NMR-driven secondary and tertiary structure model of Ca^{2+}-loaded calexcitin ☆

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Abstract

Calexcitin (CE) is a Ca^{2+}-binding protein which is expressed in neuronal cells and is a member of the sarcoplasmic Ca^{2+}-binding protein subfamily. The peptide backbone of Ca^{2+}-CE has been assigned by NMR and it shows that CE is composed of nine α-helices—forming four EF-hands and an additional helix near the C-terminus. A structural model of CE suggests the presence of a putative recessed hydrophobic pocket that may be involved in Ca^{2+}-mediated protein–ligand interactions. This feature is unique to CE and is absent in other SCPs, such as those from *Branchiostoma* and *Nereis*, and from calerythrin.
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Calcium ions (Ca^{2+}) are essential for the normal function of cells. Ca^{2+} signaling pathways often recruit specialized Ca^{2+}-binding proteins (CaBPs), including Ca^{2+} sensors and buffers. Calexcitin (CE) is a cytosolic neuronal protein which may be implicated in learning, memory, and neurological conditions, such as Alzheimer's disease and epilepsy (for review, [1]). CE is an EF-hand protein that belongs to the sarcoplasmic Ca^{2+}-binding protein (SCP) subfamily [2]. Unlike SCPs that have high Ca^{2+}-binding affinities (nM), CE has a moderate Ca^{2+}-binding constant (μM) similar to Ca^{2+} sensor proteins. Other differences between CE and other SCPs are that CE expression is neuronal, while SCPs are produced in the muscle [3], and our earlier work has also indicated that the SCP subfamily can be divided into two groups: those expressed in neurons (termed CE) and those expressed in muscles (termed SCPs) [2]. This has also been supported by computational studies [4].

CE has been suggested to interact with at least two potential targets. Nelson et al. [5] co-purified CE with the ryanodine receptor (RyR) from the squid optic lobe. A peptide region from the RyR that can bind CE has also been identified. The RyR is an important Ca^{2+} release channel within neurons that enhance the Ca^{2+} signal by releasing additional Ca^{2+} from the endoplasmic reticulum. The same group also showed that expression of CE coincides with the inhibition of K^{+} channels [1]; however, the type of K^{+} channel CE acts on is currently unknown. ANS-binding studies on CE have revealed a possible recessed hydrophobic pocket that may be indicative of the target recognition site [6].

We have previously characterized CE and have shown it to be a four EF-hand (helix–loop–helix) protein capable of binding Ca^{2+} and Mg^{2+}, like other members of the SCP family [2,6]. Furthermore, this protein exists in three distinct conformational states that interconvert upon addition of Ca^{2+} and/or Mg^{2+} [6]. In contrast to the proposal of Ascoli and colleagues that CE is an α/β protein [7], our data indicate that CE consists mainly of α-helices, with

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short β-sheets (2–3 residues), holding pairs of EF-hands together [2]. Homology models of *Drosophila melanogaster* SCP2 (dSCP2) [8] display similar amounts of overall secondary structure to CE; however, there are differences in the position and number of α-helices. To clarify the secondary structure of these related proteins, we have overexpressed, purified, and assigned the backbone ^{1}H, ^{13}C, and ^{15}N resonances of CE in the presence of Ca^{2+}. Since the detailed three-dimensional structure of CE is still not available, we also propose a working model of Ca^{2+}-CE.

Materials and methods

Expression and purification of CE. The ^{13}C- and ^{15}N-labeled sample was produced in standard M9 minimal media supplemented with ^{15}NH$_4$Cl (1 g/L) and [^{13}C$_6$]-D-glucose (2 g/L). *Escherichia coli* BL21 (DE3) cells transformed with pGEX-2TK-*Nde*I-CE were grown at 37 °C, induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 12 h, followed by further addition of 1 mM IPTG and growth for an additional 12 h. The GST–CE fusion protein was purified and the CE protein was cleaved and checked for purity as described previously [2]. CE was dialyzed against a solution containing 10 mM Tris–HCl (pH 7.5), 1 mM DTT, and 10 mM CaCl$_2$. The protein concentration was about 1 mM and ^{2}H$_2$O was added to 5% (v/v).

NMR spectroscopy. NMR spectra were acquired at 30 °C on a Varian UNITY-plus 500 or Varian INOVA 600 spectrometer equipped with pulsed field gradient triple resonance probes. Linear prediction was used in the ^{13}C and ^{15}N dimensions to improve digital resolution. Spectra were processed using the NMRPipe software package [9] and analyzed with XEASY [10]. The ^{1}H, ^{15}N, and ^{13}C backbone resonance assignments were obtained from the following experiments: ^{1}H–^{15}N HSQC, CBCA(CO)NH, HNCACB, HNCO, and HCCTOCSY-NNH [11].

Generation of the model of the three-dimensional structure of Ca^{2+}-CE. The model of the three-dimensional structure of Ca^{2+}-CE was generated using SWISS-MODEL [15], based on the structure of amphioxus SCP [12].

Results and discussion

Backbone assignments and secondary structure determination

The eight residues of the N-terminal overhang of the expression vector and nine other non-proline residues of CE are not visible in the HSQC spectrum [2]. Ninety-nine percent of the visible backbone amide resonances of CE were assigned. With regard to the other backbone resonances of CE, 96% of C$^{\alpha}$, 75% of H$^{\alpha}$, and 93% of C′ have been assigned. The quality of the HCCTOCSY-NNH spectrum is low and was unimproved by standard spectrum enhancement techniques. The secondary structure assign-
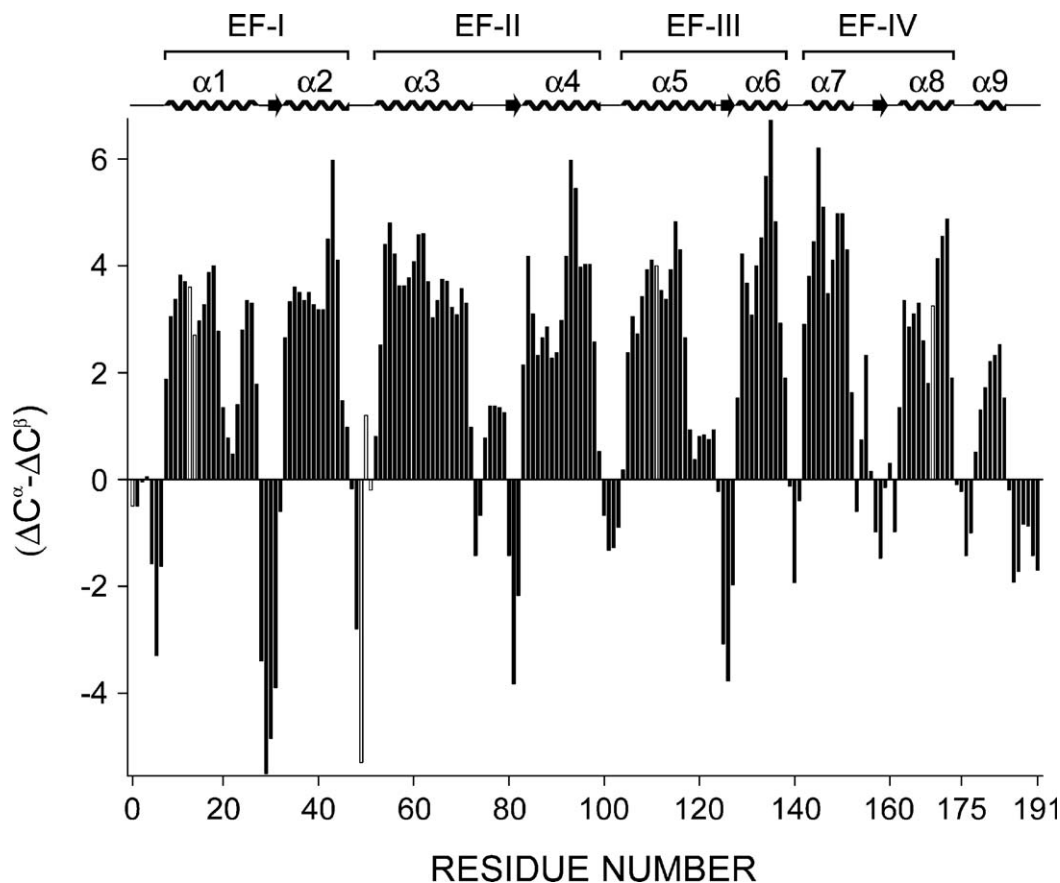

Fig. 1. Summary of NMR data for secondary structure determination. The values of $(\Delta C^{\alpha} - \Delta C^{\beta})$ are plotted against the amino acid sequence, where ΔC^{α} and ΔC^{β} were obtained by subtracting the corresponding random coil chemical shifts of ^{13}C$^{\alpha}$ and ^{13}C$^{\beta}$ from the shifts measured in the spectra. The value $(\Delta C^{\alpha} - \Delta C^{\beta})$ for a particular residue (i) represents an average of three consecutive residues: $(\Delta C^{\alpha} - \Delta C^{\beta})_i = (\Delta C^{\alpha}_{i-1} + 2 \times \Delta C^{\alpha}_i + \Delta C^{\alpha}_{i+1} - \Delta C^{\beta}_{i-1} - 2 \times \Delta C^{\beta}_i - \Delta C^{\beta}_{i+1})/4$. Residues that are not completely assigned are indicated with open bars. The cartoon shows the secondary structure elements. The EF-hand motifs are indicated on top.

ment has been based on secondary chemical shifts for C^α and C^β (Fig. 1).

CE is composed of nine α-helices, eight of which comprise the EF-hand motifs. The ninth α-helix is located near the C-terminus of the protein. This segment has been under tremendous scrutiny. Ascoli and colleagues suggested that this area contains a β-sheet on CE [7], whereas Maxwell

and colleagues proposed that this area is composed of a flexible loop in dSCP2, the *Drosophila* CE homologue [8]. However, our data show that this region contains an α-helix (Fig. 1), in agreement with secondary structure prediction [2]. The C-terminal helix may participate in interactions with the two EF-hand domains of CE to form a globular structure as a whole, in a manner similar to that seen in SCPs [12,13]. It is clear, however, that CE is predominantly an α-helical protein as suggested earlier [2], rather than a mixed α/β protein, as suggested previously [7].

A model of the three-dimensional structure of Ca²⁺-CE

There are two X-ray crystal structures of Ca²⁺-loaded SCPs [12,13] and an NMR structure of Ca²⁺-calerythrin [14], a bacterial protein which shows SCP-like characteristics. Although the primary sequences of these three proteins are quite different (amino acid identities of ∼15%), the three structures show remarkable similarities, with backbone root mean square distances (RMSDs) of 1.3–1.7 Å. Therefore, it is conceivable that the three-dimensional structure of CE would show a high degree of similarity to these proteins. Using SWISS-MODEL [15], a model of the three-dimensional structure of Ca²⁺-CE was generated, based on the structure of amphioxus SCP [12], as it showed the highest sequence similarity. The model shows that, in agreement with previous data [6], CE is composed of two independent domains, which show little interconnectivity (Fig. 2). Furthermore, the model identifies a recessed pocket that is composed mostly of hydrophobic residues (Fig. 3). This is again in agreement with previous data [6] and a common feature to several other classes of EF-hand proteins. Surprisingly, the residues that form this hydrophobic pocket are the most

Fig. 2. A ribbon diagram of the model of CE based on the three-dimensional structure of amphioxus SCP [12]. This model was generated with SWISS-MODEL. The two independent domains are visible. EF-I and II are in red and EF-III and IV are in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)
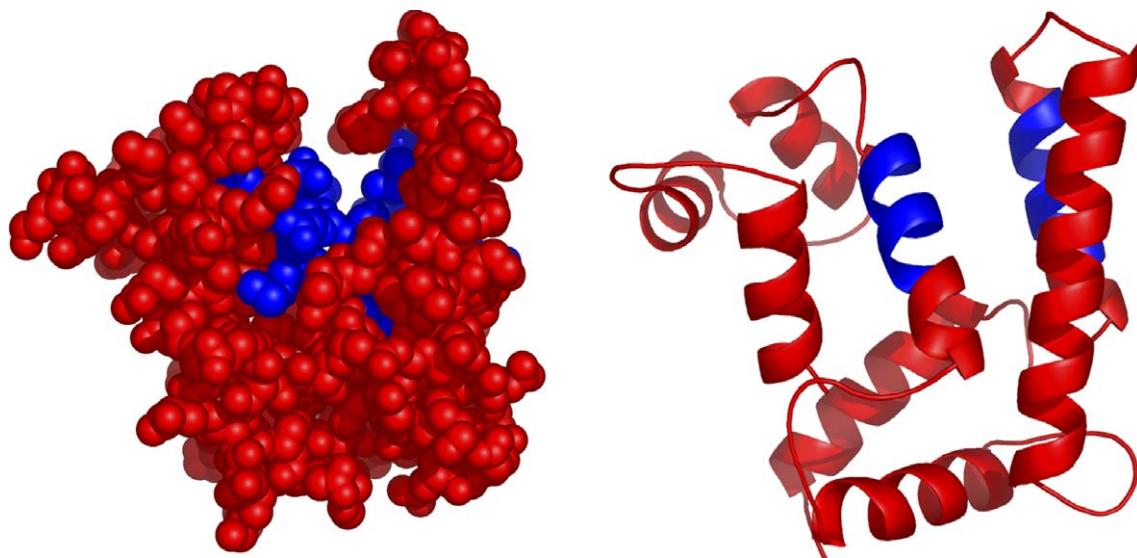

Fig. 3. Spacefilling (left) and ribbon (right) diagram of the CE structure model. The conserved residues that form the fingerprint regions of CE are identified in blue. These regions form the proposed recessed hydrophobic pocket that was postulated previously [6]. These regions map to the exiting helix of EF-I (furthest right) and the entering helix of EF-III (center of structure). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)
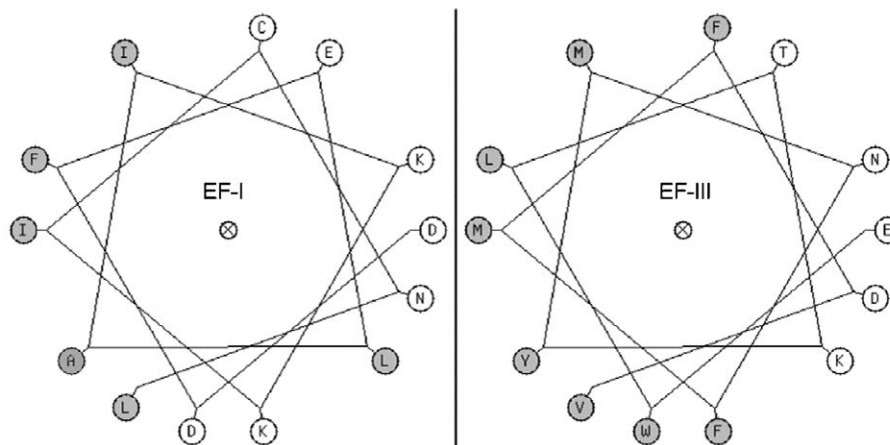
Fig. 4. Helical wheel representation of the fingerprint region of CE, showing the relevant section of the exiting helix of EF-I (DFELAIKKICNL; left) and the entering helix of EF-III (EWLTKYMNFMFDV; right). These amphipathic helices form the recessed hydrophobic target binding site of CE, predicted by the homology model.

conserved among various CEs and one region (YXNFM) has been proposed to form the fingerprint region that distinguishes neuronal CEs from muscle SCPs [2]. Examination of this model of CE suggests, however, the presence of a second fingerprint DFXLhhpphpph, where p represents any polar residue and h represents any hydrophobic residue. Furthermore, the first fingerprint can be further extended to YXNFMFph. Based on the sequence of these regions and knowing that these areas are in an α-helical conformation (Fig. 1), it is suggested that these two helices (helix 5 and 2, i.e., entering helix of EF-III and exiting helix of EF-I, respectively) are amphipathic helices (Fig. 4). Both of the fingerprint regions also contain acidic residues, which, in other EF-hand proteins, are known to interact with basic residues on the target [16]. Lastly, the fingerprint region within EF-III contains one or more Met residues, which may also be important in target interaction and allow multiple targets to interact with calmodulin [17].

It is also tempting to raise a possible structural explanation regarding the observed functional difference between CE and SCP. The residues that constitute the putative hydrophobic pocket of CE have been replaced by polar amino acids in SCPs. In CE, this putative hydrophobic pocket may serve as a binding site for an unknown ligand or possibly the suggested target proteins (RyR [5] and the K^+ channels [18]).

The backbone assignment of CE lays the foundation for future studies on the exact structural effects of ion binding and phosphorylation on CE. Since the Ca^{2+}/Mg^{2+} interplay on CE is complex, the information gathered from NMR measurements of protein motions could yield important results for the structural changes of CE upon ion binding. Similarly, phosphorylation of CE has been previously shown [1,2,18,19], but its physiological relevance has been questioned [2]. Therefore, the backbone assignment of Ca^{2+}-CE also lays the foundation to detailed structural work on the effects of phosphorylation to clarify this issue.

Our structural model of CE based on amphioxus SCP fits well with previously published results; nevertheless, to unequivocally address this issue, it will be necessary to solve the three-dimensional structure of CE.

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