The amino acid sequence of rabbit skeletal muscle calmodulin

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The amino acid sequence of calmodulin which can be extracted from rabbit skeletal muscle with low ionic strength buffer and presumably activates myosin light chain kinase has been determined. It is a single polypeptide chain of 148 residues with a blocked N terminus. The sequence of the N terminal tripeptide and residues 98 and 99 were not determined unequivocally nor were the amide assignments of residues 48, 50, 58 and 60. The protein is otherwise identical with the subunit of phosphorylase kinase and bovine uterus calmodulin and very similar to all other mammalian calmodulins.

Rabbit skeletal muscle *Calmodulin* *Amino acid sequence* *Myosin light chain kinase*

1. INTRODUCTION

The major calcium binding protein of striated muscle is troponin C, which confers calcium sensitivity on the actomyosin ATPase. Calmodulin, however, is also present in rabbit skeletal muscle [1–3], although in considerably lower concentrations. Calmodulin was originally isolated from bovine brain as the activator of phosphodiesterase [4,5] and of adenylate cyclase [6], but has since been shown to be very widely distributed. It occurs in high concentrations in all eukaryotes so far examined and acts as the calcium-binding subunit for a large number of enzymes (review [7–10]).

It has been suggested that there are at least two pools of calmodulin in rabbit skeletal muscle which may be under separate genetic control [3]. According to this view one pool consists of the subunits of phosphorylase kinase (30–35% of the total calmodulin) and cannot be extracted by low ionic strength buffers. The remainder of the calmodulin that regulates other enzymes, e.g., myosin light chain kinase [2], would constitute the second pool and can be extracted with low ionic strength buffers in the presence of EDTA or EGTA. The amino acid sequence of the δ-subunit has been determined [11] and shown to be very similar to that of calmodulin isolated from other mammalian sources; e.g., bovine brain [12], bovine uterus [13] and rat testis [14]. Minor differences between the amino acid sequences of the 4 proteins have been found, although it is probable these are due to sequencing errors, particularly in the assignments of amides rather than to genuine variations in gene coding for the various proteins. Here we present the amino acid sequence of calmodulin extracted from rabbit skeletal muscle with low ionic strength buffers, which presumably activates myosin light chain kinase, and show that it is very similar to all other vertebrate calmodulins for which amino acid sequence data are available.

2. MATERIALS AND METHODS

Calmodulin was prepared from low ionic strength extracts of rabbit skeletal muscle as in [2].

2.1. *Preparation of peptides*

2.1.1. CNBr digestion

Calmodulin (50 mg) was dissolved in 70% formic acid (8 ml) and a 100-fold molar excess of

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cyanogen bromide over the methionine residues was added. After 24 h at 20°C the digest was diluted 10-fold with water and freeze-dried.

2.1.2. Enzymic digestion

Calmodulin (20 mg) was labelled at the methionine residues with iodo[^{14}C]acetic acid as in [15] and, after dialysis against water, freeze-dried. The protein was dissolved in 50 mM NH_4CO_3 and digested with TPCK-treated trypsin (Worthington Chemical Co., Cambrian Chemicals, Croydon, Surrey) for 4 h at 37°C at an enzyme:substrate ratio of 1:50 (w/w).

Peptides resulting from digestion of calmodulin with CNBr or trypsin were purified by gel filtration, followed in appropriate cases by ion-exchange chromatography of high voltage electrophoresis at pH 6.5, 3.5 and 2.0 using Whatman 3MM or no. 1 chromatography paper.

CNBr and large tryptic peptides were further digested as appropriate with either TPCK treated trypsin, thermolysin (Calbiochem., San Diego, CA) or V8 protease (Miles Laboratories, Slough, England) at an enzyme:substrate ratio of 1:50 (w/w) at 37°C. Peptides were digested with trypsin and thermolysin for 4 h and with V8 protease for 16 h. High voltage electrophoresis was used to purify peptides resulting from enzymic digests.

2.2. Sequencing methods

The N-terminal residue of calmodulin was determined as in [16]. Peptides were sequenced manually – either by the dansyl-Edman method in [17] or by the rapid Edman procedure in [18]. Amides were assigned on the basis of electrophoretic mobility as in [19]. Trimethyllysine was detected in peptides by amino acid analysis using 'system C' described in [20]. Radioactive peptides were identified using Kodak Blue Brand X-ray plates.

3. RESULTS AND DISCUSSION

The CNBr digest of rabbit skeletal muscle calmodulin was chromatographed on a column (2.2 × 240 cm) of Sephadex G-50 equilibrated and eluted with 50 mM NH_4HO_3 pH 7.9. An elution profile very similar to that described for the CNBr fragment of bovine uterus calmodulin [13] and phosphorylase kinase δ-subunit [11] was obtained (fig.1). The two largest peptides (CNB1 and CNB2) were further purified from fractions 1 and 2, respectively, by chromatography on DEAE-cellulose equilibrated with 20 mM sodium phosphate (pH 7.0) as in [13]. Peptide CNB4 was purified by chromatography of fraction 3 (fig.1) on DEAE-cellulose. An additional peptide CNB3 was also present in fraction 3 but the yield was low when separated by this procedure. CNB3 was therefore purified by high voltage electrophoresis on Whatman 3MM chromatography paper of fraction 3 obtained from a second CNBr digest of rabbit skeletal muscle calmodulin. The smaller fragments, peptides CNB5, CNB6, CNB7 and CNB8 were purified from fractions 4, 5 and 6 by high voltage electrophoresis at pH 6.5, 3.5 and 2.0. Free homoserine was also isolated. Amino acid analyses of the CNBr fragments are given in table 1.

The sequences of the larger CNBr peptides were determined by additional digestion with trypsin and thermolysin. Peptides CNB1, CNB2, CNB3 and CNB4 were digested with both thermolysin and with V8 protease, CNB2 with trypsin and CNB5 with thermolysin. Peptides CNB6, CNB7 and CNB8 were sequenced directly. Peptide CNB1 was a partial cleavage product, spanning residues 77–124 in which the methionine–threonine bond between residues 109 and 110 did not split as has

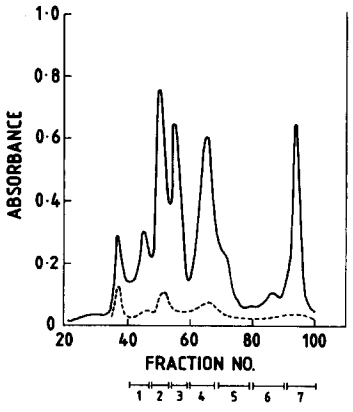

Fig. 1. Chromatography of CNBr digest of rabbit skeletal muscle calmodulin. The digest (50 mg) was applied to the column (2.2 × 240 cm) of Sephadex G-50 equilibrated and eluted with 50 mM NH_4HCO_3 (pH 7.9) Fractions of 10 ml were collected. (– – –) A_{280}, (——) A_{215}. Horizontal bars indicate the fractions pooled.

Table 1

Amino acid analysis of CNBr peptides from calmodulin isolated from low ionic extracts of rabbit skeletal muscle

	CNB1		CNB2		CNB3		CNB4		CNB5		CNB6		CNB7		CNB7a		Rabbit calmodulin	
	Exp.	Fd.	Exp.	Fd.	Exp.	Fd.	Exp.	Fd.	Exp.	Fd.	Exp.	Fd.	Exp.	Fd.	Exp.	Fd.	Exp.	Fd.[a]
Asp	8	7.3	4	4.4	4	4.3	5	4.3	2	2.2	-	-	-	-	-	-	23	22.7
Thr	3	3.4	5	4.7	-	-	2	1.6	1	1.0	-	-	1	0.9	1	1.0	12	11.4
Ser	2	2.2	1	1.1	-	-	-	-	1	1.0	-	-	-	-	-	-	4	5.3
Glu	9	9.1	7	7.0	5	5.1	2	3.6	4	3.9	-	-	-	-	-	-	27	25.9
Pro	-	-	-	-	-	-	1	0.7	1	1.9	-	-	-	-	-	-	2	1.6
Gly	3	8.4	3	3.1	2	2.5	2	2.1	1	1.2	-	-	-	-	-	-	11	11.6
Ala	3	3.4	3	2.9	1	1.2	1	1.4	1	1.1	1	1.1	1	1.0	1	1.1	11	11.0
Val	3	2.3	1	1.0	2	1.7	1	1.0	-	-	-	-	-	-	-	-	7	8.0
Ile	2	1.8	2	1.9	2	1.6	2	1.8	-	-	-	-	-	-	-	-	8	8.2
Leu	3	3.6	3	3.2	-	-	1	1.0	2	1.9	-	-	-	-	-	-	9	10.5
Tyr	1	0.9	-	-	1	1.4	-	-	-	-	-	-	-	-	-	-	2	2.6
Phe	2	2.2	3	2.9	1	1.2	2	1.6	-	-	-	-	-	-	-	-	8	7.9
His	1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1.9
Lys	2	2.2	3	2.9	-	-	-	-	-	-	1	0.9	1	1.0	1	0.9	7	7.9
Arg	3	2.7	-	-	1	0.8	-	-	1	0.7	1	0.9	-	-	-	-	6	6.1
Hse	1	+	1	+	1	+	1	+	1	+	1	+	-	-	1	1.0		
Tml	1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.6

Exp., expected; Fd., found; Tml, trimethyllysine. + indicates the presence of an amino acid, but accurate quantitation was not possible. Glu figures include Hse. The expected values for each peptide are those calculated from the sequence of bovine brain calmodulin [12]. Free Hse was also isolated.

[a] Values taken from [2]

been observed for bovine uterus calmodulin [13] and bovine brain calmodulin [21].

The CNBr fragments were overlapped by purification and analysis of the radioactive tryptic peptides obtained after labelling of the methionine residues with iodo[^{14}C]acetate. The tryptic digest was chromatographed on a column (2.2 × 240 cm) of Sephadex G-50 equilibrated and eluted with 50 mM NH_4HCO_3, pH 7.9 (fig.2). Peptides were further purified by high voltage electrophoresis. The pattern of digestion of rabbit skeletal muscle calmodulin with trypsin differed from that of bovine uterus calmodulin [13] and bovine brain calmodulin in that cleavage did not occur in the rabbit protein at Arg 126. No explanation can be offered at present for this observation, but it was a consistent result obtained on a number of occasions. The large radioactive tryptic peptides were further digested with thermolysin to facilitate sequencing.

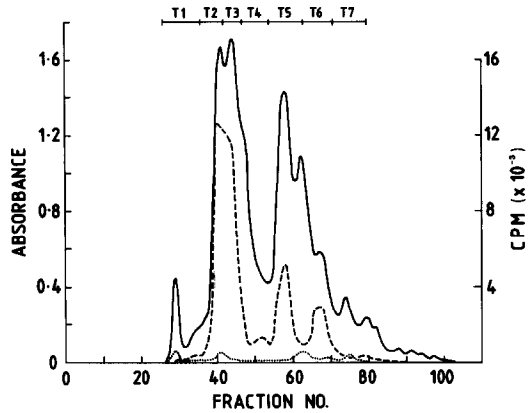

Fig. 2. Chromatography of tryptic digest of rabbit skeletal muscle calmodulin. Calmodulin (20 mg) labelled at the methionine residues with iodo[^{14}C]acetic acid was digested with trypsin, and then chromatographed on a column (2.2 × 240 cm) of Sephadex G-50 equilibrated and eluted with 50 mM NH_4HCO_3 (pH 7.9). Fractions of 10 ml were collected. (···) A_{280}, (——) A_{215}, (– – –) cpm. Horizontal bars indicate the fractions pooled.

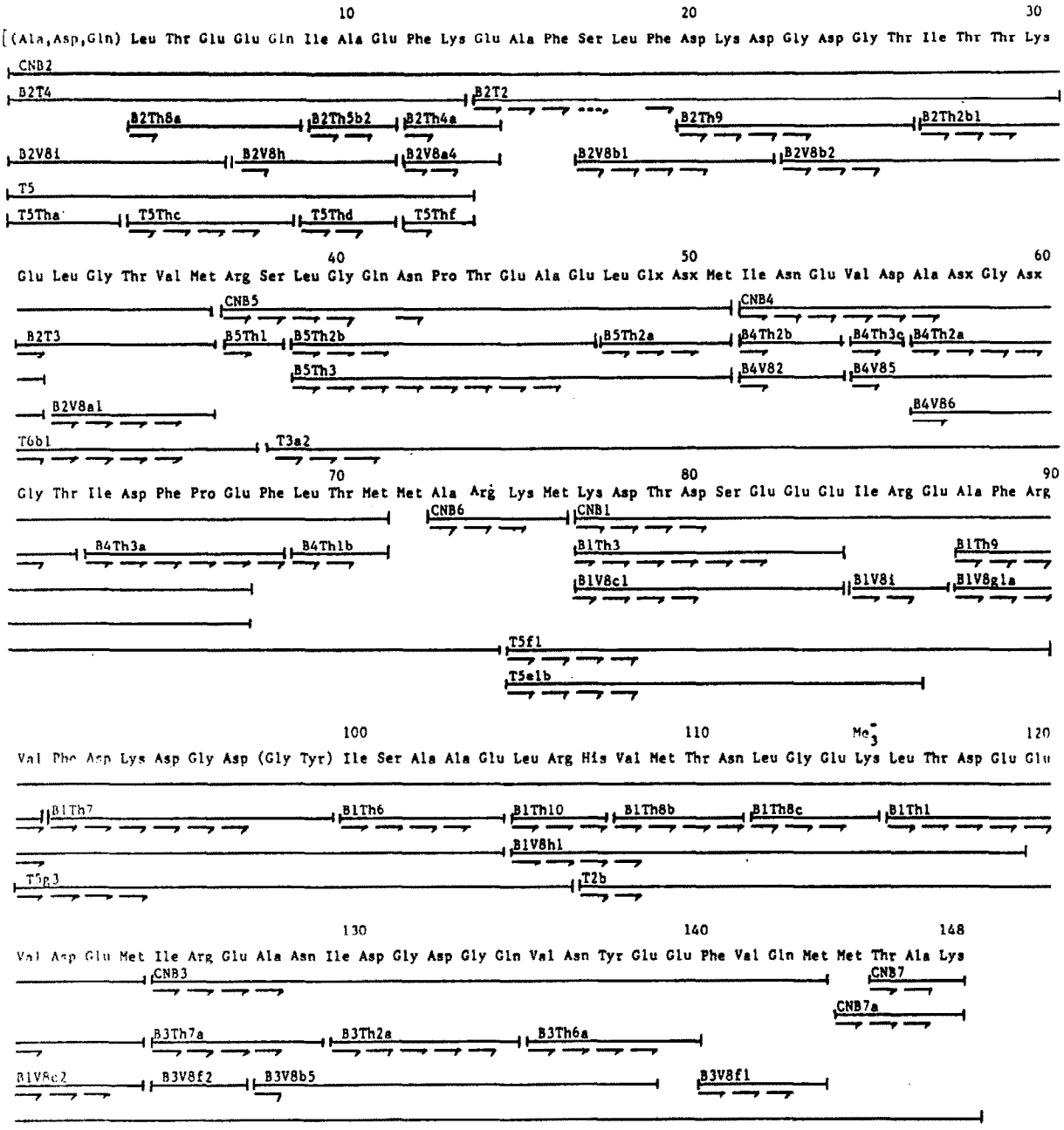

Fig. 3. Amino acid sequence of rabbit skeletal muscle calmodulin. The peptides from which the sequence was deduced are indicated by horizontal bars. Peptides bearing the prefixes CNB (or B), T, Th or V8 result from cleavage with CNBr, trypsin, thermolysin and V8 protease, respectively. (—) indicates one step of the Edman degradation. [, indicates a blocked N terminus. Me_3-Lys, trimethyllysine. The amino acid sequences of the residues in brackets were not determined unequivocally.

A large proportion of the other tryptic peptides was purified from this digest. Cleavage did not occur after Lys 21 and Lys 94 probably due to the proximity of acidic residues. Authors in [21] noted that digestion of bovine brain calmodulin with trypsin resulted in cleavage at the two Met-Met sequences (residues 71–72 and 144–145) as well as at the lysine and arginine residues. In [22] it was also found that trypsin cleaves chicken troponin C and Met-Met sequences (residues 81–82 and 157–158). However, in the case of rabbit skeletal muscle calmodulin no such cleavage occurred, probably due to modification of the methionines with iodoacetic acid. Trypsin also failed to cleave the molecule at the trimethyllysine residue (115).

The Met-Met sequences in calmodulin (residues 71–72 and 144–145) were confirmed by isolation from iodo[^{14}C]acetate-labelled calmodulin of tryptic bridge peptides containing approximately twice as much radioactivity and methionine as the other overlap peptides. The cyanogen bromide peptide CNB7 was also isolated in two forms (table 1), one having the sequence Hse-Thr-Ala-Lys and the other Thr-Ala-Lys. It is not clear how the methionine residue can be modified in such a way as to produce a homoserine residue without cleavage occurring but homoserine was shown to be present by the amino acid analysis and confirmed as the N-terminal residue by dansylation.

The sequence of rabbit skeletal muscle calmodulin (fig.3) was determined unequivocably except for the order of the residues 98 and 99 and the N-terminal tripeptide. It is probable, however, that this latter sequence is identical with that of bovine brain calmodulin [21]. The whole molecule was found to have a blocked N terminus, the nature of which was not determined, although like bovine brain calmodulin [12] it is probably an acetyl group. In addition amide groups at residues 49, 50, 58 and 60 were not assigned. Apart from these relatively minor points, the amino acid sequence was identical to that of bovine uterus calmodulin [13] and very similar to bovine brain calmodulin.

The only differences from the latter proteins were amide assignments at residues 24 and 97 (aspartic acid, in the rabbit protein, asparagine in the bovine brain protein) and 135 (glutamine in rabbit, glutamic acid in bovine brain). Residue 135 was originally assigned as glutamic acid in bovine uterus calmodulin [13], but a reconsideration of the evidence suggests that it is, in fact, glutamine as is present in the subunit of phosphorylase kinase. Thus the sequence of the calmodulin present in the sarcoplasm is identical to the bound form that that exists as the δ-subunit in phosphorylase kinase. If the bound and unbound forms in fact represent different pools of the proteins [3] they are not under different genetic control.

Rabbit skeletal muscle calmodulin consists of 148 residues, has an M_r of 16 722 and has a net charge of -24 at pH 7.0. It is similar in amino acid sequence to rabbit skeletal muscle troponin C. To obtain maximum homology between calmodulin and troponin C it is necessary to introduce 3 deletions into the calmodulin sequence (between residues 80 and 81). If this is done, the two proteins differ at 48% of the residues. Four domains can be distinguished in calmodulin which are related to each other in sequence [12,21]. These comprise residues 8–40 (first domain), 44–76 (second domain), 81–113 (third domain) and 117–148 (fourth domain). The first and third domains are homologous, as are the second and fourth with each containing one Ca^{2+} binding site.

The strongly conserved nature of the sequence of calmodulin from vertebrate and invertebrate tissues (review [10]) indicates that great constraints have been placed on the calmodulin sequence during evolution. Presumably these constraints result from the necessity to maintain precisely defined sites for binding calcium and for a number of other proteins with which calmodulin interacts. The site at which rabbit skeletal muscle troponin I binds to calmodulin has been shown to be in the area of residues 77–124 [23,24]. It seems likely, however, that this is not the site concerned in the binding to myosin light chain kinase since the addition of troponin I at a 100-fold molar excess over calmodulin caused only slight inhibition of myosin light chain kinase [25]. In addition, there may be different sites involved in the interaction of calmodulin with other enzymes and proteins, e.g., phosphorylase kinase, phosphodiesterase.

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