Comparing skeletal and cardiac calsequestrin structures and their calcium binding: a proposed mechanism for coupled calcium binding and protein polymerization

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Running Title: Structure and Function of cardiac Calsequestrin

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Summary

Calsequestrin, the major calcium storage protein of both cardiac and skeletal muscle, binds and releases large numbers of Ca\textsuperscript{2+} ions for each contraction and relaxation cycle. Here we show that two crystal structures for skeletal and cardiac calsequestrin are nearly superimposable, not only for their subunits, but also their front-to-front type dimers. Ca\textsuperscript{2+} binding curves were measured using atomic absorption spectroscopy. This method enables highly accurate measurements even for Ca\textsuperscript{2+} bound to polymerized protein. The binding curves for both skeletal and cardiac calsequestrin were complex, with binding increases that correlated with protein dimerization, tetramerization, and oligomerization. The Ca\textsuperscript{2+} binding capacities of skeletal and cardiac calsequestrin are directly compared for the first time, with \( \sim 80 \) Ca\textsuperscript{2+} ions bound per skeletal calsequestrin and \( \sim 60 \) Ca\textsuperscript{2+} ions per cardiac calsequestrin, as compared to net charges for these molecules of -80 and -69 respectively. Deleting the negatively charged and disordered C-terminal 27 amino acids of cardiac calsequestrin results in a 50% reduction of its calcium binding capacity and a loss of Ca\textsuperscript{2+}-dependent tetramer formation. Based on the crystal structures of rabbit skeletal muscle calsequestrin and canine cardiac calsequestrin, Ca\textsuperscript{2+} binding capacity data and previous light scattering data, a mechanism of Ca\textsuperscript{2+}-binding coupled with polymerization is proposed.
Calsequestrin (CSQ) binds and releases large quantities of Ca\(^{2+}\) through its high capacity (40~50 mol Ca\(^{2+}\) ion per molecule) and relatively low affinity interactions with Ca\(^{2+}\) (K\(_d\)=1 mM) (1). Due to this Ca\(^{2+}\) buffering capacity of CSQ in the luminal space, the concentration of free Ca\(^{2+}\) in the sarcoplasmic reticulum (SR) can be maintained below the inhibitory level of the Ca\(^{2+}\) pump (1 mM), and simultaneously the SR can maintain the ability to rapidly deliver a high capacity Ca\(^{2+}\) signal to the cytoplasm. Even though the luminal space is minuscule compared with the extracellular space, the high concentrations (~100 mg/ml) of CSQ make the SR an efficient storage compartment for Ca\(^{2+}\) (2).

CSQ is associated physically with the RyR protein by a nucleation event that involves CSQ binding to the basic luminal domains of triadin (3) or junctin (4). These two proteins interact with RyR in the junctional face region of the SR and this network of interacting proteins assures that high concentrations of Ca\(^{2+}\) are stored very near to the site of Ca\(^{2+}\) release. Ca\(^{2+}\) release from CSQ through the Ca\(^{2+}\) release channel is regulatory, but not limiting.

The Ca\(^{2+}\)-binding and dissociation mechanisms of CSQ are not yet clearly understood. Ca\(^{2+}\) binding sites in CSQ are supposed to be very different from those in the Ca\(^{2+}\) pump (SERCA), calmodulin (CaM) and troponinC (TnC). CSQ sites need to be made and broken, but not over the low cytosolic Ca\(^{2+}\) concentration range or with the same stoichiometry and precision as those formed and subsequently disrupted in the Ca\(^{2+}\) pump or those which are intrinsic to the EF hand structure (5). Therefore the high capacity and low affinity Ca\(^{2+}\) binding by CSQ is likely nonspecific, although the first few ions that bind at low Ca\(^{2+}\) concentrations may have some specificity. Instead of a distinct Ca\(^{2+}\)-binding site such as the EF-hand motif (6), pairs of acid residues have been proposed to bind Ca\(^{2+}\) with Ca\(^{2+}\) binding being driven to a significant degree by the entropy gain from liberation of many water molecules from the hydrated cations (7,8).
The crystal structure of rabbit skeletal CSQ (sCSQ) shows that this protein is made up of three domains, each with a thioredoxin fold, which is a five β-strand sandwiched by four α-helices (9). The individual domains of CSQ show very low sequence similarity with thioredoxins, but the locations of hydrophobic and hydrophilic residues can be overlapped after superimposing the secondary structural components of these two proteins. Each domain of CSQ has a hydrophobic core with acidic residues on the exterior, generating electronegative potential surfaces. Individual domains are connected by short sequences located interior to the domains themselves. These connecting loops and the secondary structural elements that fill the inter-domain space contain mostly acidic residues, making the overall center of the protein hydrophilic rather than hydrophobic. Therefore, cations are required to stabilize the acidic center of CSQ. Divalent cations, which can provide cross bridging, would be expected to be more effective in this regard than monovalent cations.

CSQ forms regular, highly elongated structures that appear as crystalline arrays in the lumen of the SR (10-12). We previously showed that the crystal lattice of the rabbit sCSQ contains a linear polymer joined by two distinct dimerization contacts, the front-to-front and back-to-back interfaces (9). These linear polymers provide a reasonable basis for both the crystalline arrays seen in vivo and the needle-shaped crystals often grown in vitro in the presence of Ca\(^{2+}\). We also reported that oligomerization is Ca\(^{2+}/K^+\) dependent and proposed several ways this dynamic polymerization may have biological significance (13). According to our proposal, the front-to-front and back-to-back contacts between the CSQ subunits permit formation of a Ca\(^{2+}\) dependent linear polymer that is inhibited as the concentration of monovalent ion, K\(^+\), increases. Ca\(^{2+}\) largely fills the electronegative pockets formed in these two contacts cross-bridging the subunit, which monovalent cations cannot do. This dynamic formation of CSQ
polymer can provide a highly charged surface onto which Ca\(^{2+}\) ion is adsorbed. The attractive forces exerted by such an extended surface would have a longer range than those from an isolated molecule and a sparingly soluble ion such as Ca\(^{2+}\) would tend to spread over the surface of the CSQ polymer forming a readily exchangeable film (14). Thus Ca\(^{2+}\) diffusion from CSQ to the Ca\(^{2+}\) release channel is likely to involve surface diffusion, a more rapid process than diffusion through liquid (14,15).

In mammals, two different CSQ genes, cardiac and skeletal CSQ (cCSQ and sCSQ), have been identified, which are localized in two different cellular environments that obviously require different types of calcium regulation. Apparently, the rabbit cCSQ shows a higher sequence identity with canine cCSQ (92.1 %) than with its own sCSQ (66.5 %) clearly reflecting its functional divergence. Among the ~360 residues in either isoform of CSQs from various species, more than 110 residues are either Asp or Glu, making CSQ one of the most acidic self-folding proteins in existence. For example, rabbit sCSQ has a net charge of ~80, while canine cCSQ has a net charge of ~69 at neutral pH. Despite this small overall difference in net charge, sCSQ is known to bind about twice the amount of Ca\(^{2+}\) compared to the cardiac molecule (16). In general, cCSQs have more negatively charged amino acids in its C-terminus than sCSQs, except for those from frog. For example, the C-terminal tail of the canine cCSQ has about twice as many negatively charged amino acids as does the rabbit sCSQ. Therefore, comparing the atomic structures of the two isoforms of CSQ is likely to be very informative.

CSQ has medical importance as well. Overexpression of CSQ impairs Ca\(^{2+}\) signaling, leading to severe cardiac hypertrophy (17,18), sporadic Ca\(^{2+}\) sparks (19), depressed contractility in the heart coupled with an induction of a fetal gene expression program (20), and premature death (21-23). Recently, a missense mutation in a highly conserved region of cCSQ, D306H, was
found to be the cause of the autosomal recessive form of Catecholamine-induced polymorphic ventricular tachycardia (CPVT) (24,25). Additionally, three CSQ-related CPVT families were discovered: evidently, CSQ mutations are more common than previously thought and can produce a severe form of CPVT (26). The atomic resolution structure of cCSQ can help us to understand the functional perturbations of these hereditary mutations.

In this contribution, we have completed the X-ray crystallographic analysis of cCSQ in order to perform comparative studies on the cardiac and skeletal isoforms of CSQs. As described below, the three-dimensional X-ray crystal structures of canine cCSQ was refined to 2.6 Å resolution, the structures of which now allow incisive comparative studies to be made between the cardiac and skeletal isoforms of the CSQs. We also have established accurate Ca\(^{2+}\) binding curves of both CSQs using atomic absorption spectroscopy and correlated these with their Ca\(^{2+}\)-dependent oligomerization patterns. These structural and Ca\(^{2+}\)-binding comparisons yield a mechanism for Ca\(^{2+}\)-associated CSQ polymerization that provides important new insights for understanding the structure-function relationships for this important protein.

**EXPERIMENTAL PROCEDURES**

*Plasmid constructs* – Based on our previous experiences crystallizing sCSQ and cCSQ, the last 27 residues (\[C27\]), which are all negatively charged, were removed in order to make the back-to-back contact less sensitive to divalent ions. The expression plasmid, pTYB1-cCSQ [C27] was constructed using a polymerase chain reaction (PCR) strategy. Specific regions of canine cardiac CSQ were amplified by PCR using the cDNA clone as a template. A sense primer CAL5 (5’CTGTCAACATATGGAAGAGGGCTCAACTTCCCCA3’) that contains an NdeI site and a start codon, and an antisense primer CAL3-27Im (5’CTAATGGCTCTTCGACCCGTCATCCCCCTTT3’)
that contains a SapI site were used for C27 cCSQ (amino acid 1-364). PCR amplified products were digested with NdeI/SapI, and ligated into the expression vector pTYB1 (New England Biolabs). The sequence of the construct was confirmed by DNA sequencing.

*Expression and purification* - The *E. coli* strain ER2566 transformed with pTYB1-cCSQs C27 was grown at 37 °C in LB containing 100 ug/ml ampicillin to an OD₆₀₀nm of 0.6, then induced with 1 mM IPTG for 6 h at RT. The C27 cCSQ mutant was purified through a chitin affinity column (New England Biolabs) followed by an anion exchange column. Briefly, cells were pelleted and suspended in sonication buffer (10 mM Tris-HCl (pH 9.0), 500 mM NaCl, 1 mM EDTA, 1 % Triton-X100), sonicated on ice and centrifuged for 15 min at 20,000 g. Supernatant was applied to a chitin column and washed with a ten-column volume of sonication buffer without Triton-X100. On-column cleavage was performed by incubating the chitin resin with cleavage buffer containing 10 mM Tris-HCl (pH 9.0), 1 M NaCl, 1 mM EDTA, and 90 mM DTT. After overnight incubation, the cleaved CSQ protein was eluted with elution buffer (cleavage buffer without DTT). The elution was loaded onto an Uno-Q12 (BioRad) column after concentrating and substituting the buffer to 20 mM Tris-HCl (pH7.5), 20 mM NaCl with an ultrafiltration unit (Amicon). The column was then washed with a linear gradient from 100 mM to 1 M NaCl. The mutant CSQs eluted at approximately 400 mM to 600 mM NaCl were concentrated with Centriplus-10 (Amicon). Purified protein was dialysed against 300 mM KCl with 10 mM Tris-HCl (pH 7.5) and 5 mM DTT.

*Crystallization and structure determination of cCSQ*: Crystals of C27 cCSQ were grown at room temperature by the vapor diffusion method in 5mg/ml protein, 15 % PEG 400, 50 mM Na
citrate, 0.25 % n-Dodecyl-bD-maltoside (DM) and 0.1 M Tris-HCl at pH 8.5. Drops of ~ 4 uL in size were equilibrated against a 0.5ml reservoir solution containing 30 % PEG 400, 100 mM Na citrate and 0.2 M Tris-HCl at pH 8.5.

Typically diffraction quality crystals were fully grown 3 days after setup and stored in a 4°C cold room until they are used. Crystals were briefly transferred to reservoir solution before freezing in a cryo-stream at a temperature of –170°C. The $\square$C27 cCSQ crystal belongs to the tetragonal space group I4 with two molecules in an asymmetric unit (a=b= 145.188 Å, c=99.82 Å, $\square$=g= 90°). A data set of 2.6 Å was collected on an ADSC Q315 CCD detector at the Advanced Light Source (ALS) beam line 5.0.2. The diffraction data was processed with HKL2000.

The structure of canine cCSQ was solved by molecular replacement methods using our previous rabbit sCSQ coordinates (PDB: 1A8Y) and the software package AMoRe (27). The rigid-body refinement of the initial position was carried out using 15.0 Å to 3.0 Å resolution data and produced an R-value of 29 %. After several cycles of positional refinement, temperature factor refinement, and simulated annealing omit map, we were able to fit most of the residues to the electron density. The backbones of the canine cCSQ did not change significantly from the starting coordinates of rabbit sCSQ. As in the sCSQ structure, the electron density corresponding to the C-terminal residues starting from the residue 351, was not visible from the early stage of refinement.

The R-factor for the final models containing 5731 non-hydrogen atoms for two cCSQ and solvents is 19.3 % ($R_{\text{free}}$=24.2 %). The reflection numbers above 2$\theta$ level were 25,014 (80 % completeness) between 10.0 Å and 2.6 Å resolution.
The rms deviations (from standard geometry) of the cCSQ are 0.018 Å for bonds and 3.54° for angles. The coordinate of the canine cCSQ structure has been deposited in the Protein Data Bank.

*Equilibrium dialysis:* Wild-type sCSQ, cCSQ, and a mutant cCSQ (D27C) at a protein concentration of 1-3 mg/ml were dialyzed first against distilled water followed by buffer containing 10 mM Tris (pH 7.5) with 300 mM KCl and 2 mM NaN₃. Dialysis was performed for one week in the cold room with ten reservoir changes. Upon completion of the buffer exchange, the dialysis bag was removed and the protein solution was transferred to the half cell of a modified horizontal-diffusion chamber.

One and a half ml of protein solution was equilibrated against the same volume of various concentrations of calcium chloride solution (0.1 to 40 mM) across the 12 kD MWCO dialysis membrane. Equilibrium was achieved by gentle tilt shaking for 36 hours at room temperature.

Both the protein and ligand compartments were analyzed for Ca²⁺ concentration using Atomic Absorption Spectrophotometer (Shimadzu AA-6200) at the absorption wavelength of 422.7 nm. Unlike alterative approaches for measuring Ca²⁺ ion concentrations, the complete vaporization of the sample by this method means that Ca²⁺ binding to protein, even to polymerized protein, should present no significant experimental difficulties. Protein concentration of each dialysis cell was measured again by Bradford protein assay after equilibrium dialysis. Fractional occupancy (Y = [bound Ca]/[total CSQ]) was calculated by a difference in Ca²⁺ concentration of both compartments. Even though the ligand in this experiment is an electrolyte, Donnan effect was not considered in treating the dialysis data, since
the concentration of another electrolyte (KCl, 300 mM) was already high enough in both compartments.

RESULTS

Overall structures □ The asymmetric unit of crystalline canine □C27 cCSQ has two independent molecules, virtually superimposable with a root-mean-square deviation (rmsd) of 0.87 Å between backbone atoms of two molecules. Consistent with this crystal lattice packing, our previous static light scattering experiments with □C27 cCSQ solutions gave an apparent molecular weight corresponding to a dimer (13).

The overall 3-D structure of canine cCSQ is very similar to that of rabbit sCSQ with an rmsd of 1.4 Å, reflecting the high level of sequence identity (66 %) between two CSQ isomers from two species (Fig. 1). As observed in the structure of sCSQ, the overall structure of cCSQ is composed of three thioredoxin-like domains (Fig. 1B). Individual thioredoxin domains have a five-stranded □-sheet sandwiched by four □-helices, composed of ~100 residues (residues 12-124, 125-228, 229-352). As shown in Fig. 2, the surface electric potential of cCSQ is less negative than that of sCSQ due to the lower net charge of every domain. The net charges of the domain I, II and III of the sCSQ is –21, -13 and –32 respectively, and –14 at its disordered C-terminal tail. On the other hand, for cCSQ these net charges are reduced to –8, -11, -22 in its three domains, but there are twice as many negatively charged residues at its C-terminal tail (Fig.3). All three domains of both cCSQ and sCSQ show stable hydrophobic cores with high aromatic amino acid composition, perhaps to balance the destabilizing effects of the very high negative net charge of this acidic protein. Statistical studies on peptide sequences suggest that
charge imbalance is an important factor favoring the unfolded state and that high aromatic content is an important factor favoring the folded state (28). Most of the aromatic residues, especially in domains II and III are highly conserved between cCSQ and sCSQ (Fig. 3).

Two N-terminal residues and twelve C-terminal residues starting from Asn351 are disordered and the corresponding electron densities for these regions are not visible, which is similar to the crystal structure of sCSQ. Therefore it is very likely that the C-terminal 38 amino acids of the wild-type cCSQ including the 27 truncated residues, which are composed of large numbers of aspartic acids and glutamic acids, are all disordered. The reported phosphorylation site of Thr353 is located at the beginning of the stretch of structural disorder, as in most cases of the known phosphorylation sites of other proteins (29).

In general, the loop areas connecting the helices and strands show elevated temperature factors and in many cases have different conformations in the two molecules of the asymmetric unit and also in cCSQ and sCSQ. In particular, the loop containing residues 328-333, which is disordered in the case of sCSQ, becomes ordered in cCSQ. The other four areas of partially high temperature factors are residues 38-47, 153-158, 201-204 and 257-261. The residue Asp306, which is known to be the cause of the autosomal recessive form of CPVT, is located near the front-to-front dimer interface in a solvent exposed loop (Fig. 4A).

**Dimer interface**

The asymmetric unit of ΔC27 cCSQ crystal lattice is a front-to-front dimer (Fig. 4A). This cCSQ dimer has an rmsd value of 1.4 Å with the same type of front-to-front dimers observed in sCSQ. As seen in the front-to-front interface of sCSQ, the arm exchange of the N-terminal 13 amino acids between the subunits are important in this electronegative interface of
the cCSQ dimer (Fig. 4B). Previously, we showed that deletion of the first 13 residues (DN13 cCSQ) leads to a high molecular weight aggregate probably due to random interactions among CSQ molecules (13). All but three of these 13 residues are identical between canine cCSQ and skeletal sCSQ (Fig. 3).

The back-to-back type molecular contact and consequent linear polymer observed in the crystal lattice of sCSQ are not observed in this C-terminal truncated cCSQ. This enforces the importance of the two kinds of dimer interfaces, back-to-back and front-to-front, in the polymeric behavior of this protein. Previously, we determined the cation-dependency of the apparent molecular weight of [C27 cCSQ using size exclusion chromatography equipped with a static light scattering instruments (13). This truncation mutant shows the same Ca$^{2+}$-dependent dimerization pattern following an increase in calcium ions, in spite of its 27 fewer acidic residues in its tail, but stays in the dimer state and does not show any further Ca$^{2+}$-dependent polymerization as observed in the case of wild-type cCSQ. Since this C-terminal truncation mutant has an intact N-terminus, the dimers seen on these mutants were predicted to be a front-to-front type of dimer, which is now confirmed by the crystal structure of [C27 cCSQ.

$Ca^{2+}$ binding property

In order to study the potential for coupling between Ca$^{2+}$ binding and the polymerization of CSQ, Ca$^{2+}$ binding capacities of wild-type cCSQ, [C27 cCSQ and wild-type sCSQ were analyzed by atomic absorption spectroscopy. Previously, the maximum capacity of canine cCSQ for Ca$^{2+}$ was reported as ~18 mol of Ca$^{2+}$ per molecule which is about half the capacity of rabbit sCSQ (2,16,30-34), but there has never been any report measuring Ca$^{2+}$ binding capacities of both cCSQ and sCSQ using the same method and the same binding conditions.
In our study, the buffer condition for measuring Ca\(^{2+}\) binding was chosen based on circular dichroism (CD), fluorescence spectroscopies (unpublished data) and light scattering experiments (13). At 300 mM KCl and 10 mM Tris-HCl (pH7.5), both CD and fluorescence signals, which reflect acquisition of secondary and tertiary structure respectively, reach a plateau. The molecular weight estimate from the light scattering data for CSQ at this condition is 44 (±1) kD, corresponding closely to the molecular weight of one CSQ molecule.

As shown in Fig. 5A, sCSQ shows a higher Ca\(^{2+}\) binding capacity than cCSQ along the entire range of Ca\(^{2+}\) concentration we have tested and the \(\square\)C27 cCSQ shows a significantly reduced binding capacity, approximately 50 % of its wild-type. In both wild-type sCSQ and cCSQ, there are several changes of curvature that do not fit into a simple binding model, which is clearer after converting the binding data to a Scatchard-type plot (Fig.5B). Therefore, the binding characters of both CSQs are not even throughout the Ca\(^{2+}\) concentration, instead they vary or make transitions following a change of Ca\(^{2+}\) concentration. Both cCSQ and sCSQ show sharp increases in their Ca\(^{2+}\) binding capacities between 0 and 1 mM Ca\(^{2+}\) concentration (Fig. 5A) and reach approximate values of 20 and 34 bound Ca\(^{2+}\) respectively at 1 mM. At 5 mM CaCl\(_2\) concentration, Ca\(^{2+}\) binding capacities of cCSQ and sCSQ reach the level of 34 and 45 Ca\(^{2+}\). In the equilibrium dialysis set of \(\square\)C27 cCSQ beyond the Ca\(^{2+}\) concentration of 5 mM, precipitation of this mutant protein was clearly observed, but this insoluble Ca\(^{2+}\)-protein complex was readily dissolved by dilution with 300 mM KCl buffer. This precipitation was not apparent in either wild-type sCSQ or cCSQ at the same high Ca\(^{2+}\) concentration. After 5 mM, both cCSQ and sCSQ plateau at about 36 and 50 moles of Ca\(^{2+}\) per molecule respectively, but contrary to a previous report (16), both show a sudden increase in the range of 7-10 mM Ca\(^{2+}\) and then rises slowly and steadily after that. In the same range of Ca\(^{2+}\) concentration, the \(\square\)C27 cCSQ rather
slowly increases its $\text{Ca}^{2+}$ binding capacity forming substantial amount of precipitates. We showed that neither a mutant with reduced C-terminal charges ($\square$C27) nor a mutant with its C-terminal tail completely removed ($\square$C38) is able to form $\text{Ca}^{2+}$-dependent-tetramer (13). Therefore we attribute this precipitation to $\square$C27 aggregating randomly instead of forming a linear polymer via tetramer formation in response to high $\text{Ca}^{2+}$ concentration above $\sim$5 mM.

**DISCUSSION**

Canine cCSQ and rabbit sCSQ have a very similar structure and the surface of cCSQ is less electronegative than that of sCSQ. We also found that the C-terminal tail of cCSQ, which is longer than sCSQ in general and has more negatively charged amino acids, is disordered as observed in the structure of sCSQ. Truncation of the C-terminal 27 amino acids ($\square$C27) still maintains the identical front-to-front interaction as observed in sCSQ through N-terminal arm exchange, but the back-to-back interface is not present. On the other hand, complete removal of the C-terminal tail, $\square$C38, dimerizes cCSQ at lower ionic strength in a $\text{Ca}^{2+}$-independent manner. That is, at the KCl concentration of 300-500 mM (without $\text{Ca}^{2+}$), $\square$C38 cCSQ already has a substantial dimer population (13). Based on our crystal structure of $\square$C27 cCSQ and our previous light scattering data we conclude, therefore, that the front-to-front dimer forms before the back-to-back dimer and that the dimer observed in the solution of $\square$C38 cCSQ might be a non-specific back-to-back dimer. The negatively charged C-terminal tail in wild-type cCSQ, therefore, inhibits or slows down the formation of the non-specific back-to-back dimerization. By the same logic, eleven disordered amino acids at the C-terminal tail of $\square$C27 cCSQ may not be long enough to induce (or stabilize) the back-to-back dimer interface even at high $\text{Ca}^{2+}$ concentration,
but is still long enough to provide enough charge repulsion to prevent the formation of the Ca\textsuperscript{2+}-independent (or nonspecific) back-to-back dimer.

Fig. 6 explains the possible coupling mechanisms between high capacity Ca\textsuperscript{2+} binding and the polymerization of CSQ. From our previous light scattering data (13), the dimerization of the CSQ molecule occurs in the Ca\textsuperscript{2+} concentration range of 0-1 mM and at 3 mM CaCl\textsubscript{2}, there is already a detectable tetramer population of CSQ. The part of the sharp increase in Ca\textsuperscript{2+} binding observed at the 0-1 mM concentration therefore is due to the formation of the front-to-front dimer. The next increase observed at 3-5 mM Ca\textsuperscript{2+} is probably due to the dimer-tetramer transition capturing substantial amounts of Ca\textsuperscript{2+} ion in the back-to-back interface in addition to the tetramer surface (Fig. 6). It appears that slightly higher concentration of Ca\textsuperscript{2+} ion is required for sCSQ to approach each other to form a dimer compared to the cCSQ (inner box in Fig. 6). Consequently dimers of sCSQ can sequester more Ca\textsuperscript{2+} ions than dimers of cCSQ, even though the single molecule state (Ca\textsuperscript{2+} concentration less than ~0.4 mM) of sCSQ can bind only slightly more Ca\textsuperscript{2+} ions than cCSQ (inner box in Fig. 6).

At 0-1 mM Ca\textsuperscript{2+} concentration [\textsc{c27} cCSQ already shows reduced Ca\textsuperscript{2+} binding, even though it undergoes the same transition to the front-to-front dimer as wild-type cCSQ (Fig.5A). This dimer of [\textsc{c27} cCSQ reaches the maximum binding capacity of ~25 Ca\textsuperscript{2+} ions per molecule, which matches well with the net charge of [\textsc{c27} cCSQ, –49. The sCSQ dimer can bind ~40 ions per molecule (first plateau of the slope) which is also consistent with the total net charge of the sCSQ molecules, -80. On the contrary, the maximum number of bound Ca\textsuperscript{2+} per wild-type cCSQ dimer is ~30, which is less than its charge of -69.

Both cCSQ and sCSQ bind many more Ca\textsuperscript{2+} ions than predicted by their net negative charges as they form their polymeric states. At the highest Ca\textsuperscript{2+} concentrations tested, each sCSQ
binds an excess of ~ 40 Ca$^{2+}$ ions and each cCSQ binds an excess of ~ 35 ions. The principle of electroneutrality means that polymer formation involves the binding of about 80 negative charges for sCSQ and about 69 negative charges for cCSQ. Thus, Ca$^{2+}$ regulation within the SR by CSQ polymerization and depolymerization must involve substantial anion binding. One possibility is that the transmembrane flux of a particular anion plays an additional, currently unrecognized regulatory role in binding and release of Ca$^{2+}$ via CSQ polymerization and depolymerization. If so, studies of the anion dependence of Ca$^{2+}$-associated CSQ polymerization could reveal important new insights regarding Ca$^{2+}$ regulation within the sarcoplasmic and endoplasmic reticula.

In summary, the Ca$^{2+}$-dependent and sequential formation of two different types of dimer interface, front-to-front and back-to-back, through its N-terminal arm and its acidic C-terminal tail is a key feature in the polymerization of both sCSQ and cCSQ. This dynamic polymerization can be directly linked to the high-capacity, low-affinity Ca$^{2+}$ binding of CSQ. Our studies on sequence conservation across both cCSQ and sCSQ molecules indicates that the residues involved in those two interfaces are the most highly conserved residues in the entire structure. This situation is reminiscent of the higher conservation of active-site residues of other proteins, and thus strongly supports our hypothesis that these dimer interfaces are the functional contacts involved in the coupled CSQ polymerization and low affinity Ca$^{2+}$ binding. This polymerization of CSQ is promoted by Ca$^{2+}$ and inhibited by K$^+$, which is explained well in terms of trafficking K$^+$ and Ca$^{2+}$ in SR because of the high concentration of CSQ (100 mg/ml) and physiologically varying concentrations (opposite direction) of those two ions. The likely added importance of anion binding in this process provides a new insight that requires further investigation. The use of Ca$^{2+}$ as a cross-linker rather than as a tightly-bound form free of H$_2$O, also speeds
dissociation(14). In our crystal structures, sCSQ shows lower electric potential on its surface than cCSQ. Therefore, growing sCSQ polymers provide more charged surface than cCSQ onto which more Ca\textsuperscript{2+} ions can be adsorbed.

Our data strongly imply the Ca\textsuperscript{2+}-bound state forms linear structures that can be assembled and destroyed dynamically following the flux of K\textsuperscript{+} and Ca\textsuperscript{2+}. The strong coupling between Ca\textsuperscript{2+} binding and protein oligomerization provide a distinctive mechanism for facilitated Ca dissociation and for making diffusion in CSQ even more rapid than would be the case for the free 3D diffusion of Ca\textsuperscript{2+} ions.

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References

Figure legend

**Fig. 1.** A) Stereo view of a schematic diagram showing the distribution of structural elements of rabbit cardiac calsequestrin. Three domains are indicated by DI, DII and DIII. B) Three domains of canine cCSQ C) Superimposed views of Cθ positions of cardiac and skeletal (1A8Y) calsequestrin. Cardiac and skeletal isomers are depicted in green and orange respectively.

**Fig. 2.** The molecular surface of A) canine cardiac calsequestrin and B) rabbit skeletal calsequestrin (1A8Y). Molecular surface shows the electrostatic potential from $-45.24 \ k_B T$ to $35.44 \ k_B T$ (where $k_B$ is the Boltzman constant and $T$ is the absolute temperature). Red is negative, blue is positive, white is uncharged or hydrophobic. Overall, both isomers show extreme negative values of their surface electropotential. The electrostatic potential surface was calculated by the program GRASP (35).

**Fig. 3.** Comparison of amino acids sequences for rabbit skeletal and canine cardiac calsequestrins. The individual domains, I, II and III, are marked with different colors. Non-identical residues are high lightened by bold characters.

**Fig. 4.** A) Ribbon diagram showing the distribution of structural elements of canine cardiac calsequestrin and the representation of the front-to-front interaction. The extended N-terminal residues of one subunit become inserted in the other subunit. The Asp306, which is related to the CPVT, is indicated with a ball and stick model. B) Key residues in this front-to-front interaction are indicated. The equivalent sets of residues exist in pseudo two-fold manner. This figure was prepared using WebLab™ ViewerLite 3.2.

**Fig. 5.** Ca$^{2+}$ binding properties of calsequestrins. A) Fractional occupancy ($Y=\text{[bound Ca}^{2+}\text{]/[total CSQ]}$) was plotted against unbound ligand concentration. Each point represents the average value obtained from three to four equivalent repeats of the experiment. X-error bar was not shown for the ease of view. The inset shows a magnified view on the Ca$^{2+}$ range within 0-0.7 mM. B) Scatchard-type plot of the same data that clearly shows that the dissociation constant (slope) varies according to the degree of ligand binding.

**Fig. 6.** Coupling between Ca$^{2+}$ binding capacity and polymerization of CSQs. Red for sCSQ, and Blue for cCSQ (M: single molecule, D: Dimeric, T: Tetrameric, P: Polymeric).
Fig. 1

A

B

C
Fig. 2
### Fig. 3

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Fig. 6
Comparing skeletal and cardiac calsequestrin structures and their calcium binding: a proposed mechanism for coupled calcium binding and protein polymerization
HaJeung Park, Il Yeong Park, EunJung Kim, Buhyun Youn, Kelly Fields, A. Keith Dunker and ChulHee Kang

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