CHARACTERIZATION OF SARCOTUBULAR MEMBRANE PROTEIN

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Received February 3, 1969

Most of the protein of the sarcotubular membranes of rat skeletal muscle is composed of electrophoretically identical polypeptide units with a molecular weight of $\sim$17,000. The predominant protein isolated from the sarcotubular membranes was found to be a large molecular weight aggregate of these polypeptides. It is probable that this polypeptide species is a component of sarcotubular membrane Ca^{++} transport system. Preparative gel electrophoresis, based on the analytical methods described in this report, should permit the isolation of this protein component in sufficient quantity for the molecular investigation of this transport system.

The sarcotubular membrane system of skeletal muscle serves to eject and resorb massive amounts of Ca^{++} during the contraction-relaxation cycle of muscle (1). Mommaerts (2) has suggested that a high proportion of the material comprising these membranes represents active Ca^{++}-transporting structures. This belief is based on data from studies in his laboratory, on the sulfhydryl groups essential to the Ca^{++} transport function (3), which suggest that the membranes consist of units of about 120,000 to 175,000 Daltons [an estimate supported by his recent radiation inactivation studies (4)] and Martonosi's data that led to the estimate of one Ca^{++}-binding site for each 300,000 Daltons of protein (5). Mommaerts concludes that if these estimates are reasonably accurate, there is little room in the sarcotubular membrane for protein that does not function in the Ca^{++} transport system. We felt that this conclusion points to the strong possibility that most of the protein of sarcotubular membranes is comprised of a very limited number of molecular species and that one or more of these predominant species might be considered the "Ca^{++} transport protein". This communication reports results of experiments which indicate that one polypeptide species, possibly occurring naturally in various sized molecular aggregates, accounts for most of the protein in sarcotubular membranes.

A highly purified fraction of sarcotubular membranes was prepared from rat skeletal muscle by the method of Yu et al (6). The proteins soluble at high ionic strength, which had been found by Martonosi to contaminate muscle microsomes (7), were removed from the membranes by a modification of Martonosi's

method; specifically, the membranes were extracted with a 0.6 M KCl, 80 mM Tris maleate solution (pH 7.2) for 1 hour at 2°C. The salt-treated membranes, which have full (Ca^{++} + Mg^{++})-ATPase and Ca^{++} uptake activity, were harvested by centrifugation at 105,000 x G for 60 min., washed with 0.3 M sucrose and again collected in a pellet by ultracentrifugation.

The pellet was homogenized at 2°C in 1 ml of the following medium per 3 mg sarcotubular protein: 10 mM sodium dodecylsulfate (SDS), 8 mM NaCl, 2.5 mM Tris, 0.5 mM 2-mercaptoethanol, pH 7.4 at 25°C. The homogenate became totally transparent in less than 5 minutes; this "sarcotubular solution" was dialyzed at 2°C for 36 hours against frequent changes of Solution A of the following composition: 8 mM NaCl, 2.5 mM Tris, 0.5 mM 2-mercaptoethanol, pH 7.4 at 25°C. Little loss of lipid or protein occurred during dialysis, but experiments with ^{35}S-labeled SDS showed that 97 to 98% of the detergent was removed from the dialysate during dialysis. To remove large particulate material the dialysate was then centrifuged for 30 min. at 48,000 X g, yielding a supernatant containing 95% of the protein and a precipitate with 5%.

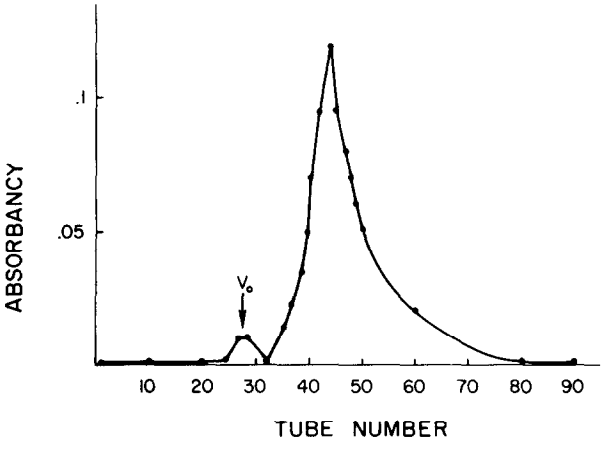
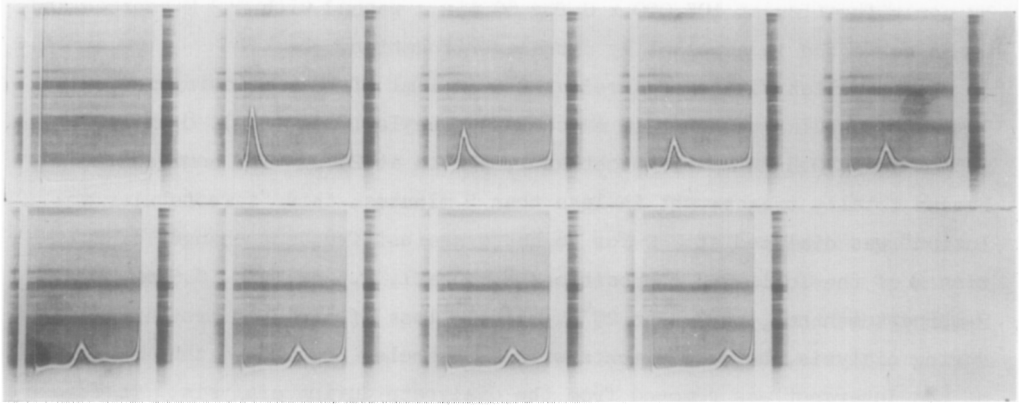
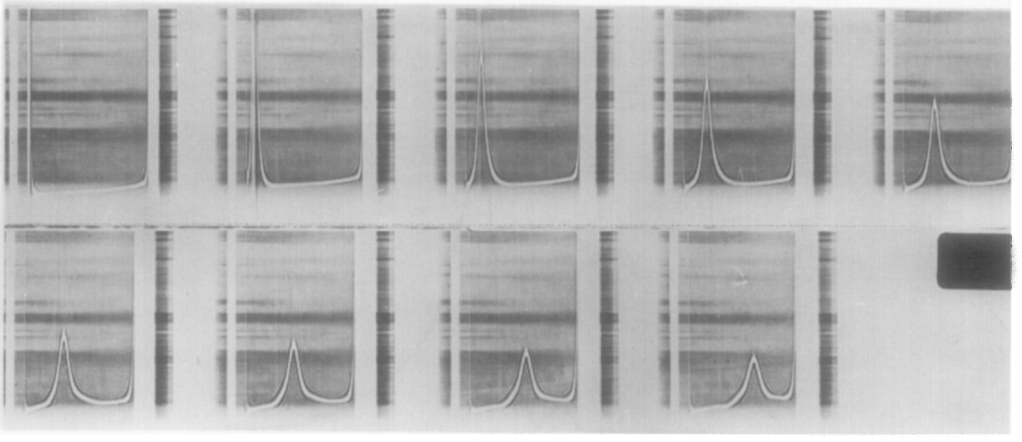


Figure 1 - Gel Filtration Analysis:

Sepharose 4B was equilibrated with Solution A and packed in a column. Solution A was also used for elution. An 1 ml aliquot of supernatant containing 2.12 mg of sarcotubular protein and 5% Ficoll was applied to the column; the elution flow rate was 6.35 ml/cm^2/hr. Each collection tube contained 1.9 ml of eluate. The protein concentration profile was roughed out by U.V. monitoring of the eluate. The protein concentration of the contents of the tubes reported in the graph was measured by the method of Lowry et al (8); the interference from tris and 2-mercaptoethanol was assessed and found not to be of a magnitude to affect the shape of the curve presented. The percent recovery of applied protein was assessed in separate experiments in which the peaks were again monitored by U.V.; the contents of the tubes making up each peak were pooled and the protein content of each pool assayed by the method of Lowry et al (8).



2a



2b

Figure 2 - Analytical Ultracentrifugation Analyses:

a) Photographs are of analytical ultracentrifugation of fraction 2 (protein concentration 6 mg/ml) in solution A. A Model E Beckman analytical ultracentrifuge was used. Sedimentation was from left to right. Speed was 56,000 rev/min. Photographs taken at 16 min. intervals after reaching full speed. Bar angle was 50°. Similar results were obtained in another run in which the protein concentration was 9 mg/ml. During the time interval required to reach 56,000 rev/min. the system was inspected visually and no evidence of rapidly sedimenting material was observed.

b) Photographs are of analytical ultracentrifugation of fraction 2 prepared in absence of 2-mercaptoethanol (protein concentration 6 mg/ml in solution A minus 2-mercaptoethanol). Ultracentrifugation and photographic procedures were identical to those described for Figure 2a.

Gel filtration was utilized for the analysis and fractionation of the supernatant. Analysis on a Sephadex G-200 column yielded two protein peaks, one with a V_e (elution volume) equal to the V_o (void volume) and another with a $V_e > V_o$; the latter contained most of the protein applied to the column and exhibited a V_e/V_o ratio of 1.27.

However, since Sephadex G-200 column chromatography did not permit good resolution of the two protein peaks, Sepharose 4B gel filtration was used to resolve them. An aliquot of the supernatant was applied to a Sepharose 4B column (see legend to Figure 1 for experimental details) and more than 90% of the protein was routinely eluted in two separate peaks. The first peak (called fraction 1) emerges at the V_o (void volume) and accounts for about 0.5% of the applied protein; the second peak (called fraction 2) accounts for 90% or more of the applied protein (Figure 1). The SDS:protein weight ratio of fraction 2 is less than 0.001; i.e., fraction 2 is either free of or contains only traces of SDS. The phospholipid:protein weight ratio of fraction 2 is 0.05 compared to a ratio of 0.49 found for intact sarcotubular membranes (9).

The proteins of fraction 2 were further studied by analytical ultracentrifugation and electrophoresis. When fraction 2 prepared as described above was investigated by analytical ultracentrifugation (see legend of Figure 2a for experimental details), it was found to contain two protein components (Figure 2a), a major one with an S_{20} value of 6.5 and a very minor component.

It was also found that 2-mercaptoethanol could be omitted from each step of the procedure without decreasing the yield of fraction 2. When analytical ultracentrifugation (see legend of Figure 2b for experimental details) was carried out with fraction 2 prepared in the absence of 2-mercaptoethanol, only a single protein component was found to be present (Figure 2b).

Fraction 2 was analyzed by cellulose acetate strip electrophoresis (Millipore Phoroslide System) at pH 6.0, 7.0, 8.6, 9.0 and 10.0. At each pH the protein migrated in a single band towards the anode.

Attempts to analyze the protein of fraction 2 by polyacrylamide vertical gel electrophoresis were unsuccessful with gels containing 4% or more polyacrylamide because most protein did not enter the gel. A 3% polyacrylamide gel was too difficult to handle with the vertical system but was manageable if 0.5% agarose were also present. With this polyacrylamide-agarose system, all protein entered the gel and migrated in two bands, a major and minor one (Figure 3, see legend for experimental details).

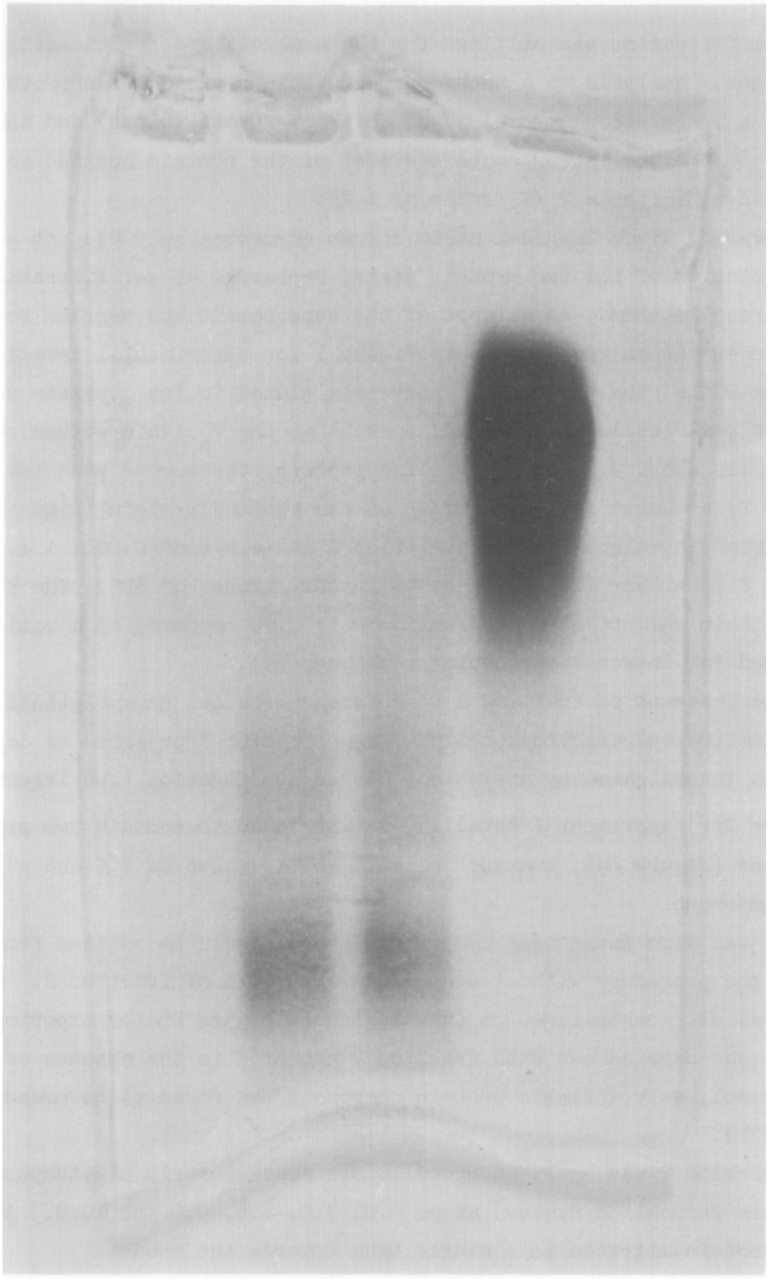


Figure 3 - Polyacrylamide - Agarose Gel Electrophoresis:

The Vertical Gel Electrophoresis Cell of the E-C Apparatus Corp. was used. A 3% cyanogum 41 (E-C Apparatus Corp.) -0.5% agarose gel was prepared in a buffer containing 85 mM tris, 80 mM boric acid, 2.5 mM Na_2 EDTA, pH 9.4. The electrode chamber contained the same buffer and migration was toward the anode. In the right lane hemoglobin was applied while in the other two lanes fraction 2 was applied. Electrophoresis occurred for 2 hours at 300 volts before the gel was fixed and dyed with Amido Black.

To dissociate the proteins of fraction 2 into polypeptide components, fraction 2 was treated with SDS and 2-mercaptoethanol by the method of Shapiro et al (10); the resulting protein containing solution was analyzed electrophoretically in a 5% polyacrylamide vertical gel containing 0.1% SDS (Figure 4, see legend for experimental details). All protein entered the gel and most migrated in a single band; a comparison of the extent of migration of this band relative to standard proteins indicates that it is composed of polypeptides of a molecular weight of $\sim$17,000. The minor band (shown in Figure 4) as well as those observed in other experiments (not shown in Figure 4) appear to be composed of protein with molecular weights that are integer multiples of that of the polypeptide species in the major band. That this dissociating system changed the sarcotubular protein so that it would enter a 5% gel is strong evidence that the protein isolated in fraction 2 is an aggregate of polypeptide units. Moreover, it seems likely that the minor band seen in Figure 4 results from the failure of the method to totally dissociate this polypeptide aggregate into single units.

The data presented above indicate that the protein of sarcotubular membrane is composed mainly of a single species of polypeptide with a molecular weight of $\sim$ 17,000. The protein solubilized from the sarcotubular membranes, isolated by gel filtration, and characterized by the data presented in Figures 2 and 3 is clearly a large aggregate of these polypeptide units. Whether these polypeptide units exist monomerically or as aggregates in the sarcotubular membrane structure remains a matter for conjecture.

Our conclusions about sarcotubular membrane protein differ markedly from those of Martonosi (7) who found that when a cholate-deoxycholate solution of muscle microsomes was analyzed by gel electrophoresis, much of the material failed to enter the gel and what did enter migrated in 8 bands. Our electrophoretic findings may differ from Martonosi's because cholate-deoxycholate yields a lipid-protein complex rather than an almost lipid-free protein or because he used a muscle microsome preparation rather than purifield sarcotubular membranes.

If Mommaerts is correct in assuming that most of the protein of sarcotubular membranes is involved in the Ca^{++} transport function, the predominant molecular species of protein identified in the present investigation must be an important component of the sarcotubular Ca^{++} transport system. Further, preparative gel electrophoresis, based on our analytical methods, should permit the isolation of this protein component in sufficient quantity for the molecular investigation of such a Ca^{++} transport system.

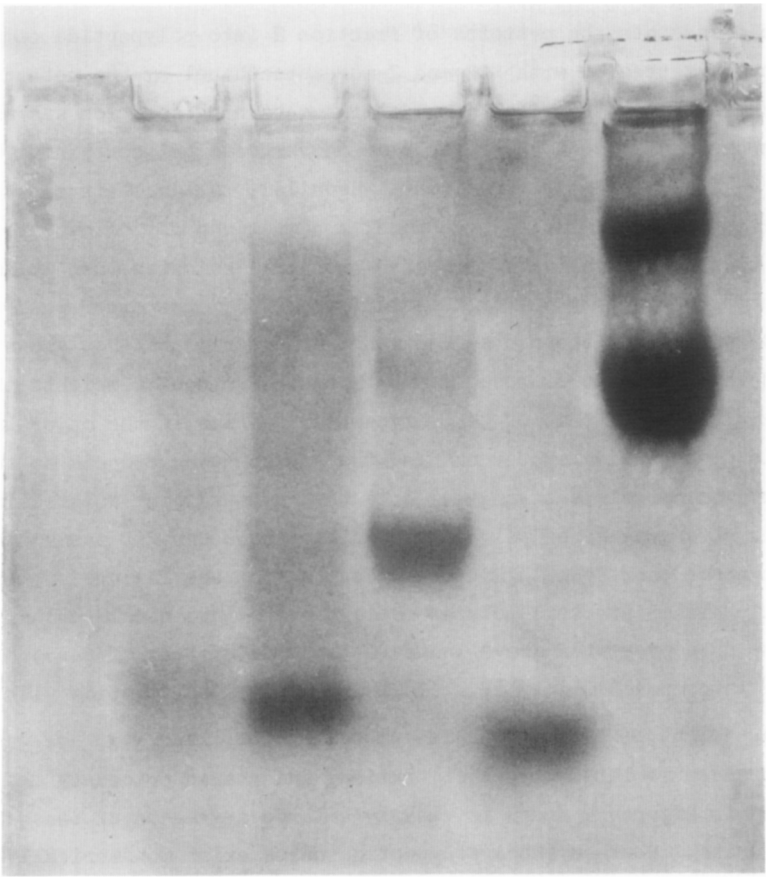


Figure 4 - Vertical Gel Electrophoresis in Presence of Dissociating Agents:

The proteins were denatured and reduced in the following way: 50 μg of protein was dissolved in 1% SDS, 1% 2-mercaptoethanol, 0.01 M phosphate buffer, pH 7.1 and incubated for 3 hours at 37°C. The denatured proteins were dialyzed for 16 hours against 0.1% SDS, 0.1% 2-mercaptoethanol and 0.01 M phosphate buffer, pH 7.1 at 20-25°C. Electrophoresis was carried out at 8 volts/cm for 3 hours in E-C Vertical Gel apparatus in 5% cyanogum 41 gel containing 0.1% SDS, 0.1 M phosphate, pH 7.1 and an electrophoresis buffer of 0.1% SDS, 0.1 M phosphate, pH 7.1; migration was toward the anode. The gel was fixed in 20% sulfosalicylic acid for 16 hours, stained in 0.25% Coomassie Blue for 5 hours and destained in 7% acetic acid. The slots are numbered 1 to 5 consecutively from left to right; slot 1 contains apo-ferritin, slot 2 Fraction 2, slot 3 pepsin, slot 4 hemoglobin and slot 5 bovine serum albumin.

We gratefully acknowledge the help of Dr. George Kaldor for carrying out the analytical ultracentrifugation measurements and the financial support of the National Science Foundation Grant # GB 5147, the Public Health Service Grant # AM 09458 and the Whitehall Foundation, Inc.

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