Biochemical Identification of Transmembrane Segments of the Ca\textsuperscript{2+}-ATPase of Sarcoplasmic Reticulum*

(Received for publication, May 4, 1994, and in revised form, June 16, 1994)

Jai Moo Shin, Masayoshi Kajimura, José M. Argüello‡, Jack H. Kaplan‡, and George Sachs§

From the Wadsworth Veterans Administration Medical Center and the Department of Medicine and Physiology, UCLA, Los Angeles, California 90073 and the §Department of Physiology, University of Pennsylvania, Philadelphia

The transmembrane segments of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase were determined by trypsinization of cytoplasmic side-out intact sarcoplasmic reticulum vesicles. The membrane portion of tryptic digest comprising the transmembrane fragments, joined by the intravesicular segments, was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after labeling with fluorescein 5-maleimide in the presence of sodium dodecyl sulfate. In this way, seven fluorescent bands of tryptic fragments below 11 kDa were observed which were derived from 4 pairs of membrane spanning segments and one hydrophobic sequence at the C-terminal end. Two peptides of 10.8 and 10.6 kDa had the identical N-terminal sequence beginning at Glu\textsuperscript{699}, representing the transmembrane segments M7 and M8 and their connecting loop. A band at 8.1 kDa contained one peptide beginning at Tyr\textsuperscript{700} (M1/loop/M2). A 7.7-kDa peptide starting at Leu\textsuperscript{721} (M3/loop/M4) and a 7.3-kDa peptide beginning at Ala\textsuperscript{722} (M5/loop/M6) were also observed. A band at 6.7 kDa contained two peptides, one beginning at Ser\textsuperscript{74} (M1/loop/M2) and another beginning at Tyr\textsuperscript{748} (M5/loop/M6). In addition, a 4-kDa peptide beginning at Met\textsuperscript{750} was observed. The size of this peptide did not allow for a complete pair of transmembrane segments, but this peptide could have been derived from trypsinolysis between the last pair of membrane spanning segments. These data therefore provide biochemical evidence for at least 8 transmembrane segments and perhaps two more at the C-terminal end of the enzyme.

The Ca\textsuperscript{2+}-ATPase of skeletal muscle sarcoplasmic reticulum is one of the P-type ATPases and is a member of the SERCA gene family (1). The P type ATPases have been predicted to have either 8 (2-4) or 10 (5, 6) transmembrane domains based on hydropathy of the amino acid sequence. All models agree in proposing four transmembrane segments in the N-terminal half and a large cytoplasmic loop in the central portion of the protein. They differ in the number of transmembrane segments (4 or 6) in the C-terminal half of the pump.

The location of both the N- and C-terminal segments of the Ca\textsuperscript{2+}-ATPase was shown to be in the cytoplasmic domain by antibody binding (7) establishing the presence of an even number of transmembrane fragments. A common difficulty with hydropathy plots is the nature of the 5th and 6th membrane spanning segments. Whereas this region was originally predicted to have a membrane spanning pair of segments in the Ca\textsuperscript{2+}-ATPase (5), hydropathy algorithms only predict a single segment and only a single segment in this region was proposed in a model of the Na,K-ATPase (2).

Antibodies against the residues between Lys\textsuperscript{770} and Ile\textsuperscript{780} react with cytoplasmic side outside vesicles only after detergent treatment, providing evidence for a luminal location of this region. Since this region is between M7 and M8, the M5/M6 region is deduced to have a pair of membrane spanning segments (8, 9). These data appear to confirm a 10-membrane segment model for the ATPase, although direct biochemical evidence for the membrane spanning segments has not been published.

There are 1 or more cysteines predicted to be present in all the putative membrane segment pairs of the 10-segment model of the ATPase. Therefore, fluorescent labeling with a cysteine-reactive probe should allow detection of these peptides after having removed the cytoplasmic regions of the enzyme by extended proteolysis of intact sarcoplasmic reticular vesicles. In this work, we describe the results obtained using this approach and fluorescein 5-maleimide as the fluorescent label. Direct evidence was obtained for the first 8-membrane spanning segments of the Ca\textsuperscript{2+}-ATPase. Experimental evidence for the presence of the putative 9th and 10th transmembrane segments was less conclusive, although a short membrane-associated fragment derived from the C-terminal region was identified.

EXPERIMENTAL PROCEDURES

Preparation of Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase—Rabbit sarcoplasmic reticulum vesicles were prepared by the procedure of Chu et al. (10). All manipulations were performed at 2–4 °C. A fraction between 32 and 34% sucrose (light sarcoplasmic reticulum vesicles) was collected and the fractions sedimented. The pellets were resuspended in a 0.3 M sucrose, 10 mM imidazole (pH 7.4) solution at a protein concentration of 12 mg/ml and stored at −70 °C. Protein was determined by the method of Lowry (11) using bovine serum albumin as a standard.

The sarcoplasmic reticulum vesicles of Ca\textsuperscript{2+}-ATPase used in this experiment were largely inside-out based on cleavage by trypsin. These vesicles were also relatively sealed prior to trypsinolysis based on the finding that their calcium uptake was equivalent to literature values (12).

The Ca\textsuperscript{2+}-ATPase activity of light sarcoplasmic reticular vesicles was about 3.2 μmol of P, mg\textsuperscript{-1} min\textsuperscript{-1}, and the Ca\textsuperscript{2+} uptake capacity, measured by a method of Meissner and Fleischer (12) using CaCl\textsubscript{2} in the presence of 5 mM oxalate, was 4.0 μmol of calcium ion/mg of protein. The intactness of the sarcoplasmic reticular vesicles was also determined by measuring Ca\textsuperscript{2+} uptake and release using a calcium dye method. The impermeant calcium chelator, Fura-2, was used to monitor changes in extravesicular Ca\textsuperscript{2+} concentration (13). The 340/380 nm excitation ratio with emission at 505 nm was used as described previously to monitor extravasicular Ca\textsuperscript{2+} concentration (13). The sarcoplasmic reticular vesicles (100 μg/ml) were preincubated in a buffer solution composed of 0.25 M sucrose, 1 mM MgCl\textsubscript{2}, 100 mM KH\textsubscript{2}PO\textsubscript{4}-K\textsubscript{2}HPO\textsubscript{4} (pH 7.0), 60 μM Fura-2, and 50 μM CaCl\textsubscript{2} at room temperature for 15 min. The sarcoplasmic reticular vesicle suspension (2 ml) was

* This work was supported by USVA-SMI and National Institutes of Health Grants DK 40615, 41301, 14752, and GM 39500. Sequencing was supported by National Institutes of Health BRS Shared Instrumentation Grant S10RR05554-01 to UCLA. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Wadsworth VA Hospital, Bldg. 113, rm. 326, Los Angeles, CA 90073. Fax: 310-312-9478.

§ This work was supported by USVA-SMI and National Institutes of Health Grants DK 40615, 41301, 14752, and GM 39500. Sequencing was supported by National Institutes of Health BRS Shared Instrumentation Grant S10RR05554-01 to UCLA. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Wadsworth VA Hospital, Bldg. 113, rm. 326, Los Angeles, CA 90073. Fax: 310-312-9478.

§ To whom correspondence should be addressed: Wadsworth VA Hospital, Bldg. 113, rm. 326, Los Angeles, CA 90073. Fax: 310-312-9478.

¶ The abbreviations used are: Fura-2, [1-(2,5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2,2'-amino-5'-methoxyphenoxymethylene-N,N,N',N'-tetraacetic acid; PAGE, polyacrylamide gel electrophoresis.

22533
Membrane Domain of Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase

stirred for 4 min in a SPEX 1681 dual wavelength spectrophotometer with dual excitation at 340 and 380 nm and emission at 505 nm, giving the initial extravesicular calcium concentration. Ca\textsuperscript{2+} uptake was started by adding ATP (1.5 mm final concentration), and was monitored for 20 min. The initial concentration of the extravesicular Ca\textsuperscript{2+} rapidly decreased as the Ca\textsuperscript{2+} uptake proceeded and, finally, the concentration of the extravesicular calcium ion became constant at 1.1 mm. After 24 min Triton X-100 (0.2% final concentration) was added to the sarcoplasmic reticulum vesicle suspension to release intravesicular Ca\textsuperscript{2+}. The concentration of external Ca\textsuperscript{2+} promptly increased. Finally, EGTA (5 mm final concentration) was added to the medium to obtain the basal fluorescence value (F\textsubscript{0}) necessary for calibration of the Ca\textsuperscript{2+} signal.

Whereas the above data demonstrate that the vesicles used here conform in terms of Ca\textsuperscript{2+} uptake to what has been described previously (12), it cannot be excluded that trypsin penetrates the vesicle interior during digestion and hydrolyzes a particularly sensitive bond on the extracytoplasmic face.

Trypsin Digestion of Sarcoplasmic Reticulum Vesicles and Labeling with Fluorescein 5-Maleimide—Proteolysis with trypsin was carried out at 1:4 (w/w) ratio of trypsin to protein at 37 °C using either light intact or calcium oxalate-loaded heavy vesicles derived from the sarcoplasmic reticulum. Light sarcoplasmic reticulum vesicles under isotonic conditions in the presence of 250 mm sucrose and the heavy sarcoplasmic reticulum vesicles as loaded with calcium oxalate are usually intact (14). Fractionation of sarcoplasmic reticulum vesicles to produce the heavy vesicles by calcium oxalate precipitation was carried out by the method of Leberer et al. (14).

The Ca\textsuperscript{2+}-ATPase of sarcoplasmic reticulum (1 mg) was suspended in a buffer (1 ml) composed of 0.25 mm sucrose, 50 mm Tris/HCl (pH 8.1), and trypsinolysis was started by adding 0.25 mg of trypsin (1 mg/ml) dissolved in the same buffer and incubating at 37 °C for intervals of 2, 5, 10, and 20 min. At each time point, 125-μl aliquots were taken and added to 37.5 μl of 10 mg/ml soybean trypsin inhibitor, 50 mm Tris/HCl (pH 8.1) on ice. The aliquots were centrifuged at 110,000 × g for 1 h. The pellet portions were washed with 50 mm Tris buffer (pH 7.4), 10 μg/ml soybean trypsin, and dissolved in 100 μl of 50 mm Tris/HCl (pH 6.8), 0.3% sodium dodecyl sulfate (SDS), and 50 μm fluorescein 5-maleimide, and incubated at room temperature for 20 min before SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The experiments described are typical of at least five experiments following an identical protocol.

Peptide Separation by SDS-PAGE and Transfer onto Polyvinylidene Difluoride Membranes—The membrane peptides were combined with 20% volume of sample buffer (0.3 x Tris, pH 7.0, 10% SDS, 50% sucrose, and 0.025% bromphenol blue), and separated by SDS-PAGE according to Laemmli (15) with some modification. In these experiments, big slab gels (size: 14 × 16 cm, thickness: 1.5 mm) were prepared as follows. Three-fourths of the gel from the top was a linear gradient from 10% (34:1, acrylamide/methylene bisacrylamide) to 21% (17:1, acrylamide/methylene bisacrylamide) and the bottom one-fourth was 21% acrylamide (17:1, acrylamide/methylene bisacrylamide). Gels were run for 19-24 h at 48 m</div>
The extracytoplasmic segments of the membrane-associated tryptic digest

<table>
<thead>
<tr>
<th>Molecular mass (kDa)</th>
<th>Sequence (N-terminal)</th>
<th>Recovery (pmol)</th>
<th>Transmembrane region</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.8</td>
<td>E955 PLIS...</td>
<td>10</td>
<td>M7/loop/M8</td>
</tr>
<tr>
<td>10.6</td>
<td>E955 PLIS...</td>
<td>37</td>
<td>M7/loop/M8</td>
</tr>
<tr>
<td>8.1</td>
<td>Y966 GHNEL...</td>
<td>65</td>
<td>M1/loop/M2</td>
</tr>
<tr>
<td>7.7</td>
<td>L971 DEFG</td>
<td>72</td>
<td>M3/loop/M4</td>
</tr>
<tr>
<td>7.3</td>
<td>A972 IYNN...</td>
<td>38</td>
<td>M5/loop/M6</td>
</tr>
<tr>
<td>6.7</td>
<td>S981 LWELVI...</td>
<td>12</td>
<td>M1/loop/M2</td>
</tr>
<tr>
<td>6.0</td>
<td>Y967 LISS...</td>
<td>17</td>
<td>M5/loop/M6</td>
</tr>
<tr>
<td>4.0</td>
<td>M983 PPWV...</td>
<td>41</td>
<td></td>
</tr>
</tbody>
</table>

* Recovery (picomoles) was semiquantitatively calculated by the N-terminal sequencing yields of the tryptic digest obtained from 110 pg of sarcoplasmic reticulum vesicles. Seven fluorescent bands of 10.8, 10.6, 8.1, 7.7, 7.3, 6.7, and 4 kDa obtained by the progressive trypsinolysis were sequenced and the results are summarized in Table I.

In this work, we have presented biochemical evidence for the identification of the first 8 transmembrane segments of the Ca\(^{2+}\)-ATPase. The Kyte-Doolittle algorithm using a moving average of 11 amino acids predicts four membrane spanning segments in the first one-third of the molecule. However, in the C-terminal one-third, only four membrane segments are predicted, since what is found to be M6 and M7 have a peak hydrophy of less than 200 (Fig. 3) and are predicted not to be membrane spanning. Other algorithms also fail to predict 10-membrane spanning segments. This uncertainty led to experiments which defined the sidedness of antibodies directed against a region between...
M7 and M8 (8, 9) as being extracytoplasmic. From this, a 10-membrane segment model could be deduced, on the assumption that M8 returned to the cytoplasmic side. If M8 remained extracytoplasmic, and M9 returned the protein back to the cytoplasm, an 8-membrane segment model could still be correct. Recently, data obtained using artificial epitope mapping for the Na,K-ATPase were interpreted as showing an 8-membrane segment model, since an epitope inserted at position 978 of the rat α1 Na,K-ATPase was shown to be cytoplasmic and the epitope inserted at position 941 was thought to be extracytoplasmic (17). These epitopes are on either side of putative M9 and the model placed M9 as the last membrane spanning segment of the Na,K-ATPase. However, the epitope at position 941 was not actually visualized, and could well also be cytoplasmic. This would place the connection between M8 and M9 and between M9 and M10 in the cytoplasmic domain, a possibility suggested by our data. A model wherein the M9 segment forms an intramembraneous loop and does not span the membrane is also a possibility to be considered (see Fig. 3).

A recent study used a set of fusion proteins to determine the topology of the Mg$^{2+}$ translocating P-type ATPase of Salmonella typhimurium. The topological mapping depended on the cytoplasmic activity of LacZ product (β galactosidase) and extracytoplasmic activity of BalM product (β lactamase) (18). The data presented were in agreement with the 10-membrane segment model of the Ca$^{2+}$-ATPase and the first 8 segments as demonstrated in this work are also in direct agreement with this. There was, however, ambiguity in the region between M6 and M7 and only one extracytoplasmic site was mapped in the M9/M10 region.

Identification of First 8 Transmembrane Segments (M1-M8)—Six peptides accounting for the first 8 transmembrane segments were readily identifiable (see Fig. 1, Table I). The model in Fig. 3B presents the arrangement of these peptides in the membrane, together with the position of the cysteine residues known to be localized in the luminal side of the vesicles. The loop between M7 and M8 has been shown to be extracytoplasmic using antibody epitope mapping (8, 9). These first 8-transmembrane domains are coincident with those predicted by the 10-transmembrane helix model based on hydrophobicity plots (5, 6).

The location of these first 8 transmembrane segments is in accord with the known positions of 8 transmembrane segments of other P-type ATPase, the H,K-ATPase and the Na,K-ATPase. In the case of the H,K-ATPase, the presence of 4 pairs of transmembrane segments were identified by the tryptic cleavage of the intact gastric vesicles with labeling by selected extracytoplasmic reagents such as an imidazopyridine (19) and the substituted benzimidazoles, omeprazole (16), lansoprazole (20), and pantoprazole (21). In the case of the Na,K-ATPase, trypsinolysis also provided evidence for the transmembrane segment pairs M1/M2, M3/M4, M5/M6 and a C-terminal 19-kDa fragment containing the M7/M8 transmembrane segments (22, 23). These data for these mammalian P-type ATPases justify a
similar topological arrangement for the first 8-membrane spanning segments.

The C-terminal Region—Beside the first four transmembrane pairs, another labeled hydrophobic peptide starting at Met926 was observed. However, this peptide extending to Lys951 depends on the apparent molecular mass of 4 kDa in SDS-PAGE. It was probably too short to account for two transmembrane segments. It would thus contain only the 9th transmembrane segment and end just before the putative 10th transmembrane segment proposed in models based on hydropathy plots (5, 6). We have found no evidence for the putative 10th transmembrane segment retained in the membrane pellet.

It has been shown that the C-terminal segment of the Ca2+-ATPase is localized in the cytosol (7). The peptide starting at Met926 then cannot cross the membrane and have its C terminus in the luminal side of the vesicles. There are several explanations which might resolve this discrepancy.

One explanation is that the peptide beginning at Met926 contains a 10th transmembrane segment (i.e. it has a molecular mass > 8800 Da) but runs with an anomalously high mobility in SDS-PAGE. This would result in a 10-transmembrane segment model as shown in Fig. 3. If this were true, if permeabilized vesicles were to be treated with trypsin, Lys960 and Lys960 (localized at the luminal surface in this model) would become accessible to trypsin and the peptide beginning at Met926 would be reduced in size. In fact, the peptide obtained after tryptic digestion of permeabilized vesicles migrates with the same mobility as the tryptic peptides obtained from the intravesicular side (6). We have found no evidence for the putative 10th transmembrane segment if trypsin had access to the intravesicular side of the vesicles (6).

A second possibility is that the peptide beginning at Met926 and extending to Lys951 or Lys960 could be produced in a 10-transmembrane segment model if trypsin had access to the intravesicular surface. Our experiments which show that the vesicles actively transport and accumulate Ca2+ ions suggest that the vesicles are not leaky to Ca2+ ions prior to trypsin treatment. Heavy (calcium oxide) and light vesicles provide the same tryptic cleavage pattern. These data may suggest that trypsin does not penetrate the vesicles to a significant extent, particularly since the 4-kDa fragment is produced rapidly and in high yield.

Another possibility is that this small 4-kDa peptide was the result of further tryptosilation that may have occurred in the gel during electrophoresis due to possible residual trypsin left with the sample. Soybean trypsin inhibitor is known to stabilize trypsin activity in the presence of SDS (24). It may be that a certain amount of trypsin complexed with soybean trypsin inhibitor remained associated with the membrane, and retained its activity in SDS during electrophoresis resulting in further cleavage. If this were true, the M10 fragment that resulted from the additional cleavage should have been found along with the M9 fragment or below the M9 fragment band on the gel. However, no significant peptide sequences were observed below the 4-kDa range. This may be due to a difficulty in detecting the M10 or its fragments following labeling with fluorescein-5-maleimide, since there are no cysteines predicted to be present in this region of the enzyme.

Another possibility is that with cleavage of the cytoplasmic domain, the peptide representing M9/M10 can rearrange by moving out of the bilayer, exposing the sequence Lys-Leu-Lys at positions 958–960 to trypsin hydrolysis. In fact the putative 10th membrane spanning segment contains hydrophilic amino acids such as Asp, Lys, and Thr and could be unstable in the membrane domain once the cytoplasmic domain disappears.

Hence, if the 10-membrane segment model is correct, the access of trypsin to the site(s) between M9 and M10 must be due either to unexpected and rapid access of trypsin to the interior of the vesicles or due to a rearrangement of the peptide fragments after cleavage of the cytoplasmic domain.

An 8-membrane segment model could also account for production of this fragment. In this model, the 9th hydrophobic sequence, M9, does not traverse the membrane but enters it from the cytosol, loops within the membrane, and returns to the cytosol (see Fig. 3). In this way, Lys951 and Lys960 are exposed to trypsin from outside vesicles. The model is then composed of only 8 transmembrane segments with a membrane-embedded loop forming a 9th membrane-associated domain and the 10th hydrophobic sequence, M10, being cytoplasmic. Recently, a membrane-associated protein, prostaglandin H2 synthase-1 was shown to be a monotopic membrane protein, while the models of this protein have been proposed requiring only one or more transmembrane segments based on amino acid sequence (25). The proposed transmembrane segments of this protein have been identified as part of the catalytic domain by x-ray crystallography, and the membrane binding surface of this compact enzyme was not deep enough to extend beyond one leaftlet of the lipid bilayer. This observation enables a possible interpretation that the hydrophobic M9 region is not deep enough to extend beyond one leaftlet of the lipid bilayer. In fact this observation provides evidence for the first 8-transmembrane segments of the Ca2+-ATPase. There is considerable sequence similarity in the family of P-type ATPases in the N-terminal regions and so this arrangement is probably true for all the members of this family in spite of widely varying amino acid composition. Our studies did not allow us to unambiguously distinguish between a modified 8-transmembrane segment model or one which contains 10 such segments which traverse the membrane.

Acknowledgments—We thank Dr. Audree Fowler for peptide sequencing and David Scott for discussions.

REFERENCES


Membrane Domain of Sarcoplasmatic Reticulum Ca2+-ATPase 22587