THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 4, Issue of February 25, pp. 1317-1326, 1980
Printed in U.S.A.

Localization of the High Affinity Calcium Binding Protein and an Intrinsic Glycoprotein in Sarcoplasmic Reticulum Membranes*

(Received for publication, June 21, 1979)

Marek Michalak,‡ Kevin P. Campbell,§ and David H. MacLennan

From the Banting and Best Department of Medical Research, Charles H. Best Institute, University of Toronto, Toronto, Ontario, Canada M5G 1L6

Several proteins in sarcoplasmic reticulum preparations move in a band with a mobility, in sodium dodecyl sulfate-polyacrylamide gels (0.1 M phosphate buffer, pH 7.0), corresponding to a molecular mass of about 55,000 daltons. Only one of these proteins is the high affinity calcium binding protein. An intrinsic glycoprotein is also present in this band, and it is this glycoprotein which is found in vesicles reconstituted after dissolution of sarcoplasmic reticulum in deoxycholate. Both of these proteins are found in rather constant ratios with the ATPase in light, intermediate, and heavy sarcoplasmic reticulum vesicles. Transverse tubular vesicles can be isolated from the heavy sarcoplasmic reticulum vesicles after disruption of the membrane in a French pressure cell (Lau, Y. H., Caswell, A. H., and Brunschwig, J. P. (1977) *J. Biol. Chem.* 252, 5565-5574). These vesicles are enriched in their content of the high affinity calcium binding and depleted of the intrinsic glycoprotein.

Cycloheptaamylose•fluorescamine complex (CFC) labels the intrinsic glycoprotein heavily indicating that it is at least partially exposed on the cytoplasmic surface of sarcoplasmic reticulum membranes. Since the carbohydrate component of the protein must lie in luminal spaces, it is inferred that the intrinsic glycoprotein is a transmembrane protein. The high affinity calcium binding protein is not labeled by CFC indicating that it is not exposed on the cytoplasmic surface of sarcotubular vesicles. The protein is also not affected by proteolytic digestion of sarcoplasmic reticulum vesicles and can be isolated intact from trypsin-digested vesicles. It is not removed from sarcoplasmic reticulum vesicles by washing with buffers containing Chelex 100 or ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA). These data show that the high affinity calcium binding protein is localized in the interior of the sarcotubular system and suggest that it might be common to both sarcoplasmic reticulum and transverse tubular membranes.

In initial investigations of the protein composition of rabbit skeletal muscle sarcoplasmic reticulum, five major protein bands were observed in SDS[1]-polyacrylamide gel electrophoresis (1). Proteins contained in four of these bands, the ATPase, M_r = 100,000 (2), the proteolipid, M_r = 12,000 (3), calsequestrin, M_r = 44,000 (4), and the high affinity calcium binding protein, M_r = 55,000 (5), have been highly purified. There is only a preliminary report of purification of material at 30,000 daltons (6). There has been increasing evidence that many of the bands observed in SDS gels contain more than a single component.

In order to understand the function of each of the sarcoplasmic reticulum proteins, their structure and localization must also be known. Much of the work related to the structure, function, and localization of the ATPase and calsequestrin has been reviewed (1, 7). The data have supported the view that the ATPase is solely responsible for calcium transport while calsequestrin acts as a calcium buffer on the interior of the membrane.

The localization of the high affinity calcium binding protein is less clear cut and its function is unknown. Many studies have suggested that its location is the same as that for calsequestrin. It is not dissociated from the membrane by chaotropic salts (3, 5); it is released by chelators at a more rapid rate than calsequestrin (8), but this could be explained by the different capacities and affinities for calcium binding of these two proteins (5); it is not reactive with antibodies acting at the exterior of the membrane (9); and it appears to be only lightly labeled with diazotized diiodosulfanilic acid (10). The protein did not appear to be digested when membranes were exposed to trypsin (11). The protein, however, was not isolated and shown to be intact. Ikemoto *et al.* (12) were unable to resolve proteins of 44,000 and 55,000 daltons in their disc gel electrophoretic analysis of their preparation of sarcoplasmic reticulum. However, electrophoresis of these membranes in our laboratory confirmed the presence of protein of 44,000 daltons corresponding to calsequestrin and 55,000 daltons corresponding to the high affinity calcium binding protein (5). Attempts were not made to purify the high affinity calcium binding protein from this preparation of sarcoplasmic reticulum.

Other studies have indicated possible differences in binding of calsequestrin and 55,000-dalton protein to the membrane. When the sarcoplasmic reticulum was dissolved in detergent and reconstituted, protein of 55,000 daltons rebound to the

* This research was supported by Grant MT-3399 from the Medical Research Council of Canada and by a grant from the Muscular Dystrophy Association of Canada. This is the 12th paper in a series on "Resolution of Enzymes of Biological Transport." The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Postdoctoral fellow of the Muscular Dystrophy Association of Canada on leave of absence from the Department of Biochemistry of Nervous System and Muscle, Nencki Institute of Experimental Biology, Warsaw, Poland.

§ Postdoctoral fellow of the Medical Research Council of Canada.

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; ATPase, Ca^{2+} + Mg^{2+}-dependent adenosine triphosphatase; CFC, cycloheptaamylose•fluorescamine complex; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; R_1W, sarcoplasmic reticulum vesicles (2).

membranes, whereas calsequestrin did not (13, 14). It was not clear whether the localization of the rebound protein was affected during this manipulation, nor was it known whether or not the rebound protein was the high affinity calcium binding protein.

Sarcoplasmic reticulum has been fractionated into light and heavy components by a variety of procedures (15–18). Sarzala and Michalak (15) carried out differential centrifugation on muscle extracts. The heavy fraction precipitated between 8,000 and 30,000 × *g* and was further purified as a band between 30 and 37% sucrose on density gradient centrifugation; the light fraction precipitated between 30,000 and 100,000 × *g* on differential centrifugation and was further purified as a band between 25 and 30% sucrose on density gradient centrifugation. Meissner (17) fractionated muscle extracts on two successive density gradients. Light, intermediate, and heavy fractions were defined as banding at 28 to 32, 32 to 39, and 39 to 43% sucrose, respectively. Lau *et al.* (18) separated the membrane fraction precipitating between 10,000 and 95,000 × *g* on a sucrose density gradient and defined light and heavy fractions as those banding between 30 and 33% sucrose and between 39 and 41% sucrose, respectively. Heavy fractions were disrupted in a French pressure cell and separated into a transverse tubule-enriched fraction banding at 24% sucrose and a terminal cisternae-enriched fraction banding at 39% sucrose.

Sarzala and Michalak (15) and Zubrzycka *et al.* (16) found that their light vesicles were enriched in protein of 55,000 daltons, whereas the heavy vesicles were enriched in protein of 44,000 daltons. A variety of morphological and biochemical data suggested that the heavy vesicles were right side out while the light vesicles were inside out. Zubrzycka *et al.* (16) incubated these vesicles with Chelex 100 and found that 55,000-dalton material was released from the heavy vesicles, whereas 44,000-dalton protein was released from the light vesicles. These data were interpreted as indicating that protein of 55,000 daltons, including the high affinity calcium binding protein, was located on the cytoplasmic surface of the membranes, whereas calsequestrin was located in the interior of the vesicles.

Meissner (17) found that the major difference among his fractions was in their content of calsequestrin. Calsequestrin was enriched in the heavy fraction and depleted in the light fraction. He concluded that the light fraction represented elements of the longitudinal reticulum, while the heavy fraction represented the terminal cisternae. Lau *et al.* (18) confirmed these observations and, in addition, showed that the transverse tubule-enriched fraction was greatly enriched in a 55,000-dalton protein.

A function for the high affinity calcium binding protein has not been found. It binds calcium with an affinity comparable to that of troponin (5). This type of calcium binding is usually found in regulatory proteins, and this is the best clue to the function of the protein. However, were the high affinity calcium binding protein located internally where Ca^{2+} concentrations remain high, regulation would be obviated. The protein might act as a Ca^{2+} receptor or concentrator in the transport process, but this has not been demonstrated.

The present study was initiated to answer some of the outstanding questions concerning the localization and function of the high affinity calcium binding protein and other 55,000-dalton proteins associated with the sarcoplasmic reticulum.

METHODS

Preparations—Sarcoplasmic reticulum fractions were isolated from rabbit skeletal muscle by published procedures. These included: R_1W (2); light and heavy fractions (15); light, intermediate, and heavy fractions (17); and, light, heavy, terminal cisternae-enriched and transverse tubule-enriched fractions (18).

The Ca^{2+} + Mg^{2+}-dependent ATPase (2), calsequestrin (4), and the high affinity calcium binding protein (5) were isolated by published procedures. However, concentrated salts were removed at various stages in the preparations, by passage of samples through a column (2.5 × 45 cm) of Sephadex G-25 in 0.1 mM EGTA, 5 mM Tris-HCl, pH 7.5, instead of dialysis.

Desmin was prepared by the method of Lazarides and Hubbard (19). Tubulin, a gift from Dr. Victor Ling, University of Toronto, was prepared by the method of Shelanski *et al.* (20).

Preparation of High Affinity Calcium Binding Protein Antibody—The high affinity calcium binding protein was purified to homogeneity by the method of Ostwald and MacLennan (5). Antiserum was raised in a mature sheep by a series of three intramuscular injections (1 mg each) of the protein in Freund's complete adjuvant. The second injection was made after 3 weeks, the third injection at 5 weeks, and the first bleed at 7 weeks. The presence of antibody was detected by the double diffusion technique in 0.75% agarose gelled from a solution of 10 mM triethanolamine KOH, pH 7.0, 0.15 M KCl, 1 mM $MgCl_2$, and 0.01% NaN_3.

Reconstitution and Extraction of Sarcoplasmic Reticulum Proteins—Reconstitution of functional vesicles from deoxycholate extracts of sarcoplasmic reticulum was carried out according to the method described by Meissner and Fleischer (13). Reconstituted vesicles were washed three times by centrifugation in Buffer A before analysis. Chelex 100 and EGTA extraction of sarcoplasmic reticulum proteins was carried out by the method of Zubrzycka *et al.* (16) and of Duggan and Martonosi (8), respectively.

Digestion with Proteolytic Enzymes—Digestion of sarcoplasmic reticulum vesicles was carried out by the method described by Stewart and MacLennan (9). Tryptic digestion was carried out both in 1 M sucrose for 15 min and in 5 mM $CaCl_2$ and 100 mM KCl for 30 min at 32°C at a substrate to enzyme ratio of 10:1 (w/w). The reactions were stopped by the addition of phenylmethanesulfonyl fluoride (PMSF) to a final concentration of 1 mM. Samples were washed three times with 100 mM KCl, 10 mM Tris-HCl, pH 7.5, and pellets were subjected to gel electrophoresis.

Labeling with Cycloheptaamylose·Fluorescamine Complex (CFC)—CFC was prepared by the method of Nakaya *et al.* (21) and assayed by the method of Hidalgo and Ikemoto (22). Light and heavy sarcoplasmic reticulum (15) or R_1W (2) was suspended in 100 mM KCl, 20 mM Hepes, pH 7.5, at a protein concentration of 5 mg/ml. In some experiments, the following additions were made; Triton X-100, 1%; SDS, 1%; $CaCl_2$, 5 mM; EGTA, 1 mM. CFC was added to a concentration of 2.5 mg/ml, and the mixture was incubated at 37°C for 30 min.

For measurement of CFC incorporation into proteins, 50 μg of vesicle protein was separated on 12.5% polyacrylamide slab gels in SDS according to the method of Laemmli (23). After electrophoresis, the slabs were cut into two parts, one part being stained with Coomassie blue and destained exhaustively for ultimate measurement of the protein content of each band and the other, unstained part being used for measurement of CFC binding. Bovine serum albumin was separated in the same slab, stained with Coomassie blue and used as a standard for protein content. For protein concentration measurements, proteins were extracted from the gel by incubation at 37°C overnight in a solution of 0.1 N NaOH and 0.2% SDS. No color remained in the gel after this treatment. The extracts were neutralized with 0.1 N HCl before absorbance was read at 590 nm. With the albumin standard, a linear relationship was observed between protein applied to the gel and absorbance at 590 nm in the range between 1 and 10 μg of protein. This method of protein estimation does not necessarily provide absolute values for different proteins since it is dependent on the ability of the protein to bind Coomassie blue. However, it is reproducible for any given protein, thereby permitting the comparative estimates required in this study. A comparison of recovery of optical density for calsequestrin, high affinity calcium binding protein and albumin showed that the extent of Coomassie blue binding by the three proteins was essentially identical. For measurement of CFC binding, unstained bands were incubated overnight at 37°C in a solution of 1% ammonium bicarbonate and 0.1% SDS. No fluorescence remained in the gel after this treatment. Extracts were used directly to measure fluorescence emission at 465 nm with an excitation wavelength at 390 nm in an Aminco-Bowman spectrophotofluorometer. A baseline level of fluorescence emission was established using extracts of the gel where no protein bands were apparent.

SDS-polyacrylamide gel electrophoresis was carried out using both methods of Laemmli (23) and Weber and Osborn (24). Two-dimensional gels used to isolate calsequestrin were prepared by layering unfixed disc or slices of slab gels prepared by the method of Weber and Osborn directly on the top of the stacking gel in a Laemmli slab gel system. Agarose, 0.5% in Tris/glycine buffer, pH 6.8, was then hardened over and around the added gel, the system was equilibrated for 15 min, and electrophoresis was begun in the second dimension.

Identification of Glycoproteins in SDS-Polyacrylamide Gels—Glycoproteins in sarcoplasmic reticulum were detected after separation in Laemmli slab gels through their reaction with ^{125}I-concanavalin A (25). Laemmli slab gels, 0.75 mm thick, were transferred to a 20-cm square plastic dish and incubated overnight in a solution of 25% isopropanol and 10% acetic acid to remove SDS. The slabs were then freed of isopropyl alcohol and acetic acid by incubation in four changes of 500 ml of 0.5 M NaCl, 0.05 M NaH_2PO_4, pH 6.5, 0.01% NaN_3 (Buffer B). Protein bands were labeled with concanavalin A by incubation for 6 to 8 h in 200 ml of Buffer B containing about 10^8 cpm of ^{125}I-concanavalin A prepared by the chloramine-T method of Greenwood *et al.* (26) but using one-half of the chloramine-T concentration and extending the time of iodination to 60 s. Free ^{125}I-concanavalin A was then removed by washing the gel with several changes of Buffer B over a period of several hours. The gel was then stained with Coomassie blue, destained, and dried according to standard procedures. The dried gel was placed under x-ray film for 1 to 3 days, and autoradiographs were developed to locate the ^{125}I-concanavalin A bound to glycoprotein.

Since this procedure led to elution of extrinsic membrane proteins, in some cases (Fig. 7) slab gels were stained with Coomassie blue and destained before treatment with Buffer B. The stained bands became diffuse during this procedure but were not eluted from the gel.

Assays—Binding of cAMP to the regulatory subunit of protein kinase was assayed by the method of Gilman (27). Phosphorylase phosphatase was assayed by the method of Haschke *et al.* (28) using [^{32}P]phosphorylase. Phosphorylase was labeled with ^{32}P by the method of Krebs and Fischer (29) and was stored at 4°C in 50% saturated ammonium sulfate. Both substrate and enzyme were dialyzed for 2 h against 10 mM Tris-HCl, pH 7.5, to remove inhibitory salts. The reaction was stopped by the addition of trichloroacetic acid to 5% to precipitate protein, and the content of radioactivity in the supernatant was measured by Cerenkov counting. Pyruvate kinase was assayed by the method of Gutmann and Bernt (30). The reaction rate was followed by measuring the change in NADH absorbance at 340 nm using a Beckman DU_2 spectrophotometer with a circulating water bath set at 24°C. Protein was determined by the method of Lowry *et al.* (31).

RESULTS

Typical electrophoretic profiles of our preparation of sarcoplasmic reticulum (2) separated on SDS-polyacrylamide gels by the method of Weber and Osborn (24) showed that the ratio of Coomassie blue staining intensity of the protein bands at 44,000 daltons containing calsequestrin and at 55,000 daltons containing the high affinity calcium binding protein was about 2:1. In SDS-polyacrylamide gels, run according to the method of Laemmli (23), calsequestrin moved with a lower mobility corresponding to M_r = 63,000. The relative staining density between the band containing calsequestrin and the band containing the high affinity calcium binding protein now changed to about 5:1. These observations suggest that the protein bands are not homogeneous in one or the other of the gel electrophoretic systems.

Since the protein mobility patterns appeared different in the two gel systems, we have asked whether it would be possible to combine the two systems to obtain a two-dimensional separation of proteins associated with the sarcoplasmic reticulum. Fig. 1 shows the type of separation that can be obtained by separating sarcoplasmic reticulum proteins first in the Weber and Osborn system (Fig. 1*A*) and then in the Laemmli system (Fig. 1*B*). Proteins with the same mobility in the two systems fell on a diagonal line. The high affinity calcium binding protein was one of these proteins. However, there were at least four proteins in the same column as the high affinity calcium binding protein which run as one protein band in the Weber and Osborn system. There were no proteins to the right or left of the high affinity calcium binding protein indicating that separation in one dimension using the Laemmli system was adequate to purify the high affinity calcium binding protein. Calsequestrin, as expected, fell far from the diagonal and was purified in the process. The fact that proteins were apparent in the same column and in the same row as calsequestrin indicates that neither system alone was adequate to purify calsequestrin but that a combination of the two systems was very effective. A protein labeled IG for intrinsic glycoprotein fell off the diagonal but on the opposite side from calsequestrin. Other proteins were observed in the same column and in the same row as the intrinsic glycoprotein. Because it fell so far from the diagonal, however, we are confident that it was purified by the two-dimensional procedure.

The heterogeneity in the 55,000-dalton region of Weber and Osborn gel systems could also be demonstrated by examination of the proteins extracted from sarcoplasmic reticulum with deoxycholate and KCl and separated on DEAE columns (3). Fig. 2*A* shows that proteins of about 55,000 daltons were eluted in different fractions from DEAE. Only the most acidic of these was the high affinity calcium binding protein, yet, all moved in the same electrophoretic band in the Weber and Osborn system (24). When these proteins were subjected to electrophoresis in the Laemmli system (23), they were separated, each having a unique mobility (Fig. 2*B*).

Having obtained adequate separation of calsequestrin and the high affinity calcium binding protein from their contaminants, we found it possible to re-examine the question of how these unique proteins would respond to surface probes. We have used three different probing methods: we have bound fluorescamine conjugated to cycloheptaamylose (CFC) to the membrane surface, we have digested the membrane surface with proteolytic enzymes, and we have extracted the membrane surface with calcium chelators such as Chelex 100 and EGTA.

Labeling of Sarcoplasmic Reticulum Proteins with CFC—Experiments with CFC are described in Fig. 3. Fig. 3*A* shows the labeling pattern of intact sarcoplasmic reticulum vesicles with CFC. In the first dimension (Weber-Osborn system (24),

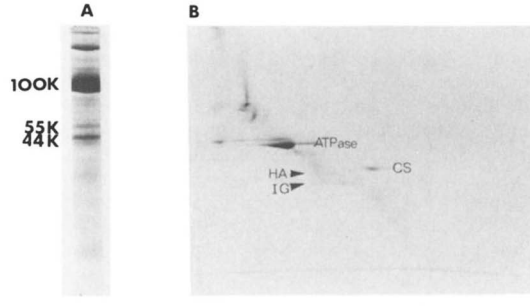

FIG. 1. **Two-dimensional SDS-gel electrophoresis of sarcoplasmic reticulum proteins.** Fifty micrograms of protein was separated first by SDS-disc gel electrophoresis according to Weber and Osborn (24). The unstained gel was then applied on the top of an SDS-slab gel prepared according to Laemmli (23). A 0.5% solution of agarose in 10 mM Tris/glycine buffer, pH 6.8, was poured around and over the gel, and it was equilibrated for 15 min and then developed by electrophoresis in the second dimension. *A*, first dimension disc gel electrophoresis of sarcoplasmic reticulum proteins according to Weber and Osborn (24). *B*, second dimension slab gel electrophoresis according to Laemmli (23). Note the separation of calsequestrin and of the protein labeled *IG* (intrinsic glycoprotein) due to the changes of mobilities in the two systems. Both gel systems contained 7.5% polyacrylamide. *CS*, calsequestrin; *HA*, high affinity calcium binding protein.

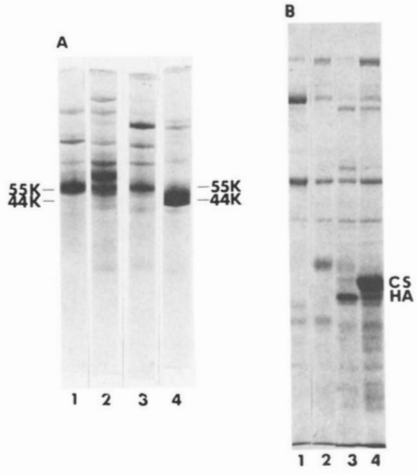

FIG. 2. **Fractionation of extrinsic sarcoplasmic reticulum proteins on a DEAE-cellulose column.** Sarcoplasmic reticulum was extracted with deoxycholate and KCl (2), and the soluble fraction was separated on DEAE-cellulose (4). *Fractions 1, 2, 3,* and *4* were eluted at concentrations of KCl corresponding to 0.20 to 0.25 M, 0.27 to 0.30 M, 0.32 to 0.34 M, and 0.40 to 0.50 M, respectively. They were concentrated, dialyzed, and analyzed by SDS-disc gel electrophoresis (7.5% polyacrylamide) according to the methods of Weber and Osborn (24) (*A*) and Laemmli (23) (*B*). Twenty micrograms of protein were applied on each gel. *CS*, calsequestrin, *HA*, high affinity calcium binding protein.

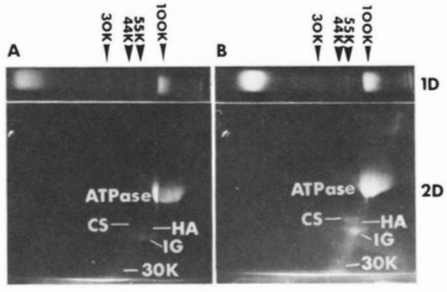

FIG. 3. **Two-dimensional gel electrophoresis of CFC-labeled sarcoplasmic reticulum.** Sarcoplasmic reticulum vesicles (5 mg of protein/ml) were incubated with cycloheptaamylose·fluorescamine complex (CFC, 2.5 mg/ml) at 37°C for 30 min in a solution containing 0.1 M KCl, 20 mM Hepes, pH 7.5, in the presence or absence of 1% SDS according to the method of Hidalgo and Ikemoto (22). Labeled proteins (100 μg) were separated by SDS-gel electrophoresis (7.5% polyacrylamide) first according to the method of Weber and Osborn (24) and then according to the method of Laemmli (23) (see the legend to Fig. 1); *A*, two-dimensional gel electrophoresis of CFC-labeled intact sarcoplasmic reticulum vesicles; *B*, two-dimensional gel electrophoresis of CFC-labeled sarcoplasmic reticulum solubilized with 1% SDS before labeling. *CS*, calsequestrin; *HA*, high affinity calcium binding protein; *IG*, intrinsic glycoprotein; *30K*, 30,000-dalton protein. The brightly staining band at the leading edge of the ATPase is phosphorylase.

the ATPase and the 55,000-dalton region were seen to be labeled. In the second dimension (Laemmli system (23)), the ATPase and the intrinsic glycoprotein were heavily fluorescent-labeled, but almost no label was detectable in either the high affinity calcium binding protein or calsequestrin. By contrast, all four proteins were heavily labeled when the labeling was carried out in the presence of SDS (Fig. 3*B*). In control experiments, we have determined that labeling of any of these four sarcoplasmic reticulum proteins with CFC has no observable effect on their mobility in one- or two-dimensional systems.

To quantitate the relative amount of CFC bound to each protein in the sarcoplasmic reticulum, labeled bands from intact and SDS-solubilized vesicles were eluted and assayed for protein and fluorescence intensity. These data are described in Table I for three preparations of sarcoplasmic reticulum vesicles: light and heavy fractions as defined by Sarzala and Michalak (15) and R_1W (2). The ATPase was labeled to the extent of about 30% of maximal in all preparations. Calsequestrin was not labeled in the heavy fraction and only to the extent of about 10% of maximal in the light fraction and in R_1W. The high affinity calcium binding protein was not labeled by CFC in the heavy fraction or in R_1W but was labeled to the extent of about 8% of maximal in the light fraction. The intrinsic glycoprotein was labeled to the extent of about 62% of maximal in R_1W. A 30,000-dalton protein was present only in the heavy fraction and in R_1W. It was labeled to the extent of 50 to 55% of maximal in these preparations.

We were concerned that calsequestrin and the high affinity calcium binding protein might be located on the cytoplasmic surface but, by virtue of folding induced by Ca^{2+} binding or of binding to the membrane surface, sites potentially available for fluorescamine binding might be covered. There is evidence that calsequestrin folds dramatically in response to ion binding (32, 33) although this is not true for the high affinity calcium binding protein (33). We reasoned that EGTA would unfold calsequestrin by removing divalent ions from its binding sites, and that this would increase its labeling if it were on the cytoplasmic surface but tightly folded. Table II shows that EGTA had no capacity for increasing the labeling of calse-

TABLE I

Incorporation of CFC into proteins in intact and SDS-solubilized vesicles

CFC-labeled proteins were separated by SDS-gel electrophoresis in 12.5% polyacrylamide gels according to the method of Laemmli (23). One-half of the slab gel was stained with Coomassie blue, and stained bands were eluted and assayed for protein content. Comparable bands were eluted from the other half of the gel and assayed for fluorescence intensity. For calculation of molar fluorescence, molecular weight values of 100,000, 44,000, 55,000, 53,000, and 30,000 for ATPase, calsequestrin (CS), high affinity calcium binding protein (HA), intrinsic glycoprotein (IG), and the 30,000-dalton protein (30K) were used, respectively. The total number of free amino groups per molecule was obtained for ATPase, calsequestrin, high affinity calcium binding protein, and intrinsic glycoprotein from Refs. 3, 4, 3, and unpublished studies of K. P. Campbell and D. H. MacLennan, respectively. Light and heavy vesicles were prepared by the method of Sarzala and Michalak (15); R_1W was prepared according to MacLennan (2).

	Membrane source					
	Light		Heavy		R_1W	
	I[a]	MF[b]	I	MF	I	MF
Intact vesicles						
ATPase	24.0	4.7	25.0	4.90	23.0	4.51
CS	10.0	1.7	0.0	0	8.0	1.40
HA	12.5	1.4	0.0	0	0	0
IG	ND	ND	ND	ND	40.0	8.4
30K	ND	ND	45.5	—[c]	45.0	—
SDS-solubilized vesicles						
ATPase	64.0	12.5	70.0	13.7	80.0	15.7
CS	70.0	12.2	90.0	13.7	74.6	13.1
HA	150.0	16.8	200.0	19.6	120.0	16.8
IG	ND	ND	ND	ND	64.0	13.5
30K	ND	ND	83.3	—	90.0	—

[a] I, intensity of fluorescence/mg of protein × 10^2.

[b] MF, molar fluorescence/total groups/molecule × 10^9. Molar fluorescence was calculated from the fluorescence intensity values divided by the protein concentration (I) and extrapolated to the fluorescence of a 1 M solution.

[c] Dashes represent values not calculated because the amino acid composition of the 30,000-dalton protein is unknown. ND, not determined.

TABLE II

Labeling with CFC of proteins in the heavy fraction and in purified calsequestrin in the presence of Triton X-100

Proteins of the heavy fraction (15) of sarcoplasmic reticulum vesicles and purified calsequestrin were labeled with CFC in the presence of 5 mM Ca^{2+} or 1 mM EGTA. Samples solubilized with 1% Triton X-100 in 0.25 M sucrose, 1 mM histidine, and 10 mM Tris-HCl, pH 8.0, were centrifuged at 100,000 × *g* for 60 min, and the supernatants obtained were labeled with CFC in the presence of 5 mM Ca^{2+} or 1 mM EGTA. For measurement of fluorescence intensity, labeled proteins of the heavy fraction as well as calsequestrin were separated by SDS-gel electrophoresis in 12.5% polyacrylamide gels according to the method of Laemmli (23). Bands were cut out and extracted from the gel for protein and fluorescence analysis (see "Methods"). Calculation of the fluorescence intensity (I) and molar fluorescence (MF) was carried out as described in the legend to Table I.

	5 mM Ca^{2+}		1 mM EGTA		5 mM Ca^{2+}, 1% Triton X-100		1 mM EGTA, 1% Triton X-100	
	I	MF	I	MF	I	MF	I	MF
Intact vesicles								
ATPase	32.0	6.3	25.0	4.9	56.0	10.1	55.0	10.8
CS[a]	0	0	0	0	30.0	5.3	37.0	6.5
HA[b]	0	0	0	0	133.0	14.5	150.0	16.3
30K[c]	45.0		48.0		100.0		95.0	
					I	MF		
Purified calsequestrin								
20 mM Hepes, pH 7.5					70.0	12.5		
1% Triton X-100					60.0	10.5		
1% Triton X-100, 5 mM Ca^{2+}					52.0	9.1		
1% Triton X-100, 1 mM EGTA					64.0	11.2		
1% SDS					90.0	15.7		

[a] CS, calsequestrin.
[b] HA, high affinity calcium binding protein.
[c] 30K, 30,000-dalton protein.

questrin or of the high affinity calcium binding protein in the heavy vesicles. We also carried out the experiment in the presence of 1% Triton X-100 to dissolve the membrane structure without unfolding calsequestrin. Calsequestrin was now labeled to the extent of 30 to 40% of maximal, and the presence or absence of Ca^{2+} in the lysis buffer did not affect the extent of labeling. Thus, calsequestrin *in situ* does appear to have sites protected against fluorescamine that are only opened up in SDS. By contrast, labeling of the high affinity calcium binding protein in the presence of 1% Triton X-100 was unaffected by the further addition of Ca^{2+} or of SDS.

Labeling of purified calsequestrin in the presence of 1% Triton was also incomplete: only about 80% of the amino groups labeled in the presence of SDS were labeled in the presence of 1% Triton. The addition of 5 mM Ca^{2+} in the presence of 1% Triton decreased the extent of labeling to about 60% of the SDS value (Table II). The labeling of the ATPase is increased in the presence of Triton X-100 (Table II) although it is not as high as in the presence of SDS (Table I). This might indicate that the ATPase has exposed sites on the luminal side of the membrane.

These data suggest that in the intact, heavy fraction of sarcoplasmic reticulum, calsequestrin and the high affinity calcium binding protein are not exposed to the cytoplasmic surface of the vesicles. There is limited exposure of the proteins in the light fraction and in R_1W. This may reflect the presence of leaky vesicles in the population or of additional, contaminating proteins in these preparations. Two-dimensional gels of R_1W and light vesicles do not indicate that there are significant additional components in these fractions, however. About 30% of the ATPase, 62% of the intrinsic glycoprotein, and 50% of the 30,000-dalton protein are exposed to the surface. The remainder of these molecules may be buried in hydrophobic regions. Hidalgo and Ikemoto (22) showed earlier that calsequestrin is not exposed to the cytoplasmic surface of the membrane while the ATPase and a mixture of proteins of M_r = 30,000 are exposed to the cytoplasmic surface. The group of proteins at 30,000 daltons were separated in this study, and we have shown that 50% of the single protein of 30,000 daltons is, indeed, exposed to the surface.

Digestion of Vesicles with Proteolytic Enzymes—Vesicles were exposed to trypsin under different conditions to see whether calsequestrin or the high affinity calcium binding protein was digested. We have previously shown that calsequestrin is not degraded by tryptic digestion of intact vesicles and that the protein can be isolated intact even after extensive tryptic digestion (9). We have also shown that material at 55,000 daltons is trypsin-resistant, but we have not shown that this is, indeed, the high affinity calcium binding protein. Fig. 4*A* shows an experiment in which sarcoplasmic reticulum vesicles in 100 mM KCl, 5 mM $CaCl_2$, and 10 mM Tris-HCl, pH 7.5, were exposed to trypsin and to papain for 30 min or to pronase for 60 min. The digested vesicles were then separated on the Laemmli one-dimensional system (23). The membrane-associated high affinity calcium binding protein was not cleaved by these proteolytic enzymes. By contrast, digestion of both purified high affinity calcium binding protein and purified calsequestrin with any of these proteolytic enzymes led to complete degradation of both proteins.

Fig. 4 (*Column 6*) shows a gel of the high affinity calcium binding protein that was purified after digestion of vesicles for 15 min with trypsin in the presence of 1 M sucrose, 100 mM KCl, and 10 mM Tris-HCl, pH 7.5. The protein could be isolated in intact form and in undiminished yield from such vesicles showing again that the high affinity calcium binding protein is not exposed to high molecular weight probes that are excluded from crossing the membrane.

Antiserum raised specifically against the high affinity calcium binding protein did react with the highly purified protein isolated from trypsin-digested vesicles (Fig. 4*B*).

Extraction of Vesicles with Chelex 100 and EGTA—We have re-examined the question of whether Ca^{2+} chelators such as Chelex 100 or EGTA can selectively remove the high affinity calcium binding protein from sarcoplasmic reticulum. Fig. 5 shows the results with Chelex 100 extraction according to the method of Zubrzycka *et al.* (16). In our hands, both Chelex 100 and EGTA extraction procedures released material of 44,000 daltons corresponding to calsequestrin rather than to the 55,000-dalton proteins observed in previous studies (8, 16). When the EGTA extract was separated on DEAE, material was eluted in the fractions expected for calsequestrin. No peak corresponding to the high affinity calcium binding

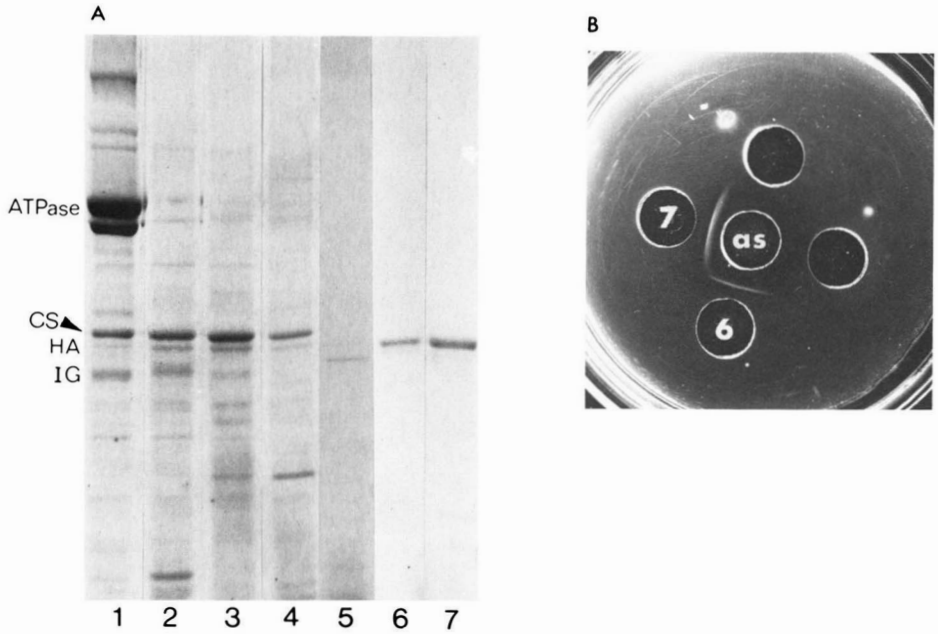

FIG. 4. **Digestion of sarcoplasmic reticulum vesicles with proteolytic enzymes.** Sarcoplasmic reticulum vesicles (10 mg/ml) were digested with trypsin, pronase, or papain in the presence of 100 mM KCl, 5 mM $CaCl_2$, and 10 mM Tris-HCl, pH 7.5 (9). The enzyme to protein ratio was 1:10 (w/w). The reaction was stopped by addition to PMSF to a final concentration of 1 mM and samples were washed three times by centrifugation at 150,000 × *g* for 60 min. The protein pattern of the digested samples was analyzed by SDS-gel electrophoresis (7.5% polyacrylamide) according to the method of Laemmli (23). Forty micrograms of protein were applied on gels 1 to 5: *A:1*, intact (original) vesicles; *2*, vesicles digested with trypsin for 30 min; *3*, vesicles digested with pronase for 60 min; *4*, vesicles digested with papain for 30 min; *5*, vesicles dissolved in 1% Triton X-100 digested with pronase for 30 min; *6*, high affinity calcium binding protein isolated from vesicles digested with trypsin for 15 min in the presence of 1 M sucrose (9); *7*, high affinity calcium binding protein isolated from intact vesicles. *CS*, calsequestrin; *HA*, high affinity calcium binding protein; *IG*, intrinsic glycoprotein. *B*, Ouchterlony double diffusion in an agarose plate. *Wells* labeled *6* and *7* contained the high affinity calcium binding protein isolated from trypsin-digested and intact vesicles, respectively. The well labeled *as* contained antiserum raised in a sheep against the high affinity calcium binding protein.

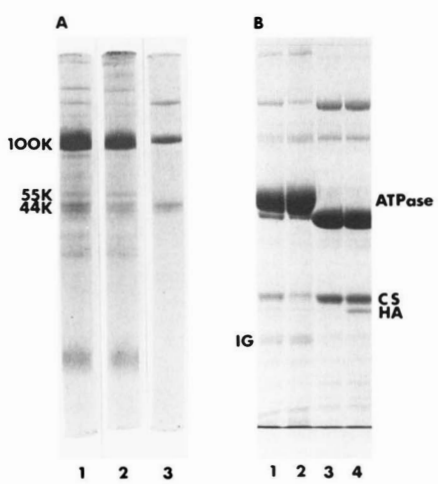

FIG. 5. **Extraction of sarcoplasmic reticulum proteins with Chelex 100.** Sarcoplasmic reticulum vesicles (R_1W) were extracted with Chelex 100 by the method described by Zubrzycka *et al.* (16), and the protein patterns of the fractions obtained were analyzed by SDS-gel electrophoresis (7.5% polyacrylamide) according to Weber and Osborn (24) (*A*) and Laemmli (23) (*B*). *1*, original preparation; *2*, pellet obtained after Chelex 100 extraction; *3*, supernatant obtained after Chelex 100 extraction; *4*, co-electrophoresis of *3*, with purified high affinity Ca^{2+} binding protein. *HA*, high affinity calcium binding protein; *CS*, calsequestrin. *IG*, intrinsic glycoprotein.

protein was observed. When the pellet, either after Chelex 100 or EGTA extraction, was extracted with deoxycholate and KCl (2), 55,000-dalton material was released into solution. When this solution was separated on a DEAE-cellulose column (5), protein eluted in the position corresponding to the high affinity calcium binding protein.

TABLE III

Muscle proteins contributing negligible mass to the 55,000-dalton band

Protein	Comments
Regulatory subunit of protein kinase	No cAMP binding by sarcoplasmic reticulum or high affinity calcium binding protein.
Pyruvate kinase, subunits of mitochondrial ATPase	Present in sarcoplasmic reticulum; accounts for <1% of the protein in 55,000-dalton region.
Desmin	Same mobility as high affinity calcium binding protein in Weber-Osborn system (24), but not in Laemmli system (23). Absent from all preparations of sarcoplasmic reticulum.
Tubulin	
Phosphorylase phosphatase	None of these activities are found in high affinity calcium binding protein.
Phosphorylase kinase	
Phosphorylase	

We have also carried out these studies with the heavy fraction prepared according to the method of Sarzala and Michalak (15). In this case, a fraction of the material staining at both 55,000 and 44,000 daltons was eluted from the vesicles (not shown), but the vesicles retained the bulk of both of these protein bands. Thus, we could not observe selective release of protein of 55,000 daltons although we could show that a protein of 55,000 daltons, not extractable from R_1W, was extractable from the heavy fraction. These studies therefore failed to confirm the observations that led Zubrzycka *et al.* (16) to propose that the high affinity calcium binding protein is located on the exterior of sarcoplasmic reticulum, on the opposite side from calsequestrin.

Analysis of Activities Associated with 55,000-dalton Proteins—A number of proteins of molecular weight near 55,000 daltons have been described in muscle. Table III lists a

number of proteins that we have examined as possible contributors to the material in the 55,000-dalton band in the Weber and Osborn electrophoretic system (24). Pyruvate kinase makes a small contribution to this band, estimated at about 1 or 2% of the protein mass. Lack of cAMP binding by sarcoplasmic reticulum indicates that the regulatory subunit of protein kinase is not present on the surface of the membrane. The purified high affinity calcium binding protein is also devoid of cAMP binding. Tubulin and desmin were found, by Laemmli gel systems (23), to be absent from sarcoplasmic reticulum. Mitochondrial F_1 subunits are present to a small extent in sarcoplasmic reticulum but could only account for a small fraction of the 55,000-dalton material. Therefore, we cannot assign known activities to the bulk of the material which contributes to the 55,000-dalton band in the Weber-Osborn (24) gel system.

Reconstitution of Vesicles—It was of interest to us to ask which protein of the 55,000-dalton band was rebinding to membranes re-formed after dissolution in deoxycholate (13, 14). We were able to confirm binding of 55,000-dalton material as illustrated in Fig. 6. We found that the material which rebound was very insoluble and resisted extraction by even high concentrations of deoxycholate in the presence of KCl or ammonium acetate although it was soluble in SDS. By contrast, the high affinity calcium binding protein is readily dissolved in deoxycholate and, once released from the membrane, remains water soluble. In the Laemmli gel system (23),

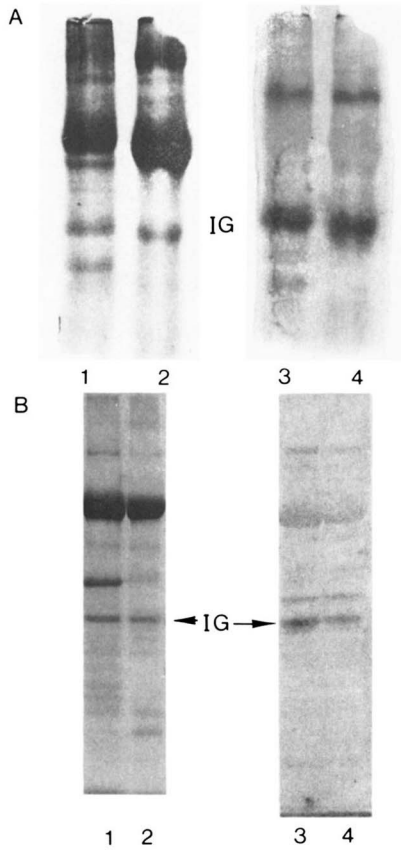

FIG. 6. **Protein pattern of reconstituted sarcoplasmic reticulum vesicles.** Sarcoplasmic reticulum vesicles (R_1W) were dissolved in deoxycholate and reconstituted according to the method of Meissner and Fleischer (13). *Columns 1* and *2* show Coomassie blue staining of original and reconstituted vesicles, respectively; *Columns 3* and *4* show autoradiograms of ^{125}I-concanavalin A bound to the stained gels seen in Columns 1 and 2. Separations were in 7.5% polyacrylamide gels according to the method of Weber and Osborn (24) (*A*) and Laemmli (23) (*B*). *IG*, intrinsic glycoprotein.

the protein did not migrate in the same region as the high affinity calcium binding protein (Fig. 6*B*). From these observations, we have concluded that the material which rebinds is not the high affinity calcium binding protein.

Identification of an Intrinsic Glycoprotein—In experiments designed to identify glycoproteins among the protein components of sarcoplasmic reticulum, we observed a strong binding of ^{125}I-concanavalin A by a protein of about 53,000 daltons in the Laemmli gel system (23) and about 55,000 daltons in the Weber and Osborn system (24) (Fig. 6). Since this protein was not eluted from the gels by the washes in Buffer B which elute calsequestrin and the high affinity calcium binding protein but not the ATPase, we have concluded that it is an intrinsic membrane protein. Examination of the gel pattern of the vesicles reconstituted by the procedure of Meissner and Fleischer (13) showed that this glycoprotein was present in undiminished amounts in the reconstituted membrane vesicles. Therefore, we conclude that it is this intrinsic glycoprotein which rebinds during reconstitution and not the high affinity calcium binding protein.

The gels shown in Fig. 1 show that the 53,000-dalton protein band is probably not homogeneous after separation in the Laemmli gel system (23) since a second protein band is seen in the same row and to the right of the intrinsic glycoprotein. However the protein, like calsequestrin, can be purified in two-dimensional gels where we have identified it by its ability to bind ^{125}I-concanavalin A. Fig. 3 shows that the intrinsic glycoprotein separated in two dimensions is heavily stained with CFC. Therefore, it is exposed, at least in part, to the membrane surface.

Fractionation of Material at 55,000 Daltons among Vesicular Fractions—Previous investigators have noted that protein corresponding to calsequestrin and to protein of about 55,000 daltons is found in different amounts among the light, heavy, and intermediate fractions of gradient-separated sarcoplasmic reticulum (15, 17, 18, 34–37). We have investigated whether these differences reflect the distribution of the high affinity calcium binding protein or the intrinsic glycoprotein.

In sarcoplasmic reticulum preparations, there are at least three clearly distinguishable fractions. On density gradients, the sarcoplasmic reticulum separates into a light fraction, apparently derived from the longitudinal sarcoplasmic reticulum and a heavy fraction apparently derived from the terminal cisternae (17, 18). The heavy fraction in turn, can be separated, after treatment with a French pressure cell, into a light fraction believed to be derived from transverse tubules and a heavy sarcoplasmic reticulum fraction representing more purified terminal cisternae.

Fig. 7 shows a separation of these fractions according to the method of Lau *et al.* (18). Coomassie blue staining (Fig. 7, *upper*) shows that the light fraction apparently derived from the longitudinal reticulum was rich in ATPase and deficient in calsequestrin and that the heavy fraction apparently derived from the terminal cisternae was rich in both ATPase and calsequestrin as previously described (17, 18). The band corresponding to the high affinity calcium binding protein was found in light (30 to 33% sucrose), intermediate (34 to 38% sucrose), and heavy (39 to 41% sucrose) fractions. The intrinsic glycoprotein was also present in all three fractions. When the heavy fraction was disrupted in a French pressure cell, the lightest transverse tubule-enriched fraction (22% sucrose) was found to be enriched in the high affinity calcium binding protein and to be deficient in the intrinsic glycoprotein. The small amount of purified transverse tubules obviated the isolation of the high affinity calcium binding protein from the preparation at the present time. However, we were able to confirm the presence of the high affinity calcium binding

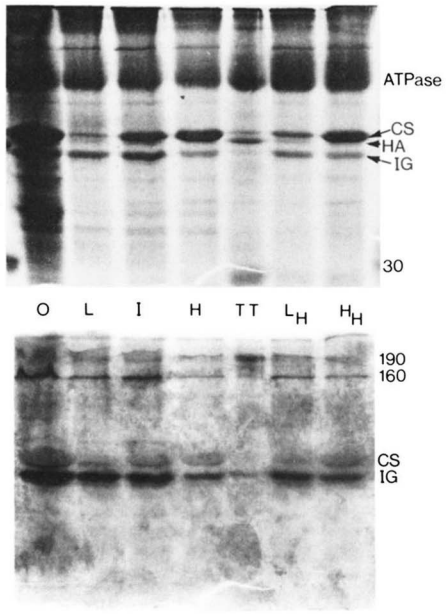

FIG. 7. **Distribution of proteins among the various fractions obtained in the preparation of transverse tubules (18).** *Upper*, Coomassie blue staining pattern of proteins separated in a 7.5% polyacrylamide gel according to the method of Laemmli (23). *Lower*, autoradiogram of ^{125}I-concanavalin A bound to the stained gels seen in the *upper panel*. *O*, unfractionated microsomes; *L*, *I*, and *H*, fractions obtained at 30 to 33%, 34 to 38%, and 39 to 41% sucrose, respectively, following density gradient centrifugation of unfractionated microsomes; *TT*, L_H, H_H, fractions obtained at 22, 30, and 39% sucrose, respectively, of Fraction *H* subjected to 5000 p.s.i. in a French pressure cell and then to sucrose density gradient centrifugation. Fraction *L* is believed to be derived largely from longitudinal elements of sarcoplasmic reticulum, Fractions *H* and H_H from terminal cisternae, and Fraction *TT* from the transverse tubules. *CS*, calsequestrin; *HA*, high affinity calcium binding protein; *IG*, intrinsic glycoprotein.

protein in this fraction using the antibody specific against the protein (not shown).

Fig. 7 (*lower*) shows the distribution of glycoproteins among the fractions. The intrinsic glycoprotein was found in relatively constant ratio with the ATPase in light, intermediate, and heavy fractions of the sarcoplasmic reticulum, and its content was sharply reduced in the transverse tubule-enriched fraction. Calsequestrin stained as a light, diffuse band, in accord with its low content of sugars (38). A bright sharp band of concanavalin A binding was observed at the leading edge of the calsequestrin band. This may reflect an artifact of calsequestrin migration or it may reflect the presence of a small amount of a unique, carbohydrate-containing protein. A glycoprotein band of about 160,000 daltons was present in sarcoplasmic reticulum but was reduced in the transverse tubular fraction. Conversely, a faint glycoprotein band of about 190,000 daltons in the sarcoplasmic reticulum was concentrated in the transverse tubular system. This pattern was also observed in the Coomassie staining patterns seen in Fig. 7. We did not observe concanavalin A binding to any proteins in the 30,000-dalton region.

After French pressure cell disruption, two fractions additional to the transverse tubule-enriched fraction were obtained. These fractions, designated L_H and H_H, banded at sucrose concentrations of about 30 to 32% and 39 to 40%, respectively. Both of these fractions also contained the high affinity calcium binding protein and the intrinsic glycoprotein. In the heavier fraction, calsequestrin was present in a very high ratio relative to the high affinity calcium binding protein.

Table IV gives the ratios of Coomassie blue bound by the

TABLE IV

Ratios between Coomassie blue bound to the ATPase and the high affinity calcium binding protein (HA) and to the ATPase and the intrinsic glycoprotein (IG) in various sarcoplasmic reticulum fractions

Fraction	Ratio[a] ATPase/HA	ATPase/IG
R_1W	8:1	4.5:1
Light fraction (17)	10:1	3:1
Heavy fraction (17)	8:1	3:1
Light terminal cisternae (18)	6:1	3.5:1
Heavy terminal cisternae (18)	8.5:1	3.5:1

[a] Ratios were determined by measuring the amount of Coomassie blue bound to each protein isolated by two-dimensional SDS-gel electrophoresis as described under "Methods." Light and heavy terminal cisternae were obtained after disruption of the heavy fraction in a French pressure cell. The light terminal cisternae banding at about 32% sucrose is not to be confused with the transverse tubule-enriched fraction which bands at about 22% sucrose.

ATPase to that bound by the high affinity calcium binding protein and to that bound by the intrinsic glycoprotein in the various fractions from sarcoplasmic reticulum. Values were obtained following separation of the proteins by two-dimensional gel electrophoresis. Both the high affinity calcium binding protein and the intrinsic glycoprotein were found to be present in fairly constant ratios with the ATPase in all fractions analyzed. The ratio of ATPase to high affinity calcium binding protein was about 9:1, while the ratio of ATPase to glycoprotein was about 3:1. We cannot determine molar ratios very accurately from these numbers because of uncertainty over the amount of Coomassie blue bound for each protein species. If the ATPase and the glycoprotein bound the same amount of Coomassie blue per mg and if the ATPase were, indeed, twice as large as the glycoprotein, then the molar ratio of ATPase to glycoprotein in all of these fractions would be about 1.5:1.

We have also examined the distribution of the high affinity calcium binding protein and the glycoprotein among the light, intermediate, and heavy fractions of Meissner (17) (not shown). Both of the proteins were found in a relatively constant ratio with the ATPase in all these fractions (Table IV). Calsequestrin varied greatly in its content, however, being low in the light fraction and greatly enriched in the heavy fraction.

DISCUSSION

This study was initiated to answer the question whether the high affinity calcium binding protein is localized in the interior or exterior of sarcoplasmic reticulum vesicles. The data on this point seem quite unequivocal: the protein is not exposed to CFC or to trypsin, and it is not released with Chelex 100 or EGTA. These data support our original contention that the high affinity calcium binding protein is in the interior of sarcoplasmic reticulum vesicles (1, 3). These studies do not support the suggestion by Sarzala and Michalak (15) and Zubrzycka *et al.* (16) that their light sarcoplasmic reticulum vesicles might be inside out relative to their heavy vesicles. Neither calsequestrin nor the high affinity calcium binding protein was exposed to CFC to any significant extent in either light or heavy vesicles. Moreover, the extent of CFC labeling of the ATPase was identical in both fractions. This would not be expected if the vesicles were inverted relative to each other since it is generally believed that the ATPase is asymmetrically localized in the membrane with a large part of the peptide chain exposed to the cytoplasmic surface and virtually none exposed on the luminal surface (1).

In the course of these studies, several other observations were made. There are several proteins associated with sarcoplasmic reticulum that have molecular weights similar to the high affinity calcium binding protein and which migrate in the same band in the Weber and Osborn disc gel electrophoretic system (24). These species can all be separated away from the high affinity calcium binding protein, however, by electrophoresis in the Laemmli gel system (23). We have not been able to assign known functions to these contaminants. At least one contaminant is water-soluble and is separated from the high affinity calcium binding protein on DEAE-cellulose. It is possible that this contaminant is external and is removed from the membrane rather readily. Since there is a report in the literature that the high affinity calcium binding protein can be removed by washing of the vesicle surfaces (16), it is possible that it was this contaminant, rather than the high affinity calcium binding protein, that was removed.

A newly recognized intrinsic glycoprotein of about 53,000 daltons is of greater interest. Jones *et al.* (39) have observed a protein of about 55,000 daltons in cardiac microsomes which stains for carbohydrate, but the protein seems not to have been previously identified in skeletal muscle. This intrinsic glycoprotein, rather than the high affinity calcium binding protein, is the one that reassociates with the membrane during reconstitution by the method of Meissner and Fleischer (13, 14). It is of particular interest that this intrinsic glycoprotein might be comparable to the intrinsic glycoprotein associated with the $Na^+ + K^+$ ATPase (40). However, in contrast to the tight association found between the glycoprotein and the $Na^+ + K^+$ ATPase, the intrinsic glycoprotein of sarcoplasmic reticulum is fractionated away from the $Ca^{2+} + Mg^{2+}$ ATPase during its purification. It is extracted from sarcoplasmic reticulum into low deoxycholate-high KCl solution and remains soluble but oligomeric upon removal of the detergent. It will be of great interest to discover a role for this protein in the sarcoplasmic reticulum and to compare its role with that of the glycoprotein in the $Na^+ + K^+$ ATPase.

We have looked for differences in the localization of proteins of 55,000 daltons among various membrane fractions. Previous investigators have found that calsequestrin does not fractionate uniformly among light, intermediate, and heavy fractions of sarcoplasmic reticulum (15, 17, 18, 34–37). We have looked specifically at the fractionation of the high affinity calcium binding protein and the intrinsic glycoprotein and have found a rather constant relationship between the amount of ATPase and the amount of glycoprotein or high affinity calcium binding protein in each of these fractions. The high affinity calcium binding protein seems to be concentrated, however, in a light fraction derived from the heavy fraction after treatment with a French pressure cell, whereas the intrinsic glycoprotein is nearly absent from this fraction. This fraction has been identified as consisting, in large part, perhaps as much as 85 to 90%, of transverse tubules (18). Therefore, we have confirmed the observations of Lau *et al.* (18) that protein of 55,000 daltons is present in the transverse tubules and, in addition, we have shown that this protein is the high affinity calcium binding protein on the basis of its mobility and antigenicity.

We have not been able to identify other proteins contained in the 55,000-dalton region or to assign any activity to the high affinity calcium binding protein. We have, however, been able to rule out a number of metabolic enzymes as contributing to protein in the 55,000-dalton band. Pyruvate kinase is present, but it only accounts for a small fraction of the protein of the band.

The fact that the high affinity calcium binding protein is internally located lends confidence to the view that it is, indeed, a part of the membrane system and is not an adherent contaminant. We have never been able to identify the protein in soluble fractions from muscle which also suggests its membrane association. The finding that the protein is concentrated in the fraction containing transverse tubules is of great interest since it may suggest that the protein is common to the two systems and may play some role in communicating signals between the two membranes.

REFERENCES

1. MacLennan, D. H., and Holland, P. C. (1975) *Annu. Rev. Biophys. Bioeng.* **4,** 377–404
2. MacLennan, D. H. (1970) *J. Biol. Chem.* **245,** 4508–4518
3. MacLennan, D. H., Yip, C. C., Iles, G. H., and Seeman, P. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37,** 469–478
4. MacLennan, D. H., and Wong, P. T. S. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 1231–1235
5. Ostwald, T. J., and MacLennan, D. H. (1974) *J. Biol. Chem.* **249,** 974–979
6. Ikemoto, N., Cucchiaro, J., and Garcia, A. M. (1976) *First International Congress of Cell Biology*, 869 abstr.
7. Tada, M., Yamamoto, T., and Tonomura, Y. (1978) *Physiol. Rev.* **58,** 1–79
8. Duggan, P. F., and Martonosi, A. (1970) *J. Gen. Physiol.* **56,** 147–167
9. Stewart, P. S., and MacLennan, D. H. (1974) *J. Biol. Chem.* **249,** 985–993
10. Yu, B. P., Masaro, E. J., and Morley, T. F. (1976) *J. Biol. Chem.* **251,** 2037–2043
11. Stewart, P. S., MacLennan, D. H., and Shamoo, A. E. (1976) *J. Biol. Chem.* **251,** 712–719
12. Ikemoto, N., Bhatnagar, G. M., Nagy, B., and Gergely, J. (1972) *J. Biol. Chem.* **247,** 7835–7837
13. Meissner, G., and Fleischer, S. (1974) *J. Biol. Chem.* **249,** 302–309
14. Repke, D. I., Spivak, J. C., and Katz, A. M. (1976) *J. Biol. Chem.* **251,** 3169–3175
15. Sarzala, M. G., and Michalak, M. (1978) *Biochim. Biophys. Acta* **513,** 221–235
16. Zubrzycka, E., Korczak, B., Sarzala, M. G., and Drabikowski, W. (1978) *FEBS Lett.* **90,** 215–217
17. Meissner, G. (1975) *Biochim. Biophys. Acta* **389,** 51–68
18. Lau, Y. H., Caswell, A. H., and Brunschwig, J. P. (1977) *J. Biol. Chem.* **252,** 5565–5574
19. Lazarides, E., and Hubbard, B. D. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 4344–4348
20. Shelanski, M. L., Gaskin, F., and Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70,** 765–768
21. Nakaya, K., Yabuta, M., Iinuma, F., Kinoshita, T., and Nakamura, Y. (1965) *Biochem. Biophys. Res. Commun.* **67,** 760–766
22. Hidalgo, C., and Ikemoto, N. (1977) *J. Biol. Chem.* **252,** 8446–8454
23. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685
24. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406–4412
25. Rostas, J. A. P., Kelly, P. T., and Cotman, C. W. (1977) *Anal. Biochem.* **80,** 366–372
26. Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963) *Biochem. J.* **89,** 114–123
27. Gilman, A. G. (1972) *Adv. Cyclic Nucleotide Res.* **2,** 9–24
28. Haschke, R. H., Heilmeyer, L. M. G., Jr., Meyer, F., and Fischer, E. H. (1970) *J. Biol. Chem.* **245,** 6657–6663
29. Krebs, E. G., and Fischer, E. H. (1962) *Methods Enzymol.* **5,** 373–376
30. Gutmann, I., and Bernt, E. (1974) *Methods of Enzymatic Analysis* (Bergemeyer, H. U., ed) Vol. 2, pp. 774–783, Verlag Chemie, Weinheim, Academic Press, New York
31. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
32. Ikemoto, N., Nagy, B., Bhatnagar, G. M., and Gergely, J. (1974) *J. Biol. Chem.* **249,** 2357–2365
33. Ostwald, T. J., MacLennan, D. H., and Dorrington, K. J. (1974) *J. Biol. Chem.* **249,** 5867–5871
34. Campbell, K. P., and Shamoo, A. E. (1977) in *Calcium Binding Proteins and Calcium Function* (Wasserman, R. H., Corradino, R. A., Carafoli, E., Kretsinger, R. H., MacLennan, D. H., and Siegel, F. L., eds) pp. 185–187, North Holland Press, New York
35. Michalak, M., Sarzala, M. G., and Drabikowski, W. (1977) *Acta*

Biochim. Pol. **24,** 105–116
36. Malan, N. T., Sabbadini, R., Scales, D., and Inesi, G. (1975) *FEBS Lett.* **60,** 122–125
37. Scales, D., Sabbadini, R., and Inesi, G. (1977) *Biophys. Biochem. Acta* **465,** 535–549
38. Jorgensen, A. O., Kalnins, V. I., Zubrzycka, E., and MacLennan, D. H. (1977) *J. Cell Biol.* **74,** 287–298
39. Jones, L. R., Besch, H. R., Fleming, J. W., McConnaughey, M. M., and Watanabe, A. M. (1979) *J. Biol. Chem.* **254,** 530–539
40. Kyte, J. (1972) *J. Biol. Chem.* **247,** 7642–7649