J. Membrane Biol. 44, 233–257 (1978)

# Purification and Characterization of the 45,000-Dalton Fragment from Tryptic Digestion of $(Ca^{2+} + Mg^{2+})$-Adenosine Triphosphatase of Sarcoplasmic Reticulum

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Received 22 February 1978; revised 15 August 1978

*Summary.* Tryptic digestion of $(Ca^{2+} + Mg^{2+})$-ATPase from sarcoplasmic reticulum of rabbit skeletal muscle has previously been shown to cleave the enzyme initially into a 55,000-dalton fragment and a 45,000-dalton fragment. In the present study the two fragments are solubilized in sodium dodecyl sulfate (SDS) and separated by preparative polyacrylamide gel electrophoresis. The 45,000-dalton fragment is found to be a relatively nonselective, divalent cation-dependent ionophore when incorporated into an oxidized cholesterol membrane (BLM). Ionophoric activity of this fragment is inhibited by low concentrations of $LaCl_3$, $HgCl_2$, and various reducing agents. There appears to be one or two relatively inaccessible disulfide bonds in the 45,000-dalton fragment that are essential for transport. Addition of reducing agents inhibits the ionophoric activity of the succinylated undigested enzyme and the 45,000-dalton fragment, but has no effect on the 55,000-dalton fragment. These experiments imply that the 45,000-dalton fragment and the 55,000-dalton fragment are in a series arrangement in the membrane.

By hydrolysis of ATP, the sarcoplasmic reticulum lowers the cytoplasmic calcium concentration in the region of the myofibrils to micromolar levels, pumping calcium against a 1000- to 3000-fold calcium gradient (Hasselbach & Makinose, 1962). This depletion of calcium leads to muscle relaxation. The main protein constituent of sarcoplasmic reticulum from rabbit white skeletal muscle is $(Ca^{2+} + Mg^{2+})$-ATPase [1] (Martonosi, 1969; Martonosi & Halpin, 1971). This enzme has been purified (MacLennan, 1970) and reconstituted into vesicles (Racker, 1972; Racker & Eytan, 1973; Meissner & Fleischer, 1974; Warren *et al.*, 1974). The recon-

1 *Abbreviations:* $(Ca^{2+} + Mg^{2+})$-ATPase, $(Ca^{2+} + Mg^{2+})$-dependent adenosine triphosphatase; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; Tris, TRIS (hydroxymethyl) aminomethane; PIPES, Piperazine-N-N′-bis [2-ethane sulfonic acid]; MES, 2[N-morpholino]ethane sulfonic acid; SDS, sodium dodecyl sulfate; BLM, black lipid membrane; DTT, dithiothreitol; SR, sarcoplasmic reticulum.

0022-2631/78/0044-0233 $05.00

stituted ATPase is capable of both hydrolyzing ATP and transporting calcium into vesicles against a concentration gradient. The complete calcium transport machinery appears to reside in the ATPase molecule.

In order to better understand the mechanism of transport through the enzyme and its localization in the membrane, sarcoplasmic reticulum has been exposed to trypsin and the proteolytic fragments of the ATPase have been examined. Upon exposure to trypsin the 102,000-dalton ($Ca^{2+} + Mg^{2+}$)-ATPase is initially cleaved into two fragments: a 55,000-dalton fragment (*A* fragment) and a 45,000-dalton fragment (*B* fragment) (Thorley-Lawson & Green, 1973, 1975; Stewart & MacLennan, 1974). Further digestion cleaves the 55,000-dalton fragment into a 30,000-dalton fragment ($A_1$ fragment), and a 20,000 dalton fragment ($A_2$ fragment). The nomenclature in parenthesis has recently been introduced by Thorley-Lawson & Green (1977). Experiments using antibodies raised against the tryptic fragments helped localize the fragments with respect to the SR (Stewart, MacLennan & Shamoo, 1976). From these studies it was deduced that the 55,000-dalton fragment is exposed to the exterior of the SR and the 45,000-dalton fragment is probably buried in the membrane. ($Ca^{2+} + Mg^{2+}$)-ATPase was exposed to trypsin, then phosphorylated with [$\gamma$-$^{32}$P]ATP, and the distribution of the phosphorylated fragments was examined (Thorley-Lawson & Green, 1973; Stewart *et al.*, 1976). The ATPase, the 55,000-dalton fragment and the 30,000-dalton fragment were all phosphorylated. From these studies it is believed that the 30,000-dalton fragment is exposed on the external surface of the SR and contains the site of ATP hydrolysis.

In order to understand the transport properties of the intact enzyme and its tryptic fragments, we incorporated these peptides into an artificial black lipid membrane (BLM) and investigated their permeability properties (Shamoo & MacLennan, 1974, 1975; Shamoo *et al.*, 1976; Stewart *et al.*, 1976). The intact enzyme, the 55,000-dalton fragment and the 20,000-dalton fragment were all found to be calcium selective and dependent ionophores.

In this paper a novel method for separating the 45,000-dalton fragment and the 55,000-dalton fragment is described, and the ionophoric properties of the 45,000-dalton fragment are investigated.

## Materials and Methods

### *Purification of Tryptic Fragments*

Sarcoplasmic reticulum from rabbit white skeletal muscle is prepared according to the method of MacLennan (1970). This sample is tryptically digested according to the method of Stewart *et al.* (1976). Extrinsic proteins are removed by addition of potassium deoxycho-

late and the digested fragments are solubilized in sodium dodecyl sulfate (SDS) and applied to a Bio-Gel A 1.5 m column (Stewart *et al.*, 1976). The fractions containing both the 45,000- and the 55,000-dalton fragments are concentrated to approximately 20 mg of protein/ml by pressure ultrafiltration with an Amicon PM 10 ultrafilter. 1.5 ml of the concentrate is added to an equal volume of 2X Weber and Osborn (1969) sample buffer (0.2 M $NaH_2PO_4$, 2% SDS, 2% $\beta$-Mercaptoethanol, NaOH, pH 7.0). Glycerol is added (10 drops) and the sample is incubated at 37 °C for 3 hr. It is this sample that is applied to the preparative gel.

*Preparative Gel*

We use a modified Shandon Southern Apparatus (Ryan *et al.*, 1976) in order to separate the 55,000-dalton and 45,000-dalton fragments. The SDS polyacrylamide gel system used is as described by Weber and Osborn (1969). 55 ml of buffer (5% acrylamide, 0.135% methylene bisacrylamide, 0.028 M $NaH_2PO_4$, 0.072 M $Na_2HPO_4$, 0.1% SDS, 0.1% TEMED, 0.05% ammonium persulfate) is added to the preparative gel column, layered with water, and allowed to polymerize. The height of the gel is approximately 12 cm. Electrophoresis is carried out at constant voltage (100 V) (ISCO model 492 Electrophoresis power supply) for approximately 24 hr.

The temperature of the gel is maintained at 20 °C by circulating ethylene glycol through the outer jacket of the preparative gel column (by using a Masterline 2095 bath and circulator). It takes approximately 12–15 hr for the 45,000-dalton fragment and the 55,000-dalton fragment to be eluted from the gel. Samples are eluted off in Weber and Osborn reservoir buffer (0.028 M $NaH_2PO_4$, 0.072 M $Na_2HPO_4$, 0.1% SDS) and collected in test tubes. Protein is assayed by absorbance at 280 nm or by the method of Lowry *et al.*, (1951). Those fractions containing the "pure" 45,000-dalton fragment (assayed by SDS gel electrophoresis using 5% Weber and Osborn gels) are combined and concentrated to approximately 0.1 mg protein/ml. SDS is removed by dialysis against 2 liters of 8 M urea, 20 mM Tris, HCl, pH 7.0, plus 10 g of Bio-Rad AG1-X2 (200–400 mesh, chloride form) anion exchange resin, for 7 days, followed by dialysis against 2 liters of 20 mM Tris, HCl, pH 7.0, plus 10 g of AG1-X2 anion exchange resin for 3 days (Weber & Kuter, 1971). Dialysis is carried out at 4 °C.

Determination of the amount of SDS bound to protein after dialysis against urea followed by buffer is made using [$^{35}$S] SDS (Shamoo *et al.*, 1976). Isotope is added to the purified 45,000-dalton fragment in Weber and Osborn reservoir buffer and equilibrated at 37 °C for 3 hr. Before commencement and upon the completion of dialysis (as described in the previous paragraph), an aliquot of the material from the inside of the dialysis bag is removed and assayed for radioactivity by using a liquid scintillation counter. The initial amount of SDS bound to the 45,000-dalton fragment is determined by equilibrium dialysis. The protein is added to a dialysis bag and equilibrated with Weber and Osborn reservoir buffer. [$^{35}$S] SDS is added to the inside of the dialysis bag and allowed to equilibrate. The difference in the number of counts per unit volume (inside the bag minus outside the bag) is easily related to the amount of SDS bound to the protein.

*Lipid Preparation and Chemical Methods*

Oxidized cholesterol is prepared by the method of Tien, Carbone, and Davidowicz (1966). SR lipids are prepared as described by MacLennan *et al.* (1971), by the method of Folch, Lees and Sloane-Stanley (1957).

Titanium (III) citrate used in all bilayer experiments is prepared by adding $TiCl_3$ to 0.2 M potassium citrate solution (Fisher Scientific Co.) in equal molar ratios. The solution is kept under a nitrogen atmosphere. The pH is adjusted to 6.8 by adding 1 M Tris base. The final concentration of titanium citrate is approximately 0.16 M. Titanium citrate is stored in liquid nitrogen until ready for use. Storing the titanium (III) citrate in the freezer overnight causes it to oxidize to titanium (IV) citrate, and appear clear.

*Conductance Measurements*

In most experiments described an oxidized cholesterol bilayer (BLM) is formed across a 1-mm diameter hole in the presence of salt solution on both sides of the membrane. Membranes are made by using a tapered Teflon brush. A function generator produces a triangular wave of amplitude $\pm 50$ mV, at a frequency of 1 cycle per min. Calomel electrodes are used (Beckman electrode #41239). The current through the BLM is measured by using a high impedance current amplifier in series with the membrane (Keithley, model 616). Solutions are buffered with 5 mM MES, Tris at pH 6.0, with 5 mM PIPES, Tris at pH 6.5 and 7.0, with 5 mM HEPES, Tris at pH 7.3, and with 5 mM Tris, HCl at pH 8.0 and 9.0

In order to measure cation *vs.* anion selectivity, a salt gradient is applied across the membrane and the voltage intercept at zero current is measured. From these voltage intercepts, using a modified Goldman equation (Shamoo & Goldstein, 1977), the relative permeability ($P_{Ca^{2+}}/P_{Cl^-}$) of the ions is derived. The relative permeability of various divalent cations is measured in a similar manner. Equal concentrations of different divalent salts on opposite sides of the bilayer yields a voltage intercept at zero current which is related to the relative permeability of the different ions using a modified Goldman equation (Shamoo & Goldstein, 1977). All solutions are made with distilled and deionized water. HEPES is obtained from Calbiochem. Tris, PIPES, and MES are obtained from Sigma. [$^{35}$S] SDS is purchased from New England Nuclear. N-Ethyl [2,3-$^{14}$C] maleimide is purchased from Amersham. Human serum $\gamma$-globulin is obtained from Sigma.

*Labeling of Reduced Disulfides with N-ethyl [$^{14}$C] Maleimide*

The method for determining the number of disulfide bonds is similar to the method of Thorley-Lawson and Green (1977). The protein at a concentration of 0.5 to 0.67 mg/ml is suspended in 50 mM Tris, HCl, pH = 7.0, 1 mM EDTA. If SDS is present, the sample is dissolved in 2% SDS. Concentrated dithiothreitol (DTT) is added to make the final concentration 0.26 mM. This represents a 45× excess of DTT over protein. The sample is then boiled for 5 min. After it has equilibrated at room temperature, a 5× molar excess of N-ethyl [$^{14}$C]-maleimide (final concentration = 1.33 mM) is added. The sample is then incubated at room temperature for 15 min at which time the solution is diluted with 3 volumes of water. It is then dialyzed against two changes of a large excess of 10 mM Tris, HCl, pH = 7.0 over a period of at least 48 hr. If SDS is present in the protein suspension, the sample is dialyzed against 0.1% SDS, 10 mM Tris, HCl, pH = 7.0. Following dialysis, protein-bound radioactivity is measured by adding 100 µl of the [$^{14}$C]-labeled protein to 10 ml of a toluene base liquid scintillation fluid and is then counted. N-ethyl [$^{14}$C]-maleimide is prepared by adding 25 µCi($\sim$100 µl in pentane) of [$^{14}$C] NEM to 200 µl of 100 mM NEM. The pentane is removed under a stream of $N_2$.

In those experiments in which titanium citrate is used, instead of DTT, as a reducing agent, titanium citrate is prepared by adding $TiCl_3$ to 0.2 M sodium citrate in equal molar ratios. The solution is then titrated to pH = 7.0 by the addition of 1 M Tris. A large molar excess of titanium (III) citrate is added to the protein. Since we have observed that titanium (III) citrate is oxidized upon the addition of NEM, the titanium citrate is allowed to air oxidize in the presence of the protein over a period of 48 hr. Upon complete oxidation of the titanium (III) citrate to titanium (IV) citrate, the pH drops to pH = 5.4. At this low pH, the sulfhydryls should not reoxidize to form disulfide bonds. The pH is now adjusted to pH = 7.0 by the addition of NaOH. The experiment is then completed by the addition of [$^{14}$C] NEM followed by dialysis to remove the unreacted [$^{14}$C] NEM.

*Organic Extraction*

The succinylated ($Ca^{2+}+Mg^{2+}$)-ATPase is suspended (0.5 ml) in 5 mM HEPES, Tris, pH = 7.3 at a concentration of 0.67 mg/ml in either the absence or presence (5 mM) of $CaCl_2$. Samples are reduced by the addition of concentrated DTT. They are then incubated at room temperature for 45 min. Three volumes of decane is added to each sample and is then vortexed for 1 min. The sample is then centrifuged at $500 \times g$ for 10 min. Aliquots from the aqueous phase are removed and a protein determination by the method of Lowry *et al.* (1951) is performed.

*Calcium Uptake*

Calcium uptake is measured at 20 °C in a medium of 0.1 M KCl, 25 mM HEPES, KOH, pH 6.8, 5 mM $MgCl_2$, 0.1 mM $^{45}CaCl_2$ at a protein concentration of 0.2 mg/ml. Reducing agent is present in the uptake medium at the concentration indicated in the text. The protein is incubated in the uptake medium for 30 min on ice, followed by incubation at 20 °C for 15 min. Uptake is initiated by the addition of $Na_2ATP$ (Sigma), KOH, pH 6.8 to a final concentration of 5 mM. The reaction is stopped by Millipore filtration through a Whatman GF/C glass fiber filter in series with a Millipore 0.45 μm type HA filter. Filtrates are collected (Martonosi & Feretos, 1964), added to a toluene base liquid scintillation cocktail, and are then counted.

## Results

### *Isolation of Fragments and Preparations of Samples for Bilayer Experiments*

Attempts have been made to separate the tryptically digested fragments of ($Ca^{2+}+Mg^{2+}$)-ATPase without solubilizing the enzyme with the anionic detergent SDS. Both nonionic detergents such as Triton X-100 and strong chaotropic agents such as guanidine-HCl, NaBr, urea, and propionic acid failed to solubilize and dissociate the fragments (Thorley-Lawson & Green, 1975). We have also attempted to dissociate the tryptic fragments using urea (1 M, 3 M and 6 M) and NaSCN (1 M, 3 M and 6 M) (Shamoo & Abramson, 1977). These attempts have not been successful. The enzyme seems to separate from the membrane before the fragments dissociate from one another.

There have been two methods reported to separate the SDS-solubilized 45,000-dalton and 55,000-dalton fragments. Stewart, MacLennan and Shamoo (1976) used column chromatography on a Bio-Gel A1.5-m column, followed by fractionation with a hydroxylapatite column. This method did not give reproducible elution patterns. Not all batches of hydroxylapatite separated the 45,000-dalton fragment from the 55,000-dalton fragment. Thorley-Lawson and Green (1975) separated the two

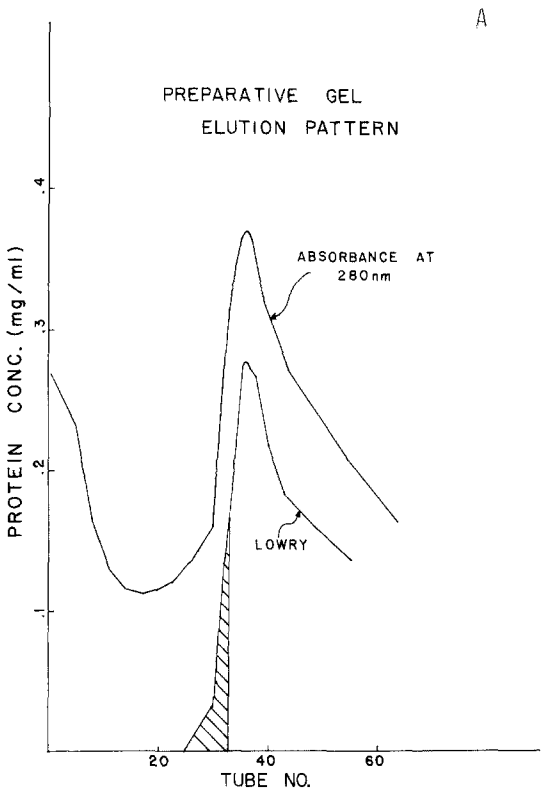


Fig. 1. Elution pattern of successive fractions collected from a 5% polyacrylamide preparative gel. (*A*): The absorbance at 280 nm and the protein concentration determined by the method of Lowry *et al.* (1951). The shaded area contains the "purified" 45,000-dalton fragment. (*B*): 5% Weber and Osborn (1969) polyacrylamide gels of some of the successive fractions eluted from the preparative gel. The leftmost gel shows the "purified" 45,000-dalton fragment. Successive fractions elute with a larger proportion of the 55,000-dalton fragment present. In all gels there is a small amount of protein visible, whose mobility corresponds to approximately 100,000-daltons. This "dimer" is present in both those fractions rich in the 45,000-dalton fragment and those rich in the 55,000-dalton fragment. Approximately 20 μg of protein is applied to each gel

fragments from the initial tryptic cleavage of the ATPase by SDS-cellulose acetate slab-gel electrophoresis. Electrophoresis proceeded for 2.5–3 days. The protein was located on the slab gel and was then cut out and squeezed from the gel.

In this paper, we report a method using SDS preparative gel electrophoresis to separate the 45,000-dalton fragment from the 55,000-dalton fragment. In Fig. 1 we show the absorbance at 280 nm and the protein concentration determined by the method of Lowry *et al.* (1951) of succes-

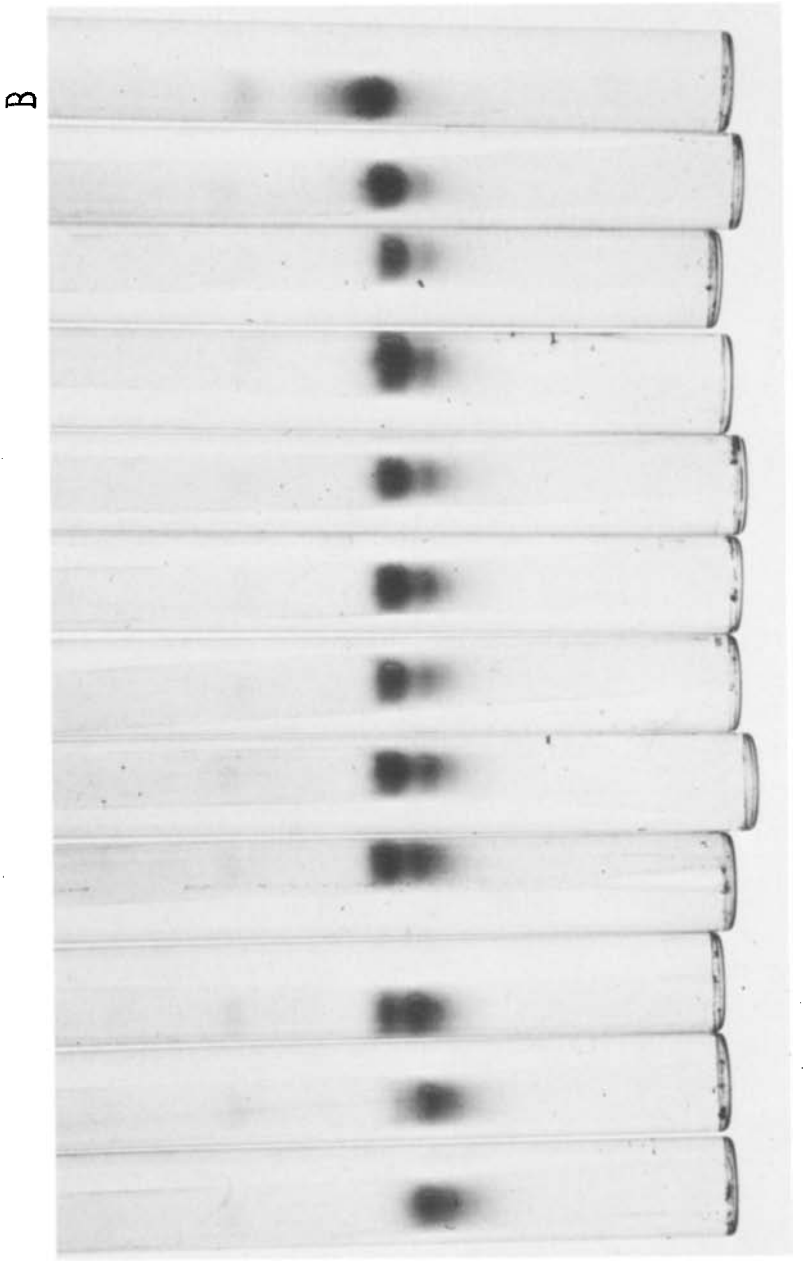


Fig. 1B

sive samples eluted from the preparative gel, and a series of 5% acrylamide, Weber and Osborn (1969) analytical gels showing the protein pattern from various fractions. The 45,000-dalton fragment is eluted first, followed by a mixture of the two fragments, and lastly by the 55,000-dalton

fragment. A small amount of protein migrates with the mobility of a dimer (~100,000 daltons). It is present in both those fractions rich in the 45,000-dalton fragment and those fractions rich in the 55,000-dalton fragment (i.e., a small fraction of the 45,000-dalton fragment forms a 45K-45K dimer and a small fraction of the 55,000-dalton fragment forms a 55K-55K dimer). By applying 30 mg of a mixture of the 45,000-dalton fragment and the 55,000-dalton fragment to the preparative gel, approximately 2 mg of the "purified" 45,000-dalton fragment and 4 mg of the "purified" 55,000-dalton fragment is eluted off the gel and collected.

In order to remove the SDS from the protein fragments, the purified peptides are pooled together and dialyzed against urea, followed by 20 mM Tris buffer as described in *Materials and Methods*. Equilibrium dialysis on the 45,000-dalton fragment yields 5.6 mg SDS bound per mg protein. This compares with 7.16 mg SDS per mg protein for the 30,000-dalton fragment and 18.4 mg SDS per mg protein for the 20,000-dalton fragment (Shamoo *et al.*, 1976). Upon completion of the dialysis procedure $7.5 \times 10^{-4}$ mg of SDS remains associated with 45,000-dalton fragment per mg protein, or 0.12 moles SDS per mole protein. This is approximately two orders of magnitude less than the amount of SDS remaining on the 20,000- or 30,000-dalton fragments after dialysis under the same conditions. Much of the apparent SDS remaining bound to the protein after extensive dialysis is a nondialyzable contaminant in the SDS that remains in the dialysis bag even in the absence of protein. Therefore, there is actually less than 0.12 moles of SDS bound per mole of the 45,000-dalton fragment upon completion of dialysis. Even with the removal of essentially all of the SDS, the protein fragment remains soluble. Assuming 0.12 moles of SDS bound per mole of protein, the concentration of free SDS, if all the detergent should dissociate from the protein, would be more than four orders of magnitude less than the concentration of SDS that is needed to cause an increase in BLM conductance (Shamoo *et al.*, 1976).

### *Ionophoric Properties of the 45,000-Dalton Fragment*

Most bilayer experiments are performed at pH 7.3 in 5 mM $CaCl_2$ using an oxidized cholesterol BLM. The ionophoric activity of the 45,000-dalton fragment is approximately the same as that of the 55,000- and 20,000-dalton fragments. At a concentration of $1.5 \times 10^{-9}$ M, the 45,000-dalton fragment increases the conductance of the oxidized cholesterol

Table 1. Calcium *vs.* chloride permeability of the 45,000-dalton fragment as a function of pH[a]

| pH | Mean voltage intercept ± SE (mV) | Mean breakline ± SE (mV) | Average $P_{Ca^{2+}}/P_{Cl^-}$ | No. of membranes |
|---|---|---|---|---|
| 6.0 | −6.47±0.61 | −4.03±0.35 | 0.350 | 8 |
| 6.5 | −2.53±0.85 | −3.38±0.21 | 0.655 | 8 |
| 7.0 | −0.83±0.87 | −4.00±0.29 | 0.866 | 6 |
| 7.3 | −0.47±0.62 | −4.59±0.44 | 0.921 | 8 |
| 8.0 | −0.59±0.51 | −3.39±0.33 | 0.903 | 15 |
| 9.0 | 1.34±0.63 | −4.45±0.36 | 1.280 | 14 |

[a] Solutions are buffered at the indicated pH with: 5 mM MES, Tris, pH 6.0; 5 mM PIPES, Tris, pH 6.5; 5 mM PIPES, Tris, pH 7.0; 5 mM HEPES, Tris, pH 7.3; 5 mM Tris, HCl, pH 8.0; 5 mM Tris, HCl, pH 9.0. In all experiments an equal amount of protein is added to both sides of the BLM. The protein concentration tested is the same at each value of pH. Both sides of the BLM are at the same pH. 10 mM $CaCl_2$ is on one side of an oxidized cholesterol BLM, 5 mM $CaCl_2$ is on the other side. $P_{Ca^{2+}}/P_{Cl^-}$, the relative permeability, is calculated from the modified Goldman equation (Shamoo & Goldstein, 1977). More positive voltage intercepts correspond to larger values of $P_{Ca^{2+}}/P_{Cl^-}$. The "breakline" is the voltage intercept at zero current after the membrane breaks. It is a measure of the mobility in free solution of the various ions.

BLM in the presence of 5 mM $CaCl_2$. At higher salt concentrations, the activity of the fragment increases (i.e., the time required to reach an arbitrary level of conductance decreases, and the final conductance level increases).

In order to measure the relative cation *vs.* anion permeability, a $CaCl_2$ gradient is formed across the BLM. Initially, 5 mM $CaCl_2$ is placed on both sides of the BLM. Concentrated $CaCl_2$ is added to one side and the solution is stirred. This yields 10 mM $CaCl_2$ on one side of the BLM and 5 mM $CaCl_2$ on the other side. As the conductance of the BLM, in the presence of the 45,000-dalton fragment increases, the voltage intercept at zero current remains unchanged. Using a modified Goldman equation (Shamoo & Goldstein, 1977), this intercept is related to the relative permeability of calcium to that of chloride. In this set-up a more positive voltage corresponds to a higher ratio of $P_{Ca^{2+}}/P_{Cl^-}$. The voltage intercepts and the derived relative permeabilities, $P_{Ca^{2+}}/P_{Cl^-}$, as a function of pH are shown in Table 1 and are illustrated in Fig. 2. At low pH, the membrane is more anion selective; at high pH it is more cation selective. In the range from pH 7.0 to pH 8.0 the membrane is relatively nonselective, $P_{Ca^{2+}}/P_{Cl^-} \simeq 1$. If the membrane,

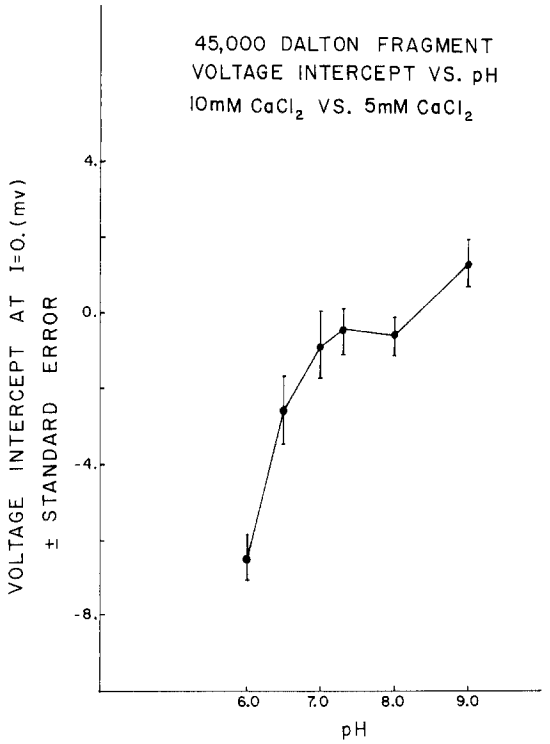


Fig. 2. Voltage intercept (mV) at zero current, as a function of pH. Conditions are identical to those described in Table 1

doped with the 45,000-dalton fragment behaved like a water-filled pore, the voltage intercept would reflect the higher mobility in free solution of chloride as compared to calcium ($P_{Ca^{2+}}/P_{Cl^-} \simeq 0.5$). The voltage intercept would be the same as the break line. At each pH, measurements are made with at least three different bathing solutions. The total number of membranes included in each measurement is indicated in Table 1.

Not only does the pH affect the cation *vs.* anion selectivity of the 45,000-dalton fragment, as shown in Fig. 3, it also affects the ionophoric activity of the fragment. In the high and the low pH range, the time required for the conductance to reach an arbitrary level ($0.5 \times 10^{-10}$ mho) increases. This plot demonstrates a combined effect of both the rate of incorporation into the BLM and the effectiveness of the protein fragment as a conducting unit. If the incorporation rate is not a function of pH, the data at low pH is consistent with a decreased flux of calcium and at high pH is consistent with a decreased flux of chloride.

In order to measure the relative permeability of the 45,000-dalton

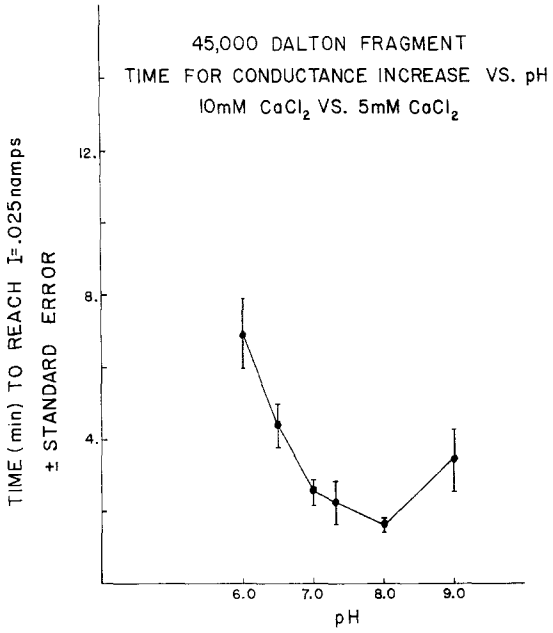


Fig. 3. Time (minutes) for the conductance to reach $5 \times 10^{-10}$ mho as a function of the pH of the bathing solution on both sides of an oxidized cholesterol BLM. Conditions are identical to those described in Table 1

fragment to various cations, 5 mM $CaCl_2$ is placed on one side of the BLM, and 5 mM of a different divalent salt is placed on the other side of the BLM. By knowing the ratio $P_{Ca^{2+}}/P_{Cl^-}$ from the previous measurements, measuring the voltage intercept at zero current, and using the modified Goldman equation (Shamoo & Goldstein, 1977), the relative divalent cation permeability of the 45,000-dalton fragment in an oxidized cholesterol BLM can be calculated. In Table 2, we show the voltage intercepts and calculated relative permeabilities for various divalent cations. As is seen in Table 3, the 45,000-dalton fragment is less selective among divalent cations than either the undigested succinylated $(Ca^{2+}+Mg^{2+})$-ATPase, or any of its previously tested fragments (i.e., the 55,000-dalton fragment and the 20,000-dalton fragment). The selectivity of the 45,000-dalton fragment differs significantly from the other fragments shown. In the case of the 45,000-dalton fragment, $P_{Mn^{2+}}/P_{Mg^{2+}}$ is greater than one. For all other fragments tested, $P_{Mn^{2+}}/P_{Mg^{2+}}$ is less than one. The 45,000-dalton fragment at pH 7.3 distinguishes very little between cations and anions and between the different divalent cations tested. Yet it is not simply a water-filled pore. The voltage inter-

Table 2. Relative divalent cation permeability of the 45,000-dalton fragment at pH 7.3[a]

| Divalent cations present | Mean voltage intercept ±SE (mV) | Mean breakline ±SE (mV) | Average $P_{Ca^{2+}}/P_{D^{2+}}$ | No. of membranes |
|---|---|---|---|---|
| $Ca^{2+}$ *vs.* $Ba^{2+}$ | 0.06±0.04 | 0.19±0.10 | 1.007 | 8 |
| $Ca^{2+}$ *vs.* $Sr^{2+}$ | 0.96±0.10 | 0 ±0 | 1.126 | 7 |
| $Ca^{2+}$ *vs.* $Mn^{2+}$ | 1.21±0.28 | 0.17±0.11 | 1.162 | 6 |
| $Ca^{2+}$ *vs.* $Mg^{2+}$ | 2.00±0.29 | 0.13±0.06 | 1.289 | 6 |

[a] 5 mM $CaCl_2$ is on one side of an oxidized cholesterol BLM, 5 mM of the other divalent cation ($D^{2+}$) chloride salt, is on the other side of the BLM. Both solutions are buffered with 5 mM HEPES, Tris, pH 7.3. More positive voltage intercepts correspond to a higher relative calcium permeability. Relative cation permeability is calculated by using the modified Goldman equation (Shamoo & Goldstein, 1977), assuming $P_{Ca^{2+}}/P_{Cl^-}=0.921$ (*see* Table 1).

Table 3. Relative divalent cation permeability ($P_{Ca^{2+}}/P_{D^{2+}}$) and cation *vs.* anion permeability ($P_{Ca^{2+}}/P_{Cl^-}$) for the undigested succinylated ($Ca^{2+}+Mg^{2+}$)-ATPase, and the various tryptic fragments[a]

| | Undigested succinylated ($Ca^{2+}+Mg^{2+}$)-ATPase | 45,000-dalton fragment | 55,000-dalton fragment | 20,000-dalton fragment |
|---|---|---|---|---|
| $P_{Ca^{2+}}/P_{Ba^{2+}}$ | 0.55 | 1.01 | 0.50 | 0.69 |
| $P_{Ca^{2+}}/P_{Sr^{2+}}$ | 1.49 | 1.13 | 1.50 | 1.10 |
| $P_{Ca^{2+}}/P_{Mg^{2+}}$ | 1.89 | 1.29 | 1.80 | 1.34 |
| $P_{Ca^{2+}}/P_{Mn^{2+}}$ | 2.04 | 1.16 | 2.00 | 1.39 |
| $P_{Ca^{2+}}/P_{Cl^-}$ | 4.30 | 0.92 | 4.30 | 2.30 |

[a] All bilayers are made with oxidized cholesterol, and the bathing solution is buffered with 5 mM HEPES, Tris, pH 7.3. The SE of each of the entries is approximately 1% of the reported value. The error is never greater than 5%.

cepts measured are not consistent with the break lines shown in Table 1 or 2.

The ion dependency of ionophoric activity is determined by measuring the time for the conductance to reach an arbitrary level, at a fixed protein concentration, in the presence of one salt on both sides of the BLM (e.g., 5 mM $MnCl_2$ on both sides of the membrane measures the $Mn^{2+}$ and $Cl^-$ dependency on ionophoric activity). Within statistical errors, there is no difference in the time for the conductance to reach

$5 \times 10^{-10}$ mho for all divalent cations tested (i.e., $Ca^{2+}$, $Ba^{2+}$, $Sr^{2+}$, $Mn^{2+}$, and $Mg^{2+}$). However, in the presence of monovalent cations (i.e., $Na^+$ or $K^+$) and the absence of divalent cations, no increase in conductance occurs. There is a requirement for divalent cations to be present in order for the 45,000-dalton fragment to act as an ionophore.

Mercuric chloride and lanthanum chloride are both potent inhibitors of the ionophoric activity of the 45,000-dalton fragment in an oxidized cholesterol membrane. In the presence of 5 mM $CaCl_2$, pH 7.3, $HgCl_2$ at concentrations $\geqq 135$ μM totally inhibits ionophoric activity. $LaCl_3$ is a slightly more effective inhibitor. 13.5 μM $LaCl_3$ totally inhibits the ionophoric activity of the 45,000-dalton fragment. If $LaCl_3$ is added to a membrane, already at a high stable level of conductance, within ten minutes the conductivity decreases to that of the undoped bilayer. The effectiveness of $HgCl_2$ at the higher concentration is not quite as dramatic as $LaCl_3$. It is only able to prevent an increase in conductance. When $HgCl_2$ is added to a membrane already at a high stable level of conductance, it shows no inhibitory effect.

In addition to studying the effects of the 45,000-dalton fragment in oxidized cholesterol membranes, the fragments were studied in phosphatidylcholine/cholesterol (5:1 mg) BLMs, and in BLMs made from sarcoplasmic reticulum lipids. SR lipids are extracted by the method of Folch *et al.* (1957). Approximately 10 mg lipid is suspended in 1 ml decane. In both lipid preparations addition of the 45,000-dalton fragment or the undigested succinylated $(Ca^{2+} + Mg^{2+})$-ATPase fails to cause an increase in conductance. This lack of an ionophoric effect is probably due to the inability of the protein to incorporate into the BLM.

### *Inhibition due to Reduction of a Disulfide Bond*

Thorley-Lawson and Green (1977) have shown that there are two or three disulfide bonds in the 45,000-dalton fragment that are not accessible to reduction without delipidation of the SR by using detergents. We have studied the effects of several reducing agents, β-mercaptoethanol, dithiothreitol and titanium (III) citrate, on the ionophoric properties of the 45,000-dalton fragment. The oxidation-reduction potential for DTT at pH 7.0 is −0.33 V (Cleland, 1964), for titanium citrate it is −0.480 V (Zehnder & Wuhrmann, 1976), and for β-mercaptoethanol it is close to that of cysteine which is −0.22 V (Konigsberg, 1972; Fruton & Clarke, 1934). When tested in a black lipid membrane, all reducing agents, especially mercaptoethanol, are added in great molar excess.

Table 4. Sulfhydryl content of the undigested $(Ca^{2+}+Mg^{2+})$-ATPase, both unsuccinylated ($R_3$) and succinylated ($R_3$-Chol-Succ) in the presence and absence of various reducing agents[a]

| Protein | Reducing agent | Number of moles SH per mole ATPase | Number of SH per ATPase liberated by reduced agent |
|---|---|---|---|
| $R_3$-Chol-Succ | none | $0.35 \pm 0.04$ ($n=4$) | – |
| $R_3$-Chol-Succ | DTT | $4.16 \pm 0.20$ ($n=4$) | $3.81 \pm 0.20$ |
| $R_3$-Chol-Succ | titanium (III) citrate | $0.20 \pm 0.03$ ($n=2$) | $-0.15 \pm 0.05$ |
| $R_3$-Chol-Succ | titanium (IV) citrate | $0.25 \pm 0.06$ ($n=2$) | $-0.10 \pm 0.07$ |
| $R_3$-Chol-Succ | titanium (III) citrate + at $t=6$ hr DTT | $0.75 \pm 0.04$ ($n=1$) | $0.40 \pm 0.06$ |
| $R_3$-Chol-Succ | DTT + at $t=6$ hr titanium (III) citrate | $1.27 \pm 0.04$ ($n=1$) | $0.92 \pm 0.06$ |
| $R_3$-Chol-Succ + SDS | none | $1.14 \pm 0.03$ ($n=2$) | – |
| $R_3$-Chol-Succ + SDS | DTT | $3.59 \pm 0.13$ ($n=2$) | $2.44 \pm 0.14$ |
| $R_3$ + SDS | none | $18.04 \pm 0.59$ ($n=2$) | – |
| $R_3$ + SDS | DTT | $20.40 \pm 0.70$ ($n=2$) | $2.36 \pm 0.91$ |

[a] All samples are suspended in 50 mM Tris, HCl, pH = 7.0, 1 mM EDTA. If SDS is present, the sample is dissolved in 2% SDS. The protein concentration is determined as described in the text. The molecular weight of $(Ca^{2+}+Mg^{2+})$-ATPase is taken as 115,000 daltons. The numbers shown represent the mean ± the SE. $n$ represents the number of totally independent experiments.

In order to confirm that the reducing agents tested are indeed reducing disulfide bonds in the undigested succinylated enzyme, a series of experiments were undertaken to label all accessible sulfhydryl groups in the presence and absence of reducing agent. The results of these experiments are shown in Table 4. The protein concentration is determined by the method of Lowry *et al.* (1951) divided by the factor 1.06 or in the case of the unsuccinylated enzyme by dividing the absorbance at 280 nm by 1.2 (Thorley-Lawson & Green, 1977). The molecular weight of the ATPase molecule is taken as 115,000 daltons (Thorley-Lawson & Green, 1977).

As we see in Table 4, the succinylated enzyme has lost all of the accessible sulfhydryl groups, either by succinylation or by air oxidation previous to succinylation. Succinylation of the cholate-treated ATPase molecule (Shamoo & MacLennon, 1974) is performed with a large excess

of succinic anhydride over protein. Addition of dithiothreitol (DTT) to the succinylated enzyme causes the reduction of two disulfide bonds.

In the presence of 2% SDS, both the succinylated and unsuccinylated enzyme, upon the addition of DTT, show the appearance of two sulfhydryl groups, or the reduction of only one disulfide bond. It seems likely that, in the presence of SDS, the exposed sulfhydryl groups can more easily reoxidize to form disulfide bonds. If the SDS-treated enzyme, either succinylated or unsuccinylated, is allowed to react with DTT and is incubated at room temperature for 48 hr before the addition of the labeled NEM, the reduction of no disulfide bonds can be measured (data not shown). Incubation at room temperature for 48 hr of the non-SDS treated succinylated enzyme still show the reduction of two disulfide bonds in the presence of DTT.

An attempt to reduce these disulfide bonds using titanium (III) citrate was less successful. As described in *Materials and Methods,* titanium (III) citrate is observed to become oxidized upon the addition of N-ethyl maleimide. In order to avoid reacting all of the [$^{14}$C]-NEM with the titanium (III) citrate, the titanium (III) citrate is allowed to oxidize in the presence of the protein over a period of 48 hr. This causes the pH to drop from pH=7.0 to pH=5.4. At this low pH the reoxidation of protein sulfhydryl groups to disulfides is inhibited. The pH is adjusted to pH=7.0 by the addition of NaOH and [$^{14}$C]-NEM is immediately added to label the resulting sulfhydryl groups. As we see in Table 4, no reduction of disulfides seems to occur.

In order to determine if titanium (III) citrate prevents DTT from reducing the two disulfide bonds in the ATPase molecule or if, upon oxidation, it is reacting with the [$^{14}$C]-NEM and hence prevents the labeling of the sulfhydryls, two additional experiments were performed. In the first experiment titanium citrate was added to the succinylated enzyme. After 6 hr DTT was added and allowed to react at room temperature for 48 hr. In the second experiment DTT was added first, followed by the addition of titanium (III) citrate after 6 hr. After 48 hr the [$^{14}$C]-NEM was added. Both experiments were then followed by dialysis, as described previously. Neither of the two disulfides are reduced in either experiment. From this we conclude that the oxidized titanium citrate is reacting with the [$^{14}$C]-NEM and preventing us from determining if titanium (III) citrate is reducing the disulfide bonds in the succinylated enzyme.

The use of titanium (III) citrate as a reducing agent is complicated by several factors. Titanium citrate has a large absorbance at 280 nm

and hence interferes with simple protein determinations. It also interferes with protein determinations by the method of Lowry *et al.* (1951). Such conventional methods of separating reacted protein from reagent as dialysis, ultrafiltration, column chromatography, and centrifugation all failed to separate titanium citrate from the protein with which it reacted.

In order to determine if titanium citrate would indeed reduce disulfide bonds, two experiments were performed. In the first experiment titanium (III) citrate and previously oxidized titanium (IV) citrate were added to 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB). The solution was buffered with 50mM Tris, HCl, pH=8.0. DTNB is an aromatic disulfide. The nitromercaptobenzoate anion formed from the reduction of the disulfide of DTNB has an intense yellow color with a molar absorptivity of 13,600 $M^{-1}$ $cm^{-1}$ at 412 nm (Habeeb, 1972). Upon reacting with DTNB approximately 15% of the titanium (III) citrate added reduced the disulfide bonds in DTNB. None of the DTNB reacted with the oxidized titanium (IV) citrate. Only a small percentage of titanium (III) citrate reduced the disulfide bond of DTNB. This is probably due to air oxidation of the majority of the added titanium (III) citrate.

Attempts to reduce interchain disulfide bonds in proteins with titanium (III) citrate were unsuccessful. When the two interchain disulfide bonds in insulin (mol wt 5764) are reduced to an A chain (mol wt=3400) and a B chain (mol wt=2300) this can easily be seen in the increased mobility of the peptides by using SDS gel electrophoresis. This is also true when the 4 interchain disulfide bonds of human serum $\gamma$-globulin (mol wt=154,000) are reduced and two heavy chains (mol wt=51,600) and two light chains (mol wt=23,500) result. When titanium citrate was used as a reducing agent the interchain disulfide bonds of $\gamma$-globulin and insulin were not reduced.

We have not been able to show that titanium (III) citrate can reduced disulfide bonds in proteins in spite of its large oxidation-reduction potential. Experiments designed to determine if the disulfide bonds in $(Ca^{2+}+Mg^{2+})$-ATPase were reduced by titanium (III) citrate were inconclusive. It is possible that the succinylated enzyme is more susceptible to reduction by titanium (III) citrate (Habeeb, 1967) than the unsuccinylated enzyme. This may explain why $\gamma$-globulin and insulin are not reduced by titanium (III) citrate. Had the proteins been succinylated, they may have been more susceptible to reduction. On the other hand, we have no positive proof that titanium (III) citrate actually reduces any disulfide bonds in $(Ca^{2+}+Mg^{2+})$-ATPase.

In Table 5, we show the results of the inhibition studies when the

Table 5. The effect of various reducing agents on the ionophoric activity of the undigested succinylated ($Ca^{2+} + Mg^{2+}$)-ATPase ($R_3$-Chol-Succ), the 45,000-dalton fragment (F45) and the 55,000-dalton fragment (F55)[a]

| Protein | Reducing agent or control | Concentration of reducing agent in incubation medium | Molar ratio of reducing agent/protein | $t_{ratio} \pm SE$ |
|---|---|---|---|---|
| F45 | Dithiothreitol | 1 mM→100 mM | $10^3 \rightarrow 10^5$ | $2.35 \pm 0.52$ |
| $R_3$-Chol-Succ | Dithiothreitol | 100 mM | $2.72 \times 10^4$ | $2.13 \pm 0.55$ |
| F45 | β-mercaptoethanol | 1.43 M→7.1 M | $1.29 \times 10^6 \rightarrow 5.36 \times 10^7$ | $6.27 \pm 1.07$ |
| $R_3$-Chol-Succ | β-mercaptoethanol | 7.1 M | $1.43 \times 10^6$ | $5.33 \pm 1.46$ |
| F55 | β-mercaptoethanol | 7.1 M | $3.27 \times 10^7$ | $1.10 \pm 0.24$ |
| F45 | Ethylene glycol | 1.43 M | $1.29 \times 10^6$ | $1.33 \pm 0.66$ |
| $R_3$-Chol-Succ | Ethylene glycol | 8.93 M | $1.79 \times 10^6$ | $1.63 \pm 0.55$ |
| F45 | Titanium (III) citrate[b] | 80 mM | $1.2 \times 10^5$ | $8.19 \pm 3.54$ |
| F45 | Titanium (III) citrate[b] | 10 mM | $8.0 \times 10^3$ | $4.46 \pm 1.45$ |
| $R_3$-Chol-Succ | Titanium (III) citrate[b] | 80 mM | $1.6 \times 10^5$ | $3.80 \pm 1.09$ |
| F55 | Titanium (III) citrate[b] | 27 mM | $8.0 \times 10^3$ | $0.90 \pm 0.19$ |

[a] Column 3 gives the concentration of the reducing agent in the incubation medium and column 4 shows the molar ratio of reducing agent to protein. $t_{ratio} \equiv$ average time required for an increase in conductance ($G \geqq 5.0 \times 10^{-10}$ mho) in the presence of the protein and the reducing agent divided by the average time in the absence of the reducing agent.

[b] In the titanium citrate experiments, the control ("without reducing agent") is in the presence of previously air-oxidized titanium citrate (titanium (IV) citrate).

reduced and nonreduced enzyme is added to an oxidized cholesterol black lipid membrane. As defined in Table 5, $t_{ratio}$ is the average time required for an increase in conductance in the presence of the protein and the reducing agent, divided by the average time in the absence of the reducing agent (the control). For all entries, except the titanium citrate data, the control is incubated with water. For the titanium citrate data, the control is incubated with previously air-oxidized titanium citrate. If $t_{ratio}$ is equal to 1, the reducing agent has no effect. If it is greater than 1, the agent is acting to inhibit transport. The numbers represent the average value of the ratio, plus or minus the standard error. Each number presented is derived from at least two experiments, and each experiment is composed of at least four bilayers in the presence and four bilayers in the absence of the reducing agent. Each experiment is done by adding the reducing agent to the protein sample in the presence of nitrogen. The two samples, with an equal amount of protein, one with and the other without reducing agent, are then incubated at room

temperature for 30 min. In the case of the titanium citrate experiments the incubation for 30 min is done on ice. The concentration of the reducing agent and the molar ratio of the reducing agent to the protein in the incubation medium are also shown in Table 5. All samples are then stored on ice and each in turn is tested by adding it to both sides of the BLM. There is approximately a 100-fold dilution when the sample is added to the bathing solution. The time is then recorded for the conductance to increase to a level which is five times that of the undoped bilayer.

All of the reducing agents tested inhibit the ionophoric activity of the 45,000-dalton fragment (F45) and the undigested succinylated enzyme ($R_3$-Chol-Succ), but they have no effect on the 55,000-dalton fragment (F55). The effectiveness of dithiothreitol (DTT) as an inhibitor of the ionophoric activity of the 45,000-dalton fragment or the succinylated undigested enzyme is less than either titanium (III) citrate or $\beta$-mercaptoethanol.

The inhibition that we see can be due to either the direct effect of the reducing agent, i.e., a disulfide bond essential for transport is reduced and transport is inhibited, or it can be due to a change in the solubility of the protein or protein fragment due to the presence of the reducing agent; i.e., the reducing agent has lowered the ability of the protein to incorporate into the bilayer. In order to test for the latter possibility, the protein is incubated in ethylene glycol, at a molar ratio of inhibitor to protein equal to or greater than that, that is used in the $\beta$-mercaptoethanol experiments. Ethylene glycol has the same structure as $\beta$-mercaptoethanol except that the sulfhydryl of mercaptoethanol is replaced by a hydroxyl group. Ethylene glycol does not show the same inhibitory effect as $\beta$-mercaptoethanol. The control in the titanium citrate inhibition experiments, oxidized titanium citrate, also shows no inhibitory effect on ionophoric activity.

Another possible explanation for this data is that the reduction of the disulfide bonds in the 45,000-dalton fragment changes the solubility of the protein or peptidic fragment and prevents the incorporation of the reduced protein into the black lipid membrane. In order to test for this possibility, an organic extraction experiment was performed as described in *Materials and Methods*. With and without reducing agent, in the presence and absence of $CaCl_2$, protein extraction from the aqueous phase into the decane phase was measured. The results of this experiment are shown in Table 6. In the presence of 5 mM $CaCl_2$, more protein is extracted from the aqueous phase into the decane phase. This is in

Table 6. Extraction of the succinylated ($Ca^{2+}+Mg^{2+}$)-ATPase from the aqueous phase (in the presence or absence of 5 mM $CaCl_2$) into a decane phase[a]

| | Reference (no organic phase) | Protein (no $CaCl_2$) | Protein +5 mM $CaCl_2$ | Reduced protein +5 mM $CaCl_2$ |
|---|---|---|---|---|
| Protein remaining in aqueous phase (mg/ml) | $0.66\pm0.01$ | 0.52 | 0.28 | $0.08\pm0.01$ |
| Percentage of total protein extracted from the aqueous phase | – | 22% | 58% | 88% |

[a] Protein remaining in the aqueous phase is determined by the method of Lowry *et al.* (1951).

agreement with our bilayer data which shows that at higher $CaCl_2$ concentrations the conductance of the BLM, in the presence of the succinylated undigested enzyme, increases more quickly and to a higher level of conductance than at lower $CaCl_2$ concentrations. Incorporation of the succinylated enzyme is $CaCl_2$ dependent.

In order to determine the effect of reduction of disulfide bonds in the succinylated enzyme on extraction from the aqueous phase, three samples were prepared; one with protein but without reducing agent, one with protein and reducing agent, and one without protein but with reducing agent. The samples were incubated for 45 min and extracted into the decane phase as described earlier. Since at this concentration DTT interferes with the Lowry protein determination, the result of reduction of the protein disulfide bonds was derived from subtracting the value in the presence of DTT without protein from the value with both protein and DTT present. As we see from the results of this experiment, more protein, not less, is extracted into the organic phase upon the reduction of the protein disulfide bonds.

In these experiments the amount of protein remaining in the aqueous phases, after addition of the decane phase, was measured. The protein does not appear to be soluble in the decane phase. It seems to precipitate at the border of the organic and aqueous phases. This is evidence against the succinylated enzyme being a mobile carrier.

In order to determine if the reduction of protein disulfide bonds could influence the Lowry protein determination, a known amount of human serum $\gamma$-globulin was solubilized in 0.1% SDS, 20 mM Tris, pH = 7.0. In one aliquot of the protein, disulfide bonds were reduced by the addition of 2 mM DTT. In a second aliquot no reducing agent was

added to the protein. In the third aliquot 2 mM DTT was present in the absence of the protein. The second sample (no DTT) was compared to the difference between the third and first samples. Reduction of the disulfide bonds caused a decrease in the Lowry protein determination by not more than 20%. This effect is far too small to explain the decreased protein remaining in the aqueous phase upon reduction of the disulfide bonds in $(Ca^{2+} + Mg^{2+})$-ATPase.

We conclude from these results that there is one or more essential disulfide bonds, localized in the 45,000-dalton fragment, whose integrity is essential for transport. Reduction of this disulfide bond disrupts the integrity of the transport machinery. It does not merely effect the solubility of the protein. Because of the lack of an inhibitory effect on the 55,000-dalton fragment, and the presence of inhibition in the undigested enzyme and the 45,000-dalton fragment, we conclude that the two fragments are in a series arrangement in the intact enzyme. Inhibition of the ionophoric properties of one fragment, by the reduction of an essential disulfide bond, results in inhibition of transport in the intact enzyme.

Attempts made to reoxidize the disulfide bond, essential for transport in the 45,000-dalton fragment, were unsuccessful. Incubation of the protein fragment, following treatment with titanium (III) citrate, in the freezer overnight does not restore ionophoric activity, in spite of the fact that titanium (III) citrate is air oxidized to titanium (IV) citrate. Adding cupric phenanthroline (which catalyzes the air oxidation of sulfhydryl groups) to the bathing solution (Kobashi, 1968; Murphy, 1976), also fails to reoxidize the disulfide bond and restore ionophoric activity. Incubation in 5 mM N-ethylmaleimide (NEM) fails to inhibit ionophoric activity of the 45,000-dalton fragment. This is in agreement with our earlier result that succinylation, which eliminates all reactive sulfhydryl groups, has no effect on the ionophoric activity of the undigested enzyme.

The effect of $\beta$-mercaptoethanol and titanium (III) citrate on active calcium uptake into fragmented sarcoplasmic reticulum (SR) has also been studied. Uptake of $^{45}Ca$ is carried out as described in *Materials and Methods*. The maximum concentrations of titanium (III) citrate and $\beta$-mercaptoethanol present in the uptake medium are 16 mM and 1.43 M, respectively. Oxidized titanium (IV) citrate and ethylene glycol, at the same molar rations of reducing agent to SR protein are tested as controls. In the case of $\beta$-mercaptoethanol, uptake (in the absence of a precipitating anion) is inhibited more than for the control, ethylene glycol. However, we find that at the high concentrations of $\beta$-mercaptoethanol tested the SR is leaky to calcium (data not shown). In the case of titanium

citrate, for both the oxidized and the reduced state, uptake is decreased by 15–30% of the untreated SR ($\sim$100 nmol/mg protein after 30 sec). There is, however, no significant difference in the $^{45}Ca$ uptake between the reduced and the oxidized titanium citrate. We find that neither reducing agents decrease active calcium uptake due to the reducing power of the agent tested. This is consistent with the Thorley-Lawson and Green (1977) finding that the disulfide bonds are not reducible in the native enzyme. They can, however, be reduced after delipidation of the enzyme with anionic detergents.

## Discussion

By SDS preparative gel electrophoresis, we have been able to separate the two tryptic fragments from the initial cleavage of ($Ca^{2+} + Mg^{2+}$)-ATPase from the sarcoplasmic reticulum of white skeletal muscle. SDS is removed by extensive dialysis. The 45,000-dalton fragment is found to be active as an ionophore in an oxidized cholesterol BLM at a concentration of $1.5 \times 10^{-9}$ M. It shows approximately the same activity as the other fragments previously tested.

One of the major differences between the 45,000-dalton fragment and the other products of tryptic cleavage is that the 45,000-dalton fragment is relatively nonselective at neutral and slightly basic pH. Of particular interest is the change in the selectivity in the region below pH 7.0 and above pH 8.0. Below pH 7.0 it appears as if the imidazole group of a histidine is being protonated. Above pH 8.0 it appears as if a sulfhydryl of a cysteine or an $\varepsilon$–$NH_3^+$ group of a lysine is being modified. Yu, Masoro and Bertrand (1974) have shown the photooxidation of histidine residues in SR causes a decrease in active calcium uptake at a rate that is faster than the loss of ATPase activity. This may well be due to the oxidation of the histidine in the 45,000-dalton fragment that influences its selectivity and ionophoric activity. Further study is necessary to clarify this point.

The 45,000-dalton fragment is shown to be a relatively nonselective ionophore (at neutral pH) when compared to the succinylated ($Ca^{2+} + Mg^{2+}$)-ATPase or any of the other fragments already studied (*see* Table 3). The relative permeabilities measured for the undigested succinylated enzyme are consistent with the two tryptic fragments being in a series arrangement in the membrane. The ionophoric activity of the 45,000-dalton fragment is also shown to be dependent on the presence

of divalent cations. In the presence of monovalent cations only, there is no increase in conductance.

In this paper we have presented data which shows the inhibition of ionophoric activity in the 45,000-dalton fragment due to the presence of $HgCl_2$ and $LaCl_3$. Methylmercuric chloride has no inhibitory effect on ionophoric activity. As shown by Shamoo and MacLennan (1975), mercuric chloride inhibits calcium uptake into fragmented sarcoplasmic reticulum at a lower concentration than it inhibits ATPase activity. This was taken as evidence for the separate location of the ionophoric site (ion translocating site) and the site of ATP hydrolysis. In this same study, $HgCl_2$ was shown to cause inhibition of ionophoric activity in the succinylated undigested enzyme, while $CH_3HgCl$ showed no inhibitory effect on ionophoric activity. The differential effect of $CH_3HgCl$ and $HgCl_2$ was taken as evidence against $HgCl_2$, causing inhibition by reacting with sulfhydryl groups. In this paper we have confirmed this observation. There are no free sulfhydryl groups in the ATPase molecule following succinylation.

The effects of both $HgCl_2$ and $LaCl_3$ are probably directly on the ionophoric sites. Both $La^{3+}$ and $Hg^{2+}$ seem to substitute for $Ca^{2+}$ and prevent the translocation of $Ca^{2+}$ ions across the membrane. The inhibition effect that we see here due to $HgCl_2$ and $LaCl_3$ has also been observed in the 20,000-dalton fragment and the 55,000-dalton fragment (Shamoo *et al.*, 1976). The existence of ionophoric activity in both the 45,000-dalton fragment and the 55,000-dalton fragment and one of its tryptic fragments, the 20,000-dalton fragment, all of which are inhibited by approximately the same dosage of $HgCl_2$ and $LaCl_3$, indicates that the ion translocation sites extend across the whole enzyme and are found in each of its fragments. The ionophoric site is not simply localized in one unit that acts as a mobile carrier. The ATPase molecule probably acts like an extended channel translocating calcium across the membrane.

Thorley-Lawson and Green (1977) have shown that there are two or three disulfide bonds in the 45,000-dalton fragment that are accessible to reduction only after delipidation by detergent. We have shown in this paper that there are two disulfide bonds accessible to reduction by DTT in both the succinylated enzyme and in the unsuccinylated $(Ca^{2+}+Mg^{2+})$-ATPase which has been solubilized with SDS. Futhermore, the enzyme has lost all of its free sulfhydryl groups during succinylation. We also show that one or more of these disulfides are essential for the 45,000-dalton fragment to act as an ionophore. Reduction of

the disulfide bonds by using dithiothreitol, $\beta$-mercaptoethanol, or titanium (III) citrate causes inhibition of ionophoric activity in both the undigested enzyme and the 45,000-dalton fragment. Reduction of these disulfide bonds disrupts the transport machinery. It does not merely lessen the solubility of the protein in the lipid phase. The protein appears to be more soluble in the lipid phase after reduction than before reduction of the disulfide bonds.

Experiments designed to determine if titanium (III) citrate reduces these protein disulfide bonds were unsuccessful. Even if the effect of titanium (III) citrate is not that of reducing disulfide bonds, the effect is still very important. Titanium (III) citrate is a very potent inhibitor of the ionophoric activity of both the 45,000-dalton fragment and of the undigested enzyme, It does not effect the ionophoric properties of the 55,000-dalton fragment.

Attempts to air oxidize the sulfhydryl groups and regain ionophoric activity have not been successful. Tests done on the 55,000-dalton fragment show its ionophoric activity to be unaffected by treatment with reducing agents. This is as expected, since there are not known to be any disulfide bonds in the 55,000-dalton fragment (Thorley-Lawson & Green, 1977). Treatment of the undigested succinylated $(Ca^{2+} + Mg^{2+})$-ATPase with each of these reducing agents causes inhibition of ionophoric activity, similar to what we see in the 45,000-dalton fragment. From these results we deduce that the 45,000-dalton fragment and the 55,000-dalton fragment are in a series arrangement as they transport calcium across the sarcoplasmic reticulum. Inhibition of one of the two tryptic fragments inhibits the transport function of the undigested enzyme.

The data lends further support to our model of active calcium transport across the sarcoplasmic reticulum (Shamoo & Goldstein, 1977). The model involves the binding of calcium to high affinity $Ca^{2+}$-binding sites located in the 30,000-dalton fragment and/or in the 20,000-dalton fragment, followed by the hydrolysis of ATP at a site located in the 30,000-dalton fragment. The energy from the hydrolysis of ATP is then funneled into moving calcium through the 20,000-dalton fragment followed by the 45,000-fragment where it is released on the inside of the SR.

This paper is based on work performed under contract with the U.S. Department of Energy (DOE) at the University of Rochester Biomedical and Environmental Research Project and has been assigned Report No. UR-3490-1338. This paper was also supported in part by NIH Grant 1 RO1 18892; A center Grant ESO-1247; and Program Project

Grant ES-10248 from the NIEHS; the Muscular Dystrophy Association (USA) and the Upjohn Company.

This work was done during the tenure of Jonathan J. Abramson as a Research Fellow of Muscular Dystrophy Association. Adil E. Shamoo is an Established Investigator of the American Heart Association.

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