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Auto-inhibition of *Arabidopsis thaliana* plasma membrane Ca^{2+}-ATPase involves an interaction of the N-terminus with the small cytoplasmic loop

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Abstract Type IIB Ca^{2+}-ATPases have a terminal auto-inhibitory, domain the action of which is suppressed by calmodulin (CaM) binding. Here, we show that a peptide (6His-^{1}M-I^{116}) corresponding to the first 116 aminoacids (aa) of *At*-ACA8, the first cloned isoform of *Arabidopsis thaliana* plasma membrane Ca^{2+}-ATPase, inhibits the activity of the enzyme deprived of the N-terminus by controlled trypsin treatment 10-fold more efficiently than a peptide (^{41}I-T^{63}) corresponding only to the CaM-binding site. A peptide (^{268}E-W^{348}) corresponding to 81 aa of the small cytoplasmic loop of *At*-ACA8 binds peptide 6His-^{1}M-I^{116} immobilized on Ni–NTA agarose. Peptide ^{268}E-W^{348} stimulates Ca^{2+}-ATPase activity. Its effect is not additive with that of CaM and is suppressed by tryptic cleavage of the N-terminus. These results provide the first functional identification of a site of intramolecular interaction with the terminal auto-inhibitory domain of type IIB Ca^{2+}-ATPases.

Keywords: Ca^{2+}-ATPase; Plasma membrane; Calmodulin; Auto-inhibition; Intramolecular interaction; *Arabidopsis thaliana*

1. Introduction

Calcium extrusion from the cytoplasm to the apoplast of plant cells is catalyzed by a Ca^{2+}-ATPase that drives a H^+/Ca^{2+} exchange. Using both the energy of ATP hydrolysis and that of the electrochemical proton gradient maintained by the plasma membrane (PM) H^+-ATPase, it works far from equilibrium under physiological conditions and the rate of Ca^{2+} extrusion can be precisely tuned by regulation of its catalytic activity [1–4].

The plant PM Ca^{2+}-ATPase is a P-type ATPase belonging to the type IIB family characterized by an extended cytosolic terminal domain containing a calmodulin (CaM)-binding site [5–10]. Cleavage of the cytosolic terminal domain by controlled proteolysis or by deletion at the gene level generates a constitutively active enzyme, indicating that it contains an auto-inhibitory domain [10–15]. These features are similar to those of animal PM Ca^{2+}-ATPase (PMCA), in which the CaM binding site and the auto-inhibitory domain have been shown to be largely, although not completely, overlapping [6,7, and references therein]. However, while the regulatory domain of PMCA is localized in the extended cytosolic C-terminal of the protein [6,7, and references therein], the regulatory domain of plant PM Ca^{2+}-ATPase is localized in the extended N-terminal domain [8–10], like in type IIB Ca^{2+}-ATPases of plant cells endomembrane [9,10, and references therein].

Different pieces of evidence indicate that the auto-inhibitory domain of PMCA extends beyond the CaM-binding site [6,7,16,17]. Synthetic peptides comprising the CaM-binding domain of an ER (*Arabidopsis thaliana* isoform *At*-ACA2) and a tonoplast (*Brassica oleracea* isoform BCA1) isoform of plant type IIB Ca^{2+}-ATPases inhibit the activity of N-truncated enzyme [18,19], indicating that also in plants the auto-inhibitory and CaM-binding domains are at least partly overlapping. However, single point mutations of *At*-ACA2 downstream the peptide sequence generate a deregulated enzyme [20], suggesting that the auto-inhibitory domain may extend beyond the CaM-binding site.

As to the mechanism of auto-inhibition, it remains to be elucidated for both the animal and the plant enzymes. Cross-linking of PMCA with a peptide corresponding to the extended CaM-binding site allowed the identification of two putative sites of intramolecular interaction for the auto-inhibitory domain within the cytoplasmic head containing the catalytic domain: one is localized in the small cytoplasmic loop connecting transmembrane (TM) domains 2 and 3 and the other in the big cytoplasmic loop connecting TM4 and TM5 [21,22]. In *At*-ACA2, mutation of single residues in the stalk connecting the cytoplasmic head to the TM domain generated constitutively active enzymes [20]. These results overall indicate that in the absence of CaM the regulatory domain of type IIB Ca^{2+}-ATPases interacts with one or more sites in the cytoplasmic head hampering the enzyme's catalytic activity.

In this work, we have investigated the mechanism of auto-inhibition of *A. thaliana* PM Ca^{2+}-ATPase and we show that (i) the N-terminus contains an auto-inhibitory domain which extends beyond the CaM-binding site and (ii) a sequence in the small cytoplasmic loop connecting TM2 and TM3 interacts with the N-terminus, hampering its auto-inhibitory action.

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Abbreviations: aa, aminoacids; CaM, calmodulin; PM, plasma membrane; TM, transmembrane domain

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2. Materials and methods

2.1. Plant material and isolation of PM vesicles

PM was purified from cell suspension cultures of *A. thaliana* (L.) ecotype Landsberg by aqueous two-phase partitioning as reported in [8]. When specified, PM (1 mg protein ml^{-1}) was treated with trypsin protease as reported in [12].

2.2. Peptide synthesis

Peptides ^{41}I-T^{63} and ^{541}S-G^{557} from *At*-ACA8 (Accession No. AJ249352) were synthesized by Primm (Milano, Italy). Identity and purity of the peptides were tested by HPLC and mass spectrometry.

2.3. Constructs and purification of peptides

Standard PCRs were used to amplify the first 116 aminoacids (aa) at the N-terminus of *At*-ACA8 using *At*-ACA8 full-length cDNA as template and the following specific oligonucleotides:

-(S) 5′GATCATATGACGAGTCTCTTGAAGTC3′

-(AS) 5′GATGGATCCTCAAATTCCAAAATCACCAGC3′

The 5′ end of the S primer contains an *Nde*I restriction site and that of the AS primer a *Bam*HI restriction site. This oligonucleotide pair was used to product His-tagged *At*-ACA8 N-terminus (6His-^{1}M-I^{116}).

The sequence ^{268}E-W^{348} of *At*-ACA8 was amplified by standard PCRs using *At*-ACA8 full-length cDNA as template and the following specific oligonucleotides:

-(S) 5′GGAGGATCCGAGATTTCGATCTATGAC3′

-(AS) 5′TCCCCCGGGTCACCATTCAGTGTTGACTCC3′

The 5′ end of the S primer contains a *Bam*HI restriction site and that of the AS primer a *Sma*I restriction site. This oligonucleotide pair was used to product ^{268}E-W^{348} of *At*-ACA8 fused to glutathione *S*-transferase (GST-^{268}E-W^{348}).

PCR was performed in a Robocycler Gradient 40 (Stratagene) using Advantage cDNA Polymerase mix (Clontech) for 25 cycles as follows: 94 °C for 90 sec, 58 °C (6His-^{1}M-I^{116}) or 65 °C (GST-^{268}E-W^{348}) for 1 min and 72 °C for 1 min. The PCR products were first cloned into the pCR2.1 vector to ensure the absence of mistakes by sequencing (Primm Milano, Italy) and then moved into a pET-15b vector (insertion at the *Nde*I–*Bam*HI sites) or into a pGEX-2TK vector (insertion at the *Bam*HI–*Sma*I sites) for construction of 6His-^{1}M-I^{116} or GST-^{268}E-W^{348}, respectively. The correct insertion in pET-15b and pGEX-2TK vectors was ensured by sequencing. Peptides were overexpressed in *Escherichia coli* strain BL21 (DE3)p LysS. Cells were grown at 37 °C until an $OD_{600}$of 0.6 was reached, then isopropyl β-D-thiogalactopyranoside was added (1 mM final concentration) and the culture grown for 2 h. 6His-^{1}M-I^{116} and GST-^{268}E-W^{348} were purified by affinity chromatography. respectively. on Ni–NTA agarose gel (QIAGEN Code No. 30210) and Glutathione–Sepharose 4B gel (Amersham Biosciences Code No. 17–0756–01). The purification procedure was performed under native conditions as described in the manufacturer's instructions except for the addition of 0.5% (v/v) Triton X-100 during cell lysis. ^{268}E-W^{348} peptide released from GST was obtained by cleavage of GST-^{268}E-W^{348} linked to the Glutathione–Sepharose 4B gel with thrombin protease: after overnight incubation at 4 °C with ca. 10 units thrombin per mg of fusion protein, the protease was inactivated by addition of heparin (Sigma Code No. H4784) and antithrombin III (from bovine plasma, Sigma Code No. A9141) in the ratio of 1.5 units of each per unit of thrombin. ^{268}E-W^{348} peptide was collected in the flow through of the column. Protein concentration in fractions from peptides purification procedures was determined using the Bradford method (Bio-Rad protein assay dye reagent, Code No. 500-0006) and γ-globulin as the standard.

2.4. Assays of Ca^{2+}-ATPase activity

Ca^{2+}-ATPase hydrolytic activity in native and trypsin-treated PM (0.1 μg of protein per μl) was measured as Ca^{2+}-dependent MgITP hydrolysis as described in [8]. Unless otherwise specified, free Ca^{2+} concentration was buffered at 10 μM with 1 mM EGTA. Bovine brain CaM (Sigma Code No. P2277) was supplied at 1 μM. To test the effect of different peptides, PM proteins were pre-incubated with the specified peptide in assay buffer for 20 min at 4 °C before starting the reaction by addition of MgITP. Samples were incubated at 25 °C for 60 min. Ca^{2+}-dependent ATPase activity was determined as the difference between the activities measured in the presence and in the absence of Ca^{2+}. All the assays were performed at least three times with three replicates; S.E. did not exceed 5%.

2.5. Electrophoresis and immunoblotting analysis

SDS–PAGE, Western blotting and immunodecoration with polyclonal antibodies against the portion ^{17}V-T^{31} of *At*-ACA8 were performed as described in [8].

Polyclonal antibodies against the sequence ^{268}E-W^{348} of *At*-ACA8 were obtained by rabbit inoculation with GST-^{268}E-W^{348} fusion protein (see below) and purified by affinity chromatography on Glutathione–Sepharose 4B gel. The blot was incubated 2 h at 25 °C with anti-^{268}E-W^{348} diluted 1:3000 in 3% (w/v) BSA, 0.1% (v/v) polyoxyethylene(20)sorbitan monolaurate, 0.15 M NaCl and 20 mM Tris–HCl, pH 7.4. Signal detection was performed with an ECL anti-rabbit IgG antibody linked to horseradish peroxidase (Amersham Biosciences Code No. NA934) diluted 1:5000 in the same solution reported above.

2.6. Binding of ^{268}E-W^{348} to gel-bound At-ACA8 N-terminus

Peptide 6His-^{1}M-I^{116} (ca. 10 nmol) was immobilized onto 350 μl of Ni–NTA agarose gel as described above and incubated for 90 min at 4 °C under gentle rotation with 3.5 nmol of ^{268}E-W^{348} in 300 μl of 40 mM BTP-HEPES, pH 7, 50 mM KCl, 3 mM $MgSO_4$, 0.1 mM ammonium molybdate, 5 mM $(NH_4)_2SO_4$ and 1 μM free Ca^{2+} (buffered with 1 mM EGTA). After extensive washing with the same solution described above without peptide ^{268}E-W^{348}, the gel was loaded in a small Polystyrene column and gel elution was performed with 1.5 ml of 300 mM imidazole in 10 mM Tris, pH 8, and 300 mM NaCl. Aliquots of 120 μl were collected and subjected to SDS–PAGE.

3. Results

3.1. Inhibitory effect of the N-terminus of At-ACA8 on the activity of the truncated PM Ca^{2+}-ATPase

We have previously shown [8, Luoni et al., unpublished results] that the CaM-binding domain of *At*-ACA8, the first PM Ca^{2+}-ATPase cloned from *A. thaliana* [8], is comprised within the sequence ^{41}I-T^{63}. To check whether the auto-inhibitory domain is localized within the same sequence, we tested the ability of a synthetic peptide corresponding to *At*-ACA8 sequence ^{41}I-T^{63} to inhibit the activity of the truncated enzyme, in comparison with that of the whole *At*-ACA8 N-terminus (peptide 6His-^{1}M-I^{116}). Truncated *At*-ACA8 was obtained by controlled proteolysis of PM isolated from *A. thaliana* cultured cells: all the sequences obtained by sequencing Ca^{2+}-ATPase purified by CaM-affinity chromatography from the PM of suspension-cultured *A. thaliana* cells uniquely matched *At*-ACA8 sequence, indicating that *At*-ACA8 is the most abundant isoform of PM Ca^{2+}-ATPase in PM isolated from this material [8]. Fig. 1 (top panel) shows that under the applied conditions trypsin treatment of the PM generated a 106 kDa protein, which was recognized by an antiserum against the small cytoplasmic loop of *At*-ACA8, but not by an antiserum generated against a sequence within *At*-ACA8 N-terminus. Thus, trypsin effectively cleaves about 17 kDa from the cytosolic N-terminus of the protein. Ca^{2+}-ATPase activity in the trypsin-treated PM fraction was about 3-fold higher than that measured in control PM in the absence of CaM and insensitive to CaM (Fig. 1, bottom panel).

Since it has been shown that the inhibitory effect of peptides corresponding to parts of the regulatory C-terminus of PMCA is strongly Ca^{2+}-sensitive [23], the effect of peptides ^{41}I-T^{63} and 6His-^{1}M-I^{116} on the activity of the truncated PM Ca^{2+}-ATPase was assayed both at 1 and at 10 μM free Ca^{2+} (Fig. 2). Both peptides inhibited the activity of the enzyme much more at 1 than at 10 μM free Ca^{2+}, but peptide ^{41}I-T^{63} was about ten times less efficient than peptide 6His-^{1}M-I^{116}: at 1 μM free

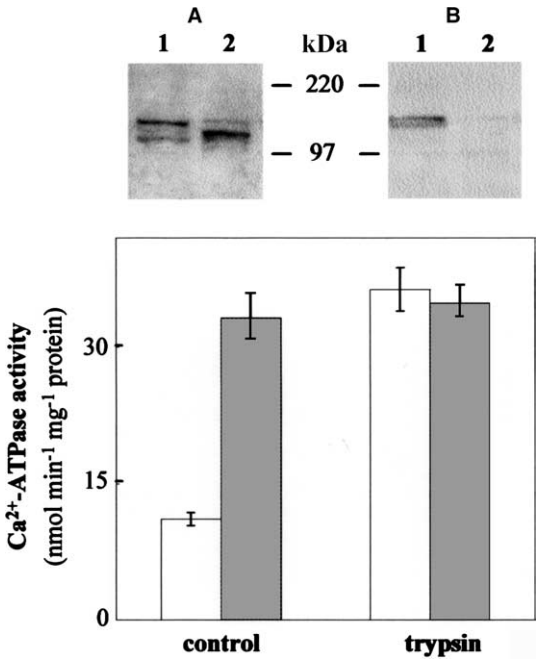

Fig. 1. Effect of controlled proteolysis on PM Ca^{2+}-ATPase. PM proteins treated with or without trypsin as described in Section 2 were either subjected to SDS–PAGE on 8% (w/v) polyacrylamide and blotted (top panel) or assayed for Ca^{2+}-ATPase activity (bottom panel). Blots were immunodecorated with antibodies raised against *At*-ACA8 (Accession No. AJ249352) sequences ^{268}E-W^{348} (A) or ^{17}V-T^{31} (B). All lanes were loaded with the same amount of protein (15 μg); lane 1: control PM; lane 2: trypsin treated PM. Ca^{2+}-ATPase activity was assayed in the presence (gray bars) or absence (open bars) of 1 μM bovine brain CaM. Results are from one experiment representative of at least two; S.E. of Ca^{2+}-ATPase activity is shown.

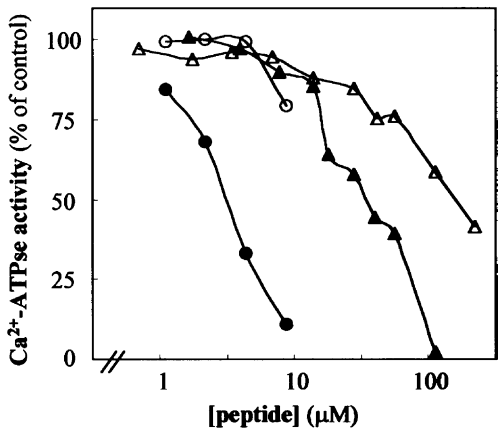

Fig. 2. Effect of peptides 6His-^{1}M-I^{116} (circles) and ^{41}I-T^{63} (triangles), reproducing the corresponding sequences of *At*-ACA8, on hydrolytic activity of N-cleaved Ca^{2+}-ATPase. Ca^{2+}-ATPase activity of trypsin-treated PM was assayed in the presence of 1 (closed symbols) or 10 (open symbols) μM free Ca^{2+} with the specified peptide concentrations. Data are expressed as percentage of activity in the absence of peptides, which was 10 nmol min^{-1} mg^{-1} protein at 1 μM free Ca^{2+} and 28 nmol min^{-1} mg^{-1} protein at 10 μM free Ca^{2+}. Results are from one experiment representative of four; S.E. did not exceed 5%.

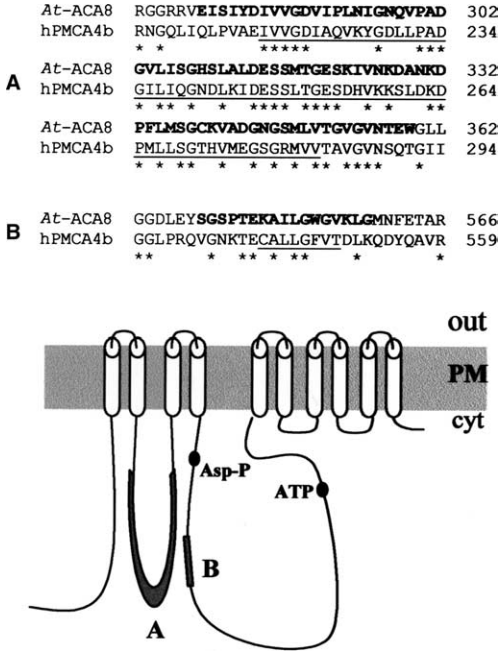

Fig. 3. Identification of putative sites of intramolecular interaction of *At*-ACA8 N-terminus. (A) and (B) represent the alignment of *At*-ACA8 and hPMCA4b regions comprising sequences of hPMCA4b identified as sites of intramolecular interaction of the C-terminal auto-inhibitory domain (underlined). Alignment was performed on the whole deduced amino acid sequence of *At*-ACA8 and hPMCA4b; asterisks mark conserved aa. Sequences of *At*-ACA8 used to design peptides ^{268}E-W^{348} and ^{541}S-G^{557} are in bold. Bottom panel shows the predicted topology of *At*-ACA8; regions corresponding to peptides ^{268}E-W^{348} (small cytoplasmic loop) and ^{541}S-G^{557} (large cytoplasmic loop) are highlighted and labeled A and B, respectively.

Ca^{2+} 50% inhibition was determined, respectively, by 3 μM 6His-^{1}M-I^{116} and 34 μM ^{41}I-T^{63}.

3.2. Identification of intramolecular interaction site of the auto-inhibitory N-terminal domain

The aa sequence of *At*-ACA8 has about 35% identity with that of different PMCA isoforms [8]. In correspondence of the sequence in the small cytoplasmic loop identified as a site of intramolecular interaction of the regulatory C-terminus of hPMCA4b [22], aa identity between *At*-ACA8 and hPMCA4b is as high as 53% (Fig. 3A). Identity is lower (37%), but similarity is high (Fig. 3B) in correspondence of the other site of intramolecular interaction of the regulatory C-terminus identified in the large cytoplasmic loop of hPMCA4b [21]. To check whether the mechanism of auto-inhibition of *At*-ACA8 is similar to that of PMCA, we designed and produced two peptides corresponding, respectively, to *At*-ACA8 sequences ^{268}E-W^{348} (in the small cytoplasmic loop) and ^{541}S-G^{557} (in the large cytoplasmic loop): peptide ^{541}S-G^{557} was synthesized, while peptide ^{268}E-W^{348}was expressed in *E. coli* as a fusion protein with GST, purified by affinity chromatography and released from GST by cleavage with thrombin protease.

The effect of the two peptides on Ca^{2+}-ATPase activity in PM purified from *A. thaliana* cells is shown in Fig. 4. Peptide ^{268}E-W^{348} stimulated Ca^{2+}-ATPase activity in a concentration dependent manner: maximal stimulation was about 50% and the effect was semi-saturated by 1 μM ^{268}E-W^{348}. On the contrary, peptide ^{541}S-G^{557} was virtually ineffective, even when supplied at 67 μM, in the presence or absence of peptide ^{268}E-W^{348}.

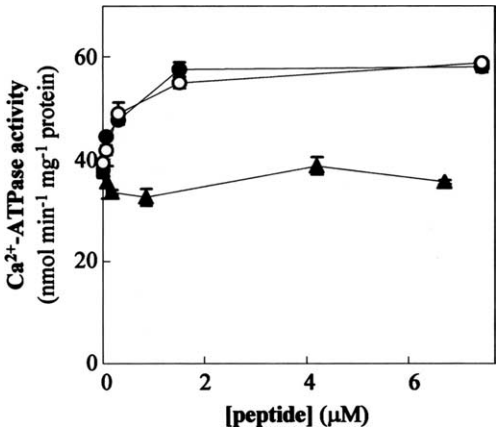

Fig. 4. Effect of peptides ^{268}E-W^{348} (circles) and ^{541}S-G^{557} (triangles), reproducing the corresponding sequences of *At*-ACA8, on PM Ca^{2+}-ATPase activity. Open circles represent the effect of increasing concentrations of peptide ^{268}E-W^{348}in the presence of 67 μM ^{541}S-G^{557}. Results (±S.E.) are from one experiment representative of three.

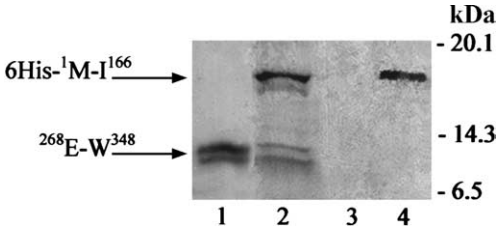

Fig. 5. Binding of peptide ^{268}E-W^{348} to peptide 6His-^{1}M-I^{116}. Peptide 6His-^{1}M-I^{116} was immobilized on Ni–NTA agarose gel (lanes 2 and 4) as described in Section 2; the gel was loaded with (lane 2) or without (lane 4) 35 nmol of peptide ^{268}E-W^{348} and after extensive washing elution was performed with 300 mM imidazole. As a control, the same amount of peptide ^{268}E-W^{348} was loaded on a void Ni–NTA agarose gel and elution performed as above (lane 3). Lanes 2, 3 and 4 were loaded with the same volume of imidazole-eluted peak fractions. Lane 1 was loaded with 0.7 nmol of ^{268}E-W^{348}. After SDS–PAGE on 18% (w/v) polyacrylamide proteins were detected by Coomassie blue staining. Results are from one experiment representative of two.

To test whether peptide ^{268}E-W^{348} bound *At*-ACA8 N-terminus, a pull-down experiment was performed by incubating a Ni–NTA agarose gel on which peptide 6His-^{1}M-I^{116} had been immobilized with peptide ^{268}E-W^{348} (Fig. 5). Upon gel elution with 300 mM imidazole, a peptide with the mass of ^{268}E-W^{348} co-eluted with 6His-^{1}M-I^{116} (Fig. 5, lane 2). No peptide of the same molecular mass was detected in mocks lacking 6His-^{1}M-I^{116} (Fig. 5, lane 3) or ^{268}E-W^{348} (Fig. 5, lane 4).

Fig. 6 shows that the effect of peptide ^{268}E-W^{348} on Ca^{2+}-ATPase activity was not additive with that of CaM, and that peptide ^{268}E-W^{348}was ineffective on enzyme activity in trypsin-treated PM.

4. Discussion

It is well known that type IIB Ca^{2+}-ATPases have an auto-inhibitory domain, the action of which is suppressed by CaM binding. However, while the site of CaM binding has been defined in some detail both in different isoforms of PMCA and in plant type IIB Ca^{2+}-ATPases localized at endomembranes

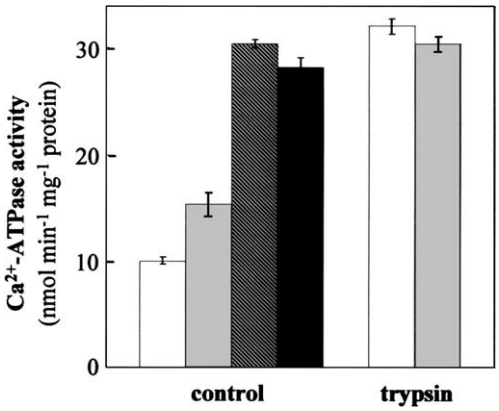

Fig. 6. Lack of effect of peptide ^{268}E-W^{348} on PM Ca^{2+}-ATPase activated by CaM or by tryptic cleavage of the N-terminus. Open bars: control; gray bars: 10 μM ^{268}E-W^{348}; dashed bars: 1 μM bovine brain CaM; closed bars: 10 μM ^{268}E-W^{348} plus 1 μM bovine brain CaM. Results (±S.E.) are from one experiment representative of three.

or at the PM (for a review see [6,7,9,10]), less is known about the mechanism of auto-inhibition.

Here, we show that peptide 6His-^{1}M-I^{116} corresponding to *At*-ACA8 N-terminus inhibits enzyme activity in PM subjected to controlled trypsin treatment, which selectively cleaves ca. 17 kDa from the N-terminal part of the protein. This result indicates that the N-terminus of *At*-ACA8, similar to the C-terminus of PMCA [21,22], interacts with one or more sites of the protein, inhibiting enzyme activity. Peptide ^{41}I-T^{63}, comprising *At*-ACA8 CaM-binding domain, inhibits as well, but with 10-fold lower efficiency. This result indicates that, like in PMCA and plant endomembrane type IIB Ca^{2+}-ATPases [6,7,16,17,20] the auto-inhibitory domain in the N-terminus of *At*-ACA8 extends beyond the CaM binding site. Further work will be necessary to precisely define the auto-inhibitory site(s) within *At*-ACA8 N-terminus.

The major output of this work is the finding that peptide ^{268}E-W^{348} corresponding to a sequence of the small cytoplasmic loop of *At*-ACA8 binds to peptide 6His-^{1}M-I^{116} and is able to stimulate PM Ca^{2+}-ATPase activity. The latter effect is not additive with that of CaM and is abolished by controlled tryptic cleavage of *At*-ACA8, indicating that it results from ^{268}E-W^{348} interaction with the N-terminus of the enzyme. Peptide ^{268}E-W^{348} corresponds to one of the sequences identified in PMCA as a site of intramolecular interaction of the regulatory C-terminus of the protein [22]. This result suggests that despite the different localization of the terminal regulatory domain, plant and animal type IIB Ca^{2+}-ATPases share a similar mechanism of auto-inhibition. In the light of our results, the simplest interpretation of the effect of single point mutations in the stalk connecting the small cytoplasmic loop to the transmembrane helices of *At*-ACA2 [20] is that they determine a long distance conformational change that disrupts the docking of the auto-inhibitory domain with the cytoplasmic head.

The small cytoplasmic loop is part of the actuator domain of P-type ATPases. Crystallographic analysis of sarcoplasmic reticulum Ca^{2+}-ATPase has shown that the actuator domain moves substantially during the catalytic cycle so that in the E2 conformation the highly conserved sequence TGES, comprised in the ^{268}E-W^{348} peptide used in this study, meets the highly conserved TGD motif in the phosphorylation domain [24].

Binding of the regulatory N-terminal domain hampers movement of the actuator domain, thus inhibiting enzyme activity. Binding of CaM or of peptide ^{268}E-W^{348} to the N-terminus would abolish its intramolecular interaction leading to enzyme activation.

Peptide ^{268}E-W^{348} does not stimulate PM Ca^{2+}-ATPase activity as much as CaM, suggesting that binding of ^{268}E-W^{348} does not suppress the auto-inhibitory action of the N-terminus as efficiently as CaM. Cross-linking of PMCA with a peptide corresponding to the extended CaM-binding site led to identification of a second site of intramolecular interaction of the regulatory domain within the nucleotide-binding domain in the large cytoplasmic loop [21]. Site-directed mutagenesis of the regulatory C-terminus of plant PM H^+-ATPase led to the identification of two distinct auto-inhibitory domains, which differently affect enzyme activity [25, and references therein]. It is thus possible that also in *At*-ACA8 the auto-inhibitory action of the N-terminus involves binding of two auto-inhibitory domains to two sites in the cytoplasmic head and that binding of peptide ^{268}E-W^{348} does not suppress N-terminus interaction with the second site.

The synthetic peptide ^{541}S-G^{557} reproducing *At*-ACA8 sequence corresponding to the site of intramolecular interaction of the regulatory C-terminus of PMCA in the large cytoplasmic loop [21] did not affect PM Ca^{2+}-ATPase activity. Further work is necessary to ascertain whether this result depends on the small size of the used peptide, or on a different localization of a second site of intramolecular interaction of the regulatory domain in *At*-ACA8. Indeed, several single point mutations within the phosphorylation domain of plant and yeast PM H^+-ATPases generated constitutively active enzymes, pointing to the phosphorylation domain as a possible site of intramolecular interaction of the regulatory C-terminus [25, and references therein].

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