COMMUNICATION

Induced Interchain Disulfide Bonding in cAMP-dependent Protein Kinase II*

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Cupric phenanthroline was used to catalyze the formation of disulfide bonds in cAMP-dependent protein kinase II. Incubation of holoenzyme alone with cupric phenanthroline resulted in no disulfide bond formation. In contrast, when holoenzyme was preincubated with cAMP prior to treatment with cupric phenanthroline, specific interchain disulfide bonding was found between the regulatory (R) and catalytic (C) subunits. Formation of the R-C dimer was independent of the phosphorylation state of R. Experiments with R that had been freed of bound cyclic nucleotide suggest that a ternary complex of R, C, and cAMP is necessary for the formation of this cross-linked species.

When the dimeric R-subunit alone was incubated with cupric phenanthroline, the two protomers of the R-dimer were frequently cross-linked. Phosphorylation of R did not affect the formation of R-R dimers. In contrast to the R-subunit of the type I protein kinase, R-R dimers of the type II protein kinase were not normally observed in the absence of an added catalyst. Factors which favor disulfide bond formation in the R-dimer have not been determined.

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CAMP-dependent protein kinase (EC 2.7.1.37) is a tetrameric enzyme consisting of 2 regulatory (R) and 2 catalytic (C) subunits. On binding CAMP, the inactive holoenzyme can be dissociated into a regulatory subunit dimer and 2 active catalytic monomers by the scheme R2C2 + 4 CAMP = R2(cAMP)4 + 2 C (1, 2).

The R-subunit in the type II holoenzyme from heart is autophosphorylated at serine 95 (3, 4), and this serine is preceded by 2 arginine residues that are characteristic of phosphorylation sites recognized by this kinase (5–7). CAMP is not required for this autophosphorylation (8, 9), suggesting that Ser 95 is at the R-C interaction site in the holoenzyme. Nelson and Taylor (10) have demonstrated that cysteine 97 in the R-subunit and cysteine 199 in the C-subunit can be alkylated when the subunits are dissociated, whereas both of these residues are selectively protected in the holoenzyme indicating that these residues, too, are at the R-C interaction site.

Limited proteolysis has been used to identify general functional domains in the R-subunit. In particular, the type II R-subunit can be cleaved by limited proteolysis with chymotrypsin into an NH2-terminal domain and a COOH-terminal domain where the NH2-terminal domain (M, = 16,000) contains the primary site for dimer interaction and the COOH-terminal domain (M, = 37,000) retains the ability to bind CAMP and also retains the site of autophosphorylation (1, 3, 11).

The above information provides some insight into the regions that are involved in subunit interactions in the holoenzyme and in the dissociated R-subunit. As a complimentary approach, various cross-linking reagents have been used as probes for studying specific regions of subunit interaction (12). In conjunction with these studies, cupric phenanthroline has been used to catalyze disulfide bond formation. The results indicate that very specific interchain disulfide bonding can be induced between the R- and C-subunits, that this interchain disulfide bonding is frequently stoichiometric, and finally that formation of R-C dimers is dependent on cAMP. These results are consistent with the previously reported observations of Huang (13) with particular emphasis on the interchain cross-linking of the holoenzyme.

EXPERIMENTAL PROCEDURES

Materials—Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was a generous gift from Dr. E. T. Kaiser (Laboratory of Bioorganic Chemistry, Rockefeller University). Reagents were obtained from the following sources: 1,10-phenanthroline, Sigma; N-ethylmaleimide, Calbiochem; CM-Sepharose CL-6B, Pharmacia; [γ-32P]ATP (28 Ci/mmole), Amersham.

Proteins—The C- and R-subunits were purified from porcine heart as described previously (14, 15). Holoenzyme (0.5–1.0 mg/ml) was prepared according to Nelson and Taylor (10). β-Mercaptoethanol was removed prior to cross-linking by dialysis of R, C, and holoenzyme against five changes (1 liter each) of buffer A (10 mM potassium phosphate, pH 6.5) at 4 °C. The initial dialysis buffer contained buffer A plus 2 mM EDTA. The buffers were degassed and nitrogen was bubbled through them during the dialysis. Holoenzyme, C, and R were used immediately following dialysis. Protein concentration was determined by the method of Bradford (16).

Autophosphorylation—Holoenzyme was phosphorylated prior to cross-linking by the addition of [γ-32P]ATP (0.05 volume, 1 mCi/ml) and 100 mM magnesium acetate containing 12 mM ATP (0.05 volume) followed by incubation for 5 min at 37 °C. Phosphorylation of R was achieved by adding 1/50 equivalents (w/w) of C-subunit/R-subunit. After bringing the mixture to 100 mM CAMP, phosphorylation was initiated by adding [γ-32P]ATP (0.05 volume) and 100 mM magnesium acetate, 12 mM ATP (0.05 volume). Incubation was continued for 5 min at 37 °C.

Preparation of Cyclic Nucleotide-free Regulatory Subunit—cGMP, bound to R2, during its purification, was removed by one of two methods. In one method, R2 was treated with urea by the procedure of Bui and Taylor (17) and is designated as R2II. Alternatively, cGMP was removed by extensive dialysis of R2 as described by Armstrong and Kaiser (18) (R2I). Both R2I and R2II bound approximately 2 mol of cAMP/mol of R monomer based on the Millipore filtration assay of Sugden, et al. (19) indicating that both cAMP binding sites were intact.

Cross-linking Conditions—After bringing the protein solutions to

1. The abbreviations used are: R2, R2-subunit in which cyclic nucleotides have been removed by treatment with urea as described under "Experimental Procedures"; R2I, R2-subunit in which cyclic nucleotides have been removed by extensive dialysis as described under "Experimental Procedures." HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
a final concentration of 30 mM HEPES (pH 7.4), disulfide bond formation was catalyzed by incubation with 0.25 mM CuSO4 and 1.3 mM 1,10-phenanthroline for 10 min at room temperature. The cross-linking reaction was quenched by the addition of 1.0 M N-ethylmaleimide (0.1 volume) and 100 mM EDTA (0.1 volume).

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis was done in the presence of sodium dodecyl sulfate with 1.5-mm thick slab gels using 7% acrylamide according to Laemmli (20). All samples, except those that were electrophoresed a second time, were denatured prior to electrophoresis by incubation at 100°C for 1 min in the presence of 1% sodium dodecyl sulfate, 0.005% phenyl red, and 10% sucrose. Selected gel bands which were initially electrophoresed in the absence of β-mercaptoethanol were excised from the unstained gel, incubated for 15 min at 100°C in 0.125 M Tris (pH 8.8), 50% glycerol, 1% sodium dodecyl sulfate, 10% β-mercaptoethanol, and re-electrophoresed on a 12% gel. Radioactive gels were dried and exposed to Kodak X-Omat R film.

RESULTS

The ability of cupric phenanthroline to catalyze the formation of disulfide bonds was characterized using both purified R-subunit and reconstituted holoenzyme. The integrity of the holoenzyme was assessed both visually by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and enzymatically by determining the dependence of holoenzyme activity on added cAMP using the coupled spectrophotometric assay of Cook (21). Disulfide bond formation was determined by polyacrylamide gel electrophoresis in the presence and absence of β-mercaptoethanol.

Incubation of holoenzyme alone with cupric phenanthroline resulted in no interchain disulfide bonding as indicated in Fig. 1, lane A. On the other hand, when holoenzyme was incubated with 1.4 × 10⁻⁶ M cAMP prior to incubation with cupric phenanthroline, a major new high molecular weight band was observed (Fig. 1, lane C). An identical band was formed when C-subunit was added to an excess of R-subunit that had been preincubated with 10⁻⁴ M cAMP and then incubated with cupric phenanthroline (Fig. 1, lane D). This new band migrated with an apparent molecular weight of 110,000 and was not observed when the samples were electrophoresed in the absence of β-mercaptoethanol. The composition of this new band was determined by excising the gel band and re-electrophoresing following incubation with β-mercaptoethanol. As indicated in Fig. 2, the Mr = 110,000 band was resolved in the presence of β-mercaptoethanol into two bands, Mr = 39,000 and 54,000, respectively, and these two bands were coincident with the mobilities of standard C- and R-subunits, respectively. No cross-linking of R- and C-subunits was observed if the incubation mixture was quenched with N-ethylmaleimide and EDTA prior to the addition of cupric phenanthroline (Fig. 1, lane B); however, addition of N-ethylmaleimide after cupric phenanthroline had no effect. All samples were quenched routinely with N-ethylmaleimide prior to electrophoresis to prevent random disulfide bond formation during the boiling procedure used for preparing the samples for gel electrophoresis. Although incubation times varied, in most instances cross-linking did not increase after 2 min.

The dependency of R-C cross-linking on cAMP is indicated in Fig. 3. Only a trace amount of cross-linking was observed when holoenzyme and cAMP were present in roughly equimolar amounts (lane D, 0.6 mol of cAMP/mol of R). However, when sufficient cAMP was present to ensure saturation of all four cAMP-binding sites (lane E, 6 mol of cAMP/mol of R), cross-linking was virtually complete.

Incubation of C-subunit with an excess of [³²P]R in the presence of cAMP and cupric phenanthroline also resulted in formation of a new cross-linked band that was ³²P-labeled (Fig. 1, lanes E and F). When this band was cut out, incubated with β-mercaptoethanol, and re-electrophoresed, two bands appeared with Mr = 56,000 and 39,000 (Fig. 2, lane D). Only the Mr = 56,000 band contained ³²P, indicating unequivocally that the Mr = 39,000 band was the C-subunit and not a proteolytic fragment of R in.

The involvement of cAMP in the cupric phenanthroline catalyzed cross-linking of R- and C-subunits was further substantiated using R² that had been depleted of cGMP by either urea treatment, R², or exhaustive dialysis, R², as described under "Experimental Procedures." When cupric phenanthroline was added to a mixture of R² and C, no R-C dimers were formed (Fig. 4, lane A). Preincubation of C-subunit with cupric phenanthroline followed by the subsequent addition of R² resulted in no cross-linking of R and C (Fig. 4, lane B). In contrast, incubation of the C-subunit with 10⁻⁴ M cAMP and cupric phenanthroline prior to the addition of R² showed almost quantitative inter-subunit cross-linking (Fig. 4, lane C). Similar results were obtained by using R² (data not shown). Also, if R² was incubated with cupric phenanthroline prior to the addition of C-subunit, no cross-linking was observed unless cAMP was present (data not shown).

As a final indication of the requirement for cAMP, C-subunit was incubated with a 10-fold excess of cupric phenanthroline after which R² was added. Once again, no significant cross-linking was found unless cAMP was included in the incubation mixture (Fig. 4, lanes D–F). Identical results were seen using R².

When R-subunit alone was incubated with cupric phenanthroline, a new band frequently was observed which had a slower mobility than the R-C band described above and which was not present prior to incubation with cupric phenanthroline (Fig. 5). Incubation of this band with β-mercaptoethanol showed only phospho- and dephospho-R-subunit following electrophoresis on 12% acrylamide gels. Addition of excess cAMP (2.5 × 10⁻⁴ M) did not alter the appearance of this band (Fig. 5, lane C). If [³²P]R-subunit was used, radioactivity was incorporated into the new band. The extent of R-R cross-
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Fig. 2 (left). Re-electrophoresis of cross-linked holoenzyme in the presence of β-mercaptoethanol. Samples A and B, C- and R-subunit standards, respectively; sample C, cross-linked M₁ = 110,000 band from Fig. 1 incubated in the presence of β-mercaptoethanol; sample D, same as sample C, using ³²P-labeled holoenzyme.

Fig. 3 (center). Dependence of holoenzyme cross-linking on cAMP. Holoenzyme (8.5 × 10⁻⁷ M) was incubated 10 min with 0.25 mM CuSO₄ and 1.3 mM 1,10-phenanthroline. The reaction was quenched as indicated in Fig. 1. All samples were identical except for the amount of cAMP present in the reaction mixture. Sample B contained no cAMP. Samples C-G contained 10⁻⁷, 10⁻⁸, 10⁻⁹, and 10⁻¹⁰ M cAMP, respectively. Sample A is untreated holoenzyme.

Fig. 4 (right). Cross-linking of C-subunit to cyclic nucleotide free R₁₀-subunit. Removal of cGMP from R and cross-linking were performed as indicated under "Experimental Procedures." Concentrations were as follows: R₁₀-subunit (4 × 10⁻⁴ M), C-subunit (5 × 10⁻⁶ M), cAMP (1 × 10⁻⁴ M). CuSO₄ (2.5 mM in lanes A-C and 25 mM in lanes D-F), and 1,10-phenanthroline (13 mM in lanes A–C and 130 mM in lanes D–F). C-subunit, R₁₀-subunit, cupric phenanthroline, and cAMP (when present) were added in the following order: lane A, C-subunit, R₁₀, CuP; lane B, C-subunit, CuP, R₁₀; lane C, C-subunit, cAMP, CuP, R₁₀; lane D, C-subunit, R₁₀, 10 × CuP; lane E, C-subunit, 10 × CuP, R₁₀, CuP; lane F, C-subunit, cAMP, 10 × CuP, R₁₀.

Fig. 5. Cross-linking of R₁₀ by cupric phenanthroline. All samples were incubated for 10 min with cupric phenanthroline and quenched with N-ethylmaleimide as described in Fig. 1. Sample A contained 0.25 mg/ml of R₁₀-subunit, 0.1 mg/ml of C-subunit, 30⁻⁴ M cAMP; samples B and C contained 0.3 mg/ml of R₁₀-subunit; and sample C contained 10⁻⁴ M cAMP.

Cross-linking was variable with different R₁₀ preparations, and the factors that favor this cross-linking are not clear.

DISCUSSION

Cupric phenanthroline has been used to investigate inter-chain disulfide bonding in R₁₀ and in the holoenzyme form of cAMP-dependent protein kinase II. The types of cross-linking observed with holoenzyme were highly specific and depended not only on the aggregate state of the protein but also on the addition of cAMP. No disulfide bonding was observed with the intact holoenzyme alone indicating that in this form all of the sulphhydril groups in both subunits are either not accessible to cupric phenanthroline or are not in a suitable orientation to form disulfide bonds in the presence of this reagent. This situation changes when cAMP is added under conditions which presumably dissociate the holoenzyme. In the presence of cAMP, cross-linking was observed between the R- and C-subunits. The reaction appeared to be highly specific since neither C-C nor R-R cross-linking occurred and in addition, only small amounts of higher molecular weight aggregates were observed.

The model RC + cAMP ⇌ R-C-cAMP ⇌ R-cAMP + C (17, 22) has been proposed for dissociation of the type I holoenzyme. The fluorescence evidence of Smith et al. (23) indicates that all four cAMP sites must be occupied prior to dissociation of the C-subunit. Armstrong and Kaiser (18) have proposed a similar ternary complex, R-C-cAMP, for the type II holoenzyme based on enhanced rates of inactivation of the holoenzyme with 5,5'-dithiobis(2-nitrobenzoic acid) in the presence of cAMP compared to the free C-subunit.

A ternary complex of R, C, and cAMP could provide an explanation for the cross-linking observed when holoenzyme is incubated with cupric phenanthroline and cAMP. The cysteines involved in the cross-linking may become reoriented in the R-C-cAMP complex, thus allowing cupric phenanthroline to catalyze the formation of a disulfide bond between them. Whether the cAMP molecule itself is essential or whether it simply stabilizes the R-subunit in a conformation that is conducive to disulfide bond formation cannot as yet be distinguished. Alternatively, these sulphhydril groups may simply be inaccessable to cupric phenanthroline in holoenzyme, even though they may be in the proper orientation for disulfide formation. The formation of a ternary complex could make the reactive sulphhydril groups accessible to the cupric phenanthroline, allowing a disulfide bond to form between...
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the R- and C-subunits. In the latter case, formation of the disulfide bond could occur either in the ternary complex or during the conversion of the R·C·cAMP complex back to the R-C complex.

To distinguish whether the R·C·cAMP ternary complex itself is important in the cross-linking reaction, or if the simple reassociation of subunits to form holoenzyme is sufficient, the cross-linking between cyclic nucleotide-free R-subunit and C-subunit was studied. The inability of C-subunit, preincubated with cupric phenanthroline, to form intersubunit disulfide bonds on the addition either of R₂ or R₃ in the absence of cAMP suggests that an R·C·cAMP ternary complex is necessary for the cross-linking to occur. A 10-fold increase in cupric phenanthroline still showed no cross-linking indicating cupric phenanthroline is not rate-limiting. On the other hand, the results cannot rule out the possibility that R and C reassociate before the cupric phenanthroline catalyzed reaction can occur and therefore do not conclusively show the involvement of a ternary complex in the cross-linking reaction.

Interchain disulfide bonding was also observed between the two protomers of the dissociated R-subunit although the extent of cross-linking was inconsistent with various R₃ samples. This cross-linking was not observed in the absence of cupric phenanthroline and should be clearly distinguished from the spontaneous interchain disulfide bonding that is observed in the type I regulatory subunit (23).

Interchain disulfide bonding in cAMP-dependent protein kinase II was reported previously by Huang (13). Huang observed R-R dimers following cupric phenanthroline treatment of R₃ and also described cAMP-dependent R-R dimers in the holoenzyme. Our results with the R-subunit are consistent with Huang (13). In our hands, R₃ samples which did not form R-R disulfide bonds were still capable of forming good holoenzyme based on the assay of Cook (22) and formed intersubunit disulfide bonds between the R₁ and C-subunits on addition of cAMP and cupric phenanthroline to the holoenzyme. In contrast to cross-linking of the free R-subunit, our results on cross-linking in the holoenzyme differ significantly from those of Huang (13). Whereas they report cAMP-dependent R-R dimers, we rarely observed such dimers and instead consistently found cAMP-dependent cross-linking of

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REFERENCES