

# Novel Details of Calsequestrin Gel Conformation *in Situ*\*

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**Background:** Calsequestrin is essential to keep a high calcium concentration inside the sarcoplasmic reticulum of muscle fibers.

**Results:** *In situ*, calsequestrin polymers appear to form a three-dimensional structure with repeated nodal points.

**Conclusion:** A three-dimensional calsequestrin polymer matrix is very suitable for its spatially confined calcium storage function.

**Significance:** The calsequestrin structure has been extensively studied in *ex vivo* systems. This approach illustrates the behavior of the protein while still in its physiological cell localization.

Calsequestrin (CASQ) is the major component of the sarcoplasmic reticulum (SR) lumen in skeletal and cardiac muscles. This calcium-binding protein localizes to the junctional SR (jSR) cisternae, where it is responsible for the storage of large amounts of Ca<sup>2+</sup>, whereas it is usually absent, at least in its polymerized form, in the free SR. The retention of CASQ inside the jSR is due partly to its association with other jSR proteins, such as junctin and triadin, and partly to its ability to polymerize, in a high Ca<sup>2+</sup> environment, into an intricate gel that holds the protein in place. In this work, we shed some light on the still poorly described *in situ* structure of polymerized CASQ using detailed EM images from thin sections, with and without tilting, and from deep-etched rotary-shadowed replicas. The latter directly illustrate the fundamental network nature of polymerized CASQ, revealing repeated nodal points connecting short segments of the linear polymer.

Calsequestrin (CASQ)<sup>3</sup> is an acidic protein that is a major component of the sarcoplasmic reticulum (SR) lumen in striated muscle cells (1–4) and also resides within the endoplasmic reticulum of other cell types (5–8). The ionic composition of the SR lumen favors the polymerized form of CASQ (9), and thus, it is not surprising that the SR domains particularly rich in CASQ, such as the junctional SR (jSR) within the triads of most skeletal muscles, contain a gel matrix responsible for imposing a wide shape to the cisternae (10–13). The luminal CASQ gel appears as a compact aggregation of structural elements that were directly identified as CASQ polymers on the basis of their disappearance when the isolated jSR was lysed to release CASQ (13), of their absence in CASQ null muscle fibers (14), and of their rescue by expression of CASQ in the null mutant (15). On

close examination of thin sections by electron microscopy, the CASQ gel is composed of slender elongated strands randomly coiled within the available space. The images of the jSR from skeletal muscle expressing CASQ1 and of large SR cisternae from cardiac muscle overexpressing CASQ2 are essentially identical (16), indicating that this is the fundamental disposition of CASQ in the SR lumen. At first approximation, the structure is consistent with that expected of linear CASQ polymers of the type described in the work of Wang *et al.* (17), which are constrained within a narrow volume. Formation of a paracrystalline arrangement has been detected only once in isolated SR vesicles (18), and thus, it is not normal under physiological conditions. However, the EM images also show evidence of a more complex network involving repeated nodal points, raising the question of possible polymer branching as recently modeled by *in vitro* studies (19). Interpretation of the images of thin sections is complicated by the overlap of details within the section thickness. An initial attempt to obtain an alternative view of CASQ in the jSR lumen using deep etching (20) did not reveal sufficient details of the structure to allow a clear-cut discrimination between linear and branched conformations of the CASQ polymer, although the latter seems to be already accepted in the literature (21). Here, we further examined the question using detailed images of thin sections with and without tilting and compared them with images of replicas of deep-etched rotary-shadowed samples. The latter approach directly illustrates the fundamental structure of polymerized CASQ, revealing repeated branching points.

## EXPERIMENTAL PROCEDURES

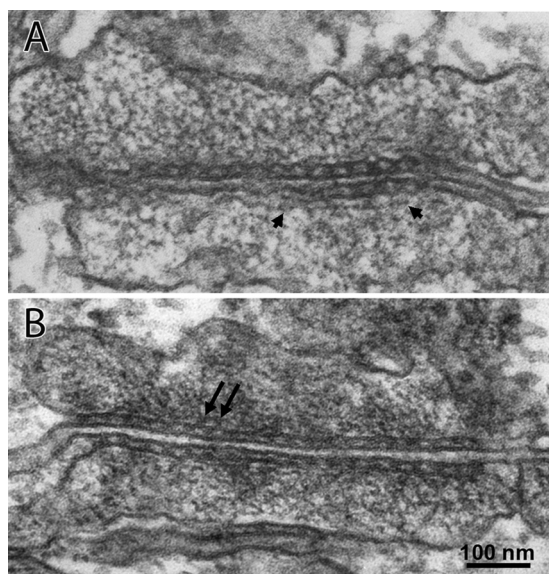
Adult northern water snakes (*Nerodia sipedon*) and frogs (*Rana pipiens*) were killed by decapitation following either deep anesthesia or stunning. The dorsal sides of the snakes were skinned, and the epaxial muscles were fixed *in situ* as described below. Frog sartorius muscles were dissected, pinned, and fixed in 3.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Tissues were post-fixed in buffered 2% OsO<sub>4</sub> for 1 h at 4 °C, stained *en bloc* with saturated aqueous uranyl acetate for 2 h at room temperature, and embedded in Epon. Thin sections were stained with lead citrate or double-stained with uranyl acetate and lead citrate.

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<sup>3</sup> The abbreviations used are: CASQ, calsequestrin; SR, sarcoplasmic reticulum; jSR, junctional SR.



**FIGURE 1. Thin sections illustrating two triads from frog (A) and snake (B) muscles.** The central T tubules are flanked by two elongated jSR cisternae, from which they are separated by two rows of evenly spaced “feet,” the cytoplasmic domains of ryanodine receptor channels. Within the SR lumen is a dense network of CASQ. In the proximity of the membrane bearing the feet, CASQ is linked to periodic triadin extensions (arrows in A) and often forms an elongated line (between arrows in B). Elsewhere, CASQ forms an apparently random network.

For freeze fracture and deep etching, small bundles of fibers were cryoprotected in 70% methanol frozen in liquid nitrogen-cooled propane, fractured at  $-110^{\circ}\text{C}$ , etched for 30 min at  $-90^{\circ}\text{C}$ , cooled to  $-110^{\circ}\text{C}$ , rotary-shadowed with platinum at  $25^{\circ}\text{C}$ , and replicated with carbon. Thin sections and freeze fractures were either examined and photographed with an AEI 6B electron microscope or examined and digitally recorded with a Philips 410 electron microscope (Philips Electron Optics, Mahwah, NJ) equipped with a tilt stage and Hamamatsu C4742-95 digital camera (Advanced Microscopy Techniques, Chazy, NY).

## RESULTS

*View of the CASQ Polymer in Thin Sections of the jSR*—The images illustrated are from frog (Fig. 1A) and snake (Fig. 1B) muscles: both present wide jSR cisternae apposed to transverse tubules containing well defined CASQ gels. CASQ has two configurations within the jSR cisternae. In close proximity and parallel to the jSR membrane facing the transverse tubules, CASQ is condensed into periodic densities (Fig. 1). Because this disposition is determined by anchorage of CASQ to triadin and/or junctin (22), two membrane-associated proteins that extend only a short distance into the SR lumen, we will consider this no further. The disposition of CASQ within the remaining jSR volume and also within the longitudinal SR of some snake muscles is presumably due to CASQ only, and thus, it is intrinsic to the protein. As already described in the literature, CASQ in the SR lumen appears as a dense aggregate of straight and curved lines oriented in all directions. In very thin sections ( $\sim 50$  nm) with contrast enhanced by double staining of the sections with uranyl acetate and lead salts, the main appearance is additionally that of a fine meshwork with some discontinuities (Fig. 2).

Parameters of the network are quite variable: it can be quite dense and difficult to resolve or less crowded. In the latter case, individual strands can be followed and shown to constitute an apparently continuous mesh with intersections and nodal points. This is best illustrated by overlaying the EM images with a colored tracing, as shown in examples from frog and snake (Fig. 2). The tracings clearly illustrate the network variability while emphasizing the apparent continuity of its components.

*Are the Intersections Real?*—Because the depth of field in transmission EM optics is large relative to the section thickness, single images cannot directly distinguish between the two possibilities that apparent branching points in electron micrographs of the CASQ polymer either are true branches of a continuous network or simply result from the overlap within the image of profiles from short segments with different orientations but located at different depths in the section. An answer to this question was obtained by two different approaches. First, we compared the appearance of small details of the network in images of the same area taken at  $20^{\circ}$  tilt angles. If a nodal point in the apparent mesh (e.g. one where two CASQ strands meet at right angles to each other) was due to the superimposition in the image of two strands separated from each other by some distance in the  $z$  axis, then the apparent nodal point would either be shifted or eliminated in the tilted images. Fig. 3 illustrates two examples. In general, we found that in the images of the network distorted by the tilt, some nodal points are lost (Fig. 3, blue hexagons), but others remain in a similar relative position (magenta circles). We argue that the latter are either at the same  $Z$  level in the section or very close to it and thus probably belong to connected branching points of the network.

*Deep-etched Images Confirm a Complex Three-dimensional Network*—Rotary-shadowed images of freeze-fractured SR in a snake muscle that is particularly abundant in CASQ offer the best opportunity for deciphering details of the protein polymers. At the edges of the fracture plane, the network is exposed and decorated by platinum shadow. The shadowing in this case was done at a relatively low angle ( $25^{\circ}$ ), so the platinum decorates only the protein strands that are very close to the surface, and as a result, within the image, there is no superimposition of structures that are located at depths other than the most superficial. Coloring of the images facilitates visualization of the linear cable-like organization of polymerized CASQ (in purple) at the edge of the fracture plane and additionally emphasizes the frequent nodal points in the network that result in a complex three-dimensional matrix (Fig. 4). As in all freeze-fracture images, the fracture plane jumps fairly capriciously at different levels, and some distortion of the structure results, so fine details of the network are well illustrated only in selected areas as shown in high magnification images (Fig. 5). These illustrate examples of repeated side lines within the network, again emphasized by a color overlay. Nodal points are formed by the convergence of several linear polymers in a random arrangement, resulting in complex ramification patterns. Multiple connections at various angles are revealed: some polymers join each other in a trigonal configuration, giving the impression of a branching of the main polymer line, but others connect in an orthogonal and even pentameric configuration with four or five polymers converging on a single nodal point. We have super-



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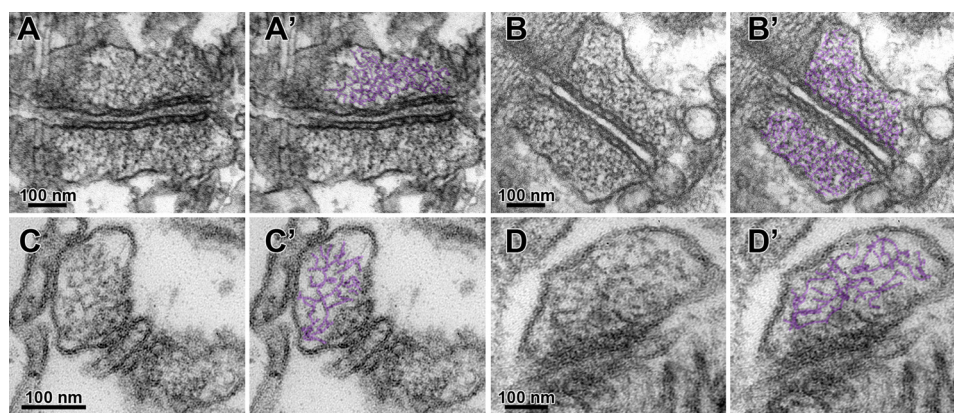


FIGURE 2. Triads from frog (A and B) and snake (C and D) muscles are shown. Each image is shown with a duplicate (A'–D') in which the thin CASQ strands are traced by colored lines. The traces indicate apparent branched networks, particularly where the density of CASQ is lower (e.g. C and C'). Note, however, that because the depth of field of the electron microscope objective lens is larger than the section thickness, the individual strands may be located at a different depth within the section and may not actually intersect.

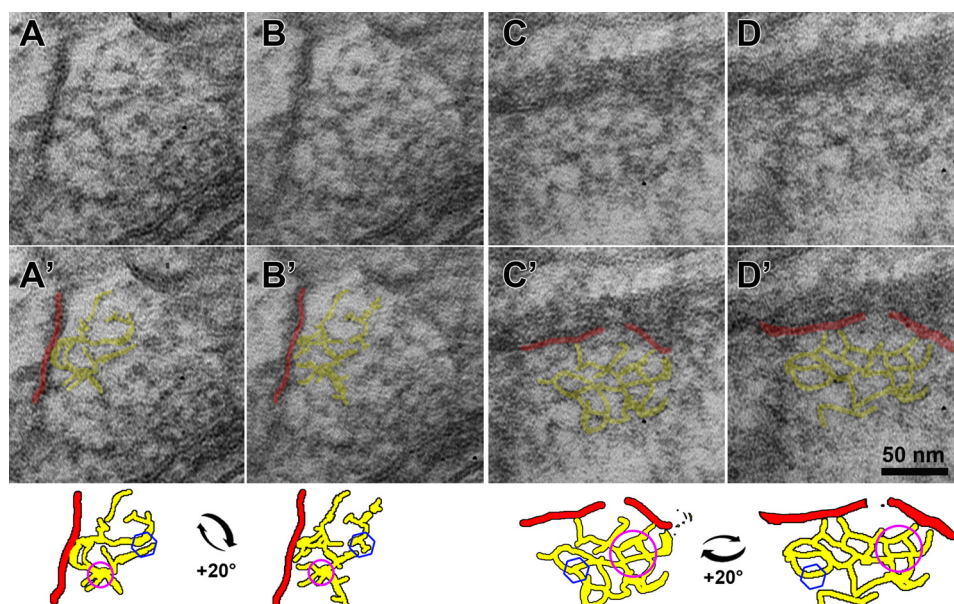


FIGURE 3. A–D, details of CASQ networks in snake viewed at two different angles with a tilt of 20°. A'–D', the thin strands are highlighted in yellow, and the yellow traces are shown separately below. Each image shows crossover points that are maintained in the tilted images (magenta circles) and others that are lost (blue hexagons). The former are real nodal points; the latter are not.

imposed an appropriately scaled model of the CASQ polymer arrangements proposed previously (19) over one of our images (Fig. 5E). The dimensions of the CASQ monomer derived from the crystal structure<sup>4</sup> are  $\sim 80 \times 65 \times 45$  Å. Here, the  $x/y$   $80 \times 65$  Å dimensions scaled to the EM image magnification fit the visible strands of the shadowed images. Clearly, the model effectively mimics the images, confirming that the observed network configuration is consistent with the proposed model of polymerized CASQ.

### DISCUSSION

CASQ is clearly polymerized within the jSR of skeletal muscle fixed under resting conditions because images of the lumen in thin sections show well detectable thin molecular strands by electron microscopy. The monomeric form, which may also be additionally present, is simply not visible. However, due to the

small dimensions of the polymer, its random arrangement, and its crowding within the narrow space of the SR lumen, it has been difficult to distinguish between the two possible configurations of polymerized CASQ that have been proposed on theoretical grounds (linear (23) or branched (19)). Our current images, particularly those using the deep-etched shadow approach, solve this question, confirming that the more complex three-dimensional network actually exists *in vivo*. The complex nodal points of the network, where several linear segments converge, indicate that it is not a simple branching. The network is entirely consistent with theoretical models, and because we detected it within the SR lumen at relatively large distances from the membrane, we can safely assume that it is not due to an association of CASQ with other jSR proteins, such as triadin and junctin. A limited view of the branched CASQ polymer has been visualized by tomography of frozen hydrated triads (24). However, these are in close proximity to the jSR membrane, and so they may be influenced by triadin/junctin.

<sup>4</sup> C. H. Kang, personal communication.



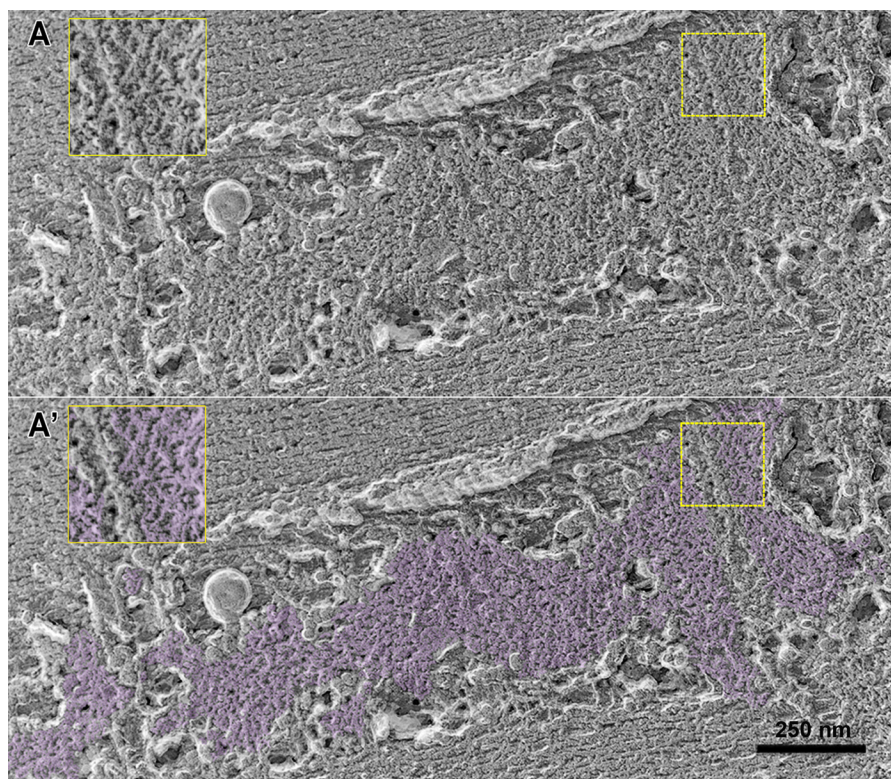


FIGURE 4. Deep-etched freeze-fractured SR in *Nerodia* muscle rotary-shadowed at 25°. CASQ extends over the entire SR lumen in this muscle (A), and tracing of the CASQ strands (A') illustrates the complex network nature of the gel matrix.

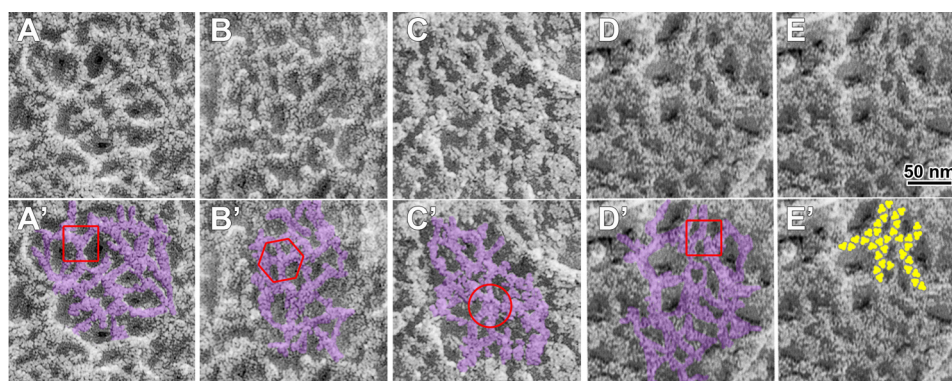


FIGURE 5. Details of CASQ networks. The images were chosen to illustrate areas in which the fracture plane followed a portion of the network that was parallel to the fractured surface. Given the fact that the platinum shadow decorates only the superficial portion of the fractured CASQ gel, and given the shallow angle of the shadow (25°), the nodal points in these images are real and demonstrate their frequency and their variety: trigonal (red squares), tetragonal (red hexagon), and even pentagonal (red circle). In E', an appropriately scaled segment of the CASQ branching network model proposed by Sanchez *et al.* (19) is superimposed on the real image, showing that the dimensions of thin strands and their connections are consistent with those of polymerized CASQ.

Similarly, atomic force microscopy images showing a CASQ meshwork under *in vitro* experimental conditions (25) are not directly relevant to this discussion because the meshwork is obtained only in the presence of junctin.

The complex arrangement of CASQ in a three-dimensional cross-linked meshwork has direct implications for structure/function relationships within the SR. It is known that CASQ is tied to the jSR membrane by its association with triadin and junctin (22, 26) and that these two proteins affect the structure of the CASQ polymer in the proximity of the jSR membrane (27). Presumably, anchorage to triadin/junctin is important in the retention of CASQ within the jSR in the proximity of T tubules. In the depth of the jSR lumen, CASQ is not directly

linked to triadin/junctin, and thus, polymerization, by linking the monomers into long chains, is responsible for retaining all CASQ within the jSR despite the fact that no barrier separates the jSR lumen from the longitudinal SR lumen. CASQ fills all available SR elements only when artificially overexpressed, *e.g.* in some cardiac muscle experiments (28), in aging and pathological mouse muscles (29, 30), or when normally present in greater abundance as in snake muscle. A complex network of the type illustrated here is more likely to be effective in restrained CASQ diffusion compared with a linear polymer that can be more easily fragmented. Polymerization may also play a role in the privileged vesicular transport and distribution of CASQ within endoplasmic reticulum/SR elements of muscle

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and other cells (31), and again, compact packaging into a dense network may be an essential element in this mechanism.

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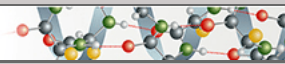
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PROTEIN STRUCTURE  
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