Mapping of Functional Domains in the Plasma Membrane Ca^{2+} Pump Using Trypsin Proteolysis†

Elena Zvaritch,‡ Peter James,§ Thomas Vorherr,§ Rocco Falchetto,§ Nikolai Modyanov,‡ and Ernesto Carafoli*,§

Laboratory for Biochemistry, Swiss Federal Institute of Technology (ETH), Universitätstrasse 16, 8092 Zurich, Switzerland, and Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, 117811 6SP Moscow, USSR

Received January 31, 1990; Revised Manuscript Received May 3, 1990

ABSTRACT: The purified erythrocyte Ca^{2+} pump has been exposed to trypsin under conditions designed to enrich the fragments of molecular mass 90, 85, 81, and 76 kDa, respectively. In SDS–polyacrylamide gels, these fragments are accompanied by a product of molecular mass about 33 kDa. N- and C-terminal sequencing of the fragments blotted on PVDF membranes has located the four high molecular mass fragments and the 33-kDa fragment within the pump structure. The work has extended previous work on the organization of the calmodulin-interacting domain of the pump (Zurini et al., 1984; Benaim et al., 1984) and has tentatively placed the domain of the pump which interacts with acidic phospholipids between transmembrane helices 2 and 3.

It has been known since 1977 that the Ca^{2+} pump in the plasma membrane is a target of calmodulin activation (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977). However, in the absence of calmodulin, the pump can be activated by a number of alternative treatments: the exposure to acidic phospholipids or long-chain polyunsaturated fatty acids (Ronner et al., 1977), a controlled proteolytic treatment with trypsin (Taverna & Hanahan, 1980; Sarkadi et al., 1980), or a phosphorylation reaction mediated by the cAMP-dependent protein kinase (Caroni & Carafoli, 1981). Experiments on the purified pump have shown that these responses are maintained in both the liposomal-reconstituted and the detergent-solubilized systems (Niggli et al., 1981a,b; Stieger & Schatzmann, 1981; Neyses et al., 1985; Enyedi et al., 1987). Although the activation consists of both a K_m and a V_{max} effect, the former is largely predominant. The K_m(Ca) of the Ca pump is of the order of 5–20 μM. Calmodulin reduces it to about 0.5–0.6 μM (Niggli et al., 1981a,b), acidic phospholipids to about 0.2–0.3 μM (Enyedi et al., 1987), and the cAMP-linked phosphorylation to 1.0–1.2 μM (James et al., 1989a,b). Thus, the treatment with acidic phospholipids (or polyunsaturated fatty acids) appears to be the most effective.

Detailed studies of the effects of trypsin on the purified erythrocyte pump (Niggli et al., 1981a,b; Zurini et al., 1984; Benaim et al., 1984) have shown that the protease progressively reduces the enzyme to fragments of 90, 85, 81, and 76 kDa (higher molecular mass fragments are also transiently produced initially). The cuts leading to the production of these fragments separate from the main body of the pump hydrophilic domains located at the C-terminal end. However, in producing the fragments of molecular masses 90–76 kDa, trypsin also cleaves at the N-terminus of the enzyme. Since in this case the fragments would be rather hydrophobic, it is doubtful whether they become actually separated in the absence of sodium dodecyl sulfate (SDS).[1] The 90-kDa fragment apparently functions as a fully competent, calmodulin- and phospholipid-stimulated Ca^{2+}-ATPase (Enyedi et al., 1987; Zurini et al., 1984). The characteristics of the 85-kDa fragment are less well established (Benaim et al., 1984, 1986): it apparently retains the ability to bind calmodulin but has a reduced ability to respond to it (Enyedi et al., 1987; Benaim et al., 1984, 1986). The 81-kDa fragment has high Ca^{2+} affinity; it does not bind calmodulin and thus fails to respond to it, but still responds to acidic phospholipids (Enyedi et al., 1987). The 76-kDa fragment is fully active in the absence of calmodulin and acidic phospholipids; i.e., it has lost all regulatory properties. In parallel with the formation of the high molecular mass fragments, a particularly hydrophobic fragment of about 33 kDa is also produced by trypsin. As mentioned, the 33.5-kDa fragment has been suggested to be located at the N-terminal side of the high molecular mass fragments, possibly extending to the N-terminus of the pump (Zurini et al., 1984). More recent work using the intracellular Ca^{2+}-dependent protease calpain (James et al., 1989a,b) has shown that the main splitting product is in this case a fragment of about 124 kDa, which in fact consists of two products of very similar molecular mass. They appear sequentially during the digestion, and both extend to the N-terminus of the pump: their C-terminus is located either at the end or at the beginning of the calmodulin binding domain. As a result, the "early" 124-kDa fragment responds to calmodulin; the other does not. The fragments of 124 kDa are not exclusive to calpain, since they are also produced by trypsin at early times of proteolysis (Zurini et al., 1984).

Recent sequencing work has clarified the primary structure of a number of human (Verma et al., 1988; Strehler et al., 1990) and rat (Shull & Greeb, 1988, 1989) plasma membrane Ca^{2+} pump isoforms. The pump contains a large C-terminal domain which protrudes into the cytosol and contains the calmodulin binding domain (James et al., 1988) and the domain which is phosphorylated by the cAMP-dependent kinase (James et al., 1989a). No information is presently available

† Supported by the Swiss National Science Foundation (Grant 31-25285.88). E.Z. was supported in part by a fellowship from the Federation of European Biochemical Societies.

* Address correspondence to this author.
‡ USSR Academy of Sciences.
§ Swiss Federal Institute of Technology.

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PVDF, poly(vinylidene difluoride); SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; DTT, dithiothreitol; CaM, calmodulin; PTC, phenylthiocarbamoyl.

0006-2960/90/0429-8070$02.50/0

on the portion of the pump responsible for the activation by acidic phospholipids and polyunsaturated fatty acids. The finding that the calmodulin response is altered in the 85- and 81-kDa fragments has led to the suggestion (Zurini et al., 1984; Benaim et al., 1984, 1986) that the trypsin cleavage sites responsible for their production are located in the region of the pump containing the calmodulin binding domain. The aim of the work described in this contribution was to provide conclusive answers on this point, as well as on the related problem of the location of the trypsin cleavage sites responsible for the generation of the 76-kDa fragment. N- and C-terminal sequencing work on PVDF membrane-blotted fragments (Matsudaira, 1987) of the erythrocyte Ca^{2+} pump treated with trypsin under selected conditions has indeed located the 90-, 85-, 81-, and 76-kDa fragments within the pump structure. The fragment of about 33 kDa which is produced in parallel with the higher molecular mass fragment has also been located. As predicted, the C-terminal cuts leading to the generation of all four high molecular mass fragments directly involve the calmodulin binding domain or its immediate vicinity. The same N-terminal cut leads to the formation of the 90-, 85-, and 81-kDa fragments, whereas the N-terminal cut leading to the 76-kDa fragment occurs somewhat downstream of the latter.

Materials and Methods

Materials

Trypsin was purchased from Boehringer Diagnostics, Mannheim, FRG. Soybean trypsin inhibitor and carboxypeptidases A, B, P, and Y were purchased from Sigma Chemical Co., St. Louis, MO. PVDF membranes (Immobilon Transfer), 0.45-μm pore size, were obtained from Millipore, Bedford, MA. The molecular mass standards were purchased from Bio-Rad Laboratories, Richmond, CA. All other reagents were of the highest purity grade commercially available.

Calmodulin was isolated from bovine brain by using the procedure described by Gopalakrishna and Anderson (1982), with some modifications, and was coupled to activated Sepharose (Niggli et al., 1979).

The Ca^{2+}-ATPase was isolated from human erythrocytes and purified to homogeneity essentially according to the procedure described by Niggli et al. (1981a,b) and Benaim et al. (1984).

Methods

Limited Trypsin Proteolysis of the Purified Ca^{2+}-ATPase. For the preparation of the high molecular mass fragments, samples of the ATPase (protein concentration 0.02 mg/mL) were digested on ice with 2 μg of trypsin/mL. The reaction was stopped by the addition of a 2-fold weight excess of soybean trypsin inhibitor. Fractions enriched in the 90- and 76-kDa fragments were obtained by performing the proteolysis in storage buffer (130 mM NaCl, 20 mM HEPES, pH 7.2, 0.05% Triton X-100, 0.05% phosphatidylcholine, 2 mM EDTA, and 1 mM $MgCl_2$) for 5–7 and 90 min, respectively. The 85- and 81-kDa fragments were enriched by 40-min trypsin digestion in the presence of 5 mg/mL calmodulin and 50 μM $CaCl_2$ (the 85-kDa fragment) and in the presence of 10 μM vanadate and 10 mM $MgCl_2$ (the 81-kDa fragment). The ATPase samples were preincubated with the effectors for 10 min at room temperature followed by 5 min on ice before trypsin addition.

To generate optimal amounts of the 33.5-kDa fragment, the ATPase was digested for 1 h at room temperature at a trypsin/ATPase ratio of 1/25 (w/w) in the storage buffer (see above); the ATPase concentration was about 150 μg/mL; 220–250 μg of ATPase was digested under these conditions, precipitated with TCA (final concentration 8%) resuspended in sample buffer, and distributed into six lanes of a 10% Laemmli (1970) gel (dimensions: 8 cm × 7 cm × 0.8 mm). After electrophoresis and electroblotting (see below), six bands corresponding to the 33.5-kDa fragment were cut out of the PVDF sheet and subjected to partial sequencing.

SDS–Polyacrylamide Gel Electrophoresis. The electrophoresis was carried out by using the discontinuous buffer system described by Laemmli (1970). The samples of digested ATPase (about 1 mg of total protein) were concentrated by incubating 10 min on ice in the presence of 0.01% deoxycholate and precipitated with ice-cold TCA. The final concentration of TCA was 10% (Bensadoun & Weinstein, 1976). The pellet was washed with ice-cold acetone containing 0.1% HCl (v/v), dried under nitrogen, and resuspended in 0.6 mL of sample buffer containing 5% glycerol, 0.031 M Tris-HCl, pH 6.8, 1.5% SDS, 2.5% (v/v) 2-mercaptoethanol, and 0.03% bromophenol blue; 150-μL aliquots were applied on a slab gel (6.5–7% polyacrylamide, 1.5 mm thick). Prior to sample application, thioglycolic acid was added to the upper electrode buffer to 0.1% final concentration to act as a radical scavenger (Moos et al., 1988). The electrophoresis was performed at 30 mA, for 2 h.

Electroblotting. The Immobilon membrane was rinsed in absolute methanol for 1 min and then immersed in transfer buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid/10% methanol, pH 11.0] as described by Matsudaira (1987). The gel with the separated fragments was sandwiched between a sheet of PVDF membrane and several sheets of blotting paper. The electrophoretic transfer was carried out for 40–60 min at 150 mA in a blotting apparatus (Biorad Mini Protean).

The portion of the membrane containing the lane with the molecular mass standards and a lane of separated ATPase fragments was cut off and stained for protein (2 min in 0.1% amido black, 20% methanol, and 7.5% acetic acid followed by destaining in 20% methanol/7.5% acetic acid). The spots or the blotted fragments were excised from the unstained lanes, and the positions were verified by comparison with the corresponding bands on the protein-stained track.

N-Terminal Sequence Determination. Four to five slices of the membrane with the blotted fragment (about 0.1 nmol of the fragment according to amino acid analysis) were washed in absolute methanol to reduce the amino acid background. The fragments were sequenced in an Applied Biosystems (Foster City, CA) 470A sequencer with a 120A phenylthiohydantoin derivative analyzer.

C-Terminal Sequencing. The membrane slices containing the blotted fragments (about 0.5 nmol of fragment according to amino acid analysis) were incubated with 0.5% poly(vinylpyrrolidone) K 30 (M_r 40000)/0.1 M acetic acid for 30 min at 37 °C. After the incubation, the slices were washed 10 times with water followed by two washes with 0.1 M pyridine–acetate buffer, pH 5.6. The slices were chopped into small pieces and suspended in 0.2 mL of pyridine–acetate buffer. From the suspension, a control aliquot of 10 μL was withdrawn. Immediately after, 50 μL of a freshly prepared mixture of the carboxypeptidases (10 units of carboxypeptidase Y, 1–2 units of carboxypeptidase D, 1 unit each of carboxypeptidases A and B) in pyridine–acetate buffer was added. The digestion was performed at room temperature under constant agitation. At time intervals of 5, 15, 30, 60, 90, 120, 150, and 180 min, 10-μL aliquots were withdrawn. To each

aliquot was added 5 μL of glacial acetic acid immediately to terminate the reaction. The aliquots were centrifuged and analyzed by using an Applied Biosystems 420A derivatizer and on-line PTC detection with a Model 130A Applied Biosystems analyzer. The gradient used was flattened slightly over the first 5 min to allow the separation of Asn and Gln.

Silver Staining of the Gels. For analytical purposes, small quantities of the fragments (about 2 μg) were analyzed in the gels by the silver impregnation method described by Merril et al. (1981), with the modifications introduced by Bio-Rad Laboratories (Bio-Rad Bulletin 1089).

Protein Determination. Protein concentration in the erythrocyte ghosts was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. In the samples of the purified ATPase, the interference by Triton X-100, DTT, and other chemicals was eliminated by using a slight modification of the Lowry et al. procedure (1951): The protein solution was incubated on ice in the presence of 0.01% deoxycholate for 10 min and precipitated with ice-cold TCA (final concentration 10% TCA). Reagent A contained 1% SDS in addition to 2% sodium carbonate, 0.4% sodium hydroxide, and 0.16% sodium tartrate (Markwell et al., 1981).

Results

Production of the Tryptic Fragments in the Molecular Mass Range 90–76 kDa. Previous work (Zurini et al., 1984) had indicated that the 90-kDa fragment is a transient product, visible only at the initial times of proteolysis of the human erythrocyte enzyme under a variety of conditions. It had also been shown (Benaim et al., 1984) that the fragments of 85 and 81 kDa could be enriched by performing the proteolysis in the presence of calmodulin and of vanadate plus Mg^{2+}, respectively. No detailed studies have so far been carried out on the conditions leading to the enrichment of 76-kDa fragment, but it had been generally observed (Zurini et al., 1984; Benaim et al., 1984) that this fragment is produced under a variety of conditions and tends to persist for a long time in the proteolytic mixture. The appearance of the 76-kDa fragment is accelerated by the presence of EDTA or EGTA, although under these conditions the fragment also disappears faster (Enyedi et al., 1987). In the present paper, conditions have been explored leading to the improved enrichment of the various fragments, and to their prolonged persistence in the proteolytic mixture. It was found that the best results were obtained by performing the proteolysis at 0 °C, at a trypsin to ATPase ratio of 1 to 10 (w/w). It was also found that the concentration of the ATPase protein in the proteolysis medium greatly influenced the cleavage rate; i.e., the reaction was slowed down considerably by decreasing the ATPase concentration in the medium from 0.1–0.2 mg/mL, as used in previous studies, to 0.02 mg/mL. This point is illustrated in Figure 1: 0.02 mg/mL was thus the concentration of ATPase routinely used in the experiments to be described.

The gels shown in Figure 2 illustrate the conditions used to enrich the 90-, 85-, and 81-kDa fragments, and shows that in all cases the spatial resolution of the corresponding bands was adequate for the blotting and sequencing procedure. As expected, the 90-kDa fragment was the most labile, i.e., it appeared earlier than the others (5 min) and already started to decrease after 10 min. Separate experiments (not presented) have shown that the fragment was hardly visible after 30 min. The 85- and 81-kDa fragments were enriched in the presence of calmodulin and of vanadate plus Mg^{2+}, respectively. It is worth noting that these effectors, while clearly promoting the temporary accumulation of the 85- and 81-kDa fragments, respectively, had essentially a quantitative effect; i.e., the two

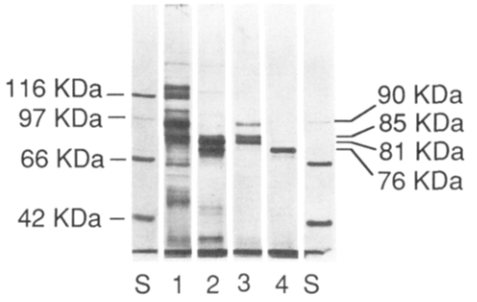


FIGURE 1: Proteolysis of the purified ATPase by trypsin at different ATPase concentrations. The figure shows 6.5% polyacrylamide gels of purified ATPase samples exposed to trypsin at 4 °C under conditions favoring the formation of high molecular mass fragments. The trypsin to ATPase ratios were always 1/10 (w/w) (for details, see Materials and Methods). Lanes 1 and 2, ATPase concentration, 0.02 mg/mL of reaction medium; lanes 3 and 4, ATPase concentrations, 0.2 mg/mL; lanes 1 and 3, proteolysis time 10 min; lanes 2 and 4, 1 h. Standards (S): myosin (200.0 kDa), β-galactosidase (116.2 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.6 kDa). The gels were stained with a silver impregnation method (Merril et al., 1981).

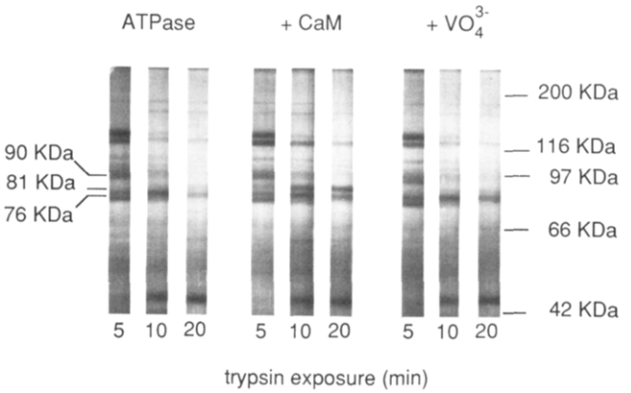


FIGURE 2: Controlled trypsin cleavage of the ATPase under conditions designed to enrich the 90-, 85-, and 81-kDa fragments. The ATPase samples (0.02 mg/mL) were digested with trypsin (2 μg/mL on ice) for the times indicated. As indicated, the proteolysis was performed without any effectors added, in the presence of 5 μg/mL calmodulin and 50 μM $CaCl_2$, and in the presence of 10 μM vanadate and 10 mM $MgCl_2$. The gels (6.5% polyacrylamide) were stained with a silver impregnation method (Merril et al., 1981).

fragments were present in both conditions. It may be of interest to note that the 76-kDa product sometimes appeared during the first minutes of proteolysis, apparently in parallel with the formation of the 90-kDa fragment. This was observed to be especially the case when ATPase samples were stored at −20 °C in the presence of EDTA for times exceeding 2 weeks: under these conditions, the ATPase gradually lost the ability to respond to calmodulin (James et al., 1989a,b), and the proteolysis pattern appeared to be much less influenced by the presence of effectors.

Determination of the N-Terminal Sequence of the Tryptic Fragments. After separation on SDS–polyacrylamide gels and blotting on PVDF membranes, the bands corresponding to the tryptic fragments obtained under the conditions described in the preceding section were excised and subjected to N-terminal sequencing (see Materials and Methods). The sensitivity of the procedures used allowed the sequencing of up to 12 amino acids from picomole amounts of protein present on the blots. Table I shows the N-terminal sequences of the four main functionally active fragments of molecular mass 90, 85, 81, and 76 kDa, of the fragment(s) of molecular mass 124 kDa produced by calpain, and of the low molecular mass, functionally inactive, tryptic fragments of 35 and 33.5 kDa. [The

Table I: N- and C-Terminal Sequences of the Tryptic Fragments of the Ca^{2+} Pump

			molecular mass
intact pump	N-term blocked (1)	C-term ETSL (1205)	132 600
127-kDa fragment	N-term blocked (1)	C-term KVV (1106)	121 660
125-kDa fragment	N-term blocked (1)	C-term LN (1097)	120 670
124-kDa fragment	N-term blocked (1)	C-term LR (1087)	119 570
90-kDa fragment	N-term TQDG (315)	C-term ASK (1161)	93 170
85-kDa fragment[a]	N-term TQDG (315)	C-term QIK (1105)	87 010
81-kDa fragment[a]	N-term TQDG (315)	C-term TTK (1066)	82 720
76-kDa fragment	N-term LAVQ (359)	C-term TTK (1066)	77 880
35-kDa fragment	N-term blocked (1)	probably KAK (314)	34 870
33.5-kDa fragment	N-term EGDF (19)	probably KAK (314)	32 230

[a] The 10% subsequences seen with these fragments begin at positions 338 and 348, respectively. The fragments of molecular mass 127 125 and 124 000 Da were obtained with calpain, the former in the presence of calmodulin (James et al., 1989a,b). The numeration of the amino acids and the molecular masses are based on the deduced sequence of hPMCA4 isoform of the plasma membrane Ca^{2+}-ATPase (Strehler et al., 1990).

improved resolution of the gels used in the present work has shown that the tryptic fragment of molecular mass about 33 kDa (Zurini et al., 1984) consists of two fragments of 35 and 33.5 kDa, respectively.] The 90-kDa fragment (12 amino acids sequenced) started at position 315 in the sequence of the human colon carcinoma Ca^{2+}-ATPase. Table I also shows that the N-terminal sequence of the 85- and 81-kDa fragments (6 and 10 amino acids were determined, respectively) coincided with that of the 90-kDa fragment. In the case of the last two fragments, however, minor sequences (ca. 10%) were found along with the main one. Their N-termini corresponded to postions 338 and 348 in the human sequence, respectively. The N-terminal cut leading to the formation of the 76-kDa fragment occurred further downstream in the human isoform of the pump now classified as hPMCA4 (Strehler et al., 1990), at leucine-359 (eight amino acids could be sequenced). The 124-kDa fragment produced by calpain was N-terminally blocked: this was expected, since previous calpain work (James et al., 1989b) had shown that this fragment had the same N-terminus as the intact pump. The same was most likely true of the 35-kDa fragment which was also N-terminally blocked, whereas the N-terminus of the 33.5-kDa fragment was at residue 19.

The N-terminal sequence of the 90-kDa fragment was confirmed on the fragment isolated by CaM affinity chromatography performed under conditions that would favor its formation (see below).

C-Terminal Analysis of the Tryptic Fragments. The fragments were separated and blotted onto PVDF membranes as described under Materials and Methods and in the preceding section. Given the general difficulties connected with carboxypeptidase analysis on very small amounts of proteins, particularly on gel-blotted proteins, the most favorable conditions were established in a series of preliminary experiments using calmodulin as a model protein. Amounts of calmodulin varying between 0.1 and 2.0 nmol were blotted onto PVDF membranes and digested with mixtures of carboxypeptidases for 2–3 h at 0, 20, and 37 °C. Optimal results, i.e., the clearest C-terminal sequences and the lowest background disturbance, were obtained by using 0.5 nmol of blotted calmodulin incubated at 20 °C with 10 units of carboxypeptidase Y, 1 unit of carboxypeptidase D, and 1–2 units of carboxypeptidases A and B. Under these conditions, three amino acid residues could be unambiguously read off the C-terminus, with a yield of about 20% (i.e., about 100 pmol out of the total 0.5 nmol applied on the blot). Other proteins, e.g., β-lactoglobulin, were completely refractory to this form of analysis. When the intact ATPase molecule was used, the results were predictably less clear, but at least two to three amino acids could nevertheless be read unambiguously. The sequence, in a series of four experiments, was found to be ETSL, corresponding to the C-terminal sequence of the minor isoform of the calcium pump of erythrocytes, which corresponds to the teratoma sequence (Verma et al., 1988) (hPMCA1b; Strehler et al., 1990). Valine, which is the C-terminal residue of the major isoform of the pump (hPMCA4), was not detected at levels above background; thus, either a single amino acid substitution has occurred or the C-terminus of the major isoform is not exposed.

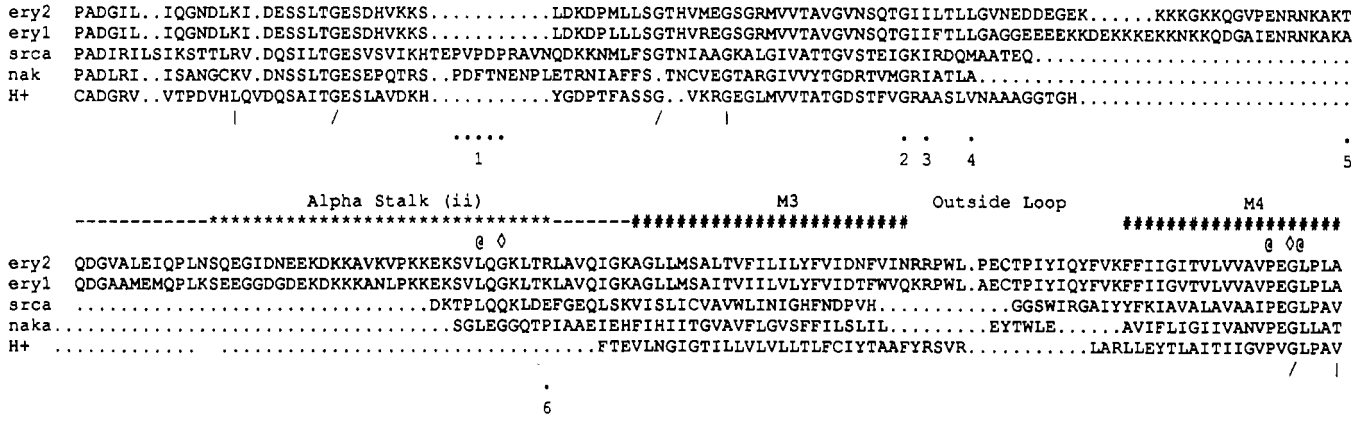
Predictions from the N-terminal results described in the preceding sections, as well as the previous finding that the 90-, 85-, 81-, and 76-kDa fragments differ essentially in their response to calmodulin (Enyedi et al., 1987; Zurini et al., 1984; Benaim et al., 1984), suggested that calmodulin binding domain and/or its immediate vicinity as the point of trypsin cleavage producing the C-terminus of the fragments studied here. Since arginines and lysines are particularly abundant in this region of the pump, the possibility existed that the blotted gel bands of the fragments would be heterogeneous, i.e., contain a mixture of products differing by only a few C-terminal residues. Thus, the determinations were repeated 4 times on each of the tryptic fragments. Table I shows that the 90-kDa fragment produced the C-terminal sequence ASK, with pronounced D and E peaks in the background: this would correspond to amino acid 1161 in the structure of the hPMCA4 isoform of the pump; i.e., the cut occurs just outside the calmodulin binding domain (Verma et al., 1988; Strehler et al., 1990). The 85-kDa fragment produced the C-terminal triplet QIK. This places the cut producing the 85-kDa fragment at residue 1105, i.e., within the calmodulin binding domain. The 81- and 76-kDa fragments yielded the same TTK C-terminal sequence, with considerable amounts of H in the background. In this case, the cut occurs at residue 1067; i.e., it completely removes the calmodulin binding domain.

As shown previously (James et al., 1989b), the calpain product of molecular mass 125 kDa (the early product of calpain proteolysis) produced the C-terminal sequence LN (1097–1098), locating the cut about 100 residues from the C-terminus of the pump at the end of the calmodulin binding domain. The later product of calpain proteolysis had the C-terminus LR (1086–1087), located at the beginning of the calmodulin binding domain. In the presence of calmodulin, the calpain cut was at 1107 (VV). The C-terminal sequences of the 35- and 33.5-kDa tryptic fragments could not be determined though it seems reasonable to place them at the N-terminal side of the cut which produces the fragments of 90, 85, and 81 kDa, since this fits in well with the observed molecular masses.

Discussion

An important point which must be made at the outset of the Discussion concerns the separation of the fragments described. The matter has already been briefly alluded to in the

a



b

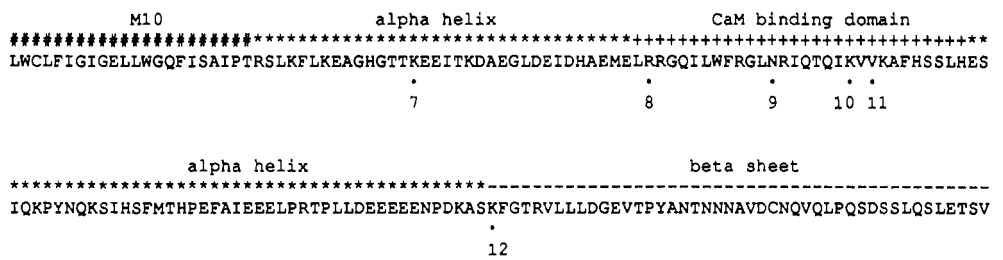


FIGURE 3: Sequence alignment at the N-terminal domains of P-type ATPases (a) and C-terminal cleavage sites in the plasma membrane pump (b). The first line is a comment on the structural domain. The second line shows the occurrence of β (–), α (*), and transmembrane stretches (#). The third line shows identical residues (@), occurring in all ATPases shown here with glycines marked as (◊). The fourth to eight lines show in order the sequences of Ery 2, the human colon carcinoma isoform of the plasma membrane Ca^{2+}-ATPase which does not contain the cAMP kinase sensitive site and contributes the major portion of the total Ca^{2+}-ATPase found in erythrocytes. Ery 1 is the sequence of the human teratoma isoform (hPMCA1b) corresponding to a minor portion of the total erythrocyte Ca^{2+}-ATPase; SR Ca is the sequence of the fast-twitch rabbit skeletal muscle form of the sarcoplasmic reticulum Ca^{2+}-ATPase; Naka is the sequence of the Na^+/K^+-ATPase from sheep kidney; H^+ is the sequence of the H^+-ATPase from *Schizosaccharomyces pombe*. The ninth line has the symbols (|) and (/) showing the positions of the intron–exon boundaries in the Na^+/K^+-ATPase and fast skeletal muscle SR Ca^{2+}-ATPase genes, respectively. The tenth line marks the positions of several important sites which are listed below [see Green (1989) for a review]: (1) peptide described by Gangola and Shamoo (1986) and T2 cut in the sarcoplasmic reticulum Ca^{2+}-ATPase (Imamura & Kawakita, 1989); (2) site of vanadate-insensitive mutation in the H^+-ATPase; (3) T3 cut in the Na^+/K^+-ATPase and T2b cut site in the sarcoplasmic reticulum Ca^{2+}-ATPase; (4) chymotrypsin cut in the Na^+/K^+-ATPase; (5) N-terminal of 90-, and 85-, and 81-kDa fragments; (6) N-terminal of the 76-kDa fragment; (7) C-terminal of the 76- and 81-kDa fragments; (8) C-terminal of the 124-kDa fragment produced at long times by calpain cutting in the absence of calmodulin (James et al., 1989a,b); (9) C-terminal of the 125-kDa fragment produced at short times by calpain cutting in the absence of calmodulin (James et al., 1989a,b); (10) C-terminal of the 85-kDa fragment; (11) C-terminal of the 127-kDa fragment produced by calpain in the presence of calmodulin (James et al., 1989a,b); (12) C-terminal of the 90-kDa fragment.

introduction: it can be safely assumed that the attack by trypsin (or by calpain) indeed leads to the removal of hydrophilic portions of the ATPase located after the last putative transmembrane helix. This conclusion has been made very likely by work on intact membrane systems (Sarkadi et al., 1980) and also by previous trypsin proteolysis work on the purified erythrocyte enzyme (Zurini et al., 1984). Whether the lower molecular mass products of trypsin attack between putative transmembrane helices 2 and 3, i.e., the fragments of 33.5–35 kDa, are removed from the main body of the pump in the absence of SDS is still an open question. In fact, two considerations would tend to make it unlikely: first, the 35–33.5-kDa fragments are very hydrophobic (Zurini et al., 1984) and tend to associate with the larger fragments in separation attempts (work in preparation). Second, the domain between putative transmembrane helices 2 and 3, where these cuts occur, is strongly preserved in the structure of ionmotive ATPases (see Figure 3). It appears unlikely that a region of strong conservation should be disposed of and still leave behind a functionally active truncated enzyme. It could also be mentioned that several functionally important sites have been located in this region in other P-type ATPases. Among them is the site of the glycine to aspartate mutation in the vanadate-insensitive mutant of *Schizosaccharomyces pombe* (Ghislain et al., 1987). A trypsin cleavage site in the Na^+/K^+-ATPase which causes 80% inhibition of the ATPase activity but causes no uncoupling is also located in this domain (Jorgensen et al., 1982). Recent work on the sarcoplasmic reticulum Ca^{2+}-ATPase has shown that tryptic cleavage at a point nearby produces a fragment which forms an aspartyl phosphate but which is not hydrolyzed. The domain where the N-terminal cleavages leading to the 90-, 85-, 81-, and 76-kDa fragments occur is thus functionally important, and it appears unlikely, albeit by no means impossible, that it separates from the main body of the pump. Thus, while the properties of the products of trypsin cleavage in the calmodulin binding domain and in its neighborhood truly reflect those of the fragments lacking the domains C-terminal to the cut(s), it appears convenient at the moment to assume that the properties of the products resulting from cleavages between transmembrane helices 2 and 3 reflect those of the entire sequence of the enzyme from the N-terminus to the cleavage point(s) in the hydrophilic domain protruding from the 10th putative transmembrane helix. Work now in progress attempts

to isolate the fragments of molecular mass 90–76 kDa free of contamination by the 33.5–35-kDa products. If the fragments will prove capable of Ca^{2+}-dependent ATP hydrolysis (and of Ca^{2+} transport in liposomes), it will obviously be necessary to modify this proposal, and to conclude that the differences between the fragments considered here reflect their actual separation from the N-terminal portion of the pump.

The data presented here provide conclusive support for the trypsin proteolysis scheme proposed previously (Zurini et al., 1984; Benaim et al., 1984). The products of molecular mass 90, 85, and 81 kDa are indeed produced by trypsin as predicted, i.e., by cuts in the region of the calmodulin binding domain. The complete preservation or loss of calmodulin reactivity in the 90- and 81-kDa fragments, respectively, is now conveniently rationalized. The properties of the 85-kDa fragment, i.e., the decreased response to calmodulin with preservation of its binding, are more difficult to interpret from a structural viewpoint. Evidently, the sequence of about 50 amino acids between the product of 85 and that of 81 kDa acts not only as the site of calmodulin binding but also as an "inhibitory" domain which somehow limits the activatory response to calmodulin (Benaim et al., 1984). Conversely, the sequence of about 50 amino acids between the products of 85 and 90 kDa apparently functions as a domain that facilitates the full expression of calmodulin stimulation after the modulator protein has been bound (Benaim et al., 1984). That the C-terminal portion of the calmodulin binding domain has no essential role in the binding of calmodulin is on the other hand also shown by previous calpain proteolysis work (James et al., 1989b), and by recent experiments on synthetic peptides corresponding to various portions of the calmodulin binding domain of the erythrocyte pump (Vorherr et al., 1990). Interestingly, the 81- and 76-kDa fragments still bind the synthetic calmodulin binding domain and are inhibited by it (Enyedi et al., 1989). Since the acidic sequence N-terminal to the calmodulin binding domain no longer exists in the 81- and 76-kDa fragments, under the experimental conditions (Enyedi et al., 1989) the calmodulin binding domain evidently interacts elsewhere in the molecule and not as previously suggested (Verma et al., 1988) by binding to this acidic domain (thus preventing Ca^{2+} from entering the ion channel of the pump). Recent work has placed the high-affinity Ca^{2+} binding sites of the sarcoplasmic reticulum Ca^{2+} pump within the membrane (Clarke et al., 1989a,b), and another contribution has shown that the previously suggested sites or "stalks", which would be formally analogous to the above-mentioned acidic sequence in the plasma membrane Ca^{2+} pump, are not important for Ca^{2+} transport (Clarke et al., 1989a,b).

The properties of the fragment of 76 kDa, i.e., the loss of sensitivity to acidic phospholipids in the 81–76-kDa transition (Enyedi et al., 1987), indicate that a domain able to bind acidic phospholipids is removed or altered in the transition. Since the C-termini of the 76- and 81-kDa fragments are identical, the difference in properties of the two fragments evidently arises from the opposite end of the fragments. It could be provisionally suggested that the attack by trypsin, in producing the 76-kDa fragment (44 amino acids dowstream of the N-terminus of the 81-kDa fragment), structurally modifies that portion of the pump, apparently preventing it from interacting with phospholipids. Alternatively, or in addition, it could confer to it a state which makes the pump optimally responsive to Ca^{2+} in the absence of phospholipids. It is interesting that the domain indicated here as the site of phospholipid interaction, i.e., the highly charged stretch of 44 amino acids separating the N-terminus of the fragment of 76 kDa from those of the 81, 85, and 90 kDa, apparently occurs only in the plasma membrane Ca^{2+} pump (Figure 3). This rationalizes the peculiar sensitivity of the latter pump to acidic phospholipids. It may be added that this domain shows propensity to form an amphiphilic helix (unpublished work): although no attempts have been made so far to investigate the location of the protruding portions of the pump with respect to the membrane environment, it is clear that the putative helical domain responsive to phospholipids must interact closely with the membrane ambient.

Proteolytic sensitivity in the region of the N-termini of the fragments of 90, 85, 81, and 76 kDa is a feature common to most of the P-type ATPases studied so far. A comparison of the aligned sequences of the N- and C-terminal portions of some of them is shown in Figure 3. The sites of trypsinolysis have been defined for the sarcoplasmic reticulum fast-twitch Ca^{2+}-ATPase and the Na^+/K^+ pumps. In the fast-twitch Ca^{2+}-ATPase pump, trypsin cleavage occurs at a point designated T2, immediately after the sequence PVPDP, which was suggested to be a possible Ca^{2+} binding site (Imamura & Kawakita, 1989). This cleavage only occurs in the E1 state and was claimed to cause partial uncoupling, from a 2/1 to a 1/1 Ca^{2+}/ATP stoichiometry (Gangola & Shamoo, 1986). However, more recent work has found no evidence for uncoupling and shown that this cleavage causes instead a drop in V_{max} by about 50%, without affecting the K_m(Ca) (Torok et al., 1988). Trypsin cleavage at a point described as T3 in the Na^+/K^+-ATPase causes an 80% inhibition of the ATPase activity without uncoupling. A conformation-sensitive chymotrypsin cleavage occurs close by, and like the T2 cut of the sarcoplasmic reticulum Ca^{2+} pump, only when the enzyme is in the E1 state. This cleavage produces complete inhibition of ATPase activity (Jorgensen et al., 1982).

The so-called transduction domain forms a region of extensive antiparallel β sheet. The glycines forming the hairpin turns are highly conserved in all ATPases and coincide with the intron–exon boundaries in the sarcoplasmic reticulum Ca^{2+}- and the Na^+/K^+-ATPase pumps genes (Korczak et al., 1988; Ovchinnikov et al., 1987). The exposed surfaces are typical for intron–exon locations since they often define junctions of high mobility between functional domains. Proteolytic cleavage sites have often been found to correspond to areas of high mobility near the surface of proteins and have been used as tools to define boundaries between the various building blocks of proteins. Examples are the cleavages of immunoglobulins by papain and of calmodulin by trypsin into functionally defined fragments. The common N-terminus of the 90-, 85-, and 81-kDa trypsin fragments of the erythrocyte ATPase as well as that of the 76-kDa fragment all occur at predicted turn sites or at β/α boundaries (this is true for all ATPases shown aligned in Figure 3), and correspond almost exactly to intron–exon boundaries in the plasma membrane Ca^{2+}-ATPase gene. The same applies also to the cleavage sites in the C-terminal domain (E. Strehler, personal communication).

Acknowledgments

We thank Dr. J. Krebs for helpful discussions.

References

Benaim, G., Zurini, M., & Carafoli, E. (1984) *J. Biol. Chem. 259*, 8471–8477.

Benaim, G., Clark, A., & Carafoli, E. (1986) *Cell Calcium 4*, 175–186.

Bensadoun, A., & Weinstein, D. (1976) *Anal. Biochem. 70*, 241–250.

Caroni, P., & Carafoli, E. (1981) *J. Biol. Chem. 256*, 9371–9373.
Clarke, D. M., Maruyama, K., Loo, T., Leberer, E., Inesi, G., & MacLennan, D. H. (1989a) *J. Biol. Chem. 264*, 11246–11251.
Clarke, D., Loo, T., Inesi, G., & MacLennan, D. H. (1989b) *Nature 339*, 476–478.
Enyedi, A., Flura, M., Sarkadi, B., Gardos, G., & Carafoli, E. (1987) *J. Biol. Chem. 261*, 9552–9557.
Enyedi, A., Vorherr, T., James, P., Krebs, J., McCormick, D. J., Carafoli, E., & Penniston, J. T. (1989) *J. Biol. Chem. 264*, 12313–12321.
Gangola, P., & Shamoo, A. E. (1986) *J. Biol. Chem. 261*, 8601–8603.
Ghislain, M., Schlesser, A., & Goffeau, A. (1987) *J. Biol. Chem. 262*, 17549–17555.
Gopalakrishna, R., & Anderson, W. B. (1982) *Biochem. Biophys. Res. Commun. 104*, 830–836.
Gopinath, R. M., & Vincenzi, F. F. (1977) *Biochem. Biophys. Res. Commun. 77*, 1203–1209.
Green, N. M. (1989) *Trans. Biochem. Soc. 17*, 970–972.
Imamura, Y., Kawakita, M. (1989) *J. Biochem. 105*, 775–781.
James, P., Maeda, M., Fischer, R., Verma, A. K., Krebs, J., Penniston, J. T., & Carafoli, E. (1988) *J. Biol. Chem. 263*, 2905–2910.
James, P., Pruschi, M., Vorherr, T., Penniston, J. T., & Carafoli, E. (1989a) *Biochemistry 28*, 4253–4258.
James, P., Vorherr, T., Krebs, J., Morelli, A., Castello, G., McCormick, D. J., Penniston, J. T., De Flora, A., & Carafoli, E. (1989b) *J. Biol. Chem. 264*, 8289–8296.
Jarrett, H. W., & Penniston, J. T. (1977) *Biochem. Biophys. Res. Commun. 77*, 1210–1216.
Jorgensen, P. L., Skriver, E., Herbert, H., & Maunsbach, A. B. (1982) *Ann. N.Y. Acad. Sci. 402*, 207–255.
Korczak, B., Zarain-Herzberg, A., Brandl, C., Ingles, C., Green, N. M., & MacLennan, D. H. (1988) *J. Biol. Chem. 263*, 4813–4819.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lowry, O. N., Rosebrough, N. J., Farr, A. L., & Randell, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Markwell, M. K., Haas, S., Tolbert, N. E., & Bieber, L. L. (1981) *Methods Enzymol. 72*, 296–303.
Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035–10038.
Merril, C. R., Dunan, M. L., & Goldmann, D. (1981) *Anal. Biochem. 110*, 201–207.
Moos, M., Jr., Nguyen, N. Y., & Liu, T.-Y. (1988) *J. Biol. Chem. 263*, 6005–6008.
Neyses, L., Reinlib, L., & Carafoli, E. (1985) *J. Biol. Chem. 260*, 10283–10287.
Niggli, V., Penniston, J. T., & Carafoli, E. (1979) *J. Biol. Chem. 254*, 9955–9958.
Niggli, V., Adunyah, E. S., & Carafoli, E. (1981a) *J. Biol. Chem. 256*, 8588–8592.
Niggli, V., Adunyah, E. S., Penniston, J. T., & Carafoli, E. (1981b) *J. Biol. Chem. 256*, 395–401.
Ovchinnikov, Y., Monastyrskaya, G., Broude, N., Ushkaryov, Y., Melkov, A., Smirnov, Y., Malzshev, I., Allikmets, R., Kostina, M., Dulubova, I., Kiyatkin, Grishin, A., Modyanov, N., & Sverdlov, E. (1987) *FEBS Lett. 233*, 87–94.
Ronner, P., Gazzotti, P., & Carafoli, E. (1977) *Arch. Biochem. Biophys. 179*, 578–583.
Sarkadi, B., Enyedi, A., & Gardos, G. (1980) *Cell Calcium 1*, 287–297.
Shull, G. E., & Greeb, J. (1988) *J. Biol. Chem. 263*, 8646–8657.
Shull, G. E., & Greeb, J. (1989) *J. Biol. Chem. 264*, 18569–18576.
Stieger, J., & Schatzmann, H. J. (1981) *Cell Calcium 2*, 601–616.
Strehler, E. E., James, P., Fischer, R., Heim, R., Vorherr, T., Filoteo, A., Penniston, J. T., & Carafoli, E. (1990) *J. Biol. Chem.* (in press).
Taverna, R. D., & Hanahan, D. J. (1980) *Biochem. Biophys. Res. Commun. 94*, 652–659.
Torok, K., Trinnaman, B., & Green, N. M. (1988) *Eur. J. Biochem. 173*, 361–376.
Verma, A. K., Filoteo, A. G., Stanford, D. R., Wieben, E. D., Penniston, J. T., Strehler, E. E., Fischer, R., Heim, R., Vogel, G., Mathews, S., Strehler-Page, M. A., James, P., Vorherr, T., Krebs, J., & Carafoli, E. (1988) *J. Biol. Chem. 263*, 14152–14159.
Vorherr, T., James, P., Krebs, J., Enyedi, A., McCormick, D. J., Penniston, J. T., & Carafoli, E. (1990) *Biochemistry 29*, 355–365.
Zurini, M., Krebs, J., Penniston, J. T., & Carafoli, E. (1984) *J. Biol. Chem. 259*, 618–627.