The Crystal Structure of the Primary Ca\textsuperscript{2+} Sensor of the Na\textsuperscript{+}/Ca\textsuperscript{2+} Exchanger Reveals a Novel Ca\textsuperscript{2+} Binding Motif

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The Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is a plasma membrane protein that regulates intracellular Ca\textsuperscript{2+} levels in cardiac myocytes. Transport activity is governed by Ca\textsuperscript{2+}, and the primary Ca\textsuperscript{2+} sensor (CBD1) is located in a large cytoplasmic loop connecting two transmembrane helices. The binding of Ca\textsuperscript{2+} to the CBD1 sensory domain results in conformational changes that stimulate the exchanger to extrude Ca\textsuperscript{2+}. Here, we present a crystal structure of CBD1 at 2.5 Å resolution, which reveals a novel Ca\textsuperscript{2+} binding site consisting of four Ca\textsuperscript{2+} ions arranged in a tight planar cluster. This intricate coordination pattern for a Ca\textsuperscript{2+} binding cluster is indicative of a highly sensitive Ca\textsuperscript{2+} sensor and may represent a general platform for Ca\textsuperscript{2+} sensing.

Rapid fluxes of Ca\textsuperscript{2+} across the sarcoplasmic membrane are an important component of cardiac excitation-contraction coupling. Ca\textsuperscript{2+} influx mediated by voltage-dependent Ca\textsuperscript{2+} channels initiates contractions, while Ca\textsuperscript{2+} efflux is dominated by the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (1). Thus, the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is an important component of regulation of cardiac contractility. Under most physiological conditions, the exchanger uses the energy stored in the inwardly directed Na\textsuperscript{+} gradient to catalyze the extrusion of Ca\textsuperscript{2+} from the cell with a stoichiometry of 3 Na\textsuperscript{+} for 1 Ca\textsuperscript{2+}.

Activity of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is modulated by the binding of Ca\textsuperscript{2+} to a high affinity regulatory site on an intracellular portion of the protein. Regulatory Ca\textsuperscript{2+} is not transported but potently activates exchange activity. Recent evidence suggests that Ca\textsuperscript{2+} may bind to and dissociate from its regulatory site during the rapid Ca\textsuperscript{2+} fluctuations that occur during a cardiac contraction cycle (2). The Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger protein is predicted to consist of nine transmembrane segments and a large intracellular loop (3, 4). The transmembrane segments translocate ions across the membrane, and the intracellular loop is largely responsible for regulation of activity. We have previously identified a region of the intracellular loop of the exchanger (amino acids 371–508) that binds Ca\textsuperscript{2+} with high affinity and mediates activation of exchange activity by Ca\textsuperscript{2+} (5, 6). This segment comprises the first of two tandem Calx-β domains (7). Mutational analysis identified two groups of three aspartate residues within the first Calx-β domain that were associated with the binding of Ca\textsuperscript{2+} (5, 6). The binding of Ca\textsuperscript{2+} to the regulatory site induces substantial conformational changes that presumably mediate regulatory function (2, 6, 8, 9).

A recent major development in the understanding of Ca\textsuperscript{2+} regulation has been the determination of the structure of the Ca\textsuperscript{2+} binding region of the large intracellular loop using NMR techniques (9). Two Ca\textsuperscript{2+} binding domains (CBD1 and CBD2) were identified that correspond to Calx-β1 and -β2. CBD1 encompasses the same region that we had identified as being responsible for Ca\textsuperscript{2+} regulation. Binding of Ca\textsuperscript{2+} to CBD1 induces a substantial conformational change consistent with earlier studies. In the presence of Ca\textsuperscript{2+}, both CBD1 and CBD2 have an immunoglobulin fold. CBD2, in the adjoining Calx-β repeat region, binds Ca\textsuperscript{2+} with substantially lower affinity and its functional role is unclear. Unlike CBD1, the removal of Ca\textsuperscript{2+} from CBD2 does not induce protein unfolding.

The NMR structure of CBD1 shows a classical immunoglobulin fold with two Ca\textsuperscript{2+} ions bound in the distal loops (9). However, the heteronuclear single quantum correlation spectra employed by Hilge and colleagues does not directly visualize the presence of Ca\textsuperscript{2+} but rather infers positions from Yb\textsuperscript{3+}-induced shifts. Here, we describe the crystal structure of CBD1 using x-ray techniques. Like the NMR structure, we find an immunoglobulin fold, and the two structures superimpose well. Strikingly, the x-ray structure reveals the presence of four Ca\textsuperscript{2+} ions bound in a unique cluster with important physiological consequences.

EXPERIMENTAL PROCEDURES

Expression and Purification of CBD1—A fusion protein encoding residues 370–509 of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) with an N-terminal extension of MGRSHTHHTHHTHTHGI was expressed using the pQE32 vector (Qiagen) and M15pRep4 Escherichia coli cells (Qiagen). Induced cell pellets were dissolved in buffer B (8 mM urea, 20 mM Tris-Cl, pH 8, 0.1 mM CaCl\textsubscript{2}, 300 mM NaCl) supplemented with 5 mM β-mercaptoethanol and EDTA-free complete protease inhibitor (Roche Applied Science), stirred for 30 min, then sonicated. Following centrifugation at 10,000 × g for 45 min the supernatant was filtered, Triton X-100 (1%), imidazole (10 mM), and nickel-nitrilotriacetic acid (Qiagen) were added and stirred for 30 min before
loading in to a column. The column was washed with buffer B followed by washes with 75% buffer B/25% wash buffer (250 mM Mes, pH 6.3, 0.3 M NaCl, 10% glycerol, 0.1 mM CaCl$_2$), 50% buffer B/50% wash buffer followed by 25% buffer B/75% wash buffer and finally with 100% wash buffer. Fusion protein was eluted from the column in wash buffer + 250 mM imidazole, pH 7.4. Fractions containing CBD1 were pooled and concentrated with Centriprep 30 (Amicon) filtered and applied to a HiPrep 16/60 Sephacryl S-100 column (Amersham Biosciences) pre-equilibrated with wash buffer 2 (20 mM Tris-Cl, pH 8, 300 mM NaCl, 0.1 mM CaCl$_2$). Peak fractions were pooled and dialyzed against five changes of 10 mM Tris-Cl, pH 7.4, 0.2 mM EGTA. Dialyzed protein was concentrated with Centricron 30 (Amicon).

**Crystal Growth and Structure Determination**—Purified CBD1 protein was maintained in a solution of 10 mM Tris-HCl, pH = 7.4, + 0.2 mM EGTA at a concentration of 25 mg/ml. This solution was screened against 480 commercially available crystallization conditions with the mosquito crystallization robot (TTP Labtech) using the hanging drop vapor diffusion technique. Crystals were obtained at 20 °C in condition number 35 of Hampton Research's Crystal Screen 2 (100 mM HEPES, pH = 7.5, + 70% 2-methylpentane-2,3-diol). These crystals were then optimized by addition of 100 mM guanidine discovered through additive screening using Hampton Research’s Additive Screen in conjunction with the Mosquito robot. The resulting crystals diffracted to 2.5 Å resolution (see Table 1 of supplemental material).

Data were collected from a cryo-cooled crystal at beamline 8.2.2 of the Advance Light Source (Berkeley, CA). The crystal belongs to the space group P2$_1$2$_1$2 with cell dimensions of $a = 59.6$ Å, $b = 45.5$ Å, and $c = 57.3$ Å. Image data were processed using the programs DENZO and SCALEPACK (10). The structure of CBD1 was phased by molecular replacement using the program PHASER (11). The coordinates of the recent NMR structure of CBD1 (PDB accession code 2FWS) were used for the search model. The structure was built using the program COOT (12) and refined using CNS (13) and REFMAC (14) with a final $R$ and $R_{free}$ of 22.2 and 28.4%, respectively.

**RESULTS AND DISCUSSION**

We sought to uncover the principles underlying Ca$^{2+}$ regulation of the NCX by resolving the crystal structures of the primary Ca$^{2+}$ binding domain (CBD1) in the Ca$^{2+}$-bound and Ca$^{2+}$-free conformations. Initial crystallization trials in the presence of 2 mM CaCl$_2$ (Ca$^{2+}$-bound) and 2 mM EGTA (Ca$^{2+}$-free) failed. To minimize the impact of these reagents on crystallization, we reduced their concentrations to 0.2 mM. An EGTA-containing sample yielded crystals diffracting to 2.5 Å. The crystal structure had a strong resemblance to the NMR structure (9) maintaining the overall immunoglobulin fold. In addition, the positions of four tightly clustered Ca$^{2+}$ ions were revealed. Further analysis confirmed a contamination of 0.12 mM Ca$^{2+}$ in condition number 35 of Hampton Research's Crystal Screen 2, which inadvertently led to the Ca$^{2+}$-bound structure.

**Structure Overview**—The NMR and crystal structures were superimposed with a root mean square difference of 1.8 for 128 Ca$_n$ atoms (Fig. 1). The overall positional alignment between the two structures coincides well including the notable β-bulge and cis-proline that disrupt the A and G strands, respectively. The striking new feature of the crystal structure is the presence of a novel Ca$^{2+}$ binding site situated in the distal loops of the β-sandwich containing four Ca$^{2+}$ ions coordinated by an extensive network of amino acids residues. The previously reported NMR structure showed two Ca$^{2+}$ ions, which approximately represent a positional average of those observed in the crystal structure (Fig. 1). This newly observed Ca$^{2+}$ binding motif was only revealed by x-ray crystallography and will provide a framework for further biochemical and mutational analysis.

There had not previously been any indication that four Ca$^{2+}$ ions were present in the Ca$^{2+}$ regulatory domain. Ca$^{2+}$ binding data had suggested the binding of two Ca$^{2+}$ ions per regulatory domain (8). Hill coefficients have been variable for binding and functional effects of Ca$^{2+}$. Values include 0.9 (5), 1.4 (15), and 2.9 (2) consistent with the involvement of multiple Ca$^{2+}$ ions, although the source of the variability is unclear.

CBD1 is arranged in a classical immunoglobulin fold, where the β-sandwich motif is formed by two antiparallel β-sheets consisting of strands A-B-E and strands D-C-F-G (Fig. 2a). The presence of a β-bulge in strand A disrupts the antiparallel hydrogen bonding pattern between strands A’ and B. Following the β-bulge, strand A’ associates with strand G’ from the opposing sheet, rather than resolving its interactions with strand B (Fig. 2c). Additionally, there is a cis-proline residue that induces an abrupt loop in the middle of strand G, but unlike strand A, strand G ressembles a normal hydrogen bonding pattern with strand F. These geometrical distortions are often observed in external strands A and G of immunoglobulin folds (16, 17) and have been suggested to be protective in preventing aggregation between multiple immunoglobulin domains by disrupting potential intermolecular hydrogen bonding surfaces (18). This suggestion seems particularly relevant based on the model presented by Hilge et al. (9), predicting that the high affinity Ca$^{2+}$ sensor (CBD1) and the low affinity Ca$^{2+}$ sensor (CBD2) form a heterodimer stacked along the A-G interface.

The coordinates for CBD1 were compared against another three-dimensional structures using the distance matrix alignment server (Dali) (19) revealing a number of structural homologues including fibronectins, cadherins, and integrins. Although there appears to be no apparent sequence identity or functional similarities, members of the immunoglobulin fold family share a common core structure (16), which is one of the most prevalent domains encoded by the human genome (20).

**Ca$^{2+}$ Coordination**—The striking difference between the crystal and NMR structures is at the Ca$^{2+}$ binding region. Hilge and colleagues (9) were able to assign the positions for two Ca$^{2+}$ ions by using a three prong approach, which included the recording of pseudo-contact shift data, obtaining spectra from the sample in the presence of Yb$^{3+}$ ions and utilizing biochemical and mutagenesis data for distance constraints. However,
the crystal structure revealed an extensive coordination scheme connecting four Ca\(^{2+}\) ions clustered in the distal loops of the β-sandwich. It appears that the two Ca\(^{2+}\) sites predicted in the NMR structure represent a positional average of those observed in the crystal structure (Fig. 1). The four binding sites are arranged in a parallelogram-like configuration, where the distances between Ca\(^{2+}\) sites 1 and 2, 2 and 3, and 3 and 4 are 4.27, 4.30, and 3.93 Å, respectively (Fig. 2, a and b). These binding sites are primarily coordinated by aspartic and glutamic acid residues forming polydentate interactions, often between two or three Ca\(^{2+}\) ions.

The majority of the residues involved in coordinating the Ca\(^{2+}\) ions are located at the C terminus (Asp\(^{498}\), Asp\(^{499}\), Asp\(^{500}\)) and in loop E-F (Asp\(^{446}\), Asp\(^{447}\), Ile\(^{449}\), Glu\(^{451}\), Glu\(^{454}\)). Additional interactions occur with Glu\(^{383}\) in the A-B loop, Asp\(^{421}\) in the C-D loop, and three water molecules. The overall coordination scheme for each Ca\(^{2+}\) site is summarized in Table 2 of the supplemental material. In short, Ca1 and Ca4 are penta-coordinated, while Ca2 and Ca3 are hexa- and hepta-coordinated, respectively. Glu\(^{451}\), Asp\(^{421}\), and Asp\(^{500}\) coordinate multiple Ca\(^{2+}\) ions and appear to be the key residues in forming a tight binding cluster of four Ca\(^{2+}\) ions. Glu\(^{451}\) is centrally located coordinating Ca1, Ca2, and Ca3. Asp\(^{421}\) coordinates both Ca1 and Ca2, while Asp\(^{500}\) coordinates Ca3 and Ca4. These three residues appear to orient the four Ca\(^{2+}\) ions into a tight binding cluster. Although never previously observed, a similar arrangement of a four Ca\(^{2+}\) ion binding cluster has been predicted for another Ca\(^{2+}\) sensor domain, the C2 domains of synaptotagmin I and phospholipase C (21).

Two acidic segments, each characterized by three consecutive aspartic acid residues (498–500 and 446–448), were previously suggested to be Ca\(^{2+}\) binding regions (6); mutations in residues Asp\(^{447}\), Asp\(^{448}\), Asp\(^{498}\), and Asp\(^{500}\) each result in an apparent 3-fold decrease in Ca\(^{2+}\) affinity (2). Additionally, a recent mutation, E454K, showed an 8-fold decrease in Ca\(^{2+}\) affinity (9). We directly visualize three residues (Asp\(^{446}\), Ile\(^{449}\), Asp\(^{499}\) and three water molecules that are ligands for Ca\(^{2+}\), which are not part of the Ca\(^{2+}\) binding structure in the NMR study (9). Conversely, Hilge et al. (9) place Asp\(^{446}\) as a Ca\(^{2+}\) ligand, but we find that this residue is not directly involved in the binding of Ca\(^{2+}\). In total, the Ca\(^{2+}\) binding region is tightly regulated through a complex coordination scheme composed mostly of carboxylate moieties.

**Comparison with Other Ca\(^{2+}\) Binding Proteins**—Analysis of sequence and structural data has revealed a number of protein modules that are widespread and repeated throughout nature (22, 23). These protein modules facilitate the regulation of numerous proteins that vary dramatically in function and impact multiple cellular processes. Analysis of the human genome revealed a number of Ca\(^{2+}\) binding modules (24). The binding of Ca\(^{2+}\) to proteins has a variety of roles. These include enhancing protein stability (25, 26) and inducing conformational changes to facilitate secondary actions as seen with calmodulin (27–29) and other Ca\(^{2+}\) sensors (30). CBD1 forms a unique binding cluster that may be utilized by other Ca\(^{2+}\) sensor proteins.

We note sequence and structural similarities between the CBD1 domain and the larger family of C\(_2\) domains. C\(_2\) domains

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**FIGURE 1. Structural alignment of the crystal and NMR structures of CBD1.** The NMR structure (yellow) was superimposed onto the crystal structure (blue) with a root mean square deviation of 1.8 Å for 128 C\(_\alpha\) atoms. The Ca\(^{2+}\) ions are represented as spheres maintaining the same color code. The two Ca\(^{2+}\) ions from the NMR structure (yellow) seem to represent a positional average of the four Ca\(^{2+}\) ions seen in the crystal structure (blue).
are the second most abundant Ca\textsuperscript{2+} binding module present in nature (24). The majority of proteins with C\textsubscript{2} domains are involved in signal transduction or membrane trafficking (31). The two C\textsubscript{2} domains that are most extensively studied on a structural level are those of synaptotagmin (32, 33) and phospholipase C (34), both of which form an eight-stranded \(\beta\)-sandwich. The \(\beta\)-sandwich scaffold permits variable loops that are widely separated in the primary sequence to facilitate the binding of multiple Ca\textsuperscript{2+} ions in a cluster. Similar to CBD1, the binding sites are comprised primarily of aspartic acid residues forming polydentate interactions between two or three Ca\textsuperscript{2+} ions. Sequence alignments (Fig. 3) between CBD1 and C\textsubscript{2} domains show a number of similarities around the first acidic segment. However, the existence of a fourth Ca\textsuperscript{2+} site, as found in CBD1, would require additional acidic coordinating residues not seen in C\textsubscript{2} domain structures. As seen in the current struc-
ture, these additional residues are located toward the C terminus in the second acidic segment but there is no structural or sequence similarity for this region in C2 domains. Although functionally diverse, the CBD1 and the C2 domains share a common Ca2+ coordination scheme that may be general for Ca2+ sensing.

The crystal structure of CBD1 reveals a new Ca2+ binding motif consisting of four Ca2+ ions arranged in a tight cluster. This coordination scheme utilizes carboxylate moieties from aspartic and glutamic acid residues to form polydentate interactions with multiple Ca2+ ions. This unique cluster facilitates the reversible binding of Ca2+ in an environment where the concentration of free Ca2+ is kept low. Further biochemical and mutational analysis based on the crystal structure and structure determinations of other components of the cytosolic loop will facilitate our understanding of the sensory mechanism of NCX.

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