A cyclic AMP-mediated intersubunit disulfide crosslinking reaction of the regulatory subunit of type II cyclic AMP-dependent protein kinase

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1. INTRODUCTION

Many physiological processes are known to be modulated via the effect of cAMP on cAMP-dependent protein kinases (EC 2.7.1.37, ATP:protein phosphotransferase) [1,2]. The enzymes are tetramers containing two regulatory and two catalytic subunits. Cyclic AMP induces dissocation of the inactive form of protein kinase and liberates the active catalytic subunit. These enzymes are classified into two main types (I,II) which differ in their regulatory subunits. In bovine heart cytosol, the major protein kinase is the type II form. Its regulatory subunit (RII) is the major cAMP-binding and phosphorylated protein in bovine heart cytosol [3]. RII has been purified and studied in much detail [3–9]. In the absence of cAMP, RII can be phosphorylated by self-phosphorylation of protein kinase II and can also be labeled with a photoaffinity analog of cAMP, 8-azido-cyclic [^{32}P]AMP [2,3,10–12]. cAMP is required to dissociate P-RII from the holoenzyme so that P-RII can be dephosphorylated by phosphatase [13].

Abbreviations: RII, regulatory subunit of type II cAMP-dependent protein kinase; P-RII; CuP, phosphorylated RII; XL-RII, crosslinked RII; XL-P-RII, crosslinked P-RII, di-1,10-phenanthroline-Cu(II) cation; DEAE-PKII, DEAE-cellulose partially purified protein kinase II; buffer A, 25 mM Tris, 1 mM EDTA and 10 mM benzamidine (pH 7.6); CRP, cAMP receptor protein; SDS, sodium dodecyl sulfate; M_r, relative molecular mass

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Very little is known about the effect of cAMP on the structure of RII. A conformational change of RII probably occurs upon binding of cAMP such that the catalytic subunit can be liberated. These data suggest that cAMP induces a conformational transition of the holoenzyme such that disulfide bond(s) linking the two RII subunits can be formed with an oxidation catalyst, di-1,10-phenanthroline-Cu(II) (CuP).

2. EXPERIMENTAL

The sources of chemicals were: benzamidine, *N*-ethylmaleimide, *o*-phenathroline, Aldrich; marker proteins ferritin, catalase, aldolase, bovine serum albumin and cytochrome *c*, Boehringer Mannheim; cAMP, myoglobin, Sigma; [^{3}H]cAMP, New England Nuclear; [γ-^{32}P]ATP was prepared as in [14]. Buffer A: 25 mM Tris, 1 mM EDTA and 10 mM benzamidine (pH 7.6). Protein kinase II, partially purified on DEAE-cellulose (DEAE-PKII) and purified RII subunit from fresh bovine cardiac muscle were prepared as in [4] with the following modifications: (a) the buffer was buffer A; (b) type II protein kinase was eluted from DEAE-cellulose and by a gradient of 2 liters each of buffer A and 0.5 M NaCl in buffer A and identified by histone protein kinase assay as in [15]. Protein concentration was determined as in [16] using bovine serum albumin as standard. The stock solution of CuP contained 13.6 mM orthophenathroline and 6.8 mM cupric sulfate in water.

2.1. *Crosslinking of DEAE-PKII*

Freshly prepared DEAE-PKII was dialyzed overnight against 50 mM sodium acetate buffer (pH 6.2). Phosphorylation was performed using 1 mg

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protein/ml of DEAE-PKII in 10 mM sodium acetate (pH 6.2), 10 mM $MgCl_2$, 1 μM [γ-^{32}P]ATP (1.5–4 × 10^7 cpm/nmol) with or without 10 μM cAMP at room temperature for 1 min. The pH of the samples was then adjusted to 8.0 by addition of 50 mM Tris–HCl to the samples. The samples were then incubated in the presence or absence of 25 μM CuP for 1 min. The reaction was terminated by addition of a concentrated 'SDS-stop solution', which yielded final conc. 15 mM EDTA, 1 mg *N*-ethylmaleimide/ml, 3% SDS (w/v), 5% glycerol and a small amount of bromophenol blue in 62 mM Tris (pH 6.7). After incubation at 37°C for 30 min the samples were applied to an SDS–polyacrylamide gel (7.5%). After electrophoresis, the gel was stained, destained, dried and autoradiographed as in [17]. It is essential to add EDTA and *N*-ethylamaleimide to the samples. EDTA chelates Cu^{2+} and quenches the crosslinking reaction. *N*-Ethylamaleimide blocks free SH groups and prevents thiol–disulfide exchange reaction [18]. All samples described were treated by this standard procedure when subjected to SDS–polyacrylamide gel electrophoresis.

2.2. *Dependency of crosslinking of RII on [cAMP] in DEAE-PKII*

DEAE-PKII was dialyzed against 50 mM potassium phosphate buffer (pH 7.8) overnight. RII was autophosphorylated in a reaction mixture of 25 mM potassium phosphate (pH 7.8), 10 mM $MgCl_2$, 20 μM [γ-^{32}P]ATP (10^6 cpm/nmol) and 1 mg protein/ml of DEAE-PKII at room temperature for 30 min. 10 mM sodium pyrophosphate was then added. The sample was divided into several fractions. Various concentrations of cAMP (10^{-8}–10^{-4} M) were then added to each fraction. Crosslinking reaction was initiated by the addition of 25 μM CuP to each fraction for 5 min. The reaction was terminated by 'SDS-stop solution'. Electrophoresis and autoradiography were performed as above. Autoradiographs were scanned with a Canalco G-II microdensitometer. The peak heights of the absorbance tracing were used as a measure of the presence of ^{32}P in XL-P-RII bands and were expressed in arbitrary units as in [10]. The peak heights of the scanning were proportional to the total radioactivity of the corresponding peaks estimated by scintillation spectrometry (cf [10]). Without addition of CuP, only trace amount of radioactivity was observed in the XL-RII band region. Addition of sodium pyrophosphate to the samples inhibits phosphatase activity [19] and dephosphorylation of P-RII was not observed.

2.3. *Crosslinking of purified RII*

RII was purified from a 2 ml column of 8-(6-aminohexyl-amino)-cAMP–Sepharose 4B as in [4], except buffer A was used for loading DEAE-PKII to the column. The affinity column was washed with 2 M NaCl in buffer A and then with 4 ml 50 mM Tris–HCl (pH 8.0). RII was eluted from the column with 4 ml 10 mM [^{3}H]cAMP (1 cpm/pmol) in 50 mM Tris–HCl (pH 8.0) at room temperature. When counts appeared in the eluate, the flow was stopped for 2 h and then ~ 1.5 ml eluate containing RII was collected.

Immediately after RII was eluted from the column, 10 mM $MgCl_2$ and 100 μM CuP were added to start the crosslinking reaction. Final protein conc. was 0.5–1.0 mg/ml. The reaction was stopped after 10 min by addition of 15 mM EDTA. The sample was then analyzed by SDS–polyacrylamide gel electrophoresis, Bio-Gel-P-300 column gel filtration and sucrose density gradient centrifugation as below. In some experiments, RII was phosphorylated before the crosslinking reaction by incubation at room temperature for 15 min with 10 mM $MgCl_2$, 20 μM ATP and 1 μg/ml purified catalytic subunit of bovine heart protein kinase II prepared as in [20]. It was found that the crosslinking reaction must be performed with freshly prepared RII to have reproducible results.

2.5. *Separation and estimation of the Stokes' radii and sedimentation coefficients of RII and XL-RII*

CuP-treated RII (1.5 ml) was applied to a long Bio-Gel-P-300 column (1.5 × 110 cm) which was equilibrated with buffer A in 50 mM NaCl. Fractions of 2.5 ml were collected and assayed for XL-RII by protein staining with Coomassie blue after SDS–polyacrylamide gel electrophoresis. The Stokes' radii of XL-RII and RII were estimated using the same column with the following marker proteins: ferritin (79 Å), catalase (52 Å), aldolase (47 Å), bovine serum albumin (35 Å) and cytochrome *c* (10 Å). The data were treated as in [21]. Estimation of the sedimentation coefficients of XL-RII and RII was done as in [22] using CuP

treated purified RII as starting material. Myoglobin (2.1 S) and bovine serum albumin (4.3 S) were used as standards.

3. RESULTS AND DISCUSSION

The major phosphoprotein obtained following incubation of the DEAE-PKII preparation with [γ-^{32}P]ATP in either the presence or absence of cAMP has M_r 56 000 (fig.1). As shown in [3] this phosphoprotein corresponds to the major cAMP-binding protein in bovine heart and appears to be the regulatory subunit (RII) of type II protein kinase. When RII was phosphorylated in the presence of cAMP, addition of CuP produced a new ^{32}P-labeled band (XL-P-RII) in SDS–polyacrylamide gel (fig.1, lanes 5,6). Densitometric scanning of the autoradiograph indicated the yield of XL-P-RII from P-RII is ~ 45%. The XL-P-RII band can be reduced back to P-RII band by treating the CuP-treated sample with β-mercaptoenthanol (fig.1, lanes 1,2). This is consistent with the fact that CuP is a crosslinking reagent which catalyzes the air-oxidation of intrinsic sulfhydryl group to form a disulfide bond [14]. Without the addition of cAMP the XL-P-RII band was not observed upon CuP treatment (fig.1, lanes 3,4). The requirement of cAMP for crosslinking of RII was also observed in crude cytosol extract of bovine cardiac muscle and with protein kinase which was further purified by ammonium sulfate fractionation and Bio-Gel-P-300 filtration from DEAE-PKII (not shown).

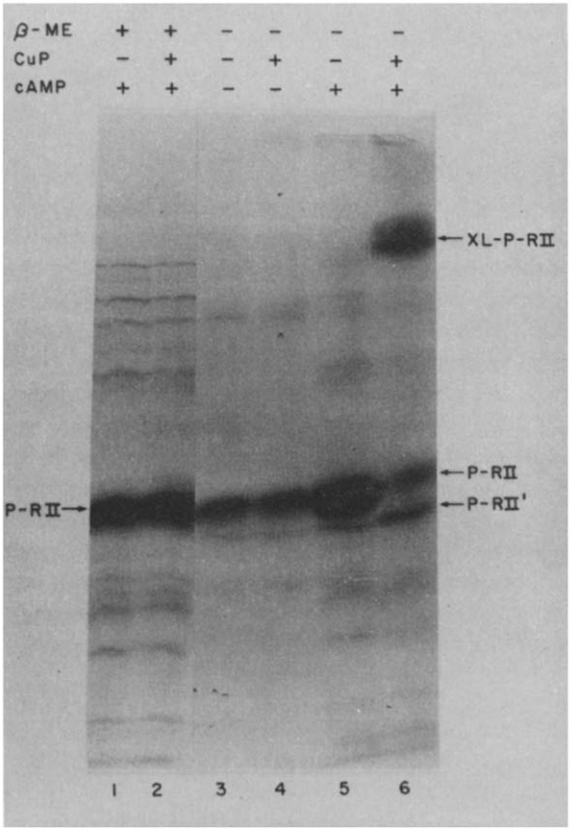

Fig.1. Autoradiograph showing the cAMP-mediated crosslinking of RII in DEAE-PKII. DEAE-PKII in 10 mM sodium acetate (pH 6.2) was phosphorylated with 10 mM $MgCl_2$, 1 μM [γ-^{32}P]ATP with (lanes 5,6) or without (lanes 3,4) the addition of 10 μM cAMP, followed with (lanes 4,6) or without (lanes 3,5) the addition of 25 μM CuP. The crosslinking reactions were stopped with a concentrated 'SDS-stop' solution and applied to SDS–polyacrylamide gel electrophoresis and autoradiographed as detailed in the text. Lanes 1 and 2 were samples from those of lanes 5 and 6, respectively, which were treated with 0.1 M β-mercaptoethanol and incubated at 37°C for 1 h before being applied to SDS gel.

The dependence of crosslinking of RII on [cAMP] was studied with DEAE-PKII (fig.2). The apparent half-maximal yield of crosslinked product occurred at ~ 1 μM cAMP. The high [cAMP] suggests that successful crosslinking requires the full dissociation of regulatory and catalytic subunits in the holoenzyme. It is known that full dissociation of the regulatory and catalytic subunits occurs at 0.5 μM cAMP with 0.02 μM purified protein kinase (cf. [2]). The crosslinking reaction may thus occur only between the RII subunits. To test this hypothesis, RII was purified by cAMP–Sepharose 4B affinity column and treated with CuP. No cAMP was added as the RII was eluted from the column with 10 mM cAMP. When CuP-treated RII was subjected to gel electrophoresis and protein staining, a protein band with M_r corresponding to XL-RII was observed (fig.3a, lanes 1,2). Densitometric scan of the gel shown in fig.3a, (lane 2), indicates that the yield of XL-RII from RII is ~ 40%.

Phosphorylation of RII by free catalytic subunit and ATP does not affect the crosslinking reaction of purified RII except that both RII and XL-RII demonstrate slightly slower mobility in gel electrophoresis when compared with unphosphorylated RII (fig.3b, lanes 1,2). The effect of phosphoryla-

tion of the mobility of RII in gel electrophoresis is well known [12,23,24]. Both in DEAE-PKII and in purified P-RII, a minor band (P-RII′) moving slightly faster than the major band (P-RII) was detectable (fig.1, lanes 5; fig.3b, lane 1). An increase in the amount of P-RII′ upon CuP treatment was observed (fig.1, lane 6; fig.3b, lane 2). Based on the observation that it disappeared upon β-mercaptoethanol treatment (cf. fig.1, lanes 2,6), P-RII′ is most likely an intrasubunit disulfide-linked P-RII. Similar crosslinking has been observed with CuP-treated rabbit muscle aldolase [25]. Intramolecular crosslinking of RII may explain the additional minor band with slightly faster mobility observed in the regions of XL-RII and XL-P-RII in SDS gel (fig.3a,3b, lane 2).

Purified RII was treated with CuP and applied to a long Bio-Gel-P-300 column, RII and XL-RII were separated by this procedure (fig.3c, lanes 1,2). The physical properties of both proteins were then examined (table 1). The data suggest that XL-RII is

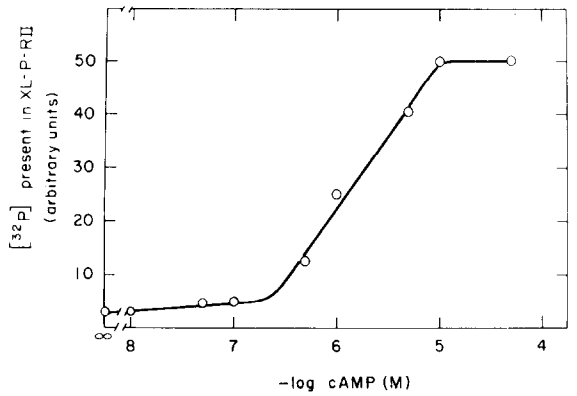

Fig.2. Dependency of crosslinking of RII on [cAMP] in DEAE-PKII. RII of DEAE-PKII was first phosphorylated in 25 mM potassium phosphate (pH 7.8), 10 mM $MgCl_2$, 20 μM [γ-^{32}P]ATP. Sodium pyrophosphate (10 mM) was then added to inhibit phosphatase activity in the sample. The sample was then divided into several fractions and various concentrations of cAMP were added to each fraction. Crosslinking reaction was initiated by the addition of CuP and terminated by 'SDS-stop solution'. Samples were subjected to electrophoresis and autoradiography. Autoradiographs were scanned with a microdensitometer. The peak heights of the absorbance were used as a measure of the presence of ^{32}P in XL-P-RII bands and were expressed in arbitrary units. Details are in the text.

Table 1
Comparison of some properties of XL-RII and RII[a]

Property	XL-RII	RII
Stokes' radius	52 Å	58 Å
Sedimentation coefficient	4.5 S	4.2 S
Frictional ratio (f/f_0)	1.69	1.86
M_r-Value (from Stokes' radius and sedimentation coefficient)	99 700	103 800

[a] Stokes' radii and sedimentation coefficients of XL-RII and RII were obtained from CuP-treated purified RII as described in the text and were used to calculate the frictional ratios and M_r-values [21]

less elongated than RII. Using values of 4.5 S for the sedimentation coefficient, 52 Å for the Stokes' radius and 0.733 ml/mg [26] for the partial specific volume, an M_r of ~ 99 700 is calculated for XL-RII [21]. This result indicates that XL-RII is a dimer of RII.

When 5,5′-dithiobis (2-nitrobenzoic) acid is used to titrate the number of SH groups in RII, the binding of cAMP to the holoenzyme or isolated RII subunit prevents the reaction of 1 SH group/RII subunit [27]. Whether this cAMP-protected SH group is also the SH group involved in the crosslinking of RII by CuP remains to be determined. Also unknown is whether the two cAMP-binding sites in each RII subunit [4–9] play equal roles in the crosslinking reaction.

It is known that membrane-associated and cytosolic protein kinases of bovine brain exhibit characteristics which closely parallel the properties of homogeneous type II protein kinase from heart cytosol [28]. The crosslinking reaction described here was also observed with RII from bovine brain synaptic membrane or with purified, Triton X-100-extracted membrane RII from the same source (unpublished).

Interestingly, a similarly cAMP-mediated disulfide crosslinking reaction also occurs in the cAMP receptor protein (CRP) of *Escherichia coli* [29]. The SH groups of CRP which respond to cAMP binding are thought to be involved in cAMP-dependent DNA binding of CRP [29]. Whether RII contains DNA binding domains remains to be studied.

These data suggest that either cAMP induces a

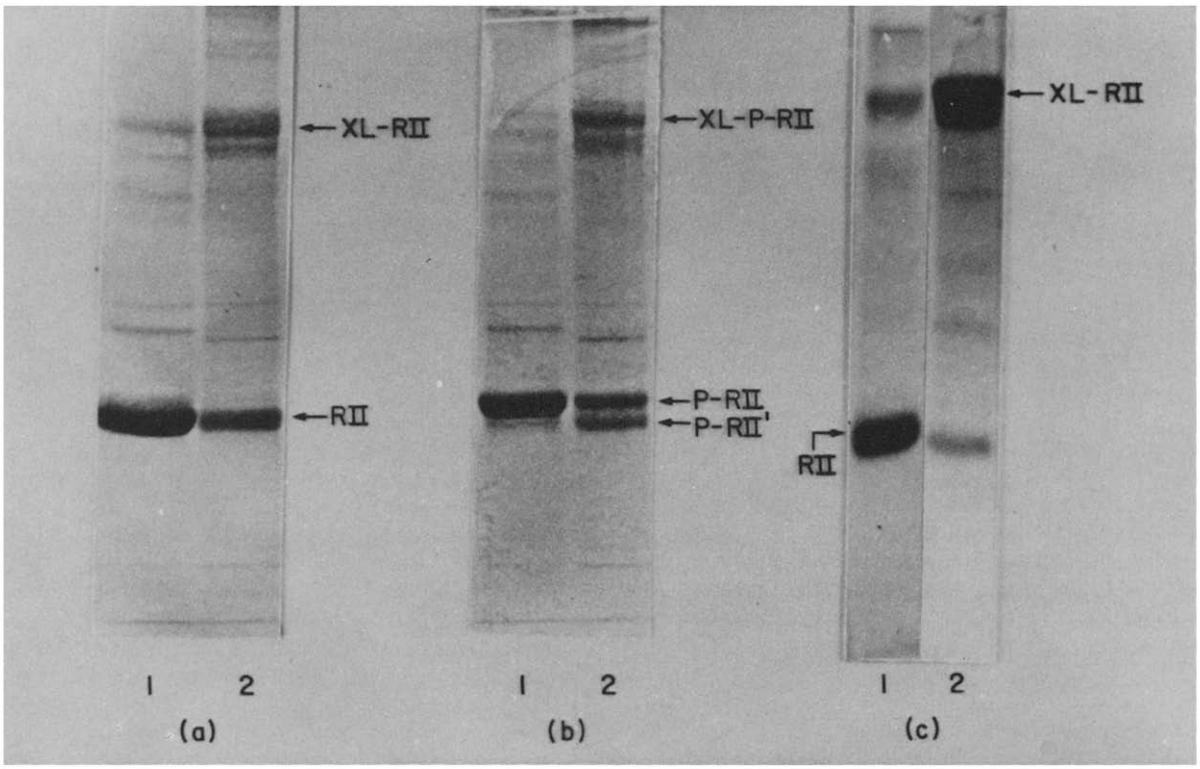

Fig.3. Crosslinking of purified RII and partial separation of RII and XL-RII by gel filtration: (a) protein staining of cAMP–Sepharose 4B affinity column purified RII (lane 1, 5 μg) and CuP-treated purified RII (lane 2, 5 μg); (b) as in (a) except that the purified RII was first phosphorylated with $MgCl_2$, ATP and catalytic subunit of cAMP-dependent protein kinase (lane 1) and then treated with CuP (lane 2); (c) protein staining of partially separated RII and XL-RII. CuP-treated purified RII (fig.3a, lane 2) was applied to a Bio-Gel-P-300 column to obtain partial separation of RII (lane 1, fraction 33, 3 μg) and XL-RII (lane 2, fraction 37, 6 μg). Details are in the text.

conformational transition of its own receptor, RII, bringing into close proximity some SH groups between two RII subunits or cAMP induces dissociation of the holoenzyme exposing buried adjacent SH groups of RII. In both cases, the disulfide bonds linking the RII subunits can be formed by CuP-catalyzed air oxidation in the presence of cAMP. Purified cAMP-free RII should be used to distinguish these two possible effects of cAMP on RII. Since the purification of cAMP-free RII involves using very harsh treatment such as urea [30] and the success of the crosslinking reaction requires the presence of closely adjacent SH groups in RII, such experiment may be difficult to succeed. Among other related protein kinases, cAMP-induced conformational changes in the regulatory subunit of protein kinase type I [30] and interchain disulfide bonding in the regulatory subunit of protein kinase type I [31] and cGMP-dependent protein kinase [32] have been reported.

This paper describes a cAMP-mediated intersubunit disulfide crosslinking reaction of RII. This reaction requires cAMP and an oxidation agent. Whether RII contains disulfide bridge in vivo in a cAMP-dependent way remains to be studied.

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