

Chapter 4

Calsequestrin

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I. SARCOPLASMIC RETICULUM

The control of muscle contraction, relaxation, and metabolism is intimately tied to the sarcoplasmic Ca^{2+} concentration (Ebashi *et al.*, 1969), and, accordingly, muscle is a rich source of a variety of Ca^{2+} -binding proteins. The contractile unit of skeletal muscle is a myofibril (Huxley, 1972; Franzini-Armstrong and Peachey, 1981) about $40\ \mu\text{m}$ in diameter and comprised of a series of longitudinal repeating elements referred to as sarcomeres (Fig. 1). The sarcomere is the region between two Z disks, rather rigid structures that contain attachment points on either side for thin filaments, which extend about $1\ \mu\text{m}$ longitudinally with opposite polarity in the two opposing filaments. Thick filaments, about $1.7\ \mu\text{m}$ long and composed largely of myosin molecules, are found in the center of the sarcomere with their ends interdigitating with both of the oppositely oriented thin filaments. The area occupied by the thick filaments and interdigitated thin filament is referred to as the A band region, the area on either side of the Z disk that contains only thin filaments as the I band region and the juncture point as the A-I junction.

The sarcoplasmic reticulum is a membranous network that surrounds each myofibril like a fenestrated water jacket around a cylinder—and

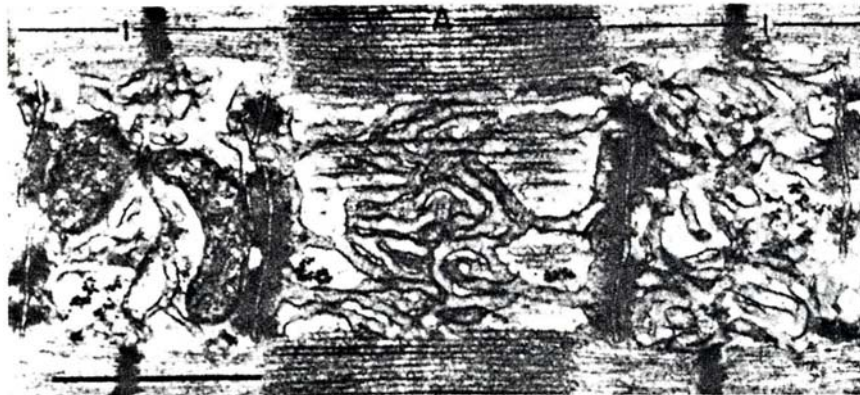


Fig. 1. A longitudinal section of extensor digitorum longus muscle (EDL) from a 30-day-old mouse (from Luff and Atwood, 1971). Thick filaments can be seen in the center of the A band region; thin filaments are seen in the I band. The transverse tubular system and the sarcoplasmic reticulum are in the center of the picture. Transverse tubules running perpendicular to the fibers can be seen near the A-I junction. The sarcoplasmic reticulum consists, in part, of convoluted tubules overlying the A and I band regions; at the A-I junction it is thickened to terminal cisternae, which abut the transverse tubular system. The matrix in the terminal cisternae is believed to consist of calsequestrin. Reproduced from *J. Cell Biol.* **51**, 369-383 by copyright permission of the Rockefeller Univ. Press.

forms a series of enclosed compartments within muscle cells (see Fig. 1). At longitudinal intervals in the sarcomere, corresponding approximately to the A-I junction, the sarcoplasmic reticulum is segmented by its intersection with a second membrane system—the transverse tubular or T system. These tubules are invaginations of the sarcolemma and their interior is continuous with extracellular fluid. The sarcoplasmic reticulum balloons in the region on either side of the transverse tubules to form a terminal sac or cisternae, but it thins and branches to form a tortuous series of longitudinal tubules extending between cisternae. In cross section, the slightly flattened transverse tubule, which is flanked on either side by two terminal cisternae, forms a tripartite structure referred to as a *triad*. The two membranes are separated by about 12 nm over most of their length with only a few points of adhesion between them (Franzini-Armstrong, 1980). These adhesions consist of plaques in which the sarcoplasmic reticulum dimples outward to form small projections whose tips are joined to the transverse tubules by amorphous structures. The projections and the amorphous material are referred to as *feet* (Franzini-Armstrong, 1970). These amorphous structures do not appear to form channels.

The two membrane systems function together to control the intracellular distribution of Ca^{2+} . In resting muscle, 60-70% of the total Ca^{2+} is found in the terminal cisternae of sarcoplasmic reticulum (Somlyo *et al.*, 1981). Electrical impulses carried to the interior of the cell by the transverse tubules (Huxley and Taylor, 1958) are the signals for the release of Ca^{2+} from the terminal cisternae. Ca^{2+} in the cytoplasm is bound to troponin C in the thin filaments to initiate muscle contraction (Greaser *et al.*, 1972b), and it is also bound to calmodulin in various sites in the cell to activate metabolic processes that regenerate the ATP, which is hydrolyzed during muscle contraction (Wang and Waisman, 1979). When electrical stimulation ceases, Ca^{2+} is pumped into the interior of the sarcoplasmic reticulum and is stored in the terminal cisternae to await the next spike of electrical stimulation.

II. ISOLATED SARCOPLASMIC RETICULUM VESICLES

After homogenizing muscle tissue, the sarcoplasmic reticulum can be readily isolated, by differential centrifugation (Hasselbach, 1964). The isolated vesicles retain the capacity to transport Ca^{2+} through the activity of a Ca^{2+} -dependent ATPase (Hasselbach, 1964). This enzyme, the major intrinsic protein of the membrane, has a very high activity and a threshold of activation by Ca^{2+} of about 0.1-0.3 μM (Hasselbach, 1972). In situ, it is

able to draw Ca^{2+} away from the sarcoplasmic Ca^{2+} -binding proteins which have K_d values for Ca^{2+} binding of about 1-5 μM (Greaser *et al.*, 1972b; Kretsinger, 1976). The contest for Ca^{2+} is, therefore, unequal and Ca^{2+} finds itself in the lumen of the sarcoplasmic reticulum. There it encounters another of the many Ca^{2+} -binding proteins of muscle, the calcium sequestering protein *calsequestrin*.

Calcium-binding sites in sarcoplasmic reticulum vesicles have been rather extensively probed. Hasselbach (1964) showed that calcium oxalate crystals are formed in the interior of isolated sarcoplasmic reticulum vesicles, when this permeant anion is added to the assay mixture. Costantin *et al.* (1965) demonstrated Ca^{2+} oxalate precipitates in the terminal cisternae of whole fibers. These studies demonstrate that Ca^{2+} can be pumped into luminal sites. The fate of Ca^{2+} in the absence of precipitating anions is not as clear, however. Carvalho (1966) and Carvalho and Leo (1967) found that sarcoplasmic reticulum contains some 350 nEq of anionic sites per milligram of protein. These sites were preferentially filled by Ca^{2+} when the sarcoplasmic reticulum was allowed to pump Ca^{2+} . In the absence of Ca^{2+} , the sites were filled with Mg^{2+} or K^+ so that the total metal content of the sarcoplasmic reticulum remained relatively constant. Chiu and Haynes (1977) used calcein and arsenazo III as spectroscopic indicators of free Ca^{2+} and Mg^{2+} , together with stopped flow rapid mixing techniques, to measure Ca^{2+} binding to sarcoplasmic reticulum. They observed two classes of binding sites—one rapidly equilibrating, which binds 35 nmole of Ca^{2+} per milligram with a K_d of 17.5 μM and one slowly equilibrating, which binds 820 nmole of Ca^{2+} with a K_d of 1.9 mM. The low affinity sites showed no specificity for Ca^{2+} over Mg^{2+} , and the time course for binding, about 4 sec, indicated that the sites were internal.

Others have measured Ca^{2+} binding either after active loading in absence of precipitating anions or after passive diffusion (Fiehn and Migala 1971, Cohen and Selinger, 1969; Chevallier and Butow, 1971; Meissner *et al.*, 1973; Miyamoto and Kasai, 1979). Careful measurement shows that there are high affinity Ca^{2+} -binding sites (K_d of 1 μM), probably externally located, that amount to about 10-15 nmole/mg. These can be accounted for by the Ca^{2+} -ATPase (Meissner *et al.*, 1973; Miyamoto and Kasai, 1979). In addition, there are less specific binding sites with a K_d of about 1 mM that account for up to 150 nmole of Ca^{2+} bound per milligram of protein. These sites are internally located (Miyamoto and Kasai 1979). The internal water volume of sarcoplasmic reticulum vesicles is about 3-4 $\mu\text{l}/\text{mg}$ (Duggan and Martonosi, 1970; Campbell *et al.* 1980). If Ca^{2+} bound at the level of 100 nmole/mg were all free inside the vesicles its concentration would be 20-30 mM. The presence of internal Ca^{2+} -binding sites to lower the internal free Ca^{2+} concentration would obviously be of

great importance because the Ca^{2+} -ATPase must pump Ca^{2+} against a concentration gradient, and any means of lowering this gradient would enhance the efficiency of Ca^{2+} pumping.

III. ISOLATION OF CALSEQUESTRIN

Calsequestrin was first isolated from sarcoplasmic reticulum by MacLennan and Wong (1971). The protein was extracted from membrane vesicles in water soluble form and purified by chromatography on DEAE cellulose, hydroxylapatite, and Sephadex. It was extremely acidic; it contained about 37% acidic amino acid residues and only 7% basic residues. It bound nearly 1000 nmole of Ca^{2+} /mg, forming an insoluble Ca^{2+} -calsequestrin complex in the process. Calculation of the concentration of the protein in the membrane (7%) and its Ca^{2+} -binding capacity showed that it could account for the 100 nmole or so of Ca^{2+} that were known to be bound per mg protein inside the sarcoplasmic reticulum. The protein was not detectable either in high speed supernatants or in nuclear or mitochondrial pellets from muscle homogenates. It was present in the microsomal fraction, but the protein was not released either by sonication or by extraction of vesicles with chaotropic salt solutions, which would normally remove extrinsic proteins from external membrane surfaces. It was released by treatment of the membranes with a low concentration of anionic detergent in the presence of 1 M KCl, conditions that led to the disruption of the membrane. These observations argued for the location of calsequestrin in the interior of the sarcoplasmic reticulum. The high Ca^{2+} -binding capacity and the internal localization led MacLennan and Wong (1971) to postulate that calsequestrin is the major site of Ca^{2+} binding in the interior of sarcoplasmic reticulum.

The studies of MacLennan and Wong (1971) were not the first to deal with the protein. Duggan and Martonosi (1970) found that extraction of sarcoplasmic reticulum with EDTA at elevated pH led to enhanced permeability of the membranes to Ca^{2+} and even to larger molecular weight compounds such as inulin. Under these conditions, two proteins designated C_1 and C_2 were released into solution. Duggan and Martonosi (1970) did not isolate proteins nor did they measure their Ca^{2+} -binding properties. Thus, they did not recognize their significance in Ca^{2+} binding. They suggested that the proteins might be involved in controlling the Ca^{2+} permeability of the membranes.

Han and Benson (1970, 1971) actually isolated calsequestrin and observed its Ca^{2+} -induced spectral changes. Their original intent, however, was to purify troponin from salt extracts of muscle that had been dehy-

drated with alcohol and water. Because calsequestrin co-purified with troponin in their procedure, Han and Benson concluded that it was associated with troponin and called it *tropocalcin*. When other preparations of troponin were found to be free of tropocalcin (Greaser *et al.*, 1972a), the protein was left in limbo. Recently, Drabikowski (1977) demonstrated the relationship between calsequestrin and tropocalcin.

Shortly after the protein was isolated by MacLennan and Wong (1971), Ikemoto *et al.* (1971) also isolated calsequestrin by different procedures. In their studies, the sarcoplasmic reticulum was dissolved in Triton X-100 and was incubated in 4 mM Ca^{2+} . The insoluble Ca^{2+} -calsequestrin complex was collected in a relatively pure form by centrifugation. Because of differences in the measured molecular weight, Ikemoto *et al.* (1972) believed that the Ca^{2+} precipitable protein was not calsequestrin, and the identity of the two proteins was not established for a few years.

IV. Ca^{2+} BINDING BY CALSEQUESTRIN

Ca^{2+} binding by calsequestrin has been measured in several laboratories. MacLennan and Wong (1971) measured Ca^{2+} binding in the presence of 10 mM Tris-HCl, pH 7.5, to be up to 970 nmole/mg with a dissociation constant of about 40 μM . In the presence of 100 mM KCl, the maximal Ca^{2+} binding was unchanged, but the dissociation constant was shifted to about 800 μM (Ostwald and MacLennan, 1974; MacLennan, 1974). Ikemoto *et al.* (1972) measured 750-1000 nmole of Ca^{2+} bound per mg of protein with a dissociation constant of 1.3 mM in the presence of 100 mM KCl. Meissner *et al.* (1973) measured Ca^{2+} binding of 900-1000 nmole/mg with a dissociation constant, in the absence of salt, of about 4 μM . These observations, which are fairly uniform, show that the Ca^{2+} -binding affinity for Ca^{2+} is intermediate between the high affinity Ca^{2+} -binding sites that have been associated with "EF hand" structures (Kretsinger, 1976) and the sites of low affinity that are commonly found in many proteins and on membrane surfaces.

The specificity of calsequestrin for Ca^{2+} is rather low. Most divalent or monovalent ions will compete for the Ca^{2+} -binding site (MacLennan and Wong 1971). Ikemoto *et al.* (1974) used tryptophan fluorescence, which was induced by calcium binding, to measure the order of affinities of various cations; the order was found to be $\text{La}^{3+} > \text{Zn}^{2+} > \text{Cd}^{2+} > \text{Mn}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+} = \text{Sr}^{2+} > \text{K}^+$ with dissociation constants ranging from 3 μM for La^{3+} through 250 mM for K^+ .

V. SIZE AND SHAPE OF CALSEQUESTRIN

The molecular weight of calsequestrin isolated from rabbit skeletal muscle is still not precisely known. Estimates of the molecular weight by SDS gel electrophoresis are dependent upon the gel system employed. Using the method of Weber and Osborn (1969), a system run at neutral pH, the molecular weight of calsequestrin was determined to be 44,000 (MacLennan and Wong, 1971), 45,000 (Sarzal *et al.*, 1974), 46,000 (MacLennan, 1974), 47,000 (Greaser *et al.*, 1972a), 52,000 (Caudwell *et al.*, 1978), and 55,000 (Ikemoto *et al.*, 1972, 1974). The molecular weights of calsequestrin determined using the Laemmli (1970) slab gel system, run at alkaline pH, were 65,000 (Meissner *et al.*, 1973) and 64,000 (Campbell *et al.*, 1980). The protein had an apparent molecular weight of 63,000 in a modified Weber and Osborn system (Bragg and Hou, 1972) run at alkaline pH (R. A. F. Reithmeier, 1983). This result suggests that the mobility of calsequestrin is sensitive to pH rather than to differences in the buffer system employed. Michalak *et al.* (1980) showed that calsequestrin could be identified in a mixture of cellular proteins if they were separated first at neutral pH and then in a second dimension at alkaline pH in a slab gel system. Under these conditions, most proteins ran in a diagonal line, because their mobilities were not pH-dependent. Calsequestrin moved off of the diagonal and could, therefore, be easily identified. This procedure has permitted the isolation of calsequestrin from muscle cell extracts on an analytical scale.

The change in mobility of calsequestrin as a function of pH is not due to anomalous binding of SDS because calsequestrin bound 1.8 mg of detergent per milligram of protein at pH 7.0 and 9.5 (R. A. F. Reithmeier, 1983). The effect of pH on the hydrodynamic behavior of calsequestrin (in the absence of SDS) revealed that the protein was a more asymmetric molecule at alkaline pH.

The molecular weight of calsequestrin has also been measured by gel filtration and analytical ultracentrifugation. The purified protein was eluted from a column of Sephadex G-200 at a position corresponding to a molecular weight of 42,000 (MacLennan and Wong, 1971). During purification, the protein was eluted from a similar column with a much higher apparent molecular weight. Caudwell *et al.* (1978) found a molecular weight of 200,000 by gel filtration, reasoning that the apparent large size of calsequestrin may be due to its asymmetric nature. Chromatography of the protein in 6 M urea or 4 M guanidine hydrochloride gave values of 55,000 (Ikemoto *et al.*, 1974) and 45,000 (R. A. F. Reithmeier, 1983), respectively. Sedimentation studies of the purified protein revealed that

the molecular weight of calsequestrin is 55,500 (Ikemoto *et al.*, 1974), 41,000 (Caudwell *et al.*, 1978), and 40,100 (R. A. F. Reithmeier, 1983).

Calsequestrin has a very elongated shape. Sedimentation analyses have shown that calsequestrin has a low sedimentation coefficient $s_{20,w} = 1.96$ (Caudwell *et al.*, 1978), $s_{20,w} = 2.20$ (R. A. F. Reithmeier, 1983) in the absence of Ca^{2+} at neutral pH. Calsequestrin has a high intrinsic viscosity ($[\eta] = 27.2$ ml/g (R. A. F. Reithmeier, 1983), which corresponds to an axial ratio of greater than 20 (Van Holde, 1971). The high intrinsic viscosity is similar to the values obtained for proteins denatured to a random-coil conformation by guanidine hydrochloride or synthetic polymers such as poly- γ -benzylglutamate in the coil form (Van Holde, 1971). Circular dichroism studies of calsequestrin (Ostwald *et al.*, 1974; Ikemoto *et al.*, 1974; R. A. F. Reithmeier, unpublished observations) have shown that calsequestrin, in the absence of Ca^{2+} , has a random-coil conformation with only 11% α -helix. Addition of guanidine hydrochloride converts the protein to a completely random coil conformation (Ikemoto *et al.*, 1974; R. A. F. Reithmeier, 1983).

One of the most dramatic properties of calsequestrin is its ability to undergo extensive conformational changes upon binding divalent or even monovalent metals (Han and Benson, 1971; Ikemoto *et al.*, 1972, 1974; Ostwald *et al.*, 1974). These conformational changes have been measured in a variety of ways, including changes in ultraviolet-difference absorption spectra, intensity of tryptophan fluorescence, and circular dichroism.

The changes in ultraviolet absorption and the increase in tryptophan fluorescence can be observed when di- or trivalent cations are bound to the protein in the presence of 0.1 M KCl. The changes in absorption and fluorescence appear to be due to transfer of aromatic residues from the polar surface of the protein to a hydrophobic interior. This process is accompanied by an increase in α -helix, which can be measured by circular dichroism. The protein free of salt has about 11% α -helix. After binding ions, this is increased to 20%, but at pH 9.5, the α -helix content can go as high as 30%. Thus the protein undergoes changes from an extended coil to a more compact structure. This change can be detected by gel filtration (Reithmeier, 1983).

Conformational changes can apparently be induced in calsequestrin by Ca^{2+} , even in the presence of SDS gel electrophoresis buffer (Campbell *et al.*, 1983a). Addition of calcium to the sample buffer caused calsequestrin to move more rapidly, thereby changing its apparent molecular weight from 63,000 to 60,000.

Calsequestrin precipitates in the presence of Ca^{2+} concentrations that are greater than about 1 mM (MacLennan and Wong, 1971; Ikemoto *et al.*, 1971). It is not known whether the Ca^{2+} -calsequestrin complex has a

regular crystal structure or whether it is merely a cross-linked aggregate.

The problems in measuring the molecular weight are probably related to the unusual shape of the protein, making comparisons to globular proteins unreliable. The conformation of calsequestrin in SDS is also quite different from that of other denatured proteins, being highly extended with an abnormally low α -helical content (Reithmeier, 1983). It is apparent that an accurate measurement of the molecular weight of calsequestrin may have to await completion of the amino acid sequence of the protein.

VI. AMINO ACID SEQUENCE

Only a small portion of the amino acid sequence of calsequestrin is known (Reithmeier and Cozens, 1982). The amino-terminal sequence of rabbit skeletal muscle calsequestrin is shown in Fig. 2. Rat skeletal muscle calsequestrin has an identical sequence except for the substitution of aspartic acid for glutamic acid at the second residue (Reithmeier *et al.*, 1980). There is a cluster of acidic residues at the amino-terminus. This is followed by a fairly hydrophobic sequence (residues 11-37) rich in tyrosine and containing only one negatively charged group. Another cluster of acidic residues follows. No sequences equivalent to high-affinity calcium-binding sites of the EF hand configuration (Kretsinger, 1976) are apparent, although residues 4-8 are homologous to a sequence in the second calcium-binding site of calmodulin (Vanaman, 1980). Because calsequestrin binds about one Ca^{2+} per 1000 daltons of its mass, there must be 1 calcium binding site every 9 or 10 amino acids. Two carboxyl groups could bind a single Ca^{2+} with additional binding energy provided by uncharged oxygens or nitrogens (Williams, 1977). Calsequestrin is composed of over one-third acidic amino acids, which are sufficient to account for the capacity of this protein to bind Ca^{2+} . The protein does not contain any γ -carboxyglutamate residues (K. P. Campbell, unpublished observations).

A second form of calsequestrin was detected by its more rapid mobility in SDS gels run at neutral pH (MacLennan, 1974). The two forms cannot

<u>Glu</u>	<u>Glu</u>	Gly	<u>Leu</u>	<u>Asp</u>	<u>Phe</u>	<u>Pro</u>	<u>Glu</u>	Tyr	<u>Asp</u> ¹⁰
Gly	Val	Asn	Arg	Val	Ile	Asn	Val	Asn	Ala ²⁰
Ile	Asn	Tyr	Tyr	Lys	Asn	Val	Phe	Lys	Tyr ³⁰
<u>Glu</u>	Val	Leu	Ala	Leu	Leu	Tyr	—	<u>Glu</u>	Pro ⁴⁰
—	<u>Glu</u>	<u>Asp</u>	—	Lys	Ala				

Fig. 2. Amino terminal sequence of rabbit muscle calsequestrin. Acidic residues are underlined. The boxed sequences are homologous to calcium binding site 2 in calmodulin.

be resolved in SDS gels run at alkaline pH. The two forms of the protein have identical amino-terminal and carboxyl-terminal sequences (Reithmeier and Cozens, 1982). The amino acid composition of the two forms are similar. Tryptic peptide maps of the two forms were identical except for two to three peptides. These results suggest that the two forms of calsequestrin have an almost identical amino acid sequence and that the difference between the two forms resides in an internal sequence. The two forms of the protein bind similar amounts of Ca^{2+} . Rabbits may contain one form or the other as well as both forms in equal amounts. Genetic studies have indicated a complex pattern of inheritance for the protein.

VII. CARBOHYDRATE CONTENT

Calsequestrin is a glycoprotein, and the earliest amino acid composition data showed the presence of glucosamine in both forms of the protein (MacLennan and Wong, 1971; MacLennan, 1974). A careful analysis of the sugar composition of the purified protein from rabbit skeletal muscle showed that it contained 52 moles of glucosamine and 76 moles of mannose per milligram of protein (Jorgensen *et al.*, 1977). This would be equivalent to one complex of 3 glucosamine: 5 mannose per protein molecule with a molecular weight of 66,000 or 2 glucosamine: 3 mannose based on a molecular weight of 41,000. Either complex would result from extensive processing of the dolichol intermediate that is initially attached to glycoproteins (Liu *et al.*, 1979).

VIII. STAINS-ALL STAINING

One of the interesting properties of calsequestrin is its dark blue or purple staining upon binding the cationic carbocyanine dye "Stains-All" (Campbell *et al.*, 1983a,b), a staining property originally attributed to sialoglycoprotein. This property, when combined with the change in mobility of the protein when two-dimensional SDS gel electrophoresis is carried out, makes it possible to identify calsequestrin readily in a mixture of proteins (Campbell *et al.*, 1983b).

Dark blue staining with Stains-All is not unique to calsequestrin or to proteolytic fragments of calsequestrin, which also stain blue with Stains All. A series of calcium binding proteins, such as troponin, calmodulin, and S-100, all stain deep blue in the presence of the dye. This simple staining test might prove very valuable for the identification of all calcium

binding proteins in complex mixture such as whole membranes (Campbell *et al.*, 1983a,b).

IX. PROPERTIES OF CALSEQUESTRIN IN OTHER MUSCLES

Calsequestrin was first found in rabbit skeletal muscle. It has been noted, but has not been studied in great detail, in other species (Louis and Irving, 1974). Yap and MacLennan (1976) isolated the protein from chicken breast muscle and found that it bound Ca^{2+} but with a lower capacity than in rabbit skeletal muscle. This protein from chicken breast muscle has been studied in more detail by Campbell *et al.* (1983b). Its mobility is identical to rabbit calsequestrin in neutral gels, but in alkaline gels, its mobility corresponds to a molecular weight of 55,000. This protein, unlike rabbit calsequestrin, appears to be a "high mannose" glycoprotein because its molecular weight is reduced by about 1000 upon incubation with endo- β -*N*-acetylglucosaminidase-H, an enzyme that specifically cleaves high mannose carbohydrate groups (Arakawa and Muramatsu, 1974).

Calsequestrin has been identified in sarcoplasmic reticulum isolated from a slow-twitch muscle (Zubrzycka-Gaarn *et al.*, 1982). Although present in lower amounts, its properties, such as apparent molecular weight changes at different pH values, blue staining with Stains All, and immunoreactivity, were identical to those of calsequestrin isolated from a fast-twitch muscle.

It has been unclear whether calsequestrin is a component of cardiac sarcoplasmic reticulum. The failure to identify calsequestrin in this tissue resulted in part from the fact that preparations of cardiac sarcoplasmic reticulum contain so many proteins and, in part, to the fact that there is a limited ability of antisera prepared against skeletal muscle calsequestrin to cross react with cardiac calsequestrin. The key to proving the existence of a cardiac form of calsequestrin came from the observation by Campbell *et al.* (1983a) that calsequestrin stained deep blue with Stains All. Using this test, a protein with a molecular weight of 55,000 at alkaline pH or with a molecular weight of 44,000 at neutral pH was observed in cardiac sarcoplasmic reticulum (Campbell *et al.*, 1983b). This protein was purified by precipitation with calcium phosphate followed by chromatography on DEAE cellulose. The protein resembled chicken breast muscle calsequestrin in apparent molecular weight and in being a high mannose glycoprotein. It was very acidic, but its amino acid composition differed in several respects from that of rabbit skeletal muscle calsequestrin. It appeared to bind less Ca^{2+} than the rabbit muscle variety.

In spite of the fact that calsequestrin from different sources has a variable apparent molecular weight in the Laemmli gel system (e.g., 66,000, rat; 63,000, pig; 55,000, chicken, cardiac), it has a rather constant apparent molecular weight of about 44,000 in the Weber and Osborn gel system.

Calsequestrin has been reported to be absent from lobster muscle sarcoplasmic reticulum (Deamer, 1973). When this preparation was examined with Stains All, blue staining was not observed in the region containing molecular weights of 50,000 to 60,000. However, there was very strong blue staining in the region below 20,000 (K. P. Campbell and D. H. MacLennan, unpublished observations). This would suggest one of two possibilities. Either there are low molecular weight Ca^{2+} -binding proteins that replace calsequestrin in lobster muscle or else calsequestrin in the preparation is degraded before or during electrophoresis. Purified preparations of rabbit skeletal-muscle calsequestrin contain a proteolytic activity that can be inhibited by phenylmethylsulfonyl fluoride. This activity, if not inhibited, will rapidly degrade calsequestrin at elevated temperatures. Indeed, these degradation products of calsequestrin were initially thought to represent low molecular weight Ca^{2+} -binding proteins of sarcoplasmic reticulum (MacLennan *et al.*, 1972; Ostwald and MacLennan, 1974; MacLennan, 1975). It is possible that this proteolytic activity is exceptionally high in lobster microsomal preparations.

X. PHOSPHORYLATION OF CALSEQUESTRIN

There are reports in the literature that calsequestrin is not only a substrate for phosphorylation by endogenous kinases (Varsanyi and Heilmeyer, 1979; Campbell and Shamoo, 1980) but that calsequestrin is a kinase itself (Varsanyi and Heilmeyer, 1980). Moreover, calsequestrin has been reported to contain bound phosphate when isolated (Varsanyi and Heilmeyer, 1980; Meissner *et al.*, 1973). It is not known whether the phosphate is stably bound or is rapidly turning over.

Campbell and MacLennan (1983) used 8- N_3 -ATP as a photoaffinity probe for ATP-binding proteins in the sarcoplasmic reticulum. This probe clearly did not bind to calsequestrin as would be expected if calsequestrin were a kinase. Campbell and MacLennan (1982) discovered a calmodulin-dependent kinase system in the sarcoplasmic reticulum that phosphorylated a protein with a molecular weight of 60,000. Under conditions where this protein was phosphorylated, calsequestrin contained only very low levels of ^{32}P , which could have been accounted for by nonspecific binding. Because the 60,000 molecular weight protein runs close to calsequestrin in the Laemmli gel system, it could have been mistaken for calseque-

trin in earlier reports, if care were not taken to separate the two proteins. Even though endogenous calsequestrin is not a substrate for the calmodulin-dependent protein kinase in intact vesicles (Campbell and MacLennan, 1982), isolated calsequestrin can serve as a substrate for the kinase. The solubilized kinase had to be mixed with soluble calsequestrin in order to demonstrate calsequestrin phosphorylation (H. Takisawa and D. H. MacLennan, unpublished observations).

XI. LOCALIZATION OF CALSEQUESTRIN

From their observation that the protein was only released after disruption of the membrane by detergents and high salt, MacLennan and Wong (1971) deduced that calsequestrin was localized in the interior of the sarcoplasmic reticulum. Confirmation of this view was provided by the work of Meissner (1975), who separated fragmented sarcoplasmic reticulum into heavy and light vesicles by sucrose density-gradient centrifugation. The heavy vesicles were enriched in calsequestrin, contained an internal matrix, and retained the capacity for binding more than 200 nmole of Ca^{2+} per milligram of protein; light vesicles were free of calsequestrin and of internal matrix and bound less than 100 nmole of Ca^{2+} per milligram of protein. Therefore, Meissner (1975) proposed that the internal matrix was comprised of calsequestrin or of a Ca^{2+} -calsequestrin complex.

Meissner (1975) also proposed that, because matrix material was found in the terminal cisternae of sarcoplasmic reticulum but not in the longitudinal elements, calsequestrin would be localized in the terminal cisternae. This hypothesis was proven by the work of Jorgensen *et al.* (1979), who used immunofluorescent staining of cryostat sectioned rat skeletal muscle to show that calsequestrin fluorescence was confined to the region of the A-I junction, whereas ATPase fluorescence was found throughout the sarcomere. More recent studies by Jorgensen *et al.* (1983), using immunoferritin labeling, have shown that a fraction of calsequestrin is also located in vesicular structures in the central region of the I band.

The finding that calsequestrin is localized in the interior of the sarcoplasmic reticulum and is concentrated in the terminal cisternae suggests not only that calsequestrin acts to lower luminal-free Ca^{2+} concentration but that it also influences the localization of Ca^{2+} in the membrane system. If calsequestrin is localized in the terminal cisternae, then Ca^{2+} should also be localized in that region. In fact, Winegrad (1968) showed that Ca^{2+} was highly concentrated in the terminal cisternae at rest and was released from that region. These observations have been extended and quantitated in electron probe analysis of Ca^{2+} localization before and after excitation in both skeletal and cardiac muscle (Somlyo *et al.*, 1981;

Chiesi et al., 1981). These authors have shown that Ca^{2+} is, indeed, localized in the terminal cisternae in resting muscle and is released from that area during a tetanus. A major function of calsequestrin, then, may be to concentrate Ca^{2+} at the region where it is most rapidly released following passage of an electrical impulse through the transverse tubular membrane.

High resolution morphological studies of the terminal cisternae provide fascinating information on the possible organization of calsequestrin near the junctional region. Fibrous material is seen to be running from internal membrane surfaces in the area near the "feet" into the lumen of the terminal cisternae (Franzini-Armstrong, 1980). The fibrous strands appear to interact with the matrix formed by calsequestrin. These morphological studies suggest a possible physical continuity between the Ca^{2+} -calsequestrin complexes and the fibrous structures that extend into, and possibly through, the membrane in the region where connections between sarcoplasmic reticulum and the electrically excitable T system occur.

When heavy sarcoplasmic reticulum vesicles were isolated in sucrose medium, they retained about 120 nmoles of Ca^{2+} per milligram of protein. A fibrous network was observed to have coalesced in a region adjacent to the "feet" (Campbell *et al.*, 1980). The network that contained calsequestrin, probably in a complex with Ca^{2+} , was readily disrupted by washing in salt, and it was not found when heavy vesicles were prepared in salt. Washing in concentrated monovalent salts reduced the Ca^{2+} content of the heavy sarcoplasmic reticulum vesicles, probably by competition for Ca^{2+} in the Ca^{2+} -calsequestrin complex. Thus, the structure seemed to depend on the association of calsequestrin with Ca^{2+} . This suggests that a physical continuity is formed between the Ca^{2+} -calsequestrin complex and the membranous elements associated with the feet structures. The physiological significance of this association is not clear.

The view that calsequestrin is localized in the lumen of the sarcoplasmic reticulum is not supported by all of the data available. Several membrane probes have been used to localize the protein. MacLennan *et al.* (1972) first showed that calsequestrin was labeled by lactoperoxidase in intact membranes. They discounted this as strong evidence that the protein was externally located (MacLennan and Holland, 1975), because the iodide ion can be mobile and, unless the interior of the membrane is highly reducing, it can label interior structures as well as external structures. The ease with which calsequestrin is iodinated might suggest that at least some of it is very closely associated with the inner surface of the sarcoplasmic reticulum membrane. Thorley-Lawson and Green (1973) reported similar observations, but they concluded that calsequestrin was externally located. Tume (1979) also reported that calsequestrin was labeled by

lactoperoxidase, but he found that the iodination was dependent on the co-factors included in the reaction mixture. In the presence of Ca^{2+} , Mg^{2+} and ATP, calsequestrin was not labeled. Tume (1979) proposed that calsequestrin may change its position in the membrane depending on the external environment. An alternate suggestion would be that the permeability of the membrane to one or more elements of the iodination system was altered. King and Louis (1976) found that lactoperoxidase, immobilized by covalent attachment to Sepharose 4B, was much less effective in labeling calsequestrin than was free lactoperoxidase. By contrast, externally located portions of the ATPase were labeled equally by both reagents. We have found (K. P. Campbell and D. H. MacLennan, unpublished data; Zubrzycka-Gaarn *et al.*, 1982) that calsequestrin in intact vesicles is heavily iodinated by an immobilized lactoperoxidase-glucose oxidase system that is commercially available (Bio Rad).

Other probes have been used for calsequestrin. Antibodies against calsequestrin do not aggregate sarcoplasmic reticulum vesicles, whereas antibodies against the ATPase do (Stewart *et al.*, 1976). Trypsin, which digests the ATPase, does not digest calsequestrin in intact vesicles (Stewart and MacLennan, 1974; Michalak *et al.*, 1980). The nonpenetrating reagent diazotized diiodosulfanilic acid labels the ATPase but not calsequestrin (Yu *et al.*, 1976). Calsequestrin in vesicles reacts with fluorescamine dissolved in acetone (Hasselbach *et al.*, 1975). When fluorescamine was bound to the large, impenetrable complex cycloheptaamylose, however, it did not label calsequestrin (Hidalgo and Ikemoto, 1977; Michalak *et al.*, 1980), suggesting that labeling with fluorescamine alone resulted from penetration of the reagent into the vesicles.

The fact that EDTA releases calsequestrin from vesicles (Duggan and Martonosi, 1970; Thorley-Lawson and Green, 1973) might suggest that it is externally located. However, it is important to recognize that treatment with EDTA greatly increases the permeability of the membrane, even to large molecules (Duggan and Martonosi, 1970), probably through removal of divalent metals from the membrane structure. The further effect of EDTA to dissolve the Ca^{2+} -calsequestrin complexes would then result in efflux of calsequestrin. Zubrzycka *et al.* (1978) used Chelex-100 to bind Ca^{2+} at the surface of vesicles and did not observe calsequestrin release. Presumably less membrane disruption occurred under these conditions.

Cross-linking experiments also bear on the location of calsequestrin. Louis *et al.* (1977) and Louis and Holroyd (1978) have studied cross-linking with dimethyl 3,3'-dithiobispropionimidate dihydrochloride and with cupric phenanthroline. They concluded that ATPase molecules were within 2 Å of each other, that calsequestrin and/or high affinity calcium-

binding protein were within 1.1 nm of each other, and that there was no indication of cross-linking between the ATPase and calsequestrin, suggesting that they were separated physically by large distances.

In summary, the internal matrix localization of calsequestrin seems well established, but its links with the membrane, particularly in the region of the feet structures, is an area that requires further investigation.

XII. BIOSYNTHESIS

The biosynthesis of calsequestrin has been studied in both cell culture and in *in vitro* systems. Because calsequestrin is a major extrinsic protein localized in the matrix and the Ca^{2+} -ATPase is an intrinsic protein comprising the bulk of the protein in the sarcoplasmic reticulum membrane, it was of interest to compare and contrast the synthesis and incorporation of these two proteins into the same membrane.

In studies of differentiating primary, rat skeletal muscle myoblasts in culture, calsequestrin synthesis was undetectable in undifferentiated cells during the first day of culture (Zubrzycka and MacLennan, 1976; Michalak and MacLennan, 1980). The synthesis of the protein was detectable at 40 hr of culture, well before the cells had begun to fuse and well before the initiation of synthesis of the ATPase (Holland and MacLennan, 1976) and other proteins whose onset of synthesis is correlated with cell fusion (Shainberg *et al.*, 1971). This biochemical finding was confirmed by immunofluorescent labeling of the ATPase and calsequestrin in developing myoblasts (Jorgensen *et al.*, 1977). Calsequestrin immunofluorescence was observed at a much earlier stage than was ATPase immunofluorescence. Moreover, calsequestrin immunofluorescence was at first confined to a small perinuclear region, which is rich in Golgi and rough endoplasmic reticulum. With time, the immunofluorescent pattern spread towards the poles of the cells and eventually was found throughout the fused myotubes. By contrast, ATPase immunofluorescence developed later, and it was found in foci throughout the cell, even at the earliest stages, where it was seen in mononucleated as well as in multinucleated cells.

These studies suggested that the ATPase and calsequestrin were synthesized noncoordinately and, moreover, that their pathways of incorporation into the same membrane probably followed different routes (MacLennan *et al.*, 1978). Knowledge of the pathways of membrane biosynthesis (reviewed in MacLennan *et al.*, 1978) suggested that both the

ATPase and calsequestrin would be synthesized on membrane-bound polysomes. However, since the sarcoplasmic reticulum seemed to “grow” out of rough endoplasmic reticulum (Ezerman and Ishikawa, 1967), it was suggested that the newly synthesized ATPase would remain in the membrane bilayer and would be laterally displaced and concentrated at a “growing point” between the rough endoplasmic reticulum and the smooth sarcoplasmic reticulum. Calsequestrin would enter luminal spaces, where it would be glycosylated, and then perhaps move in the luminal space to the Golgi apparatus where sugars would be processed. The protein would eventually be “packaged” and transported through the cell to be deposited into the terminal cisternae regions of the developing sarcoplasmic reticulum.

Parts of the hypothesis have been proven. Calsequestrin is synthesized on membrane-bound polyribosomes (Greenway and MacLennan, 1978; Reithmeier *et al.*, 1980) as is the ATPase (Greenway and MacLennan, 1978; Chyn *et al.*, 1979; Reithmeier *et al.*, 1980). Calsequestrin is synthesized with an NH₂ terminal signal sequence that has methionine at positions 1, 7, and possibly 14. By contrast, mature calsequestrin does not contain any methionine within the first 14 residues (Reithmeier *et al.*, 1980). Calsequestrin is a glycoprotein implying that it was glycosylated in the lumen of the rough endoplasmic reticulum (Hanover and Lennarz, 1980). The sugar is trimmed in the endoplasmic reticulum and may undergo further processing in the Golgi region (Harpaz and Schachter, 1980). Until the exact form of the carbohydrate in calsequestrin is defined, it will not be possible to decide how it has been processed by Golgi-specific enzymes, however. The route through which calsequestrin might pass after leaving the Golgi apparatus is, as yet, undefined, but it is likely that coated vesicles (cf. Rothman and Fine, 1980) would be involved in its transport to its final destination. The notion that calsequestrin is actively deposited in the terminal cisternae could account for the regional localization of this protein within what is normally considered to be a continuous space.

In chicken and rabbit skeletal muscle differentiating *in vivo*, calsequestrin was found at early stages of development (Martonosi, 1975; Sarzala *et al.*, 1975; Zubrzycka *et al.*, 1979). Calsequestrin was identified in the microsomal fraction isolated from muscle of newborn rabbit by its ability to bind Ca²⁺ and to react with an antibody prepared against adult rabbit muscle calsequestrin (Sarzala *et al.*, 1975; Zubrzycka *et al.*, 1979).

The studies of calsequestrin biosynthesis show that this is a potentially interesting protein for continued investigation of the ways in which a cell creates its own internal architecture.

XIII. CALSEQUESTRIN IN DISEASE

The sarcoplasmic reticulum has been analyzed for lesions in a number of disease states. In some cases, calsequestrin has been specifically analyzed. Yap and MacLennan (1976) were unable to detect any differences between calsequestrins isolated from dystrophic and from normal chicken breast muscle. The disease, muscular dysgenesis, (Gluecksohn-Waelsch, 1963; Bowden-Essien, 1972) is one in which the sarcoplasmic reticulum has been implicated, because excitation-contraction coupling is severely impaired in the diseased tissue. The rate of synthesis of calsequestrin was studied using immunoprecipitation, and the localization of calsequestrin was studied by immunofluorescence in differentiating cultures of cells isolated from neonatal dysgenic mice (Essien *et al.*, 1977). The synthesis and localization of the protein was found to be normal (Essien *et al.*, 1977).

Etienne *et al.* (1980) analyzed the turnover of sarcoplasmic reticulum proteins in dystrophic chicken muscle cells in culture. They found that a 65,000 dalton protein, tentatively identified as calsequestrin, underwent a significantly increased rate of decay when compared to normal tissue. In light of the observation (Campbell *et al.*, 1983a) that the molecular weight of chicken calsequestrin is less than 65,000, it is unlikely that the protein whose turnover was increased was, in fact, calsequestrin.

Kahn *et al.* (1982) have used antibodies against calsequestrin and the Ca²⁺-ATPase in the diagnosis of childhood rhabdomyosarcoma. Since calsequestrin synthesis is turned on earlier in differentiation than other muscle specific proteins (Zubrzycka and MacLennan, 1976; Jorgensen *et al.*, 1977), it might be predicted that it would be a better marker than other proteins for the identification of partially differentiated muscle cells. The incidence of calsequestrin accumulation in tissues tentatively identified as rhabdomyosarcomas was not higher than that observed for other muscle specific proteins. Thus calsequestrin does not appear to be an especially good marker for this disease.

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