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Isolation of a Calcium-Sequestering Protein from Sarcoplasmic Reticulum

(rabbit/deoxycholate/column chromatography/transport)

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ABSTRACT **An acidic protein has been extracted from sarcoplasmic reticulum with KCl and deoxycholate. The protein, which remains soluble after extraction, has been highly purified by fractionation on DEAE-cellulose, Sephadex, and hydroxylapatite. It has a molecular weight of 44,000 and contains 392 amino acid residues per molecule, of which 146 are either glutamic or aspartic acid. No phosphorus, sialic acid, or lipid has been detected in the preparation. The protein has been shown to bind up to 970 nmol of Ca^{++} per mg (43 mol/mol) at pH 7.5, with an apparent dissociation constant of 4×10^{-5} M. Preliminary data indicate that the protein is unique to sarcoplasmic reticulum and that it is hydrophobically bonded on the interior of these vesicles. The protein is believed to play a role in sequestering calcium within sarcoplasmic reticulum. The name Calsequestrin is suggested for the protein.**

Contraction and relaxation of muscle is controlled by the free Ca^{++} content of the tissue (1–4). Relaxation is initiated when Ca^{++} is transported into sarcoplasmic reticulum, where it is sequestered; contraction is initiated by the release of Ca^{++} from these storage points. The sites of Ca^{++} binding in sarcoplasmic reticulum have not yet been identified.

A Ca^{++}-activated ATPase has been shown to be part of the Ca^{++}-transport system of sarcoplasmic reticulum, since the activity of this enzyme can be correlated with Ca^{++} transport (5–7). We have purified the ATPase (8, 9); it consists of a single protein, of molecular weight 102,000, and an additional 45,000 daltons of lipid. The enzyme catalyzes a (Ca^{++} plus Mg^{++})-dependent ATP hydrolysis, as well as an ATP-ADP exchange, and it is phosphorylated in the presence of Ca^{++}. The three activities are purified 3- to 3.5-fold over the activities found in sarcoplasmic reticulum; on this basis, we estimate that ATPase protein makes up some 30% of the total protein of sarcoplasmic reticulum. The ATPase is a membrane-forming enzyme (9), but the membranes are not capable of storing Ca^{++} to any extent (8). In fact, ATPase membranes have virtually no capacity, other than active-site titration, to bind Ca^{++} in a form resistant to washing with isotonic saline. The ATPase may, therefore, fulfill the role of a transport protein, but it does not appear to provide binding sites for Ca^{++} in the interior of the vesicle.

The facts that Ca^{++}-binding sites have not been identified in sarcoplasmic reticulum, and that the ATPase enzyme does not seem to contain a significant number of Ca^{++}-binding sites, led us to expand our study of the components of the calcium-transport system of sarcoplasmic reticulum to include a study of calcium-binding sites. In this communication, we report the isolation of a protein whose properties lead us to believe that it is a major binding site for calcium in sarcoplasmic reticulum. We propose that the protein be designated Calsequestrin.

MATERIALS AND METHODS

Preparation of the calcium-sequestering protein

Step 1. Sarcoplasmic reticulum was prepared from two 14.4-kg albino rabbits and was extracted with deoxycholate as described (8), except that dithiothreitol (1 mM) was present during the extraction and the supernatant, after centrifugation at 165,000 $\times g$, was retained as the source of the binding protein. The deoxycholate extract (about 80 ml) was dialyzed, first for 4 hr and then for 18 hr at 12°C, against 3 liters of 5 mM Tris·HCl, pH 7.5. At 4 and 22 hr, insoluble protein was removed by centrifugation at 165,000 $\times g$ for 30 min.

Step 2. The supernatant solution (35- to 40-ml aliquot) was passed through a 1.5 $\times$ 18 cm column of DEAE-cellulose and the column was eluted with 400 ml of 5 mM Tris·HCl, pH 7.5, that contained a linear gradient of KCl from 0 to 0.7 M. The last peak to be eluted, at a KCl concentration of about 0.48 M, was collected, concentrated by ultrafiltration under pressure (Amicon Diaflo apparatus) to 8 ml, and dialyzed overnight against a solution of 0.15 M KCl–0.01 M Tris·HCl, pH 8.0.

Step 3. The dialyzed sample was passed through a 2.5 $\times$ 40 cm column of Sephadex G-200 equilibrated with 0.15 M KCl–0.01 M Tris·HCl, pH 8.0. Material absorbing at 280 nm was eluted in a sharp peak at the void volume, in a second symmetrical peak, and in a tail following the second peak. Fractions making up the second symmetrical peak were collected, concentrated by Diaflo filtration to 10 ml, and dialyzed overnight against 10 mM potassium phosphate, pH 7.0.

Step 4. The dialyzed sample was applied to a column (7 g) of hydroxylapatite (Biogel HTP) equilibrated with 10 mM potassium phosphate pH 7.0. The column was eluted with 400 ml of a linear gradient of potassium phosphate from 10 to 600 mM. The last peak to be eluted, at a phosphate concentration of about 330 mM, was concentrated to 5 ml and dialyzed overnight against a solution of 5 mM Tris·HCl, pH 7.5. The solution, containing purified protein (about 1 mg per ml), was stored at −20°C.

Analytical methods

Calcium transport was measured (10) at a protein concentration of 200 μg/ml. After 10 min at 24°C, 0.5 ml was filtered

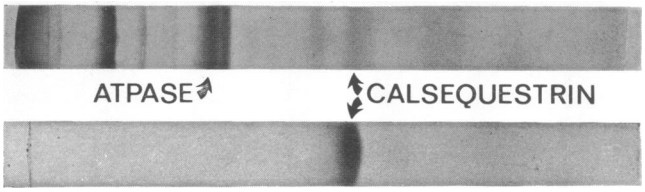

FIG. 1. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The *upper gel* is sarcoplasmic reticulum, the *lower gel* is purified Calsequestrin.

through a 0.3-μm Millipore filter. The filter was washed with 5 ml of 0.15 M NaCl and examined in a scintillation spectrometer. Ca^{++} binding was also measured by equilibrium dialysis. In a standard assay, 0.4 mg of protein in 0.4 ml was dialyzed for 40 hr at 12°C against 100 ml of a solution of 5 mM Tris·HCl (pH 7.5)–0.1 mM $CaCl_2$, to which was added $^{45}Ca^{++}$ (to a specific activity of 25–40 cpm/nmol). Samples were dissolved in "Aquasol" (New England Nuclear) and counted in a scintillation spectrometer. Protein was determined by the Lowry method (11). Under these conditions, binding of calcium was exactly proportional to protein concentration over a range of 0.25–1.0 mg/ml.

Molecular weight was measured by gel electrophoresis (12) and by gel filtration on Sephadex G-200 equilibrated with 150 mM KCl–10 mM Tris·HCl, pH 8.0 (13). Sialic acid content was measured (14) after digestion of samples in 0.1 N H_2SO_4 at 80°C for 60 min, or by the method of Svennerholm (15). Phosphorus was measured by the method of Chen *et al.* (16). Amino acid analyses were performed (17) on a Beckman 120C automatic amino acid analyser. Cysteine and methionine were analyzed as cysteic acid and methionine sulfone after performic acid oxidation (18).

RESULTS AND DISCUSSION

Table 1 shows the amount of Ca^{++} bound by sarcoplasmic reticulum and by the ATPase enzyme derived therefrom. In the presence of ATP, sarcoplasmic reticulum bound about 80 nmol of Ca^{++} per mg of protein. This Ca^{++} was not removed by washing with 0.15 M NaCl. On the other hand, the ATPase enzyme in membranous form (9) bound only 6 nmol of Ca^{++} per mg of protein that was resistant to washing with 0.15 M NaCl. The ATPase membranes may have been leaky and readily lost Ca^{++} ions stored inside, or the ATPase enzyme may lack calcium-binding sites other than those of high affinity at the active site of the enzyme [equivalent to 10 nmol/mg of protein (9)]. If the ATPase enzyme lacked calcium-binding sites, then these sites might be found in fractions removed from the ATPase during purification. Ca^{++} binding was measured for these soluble fractions.

TABLE 1. *Binding of Ca^{++} by sarcoplasmic reticulum and ATPase*

Binding system	nmol/mg of Ca^{++} bound by: Sarcoplasmic reticulum	ATPase
Complete	1880	9
−oxalate	80	6
−ATP, −oxalate	1	1

The complete reaction mixture contained, in 1.0 ml, 20 μmol histidine (pH 7.5), 50 μmol KCl, 5 μmol $MgCl_2$, 0.5 μmol ethyleneglycolbis(β-aminoethylether)-*N,N'*-tetraacetate, 0.5 μmol $^{45}CaCl_2$, 5 μmol potassium oxalate, and 5 μmol ATP. The reaction was begun by the addition of 200 μg of protein and stopped after 10 min at 24°C by filtration of 0.5 ml on a 0.3-μm Millipore filter. The filter, which retained protein and protein-bound $^{45}Ca^{++}$, was washed with 5 ml of 0.15 M NaCl and counted in a scintillation spectrometer. The ATPase enzyme was prepared (8) and converted to the membranous form (9).

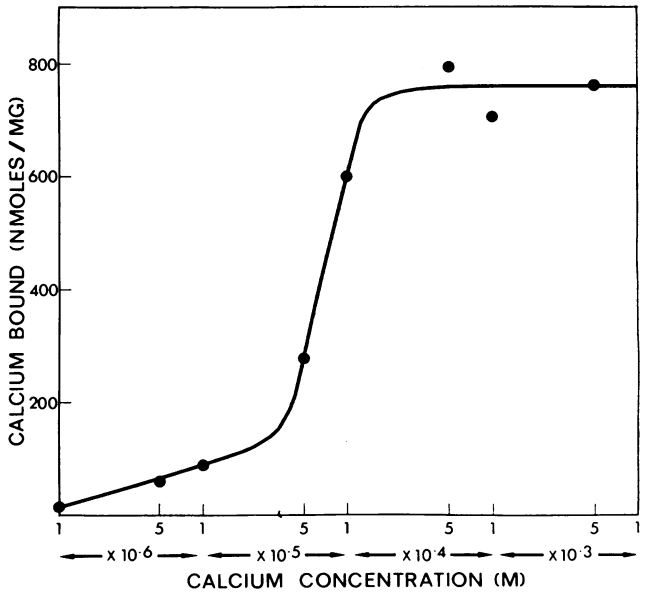

FIG. 2. Binding of Ca^{++} by Calsequestrin, as a function of the concentration of $CaCl_2$, in the presence of 5 mM Tris·HCl, pH 7.5.

Purification of the calcium-sequestering protein

In the first step of purification of the ATPase enzyme, sarcoplasmic reticulum was extracted with deoxycholate (9). The ATPase remained insoluble: Ca^{++}-binding activity was released into the soluble fraction. Binding activity remained soluble after removal of deoxycholate (Table 2). The binding activity was purified with a single protein during sequential passage through DEAE-cellulose, Sephadex G-200, and hydroxylapatite. The protein was strongly anionic; it was eluted

TABLE 2. *Recovery of protein and binding capacity during purification of Calsequestrin*

Fraction	Total protein (mg)	Total binding (nmol Ca^{++})	Total binding (%)	Specific binding (nmol Ca^{++}/mg)
Soluble extract	171	23,600	100	138
DEAE-cellulose eluate	40	19,250	82	481
Sephadex eluate	15	7,750	33	518
Hydroxylapatite eluate	6	3,750	16	625

Binding was measured by equilibrium dialysis. Protein samples (0.4 mg in 0.4 ml) were dialyzed for 40 hr against 100 ml of a solution of 0.1 mM $^{45}CaCl_2$–5 mM Tris·HCl, pH 7.5.

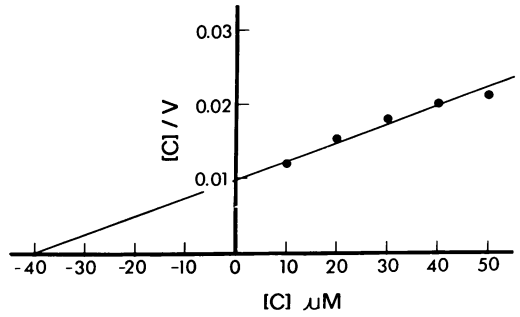

FIG. 3. Binding of Ca^{++} by Calsequestrin as a function of the concentration of $CaCl_2$ in the presence of 5 mM Tris·HCl, pH 7.5.

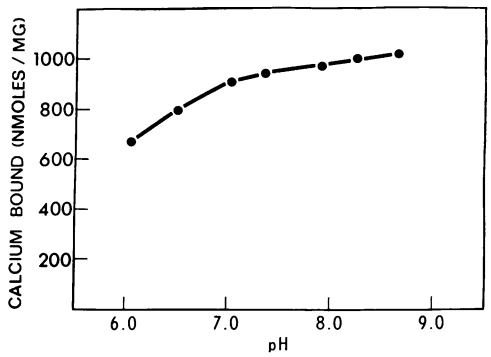

FIG. 4. Binding of Ca^{++} by Calsequestrin exposed to 0.1 mM $^{45}CaCl_2$ in the presence of 5 mM imidazole or Tris buffers of the indicated pH.

from DEAE-cellulose and hydroxylapatite only at very high salt concentration. The purity of the final product is attested to by the gel pattern shown in Fig. 1. The purified protein moved with an electrophoretic mobility identical to that of a major protein component observed in polyacrylamide gels of sarcoplasmic reticulum. About 7% of the Coomassie blue stain taken up was absorbed by this component in polyacrylamide gels of sarcoplasmic reticulum; hence, we estimate that Calsequestrin makes up about 7% of the total protein of sarcoplasmic reticulum.

Ca^{++}-binding properties

Binding of between 625 and 835 nmol of Ca^{++} per mg of protein, in the presence of 5 mM Tris·HCl (pH 7.5) and 0.1 mM $^{45}CaCl_2$, was routinely observed (Table 2, Fig. 2). Maximal binding of 970 nmol/mg of protein has been observed under these conditions. In the presence of Ca^{++} concentrations greater than 10^{-4} M, specific Ca^{++} binding reached a constant value, usually about 800 nmol/mg of protein; however, the protein became insoluble. We believe that this insolubility was due to a cross-linking of protein molecules by the divalent Ca^{++}. The insoluble complex was readily dissolved by dilution or by dialysis to remove calcium. Binding was half-maximal at about 5×10^{-5} M Ca^{++} (Fig. 2). Reciprocal plots (19) for Ca^{++}-binding activity are shown in Fig 3; a dissociation constant of 4×10^{-5} M was calculated.

Below pH 7.5, the amount of Ca^{++} bound fell rather sharply (Fig. 4). The ATPase activity of sarcoplasmic reticulum was also found to decrease rapidly at pH values below 7.5 (8). These data suggest that both calcium binding and ATP-energized Ca^{++} transport are decreased as pH values are lowered. Such conditions should lead to increased Ca^{++} release from the sarcoplasmic reticulum. The release of calcium has been reported to decrease at low external pH when the ATP supply is used up (20). However, the effect of pH changes *in vivo* in the presence of adequate ATP may differ from the effects described *in vitro*.

In vitro binding was sensitive to proteinases such as Nagarse and trypsin, but was relatively insensitive to sulfhydryl reagents and to heat (Table 3). Only a 20% loss in binding was observed after the protein was boiled for 4 min, and no precipitation was observed. Binding was strongly inhibited by equimolar concentrations of Sr^{++} and Cd^{++}. Sr^{++} is transported by sarcoplasmic reticulum (21), while Cd^{++} is a strong inhibitor of the ATPase enzyme (8). Mg^{++} and Mn^{++} were relatively ineffective competitors of Ca^{++} binding at equimolar concentrations. At concentrations near physiological, KCl, $MgCl_2$, and ATP all strongly inhibited binding of Ca^{++} to the protein. In order for the protein to play a physiological role in calcium sequestration, therefore, it would have to be screened from these ions, or else Ca^{++} would have to be present at the binding site at concentrations greater than 10^{-4} M.

Physical properties

The molecular weight of Calsequestrin, measured by mobility in polyacrylamide gels impregnated with sodium dodecyl sulfate (12), was 44,000. The molecular weight of the protein estimated by Sephadex gel filtration under nondissociating conditions (13) was about 42,000. The protein, therefore, appears to exist as a monomer in aqueous solution.

The protein was strongly anionic, as judged by its pattern of elution from DEAE-cellulose. Nevertheless, it was found to be devoid of phosphorus and to contain no material soluble in chloroform–methanol 2:1. No sialic acid was detected by the method of Warren (14) and less than 20 nmol of sialic acid

TABLE 3. *Effect of various treatments on Ca^{++} binding*

	Treatment	Binding (nmol/mg)	Inhibition %
1.	None	720	—
	Nagarse (1 mg/mg)	93	87
	Trypsin (1 mg/mg)	275	62
	Mersalyl (10^{-6} M)	620	14
	N-Ethylmaleimide (10^{-3} M)	590	18
2.	None	835	—
	60°C, 4 min	810	4
	100°C, 4 min	670	20
3.	None	530	—
	$CdCl_2$ (10^{-4} M)	90	83
	$SrCl_2$ (10^{-4} M)	218	59
	$MgCl_2$ (10^{-4} M)	420	20
	$MnCl_2$ (10^{-4} M)	550	0
4.	None	690	—
	KCl (150 mM)	60	91
	$MgCl_2$ (5 mM)	61	91
	ATP (2 mM)	7	99
	KCl, $MgCl_2$, ATP	23	97

Binding was measured as in Table 2, except that other reagents were added to the dialysis buffer as indicated. Digestive enzymes were present within the dialysis bag; heating of protein was done before dialysis.

TABLE 4. *Amino acid composition of Calsequestrin*

Amino acid	g/100 g	Minimum residues per molecule
Lysine	7.53	26
Histidine	1.66	6
Arginine	2.43	7
Aspartic acid	19.40	74
Threonine	2.44	11
Serine	2.70	14
Glutamic acid	21.19	72
Proline	4.35	20
Glycine	1.87	15
Alanine	4.18	26
Cysteic acid	0.58	3
Valine	6.00	27
Methionine sulfone	1.70	6
Isoleucine	5.18	20
Leucine	8.90	35
Tyrosine	2.12	6
Phenylalanine	7.75	24
		392

per mg of protein was detected by the method of Svennerholm (15). The bulk of the acidity could be accounted for by glutamic and aspartic acid residues in the protein (Table 4). Of the 392 residues making up the peptide chain (molecular weight 44,000), glutamic and aspartic acid together made up 146 residues (or 37% of the total). Arginine and lysine, on the other hand, made up a total of only 33 residues. Ammonia, which was present in acid hydrolysates in very low amounts, could have masked only a few of the glutamic and aspartic acid residues. After accounting for internal charge negation, the net acidic residues were calculated to be 113 or about 1 acidic residue per 3.5 residues. If two carboxyl groups bound one calcium ion, then each mole of protein could bind about 56 mol of calcium. On this basis, one mg of protein could potentially bind 1270 nmol of Ca^{++}. This figure has been approached experimentally (see Fig. 4).

Localization of Calsequestrin

We have been unable to isolate Calsequestrin from the cytoplasmic solution from which sarcoplasmic reticulum was isolated. We have also been unable to detect the protein in troponin, prepared according to the method of Yasui, Fuchs, and Briggs (22), or in deoxycholate extracts of bovine heart mitochondria. The protein appears, therefore, to be uniquely located in the sarcoplasmic reticulum of muscle tissue.

The protein was not released from sarcoplasmic reticulum by sonication in the presence or absence of 1 M KCl. This fact would suggest that it is not electrostatically bonded on the exterior or interior of the vesicle. The protein was solubilized quantitatively by deoxycholate in the presence of 1 M KCl; this would suggest a hydrophobic bonding to the sarcoplasmic reticulum membrane. It was not removed from sarcoplasmic reticulum by extraction with 3.5 M NaBr, a chaotropic solution that will selectively remove headpieces from mitochondrial inner membranes (23) and that should remove hydrophobically bonded protein from exterior surfaces of reticulum membranes. These data suggest that the protein is hydrophobically bonded on the interior of sarcoplasmic-reticulum membranes. Once freed from the membrane, the protein behaves as a highly charged, soluble molecule.

Physiological role of Calsequestrin

The physiological role of the protein is not now known. Ca^{++}-binding sites resistant to salt washing and equivalent to at least 80 nmol/mg of protein are required to account for Ca^{++} sequestration in the sarcoplasmic reticulum (see Table 1). The ATPase has 10 high-affinity sites per mg of protein (9), but it accounts for only 30% of the protein of sarcoplasmic reticulum, therefore, for only about 3 nmol/mg of sarcoplasmic-reticulum protein. The remainder (about 77 nmol/mg) is probably bound elsewhere. Calsequestrin accounts for about 7% of the protein of sarcoplasmic reticulum. From calculations of the net negative charge (see *Physical properties*), Calsequestrin could bind 1270 nmol of Ca^{++} per mg, or 89 nmol/0.07 mg, a figure more than adequate to account for calcium binding by the reticulum. Environmental conditions such as salt, nucleotide concentrations and pH would obviously affect the binding. In this regard, we might consider the internal Ca^{++} concentration of sarcoplasmic reticulum. If we assume the protein content of sarcoplasmic reticulum to be 20% of the wet weight, the binding of Ca^{++} to be 80 nmol/mg of protein, and all Ca^{++} to be in free solution, the internal calcium concentration would be about 20 mM, well above the dissociation constant for binding. Sandow (1) has also estimated internal calcium concentrations to be 10–20 mM and, on the basis of the work of Carvalho (24, 25), he had concluded that 80% of this Ca^{++} is bound.

It is probably fair to say that there are many Ca^{++}-binding sites in and on sarcoplasmic reticulum. Cohen and Selinger (26) have recognized two types of ATP-independent calcium-binding sites, accounting for 50 nmol/mg of sarcoplasmic-reticulum protein. One type, accounting for 10% of the binding, was sensitive to trypsin but not to saline, while the other type of site was insensitive to trypsin but sensitive to salt concentration. Both sites, therefore, would appear to be external binding sites and may have little relevance to the calcium-sequestering function of sarcoplasmic reticulum. Carvalho (24, 25) has shown that anionic sites in the reticulum are competed for by various cations. Only in the presence of ATP was Ca^{++} selectively bound. This observation would suggest that it is the function of the transport ATPase to move Ca^{++} and concentrate it at sites where, by virtue of its high concentration, it will compete strongly with other cations for binding sites. These physiologically important binding sites would, presumably, be found interior to the ATPase enzyme and their affinity for calcium could be significantly less than the affinity of the ATPase for Ca^{++}, since the ATPase enzyme would maintain high internal calcium concentrations.

In recent years a number of "binding proteins" have been isolated from the periplasmic space of bacteria (27, 28), from mitochondria (29), and from intestinal epithelia (30). These proteins are believed to play a role in transport or in concentrating substrate at the transport site. Calsequestrin is not a binding protein in the sense that the term has been used to describe these proteins. Calsequestrin differs in four major respects. (*a*) The molecular weight of Calsequestrin is greater than the 25,000–35,000 found for binding proteins. (*b*) The number of binding sites in Calsequestrin is far greater than the number of binding sites on binding proteins. These proteins commonly bind one molecule of substrate per molecule. (*c*) The dissociation constant for calcium binding to Calsequestrin is 4×10^{-5} M, while the dissociation constant for substrate binding to binding proteins is in the

range of 10^{-6} to 10^{-7} M. The dissociation constant for substrate binding is usually identical to the dissociation constant for substrate transport. In this case, however, the dissociation constant for the Ca^{++}-transport system is 10^{-7} to 10^{-8} M, while the dissociation constant for Ca^{++} binding is only 4×10^{-5} M. The calcium-binding function of Calsequestrin, therefore, could not aid in the process of lowering the cytoplasmic Ca^{++} concentration to the concentration of 10^{-7} M seen under conditions of muscle relaxation (1–4). The binding function could only be observed at high Ca^{++} concentrations, such as would be found in the interior of sarcoplasmic reticulum. (*d*) Calsequestrin is tightly bound to the membrane, while binding proteins are loosely bound or free in periplasmic spaces.

The apparent localization of Calsequestrin on the interior of the membrane and the ability of the protein to sequester Ca^{++} with a dissociation constant intermediate between that of the transport ATPase (less than 10^{-7} M) and the dissociation constant of a number of membrane preparations (10^{-3} M) (31) lead us to propose that the protein is a major site of calcium sequestration in the interior of sarcoplasmic reticulum membranes.

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