Rapid Purification of Calsequestrin from Cardiac and Skeletal Muscle Sarcoplasmic Reticulum Vesicles by Ca\textsuperscript{2+}-dependent Elution from Phenyl-Sepharose*

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Treatment of cardiac or skeletal muscle sarcoplasmic reticulum vesicles with 0.1 M sodium carbonate selectively extracts both the Ca\textsuperscript{2+}-binding protein calsequestrin and the two “intrinsinc glycoproteins,” while leaving the Ca\textsuperscript{2+}-dependent ATPase membrane bound. Phenyl-Sepharose chromatography in the presence of ethylene glycol bis(\(\beta\)-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA) and high salt (0.5 M NaCl) readily fractionates these solubilized proteins into a Ca\textsuperscript{2+}-elutable fraction, which contains purified calsequestrin, and a low ionic strength elutable fraction, which contains one of the two intrinsic glycoproteins. Elution of calsequestrin from phenyl-Sepharose occurs near 1 mM Ca\textsuperscript{2+}. Copurifying with calsequestrin are an homologous set of high molecular weight proteins, which like calsequestrin stain blue with Stains-All. These proteins are present in trace amounts and do not correspond to any sarcoplasmic reticulum proteins previously identified.

Elution of calsequestrin from phenyl-Sepharose is consistent with the Ca\textsuperscript{2+}-binding protein losing its hydrophobic character in the presence of millimolar Ca\textsuperscript{2+}. This behavior is converse to that observed for several calmodulin-like proteins, which are eluted from hydrophobic gels in the presence of EGTA. The high yield and purity of calsequestrin prepared by this method makes possible a unique system for studying what may be a distinct class of Ca\textsuperscript{2+}-binding proteins.

The myoplasmic Ca\textsuperscript{2+} concentration in cardiac and skeletal muscle cells undergoes rhythmic changes due in large part to the Ca\textsuperscript{2+}-handling properties of the SR.\textsuperscript{1} Ca\textsuperscript{2+} is believed to be actively transported into the SR by the Ca\textsuperscript{2+}-dependent ATPase, transiently stored therein by the relatively low affinity Ca\textsuperscript{2+}-binding protein calsequestrin, and subsequently released by a mechanism that has yet to be resolved.

Calsequestrin is a high capacity, low affinity Ca\textsuperscript{2+}-binding protein first identified in and purified from skeletal muscle (1, 2). It is observable in electron micrographs as an electron-dense matrix inside the terminal cisternae of SR (3-6), a subcellular localization supported by membrane biochemical studies (3, 6). The purified skeletal muscle protein exhibits an apparent \(M_r = 63,000\) (7) as determined by SDS-PAGE according to the method of Laemmli (8). Electron microscopic studies suggest that calsequestrin is also present in junctional SR in cardiac tissue (9); however, direct identification of the putative cardiac protein by standard biochemical methods has proven to be difficult. This is probably due to the much more complex protein composition of isolated cardiac SR vesicles, as compared to skeletal muscle vesicles (10). Jones et al. (11) first observed a prominent protein band in isolated cardiac SR vesicles which stained a characteristic dark blue with Stains-All, and which migrated with an apparent \(M_r = 56,000\) in the Laemmli gel system. More recently, Campbell et al. (7) have purified this cardiac protein and have demonstrated rather convincingly that it is the homolog of the 63-kDa form of calsequestrin observed in skeletal muscle. Both calsequestrins bind large amounts of Ca\textsuperscript{2+} and have similar acidic amino acid compositions and molecular weights; moreover, they are immunologically cross-reactive (7). In analogy to earlier biochemical studies conducted with skeletal muscle tissue (3), subcellular fractionation studies conducted with cardiac tissue also suggest that calsequestrin is localized in the junctional SR (12, 13).

Biochemical comparisons between cardiac and skeletal muscle calsequestrins have been limited in the past because no standard method exists for rapid and easy purification of the protein from either tissue. Most purification procedures reported to date are lengthy and involve an initial extraction of SR membrane proteins with detergent (1, 2, 7). In this paper, we describe a method for the rapid purification of calsequestrin to near homogeneity from both cardiac and skeletal muscle microsomal preparations following two simple steps. The essence of the procedure involves a specific Ca\textsuperscript{2+}-dependent elution of calsequestrin from phenyl-Sepharose. Copurifying with calsequestrin by this method are an apparently homologous set of higher molecular weight proteins, present in trace amounts, which have not been described heretofore. The Ca\textsuperscript{2+}-dependent elution of all of these proteins from phenyl-Sepharose represents the converse of the way in which several calmodulin-like Ca\textsuperscript{2+}-binding proteins have recently been purified.

EXPERIMENTAL PROCEDURES

Preparation of SR Vesicles and Extraction of Calsequestrin with Sodium Carbonate—Crude cardiac and skeletal muscle SR membrane vesicles were prepared from both canine ventricles and rabbit fast

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The abbreviations used are: SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(\(\beta\)-aminoethyl ether)-N,N,N’,N’-tetraacetic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Purification of Calsequestrin by Hydrophobic Chromatography — The carbonate supernatant fraction obtained as described above was made to 50 mM MOPS, 500 mM NaCl, and 1 mM dithiothreitol by addition of concentrated HCl. This mixture was then applied to a phenyl-Sepharose column (approximately 2 mg of protein/ml of gel) which was equilibrated with Buffer A (10 mM MOPS, 0.1 mM EGTA, 500 mM NaCl, 1 mM dithiothreitol, and 0.02% NaN₃, pH 7.0). The column was subsequently washed with several volumes of Buffer A. Ca²⁺-dependent elution of calsequestrin was then performed with a 100 mM Na₂CO₃ (pH 11.4) at a protein concentration of 0.25 M n-methylmannoside (data not shown).

Sodium Carbonate Extraction of SR Vesicles — Treatment of either cardiac or skeletal SR vesicles with 0.1 M Na₂CO₃ extracted 20-25% of the total membrane protein into the supernatant fraction. SDS-PAGE revealed that calsequestrin was quantitatively solubilized from both preparations by this treatment (Fig. 1A). Other SR proteins of interest extracted by carbonate included a 52- to 53-kDa glycoprotein, present in both cardiac (7, 11, 19) and skeletal SR (16, 20), as well as a 127- to 130-kDa Stains-All blue cardiac glycoprotein (7, 11), and a 160-kDa Stains-All blue skeletal glycoprotein (21) (Fig. 1A). Carbonate treatment did not solubilize the 100-kDa Ca²⁺-dependent ATPase in either membrane fraction (Fig. 1A). The minor protein band occurring at this mobility range in the cardiac supernatant fraction stained blue with Stains-All and, therefore, was probably not the Ca²⁺ pump protein, which stains pink (12).

The glycoprotein nature of several of the carbonate-solubilized SR proteins was confirmed by ¹²⁵I-concanavalin A binding to a Western blot of the protein fractions shown in Fig. 1A. Analogous sets of three concanavalin A-binding proteins were released by carbonate from both cardiac and skeletal muscle vesicles (Fig. 1B). The 53- and 130-kDa proteins from the cardiac membranes and the 53- and 160-kDa proteins from the skeletal membranes labeled by ¹²⁵I-concanavalin A corresponded to the so-called “intrinsic” SR glycoproteins (7, 21). Cardiac SR vesicles showed several additional concanavalin A-binding proteins which were not extracted by carbonate (Fig. 1B). The ¹²⁵I-labeled protein migrating at 63-kDa in the skeletal supernatant fraction corresponded to skeletal muscle calsequestrin, which has previously been reported to bind concanavalin A weakly (16). Binding of concanavalin A to the 55-kDa cardiac calsequestrin was also observed (Fig. 1B), which has not been reported previously, although the glycoprotein nature of the protein is known (7). In previous studies, we used periodic acid-Schiff staining to identify the glycoprotein nature of the protein.
the 52- to 53-kDa and 127- to 130-kDa glycoproteins in cardiac preparations (11, 19). In subsequent studies, we have observed
that cardiac calsequestrin also stains with Schiff’s reagent, although the staining is much less intense (data not shown).

Phenyl-Sepharose Chromatography of Calsequestrin—The carbonate solubilized fractions were applied to phenyl-Sepharose as described under “Experimental Procedures.” In the presence of Buffer A containing 0.5 M NaCl and no calcium (0.1 mM EGTA), most of the protein (approximately 75%) bound to the column and was not removed by up to 10 column volumes of Buffer A. Upon the addition of 10 mM CaCl2, a sharp peak of protein was eluted (Fig. 1). SDS-PAGE of this fraction, obtained from either cardiac or skeletal muscle carbonate supernatants, showed a single protein band corresponding to calsequestrin (Fig. 3). We have verified the identity of calsequestrin isolated by this method by direct measurement of Ca2+ binding to the protein using equilibrium dialysis. Cardiac calsequestrin bound approximately 650 nmol of Ca/mg of protein and skeletal muscle calsequestrin bound approximately 900 nmol of Ca/mg of protein, when Ca2+-binding was performed under saturating conditions (data not shown). Best results for the purification procedure were obtained by eluting calsequestrin with 10 mM CaCl2. Application of a Ca2+ gradient caused calsequestrin to elute more diffusely as a broad protein peak beginning near 1 mM CaCl2 (data not shown). In other studies, we have observed that calsequestrin is not eluted at concentrations of CaCl2 of 0.7 mM or lower. Interestingly, we have also observed a qualitatively identical elution using 10 mM MnCl2; 19 mM MgCl2 was not effective. Similar purification of calsequestrin was also achieved with use of phenothiazine-Sepharose. We observed no binding of calsequestrin to hexyl-, heptyl-, or octyl-Sepharose (data not shown).

Copurifying with either cardiac or skeletal muscle calsequestrin through phenyl-Sepharose chromatography was an interesting set of apparently homologous proteins which represented only about 1–2% of the total protein content (as determined by Coomassie blue staining and densitometric scanning of the purified protein fractions), and which also stained blue with Strains-All. Fig. 4 illustrates these proteins with an SDS gel grossly overloaded with respect to calsequestrin. A set of as many as eight or more high molecular weight proteins of apparent molecular masses about 110- to 160-kDa (cardiac) and about 120- to 180-kDa (skeletal) coeluted with calsequestrin. These proteins are, however, separable from calsequestrin. These proteins co-chromatograph with calsequestrin on DEAE-Sephacel and on concanavalin A-Sepharose, and preliminary data show an immunological cross-reactivity with calsequestrin. These proteins are, however, separable from calsequestrin using gel filtration on Sepharose 4B suggesting that they are not bound to calsequestrin. Moreover, following their separation from calsequestrin by gel filtration and subsequent rechromatography of calsequestrin through phenyl-Sepharose, these high molecular weight proteins are not observed. Thus, they do not appear to be artifacts of the purification method or of SDS-PAGE.

Interestingly, for the cardiac preparation, we have found a high degree of similarity between these copurifying proteins and calsequestrin beginning with the fact that these proteins, like calsequestrin, all stain blue with Stains-All. In addition, these proteins co-chromatograph with calsequestrin on DEAE-Sephacel and on concanavalin A-Sepharose, and preliminary data show an immunological cross-reactivity with calsequestrin. These proteins are, however, separable from calsequestrin using gel filtration on Sepharose 4B suggesting that they are not bound to calsequestrin. Moreover, following their separation from calsequestrin by gel filtration and subsequent rechromatography of calsequestrin through phenyl-Sepharose, these high molecular weight proteins are not observed. Thus, they do not appear to be artifacts of the purification method or of SDS-PAGE.

Another set of proteins was subsequently eluted from phenyl-Sepharose when Buffer A was replaced by buffer containing no added salt (10 mM MOPS, pH 7.0) (Figs. 2 and 3). Enriched in this salt-free fraction were the 130- and 160-kDa cardiac and skeletal muscle glycoproteins, respectively (Fig. 3). The 53-kDa glycoproteins in either tissue were not recovered in this fraction and apparently remained bound to the column. The protein in this molecular weight range eluting in the cardiac fraction (Fig. 3) did not correspond to the glycoprotein and was the same as the lower protein of the doublet visualized in both Figs. 1 and 3. We have determined that it most probably represents a mitochondrial protein contaminant (data not shown).
Purification of Calsequestrin by Hydrophobic Chromatography

**DISCUSSION**

The data presented in this study show that calsequestrin exhibits a Ca\(^{2+}\)-dependent elution from phenyl-Sepharose that leads to its rapid purification from both cardiac and skeletal muscle SR vesicles. Preparation of membranes, extraction of proteins with sodium carbonate, and elution of purified calsequestrin from phenyl-Sepharose can be accomplished within a single day. The yield of calsequestrin from cardiac SR vesicles, approximately 4 mg/g of membrane protein, is about four times the value recently reported by Campbell et al. (7). The characteristic interactions of both cardiac and skeletal muscle calsequestrins with phenyl-Sepharose provide further evidence that they are similar proteins with similar functions in both tissues.

Phenyl-Sepharose hydrophobic chromatography of calsequestrin exhibits converse behavior to that recently observed for several calmodulin-like proteins (22-24). Of the large number of proteins that constitute the calmodulin family, calmodulin, troponin C, and glial S100 protein all exhibit Ca\(^{2+}\)-dependent binding to hydrophobic matrices and EGTA-dependent elution (22-24). The name “calcimedins” has been suggested for this class of Ca\(^{2+}\)-binding proteins (25). For these proteins, particularly for calmodulin, it has been relatively well established that the binding of Ca\(^{2+}\) leads to a conformational change that exposes a hydrophobic site, which in turn is capable of interacting with appropriate receptor sites on other proteins as well as with certain amphipathic molecules (26, 27). By analogy, it would appear as if the hydrophobic moieties on calsequestrin interacting with phenyl-Sepharose are made inaccessible in the presence of Ca\(^{2+}\). To our knowledge, this peculiar Ca\(^{2+}\)-induced change in protein structure observed for calsequestrin distinguishes this protein from all other Ca\(^{2+}\)-binding proteins that have so far been described.

The Ca\(^{2+}\)-induced change in protein conformation described for calsequestrin in the present study could have physiological significance. One possibility is that the apparent association of calsequestrin with the junctional inner membrane described by others (5, 28) could vary as the Ca\(^{2+}\) concentration inside the SR changes during Ca\(^{2+}\) uptake and Ca\(^{2+}\) release. Such a dynamic interaction of calsequestrin with the SR membrane might provide a way for Ca\(^{2+}\) to traverse the junctional membrane during coupling of excitation to contraction. Alternatively, proposed interactions between calsequestrin and other proteins specifically localized to junctional SR, such as the junctional “feet” proteins (5, 28), could be regulated by Ca\(^{2+}\)-binding to calsequestrin. It is possible that the set of high molecular weight proteins copurifying with calsequestrin through phenyl-Sepharose could participate in such interactions. Recent antibody studies conducted in our laboratory suggest that these high molecular weight proteins are also localized at the junctional SR in the heart (data not shown). Thus, calsequestrin could take on a more active role in excitation-contraction coupling than has been considered previously. Although purely speculative at present, such interpretations are consistent with models for calmodulin interactions with hydrophobic portions of its many target proteins and also with observations made for glial S100 protein, which has been shown to penetrate liposomal membranes after binding Ca\(^{2+}\) and thereby to promote movement of ionic charge (29).

In this study, it was also observed that 0.1 M sodium...
carbonate extracted the two intrinsic glycoproteins as well as calsequestrin from cardiac and skeletal muscle SR vesicles. The sodium carbonate treatment of SR membrane vesicles most probably converted closed vesicles to open membrane sheets causing release of their luminal contents as reported by Fuziki et al. (14). Alkaline disruption of SR with release of calsequestrin has been noted previously (30). However, we are not aware of any studies in which the two intrinsic glycoproteins have been solubilized without the use of detergents. The nature of the apparent dissociation of these proteins from the membrane is still being investigated.

In conclusion, a number of carbonate-solubilized proteins from SR vesicles bind to phenyl-Sepharose and thus exhibit some hydrophobic character. Only calsequestrin and a homologous set of trace high molecular weight proteins elute in the presence of millimolar Ca++ . The binding of calsequestrin to phenyl-Sepharose is therefore a convenient system for studying its hydrophobic properties and may also prove useful in elucidating the possible role of calsequestrin as a transducer of Ca++ signals during the changing ionic milieu that accompanies the cardiac cycle.

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S E Cala and L R Jones

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