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STOICHIOMETRY OF cAMP BINDING AND LIMITED PROTEOLYSIS OF PROTEIN KINASE REGULATORY SUBUNITS R I AND R II

Wolfgang Weber and Helmuth Hilz

Institut für Physiologische Chemie
der Universität Hamburg, Germany

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SUMMARY

Protein kinase regulatory subunits type I (rabbit skeletal muscle) and type II (bovine heart) were isolated by a rapid two step procedure which involved affinity chromatography on an 8-thio cAMP matrix. The R proteins were analyzed for cAMP binding capacity using three different methods for the separation of bound from free cAMP, and various methods for protein determination. Regulatory subunits type I as well as type II were both found to contain two high affinity cAMP binding sites per R monomer corresponding to a formula for the native R proteins of $R_2 \cdot cAMP_4$. - Kinetic analyses of limited proteolysis by various proteases revealed striking differences between R I and R II with respect to loss of cAMP binding capacity, ability to inhibit the catalytic subunit C, and susceptibility to further degradation. Some of the products had lost about one half of the cAMP binding capacity supporting the presence of two binding sites in R while other degradation products showed no change in high affinity binding sites. By contrast, the ability to inhibit the catalytic subunit C was lost in all products of limited proteolysis except one.

INTRODUCTION

cAMP dependent protein kinases are composed of catalytic and regulatory subunits (1-5). Two types of protein kinase holoenzymes can be distinguished which differ only in their regulatory subunits (6, 7). Activation occurs by binding of cAMP to the regulatory subunits thus releasing the catalytic subunit C into an active form according to the supposed equation (8): $R_2 \cdot C_2 + 2\ cAMP_2 \rightleftharpoons R_2 \cdot cAMP_2 + 2C$. Recently, Corbin's group (9) and we (10) reported on a different stoichiometry of cAMP binding to the regulatory subunit type II. During purification of R proteins by affinity chromatography changes in R proteins were observed which appeared to be related to possible contamination with proteases. We therefore decided to analyze the number of high affinity cAMP binding sites in freshly prepared regulatory subunits using a rapid isolation procedure (10). This paper describes three independent methods for the determination of cAMP binding sites in R I and R II. Since R proteins are subject to limited proteolysis (cf. 9, 11-14), an analysis of the possible interference with cAMP binding was included.

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MATERIALS AND METHODS

cAMP was determined by a modification (M. Schumacher, unpublished data) of the method of Steiner (15): 100 µl samples (0.2 - 10 pmoles cAMP) were mixed with 100 µl 1 M $HClO_4$ and centrifuged. 150 µl aliquots of the supernatants were neutralized with 75 µl 0.68 M K_3PO_4 solution, and $KClO_4$ was removed by centrifugation. Aliquots of the supernatants were then analyzed for cAMP using highly specific antibodies. In some cases, bound cAMP was determined by quantitative exchange against (^{3}H)cAMP (10).

Protein kinase activity: 50 mM Tris/HCl pH 7.5, 10 mM acetate, 2 mM dithiothreitol, 9-10 µM (^{32}P)-ATP (1 µCi), 100 µg bovine serum albumin and 40 µg histone IIa were incubated with protein kinase (cf. Fig. 1-3) at 30° in a final volume of 200 µl for 11 min, mixed with 1.1 ml 12% trichloroacetic acid containing 0.1% cold ATP. After 15 min on ice, the samples were filtered (Sartorius, Göttingen, nitrocellulose SM 11 306, 0.45 µm), the filters washed with 20 ml 5% trichloroacetic acid - 0.01% ATP, dissolved in 0.8 ml 1 mM NaOH - 15 ml dioxan scintillator, and analyzed for radioactivity.

Regulatory subunits were prepared as described previously (10) using separation on DE 52 (Whatman), chromatography on an affinity column [8-(2'-hydroxyethylthio)]-cAMP coupled to epoxy-activated Sepharose (Pharmacia), and elution with 30 mM cAMP. Thus, 12.5 mg R I protein and 19.5 mg R II were obtained from 4.5 kg rabbit muscle and from 3.4 kg bovine heart, respectively.

Separation of free and bound cAMP

Charcoal treatment (cf. 16,17,12): 20 - 40 µl eluates from the affinity column containing the R proteins in 30 mM cAMP were diluted to 150 µl TG buffer (10 mM Tris/HCl pH 7.5 - 1 mM EDTA - 6 mM mercaptoethanol - 10% glycerol) and mixed with 80 µl 10% charcoal (E. Merck, Darmstadt) suspension, 2% serum albumin, 50 mM Tris/HCl pH 7.5. After centrifugation (4 min, 10 000 g), 170 µl aliquots of the supernatants were again mixed with 80 µl charcoal suspension, and centrifuged. Aliquots of the supernatants were analyzed for cAMP. Controls contained 30 mM cAMP but no R protein. Three instead of two charcoal treatments yielded identical values of bound nucleotide.

Ammonium sulfate precipitation: 170 - 380 µg R protein was precipitated by ammonium sulfate (75% saturation) and washed with 1 ml 70% ammonium sulfate solution. The precipitate was dissolved in TG buffer, reprecipitated and washed 3 times with 1 ml 70% ammonium sulfate solution. The precipitate was taken up in 20 mM NaOH and used for determination of protein and cAMP. Control samples with 30 mM cAMP and γ-globulin instead of R protein were subjected to the same procedure. Extension of the number of washings confirmed the validity of the procedure applied.

Dialysis: Samples of R proteins (30 mM cAMP eluates) were filled into dialysis bags and placed in a rotating cylinder containing TG buffer - 100 mM NaCl. The buffer was changed 5 times after 10 hour intervals.

All three methods yielded similar values for the stoichiometry of cAMP binding (see Table 1).

Protein determination

The Lowry method was performed according to (18), using bovine serum albumin and γ-globulin as reference standards. Absorption produced by γ-globulin was about 12% higher than by albumin.

Biuret reaction: 250 µl samples (20 - 100 µg protein) were mixed with the same volume of reagent (19). After 2 hrs at room temperature the absorption was measured at 546 nm. Serum albumin and γ-globulin served as standard proteins without significant differences in color intensities.

Table 1 Number of high affinity cAMP binding sites in R I and R II regulatory subunits as found by various procedures. - Mean values from 1-8 determinations each of two separate preparations.

Separation of Free and Bound cAMP	cAMP Binding Sites / R Monomer			
	R I		R II	
	Lowry	Biuret	Lowry	Biuret
charcoal treatment	1.97	2.12	1.92	1.95
ammonium sulfate ppt.	2.00	2.18	2.01	1.80
dialysis	2.07	2.24	1.70[a)]	--

a) 2.07 after subsequent loading with cAMP and charcoal treatment.

RESULTS AND DISCUSSION

1. Isolation of intact protein kinase regulatory subunits type I and type II with the aid of a new cAMP affinity matrix

Recently, a cAMP affinity matrix was described from this laboratory which was characterized by a hydrophilic spacer linked by a non-bleeding bond to an 8-thio cAMP derivative providing increased resistance to phosphodiesterase. In a rapid two step procedure applying DEAE cellulose and affinity chromatography on such a column, apparently homogeneous regulatory subunits R II from bovine heart, and R I from rabbit skeletal muscle could be obtained in high yield (10).

The apparent molecular weights were 49 000 for R I, and 54 000 for R II. Both proteins were able to inhibit the catalytic subunit C from bovine heart (cf. Fig. 1-3).

2. Stoichiometry of high affinity cAMP binding sites in the regulatory subunits R I and R II

Freshly prepared R proteins were loaded with excess cAMP during isolation and analyzed for cAMP binding sites as outlined in Methods.

Table 1 summarizes the results obtained with 2 separate preparations each of R I and R II using three independent procedures for the quantitation of bound cAMP. Mean values from several individual determinations are expressed as cAMP binding sites per R monomer, and based on two different protein determination

procedures. For calculation we used molecular weights of 49 000 for R I , and 54 000 for R II as determined by SDS gel electrophoresis. All these determinations yielded values close to two cAMP binding sites per R monomer. The slight tendency of the values in R I to binding sites > 2 may be due to a slight error in molecular weight determinations by SDS gel electrophoresis.

The data in Table 1 are at variance with previous reports on cAMP-binding sites in R proteins which indicated one acceptor site per R molecule (cf. 8, 20 - 22). This may in part be explained by the use of membrane filter techniques which in our hands yielded consistently lower cAMP-binding values than the charcoal method or the other procedures with all types of cAMP-binding proteins (not shown). The reason for the lower values obtained by the filter techniques is not known.

With respect to the R II type protein, the data in Table 1 confirm recent findings obtained by Corbin et al. using different techniques (9), and by ourselves (10). Since regulatory subunit type I, too, has now been shown to exhibit the same number of binding sites, a general stoichiometry of $R_2 \cdot cAMP_4$ for both R proteins in their native dimeric form (cf. (8)) is indicated. Activation of both types of protein kinase holoenzymes, therefore, may proceed according to the equation: $R_2 \cdot C_2 + 4\ cAMP \rightleftharpoons R_2 \cdot cAMP_4 + 2\ C$.

3. Limited proteolysis of regulatory subunits R I and R II

Regulatory subunits R I and R II were subjected to mild proteolytic treatment by trypsin (Fig.1). Both proteins were degraded, though at a different rate, yielding R' products of very similar size (M_r = 37 000). However, cAMP binding capacities of R I and R II were affected quite differently (Fig.1): While the degradation product of R II completely retained its cAMP binding capacity, R I immediately lost about one half of the cAMP binding sites on treatment with trypsin, which indicates loss of one of the two binding sites in R I. This loss may even precede the appearance of the 37 000 dalton R' product. Also, no correlation was seen between loss of C inhibition and the change in subunit molecular weight which was complete in 10 min.

The different susceptibilities of R I and R II towards proteolytic digestion were also seen with low concentrations of chymotrypsin (Fig.2). R II again proved to be the more susceptible polypeptide being rapidly degraded to an R II' of 37 000 dalton. This product, although of the same size as the derivative found by trypsin action, proved to be different: It was the only product of limited proteolysis, that retained at least partially the ability to inhibit the catalytic subunit C. The product also showed only a slight decrease in cAMP bin-

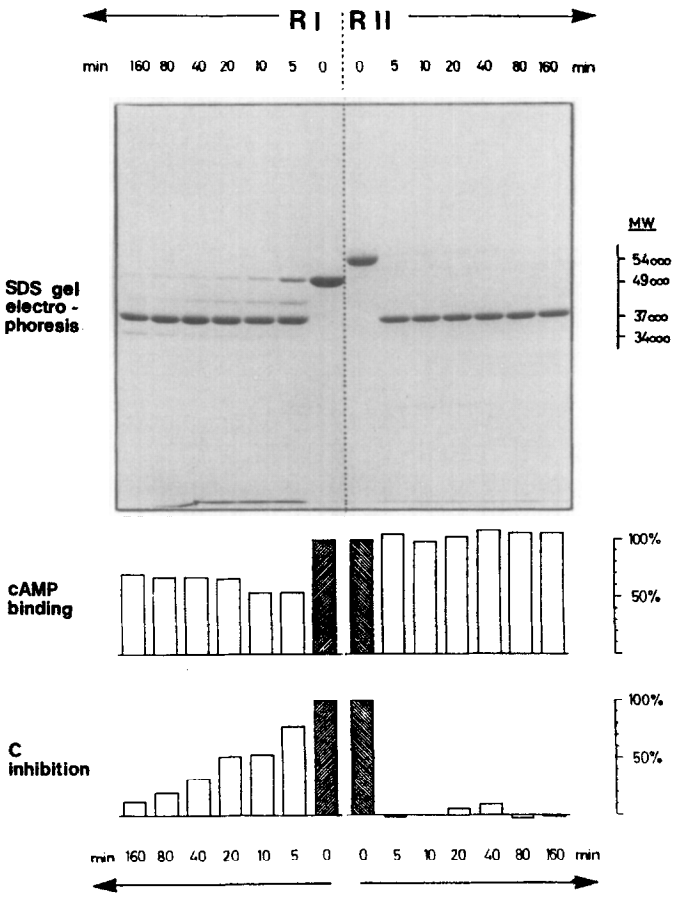

Figure 1. Kinetics of a mild trypsin treatment of regulatory subunits R I and R II. - Proteolytic treatment: 10 µl samples of R I (0.63 mg/ml) and R II (0.43 mg/ml) taken from the 30 mM cAMP eluates of the affinity chromatography were incubated at 25° with 0.008 µg trypsin in a final volume of 72 µl. At the times indicated reactions were stopped by the addition of 8 µl 50 mM phenylmethylsulfonylfluoride in isopropanol. Controls were run without proteolytic treatment. Aliquots of these reaction mixtures were analyzed by SDS gel electrophoresis acc. to (26) using 11% slab gels and the Tris/glycine system. - 25 µl samples of the incubation mixtures were diluted to 150 µl with TG buffer and treated two times with charcoal suspension to remove free cAMP as described in Methods. Supernatants were assayed for cAMP binding capacity and for the ability to inactivate the catalytic subunit of protein kinase. cAMP binding was performed by incubating samples with 0.1 µM (^{3}H)cAMP (4 Ci/mMol) for 4 - 7 hrs at 37° (10). cAMP binding capacity of control samples without proteolytic enzymes was set 100%. C inhibition was determined by incubation (1.5 hours,25°) of 2 ng pure catalytic subunit (bovine heart; kindly provided by Dr. G. Schwoch) with 1 or 2 µl charcoal treated aliquots of the proteolytic digests in a final volume of 100 µl containing 50 mM Tris/HCl pH 7.5, 1 mM EDTA, 10% glycerol and 100 µg serum albumin. The resulting kinase activity was then measured as described in Methods. C inhibition by undegraded R protein samples was set at 100%. Control experiments in the presence of 1 µM cAMP showed complete reactivation of C activity which excludes unspecific effects on C (not shown).

ding capacities. R I on the other hand appeared to be resistant to low concentrations of chymotrypsin as no change in the apparent molecular weight, and no significant change in C affinity was observed. However, cAMP binding capacity

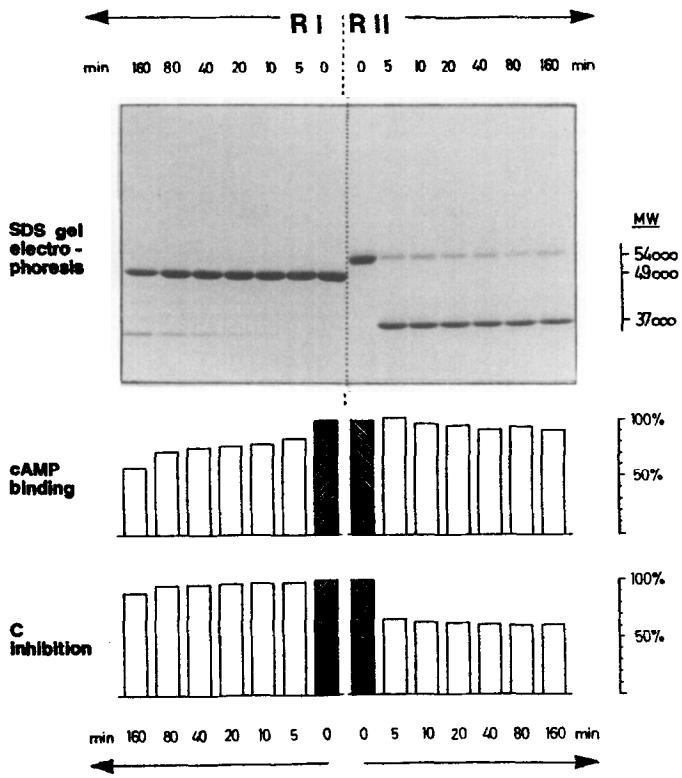

Figure 2. Chymotrypsin treatment of regulatory subunits R I and R II. - R proteins were treated with 0.008 µg chymotrypsin and analyzed as described in the legend to Figure 1.

(Fig.2) was significantly reduced already after 5 min of incubation, and decreased to about one half at 160 min, indicating subtle changes of the molecule without gross alterations of molecular size and affinity to C. Such findings may provide an alternative explanation for the diverging reports on the number of cAMP binding sites in R proteins.

Further differences in proteolytic susceptibility of R I and R II were observed by exposure to relatively high concentrations of proteinase K, trypsin and chymotrypsin, respectively, for extended periods (Fig.3): The rather unspecific proteinase K (23,24), too, had only limited access to the R proteins, yielding a 34 000 dalton product in the case of R I, and a similar product from R II with the intermediary formation of a somewhat larger precursor molecule. Trypsin at these concentrations attacked the primary products of limited proteolysis (R I' and R II' with M_r 37 000) leading to two secondary products in the case of R I, and to further, apparently unstable, degradation products in the case of R II. All these products still retained cAMP binding capacities to a considerable extent. Surprisingly, all three products of R I appearing successively during the

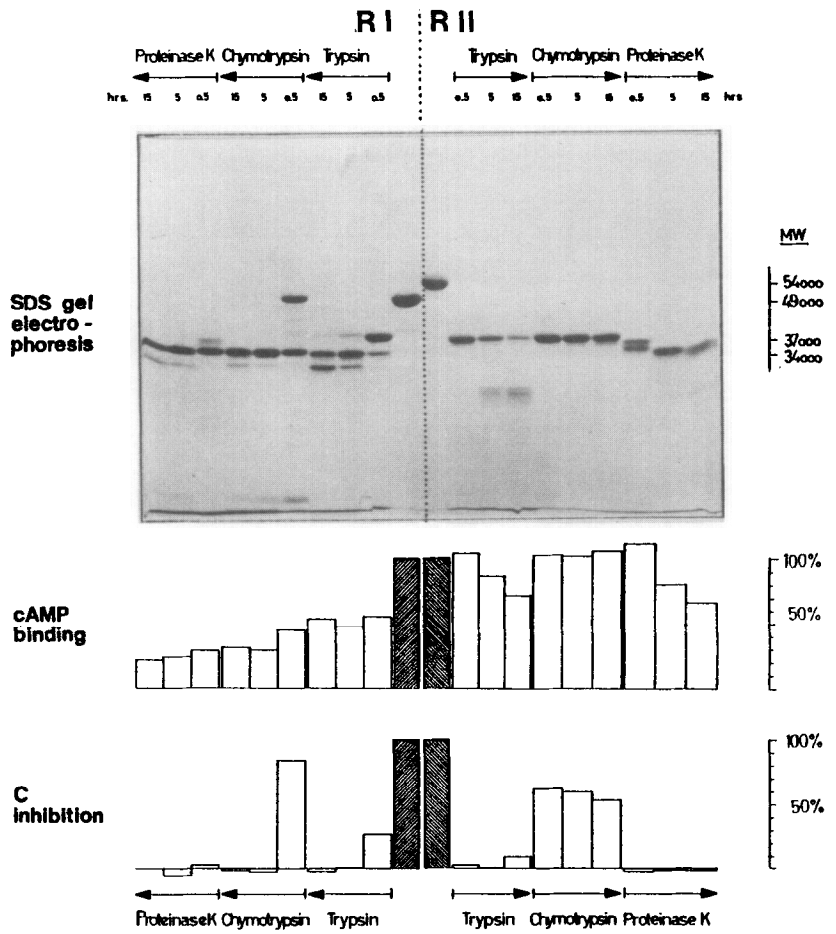

Figure 3. Extensive treatment of regulatory subunits R I and R II with different proteases. - R proteins were treated with 0.08 µg each of trypsin, chymotrypsin and proteinase K (E. Merck, Darmstadt) for 0.5, 5 and 15 hours at 25°, and analyzed as described in the legend to Figure 1.

trypsin treatment (R I' 37 000; R I' 34 000; R I' 30 000) had retained about one half of the cAMP binding capacity originally present in R I, which supports the data on two cAMP binding sites per R monomer. Chymotrypsin treatment revealed further differences of R I and R II with respect to cAMP binding capacity, the R II' product (M_r 37 000) retaining full cAMP binding capacities.

Affinity of the R proteins to the catalytic subunit C appears to be the more vulnerable property. It is abolished in all products of limited proteolysis but one: the chymotrypsin-derived R II' derivative (M_r 37 000), which retained a significant part of its capacity to inhibit the catalytic subunit C.

These data support the postulate of several structural domaines in R proteins, one being involved in cAMP binding, others in the interaction with C and in R_2

dimer formation (9,12,14,25). However, an identity of such domaines with R segments produced by limited proteolysis (cf. 25) may not be valid since conformational changes induced by the cuts may lead to secondary changes in the properties of the residual fragments. This interpretation is supported by the observation that the trypsin-catalyzed conversion of kidney R II to an R II' (M_r 37 000) led to an inaccessibility for protein kinase C of the phosphate acceptor site while phosphate groups introduced before the proteolytic conversion remained in the R II' product indicating a masking of the phosphate acceptor site in R II' (12). Conformational changes following limited proteolysis elsewhere in the molecule may also explain the loss of one cAMP binding site in R I' proteins produced by limited proteolysis.

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