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Primary structure of the regulatory subunit of type II cAMP-dependent protein kinase from bovine cardiac muscle

(regulatory enzyme/phosphoserine/metabolic control/gene duplication/cAMP)

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ABSTRACT **The complete amino acid sequence of the regulatory subunit of type II cAMP-dependent protein kinase from bovine cardiac muscle is presented. Primary fragments for the sequence determination were obtained by limited proteolysis with various proteases or by cleavage with cyanogen bromide. The sequence of the 400 amino acid residues has two homologous regions, strongly suggesting tandem gene duplication. The predicted secondary structure suggests the presence of 42% α-helix, 23% β-strand, and 23 β-turns. The molecular weight of the subunit, as derived from the sequence, is 45,084 including a phosphate group at residue 95. This is significantly less than earlier estimates based on $NaDodSO_4$ gel electrophoresis and sedimentation experiments. The structure is discussed in terms of putative sites of interaction with cAMP and with the catalytic subunit.**

The protein kinases, which catalyze reactions in which the terminal phosphate of ATP is transferred to protein substrates, constitute a class of enzymes that play an important role in the regulation of cellular functions (1). Of this group, the cAMP-dependent protein kinases, which serve as mediators for the actions of cAMP in eukaryotic cells, are the best characterized. Each is made up of regulatory and catalytic subunits, R and C, respectively, and exists as an inactive holoenzyme form, R_2C_2, that dissociates in the presence of cAMP to give two active catalytic subunits, C, and a dimeric regulatory subunit–cAMP complex, $R_2(cAMP)_4$ (2, 3). Two types of cAMP-dependent protein kinase (I and II), which differ primarily in the properties of their R subunits, are found in tissues (for review, see ref. 4); a variant of type II is present in brain (5). The two types of protein kinase have different tissue distributions (6) and vary in their relative amounts during development (7), suggesting that they may have different physiological functions. Type II cAMP-dependent protein kinase undergoes an autophosphorylation reaction (8) in which a specific site in the regulatory subunit—i.e., in R_{II}—is phosphorylated. The type I enzyme does not undergo autophosphorylation but can be phosphorylated *in vitro* in a slow reaction catalyzed by cGMP-dependent protein kinase (9).

An ever-increasing amount of structural information on the two main types of cAMP-dependent protein kinase is becoming available. Pertinent to the present paper is the finding that both R_I and R_{II} are readily cleaved into separable amino-terminal and carboxyl-terminal domains by various proteases (3, 10–12). Limited amino acid sequence data involving the hinge region of R_I and R_{II} that separates the two domains are available (12, 13). It is of interest that the autophosphorylation site of R_{II} is in this region (12, 13), as is the site on R_I that is phosphorylated by cGMP-dependent protein kinase (14). It has been shown that the cAMP binding sites on R_I and R_{II} are located in the carboxyl-terminal domain, and the sequence around one of the two binding sites (a photoaffinity-labeled tyrosine) on R_{II} has been studied (15). The complete amino acid sequence of the catalytic subunit of cAMP-dependent protein kinase has been determined (16).

The present report gives the complete amino acid sequence of beef heart R_{II} and the general strategy for its derivation. The sequence shows internal sequence homologies, probably indicative of gene duplication, as had been predicted (10). Another interesting feature is the presence of unusually acidic regions that may have specific functional roles. A molecular weight of 45,084, about 10,000 less than that previously inferred by $NaDodSO_4$ gel electrophoresis, is calculated from the amino acid sequence data. A detailed proof of this structural determination will appear elsewhere.

MATERIALS AND METHODS

R_{II} was prepared from beef heart according to Dills *et al.* (17) and stored frozen. *N*-Tosylphenylalanine chloromethyl ketone-treated trypsin and α-chymotrypsin were purchased from Worthington. A protease from *Staphylococcus aureus* V8 and proline-specific endopeptidase were obtained from Miles. Mast cell protease II (18, 19) was a gift from N. Katunuma. Thermolysin was purchased from Calbiochem–Behring. Gel filtration media (Sephacryl S-300 and S-200 and Sephadex G-50 superfine and G-25 fine) were products of Pharmacia.

Purification of peptides by reverse-phase HPLC was carried out with a Varian 5000 liquid chromatograph on columns of μBondapak C18 or CN (Waters Associates), SynChropak RP-P (SynChrom), or ODS-HC/SIL-X-1 (Perkin–Elmer) using a trifluoroacetic acid/acetonitrile elution system (20, 21). Amino acid compositions were analyzed on a Dionex D500 amino acid analyzer.

Automated Edman degradations were carried out in a Beckman sequencer model 890C according to Edman and Begg (22) using a program adapted from Brauer *et al.* (23) with double coupling for the first cycle. Three milligrams of Polybrene (24) was added to the cup in most cases to prevent peptide washout. The degradation products were identified semiquantitatively by two complementary HPLC systems (25, 26). Radioactivity of ^{14}C- and ^{32}P-labeled derivatives was measured with a Packard model B2450 scintillation counter. ^1H NMR spectroscopy (Bruker Cryospec WM 500 with a Nicolet 500-MHz magnet) and mass spectrometry (VG7070 GC/MS with a VG2035 F/B data system) were used for identification of the amino-terminal blocking group.

A computer search for a homologous sequence was carried out by the National Biomedical Research Foundation using the *Atlas of Protein Sequence and Structure* updated in September 1981. Internal sequence homology was analyzed by David

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Abbreviation: R (R_I and R_{II}), regulatory subunit of cAMP-dependent protein kinase (type I and type II).

Teller on a PDP-12 computer with a sequence comparison program (27) using the scoring tables of McLachlan (28) and Barker and Dayhoff (29). Secondary structure was predicted according to Chou and Fasman (30) using conformational parameters of Argos *et al.* (31).

Limited proteolysis was carried out as reported (12), except for the extended tryptic digest, in which the R_{II}/trypsin (wt/wt) ratio was 10 and digestion lasted for 45 min. Methionyl bonds were cleaved in S-carboxymethylated R_{II} [alkylated as described for the fragments of limited proteolysis (12)] by reaction with cyanogen bromide (Eastman) in 70% formic acid at room temperature for 3 hr. The fragments were separated on a Sephadex G-50 column equilibrated with 9% formic acid.

RESULTS AND DISCUSSION

Limited treatment of R_{II} with various proteases yielded two complementary fragments in every case (12). The region susceptible to proteolytic attack contains the site of the phosphoryl group that is enzymatically exchangeable—i.e., the autophosphorylation site (8). When R_{II} was treated more extensively with trypsin (enzyme/substrate, 1:10; 45 min), both primary fragments were further digested, yielding more than six fragments (Fig. 1). Cyanogen bromide cleavage of S-carboxymethylated R_{II} yielded 11 fragments (10 major and 1 minor overlap from incomplete cleavage) (32). Fragments that eluted together during gel filtration were purified by reverse-phase HPLC. Isolated fragments were analyzed for their amino-terminal sequences. Average repetitive yields of 90–95% were usually obtained, but progressive loss of peptide was experienced with some small peptides, even in the presence of Polybrene. The sequence positions of ≈75% of the total residues of R_{II} were determined from these primary fragments. In the analysis of fragment L_{tC}, four methionines were placed in a single sequence, thus overlapping five cyanogen bromide fragments.

To complete the sequence, some of the primary fragments were subjected to secondary enzymatic digestion or to chemical cleavage of the four acid-labile Asp-Pro bonds (33). To locate methionyl overlaps, the methionine residues in fragment L_t were labeled with [^{14}C]iodomethane (34), citraconylated (35), and digested with trypsin. The digest was fractionated by reverse-phase HPLC on a μBondapak C18 column and the radiolabeled methionine-containing peptides were selectively isolated and analyzed. The amino-terminal portion of R_{II} was one of the most difficult portions to analyze because it is blocked. Fragments with the blocked amino terminus (e.g., S_t, S_{tN}) were subdigested with thermolysin, chymotrypsin, proline-specific endopeptidase, or concentrated HCl. Integration of the data obtained from these peptides yielded the complete sequence of this region. An acetyl group was identified as the amino-terminal blocking group by the use of ^{1}H NMR and mass spectrometry of the amino-terminal dipeptide (X-Ser-His) obtained from thermolytic digestion. It was also difficult to overlap the large and small fragments obtained by limited proteolysis because the hinge region is very sensitive to the proteolytic enzymes used. The overlap was provided by a fragment (residues 62–99) isolated after Asp-Pro cleavage of fragment S_{sp}. A single major sequence was obtained from R_{II} after cleavage of the tryptophyl bond with 2-(2-nitrophenylsulfenyl)-3-methyl-3′-bromoindolenine (36).

The completed 400-amino acid residue sequence (Fig. 2) placed the only tryptophan residue at 226, the photoaffinity-labeled tyrosine residue (at a cAMP binding site) (15) at 381, and the autophosphorylation site (11, 12, 37) at serine 95. R_{II} is reported to contain another phosphate that is not enzymatically exchangeable (10), but we failed to locate this phosphate in the sequence. It is possible that the phosphorylation of the

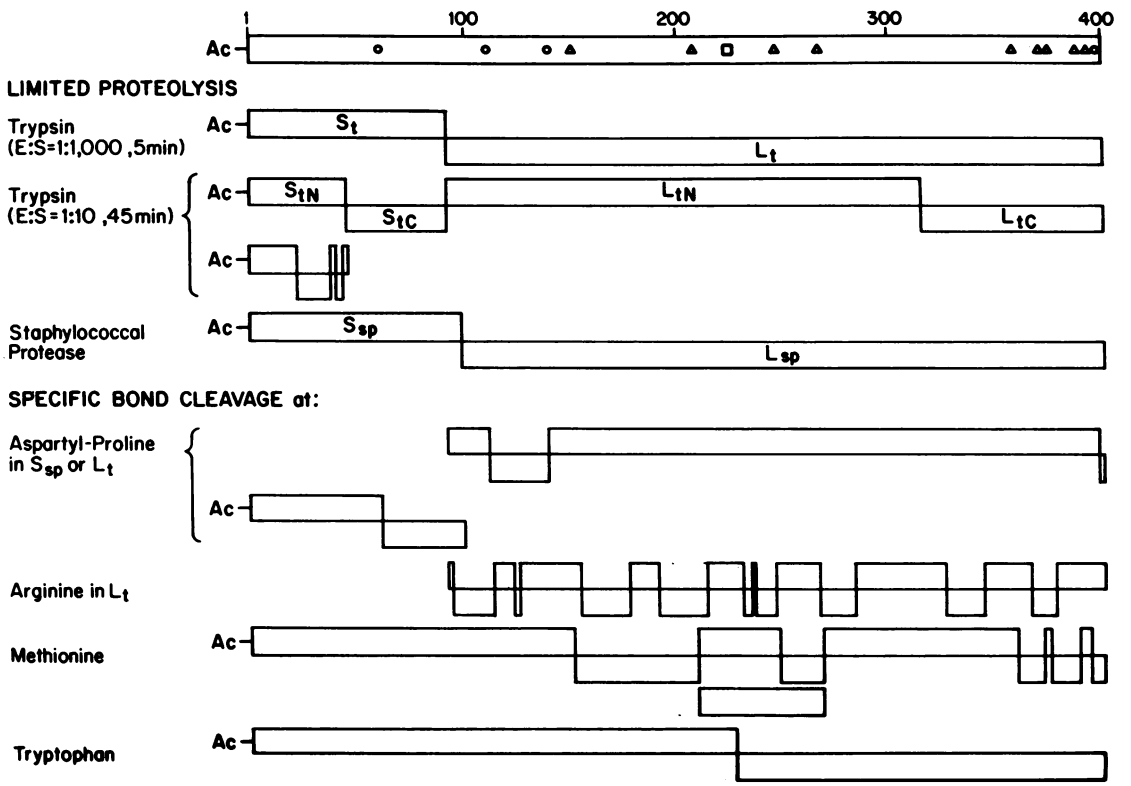

FIG. 1. Diagram indicating the origins of the primary and secondary fragments of R_{II} and their relationships to each other. The length of each bar is proportional to the length of the fragment. The top bar represents R_{II}. △, Methionine residues; □, the single tryptophan residue; ○, Asp-Pro bonds.

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                5        10        15        20        25        30
 1  Ac-S H I Q I P P G L T E L L Q G Y T V E V L R Q R P P D L V D
31    F A V D Y F T R L R E A R S R A S T P P A A P P S G S Q D F
61    D P G A G L V A D A V A D S E S E D E E D L D V P I P G R F
91    D R R V S V C A E T Y N P D E E E E D T D P R V I H P K T D
121   Q Q R C R L Q E A C K D I L L F K N L D P E Q L S Q V L D A
151   M F E R T V K V D E H V I D Q G D D G D N F Y V I E R G T Y
181   D I L V T K D N Q T R S V G Q Y D N H G S F G E L A L M Y N
211   T P R A A T I V A T S E G S L W G L D R V T F R R I I V K N
241   N A K K R K M F E S F I E S V P L L K S L E V S E R M K I V
271   D V I G E K V Y K D G E R I I T Q G E K A D S F Y I I E S G
301   E V S I L I K S K T K V N K D G E N Q E V E I A R C H K G Q
331   Y F G E L A L V T N K P R A A S A Y A V G D V K C L V M D V
361   Q A F E R L L G P C M D I M K R N I S H Y E E Q L V K M F G
391   S S M D L I D P G Q
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FIG. 2. Amino acid sequence of R_{II}. Circled P, autophosphorylation site; Ac, N^{α}-acetyl group on the amino-terminal serine. A, alanine (25 residues); C, cysteine (6 residues); D, aspartic acid (36 residues); E, glutamic acid (35 residues); F, phenylalanine (15 residues); G, glycine (25 residues); H, histidine (6 residues); I, isoleucine (24 residues); K, lysine (23 residues); L, leucine (30 residues); M, methionine (9 residues); N, asparagine (12 residues); P, proline (20 residues); Q, glutamine (18 residues); R, arginine (26 residues); S, serine (25 residues); T, threonine (18 residues); V, valine (34 residues); W, tryptophan (1 residue); Y, tyrosine (12 residues); total, 400 residues. M_r: without phosphate, 45,005; with phosphate, 45,084.

second site varies from one preparation to another or that R_{II} has several minor sites that can be phosphorylated by different kinases. It is also possible that a unique phosphorylation site is not stable during the fragmentation and isolation procedures. Although the second cAMP binding site also remains to be elucidated, the internal homology of the subunit described below does suggest a possible location of that site.

The molecular weight of R_{II} is calculated to be 45,084 including the phosphate at residue 95. This value is significantly less than the reported value of 54,000–56,000. This discrepancy may have resulted from the anomalous character of R_{II}, with its axial ratio of 12 (dimer), even though most of the reported values were determined by $NaDodSO_4$ gel electrophoresis—i.e., under conditions in which the protein is presumably completely denatured. Erlichman *et al.* (38) reported a dimeric molecular weight of 97,000 from sedimentation experiments. This value is also higher than that calculated from the sequence. In our previous paper (12), the amino acid compositions were calculated on the basis of molecular weights estimated from $NaDodSO_4$ gel electrophoresis. These molecular weights are apparently overestimated by ≈30%/20%, and 65% for R_{II} and for the large and small fragments from limited proteolysis, respectively. The small fragment is known to dimerize and is believed to be the region responsible for dimerization of R_{II} (39). Possibly the small fragment is still forming a dimeric structure in the presence of $NaDodSO_4$. The amino acid composition obtained from hydrolysates and that from the complete sequence are in good agreement when compared on the same molecular weight basis.

Secondary structure prediction indicated the presence of 42% α-helix, 23% β-strand, and 23 β-turns as shown in Fig. 3. While the relative content of α-helix agrees with that calculated from the CD spectrum of R_{II} (38.3%), the relative content of β-structure does not (30.4% from CD measurements) (unpublished results). The difference may have resulted from possible overestimation of β-turns and from the abnormal shape of R_{II} (axial ratio of 12). The hinge region was predicted to be a β-

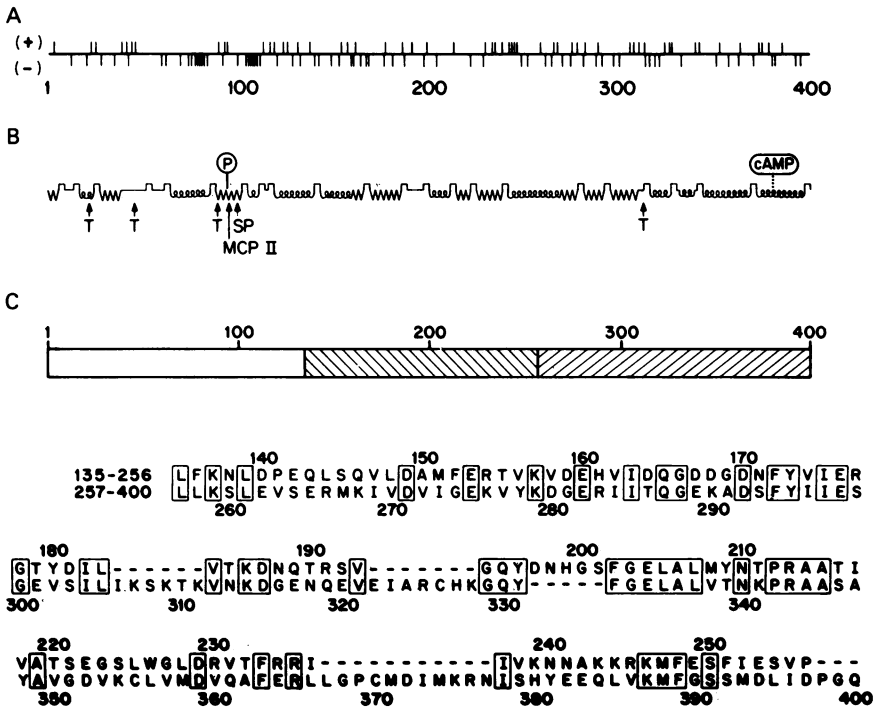

FIG. 3. Representations of R_{II} and its internal sequence homology. (*A*) Distribution of charged residues. +, Basic residues (histidine, lysine, arginine); −, acidic residues (aspartic acid, glutamic acid). (*B*) Predicted secondary structure and sites of limited proteolysis. A coil denotes an α-helix, a sawtooth pattern identifies a β-strand, a straight line represents an aperiodical structure, and a clamp indicates a β-turn tetrapeptide. Circled P and cAMP, autophosphorylation site (residue 95) and photoaffinity-labeled tyrosine (residue 381), respectively; ↑, sites of limited proteolysis by trypsin (T), staphylococcal protease (SP), and mast cell protease II (MCP II). (*C*) Internal sequence homology. ▨, Suggested regions of internal homology (residues 135–256 and 257–400); identical residues are enclosed in boxes; ---, gaps placed to optimize the homology.

structure sandwiched between two β-turns. All of the sites susceptible to limited proteolysis were located in the near vicinity of β-turns or in a β-strand.

As seen in Fig. 3, there are two extremely acidic regions (residues 73–83 and 104–111), one on each side of the autophosphorylation site (residue 95). This intervening region is accessible not only to proteases during limited proteolysis but also to the catalytic subunit during autophosphorylation. The catalytic subunit is a basic protein with a particularly basic region in the vicinity of the MgATP binding site (16), whereas all known protein inhibitors of the enzyme are acidic (40–42). The ability of histones and protamine to dissociate the holoenzyme (43) may simply be a result of competition between these basic proteins and the catalytic subunit for the regulatory subunit. Therefore, we suggest that at least one of the two extremely acidic regions may be involved in the interaction with the catalytic subunit.

Armstrong and Kaiser (44) reported that one of the six cysteine residues in R_{II} reacts with 5,5′-dithiobis(2-nitrobenzoic acid) more slowly in the holoenzyme than in isolated R_{II}. The proximity of cysteine 97 to the autophosphorylation site between the acidic regions makes it a likely candidate for this protected residue. It is not clear at this time which of the remaining five cysteine residues is the other one observed by Armstrong and Kaiser to be protected by cAMP.

On prolonged storage in a refrigerator, our preparations of R_{II} are found to be degraded by a contaminating "endogeneous protease" to a large fragment "L_e" (M_r, 37,000 by $NaDodSO_4$ gel electrophoresis) that retains regulatory activity—i.e., the ability to bind both a catalytic subunit and cAMP (unpublished results). Amino-terminal sequence analysis identified this fragment as residues 91–400 and suggested that the specificity of the endogeneous protease is similar to that of chymotrypsin. These results are consistent with the observation of Weber and Hilz (45) that chymotryptic digestion of R_{II} did not destroy the regulatory function. It is curious that fragment L_e (residues 91–400) binds the catalytic subunit whereas fragment L_t (residues 93–400) does not. This was clear because L_e both inhibits the catalytic subunit and becomes autophosphorylated (unpublished results) whereas L_t does neither to a comparable extent. Yet, the two fragments differ by only two residues (Asp-Arg at the amino terminus of L_e), suggesting the importance of residues 91 and 92 in the interaction of R_{II} and the catalytic subunit.

During the course of the sequence analysis, several remarkably similar sequences were noticed (Fig. 3). A computer analysis for internal homology indicated that residues 135–256 and 257–400 could have resulted from internal gene duplication. Of the four gaps introduced to optimize the homology, all but one (between residues 236 and 237) have statistical significance. This indication of possible gene duplication agrees with an earlier suggestion of Flockhart *et al.* (10), who offered it as an explanation of the binding of two molecules of cAMP per subunit. Assuming that those two regions were derived from tandem duplication of an ancestral gene and that elements of the original conformation were preserved in each of the duplicated regions, the residue corresponding to the affinity-labeled tyrosine (residue 381) would be expected in the region of residue 240. However, it may be more realistic to consider that the photoaffinity-labeled tyrosine 381 is not located in the cAMP binding site itself but in the vicinity of the site in the three-dimensional structure [as in the AMP binding site of phosphorylase (46)], because there is little sequential homology in the corresponding regions. The failure to label the second cAMP binding site would then be explained by the simple lack of a reactive amino acid residue (e.g., histidine, tyrosine) in the corresponding vicinity. If we assume that cyclic nucleotide binding sites are highly conserved through evolution, the homologous regions at residues 202–215 and 332–345 become likely candidates. In fact, sequences nearly identical to those are also found in the regulatory domain of cGMP-dependent protein kinase (unpublished results), which is closely related functionally to cAMP-dependent protein kinase.

Another pair of homologous regions comprises residues 60–87 and 90–117, including the autophosphorylation site and the acidic regions discussed above. Although these regions may also have resulted from gene duplication, they could simply be the result of convergent evolution to optimize electrostatic interaction with the catalytic subunit. Hashimoto *et al.* (14) reported recently the amino acid sequence around the hinge region of the type I regulatory subunit. Comparison of that sequence with the corresponding region of R_{II} shows rather poor identity (27%), suggesting a wide divergence in their evolutionary history.

A search for other sequentially related proteins among 1,666 known protein sequences, including kinases involved in transformation, did not identify homologous candidates.

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