Structure–function relationship of the human erythrocyte plasma membrane Ca^{2+} -ATPase revealed by V8 protease treatment

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Treatment of the solubilized and purified Ca^{2+} -translocating ATPase (Ca^{2+} -ATPase) (136 kDa) from human erythrocyte plasma membranes with endoproteinase Glu-C from *Staphylococcus aureus* strain V8 (V8 protease) yielded transient fragments of 96 kDa and 76 kDa and more stable fragments of 60 kDa and 37/36 kDa (doublet). The presence of calmodulin did not alter the fragmentation pattern. The 60 kDa fragment contains the protein kinase C (bovine brain) phosphorylation site(s), which we previously localized in the C-terminal region [Wang, Wright, Machan, Allen, Conigrave & Roufogalis (1991) *J. Biol. Chem.* **266**, 9078–9085]. On the other hand, the 37/36 kDa fragments possess the ability to form an acyl-phosphate intermediate. Furthermore, the presence of the 60 kDa and 37/36 kDa fragments together results in expression of calmodulin-sensitive Ca^{2+} -ATPase activity. However, further degradation of the 60 kDa fragment was coupled with the appearance of calmodulin-independent activity, whereas the 37/36 kDa fragment doublet remained stable. It was concluded that the 60 kDa and the 37/36 kDa fragments: (a) together represent the C-terminal two-thirds of the enzyme, which is functional as an Ca^{2+} -ATPase, (b) were produced by a single cleavage near the C-terminal side of the cytosolic catalytic domain, and (c) probably remain physically and functionally associated even after cleavage has occurred. At the C-terminus, the basic calmodulin-binding domain is flanked by two highly acidic regions (domains A and B). Our results indicate that domains A and B, despite containing many Asp and Glu residues, were not readily cleaved by V8 protease, which is known to cleave selectively peptide bonds at the C-terminal side of Asp and Glu. However, if the Ca^{2+} -ATPase were pre-digested with calpain I from human erythrocytes, which removed its calmodulin-binding domain (along with domain B), multiple cleavages by V8 protease in domain A were then readily observed. We propose that the calmodulin-binding domain is closely associated with the acidic domains A and B and that these acidic domains might help to co-ordinate the stimulation of the enzyme by calmodulin.

INTRODUCTION

The plasma membrane Mg^{2+} -dependent Ca^{2+} -translocating ATPase (Ca^{2+} -ATPase) has been extensively studied (Penniston, 1983; Al-Jobore *et al.*, 1984; Carafoli, 1987; Roufogalis & Villalobo, 1989). It differs from the sarcoplasmic reticulum (SR) Ca^{2+} -ATPase in several major respects, i.e. it is activated directly by calmodulin (CaM), acidic phospholipids and calpain proteolysis (Niggli *et al.*, 1981; Wang *et al.*, 1988a). The sequences of several isoforms of the plasma membrane Ca^{2+} -ATPase from rat and human have been reported (Verma *et al.*, 1988; Shull & Greb, 1988, 1989; Strehler *et al.*, 1990). Several conserved regions which are similar to regions in the SR Ca^{2+} -ATPase were identified, including ten putative transmembrane α -helices (M_1 – M_{10}), a mainly hydrophilic cytosolic loop between M_2 and M_3 (cytosolic loop 1), the cytosolically located catalytic domain (cytosolic loop 2) containing the acyl-phosphate-forming aspartate residue, and the fluorescein isothiocyanate-interacting site (involved in ATP binding) found between M_4 and M_5 . Verma *et al.* (1988) also identified several charged regions unique to the plasma membrane Ca^{2+} -ATPase: two acidic domains (A and B) flanking a positively charged domain C at the C-terminus, a mixed acidic–basic domain (D) in the middle of cytosolic loop 1, and another acidic domain (E) at the N-terminus. Domain C is now identified as the CaM-binding domain (James *et al.*, 1988), while the basic half of domain D (domain D_2) was recently

identified as an acidic-phospholipid-interacting site (Zvaritch *et al.*, 1990). The acidic half of domain D (domain D_1) and domain E were suggested to have putative Ca^{2+} -binding EF-hand structures (Verma *et al.*, 1988). On the other hand, acidic domain A, which is N-terminal to the CaM-binding domain, was originally suggested to be the high-affinity Ca^{2+} -binding site that may participate in Ca^{2+} transport (Verma *et al.*, 1988). However, this proposal now appears less likely to be the case, since more recent evidence both from site-directed mutagenesis of the SR Ca^{2+} -ATPase (Clarke *et al.*, 1989) and from limited proteolysis studies of the same enzyme (le Maire *et al.*, 1990) indicates that the high-affinity Ca^{2+} -binding sites of the SR Ca^{2+} -ATPase are located within the transmembrane helices. Based on primary structure similarity, Clarke *et al.* (1989) proposed that similar amino acid residues, which could comprise the proposed high-affinity Ca^{2+} -binding site, can be found in helices M_4 , M_5 , M_6 and M_8 of the plasma membrane Ca^{2+} -ATPase. Therefore, to date, the functions of the four acidic domains (A, B, D_1 and E) remain unclear. Since endoproteinase Glu-C from *Staphylococcus aureus* strain V8 (V8 protease) selectively cleaves peptide bonds on the C-terminal side of glutamate and aspartate residues (Houmar & Drapeau, 1972), we have used V8 protease as a tool to probe the functional significance of these acidic regions. In the present paper we report on the further elucidation of the structure–function relationship of the plasma membrane Ca^{2+} -ATPase using this method.

Abbreviations used: SR, sarcoplasmic reticulum; DG, 1-stearoyl-2-arachidonylglycerol; PS, phosphatidylserine; CaM, calmodulin; V8 protease, endoproteinase Glu-C from *Staphylococcus aureus* strain V8.

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MATERIALS AND METHODS

Materials

V8 protease (*Staph. aureus* strain V8, type XVII-B), leupeptin, Triton X-100, α -macroglobulin, ATP (disodium salt), phosphatidylserine (PS) and 1-stearoyl-2-arachidonylglycerol (DG) were from Sigma Chemical Co. CaM (bovine brain) was from Calbiochem. Asolectin was from MCB Manufacturing Chemical Inc. [γ - 32 P]ATP was from Amersham. Calpain I from human erythrocytes (calpain) was purified as described previously (Wang *et al.*, 1988a,b). Protein kinase C (bovine brain) was purified essentially as described before (Wang *et al.*, 1991), except that the hydroxyapatite column was not used and the protein kinase C preparations therefore contained a mixture of isoenzymes. The Ca^{2+} -ATPase was purified from human erythrocyte membrane as described previously (Wang *et al.*, 1988a). The enzyme was stored in 0.05 % (w/v) Triton X-100/0.1 % (w/v) asolectin/20 mM-Hepes (pH 7.4)/2 mM-EDTA/2 mM-dithiothreitol/300 mM-KCl at -80°C .

Protease digestion of the Ca^{2+} -ATPase

The purified Ca^{2+} -ATPase (6–60 μg) was either digested with V8 protease (10–100 ng, unless stated otherwise) for 1–120 min, or first digested with calpain I (1–10 μg for 120 min) followed by V8 protease in the following medium (0.1–1.0 ml) at 25°C : 50 mM-Hepes (pH 7.4), 10 mM-dithiothreitol, 0.05 % (w/v) sonicated asolectin, 0.03 % (w/v) Triton X-100, 120 μg of DG/ml, 600 μg of PS/ml (sonicated together), 1 mM-EDTA, 4 mM- CaCl_2 , 5 mM- MgCl_2 (no MgCl_2 was added if samples were to be processed for the acyl-phosphoprotein experiments) and 0.3 μM -CaM (if added). The calpain proteolysis was stopped by the addition of 200 μM -leupeptin, and V8 proteolysis was stopped by the addition of 0.1 μg of α -macroglobulin (if desired) or 15 % (w/v) ice-cold trichloroacetic acid if the samples were to be processed for SDS/PAGE.

Phosphorylation of the Ca^{2+} -ATPase by protein kinase C

The purified Ca^{2+} -ATPase (4 μg) was incubated with protein kinase C (0.04 μg) in 100 μl of the following medium: 50 mM-Hepes (pH 7.4), 20 mM- MgCl_2 , 5 mM-dithiothreitol, 0.05 % (w/v) asolectin, 0.03 % (w/v) Triton X-100, 120 μg of DG/ml, 600 μg of PS/ml, 1 mM-EDTA, 2 mM- CaCl_2 , 5 mM-sodium vanadate and 0.3 μM -CaM (if added). The phosphorylation was initiated by the addition of 50 μM -[γ - 32 P]ATP (2 mCi/mmol). After 120 min at 25°C , 10 mM non-labelled ATP was added before the samples were treated with proteases (V8 protease and calpain). The samples were eventually precipitated with 20 % (w/v) trichloroacetic acid and the pelleted protein was processed for SDS/PAGE in alkaline conditions. Under these conditions, no hydroxylamine-sensitive acyl-phosphoprotein intermediate (EP) was observed (Wang *et al.*, 1991).

Formation of the acyl-phosphoprotein intermediate

The untreated or protease-treated Ca^{2+} -ATPase (5–6 μg) was incubated at 4°C in 100 μl of the following medium at 20°C : 50 mM-Hepes (pH 7.4), 10 mM-dithiothreitol, 0.05 % (w/v) asolectin, 0.03 % (w/v) Triton X-100, 120 μg of DG/ml, 600 μg of PS/ml, 1 mM-EDTA, 4 mM- CaCl_2 and 0.3 μM -CaM (if added). [γ - 32 P]ATP (6 μM ; 6 mCi/mmol) was added to start the reaction (Wang *et al.*, 1988a). After 20 s at 4°C , formation of the phosphoprotein intermediate was arrested by the addition of 15 % (w/v) trichloroacetic acid. The precipitated proteins were then subjected to SDS/PAGE under acidic conditions (see below).

Assay for Ca^{2+} -ATPase activity

Untreated or V8 protease-treated (120 min, various concentrations) purified Ca^{2+} -ATPase (3–4 μg) was incubated at 37°C for 15 min in 400 μl of buffer containing 50 mM-Hepes (pH 7.4), 130 mM-KCl, 6.5 mM- MgCl_2 , 120 nM-CaM (if added), 2 mM-dithiothreitol, 0.1 mM-EDTA, 0.1 % (w/v) sonicated asolectin, 0.05 % (w/v) Triton X-100, 0.1 mM- CaCl_2 (free [Ca^{2+}] 1 μM) and 2 mM-ATP. For Ca^{2+} -independent ATPase activity, 5 mM-EGTA was added instead of 0.1 mM- CaCl_2 . The reaction was arrested by the addition of 2 % SDS. The P_i was subsequently determined colorimetrically (Raess & Vincenzi, 1980).

PAGE and autoradiography

SDS/PAGE (5–20 % w/v polyacrylamide slab gels) under alkaline conditions (pH 8.3) was performed as described by Laemmli & Favre (1973). For observing acyl-phosphoprotein intermediates, acid gel electrophoresis was run using a Mops buffer system (pH 6.6), as described previously (Wang *et al.*, 1988a). If desired, the gels were stained with Coomassie Blue R-250 and destained before being exposed to X-ray film for 24–72 h at -70°C . Protein concentrations were determined by a modification of the method of Lowry *et al.* (1951). Calpain and protein kinase C were assayed as described before (Wang *et al.*, 1988a, 1991). Free Ca^{2+} concentrations were determined using the computer program previously described (Goldstein, 1979; Wang *et al.*, 1988a).

RESULTS

V8 protease digestion of the purified Ca^{2+} -ATPase

In this series of experiments, in order to capture the various transient fragments, a very high substrate/protease ratio was used (600:1). Fig. 1 shows that the purified Ca^{2+} -ATPase appears as a major band of relative molecular mass 136 kDa. A small amount of the CaM-binding protein adducin (97 kDa, 103 kDa) was also present as a contaminant, as reported previously (Wang *et al.*, 1989). After 1–10 min of proteolysis, two larger fragments (96 kDa and 76 kDa) were observed. Both adducin subunits were readily and rapidly degraded by V8 protease. The 96 kDa band that was observed after proteolysis had begun was in fact slightly below the adducin doublet on the gel (Fig. 1). When preparations of the Ca^{2+} -ATPase that are essentially free of adducin contamination were used, the 96 kDa fragment was still observed (see Fig. 5b). This confirms that the 96 kDa band is indeed a fragment derived from the Ca^{2+} -ATPase. It is worth noting that the 96 kDa fragment is highly transient, and therefore it did not accumulate to any great extent at any time point of proteolysis (Fig. 1). During prolonged proteolysis (up to 120 min), two relatively stable major fragments were produced, one of 60 kDa and the other a doublet of 37/36 kDa. It is important to stress that these fragments are not authentic limit peptides. If higher concentrations of V8 protease were used these peptides were further degraded. In fact, even at low V8 protease concentrations, after prolonged incubation (120 min) several fragments of about 44–48 kDa were observed in small quantities (Fig. 1). They are probably the products of further splitting of the 60 kDa stable fragment. Since CaM is known to alter the fragmentation products of the Ca^{2+} -ATPase produced by trypsin and calpain proteolysis (Wang *et al.*, 1988a), we also examined the effect of CaM on the V8 fragmentation pattern of the Ca^{2+} -ATPase. Virtually no differences in fragmentation products or the rate of formation of the fragments were observed in the presence or the absence of CaM (Fig. 1). This suggests that the

Fig. 1. Time course of proteolysis of the Ca^{2+} -ATPase by V8 protease

Purified Ca^{2+} -ATPase (7 μg) was treated with V8 protease (11 ng) in the absence (–) or presence (+) of CaM as described in the Materials and methods section. At the indicated time, aliquots (1 μg) were taken and the protein was precipitated with 15% trichloroacetic acid and processed for alkaline SDS/PAGE. The left-hand lane contains molecular mass standards; from top to bottom: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97 kDa), BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). The apparent molecular masses (in kDa) of the Ca^{2+} -ATPase and its fragments are indicated on the right. The gel was stained with Coomassie Blue.

Fig. 2. Time course of proteolysis of the calpain-fragmented Ca^{2+} -ATPase by V8 protease

Purified Ca^{2+} -ATPase (6 μg) was first treated with calpain I (0.6 μg) in the presence (+) or absence (–) of CaM for 120 min at 25 °C. Leupeptin was then added to stop calpain proteolysis before V8 protease (10 ng) was added. At the indicated time, aliquots (1 μg) were taken and proteolysis was stopped with 15% trichloroacetic acid, and the precipitated protein was processed for alkaline SDS/PAGE. The proteolysis conditions were as described in the Materials and methods section. The gel shown was stained with Coomassie Blue. The apparent molecular masses (in kDa) of the calpain-produced fragments of the Ca^{2+} -ATPase are indicated on the left, and those of the V8-produced fragments are indicated on the right. Calpain fragments are indicated by $\triangleright$.

cleavages producing the 96 kDa, 76 kDa, 60 kDa, 37 kDa and 36 kDa fragments are not likely to be in the vicinity of the CaM-binding domain.

Origin of the 96 kDa, 76 kDa, 60 kDa, 37 kDa and 36 kDa fragments

From amino acid sequence analysis, it is now clear that the CaM-binding domain of the Ca^{2+} -ATPase is located about 10 kDa from the C-terminus (James *et al.*, 1988; Shull & Greeb, 1988; Verma *et al.*, 1988). It was also determined that the initial fragmentation of the Ca^{2+} -ATPase by calpain occurs at the C-terminus, producing large active acyl-phosphate-forming fragments. In the absence of CaM, 124 kDa and 125 kDa fragments are formed, whereas in the presence of CaM a 127 kDa fragment is formed (Wang *et al.*, 1988b; James *et al.*, 1989). It was reported that these fragments contain an unaltered N-terminus. The 127 kDa fragment retained most of the CaM-binding domain, the 125 kDa fragment only retained the N-terminal half of it, and the 124 kDa fragment essentially had the CaM-binding domain deleted (see Figs. 3b and 3c). With further proteolysis, acyl-phosphate-forming fragments of 82 kDa and 80 kDa were observed in the absence of CaM and an 85 kDa fragment was observed in the presence of CaM. Further degradation by calpain also generated non-acyl-phosphate-forming fragments of 55 kDa, 39 kDa, 37 kDa and 32 kDa [Fig. 2; Wang *et al.* (1988b)]. The formation of the 85, 82 and 80 kDa fragments parallels that of the three larger fragments (127, 125 and 124 kDa). It has been suggested that each fragment contains the same C-terminus as its larger counterpart, but their N-termini were cleaved about a further 40 kDa from the original N-terminus (Wang *et al.*, 1988b).

We performed N-terminal sequencing analysis of the 85 kDa fragment and found that its N-terminus is Ser-Gln-Thr-Gly-Ile (results not shown). The only occurrence of this sequence is at residues 282–286 [based on the deduced cDNA sequence of the human teratoma isoform (Verma *et al.*, 1988)].

To assign the origin of the five individual V8 fragments (96 kDa, 76 kDa, 60 kDa, 37 kDa and 36 kDa), we took advantage of knowledge of the fragmentation caused by calpain. First, we pre-digested the Ca^{2+} -ATPase with calpain in either the absence or the presence of CaM. The calpain proteolysis was arrested by the addition of leupeptin, which does not affect V8 protease. At this stage, in the absence of CaM, the major form of the enzyme was the 124 kDa fragment, with smaller amounts of the 82 kDa/80 kDa, 55 kDa, 39 kDa, 37 kDa and 32 kDa fragments (Fig. 2). On the other hand, in the presence of CaM, the major form is the 127 kDa fragment, with smaller amounts of the 85 kDa, 55 kDa, 39 kDa, 37 kDa and 32 kDa fragments (a small quantity of the 82 kDa fragment was also observed under these conditions) (Fig. 2). At this point the calpain-fragmented Ca^{2+} -ATPase was treated with V8 protease in the presence of CaM. As the 127 kDa fragment diminished in mass, an 85 kDa fragment, which overlaps the calpain-produced 85 kDa fragment, accumulated up to 120 min (Fig. 2). In the absence of CaM at least three fragments between 78 and 82 kDa accumulated with V8 protease treatment up to 10 min (Fig. 2). These fragments could not have resulted from further calpain proteolysis, since 200 μM -leupeptin inhibits calpain activity totally. We suggest that the 85 kDa fragment has C-terminal and N-terminal ends that are virtually almost identical to those of the calpain-produced 85 kDa fragment (Figs. 2 and 3). If this is true, 80–82 kDa

Fig. 3. Proposed relationship between the major fragments of the Ca^{2+} -ATPase produced by V8 protease and calpain I

The Ca^{2+} -ATPase is schematically presented as a rectangle with its N- and C-termini represented by N and C respectively. The letter P indicates the location of the aspartate-acyl-phosphate site. First cytosolic loop, between transmembrane α -helices M_2 and M_3 ; the cytosolic catalytic domain, between transmembrane helices M_4 and M_5 (Verma *et al.*, 1988). The CaM-binding domain is represented as the dark region. The - and + signs indicate regions enriched with acidic or basic amino acid residues respectively. The apparent molecular masses (in kDa) of the fragments are indicated.

fragments are expected when the 124 kDa calpain fragment is digested with V8 protease. Interestingly, several fragments between 78 and 82 kDa were observed (Figs. 2 and 3). With longer treatment times (10, 60 and 120 min) we observed multiple V8 protease fragments between 44 and 48 kDa when the 124 kDa calpain fragment was digested, and a 51 kDa V8 fragment was produced from the 127 kDa fragment (Figs. 2 and 3). It is important to note that the 96 kDa and 60 kDa fragments obtained with V8 protease treatment of the intact enzyme were not observed at all when the enzyme was pre-treated with calpain. This suggests that they most probably contain the intact C-terminus proper, which is cleaved by calpain. The approximate size difference between the intact enzyme (136 kDa) and its 127 kDa and 124 kDa calpain fragments is 9 kDa and 12 kDa

respectively. When these differences were subtracted from the 96 kDa V8 protease fragment, 87 kDa and 84 kDa fragments are expected. These match reasonably well with the 85 kDa and 78-82 kDa fragments obtained (Fig. 2). Similarly, subtracting 9 kDa and 12 kDa from the 60 kDa fragment gives 51 kDa and 48 kDa, which are probably the 51 kDa and 44-48 kDa fragments observed (Fig. 2). The production of multiple fragments of 44-48 kDa and 78-82 kDa indicates that the new C-terminal region of the 124 kDa calpain-generated form contains several V8-cleavable sites. In fact, based on the amino acid sequence, these multiple cleavages could occur at domain A of the new C-terminus, since it is enriched in Asp and Glu residues (Fig. 3). The 76 kDa fragment previously produced with V8 protease treatment alone was also obtained after calpain pretreatment (Fig. 2). This implies that the 76 kDa fragment must originate from the N-terminal half of the enzyme, and therefore it also retained its size when the calpain-produced 127 kDa and 124 kDa fragments were used as starting substrates for V8 protease (see Fig. 3). Lastly, the 37/36 kDa fragments previously found with V8 protease treatment alone also accumulated and were relatively stable even up to 120 min. These 37/36 kDa fragments should not be confused with the 39/37 kDa doublet fragment produced by calpain. In fact, the 37/36 kDa fragments are easily identified, as their amount is much greater than that of the 39/37 kDa calpain fragment.

It was important to confirm that the 60 kDa V8 fragment and the 51 kDa and 48-44 kDa calpain/V8 fragments originated from the C-terminal half of the enzyme. Since we know that protein kinase C phosphorylates the 10-12 kDa region of the C-terminus of the Ca^{2+} -ATPase (Wang *et al.*, 1991), ^{32}P -labelling was now used as a tag for the C-terminus proper. In these experiments the Ca^{2+} -ATPase was first phosphorylated by protein kinase C before treatment with V8 protease. A longer treatment time (60 min) was chosen, and after alkaline SDS/PAGE the major Coomassie Blue-stained bands were of 60 kDa, 37 kDa and 36 kDa, as observed before (Fig. 1, 120 min lane). A corresponding autoradiogram shows that the 60 kDa V8 fragment indeed contained the ^{32}P label (Fig. 4, lane 2). A 55 kDa minor fragment was also labelled, but the 37/36 kDa fragments were not labelled at all (Fig. 4, lane 2). On the other hand, if the ^{32}P -phosphorylated Ca^{2+} -ATPase was treated with calpain, in the absence or the presence of CaM, the 124 kDa and 127 kDa fragments were the major ones produced respectively (see Fig. 2). In our previous work it was shown that the 124 kDa fragment has lost its ^{32}P label, whereas the 127 kDa retains at least one ^{32}P -labelled site (Wang *et al.*, 1991). When the calpain-generated 127 kDa form was then subjected to V8 protease treatment (60 min), two major fragments accumulated (51 kDa and 37/36 kDa) (see Fig. 2, 60 min lane). However, only the 51 kDa fragment retained the ^{32}P label (Fig. 4, lane 4). In the absence of CaM, when the calpain-produced 124 kDa form was further digested with V8 protease the 48-44 kDa fragments as well as the 37/36 kDa fragments were formed (see Fig. 2, 60 min lane), but none of them contained ^{32}P label (Fig. 4, lane 3). These results are consistent with the proposed C-terminal origin of the 60 kDa, 51 kDa and 48-44 kDa fragments as described in Fig. 3.

In order to further understand the impact of V8 proteolysis on the function of the Ca^{2+} -ATPase, we examined Ca^{2+} -ATPase activity at different degrees of proteolysis (by using various concentrations of V8 protease). In Fig. 5, Coomassie Blue-stained fragments on SDS/PAGE gels are shown (Fig. 5b) together with their corresponding Ca^{2+} -ATPase activities (Fig. 5a), in both the absence and presence of CaM. At 10^{-5} μg of V8 protease/ml initial fragmentation started to produce trace amounts of 96 kDa and 76 kDa fragments. At 10^{-4} μg of V8 protease/ml all five major fragments were observed. Up to this

Fig. 4. V8 proteolysis of the protein kinase C-phosphorylated Ca^{2+} -ATPase

Purified Ca^{2+} -ATPase (2 μg) was phosphorylated with 2 ng of protein kinase C and [γ - ^{32}P]ATP in the absence of CaM at 25 °C for 2 h as described in the Materials and methods section. Non-labelled ATP (10 mM) was then added before the ^{32}P -phosphorylated Ca^{2+} -ATPase was subjected to no further treatment (lane 1), or to V8 protease (lane 2), or first to treatment with calpain I, in the absence (lane 3) or the presence of (lane 4) CaM, and the V8 protease treatment. Proteolysis was stopped with 15% trichloroacetic acid and the precipitated protein was processed for alkaline SDS/PAGE. An autoradiogram of the stained gel is shown. The apparent molecular masses (in kDa) of the major bands are indicated.

concentration of V8 protease, no significant change in Ca^{2+} -ATPase activity was observed (Fig. 5a). At 10^{-3} $\mu\text{g}/\text{ml}$, proteolysis had progressed further to allow accumulation of the three stable fragments (60 kDa and 37/36 kDa). At this point, to our surprise, no major change in Ca^{2+} -ATPase activity or loss of CaM-stimulation had yet occurred. This strongly indicated that the 60 kDa, the 37 kDa or the 36 kDa fragment alone, or all three fragments together, form a functional unit of CaM-stimulated Ca^{2+} -ATPase. At 0.01 μg of V8 protease/ml the 60 kDa fragment was partly degraded, probably to a 51 kDa fragment. At 0.1 μg of V8 protease/ml the 60 kDa fragment had totally disappeared (Fig. 5b). It is unclear, though, whether any remnant fragments of the 60 kDa form exist. On the other hand, the 37/36 kDa fragments persisted at 0.01–0.1 μg of V8/ml. As for the Ca^{2+} -ATPase activity, the total activity had only declined slightly at 0.01 μg of V8/ml, but the basal activity had increased and the stimulation by CaM had dropped from the original 2.3-fold to 1.2-fold. At 0.1 $\mu\text{g}/\text{ml}$ the basal activity was about the same as the total activity, and therefore the CaM-sensitivity had disappeared. This suggests that the 37/36 kDa fragments, probably along with some small remnant fragments, form a functional Ca^{2+} -ATPase unit, whereas the presence of the 60 kDa fragment provides the CaM-sensitivity. At all concentrations of protease used the ATPase activity observed was fully Ca^{2+} - and Mg^{2+} -dependent (results not shown).

To further understand the 'active fragment' concept we investigated the ability of the 37/36 kDa fragments and possibly the 60 kDa fragment to form the acyl-phosphoprotein intermediate (EP) using [γ - ^{32}P]ATP as the substrate. Under the fragmentation conditions used the 60 kDa and 37/36 kDa fragments were the only observable fragments. Fig. 6 shows that the 136 kDa untreated Ca^{2+} -ATPase forms a ^{32}P -labelled acyl-phosphate intermediate in both the absence and the presence of

Fig. 5. Effect of varying the V8 protease concentration on the ATPase activity and fragmentation of Ca^{2+} -ATPase

Purified Ca^{2+} -ATPase (3 μg) was treated with the indicated amounts of V8 protease for 120 min at 25 °C, under the conditions described in the Materials and methods section. After incubation, aliquots (1 μg) were taken for measurement of Ca^{2+} -ATPase activity in the absence (–CaM) or the presence (+CaM) of CaM for 15 min at 37 °C at 2 μM free Ca^{2+} (a). Aliquots (0.5 μg) of each sample were precipitated with trichloroacetic acid and processed for alkaline gel electrophoresis. The Coomassie Blue-stained gel is shown in (b). The apparent molecular masses (in kDa) of the fragments are indicated.

CaM, as revealed by an autoradiogram of the acid gel (Fig. 6, lanes 1 and 2). After digestion by V8 protease we observed that both the 37 kDa and the 36 kDa fragments formed ^{32}P -labelled EP, regardless of the presence of CaM, whereas the 60 kDa fragment did not form EP at all (Fig. 6, lanes 3 and 4). It is quite reasonable, then, to propose that the 37/36 kDa fragments originated from the same section at the middle of the Ca^{2+} -ATPase molecule which includes the acyl-phosphate-forming Asp residue and the ATP hydrolysis machinery (Fig. 7). It appears likely that 36 kDa fragment is produced by further cleavages at either the C- or the N-terminus of the 37 kDa fragment. In fact, while the 37 kDa fragment was consistently observed, the 36 kDa fragment was observed in small quantities on some occasions (Fig. 5). The 37/36 kDa fragments, like the intact enzyme, absolutely required Ca^{2+} for EP formation (results not shown). Also, the level of [^{32}P]EP formation by the 37/36 kDa fragment (Fig. 6, lanes 3 and 4) was severalfold higher than that of the intact enzyme (Fig. 6, lanes 1 and 2). This suggested that the 37/36 kDa fragments may have a slower rate of dephosphorylation, which leads to a higher level of EP, when compared with the intact enzyme. We also treated the Ca^{2+} -ATPase with calpain in the presence or absence of CaM before

Fig. 6. [32 P]Acyl-phosphoprotein intermediate formation of the intact Ca^{2+} -ATPase and its fragments by calpain and V8 protease

As indicated, purified Ca^{2+} -ATPase (0.5 μg) was untreated (lanes 1 and 3), incubated with CaM (lanes 2 and 4), or treated with 10 ng of calpain I in the absence (lanes 5 and 7) or the presence (lanes 6 and 8) of CaM. Samples in lanes 3, 4, 7 and 8 were further digested with 1 ng of V8 protease. After the treatment, the samples were subjected to phosphorylation with [γ - 32 P]ATP (see the Materials and methods section) at 4 $^{\circ}\text{C}$ for 20 s. After the reaction was stopped with 15% trichloroacetic acid, the precipitated ^{32}P -labelled protein was subjected to acid SDS/PAGE. Shown is an autoradiogram of the dried gel. The apparent molecular masses (in kDa) of the intact Ca^{2+} -ATPase and its fragments are indicated.

the V8 protease treatment. Again the 37/36 kDa fragments formed ^{32}P -labelled EP as readily as did the 124 kDa and the 127 kDa fragments (Fig. 6, lanes 5–8). We thus tentatively propose that the 37/36 kDa fragments cover the N -terminal two-thirds of the cytosolic catalytic loop (including the catalytic site) and have their N -termini originated from cleavages at the negatively charged region (domain D_1) in the first cytosolic loop (Fig. 3).

DISCUSSION

Several studies based on limited proteolysis of the Ca^{2+} -ATPase by trypsin have previously been reported (Zurini *et al.*, 1984; Benaim *et al.*, 1984; Sarkadi *et al.*, 1986; Enyedi *et al.*, 1987). Trypsin has selectivity for positively charged residues on the N -terminal side of the peptide bond cleaved. This placed most of the major cleavages at two basic domains: the CaM-binding domain and domain D_2 . As a result, (a) CaM-independent fragments can be generated by cleaving off the CaM-binding domain (Zurini *et al.*, 1984; Enyedi *et al.*, 1987), and (b) an acidic-phospholipid-interacting domain (domain D_2) can also be removed to generate an acidic-phospholipid-independent active fragment (Zvaritch *et al.*, 1990). On the other hand, trypsin treatment studies yield no insight into the function of the acidic domains (A, B, D_1 and E). In the present study we have chosen to study V8 protease fragmentation for its selectivity towards acidic residues. As a result the information yielded from this study is very different from that generated by trypsin treatment.

First, we found that a 60 kDa stable fragment still retained the protein kinase C phosphorylation site(s) known to occur at the C -terminal region, and thus contained the CaM-binding domain C and domain A (see Fig. 7). Among the four other major

Fig. 7. Topographical representation of the proposed structure-function relationship of the plasma membrane Ca^{2+} -ATPase

The lipid bilayer is represented by the broken lines. The N - and C -termini of the Ca^{2+} -ATPase are indicated. The ten putative transmembrane helices are denoted by M_1 – M_{10} . The cytosolic catalytic domain containing the aspartate acyl-phosphate site (Asp-P) is also illustrated. Six domains enriched in acidic ($\square$) or basic ($\blacksquare$) residues, denoted by the letters A, B, C, D_1 , D_2 and E, are indicated. Domain C is also the CaM-binding domain. The proposed V8 protease cleavage sites are indicated by the arrows. The arrows with * in domain A represent cleavage sites not readily attacked unless domain C is removed. The apparent molecular masses (in kDa) and the relative locations of various V8-protease-produced fragments are shown at the bottom of the Figure. For further details, see the text.

fragments the 76 kDa fragment is the only one likely to be complementary to the 60 kDa fragment. In fact, the sum of these two fragments (76 kDa + 60 kDa) would match perfectly the size (136 kDa) of the intact Ca^{2+} -ATPase (Figs. 3 and 7). It is therefore quite likely that the 60 kDa fragment contains most or all of the C -terminus intact. As the 60 kDa fragment is quite resistant to further proteolysis (Fig. 1), domain A, which is present in the 60 kDa fragment, must be inaccessible to V8 protease (Fig. 3). If the 60 kDa fragment does contain domain B, then this domain, like domain A, is not readily attacked by V8 protease. On the other hand, when the CaM-binding domain was first removed by calpain treatment (forming the 124 kDa fragment), no single stable fragment was produced from the C -terminus. Rather, multiple cleavages at domain A, generating fragments of 48–44 kDa, were observed (Figs. 2 and 3). If calpain pretreatment was carried out in the presence of CaM, a 127 kDa fragment retaining only the CaM-binding domain and domain A, but not domain B, was produced. Starting with this fragment, V8 protease now generated a 51 kDa fragment which retained the intact CaM-binding domain (Fig. 4), with little sign of multiple fragmentation (to 44–48 kDa) at domain A (Figs. 2 and 3). These results imply that the CaM-binding domain protects domain A against fragmentation, regardless of the absence or the presence of CaM. This is the first piece of evidence for an

association/interaction between the positively charged CaM-binding domain and the negatively charged domain A. We also speculate that negatively charged domain B probably associates strongly with the CaM-binding domain and is therefore protected against fragmentation. The fact that domain B is not readily cleaved by V8 protease when the C-terminus is still intact supports this notion (Fig. 1). These proposed interactions of domains A, B and C at the C-terminus are shown in a schematic drawing in Fig. 7. It is also tempting to suggest that domains A and B may have certain roles in co-ordinating the stimulation of the enzyme by CaM. In our previous work, when the ^{14}C -labelled NN' -dicyclohexylcarbodi-imide-treated Ca^{2+} pump was trypsin-treated, among other labelled fragments, a 23 kDa CaM-binding fragment was found to be labelled (Olorunsogo *et al.*, 1988). We also reported that CaM doubled the number of carbodi-imide-binding sites. Since carbodi-imides modify Asp and Glu residues, it is likely that domains A and/or B were labelled. This would be consistent with the proposed interactions of domains A and B with the CaM-binding domain in the present study.

In this study we have also demonstrated that (a) a 37/36 kDa fragment doublet is EP-forming, and (b) the 60 kDa C-terminal fragment probably stays associated with the 37/36 kDa fragment, as the fragments retain CaM-sensitivity of their Ca^{2+} -ATPase activity. Until now, fragments of 76–80 kDa (produced by trypsin and calpain) were thought to be the minimum active unit for the Ca^{2+} -ATPase, since no smaller EP-forming fragments had been observed (Zurini *et al.*, 1984; Sarkadi *et al.*, 1986; Wang *et al.*, 1988a). The existence of an EP-forming fragment of a mere 37/36 kDa is a surprising finding (Fig. 6). We also know that this doublet possesses Ca^{2+} -ATPase activity when associated with the 60 kDa fragment (Fig. 5). In fact, the ATPase activity is quite normal. The enzyme fragment still requires Ca^{2+} and Mg^{2+} for activity and is still sensitive to CaM (Fig. 6). Perhaps the only abnormality is the higher EP level achieved when phosphorylated at 4 °C (Fig. 6).

Calmodulin-independent high- V_{max} Ca^{2+} -ATPase activity is observed when the 60 kDa fragment is further degraded (Fig. 6). This raises the possibility that the 37/36 kDa fragments alone may be an active Ca^{2+} -ATPase unit. What is unclear is whether expression of the Ca^{2+} -ATPase activity of the 37/36 kDa fragments requires certain remnant fragments generated from degradation of the 60 kDa fragment. Also unclear is whether the 37/36 kDa and 60 kDa fragments or the 37/36 kDa fragments alone would translocate Ca^{2+} . In our model, we place the N-terminus(s) of the 37/36 kDa fragments at domain D_1 in cytosolic loop 1. This assignment is likely to be correct, since domain D_1 is negatively charged and is therefore likely to be cleaved by V8 protease. We also suggested that the 37/36 kDa fragments are produced by two adjacent cleavages in domain D_1 (Figs. 3 and 7). This arrangement would also place their C-terminal ends at the C-terminal part of the catalytic domain (based on the size of the fragments) (Figs. 3 and 7). In this case, the 37/36 kDa fragments would have only two transmembrane helices (M_3 and M_4), whereas the complementary 60 kDa fragment would contain helices M_5 , M_6 , M_7 , M_8 , M_9 and M_{10} . This suggests that the N-terminal region, including the region up to domain D_2 , is not essential for Ca^{2+} -ATPase activity. This argues against the functional significance of this region, a point unresolved from trypsin treatment studies (Zurini *et al.*, 1984). The fact that the 60 kDa and 37/36 kDa fragments together function as a Ca^{2+} -ATPase is also in agreement with the earlier speculation of Clarke *et al.* (1989) that the high-affinity Ca^{2+} -binding sites are located in helices M_4 , M_5 , M_6 and M_8 (based on inference from the site-directed mutagenesis of the SR Ca^{2+} -ATPase). If their model is correct, then the 37/36 kDa fragments alone are probably not enough to function as a Ca^{2+} -ATPase or a Ca^{2+}

pump, since only helices M_3 and M_4 are present, and certain remnants would be required containing at least helices M_5 , M_6 , M_7 and M_8 for these activities. Therefore, in order to further localize the high-affinity Ca^{2+} -binding sites in the plasma membrane Ca^{2+} pump, it will be important to investigate whether the 37/36 kDa fragments alone are active as a Ca^{2+} -ATPase as well as a Ca^{2+} pump.

In conclusion, we have demonstrated the usefulness of the Asp/Glu-selective V8 protease in studying the structure-function relationship of the plasma membrane Ca^{2+} -ATPase. The new data allow us to propose that the acidic domains A and B interact strongly with the CaM-binding domain in both the absence and the presence of CaM and may have a role in co-ordinating the activation of the enzyme by CaM. On the other hand, the roles of acidic domains D_1 and E are still unclear. We are also able to assign the functional Ca^{2+} -ATPase unit to the C-terminal two-thirds of the enzyme. The report of a low-molecular-mass EP-forming fragment (37/36 kDa) is also highly important, since further detailed examination of the 37/36 kDa fragment doublet would probably shed light on the location of the high-affinity Ca^{2+} -binding sites. During the preparation of this paper, a report was published by Falchetto *et al.* (1991) suggesting that the CaM-binding domain interacts with a sequence of the Ca^{2+} -ATPase in the vicinity of the catalytic domain, which is consistent with the data and interpretation of the present work.

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