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Purification and Characterization of the 53,000-dalton Glycoprotein from the Sarcoplasmic Reticulum*

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A 53,000-dalton intrinsic glycoprotein of the sarcoplasmic reticulum was separated from the Ca^{2+} + Mg^{2+}-ATPase by dissolution with low concentrations of deoxycholate in the presence of 1 M KCl and purified in two successive gel filtration steps. It was aggregated and eluted at the void volume when subjected to gel filtration in the presence or absence of deoxycholate. When subsequently chromatographed in the presence of sodium dodecyl sulfate, the glycoprotein eluted in pure form as a monomer. The glycoprotein contained 48% nonpolar amino acids. It also contained 4 mol of glucosamine and 18 mol of mannose per mol of protein, suggesting that it contained two chains of $(GlcNAc)_2$, $(Man)_9$. The 53,000-dalton glycoprotein was completely removed from deoxycholate extracts of sarcoplasmic reticulum by affinity chromatography on concanavalin A Sepharose. Elution of glycoproteins with α-methyl-D-mannoside and deoxycholate resulted in co-purification of the 53,000-dalton glycoprotein and 160,000-dalton glycoprotein previously observed in sarcoplasmic reticulum.

The apparent molecular weight of the glycoprotein was reduced from 53,000 to 49,000 after digestion with endo-β-*N*-acetylglucosaminidase H (Endo H) and its reactivity with concanavalin A (Con A) was lost. There was no change in molecular weight of the glycoprotein and no diminution of its reactivity with Con A when sealed vesicles of sarcoplasmic reticulum were treated with Endo H. Endo H reduced the molecular weight and the Con A reactivity of the protein when the vesicles were made permeable by detergents. These observations, together with our previous demonstration that the glycoprotein reacts with a cycloheptaamylose-fluorescamine complex in sealed vesicles (Michalak, M., Campbell, K. P., and MacLennan, D. H. (1980) *J. Biol. Chem.* 255, 1317-1326), show that the glycoprotein is a transmembrane protein.

A protein of approximately 53,000 daltons was labeled when the sarcoplasmic reticulum was reacted with the photoaffinity label [^{32}P]8-N_3-cAMP. The labeled protein was neither the glycoprotein nor the high affinity calcium-binding protein since it was not sensitive to Endo H and was sensitive to trypsin digestion.

We have used ^{125}I-concanavalin A binding to analyze the glycoprotein composition of vesicles derived from the sarcotubular system of skeletal muscle (1). We have found four glycoproteins of M_r 160,000, 63,000, 60,000, and 53,000 in the sarcoplasmic reticulum, and we showed that a 190,000-dalton glycoprotein was concentrated in fractions enriched in the transverse tubular system. The 63,000-dalton glycoprotein has been identified as calsequestrin. The 53,000-dalton glycoprotein, which bound the largest amount of Con A,[1] was found to be a major protein constituent of skeletal muscle sarcoplasmic reticulum, and it may also be a major constituent of cardiac sarcoplasmic reticulum, since Jones *et al.* (2) have found a protein of approximately 55,000 daltons in cardiac microsomes which reacts with carbohydrate stains. The 53,000-, 60,000-, and 160,000-dalton glycoproteins were found in rather constant ratios with the Ca^{2+} + Mg^{2+}-ATPase in light, intermediate, and heavy sarcoplasmic reticulum vesicles, while calsequestrin content varied among these fractions (1). The 53,000- and 160,000-dalton glycoproteins both reconstituted into vesicular structures when detergents were removed from detergent-solubilized sarcoplasmic reticulum according to methods of Meissner and Fleischer (3) or Repke *et al.* (4). About 60% of the 53,000-dalton glycoprotein was found to be exposed on the cytoplasmic surface of the sarcoplasmic reticulum (1), but the fact that the sarcoplasmic reticulum has the same orientation as the rough endoplasmic reticulum implies that the carbohydrate moieties should lie in the lumen of the sarcoplasmic reticulum (5). Thus this glycoprotein should be a transmembrane protein.

The Na^+ + K^+-ATPase is composed of two different tightly associated polypeptides (6), a 100,000-dalton protein containing the site of phosphoryl bond formation and a 53,000-dalton glycoprotein in a molar ratio of 1:1 (7). Although the function of the glycoprotein in the Na^+ + K^+-ATPase is unknown, the inhibition of Na^+ + K^+ transport, but not of ATPase activity, by wheat germ agglutinin (which binds to the glycoprotein) suggests that it may have a role in the transport of cations (8). Since the Na^+ + K^+-ATPase and the Ca^{2+} + Mg^{2+}-ATPase share properties such as molecular weight and even sequence homology (9), it is possible that they also share the property of association with a glycoprotein. The 53,000-dalton glycoprotein of the sarcoplasmic reticulum, in contrast to the 53,000-dalton glycoprotein of the Na^+ + K^+-ATPase can be separated from the ATPase in mild detergent solution, thereby making it potentially feasible to study the function of the glycoprotein by reconstitution studies.

In this study we report the purification and characterization

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[1] The abbreviations used are: Con A, concanavalin A; SDS, sodium dodecyl sulfate; Endo H, endo-β-acetylglucosaminidase H; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid.

of the 53,000-dalton intrinsic glycoprotein of the sarcoplasmic reticulum.

EXPERIMENTAL PROCEDURES

Materials—Bio-Gel A-5m, SDS, acrylamide, *N,N'*-methylenebis-acrylamide, 2-mercaptoethanol, and *N,N,N',N'*-tetramethylethylene-diamine were purchased from Bio-Rad Laboratories. ^{125}I was obtained from New England Nuclear. Concanavalin A Sepharose 4B, Sephadex G-25 (fine), Sepharose CL-6B, and concanavalin A were obtained from Pharmacia. Concanavalin A was iodinated with ^{125}I as previously described (1). HCl (constant boiling) was purchased from Pierce. Endo H (*Streptomyces griseus*) was a product of Miles Laboratories. Hexokinase, pyruvate kinase, lactate dehydrogenase, galactose dehydrogenase, and glucose-6-phosphate dehydrogenase were purchased from Sigma. Deoxycholate was purified as previously described (10). [^{32}P]8-N_3-cAMP was obtained from ICN.

Isolation of Sarcoplasmic Reticulum Vesicles—Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle according to the method of MacLennan (10) with the following modification. Vesicles pelleted at 44,000 × *g* were suspended at a protein concentration of 10 mg per ml in extraction buffer and centrifuged at 7,500 × *g* for 10 min. The flocculent pellet was discarded, and about 25 ml of the supernatant was layered on 8 ml of 25% sucrose, 10 mM Tris, and 1 mM histidine, pH 8.0. After centrifugation at 78,000 × *g* for 30 min the vesicles which passed through the single 25% sucrose step were homogenized in 0.25 M sucrose, 10 mM Tris, and 1 mM histidine, pH 8.0, and subsequently washed with 0.6 M KCl. After centrifugation at 78,000 × *g* for 1 h they were resuspended at 25 mg per ml in 0.25 M sucrose, 10 mM Tris, and 1 mM histidine, pH 8.0.

Light vesicles were pelleted from the supernatant which did not enter the 25% sucrose by centrifugation at 78,000 × *g* for 2 h and resuspended in 0.25 M sucrose, 10 mM Tris, and 1 mM histidine, pH 8.0.

Purification of Glycoprotein using SDS—Sarcoplasmic reticulum vesicles (KCl washed) were extracted with 0.1 mg of deoxycholate per mg of protein in the presence of 1 M KCl and 1 mM PMSF according to the method of MacLennan (10). The insoluble material was removed by ultracentrifugation, and the supernatant was concentrated under N_2 pressure to approximately 10 ml using an Amicon XM 50 membrane. The sample was then passed through a column (2.5 × 45 cm) of Bio-Gel A-5m equilibrated with 150 mM KCl, 10 mM Tris, pH 8.0, 0.1 mM EGTA, and 2.5 mg/ml of deoxycholate. Material absorbing at 280 nm eluted in three distinct peaks. Fractions making up the first peak were concentrated by ultrafiltration using an XM 50 membrane to approximately 5 ml and dialyzed overnight against 4 liters of 0.1 mM EGTA and 5 mM Tris, pH 7.5, to remove salts and deoxycholate. The dialyzed sample was made 2% in SDS and 1% in 2-mercaptoethanol and boiled for 5 min. It was then passed through a column (2.5 × 180 cm) of Sepharose CL 6B equilibrated with 10 mM sodium phosphate, pH 7.0, 0.5% SDS, 2.5 mM dithrothreitol, and 0.02% NaN_3. Protein in the eluate was detected by a paper spot test in which a drop of effluent applied to filter paper was stained with Coomassie blue, and protein composition was determined by slab gel electrophoresis. Pure fractions of glycoprotein were then selected and concentrated by ultrafiltration using a PM 10 membrane.

Purification of Glycoprotein using Concanavalin A Affinity Columns—Con A Sepharose 4B was either washed with 0.5 M α-methyl-D-mannoside just prior to use to remove all free or loosely bound Con A, or it was fixed with 0.03% glutaraldehyde in the presence of 0.25 M α-methyl-D-mannoside according to the method of Kowal and Parsons (11).

The deoxycholate extract of sarcoplasmic reticulum vesicles or the void peak from the Bio-Gel A-5m column was applied directly (without concentration) to a column (1.5 × 5 cm) of Con A Sepharose 4B equilibrated in a solution of 150 mM KCl and 10 mM Tris, pH 8.0, containing 2.5 mg/ml of deoxycholate. After extensive washing, the intrinsic glycoprotein was eluted from the affinity column with 0.5 M α-methyl-D-mannoside, 150 mM KCl, 10 mM Tris, pH 8.0, and 10 mg/ml of deoxycholate. The protein eluted with α-methyl-D-mannoside was concentrated using an XM 50 membrane and then dialyzed against 0.1 mM EGTA, 5 mM Tris, pH 8.0.

Endo H Removal of Carbohydrate from the 53,000-dalton Glycoprotein—Oligosaccharide chains were removed from the Con A Sepharose-purified glycoprotein by incubating the purified glycoprotein with Endo H at a concentration of 0.1 unit/mg of glycoprotein in 50 mM sodium citrate, pH 5.5, plus 0.1% SDS, according to the method of Trimble and Maley (12). After 1 h of incubation at 37°C the reaction was terminated by cooling at 0°C. The digested protein was then analyzed on SDS gels to determine its apparent molecular weight. Control incubations were carried out in the absence of Endo H.

Endo H Treatment of Sarcoplasmic Reticulum Vesicles—Endo H was used as a topological probe for determining the orientation of the oligosaccharide chains on the intrinsic glycoproteins according to the method of Hanover and Lennarz (13). Endo H, 0.1 unit/mg of protein, was added to sarcoplasmic reticulum vesicles in 75 mM sodium citrate, pH 6.5, 10 mM $MgCl_2$, 0.25 M sucrose, and 0.2 mM PMSF or the same solution containing 0.1% SDS or 0.1% Triton X-100. Incubations were carried out for 1 h at 37°C. The reactions were terminated by cooling and the vesicles solubilized in SDS-gel electrophoresis buffer. Control incubations were carried out in the absence of Endo H.

Photoaffinity Labeling of cAMP Receptor Proteins—Photoaffinity incorporation of [^{32}P]8-N_3-cAMP into sarcoplasmic reticulum proteins was carried out as described by Walter *et al.* (14). The standard reaction mixture contained 0.1 M potassium phosphate, pH 7.2, 8 mM theophylline, 10 mM $MgCl_2$, and 6×10^{-8} M [^{32}P]8-N_3-cAMP at a protein concentration of 2 mg/ml. Preincubations were carried out for 60 min in the dark at 4°C, and the samples were then irradiated for 10 min at 254 nm with a Minermite UVS-11 hand lamp at a distance of 8 cm. The labeled vesicles were analyzed by SDS-polyacrylamide gel electrophoresis, and the resulting slab gels were subjected to autoradiography. Endo H treatment of the labeled vesicles was carried out as described previously, following UV irradiation. Photoaffinity labeling was also carried out on vesicles digested with trypsin for 1 min in the presence of 1 M sucrose (15) and also on preparations of light vesicles separated from sarcoplasmic reticulum as described above.

Gel Electrophoresis—SDS-polyacrylamide slab gels were run according to the method of Laemmli (16) or of Weber and Osborn (17). Apparent M_r values were calculated from a graph of relative mobilities *versus* log M_r. M_r standards used were *Escherichia coli* RNA polymerase (165,000, 155,000, and 39,000), phosphorylase (94,600), albumin (68,000), catalase (60,000), ovalbumin (43,000), lactate dehydrogenase (36,000), carbonic anhydrase (30,000), and trypsin inhibitor (21,500).

Glucosamine Analysis—Duplicate samples (0.1 to 0.2 mg of protein) were hydrolyzed at 100°C with 4 N HCl for 4 h in N_2-flushed tubes (18). Free glucosamine was quantitated on a Durrum model D500 amino acid analyzer with ε-aminocaproic acid added as an internal standard to each sample prior to hydrolysis. Total protein was determined by amino acid analysis following hydrolysis in 6 N HCl at 110°C for 24 h.

Amino Acid Analysis—Amino acid compositions were determined after hydrolysis of the proteins in 6 N HCl for 24, 48, and 72 h in sealed, evacuated tubes at 110°C (19). Values for serine and threonine were derived by extrapolation to zero time to correct for hydrolytic losses. Values for valine and isoleucine reached plateau values at 72 h. Cystine was determined as cysteic acid, and methionine was determined as methionine sulfone after performic acid oxidation. Amino acid analyses were performed on a Durrum D500 analyzer.

Sugar Analysis—Enzymatic microsassays for mannose, glucose, and galactose were carried out on 4 N HCl protein hydrolysates of both the SDS-purified and Con A affinity-purified glycoprotein according to the method of Schachter (20). Total protein was determined by amino acid analysis following hydrolysis in 6 N HCl at 110°C for 24 h. Initial results indicated the presence of 6 mol of glucose per mol of protein in the protein hydrolysates. In order to test if this glucose was a contaminant rather than a structural component we analyzed samples of glycoprotein which were precipitated with 10% trichloroacetic acid and washed several times with H_2O. Enzymatic analysis revealed that 4 N HCl hydrolysates of the trichloroacetic acid-washed preparations of glycoprotein were free of glucose but contained normal amounts of mannose.

Assays—Protein was determined by the method of Lowry *et al.* (21) using bovine serum albumin as a standard. ^{125}I-Con A binding to Laemmli slab gels was carried out as previously described (1).

RESULTS

Glycoprotein Fractionation—A typical SDS slab gel showing the initial fractionation in the purification of the 53,000-dalton glycoprotein is shown in Fig. 1. The sarcoplasmic reticulum vesicles, after centrifugation through 25% sucrose, contained the Ca^{2+} + Mg^{2+} ATPase, calsequestrin, and the 53,000- and 160,000-dalton glycoproteins with only minor

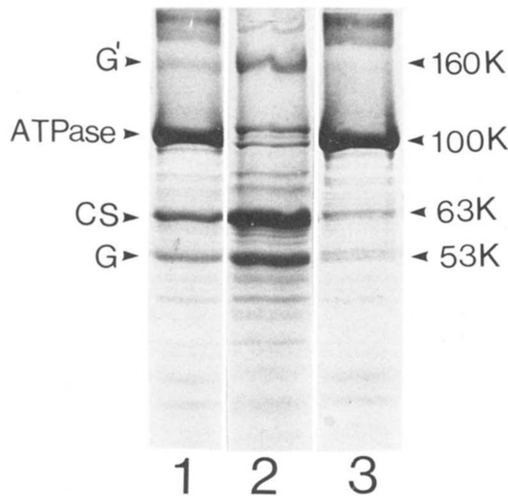

FIG. 1. **Deoxycholate solubilization of the 53,000-dalton glycoprotein.** Sarcoplasmic reticulum vesicles (lane *1*) were prepared using a 25% sucrose step gradient, washed with 0.6 M KCl, and then extracted with 0.1 mg of deoxycholate/mg of protein in the presence of 1 M KCl and 1 mM PMSF. The deoxycholate-solubilized fraction (lane *2*), obtained after centrifugation, contained calsequestrin (*CS*), the 53,000-dalton glycoprotein (*G*), and the 160,000-dalton glycoprotein (*G'*). The insoluble fraction (lane *3*) contained the ATPase as the major protein. Fifty μg of protein were applied to each well of a 7.5% Laemmli slab gel.

amounts of contaminating myosin and phosphorylase. Vesicles washed with 0.6 M KCl contained very little myosin or phosphorylase as shown in Fig. 1, lane *1*. Calsequestrin and the two glycoproteins were extracted into a solution containing 0.1 mg of deoxycholate per mg of protein and 1 M KCl (Fig. 1, lane *2*). In the insoluble fraction, the ATPase was the predominant protein together with some glycoprotein (Fig. 1, lane *3*). In the purification of the ATPase, the glycoprotein remaining was removed in the first ammonium acetate fractionation, and the resulting pure ATPase was devoid of glycoprotein (10).

The glycoprotein remained soluble after a dialysis step that removed deoxycholate and KCl, but gel filtration showed that it was aggregated whether in the presence or in the absence of deoxycholate. Fig. 2 shows the results of passing the deoxycholate supernatant through a column of Bio-Gel A-5m equilibrated with 150 mM KCl, 10 mM Tris, pH 8.0, 0.1 mM EGTA, and 2.5 mg of deoxycholate/ml. The optical density profile shows three distinct peaks. The protein profile of peaks I and II are shown in the SDS slab gel in the *inset* in Fig. 2. Peak I, the void volume peak, was enriched in two glycoproteins of M_r 53,000 and 160,000 and the ATPase. Peak II contained calsequestrin and some 53,000-dalton glycoprotein. Peak III contained low molecular weight proteins plus KCl and dithiothreitol that were eluted at the end of the column. These results indicate that the glycoprotein exists in an aggregated form that runs in peak I and a depolymerized form which runs in peak II.

^{125}I-Con A binding has confirmed that both peaks I and II contain the 53,000-dalton glycoprotein. The ratio of the amount of glycoprotein in peaks I and II was found to be dependent on the protein concentration of the supernatant that was applied to the Bio-Gel A-5m column. At protein concentrations less than 5 mg/ml the majority of the glycoprotein was found in peak II, and the total protein in peak II was approximately 4 times that found in peak I. At protein concentrations greater than 25 mg/ml the majority of the glycoprotein was found in peak I, and the total protein in both peaks was approximately equal.

SDS Purification of the Intrinsic Glycoprotein—The 53,000-dalton glycoprotein was purified from the ATPase and higher molecular weight proteins by chromatography of peak I of the A-5m column on a Sepharose CL-6B column in the presence of 10 mM sodium phosphate, pH 7.0, 0.5% SDS, 2.5 mM dithiothreitol, and 0.02% NaN_3, a solution that reduced all proteins to their monomeric forms. Fig. 3 shows fractions from the Sepharose CL-6B column. The void volume peak (fractions 2–8) contained the ATPase and higher molecular weight proteins while the retained peak contained the purified glycoprotein (fractions 12–16). After SDS gel analysis of all the glycoprotein fractions, the highly purified fractions were combined, concentrated, and dialyzed to remove SDS. Chemical analyses were carried out only on highly purified preparations of glycoprotein. Occasionally, we observed a protein band slightly below the glycoprotein. Even when it was present, it was possible to select fractions from the Sepharose CL-6B column of the glycoprotein that did not contain this band.

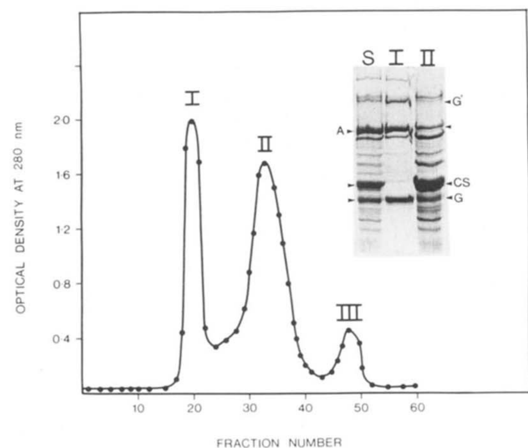

FIG. 2. **Protein elution profile from a Bio-Gel A-5m column.** The deoxycholate extract of sarcoplasmic reticulum vesicles was concentrated and applied to a column of Bio-Gel A-5m equilibrated with 150 mM KCl, 10 mM Tris, pH 8.0, 0.1 mM EGTA, and 2.5 mg of deoxycholate per ml. The profile of absorbance at 280 nm showed three distinct peaks. *Inset*, peaks I and II were analyzed on a Laemmli SDS slab gel; *S*, deoxycholate supernatant; *I*, peak I; *II*, peak II; *CS*, calsequestrin; *G*, 53,000-dalton glycoprotein; *A*, ATPase; *G'*, 160,000-dalton glycoprotein.

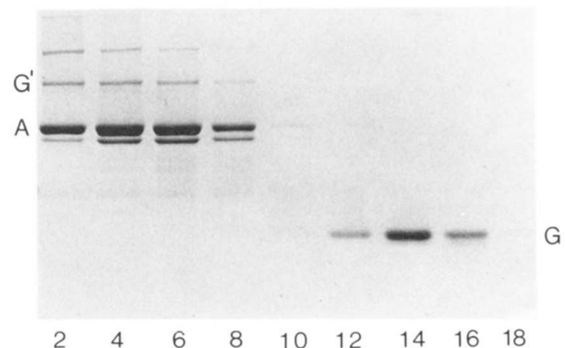

FIG. 3. **Protein elution profile from a Sepharose CL-6B column in the presence of 0.5% SDS.** Peak I of the Bio-Gel A-5m column was concentrated using an Amicon concentrator with an XM 50 membrane and dialyzed overnight against 0.1 mM EGTA, 5 mM Tris, pH 7.5. The dialyzed sample was solubilized with 2% SDS and 1% 2-mercaptoethanol, boiled, and applied to a column (2.5 × 180 cm) of Sepharose CL-6B equilibrated with 10 mM sodium phosphate, pH 7.0, 0.5% SDS, 2.5 mM dithiothreitol, and 0.02% NaN_3. Fractions containing protein were analyzed using Laemmli slab gel electrophoresis. Fractions 2–8 contained the ATPase (A), and fractions 12–16 contained the 53,000-dalton glycoprotein (*G*). *G'*, 160,000-dalton glycoprotein.

This band did bind ^{125}I-Con A, and the band did bind to Con A Sepharose. It was also seen to increase in content after storage of the glycoprotein, indicating that it was probably derived from the 53,000-dalton glycoprotein by modifications such as proteolysis or dephosphorylation.

Binding of Glycoproteins to Con A Sepharose—When sarcoplasmic reticulum vesicles were passed through a Con A Sepharose column they did not bind, showing that there was no exposure of high mannose carbohydrate chains at the vesicle surface. Fig. 4 illustrates the results of passing a deoxycholate extract of sarcoplasmic reticulum through a Con A affinity column. In order to minimize the amount of Con A leaching from the column, the gel was washed with 0.5 M α-methyl-D-mannoside just prior to the application of the deoxycholate extract. The 53,000- and 160,000-dalton glycoproteins in the supernatant were completely absorbed by the affinity column, as can be seen in the protein profile of the void fraction of the column (Fig. 4, lane *2*), but calsequestrin was only partly absorbed. Development of the affinity column with a gradient of 0 to 0.5 M α-methyl-D-mannoside in the presence of deoxycholate resulted in a broad peak of glycoprotein elution from the affinity column. Calsequestrin elution preceeded elution of the 53,000-dalton glycoprotein, but the two proteins were not clearly resolved in the eluate (Fig. 4, lanes *3* and *4*). The 160,000-dalton glycoprotein was found in both fractions, and a small amount of Con A, leaching from the column, was found in both fractions.

Con A Sepharose Purification of the 53,000-Dalton Glycoprotein—Since peak I from the Bio-Gel A-5m column contained only the 53,000- and 160,000-dalton glycoproteins, we have used it as a starting material for affinity purification of glycoproteins on a Con A column.

Fig. 5 shows the results of passing peak I from the Bio-Gel A-5m column through a Con A Sepharose column. Most of the applied protein passed through the column. The void volume fraction from the affinity column contained the ATPase and the 160,000-dalton glycoprotein (Fig. 5, *inset*, lane *2*) but very little 53,000-dalton glycoprotein. After extensive washing with loading buffer, 0.5 M α-methyl-D-mannoside was

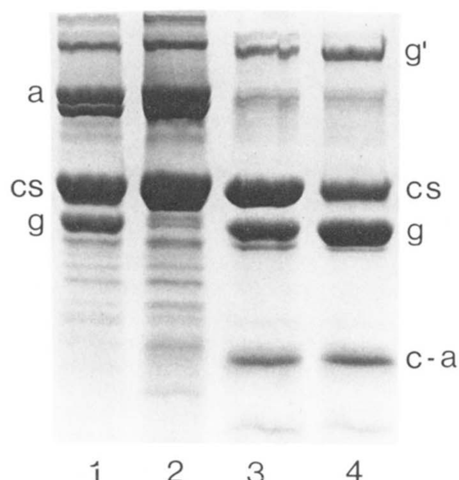

FIG. 4. **Binding of sarcoplasmic reticulum glycoprotein to Con A Sepharose 4B.** The deoxycholate extract (lane *1*) was applied to Con A Sepharose 4B column without concentration. After extensive washing, a gradient of 0 to 0.5 M α-methyl-D-mannoside in the presence of deoxycholate was applied to elute the bound glycoprotein. Lane *2*, void fraction of Con A Sepharose 4B column; lane *3*, fraction eluting with 0 to 0.25 M α-methyl-D-mannoside; lane *4*, fraction eluting with 0.25 to 0.5 M α-methyl-D-mannoside; *a*, ATPase; *cs*, calsequestrin; *g*, 53,000-dalton glycoprotein; *g'*, 160,000-dalton glycoprotein; *c-a*, Con A. Fifty μg of protein were applied to each lane of a 7.5% Laemmli slab gel.

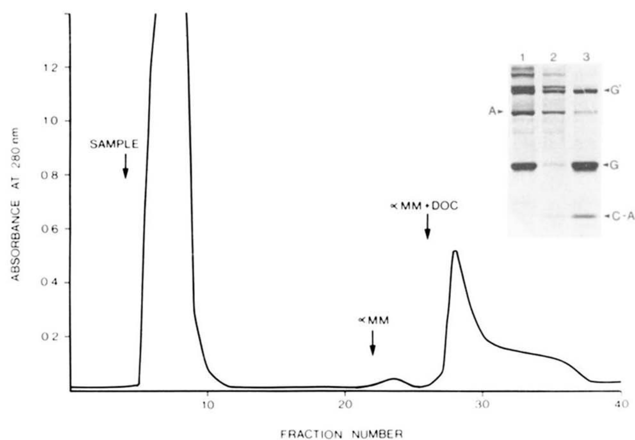

FIG. 5. **Protein elution profile from a Con A Sepharose 4B affinity column.** Peak I of the Bio-Gel A-5m column was applied directly to a Con A Sepharose 4B column. The glycoprotein was eluted with 0.5 M α-methyl-D-mannoside (*αMM*) in the presence of 10 mg/ml of deoxycholate (*DOC*). *Inset*, Laemmli SDS gel electrophoresis of *1*, peak I from Bio-Gel A-5m column; *2*, void of the affinity column; *3*, glycoproteins eluting with α-methyl-D-mannoside. *A*, ATPase; *G'*, 160,000-dalton glycoprotein; *G*, 53,000-dalton glycoprotein; *C-A*, Con A.

applied to the column. Only a small amount of Con A was eluted from the column under these conditions. The addition of deoxycholate alone did not elute any glycoprotein, but the addition of 0.5 M α-methyl-D-mannoside in the presence of 10 mg of deoxycholate/ml resulted in the elution of a large amount of protein (Fig. 5). This fraction was enriched in the 53,000-dalton glycoprotein but also contained the 160,000-dalton glycoprotein and Con A.

Since leaching of Con A from the column was a problem, we tried binding Con A to the Sepharose with glutaraldehyde. Kowal and Parsons (11) have developed a technique to prevent leaching of immunoglobulin or lactoperoxidase from Sepharose columns without destroying biological activity. Their method involved the use of glutaraldehyde, at a concentration range from 0.15 to 0.25% (v/v), to cross-link proteins which had been coupled to Sepharose by conventional methods. When Con A Sepharose was fixed with 0.1% glutaraldehyde for 30 min at 22°C, Con A no longer leached from the Sepharose, but the amount of glycoprotein bound by the affinity column was dramatically reduced. We found that 0.03% glutaraldehyde prevented leaching of Con A but did not bring about a loss of affinity for the 53,000-dalton glycoprotein. In addition, the glutaraldehyde-fixed affinity column was not able to absorb calsequestrin. Therefore, we were able to use deoxycholate supernatants directly as a starting source for purification. Fig. 6, lane *1*, shows the glycoprotein purified from the deoxycholate supernatant using a glutaraldehyde-fixed Con A affinity column. This preparation was estimated to be at least 90% pure with about 10% contamination from the 160,000-dalton glycoprotein. The 53,000-dalton glycoprotein could also be eluted from Con A Sepharose using SDS, which increased the yield but also increased the contaminating Con A.

Amino Acid Analysis—The amino acid composition of the intrinsic glycoprotein is listed in Table I. The per cent polarity of the glycoprotein was found to be 48%, assuming that the aspartic acid and glutamic acid residues are not amidated, a high value for a membrane protein (22). The polarity of the glycoprotein falls between that found for the Ca^{2+} and Mg^{2+} ATPase (42.9%) and calsequestrin (53.6%) (22). The glycoprotein polarity is similar to that of cytochrome c_1 or cytochrome b_5 (22), both of which are membrane proteins that can form

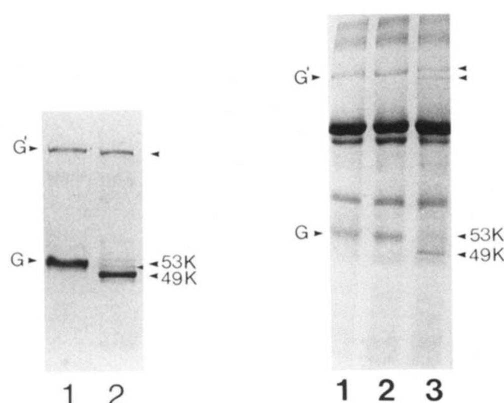

FIG. 6 (*left*). **Effect of Endo H digestion on the purified 53,000-dalton glycoprotein (*G*).** The 53,000-dalton glycoprotein was purified using glutaraldehyde-fixed Con A Sepharose. The pure glycoprotein was treated with 0.1 unit/mg of Endo H for 4 h at 37°C in the presence of 0.1% SDS. *1*, Laemmli gel electrophoresis of the purified glycoprotein; and *2*, the Endo H-treated glycoprotein. *Small arrow* indicates intermediate in Endo H digestion of the glycoprotein. Apparent M_r were calculated from a graph of relative mobilities *versus* log M_r. *G'*, 160,000-dalton glycoprotein.

FIG. 7 (*right*). **Effect of Endo H digestion on sarcoplasmic reticulum protein.** Endo H (0.1 unit/mg of protein) was added to sarcoplasmic reticulum vesicles and detergent-treated (0.1% SDS) sarcoplasmic reticulum vesicles. Control incubations were carried out in the absence of Endo H. *1*, control vesicles; *2*, Endo H treated in the absence of detergent; *3*, Endo H treated in the presence of detergent. *G*, 53,000-dalton glycoprotein; *G'*, 160,000-dalton glycoprotein.

oligomers in aqueous solutions (23, 24).

Carbohydrate Analysis—The carbohydrate composition of the intrinsic glycoprotein is shown in Table II. Enzymatic microassays were carried out on 4 N HCl hydrolysates of both the SDS-purified and Con A-affinity-purified glycoprotein. We measured approximately 18 mol of mannose and 4 mol of glucosamine per mol of glycoprotein. Since these sugars amount to a combined molecular weight of about 3700, the intrinsic glycoprotein contains about 8% carbohydrate. The carbohydrate composition is consistent with high mannose sugar chains as indicated by Endo H digestion and Con A binding.

Removal of Carbohydrate from the Purified Glycoprotein by Endo H—Treatment of the affinity-purified glycoprotein with Endo H (0.1 unit/mg of protein) for 4 h at 37°C in the presence or absence of 0.1% SDS resulted in the removal of the carbohydrate from the protein as indicated by the appearance of a single band at 49,000 daltons (Fig. 6). Endo H-treated protein no longer bound ^{125}I-Con A indicating that the high mannose chains were removed from the protein during Endo H digestion. The 160,000-dalton glycoprotein was also reduced in M_r by Endo H treatment (Fig. 6). In experiments with sarcoplasmic reticulum extracts we found that the 60,000-dalton glycoprotein was also reduced in M_r and lost the ability to bind Con A following Endo H treatment (not shown).

Endo H as a Topological Probe—In order to determine whether the 53,000-dalton glycoprotein has a transmembrane orientation, we measured the accessibility of the glycoprotein to Endo H in sealed sarcoplasmic reticulum vesicles in the absence and presence of detergent according to the method of Hanover and Lennarz (13). It is clear from the results in Fig. 7 that, in the absence of detergent, Endo H did not affect the mobility of any of the sarcoplasmic reticulum proteins, indicating that the oligosaccharide chains of the glycoproteins were not accessible to cleavage by externally added Endo H. In the presence of 0.1% SDS or 0.1% Triton X-100, which disrupts the sarcoplasmic reticulum vesicles, Endo H was able to change the mobility of the 53,000- and 160,000-dalton glycoproteins (Fig. 7, lane *3*), indicating that the oligosaccharides on both of these glycoproteins were located on the luminal face of the membrane. It has been previously shown that detergent does not stimulate the enzymatic activity of Endo H (13). The M_r of the Ca^{2+} + Mg^{2+} ATPase and calsequestrin were unaffected by Endo H treatment. A glycoprotein such as calsequestrin would not be expected to be affected by Endo H, since it does not have a high mannose sugar chain, the favored substrate for Endo H (25).

cAMP Photoaffinity Labeling—[^{32}P]8-N_3-cAMP has been used as a photoaffinity label for cAMP binding proteins in the sarcoplasmic reticulum (26, 27). Fig. 8 shows that the major band specifically labeled with [^{32}P]8-N_3-cAMP was at 53,000 daltons, in approximate agreement with previous reports (26, 27). Since the label comigrated with the glycoprotein, we

TABLE I

Amino acid composition of the 53,000-dalton glycoprotein from sarcoplasmic reticulum

Each value is the average of triplicate analyses of two preparations of glycoprotein.

Amino acid	mole %	Residues/mol[a]
Cysteine[b]	1.47	7
Aspartic acid	9.72	47
Threonine[c]	6.27	31
Serine[c]	5.69	28
Glutamic acid	12.15	59
Proline	5.29	26
Glycine	6.59	32
Alanine	6.37	31
Valine[d]	6.30	31
Methionine[e]	2.12	10
Isoleucine[d]	6.24	30
Leucine	10.91	53
Tyrosine	2.53	12
Phenylalanine	4.48	22
Histidine	2.13	10
Lysine	6.23	30
Arginine	5.51	27
% Hydrophobic, 48%		

[a] Residues per polypeptide chain assuming a molecular weight of 49,000 daltons derived from SDS gels of the carbohydrate-free protein. Values are rounded off to the nearest whole number.

[b] Determined as cysteic acid after performic acid oxidation.

[c] Estimated by extrapolation to zero time hydrolysis from 24-, 48-, and 72-h hydrolysis data.

[d] 72-h hydrolysis values.

[e] Determined as methionine sulfone after performic acid oxidation.

TABLE II

Carbohydrate composition of the 53,000-dalton glycoprotein from sarcoplasmic reticulum

Each value is the average of triplicate analyses of two preparations of glycoprotein (SDS purified and Con A purified).

Sugar	Content	
	nmol/mg[a]	mol/mol[b]
Mannose[c]	369	18
Glucosamine[d]	76	4
Glucose[c]	<10	0
Galactose[c]	<10	0
Galactosamine[d]	N.D.	0

[a] Protein was determined by amino acid analysis following 24 h of 6 N HCl hydrolysis.

[b] Moles of sugar per mol of protein assuming a molecular weight of 49,000 daltons. Values are rounded off to the nearest whole number.

[c] Determined using enzymatic microassays (20) on 4 N HCl hydrolysates.

[d] Quantitated on a Durrum model D500 amino acid analyzer with ε-aminocaproic acid added as an internal standard prior to hydrolysis. N.D., not detected.

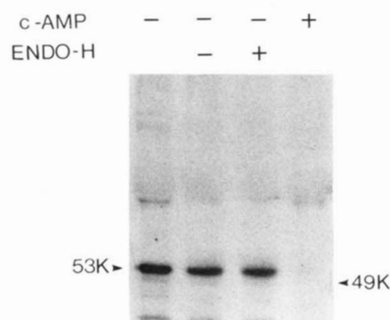

FIG. 8. **Autoradiograph showing the photoactivated incorporation of [^{32}P]8-N_3-cAMP into sarcoplasmic reticulum proteins.** The photoactivated incorporation of [^{32}P]8-N_3-cAMP was performed under conditions described under "Experimental Procedures" in the absence (–) or presence (+) of 100 μM cAMP. Endo H (+) or control (–) treatments were performed following [^{32}P]8-N_3-cAMP incorporation.

tested whether the glycoprotein was the cAMP receptor protein, using Endo H. Fig. 8 also shows that Endo H treatment of labeled vesicles in the presence of 0.1% SDS did not result in any change in M_r of the labeled protein, whereas the glycoprotein changed its M_r to 49,000.

We also examined whether the cAMP-binding protein might be the high affinity calcium-binding protein of M_r = 55,000. When sarcoplasmic reticulum vesicles are digested with trypsin for 1 min, a single proteolytic cleavage occurs in the ATPase (15), and calsequestrin and the high affinity calcium-binding protein are not digested (1, 15). We have recently noted that the 53,000- and 160,000-dalton glycoproteins also remain intact after this digestion as judged by their retention of their ability to bind ^{125}I-Con A in SDS gels. When [^{32}P]8-N_3-cAMP was added to vesicles that had been digested with trypsin for 1 min there was no specific labeling of any of the protein bands. This shows, first, that the binding was not to calsequestrin, the high affinity calcium-binding protein or the 53,000-dalton glycoprotein and, second, that the cAMP-binding protein is probably located on the surface of vesicles and that it is very susceptible to trypsin. Since the highest incorporation of [^{32}P]8-N_3-cAMP was routinely found in light vesicles, we believe that the receptor protein adheres in largest quantity to these light vesicles.

DISCUSSION

The sarcotubular system contains five glycoproteins of M_r 53,000, 60,000, 63,000, 160,000, and 190,000 (1), but since the 53,000-dalton glycoprotein is a major protein constituent of the sarcoplasmic reticulum membrane we have focused most of our attention on it in this study. The 53,000-dalton glycoprotein can be readily extracted from membranes into deoxycholate solution. However, it is aggregated, permitting its separation from calsequestrin by gel filtration. The glycoprotein can then be purified by gel filtration in the presence of a depolymerizing agent such as SDS. The pure 53,000-dalton glycoprotein contains 52% polar amino acids, a figure similar to that measured in two other membrane proteins, cytochrome c_1 and cytochrome b_5 (22), which also form oligomers in aqueous solution (23, 25). Cytochrome b_5 has been shown to exist in an equilibrium between octomer and monomer in solution and as a monomer in lipid vesicles (24). We suspect that the glycoprotein is also a monomer in the sarcoplasmic reticulum membrane and becomes an oligomer when extracted with detergent. The glycoprotein contains about 22 mole % aspartic acid (or asparagine) and glutamic acid (or glutamine) residues and about 12 mole % arginine and lysine residues. Ion exchange chromatography indicates that the glycoprotein is acidic since it is eluted from a DEAE column with about 0.3 to 0.4 M KCl.

We have used Con A affinity chromatography in the presence of deoxycholate to purify the glycoprotein under nondenaturing conditions. This preparation of the 53,000-dalton glycoprotein will be useful for future functional studies. Sarcoplasmic reticulum vesicles, when applied to Con A Sepharose 4B, passed through the column, and no protein was subsequently eluted with sugar. This is as expected since carbohydrate residues should be internal (5), and only inverted sarcoplasmic reticulum vesicles would be expected to bind to Con A columns. Following deoxycholate extraction, all five glycoproteins bound to Con A Sepharose. The 53,000- and 160,000-dalton glycoprotein had the highest affinity for Con A Sepharose and, in gel analysis, they bound the highest amount of ^{125}I-Con A (1).

When Con A Sepharose was fixed with low concentrations of glutaraldehyde to prevent any leaching of Con A from the column it retained the capacity to bind the 53,000- and 160,000-dalton glycoproteins. The eluate from this column contained about 90% 53,000-dalton glycoprotein and 10% 160,000-dalton glycoproteins. There are a number of similarities between these two glycoproteins. They are both found throughout the sarcoplasmic reticulum membrane, both have a high affinity for Con A, both are sensitive to Endo H digestion, both reconstitute with the ATPase after deoxycholate extraction, and they run together on DEAE. However, we have noted that the 53,000-dalton glycoprotein stains red with "Stains All" while the 160,000-dalton protein stains an intense blue. We do not believe that the 160,000-dalton protein is a trimer of the 53,000-dalton protein. It is also unlikely that the 160,000-dalton glycoprotein is a combination of the 100,000-dalton ATPase and the 53,000-dalton glycoprotein since the solubility and Con A binding capacity of the 160,000-dalton glycoprotein would be inconsistent with this combination. The purification of the 160,000-dalton glycoprotein, which is in progress, will answer these questions.

The 53,000-dalton glycoprotein appears to contain two high mannose sugar chains of the composition (Man)9:(GlcNAc)2. This composition is similar to the carbohydrate composition of the dolichol oligosaccharide (28) that donates $(Glc)_3$:$(Man)_9$:$(GlcNAc)_2$ to proteins. This observation indicates that the glycoprotein is processed after the oligosaccharide chains are attached to the protein only to the extent of removal of 6 glucose residues. Endo H digestion of the glycoprotein is consistent with the high mannose carbohydrate composition of the glycoprotein. Endo H splits the chitobiosyl core of *N*-linked glycoproteins and has a specificity for high mannose sugar chains (25). The M_r difference between the undigested and the Endo H-digested glycoprotein has been shown to be about 4000. The M_r of two oligosaccharides composed of Man,9:GlcNAc,2 is approximately 3700, and one would expect that Endo H would reduce the M_r of a glycoprotein with these two oligosaccharides by about 3300, a value in good agreement with our observations. Endo H digestion also provides evidence that there are, indeed, two high mannose chains since we observed a band of about 51,000 daltons appearing during our Endo H digestion (Fig. 6). This band might represent a protein with a single carbohydrate chain. The 160,000-dalton and 60,000-dalton glycoproteins were also sensitive to Endo H, and none of the Endo H-sensitive glycoproteins bound ^{125}I-Con A following digestion with Endo H.

We have previously shown that a substantial part of the glycoprotein is exposed on the cytoplasmic side of the membrane (1). Recent experiments in our laboratory with a nonpenetrating fluorescent sulfhydryl probe AEDANS (29) also indicate that there is exposure of —SH groups of the 53,000-

dalton glycoprotein on the cytoplasmic side of the membrane. In the present study we have used Endo H as a topological probe to demonstrate that the oligosaccharide moiety of the glycoprotein is susceptible to Endo H digestion only under conditions where the microsomes are made permeable to the enzyme. The results provide strong evidence that the 53,000-dalton glycoprotein is a transmembrane protein with a majority of its lysine groups exposed on the cytoplasmic surface and its oligosaccharide chains on the luminal surface. This is the first sarcoplasmic reticulum protein to be shown to be transmembrane. This result was expected since it has been previously shown that oligosaccharide chains are found exclusively on the luminal side of rough endoplasmic reticulum (5, 30) and rod outer segment discs (31). At present we would envision that the majority of the glycoprotein is exposed on the cytoplasmic surface, that it passes through the membrane only once or a few times, and that the sugar chains are on the luminal surface. The structure of transmembrane passages has recently been discussed by Kennedy (32).

Walter *et al.* (14) used [^{32}P]8-N_3-cAMP photoaffinity labeling to identify cAMP-binding proteins. They found that the type I regulatory subunit of cAMP-dependent protein kinase of 47,000 daltons and the type II subunit of 54,000 daltons were both specifically labeled by the radioactive cAMP. We have also found a 47,000-dalton cAMP-labeled protein in the supernatant fraction of muscle extracts and a 53,000-dalton cAMP-labeled protein in the membrane fraction of the muscle extracts. We have investigated whether the 53,000-dalton glycoprotein is the receptor for cAMP in our membrane fraction. Our experiments with photoaffinity labeling have shown that the 53,000-dalton cAMP-labeled protein is not the glycoprotein. We have also shown that the cAMP-binding protein is more enriched in light membranes than in sarcoplasmic reticulum membranes and that it is sensitive to mild trypsin digestion. Fractionation of muscle membranes, therefore, indicates that this labeled protein is not primarily of sarcoplasmic reticulum origin, but that it originates in a lighter membrane, possibly the T-system. It has been previously reported that the type II regulatory subunit is membrane bound, that it is sensitive to trypsin (33), and that it is probably not a glycoprotein (34). These are all characteristics of the cAMP-binding protein that we have observed in our preparations of sarcoplasmic reticulum. It should be noted that our results with Con A affinity chromatography indicate that greater than 90% of the 53,000-dalton band in the sarcoplasmic reticulum is glycoprotein. Therefore, the labeling that we observed must have been due to a very minor protein in the 53,000-dalton band.

The 53,000-dalton glycoprotein has been found in light, intermediate, and heavy sarcoplasmic reticulum vesicles and in vesicles reconstituted following deoxycholate (1, 3, 4) or Triton X-100 solubilization. Defoor *et al.* (35) have shown that a 55,000-dalton band, which almost certainly contains the glycoprotein, is present in skeletal muscle and heart muscle from rabbit, pigeon, cow, and rat. We have found that the glycoprotein is present in vesicles which have been isolated after Ca^{2+} oxalate loading of skeletal muscle sarcoplasmic reticulum vesicles, and Levitsky *et al.* (36) have found that cardiac sarcoplasmic reticulum, isolated after Ca^{2+} oxalate loading, contains the ATPase and a 55,000-dalton protein. These results indicate that the glycoprotein, like the ATPase, is present in those vesicles that are accumulating calcium in all types of muscle.

It is of interest that the Ca^{2+} transport system in sarcoplasmic reticulum consists of a 100,000-dalton catalytic subunit and a 53,000-dalton glycoprotein similar to the composition of the Na^+ + K^+ ATPase. The two glycoproteins are similar in M_r both before and after sugars have been removed (7), and they have very similar amino acid compositions. Since the 53,000-dalton glycoprotein of the sarcoplasmic reticulum can be separated from the 100,000-dalton catalytic subunit, it might be possible to use resolution, purification, and reconstitution studies to understand the function of the glycoprotein in Ca^{2+} transport. This information might then be applicable to the understanding of the function of the glycoprotein in the Na^+ + K^+ ATPase.

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REFERENCES

1. Michalak, M., Campbell, K. P., and MacLennan, D. H. (1980) *J. Biol. Chem.* **255**, 1317–1326
2. Jones, L. R., Besch, H. R. Jr, Fleming, J. W., McConnaughey, M. M., and Watanabe, A. M. (1979) *J. Biol. Chem.* **254**, 530–539
3. Meissner, G., and Fleischer, S. (1974) *J. Biol. Chem.* **249**, 302–309
4. Repke, D. I., Spivak, J. C., and Katz, A. M. (1976) *J. Biol. Chem.* **251**, 3169–3175
5. Boulan, E. R., Kreibich, G., and Sabatini, D. D. (1978) *J. Cell Biol.* **78**, 874–893
6. Kyte, J. (1972) *J. Biol. Chem.* **247**, 7642–7649
7. Craig, W. S., and Kyte, J. (1980) *J. Biol. Chem.* **255**, 6262–6269
8. Pennington, J., and Hokin, L. E. (1979) *J. Biol. Chem.* **254**, 9754–9760
9. Bastide, F., Meissner, G., Fleischer, S., and Post, R. L. (1973) *J. Biol. Chem.* **248**, 8385–8391
10. MacLennan, D. H. (1970) *J. Biol. Chem.* **245**, 4508–4518
11. Kowal, R., and Parsons, R. G. (1980) *Anal. Biochem.* **102**, 72–76
12. Trimble, R. B., and Maley, F. (1977) *J. Biol. Chem.* **252**, 4409–4412
13. Hanover, J. A., and Lennarz, W. J. (1980) *J. Biol. Chem.* **255**, 3600–3604
14. Walter, U., Uno, I., Liu, A. Y-C., and Greengard, P. (1977) *J. Biol. Chem.* **252**, 6494–6500
15. Stewart, P. S., and MacLennan, D. H. (1974) *J. Biol. Chem.* **249**, 985–993
16. Laemmli, U. K. (1970) *Nature* **227**, 680–685
17. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
18. Narasimhan, S., Harpaz, N., Longmore, G., Carver, J. P., Grey, A. A., and Schachter, H. (1980) *J. Biol. Chem.* **255**, 4876–4884
19. Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 59–62
20. Schachter, H. (1970) *Methods Enzymol.* **8**, 3–10
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
22. Capaldi, R. A., and Vanderkooi, G. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 930–932
23. Green, D. E., Jarnefelt, J., and Tisdale, H. D. (1959) *Biochim. Biophys. Acta* **31**, 34–46
24. Leto, T. L., and Holloway, P. W. (1979) *J. Biol. Chem.* **254**, 5015–5019
25. Arakawa, M., and Muramatsu, T. (1974) *J. Biochem.* **76**, 307–317
26. Lord, S. (1980) *Fed. Proc.* **39**, 1702
27. Maddock, S. W., and Jones, L. R. (1980) *Fed. Proc.* **39**, 2175
28. Liu, T., Stetson, B., Turco, S. J., Hubbard, S. C., and Robbins, P. W. (1979) *J. Biol. Chem.* **254**, 4554–4559
29. Hudson, E. N., and Weber, G. (1973) *Biochemistry* **12**, 4154–4161
30. Boulan, E. R., Sabatini, D. D., Pereyra, B. N., and Kreibich, G. (1978) *J. Cell Biol.* **78**, 894–909
31. Clark, S. P., and Molday, R. S. (1979) *Biochemistry* **18**, 5868–5873
32. Kennedy, S. J. (1978) *J. Membr. Biol.* **42**, 265–279
33. Corbin, J. D., Sugden, P. H., Lincoln, T. M., and Keely, S. L. (1977) *J. Biol. Chem.* **252**, 3854–3861
34. Corbin, J. D., Sugden, P. H., West, L., Flockhart, D. A., Lincoln, T. M., and McCarthy, D. (1978) *J. Biol. Chem.* **253**, 3997–4033
35. Defoor, P. H., Levitsky, D., Biryukova, T., and Fleischer, S. (1980) *Arch. Biochem. Biophys.* **200**, 196–205
36. Levitsky, D. O., Aliev, M. K., Kuzmin, A. V., Levchenko, T. S., Smirnov, V. N., and Chazov, E. I. (1976) *Biochim. Biophys. Acta* **443**, 468–484