RESEARCH PAPER

Phosphorylation of serine residues in the N-terminus modulates the activity of ACA8, a plasma membrane Ca\textsuperscript{2+}-ATPase of Arabidopsis thaliana

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Abstract

ACA8 is a plasma membrane-localized isoform of calmodulin (CaM)-regulated Ca\textsuperscript{2+}-ATPase of Arabidopsis thaliana. Several phosphopeptides corresponding to portions of the regulatory N-terminus of ACA8 have been identified in phospho-proteomic studies. To mimic phosphorylation of the ACA8 N-terminus, each of the serines found to be phosphorylated in those studies (Ser19, Ser22, Ser27, Ser29, Ser57, and Ser99) has been mutated to aspartate. Mutants have been expressed in Saccharomyces cerevisiae and characterized: mutants S19D and S57D—and to a lesser extent also mutants S22D and S27D—are deregulated, as shown by their low activation by CaM and by tryptic cleavage of the N-terminus. The His-tagged N-termini of wild-type and mutant ACA8 (6His-1M-I\textsuperscript{116}) were expressed in Escherichia coli, affinity-purified, and used to analyse the kinetics of CaM binding by surface plasmon resonance. All the analysed mutations affect the kinetics of interaction with CaM to some extent: in most cases, the altered kinetics result in marginal changes in affinity, with the exception of mutants S57D (\(K_D\) 10-fold higher than wild-type ACA8) and S99D (\(K_D\) about half that of wild-type ACA8). The ACA8 N-terminus is phosphorylated in vitro by two isoforms of A. thaliana calcium-dependent protein kinase (CPK1 and CPK16); phosphorylation of mutant 6His-1M-I\textsuperscript{116} peptides shows that CPK16 is able to phosphorylate the ACA8 N-terminus at Ser19 and at Ser22. The possible physiological implications of the subtle modulation of ACA8 activity by phosphorylation of its N-terminus are discussed.

Key words: Arabidopsis thaliana, Ca\textsuperscript{2+}-ATPase, calcium-dependent protein kinase, calmodulin, plasma membrane, phosphorylation.

Introduction

Cytosolic calcium is a key element in the transduction of a variety of endogenous and environmental signals in plant cells. An increasing amount of evidence indicates that signal specificity is encoded by the amplitude, frequency, and time extension of cytosolic Ca\textsuperscript{2+} waves, which in turn depend on the activity of Ca\textsuperscript{2+} channels—which when open flood the cytosol with Ca\textsuperscript{2+} from the apoplast and/or intracellular stores—and of active Ca\textsuperscript{2+} transporters—which extrude Ca\textsuperscript{2+} to the apoplast or sequester it in intracellular stores.

Fine-tuning of the Ca\textsuperscript{2+} transport systems in response to different signals is thus a crucial feature of Ca\textsuperscript{2+}-mediated signal transduction (Sanders et al., 2002; Boursiac and Harper, 2007; McAnish and Pittman, 2009; Das and Pandey, 2010; Dodd et al., 2010; Bonza and De Michelis, 2011; Pittman et al., 2011).

In plant cells, Ca\textsuperscript{2+} extrusion from the cytoplasm is accomplished either through tonoplast-localized Ca\textsuperscript{2+}–H\textsuperscript{+} antiporters powered by a proton-motive force, or through...
Ca\(^{2+}\) pumps powered by ATP hydrolysis, localized both at the plasma membrane (PM) and at intracellular membranes. PM Ca\(^{2+}\) pumps are likely to play a crucial role in re-establishing the low basal Ca\(^{2+}\) concentration especially after its increase due to opening of PM Ca\(^{2+}\) channels. Indeed, the available evidence, albeit fragmentary, demonstrates their involvement in fundamental processes such as development, hormonal regulation, and response to biotic and abiotic stresses; however, their physiological role and the mechanisms underlying their regulation in response to specific signals have not been ascertained yet (Boursiac and Harper, 2007; Bonza and De Michielis, 2011; Pittman et al., 2011).

PM Ca\(^{2+}\) pumps are calmodulin (CaM)-regulated Ca\(^{2+}\)-ATPases, belonging to the P-type ATPase superfamily: three isoforms of CaM-regulated Ca\(^{2+}\)-ATPase, all belonging to the same cluster, have been identified as PM-localized pumps in Arabidopsis thaliana: among these, the best characterized at the biochemical level is ACA8, a widely expressed isoform found in all plant organs (Bonzà and De Michielis, 2011; Pittman et al., 2011).

ACA8, like other plant isoforms of CaM-regulated Ca\(^{2+}\)-ATPases, has an extended cytosolic N-terminal domain containing an autoinhibitory domain partially overlapping the CaM-binding site: CaM binding suppresses the autoinhibitory action of the N-terminal domain and determines both the increase of \(V_{\text{max}}\) and the decrease of the \(K_{\text{0.5}}\) for free Ca\(^{2+}\) (Bonzà and De Michielis, 2011; Pittman et al., 2011).

ACA8 is also regulated by acidic phospholipids such as phosphatidylserine or phosphatidylinositol-4P, which activate the pump via two distinct mechanisms, involving their binding to different sites: acidic phospholipids binding to a site in the protein N-terminus, overlapping the autoinhibitory and CaM-binding domain, stimulates ACA8 activity similar to CaM or to cleavage of the N-terminus, while their binding to a second, as yet unidentified, site further stimulates ACA8 activity by lowering its \(K_{\text{0.5}}\) for free Ca\(^{2+}\) (Meneghelli et al., 2008).

CaM-regulated Ca\(^{2+}\)-ATPases can also be modulated by phosphorylation. In the pumps of animal cells, which have the regulatory domain localized at the extended C-terminus, the C-terminal portion is the target of phosphorylation by different protein kinases that phosphorylate different amino acids in different isoforms: each phosphorylation event has a peculiar effect on the pump activity (Enyedi et al., 1996, 1997; Penniston and Enyedi, 1998; Verma et al., 1999). In plants, it has been shown that in vitro phosphorylation of a serine residue just downstream the CaM-binding site of ACA2—an A. thaliana isoform of the endoplasmic reticulum—by a calcium-dependent protein kinase (CDPK) severely inhibits CaM-stimulated enzyme activity, without disrupting CaM binding (Hwang et al., 2000). Also the N-terminus of BCA1—an isoform of CaM-regulated Ca\(^{2+}\)-ATPase of the tonoplast of Brassica oleracea—can be phosphorylated in vitro by protein kinase C at two serine residues, one within the CaM-binding domain, but the effect of phosphorylation on pump activity was not determined (Malmström et al., 2000).

Data from large-scale phospho-proteomic studies have identified several phosphopeptides corresponding to portions of the N-terminus of ACA8. In particular (Table 1), four serine residues that are phosphorylated (S19, S22, S27, and S29) are localized ~20 amino acids upstream of the CaM-binding and autoinhibitory domain, one (S7) is within the CaM-binding site, and one (S99) is 30 amino acids downstream (Nühse et al., 2003, 2004, 2007; Benschop et al., 2007; Niittyla et al., 2007; Sugiyama et al., 2008; Whiteman et al., 2008; Jones et al., 2009; Reiland et al., 2009; Chen et al., 2010; Nakagami et al., 2010). For some of these residues, evidence has also been provided that phosphorylation is up-regulated by hormones such as abscisic acid and gibberellins (S27 and S29; Chen et al., 2010) or by elicitors such as flagellin (S27 and S99; Nühse et al., 2003, 2004, 2007; Benschop et al., 2007), or down-regulated in response to sucrose administration to cultured cells (S22; Niittyla et al., 2007). Since these serine residues are not conserved in most isoforms of A. thaliana ACA, phosphorylation of any of them could represent an isoform-specific mechanism of regulation of pump activity.

Here it is shown that substitution of any of the above-mentioned serine residues with aspartate, which mimics the effect of phosphorylation, affects the regulatory properties of ACA8, generating partially deregulated pumps (mutants S19D, S57D, and, to a lesser extent, mutants S22D and S27D), modifying the kinetics of interaction with CaM (all tested mutations), and/or changing the affinity for CaM (S57D and S99D mutants). It is also shown that the ACA8 N-terminus is phosphorylated in vitro by CDPK (Harper et al., 2004; Das and Pandey, 2010) at Ser19 and Ser22.

Table 1. Serine residues in the ACA8 N-terminus that have been found to be phosphorylated in vivo

| Phosphorylated residue | Plant material | Effectors | Reference |
|------------------------|----------------|-----------|-----------|
| Ser19                  | Cultured cells | Succrose (-) | Nühse et al. (2003, 2004) |
| Ser22                  | Cultured cells | Abascic acid (+), flagellin 22 (+) | Nühse et al. (2003, 2004); Niittyla et al. (2007); Sugiyama et al. (2008); Nakagami et al. (2010) |
| Ser27                  | Cultured cells, seedlings, shoots, leaves | (+) | Benschop et al. (2007); Sugiyama et al. (2008); Whiteman et al. (2008); Jones et al. (2009); Reiland et al. (2009); Chen et al. (2010); Nakagami et al. (2010) |
| Ser29                  | Cultured cells | Abscisic acid (+), gibberellins (+) | Nühse et al. (2003, 2004); Sugiyama et al. (2008); Chen et al. (2010); Nakagami et al. (2010) |
| Ser57                  | Cultured cells | Flagellin 22 (+) | Sugiyama et al. (2008); Nakagami et al. (2010) |
| Ser99                  | Cultured cells | (+) | Benschop et al. (2007) |
Materials and methods

Plasmid constructs

Site-directed mutagenesis of ACA8 was conducted using the Quickchange site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA, catalogue no. 200518) according to the manufacturer’s protocol using wild-type (WT) ACA8 full-length cDNA inserted in the pYES2 vector (Invitrogen, Carlsbad, CA, USA, catalogue no. V825-20v) as a template; primers are listed in Supplementary Table S1 available at JXB online. Introduction of the correct mutations and absence of errors were confirmed by sequencing.

Standard PCRs performed with GoTaq® polymerase (Promega, Madison, WI, USA, catalogue no. M3175) were used to amplify the first 116 amino acids (1T-M116) at the N-terminus of ACA8 mutants, using mutated ACA8 full-length CDNA as templates and the following specific oligonucleotides: (S) 5’ CTTGTCATAT- GACGAGTCTTGTGAGTC; and (AS) 5’ GTCTGGGAATCT- CAAATTTCAAAAATCACCAGGC. The S primer contains an NdeI restriction site and the AS primer contains a BamHI restriction site (underlined). To produce recombinant WT and mutated N-terminal domains that could later be purified by NTA affinity chromatography, the coding sequences for the N-termini of WT and mutants of ACA8, obtained using the restriction enzymes reported above, were inserted into Escherichia coli expression vector pET15b (Merck KGaA, Darmstadt, Germany, catalogue no. 69661), in this way fusing a 6His tag to the N-terminus of the peptide. Introduction of the correct mutations and absence of errors were confirmed by sequencing.

Ps 658 G-CPK1ci and Ps 652 G-CPK16-F399A plasmids encoding, respectively, calcium-independent mutants of isoforms CPK1 and CPK16 of A. thaliana CDPK, sandwiched between N-terminal glutathione S-transferase (GST) and a C-terminal 6His tag, were kindly provided by Professor J. F. Harper (University of Nevada, Reno, NV, USA).

Yeast transformation and growth media

The DNA coding for WT and mutant ACA8 proteins is inserted in the pYES2 vector (Invitrogen), under the control of a galactose-inducible promoter. Those plasmids were used for transformation of Saccharomyces cerevisiae strain K616 (MATa por1:: HIS3 pme1:: TRP1 cnb1:: LEU2, ade2, ura3; Cunningham and Fink, 1994) using a lithium acetate/polyethylene glycol method. Transformants were selected for uracil prototrophy on synthetic complete medium lacking uracil (SC-URA) nitrogen base, and 10 mM CaCl2, for 24 h at 30 °C.

Isolation of yeast microsomes

Yeast cells were homogenized and microsomes were harvested as previously reported (Bonza et al., 2004). Protein concentration was determined using the Bio-Rad assay (Bio-Rad, Hercules, CA, USA, catalogue no. 500-001).

Electrophoresis and immunoblotting analysis

SDS–PAGE, western blotting, and immunodecoration with polyclonal antibody against the ACA8 small cytoplasmic loop region were performed as described (Luoni et al., 2004); the antibody does not recognize any protein band in microsomes extracted from K616 yeast transformed with the empty vector (data not shown). Signal quantification was performed using the Fluor-Chem®SP Imaging System and AlphaEaseFC software by Alpha Innotech (MMedical, Cornaredo, MI, Italy).

Trypsin treatment

The microsomal fraction (1 mg protein ml−1) was incubated for 10 min at 25 °C in 0.1 mM EDTA, 0.5 mM ITP, 80 mM BTP (BIS TRIS propane)-HEPES pH 7.0, in the presence or absence of 150 μg ml−1 trypsin. The reaction was stopped by addition of a 100-fold excess of soybean trypsin inhibitor. Proteins were precipitated by centrifugation at 20 000 g for 1 h at 4 °C. Pellets were resuspended in 25 mM MOPS-KOH pH 7.0, 10% (w/v) glycerol, 5 μg ml−1 leupeptin, 10 mM benzamidine, 1 μg ml−1 chymostatin, 1 μg ml−1 pepstatin. Quantitative and reproducible recovery of proteins was tested using the Bio-Rad assay and western blot signal quantification.

Assays of ACA8 activity

ACA8 activity in yeast microsomes (~2.4 μg of protein per sample) was measured as eosin-sensitive MgITP hydrolysis, taking advantage of the high sensitivity of plant PM Ca2+-ATPase to this inhibitor (De Michelis et al., 1993; Bonza et al., 2004; Fusca et al., 2009). The assay medium contained 80 mM BTP-HEPES pH 7.0, 5 mM (NH4)2SO4, 50 mM KNO3, 1 μM A23187, 0.1 mg ml−1 Brij58, 1 μg ml−1 oligomycin, 2 mM phosphoenolpyruvate, 10 U ml−1 pyruvate kinase, and MgSO4 and ITP at a final concentration of 3 mM and 1 mM respectively. The free Ca2+ concentration was buffered at 10 μM with 1 mM EGTA. Unless otherwise specified, bovine tests CaM (Sigma, St. Louis, MO, USA, catalogue no. P1431) was supplied at 1 μM. Eosin-sensitive ITPase activity 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 in the assay medium. Samples 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.

Expression and purification of the WT and mutated His-tagged ACA8 N-terminus

Vectors coding for WT and mutated His-tagged ACA8 N-terminus (6His-1T-M116) were used to transform E. coli strain BL21(DE3)-pLysE (Merck KGaA, Darmstadt, Germany, catalogue no. 69389-3) by standard procedures. Purification of fusion proteins was performed as described (Luoni et al., 2004).

Surface plasmon resonance

Surface plasmon resonance spectroscopy analysis was performed with a BIAcoreX optical biosensor instrument (Biacore AB, Uppsala, Sweden) as described (Bakgaard et al., 2006; Luoni et al., 2006), but using a free Ca2+ concentration of 3.5 μM in the eluent buffer. The His-tagged ACA8 N-termini were injected into the measure flow cell of an NTA sensor chip (Biacore, AB, catalogue no. BR-1004-07) until a resonance response of 300–850 units was obtained. After changing the immobilization buffer with eluent buffer, bovine tests CaM, 50–350 nM in eluent buffer, was injected over the two flow cells. After the dissociation phase, the eluent buffer, bovine testes CaM, 50–350 nM in eluent buffer, was injected into the two flow cells. After the dissociation phase, the NTA chip was completely regenerated by injection of regeneration buffer. Results are presented as a reference cell-subtracting sensorgram, a plot of resonance signal changes as a function of time. The data were analysed using BIA evaluation 3.0 software (Biacore AB), and kinetics analyses of primary sensorgrams were carried out by global fitting using a 1:1 Langmuir binding model.

Expression and purification of CPK1 and CPK16

Vectors coding for CPK1 and CPK16 were used to transform E. coli strain DH5α by standard procedures. E. coli harbouring recombinant plasmids were grown in Luria–Bertani complete medium (GENESPIN, Milan, Italy, catalogue no. STS-LB1000) under ampicillin selection. Overnight cultures grown at 37 °C were diluted 10-fold and grown for 2 h at 28 °C (~0.6 OD600) before 0.5 mM
isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) addition, and growth was continued for 2 h more. All the following steps were carried out at 4 °C. A 400 ml aliquot of culture was centrifuged for 15 min at 3000 g and the pellet was suspended in 20 ml of lysis buffer containing 20 mM TRIS-HCl pH 7.8, 500 mM NaCl, and 1 mM phenylmethylsulphonyl fluoride (PMSF). Cells were lysed by the addition of 1 mg ml$^{-1}$ lysozyme. Incubation on ice for 15 min, and addition of 0.4% Triton X-100 followed by sonication. Cellular debris and unlysed cells were removed by centrifugation at 12 000 g for 10 min. The supernatant was incubated for 30 min with ~1 ml of nickel-NTA–agarose (Qiagen GmbH, Germany, catalogue no. 1018244) on a rocking platform. Resin was pelletted by centrifugation at 3000 g for 10 min, washed extensively with 20 mM TRIS-HCl pH 7.8 plus 500 mM NaCl, and eluted with 300 mM imidazole in 20 mM TRIS-HCl pH 6.0 plus 500 mM NaCl. The eluate was diluted 5-fold with GST binding buffer containing 50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol (DTT), and 0.4% Triton X-100, and incubated for 30 min with 2 ml of glutathione–Sepharose 4B (GE Healthcare Bio-Science AB, Sweden, catalogue no. TA3280303-2). Resin was pelletted by centrifugation at 3000 g for 10 min and washed extensively with binding buffer, followed by one wash with 50 mM TRIS-HCl pH 7.5. Protein was eluted with 10 mM glutathione in 50 mM TRIS-HCl pH 8.0 and concentrated by centrifugation in 30 000 Da cut-off VIVASPIN6 concentrators (SartoriusStedim Biotech GmbH, Germany, catalogue no. VS0621). Purified enzyme was stored at −80 °C in 50% glycerol, 20 mM TRIS-HCl pH 7.5, 100 mM NaCl, 1 mM DTT. Typically, a purification starting from 400 ml of culture yielded ~0.5–1.5 mg of pure protein, capable of phosphorylating the synthetic substrate Syntide 2 (data not shown).

**Kinase assay**

The kinase assay was performed in 20 mM TRIS-HCl pH 7.5, 6 mM MgCl$_2$, 0.5 mg ml$^{-1}$ bovine serum albumin (BSA), 1 mg ml$^{-1}$ phosphatidylcholine suspended in buffer by sonication, and 2.8 mM ATP labelled with 0.95 kBq nmol$^{-1}$ [$\gamma$-32P]ATP (Perkin-Elmer ITALIA S.p.A., catalogue no. NE302A250UC), using 2.5 μg of purified enzyme in a 25 μl reaction. Assays were initiated by the addition of 20 μM substrate and transferred from ice to 22 °C controlled temperature for 3 h. Reactions were terminated by solubilization in Laemmli buffer (Laemmli, 1970). For autoradiography, aliquots corresponding to 2–3 μg of purified substrate protein were loaded on to 20% polyacrylamide gel and subjected to electrophoresis by the addition of 20 μl of 2x SDS-PAGE sample buffer (Laemmli, 1970). Western blot of the microsomal proteins with an antiserum against a sequence in the small cytoplasmic loop of ACA8 (Luoni et al., 2004) shows that all the proteins were substantially intact (Fig. 1, top panel) and functional (Fig. 1, line a). The expression level of the mutants was evaluated by quantification of signal intensity in western blot of microsomes isolated from at least two yeast inductions (Fig. 1, line b). Expression of most mutants was similar to that of WT ACA8: only the expression level of mutants S57D and S99D was significantly different (60% and 140%, respectively, $P<0.05$) from that of WT ACA8. Molecular activities (Fig. 1, line c) were computed from the ratio between activity in the presence of CaM (Fig. 1, line a) and signal intensity in western blot (Fig. 1, line b). Molecular activities of the mutants were not significantly different from that of WT ACA8 (values ranged between 50±10% and 163±31% of the WT), indicating that the introduced mutations had no major effect on ACA8 activity.

**Effect of S/D mutations on ACA8 autoinhibition**

To test the degree of autoinhibition of ACA8 mutants, the effects of CaM on pump activity were evaluated. Figure 2 shows that under the applied experimental conditions, CaM stimulated the activity of WT ACA8 by ~300%. The response to CaM was drastically reduced ($P<0.01$) in two of the mutants, S19D and S57D, which were stimulated by ~100%; S/A mutation of the same residues only marginally affected CaM stimulation. Mutations of Ser22 and Ser27 generated proteins somewhat less stimulated by CaM (150–180%, $P<0.05$) than the WT, but in these cases the effect was independent of the substitution made. S/D mutation of residues Ser29 and S99 did not affect ACA8 response to CaM; strangely, the S29A mutant was less stimulated by CaM than the WT and the S29D mutant. This result could suggest that ACA8 was phosphorylated at Ser29 in vivo by some yeast kinase. However, mass spectrometric analysis of WT ACA8 purified from yeast microsomes by CaM affinity chromatography (Fusca et al., 2009; Bonza and Luoni, 2010) showed that the protein had not been phosphorylated in vivo under the applied yeast growth conditions, with the possible exception of Ser19, which was not identified in any tryptic peptide (data not shown). Thus, the low response to CaM of ACA8 mutants S22A, S27A, and S29A points to the relevance of these serine residues per se in determining the amplitude of the response of ACA8 to CaM.

Altogether, the results reported above suggest that the introduction of a negative charge at Ser19 or at Ser57 of ACA8—and, to a lesser extent, also at Ser22 or at Ser27—hampers the autoinhibitory action of the N-terminal domain, generating partially deregulated mutants. Alternative explanations of the low degree of CaM activation in these mutants would be a dramatic loss of affinity for CaM or the inability to shift to the active conformation upon

**Results**

Each of the serine residues of ACA8 which have been found to be phosphorylated in vivo (Nühse et al., 2003, 2004, 2007; Benschop et al., 2007; Niittylä et al., 2007; Sugiyama et al., 2008; Whitteman et al., 2008; Jones et al., 2009; Reiland et al., 2009; Chen et al., 2010; Nakagami et al., 2010) has been mutated to aspartate whose negative charge mimicks phosphorylation, or to alanine to make it non-phosphorylatable. Mutant proteins have been expressed in *S. cerevisiae* strain K616, which is devoid of endogenous Ca$^{2+}$-ATPases (Cunningham and Fink, 1994), and characterized in the isolated microsomal fraction.

Western blot of the microsomal proteins with an antiserum against a sequence in the small cytoplasmic loop of ACA8 (Luoni et al., 2004) shows that all the proteins were substantially intact (Fig. 1, top panel) and functional (Fig. 1, line a). The expression level of the mutants was evaluated by quantification of signal intensity in western blot of microsomes isolated from at least two yeast inductions (Fig. 1, line b). Expression of most mutants was similar to that of WT ACA8: only the expression level of mutants S57D and S99D was significantly different (60% and 140%, respectively, $P<0.05$) from that of WT ACA8. Molecular activities (Fig. 1, line c) were computed from the ratio between activity in the presence of CaM (Fig. 1, line a) and signal intensity in western blot (Fig. 1, line b). Molecular activities of the mutants were not significantly different from that of WT ACA8 (values ranged between 50±10% and 163±31% of the WT), indicating that the introduced mutations had no major effect on ACA8 activity.

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CaM binding. The finding that the molecular activities in the presence of CaM of the S19D, S22D, S27D, and S57D mutants are similar to that of WT ACA8 (see Fig. 1) makes the latter explanation unlikely. However, if this was the case, these mutants should be as responsive as the WT to tryptic cleavage of the N-terminus (Rasi-Caldogno et al., 1993; Luoni et al., 2004; Fusca et al., 2009; Bonza and De Michelis, 2011). Figure 3 shows that, in agreement with previously reported results (Luoni et al., 2004; Fusca et al., 2009), tryptic cleavage of the N-terminus stimulated the activity of WT ACA8 similarly to CaM and that the two effects were not additive. The same was true for all of the tested mutants, which were equally less stimulated than the WT by CaM and by tryptic cleavage of the N-terminus.

Saccharomyces cerevisiae strain K616 is unable to grow in Ca2+-deprived media (Cunningham et al., 1994; Bonza et al., 2004; Bækgaard et al., 2006; Fusca et al., 2009). None of the produced mutants was able to complement the phenotype of the K616 yeast strain (data not shown). This result confirms previous observations (Bonza et al., 2004; Bækgaard et al., 2006; Fusca et al., 2009) that only largely deregulated ACA8 mutants allow growth of K616 in the presence of very low Ca2+ concentrations.

Effect of S/D mutations on ACA8 affinity for CaM

Preliminary experiments were performed by measuring the effect of increasing concentrations of CaM on the activity of WT and mutant ACA8. Figure 4 shows the results of such an experiment conducted on the S57D ACA8 mutant. The S57D activation curve was shifted to higher CaM concentrations than that of WT ACA8, indicating a lower apparent affinity for CaM of the mutant protein. The activation curves of all the other mutants were roughly similar to that of WT ACA8 (data not shown).

It has previously been shown that mutations which weaken the autoinhibitory interaction of the N-terminus with the catalytic head of the pump can diminish steric hindrance to CaM binding, distorting evaluation of the effect of mutation on ACA8 affinity for CaM as measured by concentration dependence of activation (Fusca et al., 2009). Thus, to determine the effect of the S/D mutations on ACA8 affinity for CaM, the first 116 amino acids of WT or mutant ACA8 fused to a 6His-tag were expressed in E. coli, purified, and used for CaM binding measurements by surface plasmon resonance (Bækgaard et al., 2006; Luoni et al., 2006), a technique which allows one to measure not only the affinity of the partners but also the kinetics of interaction.

Figure 5 shows the results of a representative experiment performed on the N-terminus of WT and S57D ACA8: the S57D mutation had no major effect on the rate of complex formation, but drastically increased the rate of complex dissociation, resulting in an increase of the dissociation constant ($K_D$) from 19 nM to 165 nM (Table 2). All of the
other analysed mutations had less dramatic effects on the interaction of the ACA8 N-terminus with CaM (Table 2). The association rate constant ($k_a$) values of the mutants ranged between half (S22D) and about twice (S19D) that of the N-terminus of WT ACA8. Values of the dissociation rate constant ($k_d$) of the mutants ranged between about half (S22D and S99D) and about twice (S27D) that of the N-terminus of WT ACA8. For most mutants, the changes of the kinetic parameters brought about only minor changes of the $k_d$ values (e.g. in mutant S22D, the decrease in $k_a$ was largely compensated by the decrease in $k_d$): only in mutant S99D did the decreased rate of dissociation determine a decrease of the $K_D$ value to about half that of the WT.

**Phosphorylation of the ACA8 N-terminus by CDPK**

Evidence has been presented that CPK1, an *A. thaliana* isoform of CDPK, phosphorylates a serine residue in the N-terminus of ACA2, an isoform of *A. thaliana* CaM-regulated Ca$^{2+}$-ATPase localized at the endoplasmic reticulum (Hwang et al., 2000). It was checked whether CPK1 was also able to phosphorylate the N-terminus of ACA8; since CPK1 is localized at peroxisomes, CPK16, a PM-localized *A. thaliana* isoform, was also tested (Dammann et al., 2003). Ca$^{2+}$-independent mutants (Harper et al., 1994; Vitart et al., 2000) of CPK1 and CPK16 were expressed in *E. coli* sandwiched between N-terminal GST and a C-terminal 6His-tag and purified by two-step affinity chromatography (Harper et al., 1994). Figure 6 shows that both kinases were able to phosphorylate the His-tagged ACA8 N-terminus: upon phosphorylation with CPK16 under the applied conditions, phosphate incorporation was 0.60 ± 0.08 nmol nmol$^{-1}$ of ACA8 N-terminus.

### Table 2. Kinetics of binding of bovine testes CaM to the N-terminus of WT and mutant ACA8 measured by surface plasmon resonance

|        | $k_a$ ($\times 10^5$ M$^{-1}$s$^{-1}$) | $k_d$ ($\times 10^{-3}$ s$^{-1}$) | $K_D$ (nM) |
|--------|--------------------------------------|---------------------------------|-------------|
| WT     | 1.27±0.02                            | 2.44±0.01                       | 19.3±0.3    |
| S19D   | 2.24±0.06                            | 3.25±0.02                       | 14.5±0.4    |
| S22D   | 0.58±0.02                            | 1.39±0.01                       | 22.9±0.6    |
| S27D   | 1.96±0.03                            | 5.20±0.03                       | 26.5±0.4    |
| S29D   | 1.71±0.02                            | 3.92±0.02                       | 22.3±0.2    |
| S57D   | 1.54±0.01                            | 25.5±0.22                       | 165±1.8     |
| S99D   | 1.21±0.04                            | 1.37±0.01                       | 11.3±0.4    |

**Fig. 3.** Effect of controlled proteolysis on the activity of WT and mutant ACA8. The microsomal fraction purified from yeast expressing WT or mutant ACA8 was treated with (white and grey bars) or without (black bars) trypsin as detailed in the Materials and methods; ACA8 activity was measured in the presence (black and grey bars) or absence (white bars) of 1 mM CaM. Results are shown as percentage stimulation over the activity measured in control membranes in the absence of added CaM. Values reported are the mean of 3–5 experiments ±SEM.

**Fig. 4.** Stimulation of WT and S57D ACA8 as a function of CaM concentration. Eosin-sensitive ITPase activity of microsomal fractions (2–4 μg of total proteins) from yeast expressing WT (filled circles) and S57D (open circles) ACA8 mutant was measured in the presence of increasing concentrations of exogenous CaM. Activation of WT and S57D ACA8 ($f/f_{\text{max}}$) is expressed as the ratio between stimulation by CaM at the indicated CaM concentration ($f$) and maximal stimulation ($f_{\text{max}}$). $f_{\text{max}}$ values were 417±23% for WT ACA8 and 93±1% for the S57D mutant. Values reported are the mean of three experiments ±SEM.

**Fig. 5.** Kinetics of CaM binding to the N-terminus of WT and S57D ACA8. Phases of the interaction between CaM and peptides 6His$^{-1}$M$^{-1}$I116 derived from WT and S57D ACA8 were registered by surface plasmon resonance spectroscopy, using different concentrations of bovine testes CaM (a=350 nM; b=200 nM; c=100 nM; d=50 nM) as the flowing analyte. The reported signal (RU) is the difference in resonance units between the signal recorded in the measuring cell, with immobilized analyte, and the signal recorded in the peptide-free reference cell.
phosphorylated by CPK16, phosphorylation assays were performed on the His-tagged N-termini of ACA8 S/D mutants (Fig. 7, top panel). While mutants S29D, S57D, and S99D were phosphorylated similarly to WT ACA8, the phosphorylation level was drastically reduced in mutant S22D and lower than that of the WT also in mutants S19D and S27D. The fact that none of the mutations abolished ACA8 phosphorylation by CPK16 indicates that the enzyme is able to phosphorylate the ACA8 N-terminus at more than one residue. Since the introduction of the negative charge of the aspartate residue may affect phosphorylation of the neighbouring serine residues, phosphorylation assays were performed on the His-tagged N-termini of ACA8 S/A mutants. The bottom panel of Fig. 7 shows that phosphorylation of ACA8 by CPK16 was strongly reduced by mutations S19A and S22A, while mutant S27A was phosphorylated similarly to the WT.

**Discussion**

The finding that ACA8, a widely expressed isoform of PM Ca^{2+}-ATPase, is phosphorylated in vivo at serine residues localized within or close to the autoinhibitory and CaM-binding domain, and that phosphorylation of at least some of these residues is responsive to nutritional, hormonal, and pathogenic signals (Nühse et al., 2003, 2004, 2007; Benschop et al., 2007; Niittylä et al., 2007; Sugiyama et al., 2008; Whiteman et al., 2008; Jones et al., 2009; Reiland et al., 2009; Chen et al., 2010; Nakagami et al., 2010) opens up a new avenue for fine-tuning of its activity. To investigate the possible effect of phosphorylation on ACA8 activity, each of these residues was mutated to aspartate, which introduces a negative charge mimicking the effect of phosphorylation. The results obtained by biochemical analysis of these mutants indicate that phosphorylation of serine residues can affect ACA8 activity both by hampering the autoinhibitory action of the N-terminus and by changing the kinetics of activation by CaM and de-activation by CaM release, and, thus, at least in some instances, ACA8 affinity for CaM.

Mutation to aspartate of several phosphorylatable serine residues in the N-terminus of ACA8 generates partially deregulated proteins, with higher basal activity, less responsive to activation by CaM, and less stimulated by tryptic cleavage of the N-terminus. The effect is strongest in mutants S19D and S57D, but significant (P < 0.05) also in mutants S22D and S27D; only ACA8 mutants S29D and S99D are autoinhibited similarly to the WT protein. The ACA8 N-terminal autoinhibitory domain, which is enriched in basic residues (Bækgaard et al., 2006), interacts with a sequence in the small cytoplasmic loop rich in acidic residues (Luoni et al., 2004). Alanine scanning mutagenesis of these acidic residues generates partially deregulated ACA8 mutants, indicating that the electrostatic interaction between the positively charged N-terminal autoinhibitory domain and the negatively charged domain in the small cytoplasmic loop plays a role in ACA8 autoinhibition (Fusca et al., 2009). Thus, the negative charge introduced by the S/D mutations—or by serine phosphorylation—in the N-terminus would hamper its autoinhibitory interaction with the small cytosolic loop. However, in the case of Ser22 and Ser27, the phenotype of the S/A ACA8 mutants is similar to that of the S/D mutants, suggesting that the -OH group of serine is also important per se in the autoinhibitory mechanism.

All the analysed mutations affect the kinetics of interaction with CaM to some extent. The strongest phenotype is that of mutant S57D, which has a $k_d$ value ~10-fold higher than that of WT ACA8, but 2-fold changes in one or both of the kinetic constants of the interaction are also evident in mutants S19D, S22D, S27D, and S99D. Despite the different kinetics, the affinity for CaM of most mutants is fairly similar to that of WT ACA8 ($K_D$ values ranging between 15 nM and 26 nM for the mutants, versus 19 nM for the WT), with two exceptions: mutant S99D which has...
a $K_D$ value of 11 nM and mutant S57D which has a $K_D$ value of 165 nM, nearly 10-fold higher than that of WT ACA8, largely due to its higher $K_d$ value. The strongest phenotype of mutant S57D can be easily explained by the localization of this serine residue within the sequence defining the ACA8 CaM-binding site (Bonza et al., 2000; Bækgaard et al., 2006); interestingly, modification of ACA8 Ser57 to alanine determines a decrease in the $K_d$, resulting in a $K_D$ value about half that of the WT (Bækgaard et al., 2006). Thus residue Ser57 in ACA8 CaM-binding domain plays a crucial role in determining the stability of its interaction with CaM.

Based on these results, a major effect of phosphorylation of serine residues in the ACA8 N-terminus would be to modify the rate of pump activation following an increase of cytoplasmic Ca$^{2+}$ concentration which increases the concentration of the active Ca$^{2+}$–CaM complex or of its de-activation when the return of the cytosolic free Ca$^{2+}$ concentration toward basal levels drastically lowers the concentration of Ca$^{2+}$–CaM. The rate of activation would be halved upon phosphorylation of Ser22 and nearly doubled upon phosphorylation of Ser19 or Ser27; the rate of de-activation would be halved in the case of phosphorylation of Ser99 and would increase from 2- to 10-fold following phosphorylation of Ser27 or of Ser57 (see Table 2).

Phosphorylation of some of these serine residues affects both the kinetics of CaM activation and de-activation and the autoinhibitory action of the ACA8 N-terminus. The two effects may exert a similar or contrasting effect on the autoinhibitory action of the N-terminus. Upon phosphorylation of Ser22, the rate and extent of ACA8 activation would probably last longer, despite the slightly increased rate of CaM release, unless de-phosphorylation intervenes. Similarly, in the case of Ser57, the destabilizing effect of phosphorylation on ACA8 interaction with CaM might be largely counteracted by the inhibition of the autoinhibitory action of the N-terminus. Upon phosphorylation of Ser22, the rate and extent of ACA8 activation following an increase of cytosolic Ca$^{2+}$ would be the result of its opposite effects on the autoinhibitory action of the N-terminus and on the rate of CaM binding; conversely, the moderate decrease of autoinhibition and the decrease of the CaM dissociation rate would both contribute to keep the pump active longer.

Altogether, the subtle effects of phosphorylation of one or more serine residues in the N-terminus on ACA8 activity may have important consequences on the spatio-temporal characteristics of cytoplasmic Ca$^{2+}$ waves, and thus participate in deciphering the Ca$^{2+}$ signal. This makes identification of protein kinase(s) and phosphatase(s) controlling the phosphorylation state of ACA8 in response to different signals an important goal for future research. Here it has been shown that two isoforms of A. thaliana CDPK—CPK1 and CPK16—are able to phosphorylate the ACA8 N-terminus in vitro. The effect of single point mutations on phosphorylation indicates that CPK16, the more efficient of the two isoforms tested, phosphorylates the ACA8 N-terminus at two different serine residues: Ser19—which is part of a consensus motif recognized by CDPKs (Cheng et al., 2002)—and Ser22. Further work is needed to determine which isoform(s) of CDPK phosphorylate ACA8 in vivo and under which conditions phosphorylation occurs.

Supplementary data

Supplementary data are available at JXB online.

Table S1. Pairs of primers used for site-directed mutagenesis of ACA8.

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