THE AMINO ACID SEQUENCE OF A HINGE REGION IN THE REGULATORY SUBUNIT OF BOVINE CARDIAC MUSCLE CYCLIC AMP-DEPENDENT PROTEIN KINASE II

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1. Introduction

Cyclic AMP-dependent protein kinases (EC 2.7.1.37; ATP:protein phosphotransferase) are widely distributed and are known to play important roles in the regulation of metabolism and other cellular functions [1,2]. In each case examined, the enzyme consists of two catalytic and two regulatory subunits. Two types of enzyme appear to differ only in the regulatory subunit. Type I is the predominant form in skeletal muscle and type II in cardiac muscle. Upon binding cAMP, the enzyme dissociates into two catalytic subunits and a dimer of the regulatory subunit. Type II regulatory subunit (R_{II}) contains two covalently bound phosphates, of which one is enzymatically exchangeable [3]. This phosphate is partially lost by phosphatase action during isolation, but can be re-introduced from ATP by the catalytic subunit. The amino acid sequence around this 'autophosphorylation' site has been reported to be Asp–Arg–Arg–Val–Ser(P)–Val in bovine R_{II} [4].

Limited proteolysis of R_{II} has been studied by several investigators and found to generate a large fragment (~39 000 dalton) which binds cAMP but does not dimerize and a small fragment (~17 000 dalton) which retains the ability to dimerize but does not bind cAMP [5–8]. A smaller cAMP binding fragment (14 000 dalton) was obtained by tryptic digestion [9]. A partial amino-terminal sequence of the large fragment was obtained from porcine R_{II} by chymotryptic digestion [10]. Here we have examined the products of limited proteolysis of bovine R_{II} by three different proteases in order to define the 'hinge region' between domains and to provide a practical starting point for sequence analysis of the whole subunit. We establish a 26-residue sequence which includes the site of the autophosphorylation.

2. Materials and methods

The regulatory subunit of the cyclic AMP-dependent protein kinase (R_{II}) was prepared from beef heart according to [11]. TPCK-trypsin and staphylococcal protease (*Staphylococcus aureus* V_8) were purchased from Worthington, and Miles Labs., respectively. Mast cell protease II [12] was a generous gift from Dr N. Katunuma (Tokushima University, Japan). Reagents and solvents used for the automated sequence analysis were purchased from Beckman and Burdick, and Jackson, respectively.

The phosphorylation of R_{II} was carried out as follows: The protein (1 mg/ml) was treated at 21°C for 20 h, or at 4°C for 3 days with 0.1 mM [γ-^{32}P]-ATP in 5 mM 3-(*N*-morpholino)-propanesulfonate buffer (pH 7.0) containing 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 100 mM NaCl and 12.5 mM magnesium acetate. Since our RII preparations usually contain a trace amount of catalytic subunit, exogeneous catalytic subunit was not added.

To follow the time course of limited proteolysis at 21°C, proteases were added to phosphorylated R_{II} at a substrate:enzyme weight ratio of 100 except for trypsin which was used at a ratio of 1000. Proteolysis was terminated by mixing 25 μl aliquots of the digest with 5 μl of 1% phenylmethanesulfonyl fluoride in ethanol. SDS gel electrophoresis was carried out in a horizontal slab gel with a pH 8.0 disc gel electrophoresis system [13] modified to contain 0.1% SDS in the gel solution and electrophoresis buffer. Samples were mixed with 5 μl of 10% SDS and heated in a

boiling water bath for 5 min. Gels were stained with Coomassie brilliant blue. Autoradiograms were made from the gel after drying.

The proteolytic fragments were isolated as follows: Phosphorylated R_{II} (10 mg) was digested with trypsin, staphylococcal protease or mast cell protease II as above for 5 min, 2 h and 20 h, respectively. Digestion was terminated by the addition of trichloroacetic acid to 10%. The precipitate was collected and dissolved in 1 ml saturated guanidine hydrochloride. After addition of 1 ml 1.5 M Tris–HCl (pH 8.5) containing 7 M guanidine hydrochloride, iodo[^{14}C]acetic acid (20 μCi/1.54 μmol) was added and the solution was maintained for 30 min at 21°C. For complete carboxymethylation, the reaction mixture was treated with 8 mg dithioerythritol for 2 h followed by 20 mg iodoacetic acid. After 30 min the reaction mixture was applied directly to a Sephacryl S-200 column. Carboxymethylation and column chromatography were carried out in the dark.

Amino acid analysis was performed on a Dionex D500 amino acid analyzer. The amino-terminal sequence of the isolated fragments were analyzed with a Beckman Sequencer model 890C according to [14] using a program adapted from [15] with double coupling for the first cycle. The degradation products were identified by two high pressure liquid chromatography systems [16,17]. Radioactivities of ^{14}C and ^{32}P were also measured for the large fragment of the tryptic digest with a Packard model B2450 scintillation counter.

3. Results

Representative results of the time course of limited proteolysis of R_{II} are shown in fig.1A. Regardless of

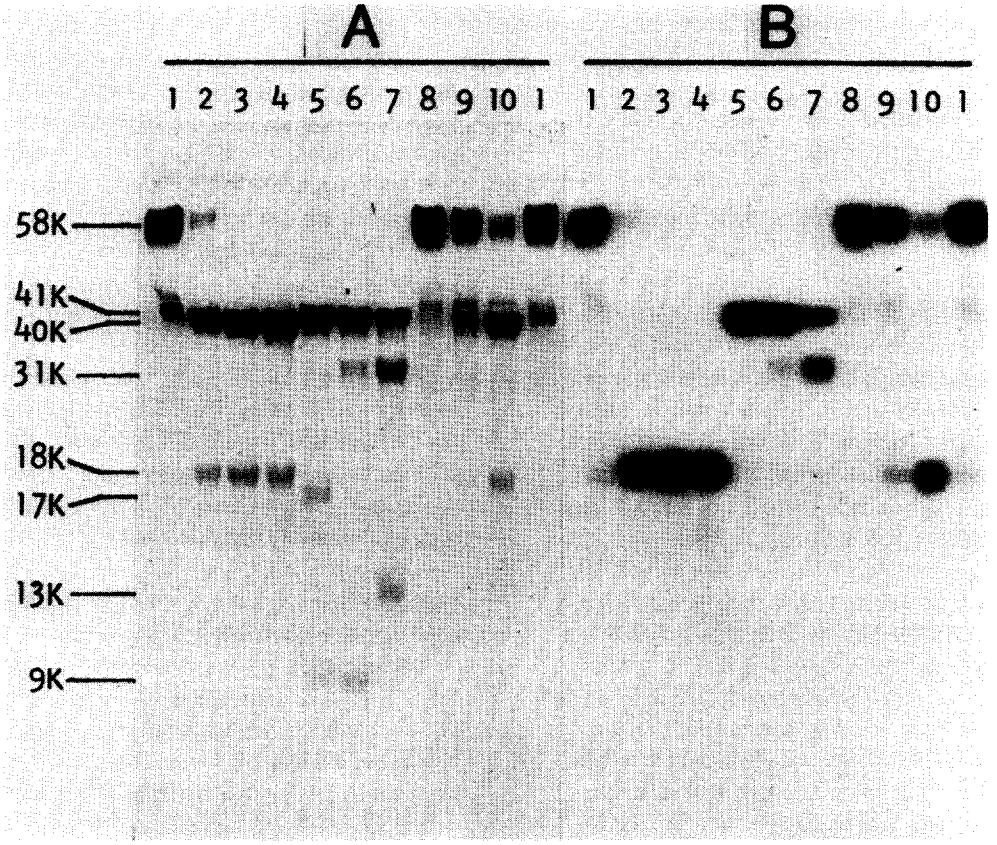

Fig.1. SDS gel electropherogram of the products of limited proteolysis of the [^{32}P]autophosphorylated regulatory subunit (type II). Panel A shows the SDS gel stained with Coomassie brilliant blue and panel B shows the autoradiogram of panel A. Proteolysis was carried out with staphylococcal protease (lanes 2–4), trypsin (lanes 5–7) and mast cell protease II (lanes 8–10) for 5 min (lanes 2,5,8), 2 h (lanes 3,6,9) and 20 h (lanes 4,7,10). Lanes 1 show regulatory subunit kept at the same conditions as the 20 h digestion, but without protease.

the proteolytic enzyme used, two primary products of cleavage were observed, a larger one (L) of ~40 000 daltons and a smaller one (S) of ~18 000 daltons. However, the time courses of the three digests were quite different. Digestion by staphylococcal protease was essentially complete in 5 min (lane 2) with a substrate:enzyme weight ratio of 100. The two products of digestion appeared to be stable for 20 h (lane 4). Digestion of R_{II} by mast cell protease II at the same substrate:enzyme ratio proceeded much more slowly (lanes 8–10). In contrast, digestion by trypsin was very rapid, yielding in <5 min (lane 5) products of similar size which appeared to degrade slowly to fragments of 31 000 and 9000 daltons (lane 7). Control incubation of R_{II} (20 h) in the absence of exogeneous protease (lanes 1) suggests a trace contamination with an unidentified endogeneous protease. Closer examination of the molecular weights of the products in fig.1 indicates that the primary site of cleavage by trypsin is significantly different from those by the other two proteases. The large fragment of the tryptic digest is ~1000 daltons larger and the small fragment ~1000 daltons smaller than the corresponding products in the other two digests.

A striking difference between the three digests was seen in the autoradiograms (fig.1B). Radioactive phosphate was observed to migrate with the small fragments in the staphylococcal and mast cell protease digests but with the large fragment in the tryptic digest. The 20 h degradation product (~31 000 dalton) of the tryptic digest was also labeled, indicating that it was derived from the large fragment. These data are consistent with a single site of autophosphorylation in a 1000 dalton region of the primary sequence which is uniquely susceptible to cleavage by proteases.

To clarify the relationship of these fragments to each other and to the whole molecule, the products of digestion were [^{14}C]carboxymethylated and separated on Sephacryl columns (fig.2 illustrates a tryptic digest). Intact R_{II} was eluted on the leading edge of the first peak so that pools of the large fragment of the staphylococcal and mast cell protease digests contained a small amount of undigested protein. The second peak eluted contained the small fragment. The very low absorbance of the small fragments at 280 nm indicates that the two tryptophan residues of R_{II} were contained in the large fragment in each case. As expected from the gels (fig.1) the ^{32}P was not associated with the large fragment of the tryptic digest but with the small fragments produced by the other two proteases (data not shown). Table 1 compares the amino acid composition of R_{II} with those of the 6 primary fragments thus obtained. The sum of the compositions of the two primary products of each digest approximates the composition of intact R_{II}. Some amino acids especially lysine, methionine and cysteine are distributed between the two fragments quite differently, verifying that they are derived from different portions of R_{II}.

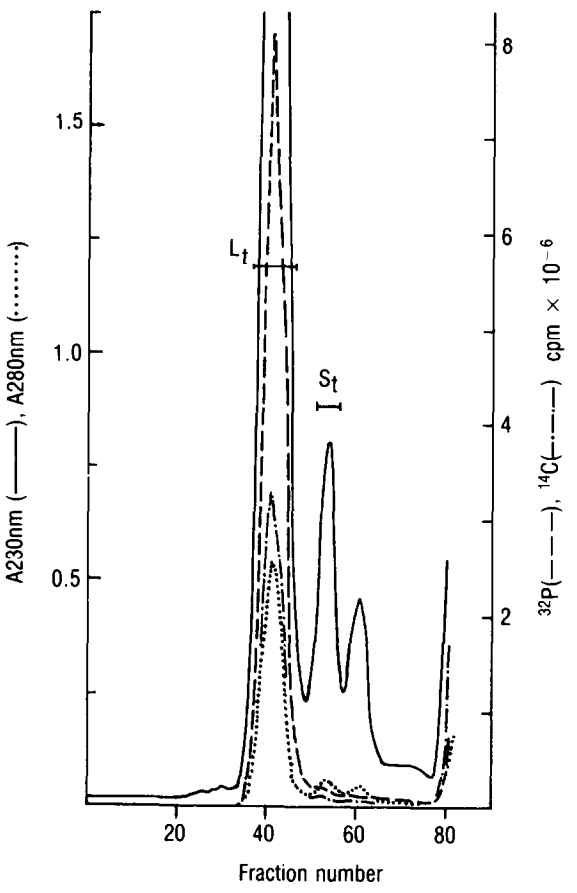

Fig.2. The separation of the fragments generated by tryptic digestion. The tryptic digest was [^{14}C]carboxymethylated, applied to a Sephacryl S-200 column (1.5 × 89 cm) and eluted at 6.0 ml/h with 7 M urea containing 10 mM HCl. Effluent was monitored by absorbance at 280 nm and 230 nm and by radioactivity.

Sequence analysis of the three small fragments indicated that their amino termini were blocked, as is the amino terminus of intact R_{II}. Similar analysis of the three large fragments identified the sequences summarized in fig.3. In each case an unusual acidic hexapeptide (Asp–Glu–Glu–Glu–Glu–Asp) served

Table 1
Amino acid compositions of regulatory subunit of bovine cardiac muscle protein kinase II and its fragments generated by limited proteolysis[a]

Amino acid		R_{II}	L_t	S_t	$(L+S)_t$	L_{mcp}	S_{mcp}	$(L+S)_{mcp}$	L_{sp}	S_{sp}	$(L+S)_{sp}$
Asp	(D)	62.1	44.4	18.4	62.8	43.7	18.3	62.0	44.1	18.4	62.5
Asn	(N)										
Thr	(T)	24.0	16.9	6.5	23.4	15.9	6.5	22.4	16.1	6.4	22.5
Ser	(S)	33.4	19.9	10.1	30.0	18.6	12.0	30.6	18.5	10.7	29.2
Glu	(E)	64.9	51.0	18.9	69.9	49.3	19.5	68.8	48.1	20.6	68.7
Gln	(Q)										
Pro	(P)	25.1	12.5	14.9	27.4	11.9	17.4	29.3	13.6	16.2	29.8
Gly	(G)	33.2	23.4	10.3	33.7	23.0	11.2	34.2	22.9	10.4	33.3
Ala	(A)	33.5	21.4	14.1	35.5	20.2	15.9	36.1	20.5	15.6	36.1
Cys	(C)	7.5	8.2	0.6	8.8	7.0	1.3	8.3	6.8	1.5	8.3
Val	(V)	42.9	30.1	11.7	41.8	28.2	14.0	42.2	28.8	14.2	43.0
Met	(M)	9.8	6.9	0.0	6.9	8.1	0.0	8.1	6.8	0.0	6.8
Ile	(I)	29.7	21.3	5.8	27.1	21.5	5.4	26.9	20.9	5.8	26.7
Leu	(L)	39.9	27.1	13.6	40.7	26.7	13.3	40.0	27.0	13.5	40.5
Tyr	(Y)	11.7	9.6	2.5	12.1	11.0	3.1	14.1	10.6	2.8	13.4
Phe	(F)	19.3	13.2	6.1	19.3	13.0	6.4	19.4	13.5	6.4	19.9
Trp	(W)	2.2	+	–		+	–		+	–	
His	(H)	8.8	5.6	2.3	7.9	5.7	1.9	7.6	5.6	2.0	7.6
Lys	(K)	31.0	26.3	2.3	28.6	26.1	1.3	27.4	25.1	2.1	27.2
Arg	(R)	35.1	21.1	13.0	34.1	20.1	12.7	32.8	21.4	13.7	35.1
Total		514.1	358.9	151.1	510.0	350.0	160.2	510.2	350.3	160.3	510.6
Estimated mol. wt.[b]		58 000	41 000	17 000		40 000	18 000		40 000	18 000	

[a] Data are expressed as residues per molecule. Subscripts, t, mcp and sp, identify large (L) and small (S) fragments of digestion by trypsin, mast cell protease and staphylococcal protease, respectively. One letter amino acid abbreviations are indicated in parentheses. Calculations were based on the molecular weight estimated from SDS gel electrophoresis. Amino acid composition of the whole protein was obtained from triplicate samples of 21, 45 and 90 h acid hydrolysates. Values for Thr and Ser were extrapolated to zero time and those of Val and Ile were taken from the 90 h hydrolysates. Amino acid compositions of the fragments were calculated from 20 h acid hydrolysates. Cys was determined as carboxymethylcysteine [18] and Trp was determined photometrically by the method in [19]

[b] Estimated by SDS gel electrophoresis

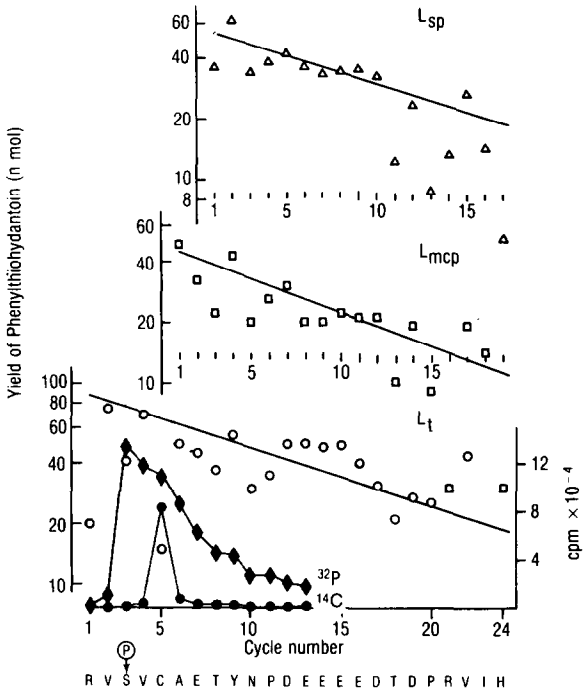

Fig.3. Sequence analyses of the three large fragments. The deduced sequence is indicated at the bottom with one letter amino acid abbreviations (see table 1). The circled P indicates the site of [^{32}P]phosphorylation.

to overlap the three sequences. The initial yields for the three analyses were 61, 95 and 65% for the fragments L_t, L_{mcp} and L_{sp}, respectively and the repetitive yields were ~93% each. The fragment L_t was larger than the other two and released both ^{32}P and the phenylthiohydantoin of dehydroalanine in the third cycle of the Edman degradation, indicating *O*-phosphoserine at that position. This fragment also yielded [^{14}C]carboxymethylcysteine at the fifth cycle. The radioactive ^{32}P did not dissolve in acetonitrile, the normal solvent for the phenylthiohydantion derivatives, but did dissolve in water.

The sequence data (fig.4) suggests that the small fragments represent the amino-terminal portions of the regulatory subunit and that a narrow region of susceptibility to limited proteolysis is found ~18 000–19 000 daltons from the amino terminus. The autophosphorylation site is located between the sites of cleavage by trypsin and by the other two enzymes. These data are consistent with the autoradiograms in fig.1.

4. Discussion

The present studies of limited proteolysis indicate that the regulatory subunit of protein kinase II must possess a surface region which is available to proteases and to phosphorylating and dephosphorylating enzymes. This region is ~1/3 rd of the linear distance from the amino to the carboxyl terminus of the intact subunit. The site of autophosphorylation in a small peptide has been identified (Asp–Arg–Arg–Val–Ser(P)–Val) from the bovine subunit [4]. In porcine R_{II}, 7 residues of the corresponding site (Asx–Arg–

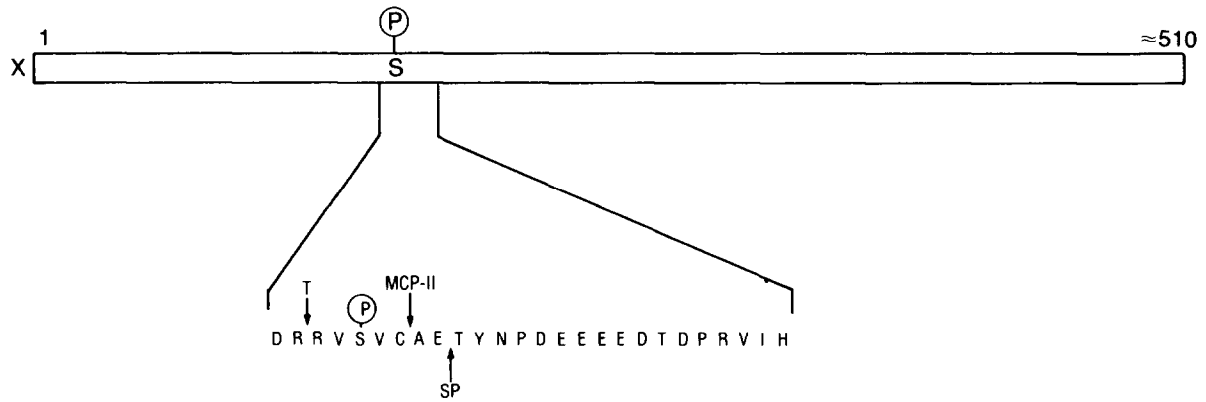

Fig.4. Amino acid sequence around the site of 'autophosphorylation' and of limited proteolysis in the regulatory subunit of bovine cardiac muscle protein kinase (type II). The top bar represents the whole molecule and the approximate location of the deduced sequence. Arrows indicate the bonds cleaved by trypsin (T), mast cell protease II (MCP-II) and staphylococcal protease (SP). One letter amino acid abbreviations are used (see table 1). The site of phosphorylation is indicated by a circled P. The amino terminal two residues are provided by overlapping the data in [4].

Arg–Val–Ser(P)–Val–X–Glu) have been identified at the amino terminus of a cAMP binding fragment derived by chymotryptic digestion [10], which corresponds to the large fragment in this report. The porcine data indicate that this site is largely conserved in the two species. The bovine data [4] provide a two residue extension towards the amino terminus from the present site of tryptic cleavage.

These data provide not only information regarding the sequence surrounding the *O*-phosphoserine residue, but also a useful first step in the strategy of determining the complete amino acid sequence of the bovine regulatory subunit by dividing the sequence problem into discrete sub-projects. These fragments are now serving as starting material for a detailed analysis of the sequence of bovine R_{II}.

The restricted specificity of cleavage of the three proteases provides some insight into the nature of the subunit. Observations of many systems now have shown that native proteins are rather resistant to proteolytic attack except for 'hinge regions' between domains or 'fringe regions' on exposed surfaces which are susceptible to limited proteolysis [20]. The regulatory subunit of protein kinase type II appears to contain such a susceptible heptapeptide region and this region seems critical for the interaction of the regulatory and the catalytic subunits. Significantly, only one of the 5 glutamyl bonds in the sequence shown in fig.4 appears to be cleaved by the glutamyl-specific protease of staphylococcus aureus and only one of the 3 arginyl bonds by trypsin. The mast cell protease II is known to cleave tyrosyl bonds [12] but the present data indicate that the tyrosyl bond near the susceptible region in R_{II} is not cleaved, whereas only one of 8 cysteinyl residues in R_{II} provides a cleavage site.

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