Intramolecular Interactions of the Regulatory Region with the Catalytic Core in the Plasma Membrane Calcium Pump*

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The access of three proteases to their sites of cleavage was used as a measure of regulatory interactions in the plasma membrane Ca\(^{2+}\) pump isoform 4b (PMCA4b). When the proteases could not cut at their sites in the C-terminal regulatory region, the interaction was judged to be tight. This was the case in the absence of Ca\(^{2+}\), when chymotrypsin and caspase cut PMCA only very slowly. Ca\(^{2+}\) accelerated the fragmentation, but the digestion remained incomplete. In the presence of Ca\(^{2+}\) plus calmodulin, the digestion became nearly complete in all cases, indicating a more flexible conformation of the carboxyl terminus in the fully activated state. The acceleration of proteolysis by Ca\(^{2+}\) or Ca\(^{2+}\) plus calmodulin occurred equally at the caspase site upstream of the calmodulin-binding domain and the chymotrypsin and calpain sites downstream of that domain. Replacing Trp\(^{1093}\) (a key residue within the calmodulin-binding domain) with alanine had a much more specific effect, because it exposed only proteolytic sites within the calmodulin-binding domain that had previously been shielded in the native protein. At these sites, both calpain and chymotrypsin cut the Trp\(^{1093}\) → Ala mutant in the absence of calmodulin. These data indicate that, in the auto-inhibited conformation, the calmodulin-binding/auto-inhibitory sequence and the regions both upstream and downstream are in close contact with the catalytic core. Trp\(^{1093}\) plays an essential role not only in stabilizing the Ca\(^{2+}\)-calmodulin/calmodulin-binding domain complex but also in the formation or stability of the inhibitory conformation of that domain when it interacts with the catalytic core of PMCA4b.

The plasma membrane Ca\(^{2+}\) ATPase or pump (ATP2B1-4, referred to here as PMCA\(^{3}\)) is a P-type ATPase that is an essential element of cellular Ca\(^{2+}\) homeostasis. Its role is to remove excess Ca\(^{2+}\) from the cell in order to maintain the large Ca\(^{2+}\) concentration gradient existing between the cytosol and the extracellular space. This pump is encoded by four different genes, and further diversity is produced by alternative splicing of the primary transcripts at two different sites (1). Isoforms PMCA1 and 4 are ubiquitous, whereas the expression of PMCA2 and 3 are more cell- and tissue-specific.

The activity of the plasma membrane Ca\(^{2+}\) pump is regulated by calmodulin. It has a unique carboxyl terminus that is responsible for the regulation. A high affinity, ~28-residue calmodulin-binding sequence is located within this region, which is also a built-in inhibitor of the enzyme (2, 3) (Fig. 1). Removing the carboxyl terminus either by proteolysis or truncation by mutagenesis causes full activation of the pump similar to that caused by calmodulin (4). Synthetic peptides representing the calmodulin-binding sequence were able to restore the inhibition when added to the fully active forms (2, 4). Cross-linking experiments using synthetic peptides have revealed that the N-terminal half of this sequence interacts with the large cytoplasmic loop (N domain), whereas the C-terminal part interacts with the small cytoplasmic loop (A domain) of the catalytic core (5–7). These interactions must interfere with the movements of the A and N domains during the reaction cycle so that, in the absence of calmodulin, the pump has low affinity for its substrate Ca\(^{2+}\) and a low maximal turnover rate. Binding of Ca\(^{2+}\)-calmodulin to the pump removes the inhibitor from this interaction, allowing high affinity substrate binding and the reaction cycle to proceed with full speed.

The PMCA isoforms show great diversity in their regulation with calmodulin. They have different basal activities, different affinities for calmodulin, and different rates of activation and inactivation in response to calmodulin binding and dissociation (8–10). Among the isoforms, PMCA4b has the lowest basal activity and the greatest stimulation by calmodulin. An important feature of this pump is that binding of calmodulin followed by activation is very slow, with a half-time of ~20–60 s depending on the Ca\(^{2+}\) concentration (11). The inactivation by calmodulin removal is even slower, with a half-time of about 20 min. These features of PMCA4b suggest that it is actively involved in the developing, shaping, and duration of the Ca\(^{2+}\) signal; it allows the first Ca\(^{2+}\) spike to develop for several seconds before becoming activated and remains activated for several minutes after the spike is dissipated so that it will respond to the next spike much faster.

The low basal activity of the PMCA4b isoform indicates that the interaction between the carboxyl terminus and the catalytic core is very strong. We have shown that Trp\(^{1093}\) within the calmodulin-binding sequence is an essential anchor for the auto-inhibitory interaction as well as for the interaction with calmodulin. Changing this residue to an alanine increased the basal activity in the absence of calmodulin, the rate of calmod-
ulin activation, and, more dramatically, the rate of inactivation by calmodulin removal (12). In addition to the calmodulin-binding sequence, regions both upstream and downstream provide additional anchor points for the auto-inhibitory interaction. Replacing Asp1080 by five residues upstream of the calmodulin-binding sequence (13) or removing the downstream inhibitory region (5) caused substantial activation of the pump. We have to emphasize that any change made within the carboxyl terminus that increased the basal activity also increased the rate of activation by calmodulin.

Controlled proteolysis of the purified and membrane-bound erythrocyte Ca\(^{2+}\) pump (which is mainly PMCA4b) has revealed several sites at the carboxyl terminus that are cut by proteases such as trypsin (14), chymotrypsin (2), calpain (15-16), V8 protease (17), and, more recently, caspase-3 (18). Among these proteases, chymotrypsin, calpain, and caspase-3 cut the protein preferentially at the carboxyl terminus. The N-terminal fragments were all fully or partially active depending on what portion of the carboxyl terminus was removed. Irreversible activation of the erythrocyte Ca\(^{2+}\) ATPase by calpain has been reported (15). In the absence of calmodulin, the pump was proteolyzed to a group of fragments of 124-125 kDa, whereas in the presence of calmodulin a 127-kDa fragment was identified. A later study using a purified erythrocyte pump or synthetic peptides determined the cleavage sites and found that all were located within the calmodulin-binding domain (16). In more recent experiments, we have demonstrated that PMCA4b is also cut by caspase-3 during the early phase of apoptosis (18). This cleavage occurs immediately downstream of Asp1080, removing the whole regulatory region and producing a fully active 120-kDa fragment.

In this study three different proteases, i.e. calpain, chymotrypsin, and caspase-3, were used to digest PMCA4b in membrane preparations from COS cells over-expressing PMCA4b. Because all three proteases cut the pump at the carboxyl terminus, conformational changes of this region induced by Ca\(^{2+}\) and Ca\(^{2+}\)-calmodulin could be monitored by limited proteolysis. We found that the accessibility of the proteolytic sites depended strongly on the conformational state of this protein. The sites within the calmodulin-binding domain were all shielded either by the autoinhibitory interactions with the catalytic core or the interaction with calmodulin. Sites within this sequence became accessible only when a key residue, Trp1093, was mutated to an alanine. The proteolytic sites upstream and downstream of the calmodulin-binding domain were somewhat more exposed in the auto-inhibited state; however, they became fully accessible only after calmodulin binding.

**MATERIALS AND METHODS**

Chemicals—Calmodulin and α-chymotrypsin were obtained from Sigma. Recombinant caspase-3 was from Upstate Biotechnology, and µ-calpain was obtained from Calbiochem. All other chemicals used for this study were of reagent grade.

**Construction of Trp1093→Ala—** The construction of the Trp1093→Ala mutant is described in our previous paper (12). The expression vector used was pMM2, which was derived from pMT2PC (19) and uses Ala mutant is described in our previous paper (12). The expression a total of 24 h. The medium containing the DNA-LipofectAMINE complex was supplemented with 10% fetal bovine serum, the vector used was pMM2, which was derived from pMT2PC (19) and uses Ala mutant is described in our previous paper (12). The expression

**RESULTS**

PMCA4b was expressed in COS cells, and limited proteolysis was performed in native COS cell membranes at 37 °C. We used caspase-3, chymotrypsin, and calpain digestion of PMCA4b to study conformational changes induced by Ca\(^{2+}\) and Ca\(^{2+}\)-calmodulin. These proteases cut the C-terminal regulatory region of PMCA4b within a short segment (about 40-50 residues) downstream of Asp1080. Following proteolysis, the samples were run on SDS-gels and immunoblotted. The immunoblots shown in the figures were stained with the antibody 5F10. 5F10 recognizes a linear epitope from residues 719-738 that is common to all known human PMCA gene products and splicing variants. To verify that the pump was cut C-terminally, Western blot analysis was also performed with two PMCA4-specific antibodies (data not shown). The monoclonal antibody J49 reacts specifically with PMCA isoform 4 near the N terminus within residues 51-75, whereas monoclonal antibody J33 binds near the C terminus from residues 1156-1180 (23). All of the proteolytic fragments detected by 5F10 were also detected by J49; however, J33 did not recognize any of the fragments detected from 5F10.
fragments. The antibody results indicate that all three proteases cut PMCA4b at the C terminus upstream of the JA3 recognition sequence.

The sizes of the fragments were determined by comparing their electrophoretic mobilities to those of C-terminally truncated mutants ct120, ct92, and ct71 (Fig. 1). These PMCA4b mutants are missing 120, 92, or 71 residues from the C terminus (24). Fig. 2A shows the fragmentation of PMCA4b by the different proteases, and Fig. 2B shows the relative electrophoretic mobility of the same fragments as compared with that of the truncated mutants. The calpain digest produced two N-terminal fragments. The larger fragment migrated close to the position of ct71, whereas the smaller fragment migrated close to that of ct92. Based on relative mobility, the calculated molecular masses of the fragments were 125 and 124 kDa, respectively. These results indicated that one calpain cut site is located five residues upstream of the calmodulin-binding domain, and a second site is located close to the C-terminal end of the calmodulin-binding domain (Fig. 1). The chymotrypsin digest produced one major N-terminal fragment of 124.5 kDa, which is smaller than ct71 but larger than ct92. Thus, the chymotryptic cut site must also be located downstream of the calmodulin-binding domain (Fig. 1). The chymotryptic cut site is after Tyr1122 as indicated in Fig. 1. A more precise determination of the cut sites by N-terminal sequencing of the small fragments was not possible, because <1% of the total membrane protein is accounted for by the expressed PMCA. We have to emphasize that mild conditions were used to produce these fragments. In the case of chymotrypsin especially, increasing concentrations of the protease or longer digestions induced further fragmentation of the protein. As also shown in Fig. 2, the caspase-3 digest produced a single N-terminal fragment of 120 kDa even after prolonged incubations, because PMCA4b contains only one caspase consensus site, Asp\textsuperscript{1077}-Glu-Ile-Asp\textsuperscript{1080}, which is located five residues upstream of the calmodulin-binding domain (Fig. 1). In a previous paper, we have demonstrated that caspase-3 cuts immediately after Asp\textsuperscript{1080} (18).

Next, we studied the effects of Ca\textsuperscript{2+}-calmodulin and free Ca\textsuperscript{2+} on the fragmentation of the pump. In the absence of Ca\textsuperscript{2+}-calmodulin, the pump is in an inhibited (closed) state and has very low activity and low affinity for its substrate, Ca\textsuperscript{2+}. In the presence of Ca\textsuperscript{2+}-calmodulin, the pump is in an activated (open) state with high activity and high Ca\textsuperscript{2+} affinity. In addition to the open and closed states of the pump, an equilibrium exists between the E2 and E1 catalytic conformations. Low concentrations of Ca\textsuperscript{2+} shift the E2-E1 equilibrium more toward E1 (25). Figs. 3 and 4 show the fragmentation of PMCA4b with caspase-3 and chymotrypsin. These proteases do not require Ca\textsuperscript{2+} for activity; therefore, we could study the fragmentation of PMCA4b in the absence of Ca\textsuperscript{2+} (in the presence of EGTA). These figures show that, in the absence of Ca\textsuperscript{2+}, both caspase-3 and chymotryptic cleavage sites are protected from proteolysis because very little fragmentation was seen under
these conditions. The addition of Ca\(^{2+}\) to the incubation media substantially increased the fragmentation. However, only \(30-40\%\) of the protein could be digested even after prolonged incubation, indicating that \(60-70\%\) of the protein remained protected from cleavage. Binding of Ca\(^{2+}\)-calmodulin, on the other hand, exposed the cleavage sites, and fragmentation became complete with both caspase-3 and chymotrypsin. Fig. 3 shows some intact protein remaining at 3 h of digestion with caspase-3; this intact material disappeared completely after longer exposure to the protease (not shown). Fig. 3B shows the progressive formation of the 120-kDa fragment in the presence of calmodulin by caspase-3. Fig. 4 also shows a more progressive time-dependent accumulation of the chymotryptic 124.5-kDa fragment in the presence of calmodulin; however, only a maximum of 60% of the total protein was converted to this fragment because of further degradation (smaller fragments are not shown).

Fig. 5 shows the degradation of PMCA4b by \(\mu\)-calpain. Calpain requires Ca\(^{2+}\) for activity; therefore, only the effect of Ca\(^{2+}\)-calmodulin on digestion could be tested. In the absence of Ca\(^{2+}\)-calmodulin, calpain produced two fragments (125 and 124 kDa); however, the digestion was incomplete. In the presence of Ca\(^{2+}\)-calmodulin, the 124-kDa fragment was not seen, although the formation of the 125-kDa fragment became more pronounced, resulting in a nearly complete disappearance of the intact protein by the end of the digestion. These data indicate that Ca\(^{2+}\)-calmodulin binding protects the calmodulin-binding site from calpain cleavage but exposes the cleavage site downstream.

Mutating Trp\(^{1093}\) to an alanine has been shown to increase the basal activity of PMCA4b by 3-fold (12). In Fig. 6 we show that chymotrypsin and calpain cut the Trp\(^{1093}\) mutant more readily in the absence of calmodulin and that its cleavage pattern is rather different from that of the wild type. With calpain, the 124-kDa fragment became predominant in the absence of calmodulin, indicating that the cleavage site within the calmodulin-binding domain is more exposed in the mutant. With chymotrypsin, the change in the pattern was even more dramatic. Presumably, the mutation of Trp\(^{1093}\) exposed a site that was otherwise inaccessible in the wild type pump. The new chymotryptic fragment is slightly smaller than ct92, with an estimated molecular mass of 123 kDa. The most likely cleavage site is after Phe\(^{1094}\) within the calmodulin-binding domain that
is protected from proteolysis when Ca\(^{2+}\) is bound to the mutant.

We also tested whether mutation at Trp\(^{1093}\) affected the accessibility of the cleavage sites upstream (caspase-3) or downstream (calpain and chymotrypsin) of the calmodulin-binding domain. The digestion of the mutant with caspase-3 was very similar to that of the wild type (not shown), as the fragmentation of the mutant was highly dependent on the presence of Ca\(^{2+}\) and calmodulin. This indicated that mutation at Trp\(^{1093}\) did not affect the accessibility of the caspase-3 cleavage site. To test the downstream cut sites, we studied the digestion of the proteins by calpain in the absence of calmodulin. Fig. 7 shows a time course of the formation of the 125-kDa fragment is shown in the presence of Ca\(^{2+}\)-calmodulin (solid circles) and Ca\(^{2+}\) (solid triangles). Means ± S.E. of two independent determinations are shown.

**DISCUSSION**

It is widely known that the plasma membrane Ca\(^{2+}\) pump is regulated by calmodulin. At its carboxyl terminus it has a high-affinity calmodulin-binding sequence that also serves as an auto-inhibitor. Previous studies have predicted that in the absence of calmodulin, i.e. in the inhibited or closed conformation, the calmodulin-binding inhibitory sequence interacts with the catalytic core. By using site-directed mutagenesis it has been demonstrated that regions both upstream and downstream of this sequence are also involved in the auto-inhibition. The limited proteolysis experiments described above were aimed at identifying the conformational changes accompanying Ca\(^{2+}\)-calmodulin binding to PMCA4b in order to further understand the role of the carboxyl terminus in the regulation of the activity of this pump. Conformational changes induced by Ca\(^{2+}\)-calmodulin binding to the calmodulin-binding site, predicted on the basis of these experiments, are illustrated in Fig. 8.

Three proteases with different substrate specificities were used to digest PMCA4b in its natural membrane environment. It has been demonstrated that all three proteases cut the pump near the carboxyl terminus (2, 14, 18). Previously, we showed that caspase-3, a key protease of apoptosis, cuts
the protein five residues upstream of the calmodulin-binding sequence after Asp^{1080}. This cut removes the whole inhibitory region and produces a single 120-kDa N-terminal fragment that is fully active without calmodulin (18). We also showed that Asp^{1080} is essential in auto-inhibition, because mutation of this residue increased the basal activity substantially (13). Here we demonstrated that digestion of PMCA4b at this site is highly conformation-dependent. In the presence of EGTA, when the E2-E1 equilibrium is shifted more toward the E2 conformation, this site is completely protected. In the presence of Ca^{2+} in the E1 conformation, this site seems more exposed, and the pump becomes partially degraded by the protease. Binding of Ca^{2+}-calmodulin to the calmodulin-binding sequence induces a larger conformational change that fully exposes Asp^{1080} to the protease so that the digestion is complete. This suggests that the carboxyl terminus of PMCA4b should be protected from caspase-3 like proteases in non-stimulated cells at low intracellular Ca^{2+} concentrations and that an increase in cytosolic Ca^{2+} followed by calmodulin binding is needed to cleave PMCA4b by caspase-3 during apoptosis.

Calpain and chymotrypsin were used to map the region downstream of the calmodulin-binding sequence of PMCA4b. Within this region, between residues Ser^{1113} and Pro^{1134}, we identified one major cut site for each protease. Digestion of the protein at these sites also depended on the apparent conformational state similar to that seen with caspase-3. In the presence of EGTA, no digestion with chymotrypsin could be seen, whereas in the presence of Ca^{2+} the cut sites were more exposed, and the pump became partially degraded by either calpain or chymotrypsin. Full digestion, however, was observed only in the presence of Ca^{2+}-calmodulin. These data suggest that, in the auto-inhibited or closed conformation, the sequences both upstream and downstream of the calmodulin-binding domain interact with the catalytic core that makes the proteolytic sites fully or partially resistant to proteolysis, depending on the equilibrium between the E1 and E2 configurations. In addition to the major cut site, digestion with calpain has revealed another site closer to the calmodulin-binding sequence. This cut site, however, is only weakly exposed in the absence of calmodulin, whereas it is completely shielded by calmodulin binding. Based on the size of this fragment we concluded that the cut site must be located near the end of the calmodulin-binding sequence.

Carboxyl-terminal sequencing of the calpain fragments derived from the erythrocyte Ca^{2+} pump (mainly PMCA4b) has revealed two additional sites within the calmodulin-binding sequence (16). In those experiments, the digestion was carried out in the absence of calmodulin on ice and for long incubation times. When we digested the erythrocyte pump in its natural environment in the membrane under the same conditions used in our experiments, the sizes of the fragments were identical to those of the fragments of the over-expressed protein in COS cells (not shown in detail). Thus, the over-expressed protein showed a similar proteolytic pattern as the pump native to erythrocytes. To determine why we did not see the additional fragments seen by others, we also digested these pumps on ice for 30 min (not shown in detail) and found that more fragments smaller than ct92 but bigger than ct120 were formed when the protease was calpain. When chymotrypsin was used, one fragment migrating faster than ct92 could be identified in addition to that seen at 37°C. These results indicated that, at low temperatures, the sites within the calmodulin-binding sequence are more exposed to proteolysis than at 37°C. It has to be emphasized that in the presence of calmodulin, these smaller fragments could not be detected even after digestion at 0°C, and only the typical fragments that were seen at 37°C were detected using either calpain or chymotrypsin. In previous experiments the calpain cut site in the presence of calmodulin was identified using synthetic peptides representing the calmodulin-binding sequence (16). In that case it is evident that calpain cut the enzyme within this sequence, because the native cut site was missing from the peptides. Our data show that the whole calmodulin-binding sequence is protected against proteolysis in the native enzyme at 37°C either in the absence or presence of calmodulin. In the absence of calmodulin this sequence must be shielded by intra-molecular interactions with the catalytic core. Binding of calmodulin induces a conformational change, allowing activation of the pump and exposing sites both upstream and downstream of the calmodulin-binding sequence, although it remains protected by the interaction with calmodulin.

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the auto-inhibitory interaction of the calmodulin-binding sequence with the catalytic core.

Comparing the crystal structure of the SERCA pump in the E1 versus E2 conformation (21, 22), it is evident that the pump undergoes large-scale movements involving both transmembrane and cytoplasmic domains. Based on the structural homology between SERCA and PMCA, we may hypothesize how binding of the inhibitory calmodulin-binding sequence to the catalytic core inhibits the activity of PMCA. Cross-linking experiments using synthetic peptides representing the sequence of the calmodulin-binding region of PMCA have identified two sites for the interaction; one is located within the nucleotide binding or N domain downstream of the phosphoenzyme forming aspartic acid (between residues 537–544), and the other is between residues 206–271 within the actuator or A domain (5, 6). These two binding sites are in regions that are homologous between SERCA and PMCA, so it is possible to draw valid conclusions by consideration of a model of PMCA based on the structure of SERCA. When such a model is constructed, it is evident that the two binding sites move relative to each other during the enzyme cycle. The binding site in the N domain is in a region whose maximum dimension is about 1.8 nm. Because the exact location of the binding site in the A domain was not well defined by the cross-linking study, the binding site could be anywhere within a region defined approximately by a sphere of 3.2 nm diameter. In the E2 state, this large sphere of possible binding locations in the A domain comes within 1.2 nm of the binding site in the N domain. In the E1 state the binding sites move apart, and the minimum distance between them is 2.5 nm. Although our present uncertainty about the location of the binding site in the A domain does not allow us to define more accurately the distances between the sites, it is clear that they move relative to one another during the enzyme cycle. Binding of the inhibitory sequence to the N domain and the A domain would interfere with the movements of these domains and slow down the reaction cycle, thus inhibiting the activity of PMCA. The possible role of the regions upstream and downstream of the calmodulin-binding sequence is to stabilize the auto-inhibited conformation. This model is also supported by previous observations that synthetic peptides similar to those used in the cross-linking experiments were able to inhibit efficiently the activity of the SERCA pump (26).

The proteolytic patterns obtained with the three proteases verify the conformational changes associated with E1/E2 conformation. We found that, in the presence of EGTA (which favors the E2 conformation), the carboxyl terminus of PMCA was completely resistant to proteolysis, whereas in the presence of Ca²⁺ the cut sites were somewhat more exposed. Full digestion was observed only in the presence of Ca²⁺-calmodulin. Our data agree very well with the idea that the A and N domains move relative to one another as the enzyme goes between the E1 and E2 conformation. In the E2 conformation, the inhibitory region is tightly bound to the catalytic core so that it forms a compact structure together with the A and N domains, preventing access of these proteases to their cut sites. In the E1 configuration, where the A and N domains are more separated, this binding could be weaker, allowing some flexibility of the C-terminal region and thus exposing more of the proteolytic sites. Removal of the contact points that anchor the C terminus to the catalytic core by site-directed mutagenesis (changing Asp¹⁰⁸⁰ or Trp¹⁰⁹⁰ to alanine or truncating the downstream inhibitory sequence) may perturb this interaction, resulting in a higher basal activity as has been observed (3, 12, 13). The effect of these mutations appeared to be very local and specific, because mutation of Asp¹⁰⁸⁰ did not affect the proteolytic sites downstream, and mutation of Trp¹⁰⁹⁰ exposed cut sites only in its immediate surroundings. Binding of Ca²⁺-calmodulin to the calmodulin-binding sequence has a more global effect on the structure; it displaces the whole inhibitory region, making the carboxyl terminus more flexible. As a result, in the presence of Ca²⁺-calmodulin the proteolytic sites are more sensitive to proteases, and the pump is fully active.

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