Polymerization of Calsequestrin

IMPLICATIONS FOR Ca\(^{2+}\) REGULATION* [S]

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Two distinct dimerization contacts in calsequestrin crystals suggested a mechanism for Ca\(^{2+}\) regulation resulting from the occurrence of coupled Ca\(^{2+}\) binding and protein polymerization. Ca\(^{2+}\)-induced formation of one contact was proposed to lead to dimerization followed by Ca\(^{2+}\)-induced formation of the second contact to bring about polymerization (1). To test this mechanism, we compared canine cardiac calsequestrin and four truncation mutants with regard to their folding properties, structures, and Ca\(^{2+}\)-induced polymerization. The wild-type calsequestrin and truncation mutants exhibited similar K\(^{+}\)-induced folding and endpoint structures as indicated by intrinsic fluorescence and circular dichroism, respectively, whereas the polymerization tendencies of the wild-type calsequestrin differed markedly from the polymerization tendencies of the truncation mutants. Static laser light scattering and 3,3'-dithiobis sulfoacryimidyl-propionate cross-linking indicated that wild-type protein exhibited an initial Ca\(^{2+}\)-induced dimerization, followed by additional oligomerization as the Ca\(^{2+}\) concentration was raised or as the K\(^{+}\) concentration was lowered. None of the truncation mutants exhibited clear stepwise oligomerization that depended on increasing Ca\(^{2+}\) concentration. Comparison of the three-dimensional structure of rabbit skeletal calsequestrin with a homology model of canine cardiac calsequestrin from the point of view of our coupled Ca\(^{2+}\) binding and polymerization mechanism leads to a possible explanation for the 2-fold reduced Ca\(^{2+}\) binding capacity of cardiac calsequestrin despite very similar overall net negative charge for the two proteins.

The sarcoplasmic reticulum (SR), which is a specialized elaboration of the endoplasmic reticulum, is adapted to supply or remove Ca\(^{2+}\) rapidly to or from the myofilaments of atrial muscle. The total concentration of Ca\(^{2+}\) in the SR is as high as 50 mM (2), but a large portion of this Ca\(^{2+}\) is bound to a specific protein, calsequestrin.

Calsequestrin binds and releases large quantities of Ca\(^{2+}\) rapidly through its high capacity and relatively low affinity interactions with Ca\(^{2+}\) (3). Due to this luminal buffering system, the concentration of free Ca\(^{2+}\) in the 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 the Ca\(^{2+}\) signal to the cytoplasm. Even though the luminal space is minuscule compared with the extracellular space, the high concentrations of calsequestrin (100 mg/ml) make the SR an efficient storage compartment for Ca\(^{2+}\) (4).

Calsequestrin is associated physically with the ryanodine receptor (RyR) by a nucleation event that involves calsequestrin binding to the basic luminal domains of triadin (5) or junctin (6). 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 (1, 7–9).

The crystal structure of rabbit skeletal calsequestrin shows that this protein is made up of three domains, each with a thioether domain, which is a five \(\beta\)-strand sandwich by four \(\alpha\)-helices (1). Each domain of calsequestrin 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 interdomain 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 calsequestrin. Divalent cations, which can provide cross-bridging, would be expected to be more effective in this regard than monovalent cations. This cationic stabilization of the acidic core likely provides at least part of the explanation for observations indicating that calsequestrin is more extended and more susceptible to protease digestion at low salt concentrations and then collapses as the ionic strength is raised (1, 3, 10, 11).

So far two isoforms of calsequestrin have been identified in mammalian tissues, cardiac and skeletal. Among the -360 residues in either isoform of calsequestrins from various species, more than 110 residues are either Asp or Glu, making calsequestrin one of the most acidic self-folding proteins in existence. Despite the very similar net negative charge for the two isoforms, cardiac calsequestrin appears to bind only about half as much Ca\(^{2+}\) as does the skeletal protein (12).

Previous studies (13) demonstrated that high capacity Ca\(^{2+}\) binding by calsequestrin is established by the formation of Ca\(^{2+}\)/protein complexes at relatively high calsequestrin and Ca\(^{2+}\) concentrations. Under such conditions, two-thirds of the total bound Ca\(^{2+}\) was associated with Ca\(^{2+}\)/calsequestrin aggregates, while one-third was associated with the soluble form of calsequestrin. Even though weak cooperativity of Ca\(^{2+}\) bind-
C-terminal 38-amino acid truncation, respectively. N-terminal 13-amino acid truncation, C-terminal 27-amino acid truncation, and C38 each represents N-terminal two-amino acid truncation, N-terminal arm and C-terminal DE-rich tail. N-terminal arm and C-terminal DE-rich tail.

Identifying the disordered tails at the C termini (14), strong Ca²⁺ binding cooperativity accompanies the polymerization of calsequestrin into the insoluble forms (13).

This Ca²⁺-induced precipitation leads to fibrils if induced rapidly or to needle-shaped crystals if induced slowly (15–17). Likewise, electron microscopy reveals the presence of electron-dense fibrous arrays in SR microsomes at the junctional membranes, and these arrays are believed to be calsequestrin in its calcium-bound form (15, 18–21). Cross-linking SR luminal proteins strongly suggest that most of the calsequestrin in the SR microsomes is involved in direct intermolecular calsequestrin/calsequestrin contacts (19, 20).

The Ca²⁺/calsequestrin aggregates are easily dissociated by K⁺ (12, 22–24). pH changes may also be an important regulator of aggregation in calsequestrin (25, 26). However, the mechanistic dependencies among Ca²⁺, K⁺, H⁺, and calsequestrin are poorly understood, and the role of ion-induced structural changes in calsequestrin remains to be elucidated.

The association of calsequestrin monomers to form macro-molecular precipitates has a character similar to condensation or crystallization, which provides an avenue for us to determine whether the calsequestrin contacts in the crystal lattice are the same as the contacts in SR membrane preparations. In the crystal, two different dimerization contacts are observed: front-to-front and back-to-back. These form the basis of a continuous, linear polymer that contains the major stabilizing forces in the crystal lattice. Not only does the polymer in the crystal lattice have the linear morphology inferred for the physiologically relevant aggregation, but also the two contacts that stabilize this polymer have structural details that lend themselves to control Ca²⁺ binding. That is, both of the dimerization interfaces sequester large numbers of acidic residues and, in addition, the back-to-back interface has a nearby polyanionic, disordered tail. Furthermore, these dimerization interfaces do not look like typical crystal contacts because large surface areas are buried in the formation of intricate interactions. Given the intricacy, the sequestration of so many negative charges, and the nearby polyanionic tail, we proposed that these interfaces provide the basis for the physiological mechanism of coupling Ca²⁺ binding with polymerization and Ca²⁺ release with depolymerization (1, 8, 9).

Although the front-to-front and back-to-back contacts in our calsequestrin crystals would appear to be favored by Ca²⁺ binding, attempts to directly visualize Ca²⁺ bound within these interfaces have failed for technical reasons. Addition of Ca²⁺ during crystallization leads to instant formation of numerous needles (15–17) that have so far not been adequate for structure determination. Therefore, we turned to alternative approaches to test our hypothesis. If the residues involved in the front-to-front and back-to-back interfaces are indeed involved in coupling Ca²⁺ binding with calsequestrin polymerization, then mutants lacking the key residues should show altered tendencies for calcium-induced aggregation. Thus, we made a series of truncation mutants of dog cardiac calsequestrin and compared their properties when subjected to different Ca²⁺ levels.

**MATERIALS AND METHODS**

**Plasmid Constructs**—Expression plasmids, pET24b-cSQ and pTYB1-cSQ were constructed using PCR strategy. Specific regions of

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Rabbit cSQ</th>
<th>Canine cSQ</th>
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<tbody>
<tr>
<td>Asp</td>
<td>51</td>
<td>58</td>
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<tr>
<td>Glu</td>
<td>59</td>
<td>51</td>
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<td>Lys</td>
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<td>Arg</td>
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<td>9</td>
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<td>His</td>
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<td>5</td>
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<tr>
<td>Total net charge</td>
<td>-75</td>
<td>-64</td>
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**TABLE I**

Total numbers of charged amino acids appear on the sequence of rabbit skeletal calsequestrin and canine cardiac calsequestrin
canine cardiac calsequestrin were amplified by PCR using the cDNA clone as a template. A sense primer CAL5 (5'-CTGTACACATATGGGAGGGGGCTCAACCTTCCCCCA-3') that contains an Ndel site and a start codon, and an antisense primer CAL3 (5'-CTGATGGCTCTTGCCGCAACCTTTCACTGACACTGTCACA-3') which contains a SalI site followed by two stop codons were used for wild-type calsequestrin construct. A sense primer CAL5 (5'-CTGTACACATATGGGAGGGGGCTCAACCTTCCCCCA-3') and an antisense primer CAL3 (5'-CTGATGGCTCTTGCCGCAACCTTTCACTGACACTGTCACA-3') and a sense primer CAL5 (5'-CTGATGGCTCTTGCCGCAACCTTTCACTGACACTGTCACA-3') and CAL3 (5'-ACGTGCAACATATGGGTACGTTACTCACTGAGATCCA-3') and CAL3 (5'-ACGTGCAACATATGGGTACGTTACTCACTGAGATCCA-3') were used for ∆C27 (amino acids 3-391) and ∆N13 (amino acids 14-391), respectively. CAL5 and an antisense primer CAL3 (5'-CTAATGGCTCTTGCCGCAACCTTTCACTGACACTGTCACA-3'), and CAL5 and an antisense primer CAL3 (5'-ACGTGCAACATATGGGTACGTTACTCACTGAGATCCA-3') were used for ∆C27 (amino acids 1-364) and ∆C38 (amino acids 1-353), respectively (Figs. 1 and 2A). Ndel site for all sense primers and Sspl site for all antisense primers were introduced to make truncation mutants. PCR-amplified products were digested with Ndel/SstI or Ndel/Sspl and ligated into the expression vector pET24b (Novagen) for the wild-type calsequestrin and pTYB1 (New England Biolabs) for the truncation mutants. The sequence of each construct was confirmed by DNA sequencing.

Expression and Purification—For the wild-type calsequestrin, the Escherichia coli strain BL21 (DE3) transformed with pET24b-cSsq was grown at 37 °C in LB containing 50 µg/ml kanamycin to an A600nm of 0.6 and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h. The expressed wild-type calsequestrin was purified as previously described (11).

For the truncation mutants, the E. coli strain ER2566 transformed with pTYB1-cSsq were grown at 37 °C in LB containing 100 mg/ml ampicillin to an A600nm of 0.6 and then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 6 h at room temperature. The truncation mutants were 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 10-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 mM NaCl, 1 mM EDTA, and 90 mM DTT. After overnight incubation, the cleaved calsequestrin proteins were 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 (pH 7.5), 20 mM NaCl with an ultrafiltration unit (Amicon). The column was then washed with a linear gradient from 100 mM to 1 mM NaCl. The mutant calsequestrins eluted at ~400–600 mM NaCl were concentrated with Centriplus-10 (Amicon).

Purified wild-type and mutant calsequestrins were dialyzed against 300 mM KCl with 5 mM Tris-HCl (pH 7.5). The salt and buffer concentration was gradually reduced in a stepwise manner. The final stage of dialysis was performed against distilled water.

Fluorescence Spectroscopy—The intrinsic fluorescence of wild-type and mutant calsequestrins was measured using 90 µl of sample containing 2 µM of protein buffered in 10 mM Tris-HCl (pH 7.5). KCl ranging from 0 to 600 mM were used to assess the K0.5 dependence of fluorescence. Measurement was performed with a MOS-250 spectrometer (Bio-Logic) at 22 °C. Each protein was excited at 282 nm using 5-nm slit width, and emission was measured at 331 nm using 20-nm slit width.

Circular Dichroism (CD)—CD spectra were measured with an Aviv 202SF spectropolarimeter (Protein Solutions, Inc.) in a 2-mm path length cell at 22 °C. 5 µM of protein was buffered in 10 mM Tris-HCl (pH 7.5) with 300 mM KCl and 1 mM DTT. Spectra were recorded from 200 to 260 nm and corrected for buffer contributions.

Size Exclusion Chromatography—Prior to the experiment, a TSK G3000SW column (TosoHaas) was equilibrated with running buffer containing 10 mM Tris-HCl, pH 7.5, and 1 mM DTT with varying concentrations of KCl and CaCl2. 100 µl of each protein at 1 mg/ml, which was preincubated with the running buffer, was injected onto a TSK G3000SW column (TosoHaas). The chromatography was carried out at a flow rate of 1 ml/min using a Acuflow series IV pump (Analytical Scientific Inst.). The eluate was passed in tandem through an UV detector (GILSON), a refractometer (Optilab DSP, Wyatt Tech.), and a multiangle laser light-scattering detector (Dawn EOS, Wyatt Tech.). The chromatographic experiments were performed at 22 °C.

Molecular Mass Determination by Multiangle Light-scattering—Scattering data were collected and analyzed using the software ASTRA (Wyatt Tech.) supplied with the instrument. Relative weight-averaged molecular masses were determined from the scattering data collected for a given condition using the Zimm fitting method, in which Kc2c/R(Q)2 is plotted against sin2(Q2), where Q is the scattering angle, R(Q) is the excess intensity (I) of scattered light at the angle Q, c is the concentration of the sample, and Kc2 is a constant equal to 4π²n²/(dn/dc)²ρsNp (where n = solvent refractive index, dn/dc = refractive index increment of scattering sample, ρs = wavelength of scattered light and Np = Avogadro’s number). Extrapolation of a Zimm plot to zero angle gives an estimate of the weight-averaged molecular mass (Mw). Mw is defined in Equation 1 as:

\[
M_w = \frac{\sum (c_i M_i)}{\sum c_i}
\]

for c moles of i different species with an individual molecular weight Mi.

Cross-linking—20 µM of purified proteins were preincubated for 10 min on ice with KCl and/or CaCl2 in the presence of 150 mM KCl and 200 mM sodium phosphate buffer (pH 7.5). The samples were then further incubated with 400 µM 3,3'-dithiobis(5-sulfosalicylpyridine) (DTSSP) (Pierce) for 2 h at 4 °C. The reactions were then rapidly quenched through the addition of concentrated Tris-HCl (pH 8.0) to a final concentration of 50 mM, followed by incubation for another 10 min. Reactions were mixed 1:1 ratio with 2 × SDS-PAGE sample buffer in the absence of any reducing agent and subjected to electrophoresis in 8% SDS-PAGE gels.

RESULTS

Calsequestrin Structure and Mutants Construction—We previously showed that our rabbit skeletal calsequestrin crystal contains a ribbon-like linear polymer joined by two distinct dimerization contacts. A short, four-molecule segment of this polymer is reproduced here (Fig. 2B), with annotation of several key features, including the following: (i) the front-to-front and back-to-back interfaces; (ii) the arm exchange, which is part of the front-to-front interface; (iii) the cavities in the two interfaces; (iv) the short (two-residue) disordered N-terminal residues, and (v) the long (15-residue) disordered C-terminal tail. Both the N-terminal two residues and the C-terminal tail are called disordered because of the lack of sufficient electron density to trace the chain. Such lack of electron density arises
from structural inhomogeneity, which could have static contributions, dynamic contributions or both.

Because a clone of rabbit skeletal calsequestrin was not available when this research was initiated, we used a clone of canine cardiac calsequestrin instead. A sequence alignment of these two proteins is shown in Fig. 2A. The regions associated with the arm, domains I, II, and III, and the disordered tail are denoted. The overall sequence identity between these two proteins is 70% (similarity 86%), suggesting with a near certainty that the two proteins have the same overall three-dimensional structure. Also, indicated are the negatively charged residues involved in front-to-front (f or F) and back-to-back dimerization (b or B) interfaces, with cavity residues (f or b) distinguished from non-cavity interfacial residues (F or B).

Both the front-to-front and back-to-back interfaces contain cavities that are fairly large, irregularly shaped, and open to the exterior. The openings make it difficult to define the cavity volumes, but approximate estimates indicate that the cavity within the front-to-front interface is about 1600 Å³ (large enough for about 50 water molecules), while that within back-to-back interface is about 2400 Å³ (large enough for about 80 water molecules). The back-to-back cavity contains the two negatively charged residues at the tip of the arm as well as uncertain portions of the two disordered tails.

The acidic and basic residues within the dimerization contacts were determined by visual inspection of the three-dimensional structure for the rabbit skeletal calsequestrin and by homology for the canine cardiac calsequestrin (Fig. 2, Tables I and II). The excess of 29 negative charges in the front-to-front interface and the excess of 21 negative charges in the back-to-back interface for rabbit skeletal calsequestrin support the likely sensitivity of these interfaces to the presence of divalent cations. Interestingly, the interfaces of canine cardiac calsequestrin are also significantly negative, but greatly reduced in net negative charge, −16 for the front-to-front interface and just −11 for the back-to-back interface. Of the 50 (29 + 21) negative charges in the two interfaces of rabbit skeletal calsequestrin, 18 are changed in the canine cardiac calsequestrin. Of these changes, two conserve the negative charge (one Asp to Gly and one Gly to Asp, seven are negative to polar or non-polar (Asp or Glu to Asn, Thr, Ser, Gln, or Gly), and nine are negative to positive (Asp or Glu to Arg, Lys, or His).

The alignment of the two sequences coupled with the three-dimensional structure for rabbit skeletal calsequestrin provided the rationale for the construction of four truncation mutants. The regions deleted for each of these are indicated in Fig. 1. The deletion of the first two residues (ΔN2) removes two residues negatively charged residues (EE) that are both disordered and located within the back-to-back cavity. Deletion of the first 13 residues (ΔN13) leads to removal of the arm and therefore would be expected to disrupt the major interaction for the front-to-front contact. Deletion of the last 27 residues (ΔC27) would remove a large portion of the disordered tail, which is mostly negatively charged. Finally, deletion of the last 38 residues (ΔC38) would remove almost all of the disordered tail. These two C-terminal truncation mutants would be expected to make the back-to-back contact less sensitive to divalent cations.

**Intrinsic Fluorescence and CD**—Monovalent or divalent cations induce skeletal calsequestrin to undergo a conformational change from an extended, random coil-like molecule to a compactly folded form. This conformational change has been mon-
itored both by circular dichroism and by intrinsic fluorescence (3, 27). Here we compare the wild-type calsequestrin and three of the truncation mutants by these two spectroscopic methods.

Increasing the salt concentration using KCl caused a similar increase in intrinsic fluorescence for the wild-type and the three truncation mutants. Approximately 100 mM KCl was required for the half-maximal increase in fluorescence, and the maximal signal was achieved at about 300 mM KCl (see Supplementary Data at http://www.jbc.org). These data suggest that wild-type and all three truncation mutants are fully folded at the latter concentration. It is unclear whether the slight differences observed at low salt concentrations are indicating slight differences in the early folding steps or are simply due to experimental variations.

The far UV-CD spectra of the wild-type calsequestrin and the three truncation mutants were compared in the same condition of 300 mM KCl, a concentration at which all should be fully folded (see Supplementary Data). The similar shapes and intensities for the far UV CD spectra indicate that the wild-type calsequestrin and three truncation mutants do indeed fold into a similar three-dimensional structure.

Static Light-scattering Experiments—Wild-type calsequestrin and the four truncation mutants were studied by light scattering to determine their tendencies to form oligomers. These light scattering experiments were performed in 300 mM KCl, 10 mM Tris-HCl (pH 7.5), so all the proteins are in fully folded state. Variable concentrations of CaCl₂ were included to assess the effect of Ca²⁺ on polymerization.

Static Light-scattering of Wild-type Calsequestrin—In 300 mM KCl without Ca²⁺, most of the wild-type calsequestrin was monomeric with less than 1% of total population in the form of dimers or higher oligomers (Fig. 3A). In 300 mM KCl and 1 mM CaCl₂, most of the population was in the dimeric form (Fig. 3B). When the Ca²⁺ concentration was increased to 3 mM, most of the calsequestrin population was in dimeric form, with small amounts of tetramer starting to appear (Fig. 3C). The overall dimer/tetramer pattern in 300 mM KCl, 3 mM CaCl₂ was unchanged up to 5 mM CaCl₂. Beyond this CaCl₂ concentration, the calsequestrin starts to precipitate clogging the column and thus makes further experiments impossible. This suggests that wild-type calsequestrin has a transition point approximately at 5 mM CaCl₂.

When KCl was decreased to 70 mM with 1 mM Ca²⁺, however, various oligomeric species up to hexamer became evident (Fig. 3D). As a control experiment, the condition of 500 mM KCl but without Ca²⁺ ions was also used. All populations in this condition were monomers (Fig. 3E). The solution containing 700 mM KCl also showed the same result (data not shown).

Static Light-scattering of Mutants—The ΔC38 mutant showed a consistent pattern of monomeric and dimeric populations throughout the conditions that were tested. The monomer peaks from 300 mM (or 500 mM) KCl solution without Ca²⁺ ions were broad and delayed when they were compared with those with 300 mM KCl with Ca²⁺ (Fig. 4A). The ΔC27 mutant showed Ca²⁺-dependent dimerization in both 1 and 3 mM CaCl₂ with 300 mM KCl, but it did not polymerize further as the wild-type protein did (Fig. 4B). Consistent dimer peaks indicate that dimerization is not dependent on the concentration of Ca²⁺. The dimers seen on these mutants seem to be a front-to-front type of dimer because both C-terminal truncation mutants have intact N termini. But we could not rule out the possibility of back-to-back dimers. Since C-terminal truncation greatly reduces the charge repulsion on back-to-back interaction, its dimerization could be very well independent of Ca²⁺ as shown and might be due to intermolecular helix-helix interaction as seen in the crystal structure of rabbit skeletal calsequestrin.

![Fig. 4. Multiangle light scattering of ΔC38 (A), ΔC27 (B), ΔN13 (C), and ΔN2 (D) mutants.](http://www.jbc.org/)

Elution profiles are shown as molecular weight versus elution volume. The solid lines represent refractive index on an arbitrary scale, which is proportional to protein concentration, and the dots indicate calculated molecular mass. Each color represents different salt conditions in addition to the running buffer as indicated in the caption.
Despite the small size of the deletion, ΔN2 mutant exhibited only a slight tendency to undergo Ca\(^{2+}\)-induced dimerization (Fig. 4C). Furthermore, unlike wild-type protein, the monomer and dimer peaks were not distinct following the addition of Ca\(^{2+}\), suggesting that the removal of these two residues leads to destabilization of the Ca\(^{2+}\)-induced dimer and a resulting rapid equilibrium between the monomer and dimer forms.

The ΔN13 mutant formed high molecular weight polymers (~16–20 mer), which elute from the column after only ~11 min. This profile was consistent throughout the various conditions that we have tested (Fig. 4D).

Cross-linking.—To confirm the results of the light-scattering experiments, we performed cross-linking experiments of ΔN13 mutant, ΔC38 mutant, and wild-type calsequestrin. The wild-type protein clearly showed Ca\(^{2+}\)-dependent polymerization. 2 mM CaCl\(_2\) without KCl produced an increased amount of very large molecular weight polymers compared with other conditions. Addition of 150 mM KCl inhibits this polymerization (Fig. 5). Cross-linking buffer alone produced very weak dimer and tetramer bands. Both C- and N-terminal truncation mutants do not show any Ca\(^{2+}\)-dependent polymerization, which is inferred from the same band patterns in all conditions. However, compared with the band patterns of the ΔC38 mutant, those of the ΔN13 mutant indicate the existence of very high molecular polymers together with a reduced intensity of the monomer band. These results are consistent with the results of the light-scattering experiments where very high molecular weight aggregates are apparent in ΔN13 mutant and no polymerization further than dimers are apparent in ΔC38 mutant.

**DISCUSSION**

Mechanisms of Ca\(^{2+}\) binding by calsequestrin have no reason to mimic the Ca\(^{2+}\) binding mechanism of either the Ca\(^{2+}\) pump or TnC (9). Ca\(^{2+}\) binding sites of calsequestrin 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 that are intrinsic to the EF hand structure of TnC. Ca\(^{2+}\) binding by calsequestrin is very likely nonspecific. It is, therefore, unlikely that there are specific Ca\(^{2+}\) sites, although the first few ions that bind at low Ca\(^{2+}\) concentrations may have some specificity.

We are proposing that Ca\(^{2+}\) regulation by calsequestrin involves an interplay among protein folding, Ca\(^{2+}\) binding, and calsequestrin polymerization. This interplay is shown schematically in Fig. 6. In summary, the polymerization of calsequestrin is promoted by Ca\(^{2+}\) and inhibited by K\(^{+}\). Both the N-terminal arm and the acidic, C-terminal tail are necessary for the Ca\(^{2+}\)-dependent linear polymerization. N-terminal arm exchange inhibits random aggregation of the protein. Deletion of the N-terminal arm may disrupt specific orientation of the front-to-front interaction, resulting in random interactions among calsequestrin monomers. Deletion of the acidic, C-terminal tail reduces the negative charge in the back-to-back interface, resulting in low concentrations of counter ion for establishing the back-to-back interactions.

From Table I, we notice that rabbit skeletal calsequestrin has a net charge of ~75, while canine cardiac calsequestrin has a net charge of ~64. Despite this small overall difference in net charge, skeletal calsequestrin binds about twice the amount of Ca\(^{2+}\) compared with the cardiac calsequestrin (12). If our coupled binding and polymerization mechanism were correct, then one would expect to observe 2-fold more negative charge within the skeletal as compared with the cardiac calsequestrin interfaces. Table II shows this expectation to be true: skeletal calsequestrin has a net charge of ~18 and ~32 for a total of ~50 within the front-to-front and back-to-back interfaces (including the cavity-lining residues), while cardiac calsequestrin has just ~7 and ~20 for a total net charge of ~27 within the two interfaces.

Since about 40 calcium ions are bound by skeletal calsequestrin compared with about 20 calcium ions by the cardiac calsequestrin, the interfacial charges of ~50 and ~27 would be insufficient to balance the positive charges arising from the bound calcium ions. Charge balance could result to a significant degree from the polyanionic C-terminal tails. In addition, the large cavities observed within the front-to-front and back-to-back interfaces could play a crucial role by providing the space needed for negative counter ions to help balance the positive charge. If charge balance by negative counter ions were indeed significant, then the amount of bound Ca\(^{2+}\) would be reduced if a large counter ion such as gluconate were used in place of a smaller ion such as chloride. This conjecture could be tested by measuring the number of (radioactive) Ca\(^{2+}\) ions bound per calsequestrin molecule with either gluconate or chloride as the sole anion.

The biological significance of this dynamic polymerization of calsequestrin can be explained in several ways. The front-to-front and back-to-back contacts between the calsequestrin monomers permit formation of a ribbon-like linear polymer. Ca\(^{2+}\) largely fills the electronegative pockets formed in these two contacts cross-bridging the monomers. In this paper, we...
report Ca\(^{2+}\)-dependent oligomerization. Considering the high concentration of calsequestrin (100 mg/ml) and physiologically varying concentrations (opposite direction) of K\(^-\) and Ca\(^{2+}\) in SR, the dynamic formation and destruction of calsequestrin oligomer in SR can be well explained from our experimental data. Growing calsequestrin polymers provides a highly charged surface onto which calcium can be adsorbed. The attractive forces exerted by such an extended surface would have a longer range than those from an isolated molecule. A sparingly soluble ion such as Ca\(^{2+}\) would tend to spread over the surface of the calsequestrin ribbon forming a readily exchangeable film. The propensity of Ca\(^{2+}\) to involve surface diffusion, a more rapid process than diffusion through liquid (28). Calsequestrin, probably in a Ca\(^{2+}\) bound form, forms regular arrays that appear crystalline in the lumen of the sarcoplasmic reticulum (18, 19, 29). Calsequestrin is associated physically with RyR by a nucleation event that involves calsequestrin binding to the basic lumenal domains of triadin (5) or junction (6). These two proteins interact with RyR in the junctional face region of the sarcoplasmic reticulum, and the 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 calsequestrin through the Ca\(^{2+}\) release channel is regulatory but not limiting.

REFERENCES

Additions and Corrections


Cytochrome oxidase assembly does not require catalytically active cytochrome c.

Antoni Barrientos, Danielle Pierre, Johnson Lee, and Alexander Tzagoloff

The following errors escaped our notice and may have caused problems in the interpretation of the results presented in the paper.

Page 8885, 1st paragraph, left column, line 4: “upper panel” should be “lower panel.”

Page 8885, 1st paragraph, left column, line 10: “lower panel” should be “upper panel.”

Legend to Fig. 6, line 8: “COX (dashed bars)” should be “COX (open bars).”

Legend to Fig. 6, line 9: “reductase (open bars)” should be “reductase (dashed bars).”

Page 8886, 1st paragraph, left column, lines 1 and 2: Fig. 3B should be Fig. 5.


Voltage dependence of the Ca$^{2+}$-activated cation channel TRPM4.

Bernd Nilius, Jean Prenen, Guy Droogmans, Thomas Voets, Rudi Vennekens, Marc Freichel, Ulrich Wissenbach, and Veit Flockerzi

Page 30820, lines 13 and 17: The citation should be (12), instead of (6).


Polymerization of calsequestrin. Implications for Ca$^{2+}$ regulation.

HaJeung Park, Si Wu, A. Keith Dunker, and ChulHee Kang

Page 16178, left column, line 11: ΔC27 should be changed to ΔN2.

Page 16178, left column, line 30: 100 mg/ml should be changed to 100 μg/ml.

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