Mastoparan binding induces a structural change affecting both the N-terminal and C-terminal domains of calmodulin

A ^{113}Cd-NMR study

Sara Linse, Torbjörn Drakenberg and Sture Forsén

Physical Chemistry 2, Chemical Center, PO Box 124, 221 00 Lund, Sweden

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^{113}Cd-NMR studies of solutions of cadmium-loaded calmodulin (Cd_4CaM) and the tetradecapeptide mastoparan in different ratios show that mastoparan binds to Cd_4CaM with high affinity. The off-rate of protein-bound mastoparan is found to be 40 s^{-1} or less. The binding of one molecule of mastoparan to Cd_4CaM is observed to affect all four metal-binding sites, indicating that both the N-terminal and C-terminal globular domains of the protein undergo conformational changes.

Mastoparan *Calmodulin* *Peptide-calmodulin interaction* *Calmodulin-binding peptide* *^{113}Cd-NMR*

1. INTRODUCTION

Calmodulin (CaM) is a ubiquitous Ca^{2+}-binding protein that is known to regulate a wide variety of cellular functions [1]. On a molecular level the regulating effect is accomplished through the interaction of CaM with enzymes, the activity of which is then increased or decreased [2]. There are few details available on these interactions, although several lines of evidence have suggested that they depend to a large extent on hydrophobicity. It has for example been noted that a number of hydrophobic organic compounds, e.g. fluorescent molecular probes (9AC, ANS and TNS) and drugs (review [3]), bind to CaM in a Ca^{2+}-dependent manner. Also it has been observed that CaM interacts with the hydrophobic support phenyl-Sepharose only in the presence of Ca^{2+} [4].

In addition to these hydrophobic organic compounds, a number of small naturally occurring peptides with hydrophobic and basic properties bind to CaM with high affinity in a Ca^{2+}-dependent manner, e.g. β-endorphin [5], dynorphin and mastoparan [6], vasoactive intestinal peptide (VIP), glucagon and ACTH [5], and also some venom peptides like melittin [7].

Although some of these peptide-CaM interactions may not play a physiological role, these peptides have nevertheless been considered as models for the CaM-binding regions on CaM-activated enzymes. The recent discovery of what appears to be the calmodulin-binding domain of skeletal muscle myosin light chain kinase (MLCK) – an enzyme activated by CaM – has a bearing on this point [8]. A peptide, denoted M13, 27 residues in length, which represents the carboxy-terminal end of the enzyme, was found to bind to CaM in a Ca^{2+}-dependent manner with a 1:1 stoichiometry. Addition of the M13 peptide was found to inhibit the activation of MLCK by CaM with a K_i of 0.9 nM [8]. Although the M13 peptide sequence does not have a high homology with the naturally occurring peptides mentioned above, they all share three structural features that appear to be of importance for interaction with CaM in its

Abbreviations: ACTH, adrenocorticotropic hormone; 9AC, 9-anthroylcholine; ANS, 8-anilinonaphthalenesulfonate; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate

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Ca^{2+}-loaded form: (i) clusters of basic residues; (ii) hydrophobic residues adjacent to the basic residues [5]; and (iii) a predicted high α-helical content [6,9,10]. In addition, the peptides compete for binding to CaM both with each other [5] and with CaM-regulated enzymes, e.g. MLCK [11] and brain phosphodiesterase [10]. It is therefore highly likely that these peptides and the CaM-regulated enzymes interact with CaM in a similar manner. It is thus of obvious interest to try to localize the peptide-binding sites on CaM and to assess possible structural rearrangements accompanying this interaction. Circular dichroism (CD) studies of Ca_4CaM-peptide complexes, e.g. with the peptides mastoparan [12], M13 [13] and melittin [14], have revealed a higher α-helix content than the sum of that observed for Ca_4CaM and free peptide, separately. It is, however, difficult to separate a structural change occurring in the bound peptide from one taking place in CaM. Similar problems arise in ^{1}H-NMR studies where signals from the peptide will partly overlap and obscure those from CaM.

Here we have employed ^{113}Cd-NMR to follow the structural changes in the CaM molecule following the binding of the peptide mastoparan, a tetradecapeptide from vespid wasp [15]. This approach is based on the fact that Cd^{2+} can replace Ca^{2+} in its 4 binding sites without significant changes in the protein tertiary structure. In fact Cd_4CaM has been shown to activate a number of enzymes just like Ca_4CaM [16]. ^{113}Cd-NMR spectral shifts are very sensitive to changes in the local structure around the metal ion, and furthermore, since $^{113}Cd^{2+}$ is bound only to CaM, no problem of spectral overlap between signals from protein and peptide arise.

2. MATERIALS AND METHODS

CaM was isolated from bovine testes as described [17]. The purity of CaM was checked by SDS gel electrophoresis, agarose gel electrophoresis and FPLC (Pro-RPC column, Pharmacia).

The calcium-free form was prepared by passing a solution of 447 mg CaM in 3 ml H_2O through a 20 ml Chelex-100 (Bio-rad) column at pH 8. The residual calcium content was 0.05 mol Ca/mol CaM, as determined by atomic absorption spectroscopy. The calcium-free protein was first freeze-dried and then redissolved in D_2O at pH 7.

Mastoparan was obtained from Bachem (Torrance, CA) (lot no.7218) and used without further purification.

^{113}Cd experiments were performed at 24 ± 1°C on a home-built 6 T NMR spectrometer (for a description see [18]), at 56.55 MHz using routine acquisition parameters described elsewhere [19]. A 30 Hz line-broadening function was used to improve the signal-to-noise ratio. Spectral shifts are reported relative to 0.1 M $^{113}CdClO_4$ at pH 7.

^{113}Cd was added to CaM in the form of $CdNO_3$ solution, prepared from Cd metal (US Services Inc.) and HNO_3. The isotope enrichment of ^{113}Cd was 93.4%.

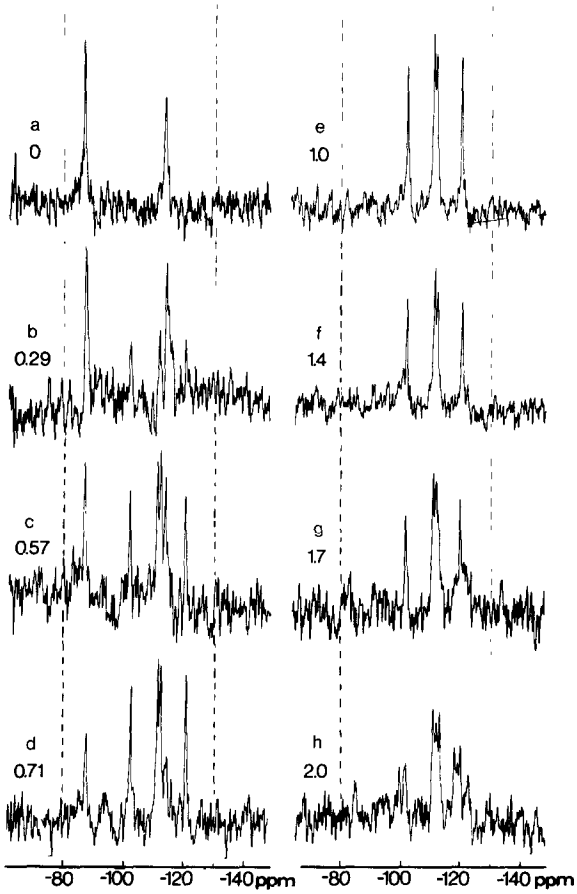

Fig.1. ^{113}Cd-NMR spectra of a 1 mM solution of Cd_4CaM with increasing amounts of mastoparan added. The molar ratio of mastoparan to CaM is indicated on each spectrum.

Table 1

Amino acid sequences of the CaM-binding peptides M13, mastoparan and mastoparan X

M13	K-R-R-W-K-K-N-F-I-A-V-S-A-A-N-R-F-K-K-I-S-S-S-G-A-L-M
Mastoparan	I-N-L-K-A-L-A-A-L-A-K-K-I-L-NH_2
Mastoparan X	I-N-W-K-G-I-A-A-M-A-K-K-L-L-NH_2

One-letter codes used: A (alanine), R (arginine), N (asparagine), G (glycine), I (isoleucine), L (leucine), K (lysine), M (methionine), F (phenylalanine), S (serine), W (tryptophan) and V (valine). Basic residues are underlined

Titration of Cd_4CaM with mastoparan was performed in a 3 ml D_2O solution containing 1 mM Cd_4CaM at pH 7. Mastoparan was added in aliquots of 20 μl, corresponding to 1/7 mol peptide per mol protein, up to a molar ratio of 1:1. Two independent titrations gave virtually the same spectral changes. The second titration was continued up to 2 mol peptide per mol Cd_4CaM.

3. RESULTS

In the absence of peptide, two ^{113}Cd-NMR signals (at -87 and -114 ppm) are observed for a solution of Cd_4CaM (fig.1a). These signals arise from $^{113}Cd^{2+}$ bound to the two strong binding sites III and IV. The $^{113}Cd^{2+}$ in the weaker binding sites I and II gives no signals, probably due to rapid interconversions on the time scale of at least 10^3–10^4 s^{-1} between two different conformations in the N-terminal domain containing sites I and II [20].

When mastoparan is added 4 new signals (at -102, -111, -112 and -121 ppm) start to appear in the ^{113}Cd-NMR spectrum (fig.1b–e). At intermediate ratios of peptide to Cd_4CaM, 6 sharp signals are observed, and at molar ratios of 1:1 and higher, only the 4 new signals of approximately the same intensity remain in the spectrum. The relative intensities of the two sets of signals, corresponding to Cd_4CaM with and without mastoparan bound, are linearly related to the molar ratio of mastoparan to Cd_4CaM.

At a molar ratio of peptide to Cd_4CaM of 2:1, all 4 signals in the ^{113}Cd-NMR spectrum are broadened, but remain at the same shifts as after addition of the first equivalent (fig.1f–h).

4. DISCUSSION

The linear dependence of the intensities of the two sets of signals in the ^{113}Cd-NMR spectra on the molar ratio of mastoparan to Cd_4CaM observed when mastoparan is successively added to Cd_4CaM clearly indicates that mastoparan is bound to CaM with high affinity. This is in good agreement with a dissociation constant of 0.3 nM for the mastoparan-Ca_4CaM complex [6], determined by competition experiments with smooth muscle MLCK. The Ca_4CaM-bound MLCK was monitored by 9AC fluorescence.

The exchange of free and protein-bound peptide is slow on the NMR time scale. The ^{113}Cd-NMR signal linewidths give an upper limit of the exchange rate of 40 s^{-1}.

The finding that 4 new signals appear in the ^{113}Cd-NMR spectrum when 1 mol mastoparan/mol Cd_4CaM is added clearly shows that a bound mastoparan molecule is able to induce conformational changes that affect all 4 Ca^{2+}-binding sites in CaM. ^{1}H-NMR studies have shown that binding of M13 to Ca_4CaM causes dramatic changes in the ^{1}H-NMR spectrum, with signals arising from protons in both halves of CaM affected [13].

In this respect, the peptides differ markedly from different small drug molecules like trifluoperazine, felodipine and diltiazem, for which both halves of CaM are affected only after addition of 2 or more mol drug/mol Cd_4CaM [21].

The unavailability of high-resolution X-ray coordinates for CaM makes it difficult to propose a detailed model for the mastoparan-Ca_4CaM complex. However, inspection of similarities with small drugs and other peptides that bind to CaM with high affinity could give some clues to the

specific interactions involved. It has been proposed that the major tranquillizers interact with both negative charges and hydrophobic regions of the amphiphilic helices of CaM [22], for example the amphiphilic helix between residues 82 and 92 of bovine brain CaM, which contains 4 glutamic acid and 2 phenylalanine residues. This region makes up about half of the long helix connecting the 2 Ca^{2+}-binding domains of CaM, as seen in the crystal structure [23].

In an attempt to localize the peptide-binding site of Ca_4CaM, the tryptophan-containing peptide mastoparan X was titrated with CaM or 1 of the 3 CaM fragments containing residues 1–106, 72–148 and 107–148, in the presence of Ca^{2+} [7]. The complex formation was monitored by fluorescence anisotropy and it was suggested that the CaM region made up of residues 72–106 contains a major portion of the peptide-binding site of Ca_4CaM [7]. This region includes most of the long α-helix (residues 65–92) connecting the 2 globular domains of Ca_4CaM. The anionic sequence at positions 78–84, which contains 2 aspartic acid and 3 glutamic acid residues, has been proposed to interact with the basic regions found in all high-affinity CaM-binding peptides, permitting interaction between hydrophobic peptide regions and the non-polar CaM residues 88–92 [7]. Also, significant reduction of the reactivities of the amino groups of Lys 75 and 148 of CaM has been observed upon binding of β-endorphin to Ca_4CaM at a molar ratio of 2.5:1 [24].

The 14-residue peptide mastoparan is sufficiently large to bind to Ca_4CaM in such a way that it is in contact with both the N-terminal and C-terminal domains of Ca_4CaM simultaneously. Mastoparan has a predicted high α-helical content in aqueous solution [6]. CD studies have indicated that the α-helical content of mastoparan is low in aqueous solution, but 55% in 70% trifluoroethanol/water mixture and the total α-helical content of Ca_4CaM and the peptide has been shown to increase upon formation of a complex [12]. It therefore seems reasonable to assume that the secondary structure of Cd_4CaM-bound mastoparan is mostly α-helix, which implies that the peptide would have a length of 21 Å in its protein-bound form. From the X-ray crystal structure of Ca_4CaM [23], the minimum separation between the van der Waals' surfaces of the two globular domains may be estimated to about 11 Å [25]. If the long α-helix connecting the two globular domains in the crystal structure of Ca_4CaM [23] is also present in solution, interaction of the basic, amphipathic peptide mastoparan with the acidic and hydrophobic regions of this long α-helix in Ca_4CaM would permit mastoparan to interact with both globular domains.

As mentioned above, the peptides M13, representing the CaM-binding region of MLCK, and mastoparan are similar in many respects, i.e. the occurrence in both of hydrophobic regions, positively charged patches and a high tendency of α-helix formation, together with comparable dissociation constants, Ca^{2+}-dependent and competitive binding to Ca_4CaM and 1:1 stoichiometry of the peptide-Ca_4CaM complexes. This suggests that mastoparan could be used as a model peptide in the study of CaM-enzyme interactions.

The observation that all 4 Ca^{2+}-binding sites in CaM are affected by mastoparan binding indicates that both globular domains are involved in CaM-target enzyme interactions. It has indeed been shown that none of the tryptic CaM fragments 1–77, 1–90, 1–106, 78–148 or 107–148 are able to activate the protein phosphatase, calcineurin or the Ca^{2+}-dependent cAMP phosphodiesterase. However, the fragment 78–148 was found to activate fully phosphorylase kinase, but with lower affinity than CaM [26].

The changes observed in the ^{113}Cd-NMR spectrum on continued titration of Cd_4CaM from 1 to 2 mol mastoparan/mol Cd_4CaM show that a second peptide molecule may be bound to CaM. The shifts of the ^{113}Cd-NMR signals are not affected by this second equivalent of mastoparan, indicating that the conformation of CaM remains the same as with only one peptide molecule bound. The broad signals suggest either or both weaker binding and faster exchange of the second equivalent of mastoparan than of the first. Binding of a second equivalent of peptide has been detected in covalent cross-linking experiments with β-endorphin and CaM [27].

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