclusion for the regulatory subunit of protein kinase isolated from pig skeletal muscle.

In contrast with the results obtained with the regulatory subunit, no detectable higher-molecular-weight species can be shown in the SDS/polyacrylamide-gel electrophoretogram after cross-linking of the catalytic subunit with dimethyl suberimidate. Since there are 34 mol of lysine residues per mol of catalytic subunit (Bechtel *et al.*, 1977), it should be quite possible that higher-molecular-weight species could be formed by cross-linking with dimethyl suberimidate provided that one catalytic subunit is topologically in close proximity to another catalytic subunit. The absence of interchain cross-links of higher-molecular-weight species thus im-

plies, though albeit does not prove, that catalytic subunit at the concentration under study is present primarily in the monomeric form.

From either the native or the reconstituted protein kinase, cross-linked hybrid R_2C_2 and R_2C species with apparent M_r 186000 and 145000 can be detected in denaturing gels after the enzyme is treated with the lysine-specific bifunctional cross-linking reagent dimethyl suberimidate under appropriate conditions. Our cross-linking data thus confirm the conclusion of many other studies that the holoenzyme of type-I cyclic AMP-dependent protein kinase is a tetrameric enzyme of subunit composition R_2C_2 (Cohen, 1978; Larner *et al.*, 1978; Krebs & Beavo, 1979). The success in cross-linking the dissimilar subunits of cyclic AMP-dependent protein kinase with dimethyl suberimidate can be attributed to at least two factors. First, the dissimilar subunits R_2 and C must be topologically in close proximity with each other. Secondly, cyclic AMP-dependent protein kinase must contain lysine residues near to or at the subunit-subunit interface. In fact, lysine is the most abundant amino acid residue in the overall amino acid composition of the catalytic subunit (Bechtel *et al.*, 1977); it is also present in significant amounts in the regulatory subunit (Zoller *et al.*, 1979).

Cyclic AMP has been known for some time to promote the dissociation of cyclic AMP-dependent protein kinase. Our cross-linking data as described in Fig. 2 show that cyclic AMP at molar concentrations much higher than that of the enzyme inhibits the generation of cross-linked hybrid species R_2C_2 and R_2C from the native holoenzyme. The absence of cross-linked hybrid species suggests strongly that the two dissimilar subunits R_2 and C are no longer in close proximity in the presence of cyclic AMP, implying that cyclic AMP promotes dissociation of the holoenzyme.

The formation of cross-linked hybrid species R_2C_2 and R_2C from the reconstituted holoenzyme, as summarized in Fig. 1(c), requires MgATP. This result is consistent with the view that MgATP promotes the association of R_2 and C (Chau *et al.*, 1980). The effect of cyclic AMP to promote the dissociation of the reconstituted holoenzyme is also demonstrated in Fig. 1(c), since the amount of R_2C_2 species in the reaction mixture containing cyclic AMP is appreciably less than in that containing no cyclic AMP [cf. curve and — curve in Fig. 1(c)].

Finally, it should be pointed out that MgATP is not absolutely required for the native holoenzyme to form cross-linked hybrid R_2C_2 and R_2C species by

treatment with dimethyl suberimidate. This contrasts with what is found for the dissimilar subunits, where MgATP is required to form hybrid R_2C_2 and R_2C species. A possible explanation is that the native holoenzyme may already have bound endogenous MgATP, whereas the dissimilar subunits in the highly purified form are free from MgATP. Nevertheless, the effect of MgATP on the cross-linking of native holoenzyme in the presence of various concentrations of cyclic AMP is clearly demonstrated in Fig. 2. Our data suggest that the native holoenzyme is dissociated by cyclic AMP, but the extent of dissociation is markedly decreased by the presence of added MgATP.

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