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ISOLATION AND CHARACTERIZATION OF DES(ALA-LYS)CALMODULIN IN PORCINE BRAIN

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SUMMARY: A calmodulin-like protein -des(Ala-Lys)calmodulin- was isolated from porcine brain extract, and was characterized in comparison to porcine brain calmodulin. Des(Ala-Lys)calmodulin was distinguishable from calmodulin by its slightly faster mobility in 10 % polyacrylamide gels without sodium dodecyl sulfate. The protein gave an amino acid composition very similar to calmodulin, and contained one ε-N-trimethyllysyl residue. Comparative peptide mapping of calmodulin and des(Ala-Lys)calmodulin by high performance anion-exchange liquid chromatography, and the subsequent analyses of the isolated peptides, have indicated that des(Ala-Lys)calmodulin lacks the Ala(147)-Lys (148) sequence at the C-terminus of calmodulin. The content of des(Ala-Lys)-calmodulin was about one-tenth of calmodulin.

INTRODUCTION: Calmodulin mediates a number of intracellular Ca^{2+} functions that are responsible for various stimulus-coupled cellular events. Now, a variety of enzymes and proteins have been identified as the target of calmodulin (1).

Bovine brain calmodulin contains a total of 148 residues, and consists of four homologous domains each domain containing a single Ca^{2+} binding site (2, 3). The protein exhibits obvious sequence homology and a close evolutionary relationship to troponin C (2,3) and to other Ca^{2+} binding proteins (4,5). One of the characteristics of the calmodulin sequence is the presence of ε-N-trimethyllysyl residue at position 115, with exceptions for calmodulins from a few sources (6,7). It is reported (6) that the trimethyllysine-free calmodulin is less effective than the trimethyllysine-containing calmodulin in activating myosin light chain kinase and phosphodiesterase. Although this observation, concerning with the biological significance of the methylation, is still a matter of controversy (7), such a post-translational modification of calmodulin indicates presumably that the metabolism of calmodulin is under strict control in cells.

This communication describes the isolation of a protein which is very similar to calmodulin, including the presence of one trimethyllysyl residue/

Abbreviations used: SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

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mol of protein. This protein has been designated as des(Ala-Lys)calmodulin because it appeared to have an amino acid sequence identical to calmodulin except for the lacking of the dipeptide sequence, Ala(147)-Lys(148), at the C-terminus of calmodulin. The removal of the C-terminal dipeptide may be one of the control mechanisms that regulate the biological activity of calmodulin.

MATERIALS AND METHODS: Porcine brains (20 kg) were obtained fresh from a slauter house, and the soluble proteins immediately extracted with 0.1 M potassium phosphate buffer (pH 7.1) containing 1 mM EDTA and 2.66 M ammonium sulfate. The calmodulin preparation, containing des(Ala-Lys)calmodulin, was obtained from the brain extract as described in the previous papers (2,8) for bovine brain calmodulin. The method consisted of ammonium sulfate precipitation of the brain extract (85 % saturation) and the following two column chromatography steps on DEAE-Sephadex A-50 and on Sephadex G-75. The calmodulin preparation showed a single band by SDS-polyacrylamide gel electrophoresis performed as described by Laemmli (9).

Amino acid compositions of proteins and peptides were determined on a Hitachi model KLA-5 analyzer equipped for two column methodology (10). The analyses of trimethyllysine were achieved on a column (8 x 300 mm) of Shodex HC-125s resin (Showa Denko Co., Tokyo) eluted by 0.2 M Na citrate-HCl buffer, pH 4.25, containing 0.5 M NaCl at 57 °C with a flow rate of 60 ml/h. Samples were hydrolyzed with constant-boiling HCl for 24 or 72 h at 110 °C in evacuated, sealed tubes.

Tryptic digestions of calmodulin and des(Ala-Lys)calmodulin were carried out using 1/50 amount (w/w) of TPCK-trypsin (Worthington, NJ) in 10 mM ammonium hydrogen carbonate (pH 8.0) at 37 °C for 4 h. Digestions were stopped by addition of lima bean trypsin inhibitor (ICN, OH), and the mixtures were injected into a liquid chromatograph without additional manipulation.

High performance anion-exchange liquid chromatography was performed on a column (4 x 250 mm) of Hitachi-Gel 3013N (5 μm; Hitachi Ltd., Tokyo) essentially as described in the previous paper (11), except that peptides were eluted at 70 °C by a linear gradient from water to 0.25 M methanesulfonic acid containing 50 % (v/v) acetonitrile and 25 % (v/v) isopropanol (adjusted to pH 2.8 with aqueous ammonia) at a flow rate of 0.5 ml/min. Other details are given in the legend to Fig. 2. High performance reverse-phase liquid chromatography was performed on a column (4.6 x 150 mm) of Zorbax ODS (5 μm; Du Pont, CA). Peptides were eluted at 50 °C by a linear gradient from 0.1 % trifluoroacetic acid to the same solvent containing 60 % (v/v) acetonitrile at a flow rate of 1 ml/min.

RESULTS: Des(Ala-Lys)calmodulin was co-purified in the crude calmodulin preparation in our procedure, and was first recognized upon column chromatography of the calmodulin preparation on DEAE-Sephadex A-50 as an unknown protein that eluted following calmodulin. The separation was complete when the column was eluted isocratically with 0.1 M potassium phosphate buffer (pH 7.1) containing 0.28 M NaCl and 1 mM EDTA (Fig. 1). The content of des(Ala-Lys)calmodulin was about one-tenth of calmodulin as estimated from Fig. 1.

Examination of des(Ala-Lys)calmodulin by polyacrylamide 10 %-gel electrophoresis without SDS (Fig. 1) indicated that it was different from calmodulin as judged by a single band that migrated slightly faster than calmodulin. In

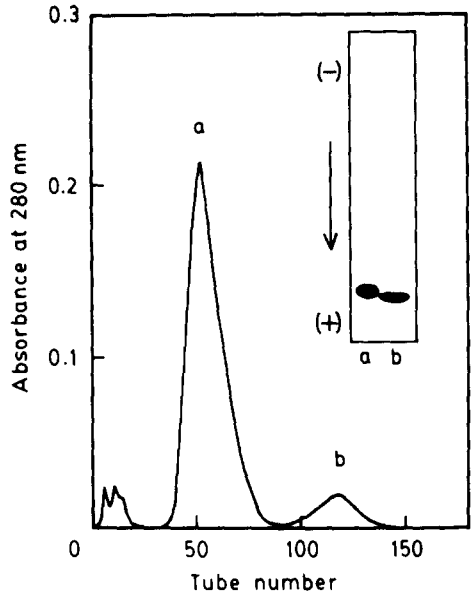

Fig. 1. Separation of porcine brain calmodulin (a) and des(Ala-Lys)calmodulin (b), and polyacrylamide gel electrophoresis of the isolated proteins. The calmodulin preparation (120 mg) was applied to a column (1.0 x 36 cm) of DEAE-Sephadex A-50 equilibrated with 0.1 M potassium phosphate buffer (pH 7.1) containing 1 mM EDTA and 0.28 M NaCl, and eluted with the same solvent at a flow rate of 3.2 ml/h. 3.2 ml-fractions were collected. The electrophoresis was performed as described by Davis (12) in a 10 %-gel slab without SDS.

polyacrylamide gels containing 0.1 % SDS, it migrated as a single band with a mobility identical to calmodulin (Mr = 16 700).

The amino acid analysis of des(Ala-Lys)calmodulin (Table 1) resulted in a composition that is very similar to calmodulin, including the presence of one mol of trimethyllysyl residue/mol of protein. In fact, both the compositions were hardly distinguishable, except that des(Ala-Lys)calmodulin apparently contained six lysyl residues instead of seven in calmodulin. Such a small difference, however, seemed to be even significant as the composition of porcine brain calmodulin, obtained by the pararell analysis, was accounted for by the one computed from the amino acid sequence of bovine brain calmodulin (Table 1).

To elucidate the structural difference between calmodulin and des(Ala-Lys)calmodulin, peptide mapping was then performed for both the proteins. The two proteins were digested with trypsin in pararell and the digests applied to the high performance liquid chromatography on Hitachi-Gel 3013N (Methods). Porcine brain calmodulin gave rise to a peptide map (Fig. 2A) that was essentially identical to the map for bovine brain calmodulin described in the previous paper (11). Here, the twelve tryptic peptides covering the total

Table 1. Amino Acid Compositions of Des(Ala-Lys)calmodulin and Porcine Brain Calmodulin[a]

Amino acid	Des(Ala-Lys) calmodulin	Porcine calmodulin	Bovine calmodulin[b]
Lys	5.9 (6)	6.8 (7)	7
TML[c]	1.1 (1)	1.0 (1)	1
His	1.2 (1)	1.2 (1)	1
Arg	5.6 (6)	6.1 (6)	6
Asx	22.1 (23)	22.5 (23)	23
Thr	11.1 (12)	11.2 (12)	12
Ser	4.5 (4)	4.0 (4)	4
Glx	27.5 (27)	27.3 (27)	27
Pro	2.3 (2)	2.1 (2)	2
Gly	11.4 (11)	10.8 (11)	11
Ala	10.3 (10)	10.9 (11)	11
Cys[d]	0.0 (0)	0.0 (0)	0
Val[d]	6.5 (7)	6.7 (7)	7
Met[d]	8.5 (9)	8.7 (9)	9
Ile[d]	7.5 (8)	7.4 (8)	8
Leu	8.7 (9)	8.9 (9)	9
Tyr	1.9 (2)	2.0 (2)	2
Phe[e]	7.6 (8)	7.8 (8)	8
Trp[e]	0.0 (0)	0.0 (0)	0
Total	146	148	148

a; From duplicate analyses of 24- and a single analysis of 72-h hydrolysate. The numbers in parentheses are the numbers of residues found by peptide mapping of the tryptic digests. b; Computed from the sequence. c; ε-N-trimethyllysine. d; From the 72-h hydrolysis value. e; From spectroscopic evidence.

sequence of calmodulin were recovered in ten peaks, 1 to 9 and X (the peaks 2 and 3 consisted of two peptides respectively). Assignments of the peptides in the calmodulin sequence are given in Fig. 2A. The peptide map of des(Ala-Lys) calmodulin (Fig. 2B) shared all of the peaks found in calmodulin with an apparent deletion of the peak X which corresponds to the C-terminal 22-residue peptide. Instead, an additional peak, Y, was detected following the peak 9.

The amino acid compositions of all the peptides derived from des(Ala-Lys) calmodulin are presented in Table 2. These compositions were obtained by the analyses of the eluted peaks, except that the peak 3 was further separated into 3a and 3b by the reverse-phase liquid chromatography. As is evident from the table, the compositions of the peaks 1 to 9 peptides were consistent to the equivalent peptides of porcine brain or bovine brain calmodulin. The peak Y peptide, on the other hand, gave rise to a composition that was similar to but not identical to the peak X peptide derived from calmodulin (included in Table 2), where the clear differences lay in the absence of lysyl residue and the presence of only one alanyl residue (versus 2 in calmodulin).

Table 2. Amino Acid Compositions of Tryptic Peptide of Des(Ala-Lys)calmodulin[a]

Amino acid	peak number in Fig. 2											
	1	2[b]	3a	3b	4	5	6	7	8	9	Y	X[c]
Lys	1.0 (1)	1.1 (1)			1.2 (1)[d]	2.1 (2)	0.9 (1)	1.0 (1)				1.2 (1)
His							1.1 (1)					
Arg		0.9 (1)	1.0 (1)	1.0 (1)	1.0 (1)		1.0 (1)		1.0 (1)			
Asx					2.8 (3)	3.0 (3)	3.0 (3)	1.4 (1)	2.0 (2)	7.0 (7)	4.0 (4)	4.1 (4)
Thr				0.9 (1)		2.7 (3)	1.9 (2)	1.1 (1)	0.9 (1)	2.9 (3)	1.0 (1)	1.3 (1)
Ser		0.3 (0)			0.9 (1)	1.1 (1)			1.2 (1)	1.3 (1)	0.3 (0)	0.3 (0)
Glx			0.9 (1)	1.1 (1)	1.0 (1)	1.0 (1)	3.8 (4)	5.0 (5)	3.1 (3)	5.6 (6)	5.1 (5)	4.7 (5)
Pro										2.0 (2)		
Gly		0.3 (0)	0.4 (0)	1.1 (1)	2.1 (2)	2.1 (2)	1.3 (1)		0.5 (0)	3.1 (3)	2.2 (2)	2.1 (2)
Ala		1.0 (1)	1.1 (1)		2.0 (2)	1.2 (1)		1.8 (2)		2.3 (2)	1.0 (1)	2.0 (2)
Val				0.9 (1)	0.8 (1)		1.6 (2)			1.1 (1)	1.7 (2)	1.9 (2)
Met		1.6 (2)		0.8 (1)			1.6 (2)			1.9 (2)	1.8 (2)	1.9 (2)
Ile					0.9 (1)	1.2 (1)	1.0 (1)	1.2 (1)	0.8 (1)	2.0 (2)	0.9 (1)	1.1 (1)
Leu				1.0 (1)	1.0 (1)	1.1 (1)	1.8 (2)	1.0 (1)		2.5 (3)		0.4 (0)
Tyr					0.8 (1)						1.0 (1)	1.0 (1)
Phe			1.0 (1)		0.9 (1)	1.7 (2)		0.9 (1)		1.9 (2)	1.0 (1)	1.1 (1)
Total residues	1	2,3	4	7	16	17	20	13	9	34	20	22
Positions in sequence[e]	75	72-74 76-77	87-90	31-37	91-106	14-30	107-126	1-13	78-86	38-71	127-146	127-148

a; Amino acids present at a level of less than 0.2 mol/mol of peptide are not shown. Numbers in parentheses are the numbers of residues computed from the sequence of bovine brain calmodulin, except those for the peak Y are the numbers of residues predicted from the composition. b; A mixture of 2- (Lys,Met: 76-77) and 3- (Arg,Ala,Met: 72-74) residue peptides. c; Derived from calmodulin. d; ε-N-trimethyllysine. e; Predicted from the calmodulin sequence.

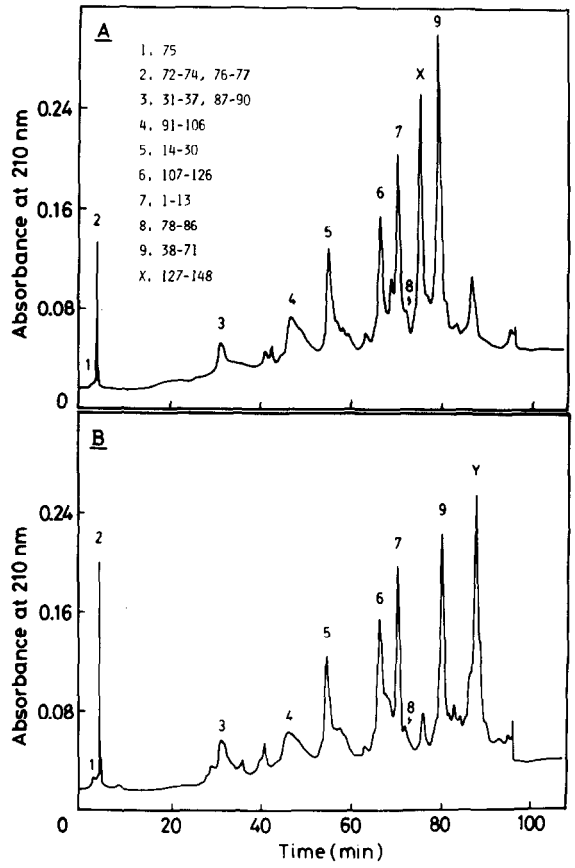

Fig. 2. Peptide maps of calmodulin (A) and des(Ala-Lys)calmodulin (B). The tryptic digests (c.a. 50 μg each) were applied to a column (4 x 250 mm) of Hitachi-Gel 3013N and eluted as described in Methods at a flow rate of 0.5 ml/min. The gradient was formed by placing 30 ml of water in a gradient mixer into which the final solvent was added at a flow rate of 0.25 ml/min. A detector (8 mm-light path) was set at 0.32 a.u.f.s. and a recorder, 1 mV. Assignments were made for the calmodulin peptides 1 to 9 and X, and their positions in the sequence are shown in (A). Amino acid compositions of the 1 to 9 and Y peptides of des(Ala-Lys)calmodulin are given in Table 2.

DISCUSSION: A calmodulin-like protein was purified to homogeneity from porcine brain extract and characterized in comparison with porcine brain calmodulin, which was also isolated in the present study. The porcine calmodulin appeared to have an amino acid sequence identical to bovine brain calmodulin because it shared all the characteristic properties of the bovine protein in terms of electrophoretic behaviour, molecular weight, amino acid composition and fragments derived by tryptic cleavage. Thus, calmodulin seems to be a remarkably conservative protein as noted in the previous papers (13,14).

The calmodulin-like protein, on the other hand, showed the properties that were very similar to but not identical to calmodulin upon chromatography and electrophoresis (Fig. 1) and in amino acid analysis (Table 1). The peptide mapping (Fig. 2) and the subsequent analyses of the isolated peptides (Table 2) have indicated that the only difference between calmodulin and the calmodulin-like protein lies in the C-terminal peptide, where the one from the calmodulin-like protein is two residues (Ala and Lys) shorter than the equivalent peptide of calmodulin. Taking into account the calmodulin sequence it seems likely that the calmodulin-like protein lacks the dipeptide sequence, Ala(147)-Lys(148), that locates at the C-terminal end of calmodulin. Therefore, this protein was designated as des(Ala-Lys)calmodulin. Des(Ala-Lys)calmodulin behaves as more acidic than calmodulin on DEAE-Sephadex A-50 and in gel electrophoresis (Fig. 1). This is probably due to the absence of one lysyl residue in des(Ala-Lys)calmodulin.

The content of des(Ala-Lys)calmodulin is about one-tenth of calmodulin, corresponding approximately to 40 mg/kg of brain tissue. According to preliminary experiments, it occurs not only in porcine brain but also in other mammalian brains such as human and bovine. The relatively high content of this protein and its wide distribution suggest that it has an unique function in cells. The compiling papers (for review see ref. 1) show that calmodulin exhibits its activity against variety of target proteins and enzymes. It is possible, however, that some of these activities are responsible for des(Ala-Lys)calmodulin that might be present in calmodulin preparations, because the separation of the two proteins is rather difficult when the conventinal procedures are employed and des(Ala-Lys)calmodulin is hardly distinguishable from calmodulin when the protein is present in one-tenth quantity of calmodulin.

We assume that des(Ala-Lys)calmodulin is derived by enzymatic removal of the C-terminal dipeptide of calmodulin, or by a different-type cleavage of "proto-calmodulin" which is not detected yet. This would imply that several post-translational modifications, not only methylation of lysyl residue but also specific peptide bond cleavages, are involved in the control mechanisms that regulate the biological activity of calmodulin. The argument may be that des(Ala-Lys)calmodulin has been produced during the purification of calmodulin. However, we have no experiences for such artificial protein cleavages in purifying other brain proteins by the similar purification procedures (4,5,7, 15,16). Thus, the possibility remains the presence of a proteinase, in vivo, which is highly specific to calmodulin and cleaves at the Thr(146)-Ala(147) bond or removes successively the Ala(147)-Lys(148) dipeptide. The removal of the C-terminal dipeptide does not affect on the Ca^{2+} binding ability of calmodulin as this sequence region locates far from the Ca^{2+} binding site (2,3),

but may play a role in alteration of the interaction between calmodulin and its target proteins.

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