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Conversion of Bovine Cardiac Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinase to a Heterodimer by Removal of 45 Residues at the N-Terminus of the Regulatory Subunit†

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ABSTRACT: The type II adenosine cyclic 3′,5′-phosphate (cAMP) dependent protein kinase from bovine heart, consisting of a dimeric regulatory subunit and two catalytic subunits, was converted to a heterodimer by limited tryptic digestion. Loss of the tetrameric structure was accompanied by proteolysis of the regulatory subunit to a form with an apparent molecular weight of 45 000 vs. 52 000 for the native subunit. The proteolyzed subunit behaved as a monomer, in contrast to the dimeric native subunit. Amino acid sequence analysis established that proteolysis removed 45 residues at the N-terminus, indicating that these 45 residues constitute the dimerizing domain of this protein. The kinetic properties of this heterodimer were indistinguishable from those of the native tetramer: half-maximal kinase activation occurred at 48 nM cAMP with a Hill coefficient of 1.45, the regulatory subunit bound 1.5 equiv of cAMP with half-maximal binding occurring at 33 nM, and kinetics for dissociation of bound cAMP were biphasic, indicating the presence of two different binding sites. These observations suggest that residues 1–45 function only in the formation of dimers and that dimerization has little influence on other functional properties of the regulatory subunit. More extensive proteolysis cleaved the monomeric fragment at Lys-311. The fragments resulting from this second cleavage did not dissociate, and the complex inhibited the catalytic subunit in a cAMP-dependent manner.

The adenosine cyclic 3′,5′-phosphate (cAMP) dependent protein kinases play an important role in mediating the effects of hormones that increase intracellular levels of cAMP (Krebs & Beavo, 1979; Flockhart & Corbin, 1982). These kinases exist in the form of a tetramer composed of a regulatory subunit, which is a homodimer, and two catalytic subunits. Addition of cAMP to the inactive holoenzyme results in dissociation of the complex into two catalytic subunits and a regulatory subunit dimer that binds 4 equiv of cAMP (Flockhart & Corbin, 1982). There are two major forms of the holoenzyme: type I and type II. These two forms have different regulatory subunits but the same catalytic subunits. Recently the amino acid sequences have been elucidated for the catalytic subunit from bovine heart (Shoji et al., 1983), for the type I regulatory subunit from bovine skeletal muscle (Titani et al., 1984), and for the type II regulatory subunit from bovine heart (Takio et al., 1984a). Comparison of the amino acid sequences (Takio et al., 1984b) has shown that the two types of regulatory subunit are structurally homologous as predicted from their functional similarity. The type II regulatory subunit has 400 residues, with residues 135–256 and 257–400 constituting the cAMP binding domains (Takio et al., 1984a).

The functional domains of both types of R[1] must include the following: two binding sites for cAMP, a domain that interacts with and inhibits C, and a domain responsible for dimer formation. The dimerizing domain is within the N-terminal 90 residues, since limited proteolysis of the regulatory subunit generates a monomeric C-terminal fragment of about 37 000 daltons (Corbin et al., 1978; Zoller et al., 1979) and a dimeric N-terminal fragment (Potter & Taylor, 1980). Additional evidence regarding the dimerizing domain comes from purified heterodimer forms, which can arise from proteolysis during purification of the cAMP-dependent protein kinase (Reimann & Rapino, 1974; Sugden & Corbin, 1976; Taylor & Stafford, 1978; Vogel & Heinz, 1980; Rannels et al., 1985). The heterodimer forms are cAMP-dependent and retain both binding sites for cAMP (Vogel & Heinz, 1980; Rannels et al., 1985). However, the size of the fragment

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[1] Abbreviations: AMP, adenosine 5′-monophosphate; cAMP, adenosine cyclic 3′,5′-phosphate; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; R, regulatory subunit of the cAMP-dependent protein kinase; C, catalytic subunit of the cAMP-dependent protein kinase; R^T, the 45 000-dalton form of the regulatory subunit obtained by proteolysis of the holoenzyme with trypsin; R^S, the 36 000-dalton form of the regulatory subunit obtained by proteolysis of the holoenzyme with trypsin; SDS, sodium dodecyl sulfate; CAP, catabolite gene activator protein of *Escherichia coli*; Tris, tris(hydroxymethyl)-aminomethane; HPLC, high-performance liquid chromatography; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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removed during proteolysis of the holoenzyme has not been established for any of these heterodimers. We, therefore, set out to characterize the fragment derived from tryptic digestion of the bovine type II holoenzyme, which has been sequenced (Takio et al., 1982), with the intent of identifying the site of cleavage and hence the dimerizing domain. While this paper was in preparation, Rannels et al. (1985) presented results similar to ours regarding kinetic properties of this bovine heterodimer. They also described the N-terminal amino acid sequence of a heterodimer derived from rabbit muscle type II holoenzyme, but because little is known about the sequence of the rabbit protein, the precise point of cleavage remains uncertain. We report here that trypsin converts the tetramer to a heterodimer as a result of removal of 45 residues from the N-terminus, indicating that the dimerizing domain is much smaller than previously recognized. We also report that more extensive proteolysis of the holoenzyme cleaves the regulatory subunit at a second site to generate a 36000-dalton fragment. However, amino acid sequence analysis showed that this fragment is not the same as the 37000-dalton fragment that occurs when R_2cAMP_4 is treated with low concentrations of trypsin.

Materials and Methods

Materials. Kemptide, cAMP, and other common reagents were purchased from Sigma. TPCK–trypsin was from Millipore. The Mono-Q column, the Superose-12 column, and Sephadex G-25 were from Pharmacia. The TSK 4000 column was from LKB. [^{3}H]cAMP was from ICN. [γ-^{32}P]ATP was prepared as described by Walseth & Johnson (1979).

Preparation of Enzymes. The protein kinase catalytic subunit was prepared from bovine heart (Reimann & Beham, 1983). The type II regulatory subunit was prepared as R_2cAMP_4 from bovine heart (Corbin et al., 1978). The type II holoenzyme (1–10 mg) was prepared at 4 °C by gel filtration of a mixture of R and a 10% molar excess of C on a Sephadex G-25 superfine column (1.5 × 110 cm) equilibrated in 25 mM Tris–SO_4 and 1 mM EDTA, pH 7.3 (G-25 buffer), at a flow rate of ~6 mL/h. In some preparations the excess C was then removed by ion-exchange chromatography on a 5 × 50 mm Mono-Q column developed with a gradient of 0–0.5 M $(NH_4)_2SO_4$ in 25 mM Tris–SO_4 (Mono-Q buffer) or on a 7.5 × 600 mm TSK-G 4000 SW column with a 7.5 × 75 mm TSK-GSWP precolumn equilibrated in 50 mM potassium phosphate and 1 mM EDTA, pH 6.8 (TSK buffer). Both columns were developed at 20 °C with a flow rate of 1 mL/min. Unless otherwise indicated, limited proteolysis of the holoenzyme (trypsin:holoenzyme = ~1:1000) was carried out at 30 °C for 10–30 min in G-25 buffer, Mono-Q buffer, or TSK buffer. The three buffers gave similar results. The reaction was terminated by the addition of a 5–20-fold excess of soybean trypsin inhibitor. The proteolyzed enzyme was separated from native holoenzyme and low molecular weight contaminants by gel filtration on a TSK 4000 column as described above or by gel filtration on a 10 × 300 mm Superose-12 column equilibrated in G-25 buffer.

Analytical Methods. Protein kinase activity was determined by a modification of the method of Roskoski (1983) in a reaction mixture containing 50 mM potassium phosphate, pH 6.8, 0.5 mM Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly), 0.2 mM [γ-^{32}P]ATP, 10 mM magnesium acetate, and 0.3 mg of gelatin/mL. Phosphorylated peptide was isolated on phosphocellulose papers washed in 75 mM phosphoric acid to remove unreacted ATP. cAMP binding activity was measured as described by Corbin et al. (1978). Protein concentration was measured by the method of Bradford (1976) or by absorbance at 280 nm based on the published $E^{0.1\%}$ values of 0.68 for R_2cAMP_4 (Corbin et al., 1978), 1.42 for C (Sugden et al., 1976), and 1.00 for R_2C_2 (derived from the values for C and nucleotide-free R).

Kinetic Analyses. The kinetics of cAMP activation were measured in the standard kinase reaction mixture with varied concentrations of cAMP. Reaction rates were determined at 30 °C over a 10-min time course. These rates were used to determine V_{max}, $A_{0.5}$, and Hill coefficient values by a weighted nonlinear regression analysis (Cleland, 1967) to fit the data to the equation:

$$v = V_{max}A^n/(A_{0.5}^n + A^n) \quad (1)$$

where v = velocity, V_{max} = velocity at saturating cAMP, A = cAMP concentration, $A_{0.5}$ = the concentration of cAMP giving half-maximal velocity, and n = the Hill coefficient.

Dissociation rates for cAMP were determined essentially as described by Rannells & Corbin (1980). Briefly, this involved incubating the holoenzyme (5.2 μg/mL) or proteolyzed holoenzyme (6.7 μg/mL) with 110 nM [^{3}H]cAMP for 60 min at 30 °C followed by the addition of 2.2 mM unlabeled cAMP and continued incubation at 30 °C. The amount of bound cAMP was determined immediately before addition of unlabeled cAMP and at the indicated times thereafter.

Molecular Weight Determinations. Subunit molecular weight values were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described by Laemmli (1970). Molecular weight values for native proteins were calculated from the relationship between gel concentration and mobility during nondenaturing polyacrylamide gel electrophoresis (Hedrick & Smith, 1968). The buffer system was Tris–asparagine (Davis et al., 1967). For each protein, the log of the relative mobility was plotted against gel concentration. A straight line was obtained when the slopes of these plots were graphed against $M_r^{1/2}$ for the following standards (M_r in parentheses): catalase (232000), bovine heart lactate dehydrogenase (140000), human transferrin (80000), ovalbumin (43000), and soybean trypsin inhibitor (21000).

Amino Acid Sequence Analysis. Amino acid sequence analyses were performed on a Beckman sequencer (Model 890B), and phenylthiohydantoin derivatives of amino acids were identified as described previously (Reimann et al., 1984).

Results

Effect of Trypsin Concentration on Proteolysis of the Holoenzyme. Polyacrylamide gel electrophoresis of the holoenzyme in the presence of SDS typically revealed bands corresponding to R phosphorylated at Ser-95 (M_r 56000), dephosphorylated R (M_r 52000), and C (M_r 40000). It should be noted that phosphorylation of R significantly reduces its electrophoretic mobility (Hofmann et al., 1975) and that mobility of R in this electrophoretic system is anomalous, since amino acid sequence analysis has demonstrated that the M_r is 45004 (Takio et al., 1984a). Figure 1 shows the effect of trypsin on a preparation of dephosphoholoenzyme. Electrophoresis after incubation with a low concentration of trypsin revealed a decrease in the amount of native R and the appearance of a new band at M_r 45000. With the highest concentration (10 μg/mL) of trypsin the 45000-dalton band virtually disappeared and another band appeared at M_r 36000 (Figure 1). When the holoenzyme was dissociated into R and C by the addition of cAMP before incubation with 1 μg of trypsin/mL, a 37000-dalton form appeared (Figure 1, right lane), consistent with previous observations showing that treatment of R_2cAMP_4 with low concentrations of trypsin, chymotrypsin, or *Staphylococcus aureus* protease generates

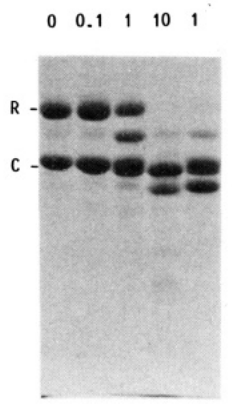

FIGURE 1: Effect of trypsin concentration on proteolysis of holoenzyme. Holoenzyme (750 μg/mL) was incubated with the indicated concentrations of trypsin for 15 min at 30 °C. The reactions were terminated by adding SDS and heating. Electrophoresis was on 10% polyacrylamide gels in the presence of SDS. Protein bands were identified by staining with Coomassie Blue. The numbers above each lane are the trypsin concentrations in micrograms per milliliter. For lane 5, 2.2 mM cAMP was included during the incubation with trypsin.

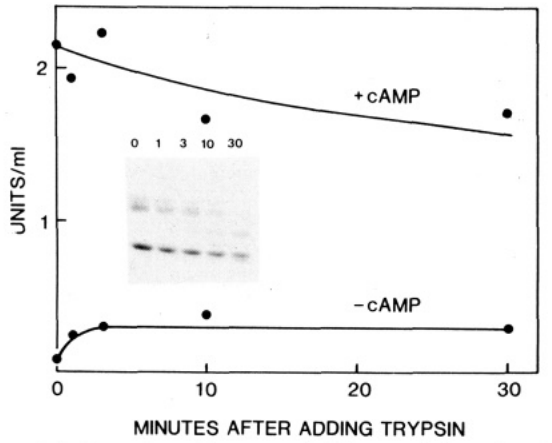

FIGURE 2: Effect of trypsin on protein kinase activity ratio. Holoenzyme (400 μg/mL) was incubated with trypsin (0.5 μg/mL) at 30 °C for the indicated times. The reactions were terminated by addition of soybean trypsin inhibitor (10 μg/mL) prior to electrophoresis or measurement of protein kinase activity with or without cAMP. Lanes 1–5 in the inset represent 8 μg of the holoenzyme incubated with trypsin for the indicated number of minutes.

a 39 000-dalton fragment (Takio et al., 1980). Treatment of the holoenzyme with low concentrations of chymotrypsin, *S. aureus* protease, or endoproteinase Lys C did not generate a 45 000-dalton fragment at any concentration tested (data not shown). The phosphorylated and dephosphorylated forms of the holoenzyme appeared to have similar susceptibility to trypsin.

Effect of Trypsin on Kinase Activity. The holoenzyme remained cAMP dependent after treatment with low concentrations of trypsin (Figure 2), showing that the 45 000-dalton fragment (R^T) retains the ability to interact with C in a cAMP-dependent manner. Prolonged trypsin treatment resulted in some loss of kinase activity measured in the presence of cAMP. This was accompanied by a decrease in the amount of C visible after staining for protein (Figure 2).

Effect of Trypsin on the Molecular Weight of the Holoenzyme. Trypsin greatly reduced the apparent molecular weight of the RC complex (Figure 3). The reduction in molecular weight paralleled the appearance of the 45 000-dalton form of R (data not shown). The retention times on the TSK 4000 column were 19.5 min for native holoenzyme (M_r 172 000), 22 min for the trypsin-treated enzyme, and 24.6 min for C (M_r 41 000). Lactate dehydrogenase (M_r 140 000)

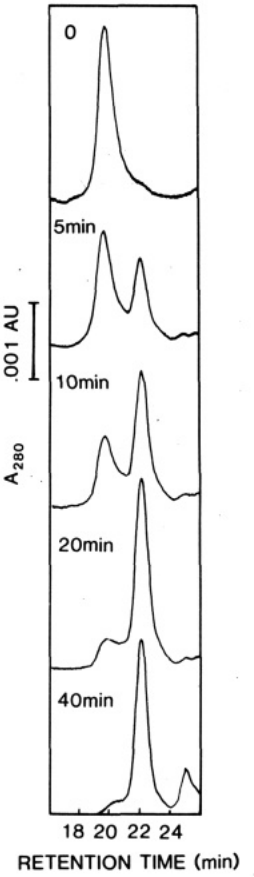

FIGURE 3: Effect of limited trypsin digestion on molecular weight of holoenzyme. The holoenzyme (210 μg/mL) was incubated with trypsin (0.9 μg/mL) for the indicated times. The reaction was stopped at the indicated times by adding soybean trypsin inhibitor (4.6 μg/mL), and 30-μL aliquots were analyzed by gel filtration on a TSK 4000 column as described under Materials and Methods.

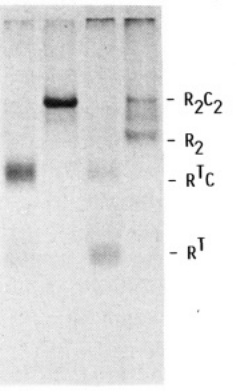

FIGURE 4: Nondenaturing polyacrylamide gel electrophoresis of native and proteolyzed holoenzyme. Electrophoresis was performed in 7.5% polyacrylamide gels as described under Materials and Methods, and proteins were identified by staining with Coomassie Blue. Lanes 1 and 3 represent proteolyzed holoenzyme and lanes 2 and 4 native holoenzyme. cAMP was added to the samples in lanes 3 and 4.

also eluted at 22 min, suggesting that the M_r of the trypsin-treated holoenzyme was ~140 000.

Purified preparations of trypsin-treated holoenzyme gave a single diffuse band when subjected to nondenaturing electrophoresis in the absence of cAMP (Figure 4, lane 1). When cAMP was added to the sample, a new band of much greater mobility appeared and the original band was almost eliminated (Figure 4, lane 3), indicating that the complex had dissociated into subunits. For comparison this gel also shows the mobility of holoenzyme before (lane 2) and after (lane 4) addition of

cAMP. The band of highest mobility in lane 4 had the same mobility as R_2 (data not shown). In this system, C does not move toward the anode and therefore is not seen on the gel. As described under Materials and Methods, this electrophoresis system was used to calculate the following M_r values: R^T = 44 500, R^TC = 87 100, R_2 = 94 000, and R_2C_2 = 174 700. (From amino acid sequence analysis R_2 = 90 000 and R_2C_2 = 172 000.) Thus, the holoenzyme preparation used was indeed the tetrameric form, and trypsin converted this tetrameric holoenzyme to a heterodimer, R^TC.

Isolation of R^TC. Purification of R^TC from the unproteolyzed holoenzyme and from low molecular weight fragments was achieved by gel filtration as described under Materials and Methods. Figure 4, lane 1, shows the appearance of a typical preparation on nondenaturing electrophoresis. Electrophoresis in the presence of SDS showed bands corresponding to C, R^T, and smaller amounts of the 36 000-dalton band (not shown). The identity of the 36 000-dalton band is discussed later.

Interaction of R^TC with cAMP. The cAMP dependence of purified R^TC was confirmed by measuring the kinase activity ratio (activity without cAMP/activity with cAMP). For two separate preparations, the activity ratios were 0.065 and 0.058. The kinase activities were 7.1 and 12.7 units/mg for these two preparations, compared to 4.7 and 7.5 units/mg for the respective holoenzymes before trypsin treatment. Measurement of cAMP binding to R^TC gave dissociation constants ranging from 27.4 to 40 nM (average 32.9 nM) in three separate experiments (data not shown). In parallel experiments, the corresponding values for native holoenzyme were 32.7–34.9 nM (average 34 nM). The kinetics of either form did not deviate significantly from those expected for a single class of noninteracting sites; i.e., the Hill coefficient was not significantly different from unity. At saturating concentrations of cAMP the mole ratio of cAMP bound per R monomer averaged 1.5 for R^TC and 1.6 for R_2C_2. The value of 1.6 for the holoenzyme is consistent with previously reported values (Rannels & Corbin, 1980). Thus the proteolyzed R monomer appears to retain both binding sites for cAMP.

It is possible to distinguish two kinetically different binding sites by using an exchange reaction in which the dissociation rate for [^{3}H]cAMP is measured by determining the amount of bound radioactivity remaining as a function of time after adding a large excess of unlabeled cAMP. With this method it has been shown that the dissociation of cAMP from the cAMP-dependent protein kinase exhibits biphasic kinetics indicating different dissociation rates for the two binding sites (Rannels & Corbin, 1980). Application of the method to R^TC likewise gave kinetics that were indistinguishable from those for R_2C_2 (Figure 5), again indicating that proteolysis produced minimal perturbation of the cAMP binding sites.

The effect of cAMP concentration on kinase activity of R^TC was measured to determine if proteolysis altered the sensitivity of the enzyme to activation by cAMP. In two separate experiments the activation of cAMP showed positive cooperativity with Hill coefficients of 1.2 and 1.7. The native holoenzyme gave values of 1.25, 1.33, and 1.44 under the same conditions. The average cAMP concentration for half-maximal activation was 51 nM for R^TC and 48 nM for holoenzyme. The results of one experiment on the proteolyzed enzyme are shown in Figure 6. These kinetic experiments give additional evidence that limited trypsin treatment did not significantly alter the cAMP binding sites.

Characterization of a 36 000-Dalton Tryptic Fragment (R^S). Proteolysis of the holoenzyme was generally accompanied by

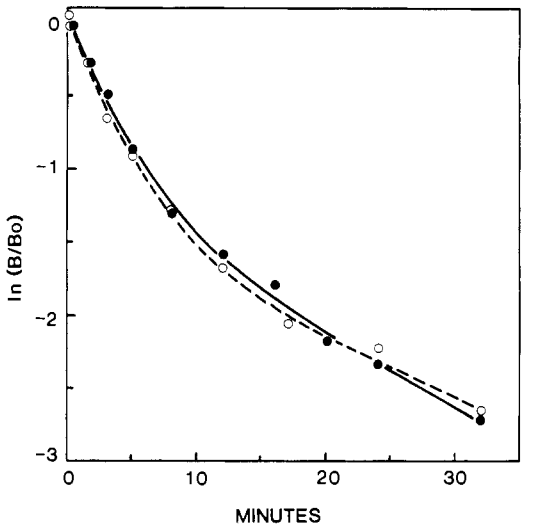

FIGURE 5: Kinetics for dissociation of cAMP. The dissociation rate for cAMP was measured as described under Materials and Methods. B refers to the amount of bound radioactivity at the indicated time points, and B_0 refers to the amount of radioactivity bound before the addition of unlabeled cAMP. The lines are computer plots of the equation $B = B_1e^{-K_1t} + B_2e^{-K_2t}$, where B_1 and B_2 refer to the amount of radioactivity bound to sites 1 and 2, respectively, and K_1 and K_2 refer to the dissociation rate constants for site 1 and site 2, respectively. For the holoenzyme (●), K_1 and K_2 = 0.048/min and 0.267/min, respectively, with 31% of the radioactivity bound to site 1 at t = 0. For R^TC (○), K_1 and K_2 = 0.040/min and 0.277/min, respectively, with 25% of the radioactivity bound to site 1 at t = 0.

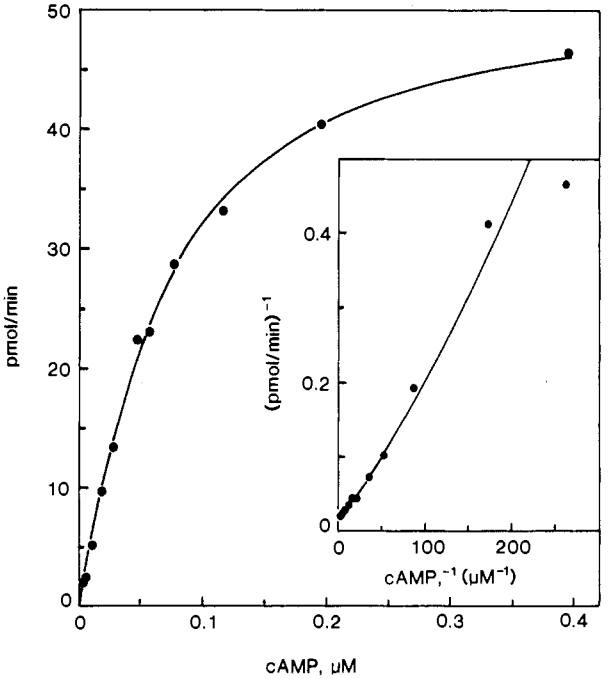

FIGURE 6: Effect of cAMP concentration on kinase activity of the heterodimer. The kinetics for cAMP activation were determined as described under Materials and Methods. For the data shown, n = 1.20, V_{max} = 51.3 pmol/min, and $A_{0.5}$ = 65.3 nM. The inset represents the data plotted in the form of a double reciprocal plot.

formation of a 36 000-dalton form in addition to the 45 000-dalton fragment (Figure 1). Extended proteolysis demonstrated that the amount of this form, subsequently referred to as R^S, was maximal when virtually all of the native R and R^T had disappeared, suggesting that it might be a further degradation product of R^TC. Initially it was assumed that R^S was the same as the 37 000-dalton fragment (residues 93–400) that results when R_2cAMP_4 is treated with trypsin (Takio et

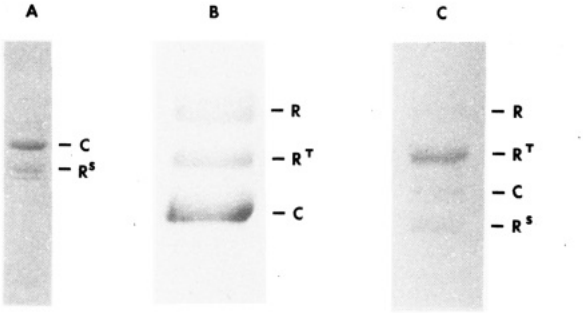

FIGURE 7: Electrophoresis of preparations of proteolyzed holoenzyme. Electrophoresis was carried out in the presence of SDS as described under Materials and Methods. (A) Electrophoresis of R^SC obtained by treating the holoenzyme with trypsin (E:S = 1:20) for 10 min. (B) Electrophoresis of the holoenzyme after partial conversion to R^TC by digestion for 5 min with trypsin (E:S = 1:1000). (C) Electrophoresis of the mixture of R^TC and R^SC obtained by treating the holoenzyme with trypsin (E:S = 1:750) for 16 min, followed by column chromatography as described in the text. The preparations analyzed in (B) and (C) were used for amino acid sequence analysis after precipitating the proteins with 10% trichloroacetic acid.

al., 1984a; Takio et al., 1982). Since fragment 93–400 does not combine readily with C and since it is monomeric, it should separate readily from R^TC on gel filtration. However, gel filtration failed to resolve R^S and R^TC, suggesting that R^S might be associated with C.

To characterize R^S in more detail, a preparation of the holoenzyme was digested with trypsin (E:S = 1:20) for 10 min, followed by addition of soybean trypsin inhibitor. Under these conditions all of the regulatory subunit was converted to fragments with M_r <40 000 (Figure 7A). Nonetheless, the preparation still showed stimulation by cAMP. Gel filtration on a Superose-12 HPLC column yielded a protein peak that contained both C and R^S as assessed by electrophoresis in the presence of SDS. The protein kinase activity ratio of this preparation was 0.12, indicating that R^S still functioned as a cAMP-dependent regulatory subunit. Electrophoresis of this preparation under nondenaturing conditions gave a result similar to that shown in Figure 4 (lanes 1 and 3); i.e., after cAMP addition the major band decreased in intensity and a new band of higher mobility appeared, suggesting that the band of lower mobility was R^SC and the higher mobility band was R^S. For comparison the tryptic fragment 93–400 was prepared by limited proteolysis of R_2cAMP_4. The mobility of this fragment was not affected by cAMP and was significantly greater than the mobility of R^S. Interestingly, the mobility of the R^S and R^T forms was very similar, suggesting that the fragments generated upon conversion of R^T to R^S remain associated except under denaturing conditions. Thus, R^S may actually consist of a complex between a 36 000-dalton fragment and another fragment too small to detect by electrophoresis in the presence of SDS. Support for this model comes from amino acid sequence analysis (see below).

Sites of Proteolysis by Trypsin. It was of particular interest to identify the site of cleavage that results in formation of the 45 000-dalton fragment since this cleavage abolishes dimerization without noticeably affecting the other properties of the enzyme. One would predict that the site of proteolysis described in the present study is near the N-terminus, since previous studies have suggested that the dimerizing domain is located within the N-terminal 90 residues (Potter & Taylor, 1980; Zoller et al., 1979). It seems unlikely that cleavage occurs at the C-terminus since cleavage at the C-terminus would probably perturb the cAMP binding site located at the C-terminus (Takio et al., 1984a,b). Furthermore, it seems unlikely that the C-terminal domain would be involved in both cAMP binding and dimer formation.

The approximate site of cleavage can be predicted from the apparent molecular weight of R^T compared to those of the native protein (apparent M_r 52 000) and fragment 93–400 (apparent M_r 37 000). Interpolation suggests that R^T lacks approximately 40 residues, possibly due to cleavage at Arg-38, Arg-40, Arg-43, or Arg-45. The actual site of cleavage was established as Arg-45 by automated Edman degradation. For this analysis a sample of holoenzyme (4.5 nmol of R monomer) was exposed to trypsin (E:S = 1:1000) for only 5 min in order to minimize the possibility of secondary cleavage sites. Gel electrophoresis showed that about 50% (2.3 nmol) of the regulatory subunit was cleaved in this incubation (Figure 7B). Since both subunits of the enzyme have blocked N-termini (Shoji et al., 1983; Takio et al., 1982), the mixture was precipitated with 10% trichloroacetic acid, extracted with ether, and subjected to N-terminal sequence analysis without further purification. Edman degradation unambiguously identified the N-terminal residue as alanine. The yield was 1.2 nmol, indicating a typical yield of 50% in the Edman degradation. Lesser amounts (0.3–0.5 nmol) of valine, serine, and lysine were also observed, with serine apparently the most abundant among these. The second cycle of Edman degradation yielded serine as the predominant residue with significant amounts of arginine, leucine, and glycine also present. Examination of the published amino acid sequences of the catalytic subunit and the type II regulatory subunit from bovine heart revealed that the only occurrence of the sequence Lys/Arg-Ala-Ser is at Arg-45 in the regulatory subunit, thereby identifying this as the primary cleavage point. Subsequent cycles of Edman degradation were consistent with this sequence in that significant amounts of threonine, proline, and alanine were observed in cycles 3, 4, and 6, respectively, as would be expected for the known sequence Arg-Ala-Ser-Thr-Pro-Pro-Ala. The presence of some serine in cycle 1 and arginine in cycle 2 suggested that some of the regulatory subunit may have been cleaved at Arg-43 yielding a second N-terminal sequence, Ser-Arg. However, the presence of other residues in cycles 1 and 2 precludes definitive identification of sequences other than the major sequence.

The analysis was repeated with a second sample that had been exposed to trypsin for 16 min (E:S = 1:750). The reaction was stopped by addition of soybean trypsin inhibitor. cAMP was added, and the mixture was passed over a phosphocellulose column to remove the catalytic subunit. This was followed by gel filtration on a TSK 4000 column to remove low molecular weight fragments. SDS gel electrophoresis revealed that this preparation contained both R^S and R^T as indicated by the presence of 45 000- and 36 000-dalton fragments. In addition, small amounts of C and unmodified R were present (Figure 7C). Sequence analysis of this sample gave two major residues in each cycle. For the first six cycles the pairs of residues were as follows: alanine/valine, serine/asparagine, threonine/lysine, proline/aspartic acid, proline/glycine, and alanine/glutamic acid, suggesting a mixture of the two sequences Ala-Ser-Thr-Pro-Pro-Ala and Val-Asn-Lys-Asp-Gly-Glu corresponding to the sequences beginning at Ala-46 and Val-312. Significantly, there was no indication of cleavage at Arg-92, which occurs upon tryptic cleavage of free regulatory subunit (Takio et al., 1982) to yield the sequence Arg-Val-Ser-Val-Cys-Ala. This analysis indicates that the 36 000-dalton fragment derived by tryptic digestion of the holoenzyme consists of residues 46–311 and that R^S consists of a complex between fragments 46–311 and 312–400. It should be emphasized that these data do not rule out loss of

small peptides from the C-terminal of these two fragments.

Discussion

Previous studies have demonstrated that the cAMP-dependent protein kinase can exist in the form of a heterodimer (Reimann & Rapino, 1974; Sugden & Corbin, 1976; Taylor & Stafford, 1978; Vogel & Heinz, 1980; Rannels et al., 1985). The data presented here show that the heterodimer formed by trypsin treatment of the holoenzyme retains two binding sites for cAMP in agreement with previous observations on heterodimer forms of this enzyme (Vogel & Heinz, 1980; Rannels et al., 1985). In addition, the data reported here demonstrate that the dimerizing domain of bovine type II R is much closer to the N-terminus than expected. Specifically, removal of only 45 residues is sufficient to eliminate dimer formation. Rannels et al. (1985) reported the N-terminal sequence of a nondimerizing proteolytic fragment derived from type II R from rabbit skeletal muscle. The N-terminus of the latter fragment was aligned with residue 89 of both type I and type II R from bovine muscle, suggesting that the dimerizing domain is limited to about 88 residues at the N-terminus. Although more information about the primary sequence is needed to identify the precise site of cleavage in the rabbit enzyme, it would appear that the monomeric regulatory subunit derived from the rabbit enzyme is shorter than the tryptic fragment generated from the bovine enzyme. Of the 45 residues in the dimerizing domain of the bovine protein, 20 have hydrophobic properties, suggesting that hydrophobic interactions may be important in maintaining the dimeric structure. However, it should be noted that type I R and cGMP-dependent protein kinase, which are also dimers, are much less hydrophobic in this region (Titani et al., 1984; Takio et al., 1984b). The region around Arg-45 must be on the surface as it is readily accessible both to trypsin and to glycogen synthase kinase 3, which phosphorylates Ser-44 and Ser-47 (Hemmings et al., 1982).

In addition to establishing the location of the dimerizing domain, these studies demonstrated that more extensive tryptic digestion resulted in formation of a 36 000-dalton species, which apparently represents residues 46–311. A preparation of R^T that contained both R^T and R^S (Figure 7C) was subjected to amino acid sequence analysis. This sample yielded two sequences beginning at Arg-46 and Val-312, indicating that the two fragments produced by cleavage at Lys-311 remain associated.

The crystal structure of a cAMP-binding protein from *Escherichia coli*, the catabolite gene activator protein (CAP), has been determined with a resolution of 2.9 Å. On the basis of this structure and on amino acid sequence homologies between it and the regulatory subunit, Weber et al. (1982) have predicted the three-dimensional structure of the cAMP binding domains of the regulatory subunit. This structure predicts that residues 308–319 form a loop between β strand 4 and β strand 5. The fact that Lys-311 is highly susceptible to tryptic attack suggests that this loop is on the surface of the protein. In addition, it appears that this loop is not directly involved in binding to the catalytic subunit, since cleavage occurs even when the catalytic subunit is bound to the regulatory subunit. The structure of CAP further predicts an association between the following antiparallel β strands: 281–290 and 342–350, 301–307 and 320–328, and 294–298 and 229–233. From this, one would predict that fragments 46–311 and 312–400 would remain associated even after cleavage at Lys-311. This is consistent with the observation that the C-terminal fragment beginning at Val-312 copurified with the 36 000-dalton fragment during gel filtration and with the finding that cleavage at Lys-311 was not associated with significant changes in electrophoretic behavior under nondenaturing conditions. Proof of this postulated structure must of course await determination of the crystal structure of this protein.

It is interesting to compare limited proteolysis of R_2C_2 and R_2. Low concentrations of any one of several proteases cleave R_2 at the "hinge" region to yield fragment 93–400 (Takio et al., 1980; Takio et al., 1982). This fragment has an apparent M_r of ~37 000. In contrast, the holoenzyme is resistant to proteolysis at the hinge region; i.e., the presence of the catalytic subunit protects the hinge region from proteolysis. This is consistent with other data which suggest that the hinge region interacts with the catalytic subunit (Flockhart & Corbin, 1982; Takio et al., 1982). Although treatment of holoenzyme with higher concentrations of trypsin generates a fragment of M_r 36 000, this fragment is not the same as that generated from R_2. Another contrast between R_2 and R_2C_2 exists in that R_2 is cleaved at Lys-315 (Takio et al., 1984a) rather that at Lys-311, indicating that the conformation of this region is altered when either C or cAMP binds to the protein.

As noted by Rannels et al. (1985), one can infer that site–site interactions are similar for native tetrameric holoenzyme and the heterodimer obtained after trypsin treatment since the kinetics for cAMP binding, cAMP dissociation, and kinase activation are indistinguishable. If this inference is correct, then R–R interactions must have little influence on activation of the tetrameric holoenzyme by cAMP. However, it is possible that subunit interactions are important for other properties of the enzyme, e.g., thermal stability or sensitivity to covalent modification. The conclusion that there is little interaction between cAMP binding sites on different subunits is consistent with previous observations showing significant accumulation of a trimer species R_2C (Rangel-Aldao & Rosen, 1977; Connelly et al., 1986); i.e., accumulation of trimer would be expected only if positive homotropic interactions between regulatory subunits are weak. Thus, the positive Hill coefficient for stimulation of kinase activity in the case of the tetramer may derive from homotropic interactions on the same chain as is observed for the heterodimer described here.

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Fractional Diffusion-Limited Component of Reactions Catalyzed by Acetylcholinesterase†

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ABSTRACT: The values of k_{cat}/K_m for the reactions of four substrates, *p*-nitrophenyl acetate (PNPA), propionyl-β-methylthiocholine (PrMSCh), 3,3-dimethylbutyl thioacetate (DBTA), and acetylthiocholine (AcSCh), with acetylcholinesterase were determined as a function of increasing viscosity (η_{rel}) in sucrose-containing and in glycerol-containing buffers. Glycerol, or possibly some contaminant of it, was found to be a nonspecific inhibitor and sucrose a nonspecific activator of the enzyme as reflected in the dependence of k_{cat}/K_m values measured for PNPA and PrMSCh upon the concentration of these reagents. The rates of reactions of these two substrates, the first neutral and the second cationic, are chemically limited rather than diffusion limited, and they thus serve as quantitative controls or internal standards to monitor the effects of the viscosogens on the enzyme, which are not related to diffusion. The additional effect on k_{cat}/K_m over the controls observed for the rapidly reacting substrates AcSCh (cationic) and DBTA (neutral) serves as a measure of the extent to which these values of k_{cat}/K_m measure diffusion-controlled processes. The reaction rate of DBTA with the enzyme is 24% diffusion controlled as measured in glycerol-containing buffers and 16–20% as determined in sucrose-containing buffers, while that for AcSCh is 100% (in glycerol) and 24–40% (in sucrose) diffusion controlled.

The reactions of acetylcholinesterase, the enzyme that catalyzes the hydrolysis of acetylcholine (AcCh)[1] at neuromuscular junctions, with good substrates exhibit some of the highest values of k_{cat}/K_m known, e.g., 1.6×10^9 M^{-1} s^{-1} for AcSCh at ionic strength = 0.0032, decreasing to 2×10^8 M^{-1} s^{-1} at ionic strength = 0.13 (Nolte et al., 1980). The magnitude of this figure has suggested that the rate of the reaction may be diffusion controlled (Rosenberry, 1975; Nolte et al., 1980; Hasinoff, 1982). Further evidence in support of this conjecture includes the lack of a solvent kinetic effect on k_{cat}/K_m for AcCh (Rosenberry, 1975) and the sensitivity of this parameter to increases in viscosity (Hasinoff, 1982). On the other hand, the correlation demonstrated by Hasan et al. (1980) of k_{cat}^n/K_m, where k_{cat}^n is the value of k_{cat} normalized

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); AcSCh, acetylthiocholine; PrMSCh, propionyl-β-methylthiocholine; PNPA, *p*-nitrophenyl acetate; DBTA, 3,3-dimethylbutyl thioacetate; AcCh, acetylcholine.

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