Single Point Mutations in the Small Cytoplasmic Loop of ACA8, a Plasma Membrane Ca\(^{2+}\)-ATPase of Arabidopsis thaliana, Generate Partially Deregulated Pumps*\(^{[S]}\)

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A CA8 is a type 2B Ca\(^{2+}\)-ATPase having a regulatory N terminus whose auto-inhibitory action can be suppressed by binding of calmodulin (CaM) or of acidic phospholipids. ACA8 N terminus is able to interact with a region of the small cytoplasmic loop connecting transmembrane domains 2 and 3. To determine the role of this interaction in auto-inhibition we analyzed single point mutants produced by mutagenesis of ACA8 Glu\(^{252}\) to Asn\(^{345}\) sequence. Mutation to Ala of any of six tested acidic residues (Glu\(^{252}\), Asp\(^{273}\), Asp\(^{291}\), Asp\(^{303}\), Glu\(^{302}\), or Asp\(^{332}\)) renders an enzyme that is less dependent on CaM for activity. These results highlight the relevance in ACA8 auto-inhibition of a negative charge of the surface area of the small cytoplasmic loop. The most deregulated of these mutants is D291A ACA8, which is less activated by controlled proteolysis or by acidic phospholipids; the D291A mutant has an apparent affinity for CaM higher than wild-type ACA8. Moreover, its phenotype is stronger than that of D291N ACA8, suggesting a more direct involvement of this residue in the mechanism of auto-inhibition. Among the other produced mutants (I284A, N286A, P289A, P322A, V344A, and N345A), only P322A ACA8 is less dependent on CaM for activity than the wild type. The results reported in this study provide the first evidence that the small cytoplasmic loop of a type 2B Ca\(^{2+}\)-ATPase plays a role in the attainment of the auto-inhibited state.

\(\text{Ca}^{2+}\text{-ATPases responsible for Ca}^{2+}\text{ extrusion from the cytoplasm through intracellular membranes or through the plasma membrane (PM)}\)\(^{2}\) of plant and animal cells are members of the P-type ATPases superfamily (1–6). Among these, type 2B Ca\(^{2+}\)-ATPases are characterized by an extended cytosolic regulatory domain whose auto-inhibitory action can be suppressed by binding of calmodulin (CaM) (1–5) or of acidic phospholipids (2, 5, 7, 8). Localization of this terminal regulatory domain is the main structural difference between plant and animal members of the type 2B group of Ca\(^{2+}\)-ATPases. In fact, while in the animal isoforms (PMCA) this domain is localized in the extended C terminus of the protein, in plant isoforms, which have a very short cytosolic C terminus, the auto-inhibitory, CaM- and phospholipid-binding domain is localized in the extended cytosolic N terminus preceding the first transmembrane domain (2–5, 7, 8). Despite this structural difference, animal and plant type 2B Ca\(^{2+}\)-ATPases share a number of catalytic and regulatory features (2–5).

Fine regulation of type 2B group of Ca\(^{2+}\)-ATPases activity plays an essential role in cell physiology: both the extent and the rate of activation contribute to determine the size and shape of cytoplasmic Ca\(^{2+}\) waves induced by different stimuli and thus realize cellular response (6, 9, 10).

Although the terminal regulatory domain of both animal and plant members of type 2B Ca\(^{2+}\)-ATPases have been described in detail (2–5, 11–14), much less is known about how its auto-inhibitory action is exerted. Cross-linking of PMCA with a peptide corresponding to the extended CaM-binding site allowed the identification of two putative sites of intramolecular interaction within the cytoplasmic head containing the catalytic domain: one is localized in the big cytoplasmic loop connecting transmembrane domains (TMs) 4 and 5 and the other in the small cytoplasmic loop connecting TM2 and TM3 (15, 16). The latter region, which is part of the actuator domain, is highly conserved between members of the type 2B Ca\(^{2+}\)-ATPase group, and a peptide reproducing this region of ACA8, a PM-localized isoform of Arabidopsis thaliana type 2B Ca\(^{2+}\)-ATPase (17), has been shown to interact with ACA8 N terminus in pulldown experiments (12).

Regulation by an auto-inhibitory terminal domain is a feature shared by other members of the P-type ATPases superfamily, such as for example the PM H\(^{+}\)-ATPase of plants, which has an extended C terminus containing an auto-inhibitory domain whose action can be suppressed by binding of 14-3-3 proteins or by lysophosphatidylcholine (for a review see Ref. 18). Several single point mutations resulting in pump activation have been identified in Np-PMA2, an isoform of Nicotiana plumbaginifoila H\(^{+}\)-ATPase: many of the residues identified in this way cluster in the C-terminal regulatory domain, but, in addition, several residues have been identified in other regions of the pump molecule, including the small cytoplasmic loop (19, 20).

To investigate the role of the small cytoplasmic loop connecting TM2 and TM3 in ACA8 auto-inhibition we have
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started a site-directed mutagenesis project. Because alanine-scanning mutagenesis of the N-terminal auto-inhibitory domain of plant isoforms of type 2B Ca\(^{2+}\)-ATPases suggests that a positively charged surface area of this domain could be an important feature of the auto-inhibitory interaction (11, 13), we started by mutating acidic residues within the small cytoplasmic loop. The reported results show that in all the tested positions substitution of Asp or Glu residues with Ala generates a partially deregulated Ca\(^{2+}\)-ATPase. Moreover, we also found that substitution of residue Pro\(^{322}\) with Ala generates a partially deregulated enzyme.

**EXPERIMENTAL PROCEDURES**

*Plasmid Constructs—*Site-directed mutagenesis of ACA8 was conducted according to the manufacturer’s protocol (QuikChange site-directed mutagenesis, Stratagene, catalog no. 200518) using primers listed in *supplemental Table 1*. Introduction of the correct mutations and absence of errors were confirmed by sequencing. The DNA coding for WT and mutant ACA8 was inserted between KpnI and XhoI in the pYES2 vector (Invitrogen), under the control of a galactose-inducible promoter. The resulting plasmids were used for yeast transformation.

*Yeast Transformation, Complementation, and Growth Media—*Saccharomyces cerevisiae strain K616 (MAT\(\alpha\) pmr1::HIS3 pmc1::TRP1 cnb1::LEU2, ade2, ura3) was transformed with the empty pYES2 vector (Invitrogen), containing 2% (w/v) galactose, 1% (w/v) raffinose, 50 mM succinic acid/tris (pH 5.5), 0.7% (w/v) yeast nitrogen base, and 10 mM CaCl\(_2\), for 24 h at 30 °C. After growth medium separation by centrifugation, microsomes were harvested as described (12). Western blotting, and immunodecoration with polyclonal anti-ACA8 antibodies against ACA8 sequences Glu\(^{268}\)-Trp\(^{348}\) and Val\(^{17}\)-Thr\(^{31}\) were performed as described (12). Signal quantification was performed using the Fluor-Chem™SP Imaging System and AlphaEaseFC software by Alpha Innotech.

*ACA8 Purification by CaM-affinity Chromatography—*Yeast microsomes expressing WT or D291A ACA8 mutant were incubated with n-dodecyl \(\beta\)-d-maltoside (4 mg of detergent ml\(^{-1}\); 4 mg of protein ml\(^{-1}\)) at 25 °C for 30 min in a solubilization medium reported in Ref. 17 with the addition of 5 μg ml\(^{-1}\) pepstatin and 5 μg ml\(^{-1}\) chymostatin. The supernatant was recovered after centrifugation at 110,000 \(\times\) g for 1 h.

The solubilized proteins (~150 μl) were incubated overnight under gentle rotation at 4 °C on 0.3 ml of CaM-agarose gel (Sigma catalog no. P4385), equilibrated with solubilization medium added with 37.5 μg ml\(^{-1}\) Brij 58. After removal of the unbound fraction the solid phase was washed with 0.6 ml of washing medium containing 10% (v/v) glycerol, 20 mM Mops-KOH, pH 7.0, 1 mM \(p\)-aminobenzamidine, 2 mM dithiothreitol, 0.25 mM NaBr, 37.5 μg ml\(^{-1}\) Brij 58, 1 mM ITP, 100 μM CaCl\(_2\), 100 μM MgSO\(_4\); a second wash was performed in the same medium but in the absence of CaCl\(_2\) and MgSO\(_4\). The third wash was carried out with 0.15 ml of a solution containing 10% (v/v) glycerol, 1 mM Mops-KOH, pH 7.0, 37.5 μg ml\(^{-1}\) Brij 58, 0.1 mM EGTA. CaM-bound proteins were eluted in the same solution of the last wash except for the presence of 5 mM EDTA instead of EGTA. The eluted fraction was added with stoichiometric CaCl\(_2\) to neutralize EDTA, and immediately used for assay of Ca\(^{2+}\)-ATPase activity. An aliquot (~1 μl) of the eluate was solubilized (17) and loaded onto a precast Tris-glycylate gel (4–20% linear gradient, Anamed, catalog no. TGF42010); after electrophoresis the gel was stained with a silver impregnation method (Sigma, cat PROTSIL-1KT).

*Trypsin Treatment—*Endoplasmic reticulum-enriched fraction (1 mg of protein ml\(^{-1}\)) was incubated for 10 min at 25 °C in 0.1 mM EDTA, 0.5 mM ITP, 40 mM BTP Hepes pH 7.0, in the presence or absence of 150 μg ml\(^{-1}\) trypsin. The reaction was stopped by addition of 100-fold excess of soybean trypsin inhibitor.

*Assays of ACA8 Activity—*ACA8 activity was measured either as Ca\(^{2+}\)-dependent MgITP or MgATP hydrolysis as previously described (23) or as eosin-sensitive MgATP or MgITP hydrolysis, taking advantage of the high sensitivity of plant PM Ca\(^{2+}\)-ATPase to this inhibitor (22, 24). The latter method was routinely used for assays performed on microsomes to minimize artifacts due to the inhibiting effect of Ca\(^{2+}\) on yeast endogenous ATPase activities: in fact (supplemental Fig. 1), 10 μM free Ca\(^{2+}\) slightly, but significantly inhibited ATPase activity in microsomes from K616 yeast transformed with the empty vector, which was hardly affected by 0.2 μM eosin Y. The inhibiting effect of Ca\(^{2+}\) on yeast endogenous ATPase led to an underestimation of ACA8 activity measured as Ca\(^{2+}\)-dependent MgITP or MgATP hydrolysis and overestimate its stimulation by CaM, specially in the case of poor expression or of low activity mutants.

In all cases free Ca\(^{2+}\) concentration was buffered at the specified concentration with 1 mM EGTA; ITP was supplied at 1 mM, in the presence of 3 mM MgSO\(_4\), whereas ATP at 0.2 mM was supplied in the presence of 2.2 mM MgSO\(_4\); unless otherwise specified, bovine testes CaM (Sigma, catalog no. P1431) was supplied at 1 μM. Ca\(^{2+}\)-dependent ATPase/ITPase activity was evaluated as the difference between activity measured in the presence of the specified free Ca\(^{2+}\) concentration and that measured in the absence of added Ca\(^{2+}\); eosin-sensitive ATPase/ITPase activity at the specified free Ca\(^{2+}\) concentrations was evaluated as the difference between activity measured in the absence of inhibitor and that measured in the presence of 0.2 μM eosin Y. When the effect of acidic phospholipids was

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FIGURE 1. Single point mutation of acidic residues in the small cytoplasmic loop of ACA8. Top panel, alignment of ACA8 sequence Thr294—Tryp349 with PMCA1 and ACA2; the sequence of the peptide that has been shown to interact with ACA8 N terminus is underlined, and the amino acids selected for mutation are in bold. Middle panel, complementation of the K616 phenotype by WT and mutant ACA8. The Ca\(^{2+}\)-ATPase-deficient yeast strain K616 transformed with WT or mutant ACA8 was grown in synthetic complete medium lacking uracil, 2% (w/v) glucose, and 10 mM CaCl\(_2\). 5-µl drops of yeast culture (A\(_{600} = 1\)) were spotted on plated synthetic complete medium lacking uracil, 2% (w/v) galactose, 1% (w/v) raffinose, 5 mM EGTA, and incubated for 3–4 days at 30 °C. Results are from one experiment representative of four. Bottom panel, expression of WT and mutant ACA8 in yeast strain K616 as detected by immunodecoration of microsomal proteins (4 µg per lane) with an antiserum against ACA8 N terminus following SDS-PAGE and blotting. Results are from one experiment representative of three.

### TABLE 1

Relative molecular activities of ACA8 mutants

|          | ACA8 protein | ACA8 activity plus CaM | Molecular activity |
|----------|--------------|------------------------|-------------------|
|          | Arbitrary units mg\(^{-1}\) membrane protein | nmol min\(^{-1}\) mg\(^{-1}\) membrane protein | % of WT |
| WT       | 1.0          | 319 ± 11               | 100               |
| D239A    | 0.6 ± 0.1    | 81 ± 2                 | 46 ± 8            |
| E252A    | 1.2 ± 0.1    | 202 ± 3                | 54 ± 5            |
| D273A    | 0.7 ± 0.1    | 296 ± 8                | 126 ± 13          |
| D291A    | 0.6 ± 0.1    | 131 ± 2                | 74 ± 7            |
| D291N    | 0.9 ± 0.2    | 329 ± 2                | 121 ± 27          |
| D303A    | 0.9 ± 0.3    | 211 ± 1                | 76 ± 23           |
| E304A    | 1.4 ± 0.4    | 332 ± 6                | 75 ± 19           |
| D332A    | 0.5 ± 0.1    | 110 ± 1                | 69 ± 7            |

tested, assays were performed in the absence of Brij 58. Samples (3–6 µg of membrane protein or 5 µl of purified enzyme) were incubated at 25 °C for 60 min, during which the reaction proceeds linearly. All the assays were performed at least three times with three replicates.

## RESULTS

Site-directed Mutagenesis of Acidic Residues in the Small Cytoplasmic Loop of ACA8—Alignment of the segment of ACA8 small cytoplasmic loop, which has been shown to interact with the N-terminal regulatory domain of the pump (12), with the corresponding region of PMCA1 and ACA2, reveals the presence of several conserved acidic residues (Fig. 1, top panel), some of which, such as Asp\(^{278}\), Asp\(^{291}\), and Glu\(^{310}\) (numbers refer to ACA8 sequence), are part of P-type ATPase signature sequences (1). We produced single point mutants in which most of the conserved acidic residues were mutated to Ala and, for Asp\(^{278}\) and Asp\(^{291}\), also to Asn. No mutant was produced for Glu\(^{310}\), because this residue has been shown to play an essential role in the catalytic cycle of the related sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, and even its mutation to Gln generates a drastically inactivated pump (25, 26).

The DNA coding for WT or mutant ACA8 was used to transform Saccharomyces cerevisiae strain K616, which lacks endogenous Ca\(^{2+}\)-ATPases (21) and is unable to grow in Ca\(^{2+}\)-deprived media, unless it expresses a fully deregulated Ca\(^{2+}\) pump (11, 13, 21, 22). When the ability of the produced ACA8 mutants to complement K616 phenotype was tested (Fig. 1, middle panel), only mutant D239A allowed K616 growth in the Ca\(^{2+}\)-depleted medium, similar to the N-deleted mutant ΔT4 ACA8 (22). This result confirms the relevance of this conserved residue localized in the stalk of the small cytoplasmic loop in the mechanism of auto-inhibition of type 2B Ca\(^{2+}\)-ATPases (11, 27).

Protein expression was checked by Western blot of the microsomal fraction with an antiserum against the enzyme N terminus (17) (Fig. 1, bottom panel). No signal could be detected for mutants D278A and D278N suggesting that this residue is essential for ACA8 folding and stability. All of the other mutants were expressed: quantification of signals from three different blots indicated that the expression level varied ~2-fold between the least expressed mutant and the WT (Table 1).

To check whether the introduced mutations affect the enzyme catalytic activity, hydrolytic activity of WT and mutated ACA8 was measured in the presence of CaM (Table 1). Also CaM-stimulated Ca\(^{2+}\)-ATPase activity varied among different mutants, being lowest in the least expressed mutants. Molecular activity of the mutants was evaluated, as a percentage of that of WT ACA8, by the ratio between activity in the presence of CaM and signal intensity in the immunoblot (Table 1). All of the expressed mutants have a molecular activity at least half that of the WT, indicating that the mutations have no dramatic effect on the enzyme catalytic activity.

To test the degree of auto-inhibition of the mutants, we evaluated ACA8 basal activity in the absence of added CaM: for each mutant results are expressed as a percentage of activity measured in the presence of CaM. Fig. 2 shows that under the applied experimental conditions basal activity of WT ACA8 was ~20% of that measured in the presence of CaM (i.e. CaM...
stimulated the activity of WT ACA8 (~5-fold). In agreement with its behavior in the complementation test and with reported data on other isoforms of type 2B Ca\(^{2+}\)-ATPase (11, 27), mutant D239A had high basal activity, which was almost insensitive to CaM. All of the other mutants had basal activities significantly \((p < 0.01)\) higher than that of the WT, although none were completely CaM-insensitive: basal activity of the least auto-inhibited mutants, as for example D291A, was about half of that measured in the presence of CaM. These results suggest that the negative charge of acidic residues plays a role in ACA8 auto-inhibition. Interestingly, mutant D291A was significantly \((p < 0.05)\) less auto-inhibited than mutant D291N.

**Characterization of D291A ACA8 Mutant**—To characterize mutant D291A, we purified by sucrose density gradient the endoplasmic reticulum-enriched fraction, in which overexpressed ACA8 accumulates (22): D291A ACA8 distributed as the WT, ruling out the possibility of altered mutant localization (data not shown). Basal ACA8 activity was somewhat lower than in the microsomal fraction, but the difference between WT and mutant D291A persisted (Fig. 3). Fig. 3 also shows the response of WT and D291A ACA8 to controlled proteolysis with trypsin, which selectively cleaves the N terminus of plant PM Ca\(^{2+}\)-ATPase (12, 28, 29). Under the applied conditions tryptic treatment of the membranes effectively, albeit not completely, cleaved the N terminus of WT and D291A ACA8 (Fig. 3, bottom panel), increasing the activity of both enzymes nearly as much as CaM addition. As a consequence of its higher basal activity, mutant D291A was much less stimulated than WT ACA8 also by tryptic cleavage of the N terminus.

The auto-inhibitory action of ACA8 N terminus can be suppressed, besides by CaM, also by acidic phospholipids (APLs) such as phosphatidylserine (PS) or phosphatidylinositol-4P (PI-4P). As for PMCA, APLs activate ACA8 via two distinct mechanisms involving their binding to different sites (2, 5, 7, 8): APL binding to a site in the protein N terminus, overlapping the auto-inhibitory and CaM-binding domain, stimulates ACA8 activity similar to CaM or to cleavage of the N terminus, whereas binding to a second, as yet unidentified, site further stimulates ACA8 activity by lowering its \(K_{0.5}\) for free Ca\(^{2+}\). When ACA8 activity is assayed in the absence of CaM, the strong activation observed mainly reflects the effect of APL bound to the site in the N terminus, whereas activity assays performed in the presence of CaM at low free Ca\(^{2+}\) concentration highlight the effect of APL bound to the second site (7). Fig. 4 shows the effect of PS and PI-4P (inset) on the activity of WT and D291A ACA8. In the absence of CaM (top panel) increasing concentrations of PS stimulated the WT enzyme much more than the D291A mutant; data analysis indicates that WT and D291A ACA8 have similar affinity for PS (half-maximal stimulation was reached at 228 ± 17 \(\mu\)M and 205 ± 21 \(\mu\)M PS, respectively), but maximal stimulation is about twice higher for the WT than for the D291A mutant (509 ± 27% versus 255 ±...
21%). Under these conditions D291A ACA8 mutant was also less stimulated by 50 μM PI-4P (inset). Conversely, in the presence of CaM (Fig. 4, bottom panel) WT and D291A ACA8 were similarly stimulated both by PS (half-maximal stimulation at 82 ± 6 μM and 97 ± 11 μM; maximal stimulation 91 ± 5% and 83 ± 6%) and by PI-4P (inset). Thus, mutation D291A only decreases the extent of ACA8 activation due to APL binding to the enzyme N terminus.

The fact that ACA8 mutant D291A has high basal activity and is less stimulated by all treatments known to suppress the auto-inhibitory action of the N terminus, CaM binding, tryptic cleavage, and acidic phospholipids, suggests that the mutation may loosen the auto-inhibitory interaction of the N terminus with the catalytic head and thus possibly make the CaM-binding site more accessible. To test this hypothesis we analyzed the dependence of stimulation of WT and D291A ACA8 activity on CaM concentration. The results reported in Fig. 5 show that mutant D291A required lower CaM concentrations to be activated: analysis of results from three independent experiments indicates that half-maximal activation of WT and D291A ACA8 is attained at 33 ± 2 and 11 ± 2 nM CaM, respectively.

In a final set of experiments we checked the auto-inhibitory state of D291A ACA8 mutant following enzyme solubilization and purification by CaM-affinity chromatography (17, 30). The high affinity of ACA8 for CaM, together with the high expression level of ACA8 in yeast, allowed single step enzyme purification from microsomes to virtual homogeneity (Fig. 6, left panel) both for the

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**FIGURE 4.** Effect of acidic phospholipids on the activity of WT and D291A ACA8. ACA8 activity of the endoplasmic reticulum-enriched fraction purified from yeast expressing the WT (open symbols) or D291A mutant (closed symbols) protein was measured as eosyn-sensitive ITPase activity in the presence of 5 μM free Ca²⁺ and of the specified PS concentrations. Results are shown as percent stimulation over the activity measured in the absence of added phospholipids in the absence (top panel) or in the presence (bottom panel) of 1 μM CaM. The insets show the effect of 50 μM PI-4P measured under the same conditions. Results (± S.E., n = 3) are from one experiment representative of three.

**FIGURE 5.** Stimulation of WT and D291A ACA8 as a function of CaM concentration. ACA8 activity of the endoplasmic reticulum-enriched fraction purified from yeast expressing the WT (open symbols) or D291A mutant (closed symbols) protein was measured as eosyn-sensitive ITPase activity in the presence of 10 μM free Ca²⁺ at the specified CaM concentrations. Results (± S.E., n = 3) from one experiment representative of three are shown as percent stimulation over the activity measured in the absence of added CaM.

**FIGURE 6.** Basal activities of WT and D291A ACA8 purified by CaM-affinity chromatography. Left panel, protein fraction purified by CaM-agarose affinity chromatography as described under “Experimental Procedures” was subjected to SDS-PAGE and stained with silver impregnation method; numbers on the left indicate the mass of markers in kilodaltons. Right panel, ACA8 activity in the purified fraction was measured as Ca²⁺-dependent ATPase activity in the presence of 10 μM free Ca²⁺ plus or minus 1 μM CaM. Basal ACA8 activity in the absence of CaM is expressed as the percentage of that measured in the presence of CaM. Results are the mean of three experiments; bars represent ± S.E.
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WT enzyme and for the D291A mutant. Both enzymes, purified in the absence of added phospholipids, had very low basal activity, but also in this case basal activity of the D291A mutant was about twice that of the WT (Fig. 6, right panel).

**Double Mutants in the Small Cytoplasmic Loop of ACA8**—The results reported so far indicate that mutation of acidic residues within the small cytoplasmic loop of ACA8 generates partially deregulated pumps. However, with the exception of mutant D239A, which confirms the relevance of this conserved acidic residue in the stalk of the small cytoplasmic loop in auto-inhibition of type 2B Ca\(^{2+}\)-ATPases (11, 27), none of the generated mutants were fully deregulated. To further investigate the role of the small cytoplasmic loop in ACA8 auto-inhibition, a new set of mutants was generated. To identify residues, which may contribute to generate a binding site for the N terminus, we used the structure of rabbit sarcoplasmic reticulum Ca\(^{2+}\)-ATPase isoform 1a (SERCA1a) determined at 3.1-Å resolution in a Ca\(^{2+}\)-free (E2) state (31) to build an approximate homology model (32, 33) of ACA8 small cytoplasmic loop. Despite the low similarity of the two primary sequences SERCA is at present the best available structural model, which has been widely used to model the structure of other, even less related, P-type ATPases (e.g. Ref. 18). Moreover, SERCA-type ATPases of both plant and animal origin can interact with peptides reproducing the auto-inhibitory CaM-binding domain of type 2B Ca\(^{2+}\)-ATPases attaining an auto-inhibited conformation (34–36): thus it is conceivable that the auto-inhibitory CaM-binding domain of type 2B pumps interacts with regions of the pump that are structurally conserved in distantly related type 2A pumps. Residues Ile\(^{284}\), Asn\(^{286}\), Pro\(^{289}\), and Pro\(^{322}\), putatively exposed to the external environment and proximal to Asp\(^{291}\) in the model shown in Fig. 7 (top panel), were selected and mutated to Ala both in WT ACA8 and in the D291A mutant. Moreover, mutants V344A and N345A were generated, because these residues align with two residues of *N. plumbaginifolia* H\(^+\)-ATPase isofrom PMA2, which when mutated give rise to pump activation (19, 20).

All of the newly generated mutants were expressed in *S. cerevisiae* strain K616, albeit to variable extent (Fig. 7, middle panel). Most of the single mutants had molecular activities in the presence of CaM (Fig. 7, middle panel) similar to that of the WT enzyme, indicating that the mutations had not seriously compromised enzyme activity. Conversely, all of the double mutants, albeit expressed as much as or more than the corresponding single mutants, had much lower molecular activities in the presence of CaM. None of the mutants were able to complement the phenotype of *S. cerevisiae* strain K616 restoring its growth in a calcium-depleted medium (data not shown).

To test the degree of auto-inhibition of the mutants, we measured basal ACA8 activity in the microsomal fraction. The results reported in Fig. 7 (bottom panel) show that among the three experiments: standard error of the mean ranged between 6 and 29%. *Bottom panel*, basal activities of single and double ACA8 mutants in the absence of CaM. ACA8 activity was measured in the microsomal fraction of yeast expressing WT or mutant ACA8 as eosin-sensitive ATPase activity in the presence of 10 \(\mu\)M free Ca\(^{2+}\) plus or minus 1 \(\mu\)M CaM: basal ACA8 activity is expressed as the percentage of that measured in the presence of CaM. Results are the mean of 3 to 12 experiments; bars represent ±S.E.
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newly generated single mutants only mutant P322A had a basal activity higher than that of the WT (44% of that measured in the presence of CaM, versus 20% for WT, p < 0.01). The phenotype of the double mutants was roughly similar to that of mutant D291A, as expected from the lack of effect of the newly introduced mutation, with the possible exception of mutant P322A/D291A: this might have a basal activity higher than that of each of the corresponding single mutants, but its low molecular activity hampers a precise analysis of its activation state.

DISCUSSION

Different subfamilies of P-type ATPases are characterized by extended cytosolic terminal domains with regulatory role, which can exert an auto-inhibitory action by interacting with the catalytic head of the enzyme (1–5, 17). However, although the regions of these terminal domains involved in auto-inhibition as well as modifications modulating or suppressing their auto-inhibitory action have been described in detail for different members of both the type 2B Ca	extsuperscript{2+}-ATPase subfamily and the type 3A H	extsuperscript{+}-ATPase subfamily (2–5, 11–14, 17–19), little is known about how the auto-inhibitory action is exerted. Evidence has been presented that the terminal auto-inhibitory domain of both animal and plant type 2B Ca	extsuperscript{2+}-ATPases is able to interact with a region of the small cytoplasmic loop connecting TM domains 2 and 3 (12, 15, 16), but the role of such an interaction in auto-inhibition remains to be elucidated.

The analysis of single point mutants of ACA8, an isoform of A. thaliana PM Ca	extsuperscript{2+}-ATPase (17), reported herein provides the first straightforward evidence that the small cytoplasmic loop and in particular its Glu	extsuperscript{252}–Trp	extsuperscript{258} sequence plays a role in the attainment of the auto-inhibited state. Mutation to Ala of any of six tested acidic residues (Glu	extsuperscript{252}, Asp	extsuperscript{273}, Asp	extsuperscript{291}, Asp	extsuperscript{303}, Glu	extsuperscript{302}, and Asp	extsuperscript{332}) results in a partially deregulated enzyme with higher basal activity in the absence of added CaM. Although none of these single point mutants are fully deregulated, these results point out the relevance of acidic residues in this region in ACA8 auto-inhibition. Because alanine scanning mutagenesis of the N terminus of plant isoforms of type 2B Ca	extsuperscript{2+}-ATPases reveals the involvement of basic residues in the auto-inhibitory domain (11, 13), it is possible that the negative charge conferred by acidic residues to the surface area of the small cytoplasmic loop favors and/or stabilizes its auto-inhibitory interaction with the N-terminal auto-inhibitory domain.

D291A ACA8, which has the highest basal activity, has been characterized in some detail: this mutant is less stimulated by CaM. The lack of effect of the other single point mutations on ACA8 auto-inhibition might be an outcome of the poor fit of the model: because no better reference comes of the poor fit of the model: because no better reference exists at present, we will have to wait for definition of the three-dimensional structure of type 2B Ca	extsuperscript{2+}-ATPases, which promises to be a difficult task. All of the double mutants of ACA8 containing mutation D291A have the partially deregulated phenotype of the D291A mutant: unfortunately, the double mutant D291A/P322A ACA8 has a very low molecular activity, which makes it difficult to determine to what extent the effect of the two mutations sums up to generate a stronger phenotype. Moreover, preliminary results indicate that the apparent affinity for CaM of ACA8 is not affected by the P322A mutation (data not shown). This result suggests that the P322A mutation might destabilize the auto-inhibited conformation of ACA8 without directly affecting the interaction between the auto-inhibitory terminal domain and the catalytic head. It has recently been shown, both by intramolecular fluorescence resonance energy transfer and by measuring phospholipid binding, that transition of PMCA between the auto-inhibited and the fully active state involves substantial conformational rearrangements of both the cytoplasmic regions and the transmembrane segments (36, 37). The latter may explain why single point mutations of residues localized at the cytoplasmic end of TM2 domain, such as residue Asp	extsuperscript{290} in this work or the corresponding Asp residue of ACA2 or PMCA4b (11, 27), generate deregulated pumps almost insensitive to further activation by calmodulin, possibly without a full disengagement of the auto-inhibitory terminal domain from the catalytic core (27, 37).

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