The Cytoplasmic Loop between Putative Transmembrane Segments 6 and 7 in Sarcoplasmic Reticulum Ca$^{2+}$-ATPase Binds Ca$^{2+}$ and Is Functionally Important*

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Limited proteolysis by proteinase K of rabbit SERCA1 Ca$^{2+}$-ATPase generates a number of fragments which have been identified recently. Here, we have focused on two proteolytic C-terminal fragments, p20C and p19C, starting at Gly-808 and Asp-818, respectively. The longer peptide p20C binds Ca$^{2+}$, as deduced from changes in migration rate by SDS-polyacrylamide gel electrophoresis performed in the presence of Ca$^{2+}$ as well as from labeling with $^{45}$Ca$^{2+}$ in overlay experiments. In contrast, the shorter peptide p19C, a proteolysis fragment identical to p20C but for 10 amino acids missing at the N-terminal side, did not bind Ca$^{2+}$ when submitted to the same experiments. Two cluster mutants of Ca$^{2+}$-ATPase, D813A/D818A and D813A/D815A/D818A, expressed in the yeast Saccharomyces cerevisiae, were found to have a very low Ca$^{2+}$-ATPase activity. Region 808–818 is thus essential for both Ca$^{2+}$ binding and enzyme activity, in agreement with similar results recently reported for the terminal side, did not bind Ca$^{2+}$ labeling with $^{45}$Ca$^{2+}$

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P-type transport ATPases are members of a large family of procaryotic and eucaryotic proteins, specialized in active transport of various cations such as Na$^{+}$, K$^{+}$, Ca$^{2+}$, and Cu$^{+}$ (1–6) and perhaps also aminophospholipids (7). Based on sequence homologies and transport specificities, these proteins can be divided into the following main groups: types I ATPases (heavy metal transporters), IIA ATPases (e.g. Na$^{+}$,K$^{+}$-ATPase or SERCA$^{1}$ Ca$^{2+}$-ATPase), and IIB ATPases (e.g. H$^{+}$-ATPase or plasma membrane Ca$^{2+}$-ATPase) (6). Ordered two-dimensional or three-dimensional membrane crystals have provided information on the overall shape of SR Ca$^{2+}$-ATPase (8–10), the H$^{+}$-ATPase of Neurospora crassa (11), Na$^{+}$,K$^{+}$-ATPase (12, 13), and H$^{+}$,K$^{+}$-ATPase (14), but the level of resolution obtained at present (down to 10 Å) does not allow a precise description of the topology. Thus the detailed organization of cytoplasmic regions and transmembrane segments in domains with defined functional properties still remains a matter of speculation. Based on amino acid similarities, it is reasonable to postulate that, within each group, the topology and the fundamental reaction mechanism exhibit common features (6, 15).

Directed mutagenesis and various biochemical techniques have been successfully used to pinpoint individual amino acid residues or group of residues important for transport activities. In the case of SR Ca$^{2+}$-ATPase it was initially demonstrated by Clarke et al. (16) and later considerably refined (17–20) that 6 amino acid residues, presumably located in the transmembrane segments, are of primary importance for Ca$^{2+}$ binding to the ATPase and/or Ca$^{2+}$-dependent phosphorylation from ATP. These residues are Glu-309, Glu-771, Asn-796, Thr-799, Asp-800, and Glu-908, located in the putative transmembrane segments M4, M5/M6, and M8. A model was built in which these transmembrane segments are clustered and form a channel that can admit two Ca$^{2+}$ in a single row (since these bound ions appear to be stacked inside the ATPase structure (21, 22)). All of these residues, with the exception of Glu-908 (18, 23), have been found to be essential for occlusion of Ca$^{2+}$. In Na$^{+}$,K$^{+}$-ATPase and H$^{+}$,K$^{+}$-ATPase the homologous residues located in the M5/M6 segments have recently been demonstrated to be of crucial importance for cation binding and transport (19, 24–29). Thanks to these and other efforts (e.g. see Refs. 30 and 31) a consensus picture of what could be the ion pathway in the membrane is gradually emerging.

An unresolved question is whether the membranous amino acid residues considered so far are the only ones that are directly involved in translocation of cations. Recently, in H$^{+}$,K$^{+}$-ATPase Swarts et al. (27) have identified 3 new functionally important amino acid residues (Glu-834, Glu-837, and Glu-838).
Electrophoresis, Blotting, and 45Ca2+-Overlay—SDS-PAGE gels (48) were prepared with inclusion of either 1 mM Ca2+ or 0.02–0.1 mM EGTA in the stacking and separation gels (49). EGTA gels were made with 12.5 or 15% acrylamide instead of 11.5% to allow for less efficient polymerization (as indicated from migration of standard proteins). The protein samples, prepared without the addition of Ca2+ or EGTA (49), were treated with concentrated urea to prevent aggregation (50). Gels were stained with Coomassie Blue. Electrophoresis of peptides was performed as described (51). 45Ca2+ overlay (52) was performed as described in Ref. 49. Western blotting was performed as described (37) with visualization by the ECL kit (Amersham Corp.). Densitometric measurements were performed on a GS-700 imaging densitometer (Bio-Rad Laboratories).

Mutations and Expression of Ca2+-ATPase in Yeast—Site-directed mutagenesis was performed with the Exsite kit (Stratagene Inc.) on Ca2+-ATPase SERCA1a cDNA (53). One single mutation E309Q, one double mutation D813A/D818A (referred to later as ADA), and one triple mutation D813A/D815A/D818A (AAA) were introduced. The presence of these mutations and the absence of unexpected mutations due to polymerase chain reaction were verified by DNA sequencing. Wild type and mutant cDNAs were inserted into the yeast expression vector pYeDPl60 (a gift of Dr. D. Pompon CNRS, Gif sur Yvette). Saccharomyces cerevisiae W303.1B (a, leu2, his3, trpl, ura3, ade1-2, can1, cyr1) was transformed (53, 54) and selected using ura3 complementation. Growth conditions and criteria for the Ca2+-ATPase were as in Ref. 53 for the test of individual clones and as in Ref. 55 for the large scale expression and crude extract preparation. The crude extract was first centrifuged at 900 × g, for 15 min at 4 °C and then centrifuged at 10,000 × g for 15 min, 4 °C. The supernatant was submitted to a second centrifugation at 120,000 × g for 45 min (4 °C) in 10 mM Hepes (Tris), pH 7.4, 0.3 mM sucrose, 0.1 mM CaCl2 to pellet a “light” membrane fraction which was homogenized and adjusted to a protein concentration of about 10 mg/ml. Protein concentrations (56) were measured in the presence of 0.5% SDS. The amount of Ca2+-ATPase was estimated by quantitative Western blotting as in Ref. 53 using the polyclonal antibody 577–588 (37). Typically, the light membranes expressed wild type or mutant Ca2+-ATPase at about 1 mg of Ca2+-ATPase per 100 mg of membrane proteins.

ATPase Assay—ATP hydrolysis was assayed at 30 °C using an ATP-regenerating coupled enzyme system as in Ref. 53, with some modifications. To a buffer containing 10 mM Tris (pH 7.5, 50 mM KNO3, 1 mg/ml CaCl2, 7 mM MgCl2, 0.1 mM CaCl2, 0.1 mg/ml lactate dehydrogenase (Boehringer Mannheim, catalog no. 127221), 50 mM phosphoric acid, and 0.25 mM NADH, we added 50 μg of protein/ml of yeast light membranes. This mixture was preincubated for 10 min at 30 °C in the presence of 5 mM NaN3, 0.05 mg/ml bafilomycin A, 0.1 mM amionomolubate as inhibitors of other ATPases and 0.1 μM N-aminocaproic acid, 0.1 μM phenylmethylsulfonyl fluoride, and 2.8 mM β-mercaptoethanol as antioxidants. After addition of 1 μM phosphoenolpyruvate and 0.1 mg/ml pyruvate kinase (Boehringer Mannheim, catalog no. 109045), the reaction was started by the addition of 1 mM Na4-ATP. Thapsigargin (1 μg/ml) was added after 100 s, and the rate of hydrolysis was followed for an additional 100 s. The Ca2+-ATPase activity was calculated as the difference between the slopes obtained in the presence and in the absence of thapsigargin and was corrected for a small background activity obtained with control yeast light membranes.

RESULTS AND DISCUSSION

It has previously been documented in a number of investigations that changes of electrophoretic mobility in Ca2+-containing SDS gels can be used for the detection of Ca2+-binding proteins or peptides (57–61). Examples of such changes in migration rate are shown in Fig. 2, A and B, where it can be seen that calmodulin in a Ca2+-containing gel (lane 4, Fig. 2B) migrates at a much faster rate than in a gel containing 0.1 mM EGTA (lane 4, Fig. 2A). Intact Ca2+-ATPase from sarcoplasmic reticulum (lanes 2) move slightly faster in the presence of Ca2+ than in its absence (compare with the 95-kDa molecular mass marker). An even more pronounced increase in migration rate is noted for fragment B (lanes 3), the C-terminal polypeptide (sequence 506–994) produced by tryptic cleavage of Ca2+-ATPase, which co-migrates with the N-terminal fragment A in the presence of EGTA but not in the presence of Ca2+. Fragment A is further cleaved to A1 (peptide 199–505) and A2 (peptide 1–198). Some increase in migration rate in the

EXPERIMENTAL PROCEDURES

Ca2+-ATPase Preparations and Proteolysis—Proteolytic digestion of SR (45) or purified Ca2+-ATPase vesicles (46) with trypsin was performed as described in Ref. 47. Proteinase K digestion, in 100 mM bis-Tris, pH 6.5, and 0.3 mM Ca2+, was performed essentially as described in Ref. 37 except that, in some cases, proteolysis took place for 4 min at 37 °C instead of 30 min at 20 °C, a modification which generated a higher amount of peptide p20C relative to p19C.
FIG. 2. Effect of Ca$^{2+}$ on the electrophoretic migration of various proteins and peptides. SDS-PAGE was performed with either 0.1 mM EGTA (A and C) or 1 mM Ca$^{2+}$ (B and D, see "Experimental Procedures") in the gels. The proteins were revealed by Coomassie Blue staining. Lanes 1 and 5 (A and B) or 1 and 6 (C and D), molecular mass markers (LMW Pharmacia Kit). Lanes 2, uncleaved purified Ca$^{2+}$-ATPase (A and B) or SR (C and D); 10 μg of protein was deposited in each well. Lanes 3, Ca$^{2+}$-ATPase cut with either trypsin (Tr) or proteinase K at 37 °C (Tr K); 10 μg of protein was deposited in each well. Lanes 4 in A and B, calmodulin (Calmod) 3 μg. Lanes 4 and 5 in C and D, peptides p20C and p19C, respectively, purified by electroelution (about 2 μg, see "Experimental Procedures"). Note that in gels C and D the extrinsic proteins, calxequarterin (CS) and glycoprotein (G), are present with Ca$^{2+}$-ATPase in lanes 2 and 3. Tr I, trypsin inhibitor.

The presence of Ca$^{2+}$ is observed for A2 (compare with the 20-kDa molecular mass marker), whereas A1 is not affected by Ca$^{2+}$ (compare with the 30-kDa molecular mass marker). The effect of Ca$^{2+}$ on the migration rate of Ca$^{2+}$-binding proteins and some of their peptide fragments is due probably to retention of a more compact and native-like structure in the presence of SDS, resulting from the binding of Ca$^{2+}$ (49, 61, 62).

In Fig. 2, C and D, we show that still shorter C-terminal Ca$^{2+}$-ATPase fragments produced by proteolysis with proteinase K (p28C, p27C, and p20C) also change their mobility in Ca$^{2+}$-containing gels, whereas other fragments (p29/30, p19C) are unaffected by the presence of Ca$^{2+}$. As illustrated in Fig. 3, p20C starts at Gly-808, a residue which was predicted to be close to the C-terminal border of the M5/M6 transmembrane region, and extends to the C-terminal Gly-994. Asp-818 (therefore in the L6–7 loop) and also ends at Gly-994. Thus a marked difference in peptide behavior in SDS gels is observed after removal of only 10 amino acids from p20C (Fig. 3B). The Ca$^{2+}$-dependent change in migration rate of p20C is observed not only in the mixture of peptides obtained after limited proteolysis (lanes 3, Fig. 2, C and D), but also after isolation of p20C by electroelution from the gel and renewed electrophoresis (lanes 4), indicating that Ca$^{2+}$ binding is an intrinsic property of the peptide. After electroelution p19C is still unaffected by the presence of Ca$^{2+}$ in the gel (lanes 5).

FIG. 3. Topological model of Ca$^{2+}$-ATPase, location of characteristic proteinase K splits, and a focus on two C-terminal membrane split products, p20C and p19C. A, the Ca$^{2+}$-ATPase model is based on 4 N-terminal (M1–M4) and 6 C-terminal (M5–M10) intramembranous helices (33). Numbers refer to the location of proteinase K cleavage sites (shown by long arrows; Ref. 37); the numbers for the residues predicted to lie at the membrane/water interface are indicated by italic numbers and are taken from Ref. 63. The broken line is considered to indicate the top of the stalk. P indicates the location of the phosphophorylatable amino acid residue (Asp-351), and T1 and T2 (short arrows) the primary tryptic cleavage sites. T1 generates fragments A (1–505) and B (506–994), while T2 generates fragments A2 (1–198) and A1 (199–505). Open boxes in the membranous domain correspond to critical residues, Glu-309, Glu-771, Asn-796, Thr-799, Asp-800, and Glu-908. B, a focus on the two smallest C-terminal peptides, p20C and p19C. Note that p20C is not particularly rich in acidic residues; its glutamate plus aspartate content is 10.7% (mol/mol), as compared with 17.1% for the tryptic peptide A2 or 12.4% for Ca$^{2+}$-ATPase (47).
mobilon membranes is a complementary technique with which to detect Ca\(^{2+}\) binding to proteins or peptides with various affinities for Ca\(^{2+}\) (49). For instance, this strategy has been successfully used to localize a Ca\(^{2+}\)-binding region which does not contain an EF-hand motif in the cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger (65). In Fig. 4A we show the results of a 45Ca\(^{2+}\) overlay experiment performed on purified p20C and p19C. In these experiments, the 20-kDa molecular mass marker, trypsin inhibitor (lane 3), also binds 45Ca\(^{2+}\) (49, p20C), providing a convenient reference in this region of the gel. It is clear that only p20C binds 45Ca\(^{2+}\) (Fig. 4A, lane 1), while p19C is not labeled (lane 2), although present in comparable amounts, as seen by Coomassie Blue staining of the blot (Fig. 4B). Note that these experiments are performed after extensive washing of the membrane with a non-ionic and non-denaturing detergent, C\(_{12}\)E\(_{8}\), which should help to remove SDS. In addition, 45Ca\(^{2+}\) overlay is performed at low Ca\(^{2+}\) concentration (10–15 \(\mu\)M) and in the presence of 5 mM Mg\(^{2+}\), which suggests that the partially refolded binding site in p20C has retained some specificity for Ca\(^{2+}\) over Mg\(^{2+}\).

The combined demonstration of a Ca\(^{2+}\)-dependent change in migration rate and of 45Ca\(^{2+}\) overlay labeling is a good indication of Ca\(^{2+}\) binding by p20C and not by p19C. However, this does not necessarily imply that Ca\(^{2+}\) is bound at functionally important sites. By inspection of the 808–818 sequence we see that the side chains of Asp-813 and Asp-818, together with the peptide carbonyl group between Asp-815 and Ile-816 could be involved in Ca\(^{2+}\) binding to the L6–7 loop. 2 We then took advantage of the functional expression of Ca\(^{2+}\)-ATPase in yeast (53) to test the activity of two cluster mutants in this region, i.e. D813A/D818A (ADA) and D813A/D815A/D818A (AAA). Note that residues Asp-813 and Asp-815 correspond to residues found to be important in the case of the H\(^{+}\)-K\(^{+}\)-ATPase (Fig. 1A). After purification of a light membrane fraction from the ATPase-expressing yeasts, we verified by Western blotting and densitometric measurements that mutant and wild type ATPases had been expressed to the same extent (data not shown). To quantify ATP hydrolysis, we used a coupled system and followed the disappearance of NADH in the absence or presence of thapsigargin, a specific inhibitor of SERCA ATPase. In Fig. 5 we present histograms of specific Ca\(^{2+}\)-ATPase activity based on several experiments. It is seen that activity in the double mutant is reduced by more than 90 %, and activity in the triple mutant drops to a negligible level, comparable to that seen after mutation to glutamine of the essential glutamate in M4 (E309Q; Ref. 16). These activity measurements do not point to any particular mechanism for inactivation, but phosphorylation experiments suggest that the Ca\(^{2+}\) binding step is indeed affected by the mutations. The inset of Fig. 5 shows that phosphorylation by \(^{32}\)P\(^{\text{P}}\), while inhibited by Ca\(^{2+}\) at 126 \(\mu\)M and 1000 \(\mu\)M for wild type ATPase and SR + control yeast membranes, is still possible for the ADA mutant despite these high Ca\(^{2+}\) concentrations. Based on the rationale of Clarke et al. (16), for the six mutations cited in the introduction, this shows that the ADA mutant is Ca\(^{2+}\)-insensitive.

\(^{2}\) T. Menguy and J. Smith, unpublished observations.
Our study demonstrates the involvement of the region corresponding to the L6–7 loop in the reaction mechanism of Ca\(^{2+}\)-ATPase. It should be noted that this region is fairly well conserved, in particular among type IIA ATPases. Therefore, the previous findings with the corresponding residues in H\(^{-}\),K\(^{-}\)-ATPase (27) probably are of wider significance and indicate common features in the transport mechanism. However, in contrast with the interpretation proposed for H\(^{-}\),K\(^{-}\)-ATPase in the latter work, there is convincing evidence for cystolic exposure of this region in Ca\(^{2+}\)-ATPase, since it is an easy target for proteolytic cleavage (37, 38) and it reacts with sequence-specific antibody 809–827 (67). Therefore, we do not consider it likely, as suggested by Swarts et al. (27), that these residues are embedded inside the membrane structure, but we favor a probable location of the Ca\(^{2+}\) -binding sites at the cytosol/membrane border. We suggest that residues of the L6–7 loop could be involved in the initial processes of Ca\(^{2+}\) transport. Specifically, they could act as a site for ion approach toward the high affinity binding pocket inside the membrane and/or be part of the gate operating during calcium occlusion (70, 71), preventing release of bound Ca\(^{2+}\) toward the cytosolic side. Exploration of these possibilities requires further studies.

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