Molecular Dissection of the C-Terminal Regulatory Domain of the Plant Plasma Membrane H^+-ATPase AHA2: Mapping of Residues that When Altered Give Rise to an Activated Enzyme†

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ABSTRACT: The plasma membrane H^+-ATPase is a proton pump belonging to the P-type ATPase superfamily and is important for nutrient acquisition in plants. The H^+-ATPase is controlled by an autoinhibitory C-terminal regulatory domain and is activated by 14-3-3 proteins which bind to this part of the enzyme. Alanine-scanning mutagenesis through 87 consecutive amino acid residues was used to evaluate the role of the C-terminus in autoinhibition of the plasma membrane H^+-ATPase AHA2 from *Arabidopsis thaliana*. Mutant enzymes were expressed in a strain of *Saccharomyces cerevisiae* with a defective endogenous H^+-ATPase. The enzymes were characterized by their ability to promote growth in acidic conditions and to promote H^+ extrusion from intact cells, both of which are measures of plasma membrane H^+-ATPase activity, and were also characterized with respect to kinetic properties such as affinity for H^+ and ATP. Residues that when altered lead to increased pump activity group together in two regions of the C-terminus. One region stretches from K863 to L885 and includes two residues (Q879 and R880) that are conserved between plant and fungal H^+-ATPases. The other region, incorporating S904 to L919, is situated in an extension of the C-terminus unique to plant H^+-ATPases. Alteration of residues in both regions led to increased binding of yeast 14-3-3 protein to the plasma membrane of transformed cells. Taken together, our data suggest that modification of residues in two regions of the C-terminal regulatory domain exposes a latent binding site for activatory 14-3-3 proteins.

The plasma membrane H^+-ATPase belongs to the superfamily of P-type ATPases, comprising primary ion pumps such as the Na^+/K^+-ATPases, Ca^{2+}-ATPases, and Cu^{2+}-ATPases (*1*, *2*). P-type ATPases are relatively small ion pumps with a catalytic subunit of ∼100 kDa and are characterized by having a phosphorylated (hence P-type) intermediate during the reaction cycle.

The plasma membrane H^+-ATPase is responsible for the ATP-fuelled ejection of protons from the cell and is an important component in the generation of the trans-membrane H^+ gradient in plant cells (*3*, *4*). The energy stored in this gradient is used to drive the uptake of nutrients through channels and H^+-coupled carrier proteins. The activity of the H^+-ATPase is regulated by the C-terminus of the enzyme. 14-3-3 proteins, which are soluble proteins playing important roles in eukaryotic signaling pathways, bind to this domain resulting in increased pump activity (*5*). Little is known regarding the mechanism by which physiological factors regulate the plasma membrane H^+-ATPase, but the fungal toxin fusicoccin activates H^+-ATPase activity in vivo by a mechanism involving the C-terminal regulatory domain and 14-3-3 protein (*6*−*7*).

The way in which the C-terminal regulatory domain of the plasma membrane H^+-ATPase regulates activity is not known. Other membrane-bound transport proteins such as the plasma membrane Ca^{2+}-ATPase (*8*), plant endomembrane Ca^{2+}-ATPases (*9*−*10*), the Na^+/H^+ exchanger NHE1 (*11*−*13*), and cyclic nucleotide-gated channels (*14*) are regulated by terminal autoinhibitory domains. In these transporters the autoinhibitory domains are thought to interact with a putative intramolecular acceptor in a sequence-specific manner. In the case of the plasma membrane Ca^{2+}-ATPase, the C-terminus contains two autoinhibitory domains (*15*−*17*). In the presence of Ca^{2+}, the first inhibitory domain (the C-domain) binds calmodulin with high affinity, resulting in complete neutralization of autoinhibition and increased pump activity. In the presence of diacylglycerol, activated protein kinase C phosphorylates a residue in the second inhibitory domain (the I-domain), resulting in partial activation of the Ca^{2+}-ATPase. Thus, two consecutive autoinhibitory domains allow for flexible regulation of ATPase activity.

One way of studying the effect of the C-terminal domain on pump activity is through proteolytic cleavage of the H^+-ATPase. Incubation of the pump with proteolytic enzymes such as trypsin or chymotrypsin removes the C-terminal domain and results in a pump with increased maximal velocity and an increased apparent affinity for ATP (*18*−*19*). Expressing the plant plasma membrane H^+-pump in the

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yeast *Saccharomyces cerevisiae* has made possible a genetic study of the C-terminal regulatory domain. Deletion of 38 amino acids from the C-terminal end of the *Arabidopsis* plasma membrane H^+-ATPase AHA2 is sufficient to produce a lowered K_m (ATP) and an alkaline shift in pH optimum (*20*). However, deletion of 66 amino acid residues is required to produce an optimal increase in V_{max} (*20*). These results suggest that residues involved in the regulation of V_{max} are separated in space from those regulating K_m (ATP) and pH optimum. In spinach, in vivo treatment of roots with fusicoccin affects V_{max}, K_m (ATP), and pH optimum of the plasma membrane H^+-ATPase, but similar treatment of leaf tissues affects only V_{max} (*21*). On the basis of these results, it was suggested that there might be more than one regulatory site in the C-terminus, allowing for V_{max} to be regulated independently of K_m (ATP) and pH optimum (*21*).

Previous work has pointed to the presence of several residues in the C-terminus and in other regions of the plasma membrane H^+-ATPase that when altered give rise to an enzyme with increased rates of ion pumping (*22*, *23*). In the present study we have performed a systematic analysis of the C-terminal regulatory domain of the *Arabidopsis thaliana* plasma membrane H^+-ATPase isoform AHA2. Employing alanine scanning mutagenesis, we individually changed the last 87 C-terminal residues to alanines (except alanines themselves, which were changed to serines and threonines). The activity state of each of the mutants was then analyzed. The results of the study reveal the presence of two regions of residues within the C-terminal domain that when altered increase pump activity and the association of the C-terminal with 14-3-3 proteins.

EXPERIMENTAL PROCEDURES

Construction of Mutants. The yeast multicopy vector YEp-351 (*24*) containing the full-length cDNA of the *A. thaliana* plasma membrane H^+-ATPase isoform *AHA2* under control of the *PMA1* promoter (*25*) was used. Site-directed mutagenesis was executed by overlap extension polymerase chain reactions (*26*) using standard methods (*27*). To facilitate mutagenesis of *AHA2*, we generated a number of silent mutations, resulting in the insertion of eight new restriction enzyme cleavage sites in the coding region of *AHA2* (Table 1), resulting in plasmid pMP-658. Mutations in the 3′-end of the coding region were produced by cassette mutagenesis, as follows: oligonucleotide primers containing mismatches were employed in a polymerase chain reaction and the resulting products digested with restriction enzymes and substituted with the corresponding fragment in pMP-658 (Table 2). All mutations were verified by DNA sequencing of the cassettes.

Expression in Yeast. S. cerevisiae RS-72 was transformed and cultured essentially as described previously (*20*). In RS-72 (*MATa ade1−100 his4−519 leu2−3,112*), the natural constitutive promoter of the endogenous yeast plasma membrane H^+-ATPase *PMA1* has been replaced by the galactose-dependent promoter of *GAL1*. Since *PMA1* is essential for yeast growth, this strain only grows on galactose medium. By using this strain, we can test plant H^+-ATPases brought under control of the constitutive *PMA1* promoter for their ability to rescue *pma1* mutants on glucose medium. Yeast cells were grown in 0.7% yeast nitrogen base (without amino acids; Difco) supplemented with adenine (40 μg/mL), L-histidine (30 μg/mL), and 2% galactose (SGAH) or glucose (SDAH). Before harvesting, first a saturated preculture was made from a fresh colony in 10 mL of SGAH. Thereafter, 100 mL of SGAH was inoculated with the preculture and grown for 2 days at 28 °C. Finally the cells were transferred to 1 L of medium containing 2% glucose, 1% yeast extract, and 2% bacto peptone (YPD) and grown for 20 h at 28 °C. The cells were harvested and plasma membranes isolated as described (*20*). Yeast microsomal membranes resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, and 20% [w/w] sucrose were centrifuged for 16 h at 30 000 rpm (SW 41 Ti rotor; Beckmann Instruments International S. A., Geneva, Switzerland) through a 12 mL sucrose step gradient (6 mL each of 43% and 53% [w/w] sucrose in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT), and plasma membranes were collected from the 43%/53% interface. After 2-fold dilution in 10 mM Tris-HCl, pH 7.5, 1 mM

Table 1: Mutagenic Oligonucleotides Used To Introduce New Restriction Sites in the Sequence of *AHA2* without Changing the Codon Specificity

	oligonucleotide[a]	enzyme[b]
	GGT	
838	C CTC TTG ATC GGT GGG ATC CCC ATT GCT AT	*Bam*HI
280	L L I G G I P I A	
	CTG	
965	CGA ATG GAT GTC CTC TGC AGT GAC AAA ACC	*Pst*I
322	G M D V L C S D K T	
	GTG	
1008	AAC AAA TTG AGT GTC GAC AAA AAC TTG GTC	*Sal*I
387	N K L S V D K N L V	
	AGC AGA	
1891	GT GCT GTT CTC ACC TCA CGT GCT ATT TTC	*Pml*I
631	A V L T S R A I F	
	GCC	
2004	TTT GAC TTC TCA GCA TTC ATG GTT CTG ATC	*Bsm*I
669	F D F S A F M V L I	
	AAA	
2100	CCT GAT AGC TGG AAG CTT AAA GAA ATT TTT GC	*Hin*dIII
701	P D S W K L K E I F	
	GCT GCA	
2603	GCG AGA GAG GCC CAA TGG GCC CTT GCT CAA AGG	*Sfi*I/*Apa*I
869	E R E A Q W A L A Q R	
	CTG	
2748	ACG CTT AGG GAG CTC CAC ACA CTC AAG GGA	*Sac*I
280	R L R E L H T L K G	

[a] The wild-type sequences are indicated above the oligonucleotide sequences. Nucleotides that have been subject to alteration are underlined. [b] The indicated restriction enzymes cut the modified sequence only.

Table 2: Alanine Scanning Mutagenesis of Residues M862 to V948 in AHA2

positions	resulting strains	method[a]	junction sites	cassette length (nucleotides)
Y^{862}-K^{864}	MP701-MP703	SP	*Hind*III; *Apa*I	516
D^{865}	MP704	SP	*Bsm*I; *Apa*I	611
Y^{866}-Q^{874}	MP705-MP713	SP	*Hind*III; *Apa*I	516
W^{875}-A^{876}	MP714-MP715	SP	*Hind*III; *Sac*I	652
L^{877}-A^{878}	MP716-MP717	SP	*Sfi*I; *Sac*I	141
Q^{879}-E^{889}	MP718-MP728	SP	*Apa*I; *Sac*I	136
A^{890}-A^{910}	MP692-MP693 + MP729-MP749	OE	*Apa*I; *Sac*I	136
K^{911}-R^{920}	MP750-MP759	SP	*Apa*I; *Sac*I	136
E^{921}-L^{922}	MP760-MP761	SP	*Apa*I; *Spe*I	226
H^{923}-L^{938}	MP762-MP772 + MP819-MP823	SP	*Sac*I; *Spe*I	90
D^{939}-V^{948}	MP824-MP833	SP	*Apa*I; *Spe*I	226

[a] SP: Cassette mutagenesis involving a single polymerase chain reaction. One junction site and the mutational mismatch were present in the same oligo. OE: Cassette mutagenesis involving three polymerase chain reactions. The products of the first two reactions were fused in the third reaction using the overlap extension method (*26*).

EDTA, and 20% [w/w] sucrose, the membranes were pelleted by centrifugation at 30 000 rpm (50.2 Ti rotor; Beckmann Instruments International S. A., Geneva, Switzerland) for 45 min, and then resuspended in the same buffer supplied with 1 mM phenylmethylsulfonyl fluoride and 0.1 mg/mL chymostatin.

ATPase Assay. ATPase activity was determined as described (*20*). The assay medium (300 μL) contained 20 mM 2-(*N*-morpholino)ethane-sulfonic acid, 20 mM 3-(*N*-morpholino)propanesulfonic acid, 50 mM KNO_3 (to inhibit vacuolar H^+-ATPase), 5 mM KN_3 (to inhibit mitochondrial F_oF_1-ATPase), 3.5 mM sodium molybdate (to inhibit acid phosphatase), 8 mM $MgCl_2$, and 2 mM ATP. The pH was adjusted with *N*-methyl-D-glucamine. The reaction was carried out at 30 °C and initiated by the addition of 1−3 μg of plasma membrane protein. After 30 min the reaction was stopped by the addition of 300 μL of an ice-cold solution containing 93 mM ascorbic acid, 0.273 N HCl, 0.059% SDS, and 5 mM NH_4-heptamolybdate. The tubes were incubated for 10 min on ice to allow formation of the P_i−molybdate complex. Excess molybdate was complexed by the addition of 450 μL of a solution containing 154 mM $NaAsO_2$, 68 mM trisodium citrate, and 350 mM acetic acid. After 30 min at 30 °C, a stable blue color had developed, and absorbance at 860 nm was determined spectrophotometrically.

The pH curves were fit to the following equation:

$$v = k_{\mathrm{deph},0}\mathrm{EP}_0(1/(1 + 10^{(\mathrm{p}K_1 - \mathrm{pH})}))(1/(1 + 10^{(\mathrm{pH} - \mathrm{p}K_2)}))$$

(*38*), where $k_{\mathrm{deph},0}$ is the intrinsic rate constant for the unprotonated phosphorylated intermediate, EP_0 is the amount of phosphorylated intermediate when the relevant group is fully protonated, pK_1 is the dissociation constant of the group that is deprotonated and affects dephosphorylation, and pK_2 is the dissociation constant for protonation of a certain group.

Drop Tests. Yeast was grown for 3 days at 30° C in liquid medium containing 2% galactose. Approximately 10^3 cells in 10 μL were spotted onto synthetic minimal media plates containing either 2% galactose at pH 5.5 or 2% glucose at pH values of 5.5, 4.1, 3.6, and 3.1. Growth was recorded after incubation for 3 days at 30° C.

Gel Electrophoresis and Western Blotting. Plasma membrane proteins were separated by SDS−PAGE on 10% acrylamide using the system of Fling and Gregerson (*28*). Western blotting was performed as described (*6*). After electrotransfer of the proteins to a Protran nitrocellulose transfer membrane (Schleicher & Schuell), the membrane was cut in two, and the half containing high molecular weight (>60 kDa) proteins was incubated with an antibody raised against the H^+-ATPase, while the other half (containing lower molecular weight proteins) was incubated with an antibody against a barley 14-3-3 protein.

Proton Extrusion. Glucose-induced acidification of the external medium was performed essentially as described (*22*). Yeast cells were prepared by growing yeast for 3 days in rich 2% galactose medium, transferring the cells to rich 2% glucose medium for 20 h, and washing them four times in ice-cold water. The yeast cells were kept on ice until use. For each experiment 4.5×10^9 cells in 20 mM KCl were equilibrated to room temperature for 20 min with stirring. After equilibration, glucose was added to a final concentration of 2% (w/v). The change in pH of the medium was followed with a pH electrode for 12 min.

RESULTS

Identification of Amino Acids in the C-Terminus that When Altered Resulted in Increased ATPase Activity. The yeast strain, RS-72, which has its own plasma membrane H^+-ATPase gene *PMA1* put under control of a galactose-dependent promoter, was transformed with a yeast multicopy vector carrying the *Arabidopsis* plasma membrane H^+-ATPase gene *AHA2* under control of the constitutive *PMA1* promoter. On galactose medium the transformed yeast strain expresses both PMA1 and AHA2, whereas on glucose medium growth is dependent on AHA2 only.

In the absence of endogenous PMA1, wild-type *Arabidopsis* AHA2 supported only very slow growth of yeast and only at pH values of pH 5.5 or above (Figure 1; ref *20*). The last 87 C-terminal amino acids of AHA2 were changed individually into alanines (alanines were changed into serines and threonines), and the corresponding enzymes were expressed in yeast. Transgenic yeast cells were plated on acidic medium (pH 5.5, 4.1, 3.6, and 3.1), and growth was recorded after 3 days.

Figure 1 demonstrates that in the absence of PMA1, several mutant enzymes increased the growth of *S. cerevisiae* compared to wild-type AHA2. While wild-type AHA2 supported slow growth at medium pH 5.5, it did not support growth at lower pH values. Three classes of mutant enzymes were observed. Class A mutants (64 positions out of 87) were those in which alteration of single residues did not affect the ability of the enzyme to substitute for PMA1, growth being negligible below pH 5. M862A, E872A, P887A, G898A, A907T, A914S, A917S, and S931A are shown in Figure 1. Class B mutants (K863A, K864A, D865A, Y866A, K868A, R871A, A873S, A876S, Q879A, G884A, S904A, I906A, A907S, A910S, A910T, K911A, R912A, I916A, and L919A) represent substitutions that resulted in enzymes conferring growth at pH 4.1. Finally, mutations at 5 positions (class C mutants: G867A, W875A, R880A, L885A, and R913A) resulted in enzymes that supported growth at pH 3.1. The growth rates at pH 3.1 compared well with those

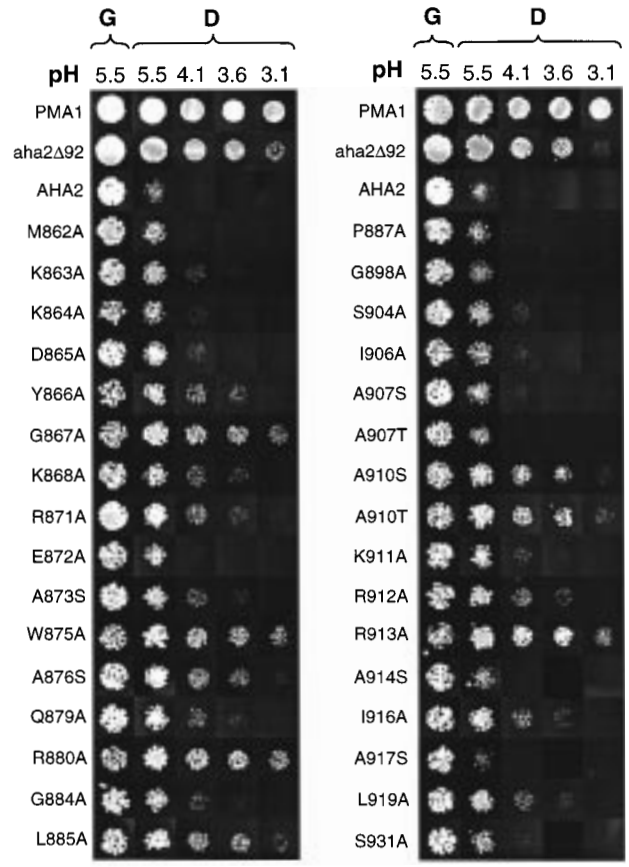

FIGURE 1: Growth of yeast strains expressing various mutated AHA2 proteins. Each strain was grown at five different conditions: galactose (G) medium at pH 5.5 and glucose (D) medium at pH 5.5, 4.1, 3.6, and 3.1. The strain marked PMA1 harbors the endogenous yeast plasma membrane H^+-ATPase on a plasmid. AHA2 is the wild-type *Arabidopsis* enzyme, and aha2Δ92 is a mutant lacking the C-terminal 92 amino acids (*37*). On the basis of the growth capabilities of the different strains, the mutations were divided into three classes (see text for details).

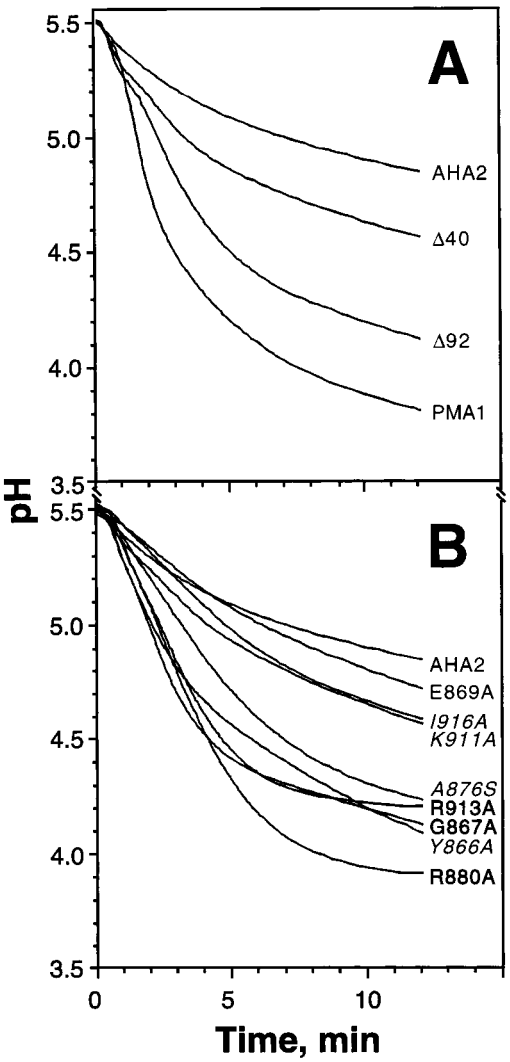

FIGURE 2: Glucose-induced acidification of the external medium by yeast cells expressing wild-type and mutated AHA2 proteins: Δ40, C-terminally truncated AHA2 lacking 40 amino acid residues; Δ92, C-terminally truncated AHA2 lacking 92 amino acid residues. In B, wild-type AHA2 and class A mutants are in normal typeface, class B mutants are in italics, and class C mutants are in bold typeface. Initial rates of H^+ extrusion are quantified in Table 3.

of yeast cells expressing an enzyme completely devoid of its C-terminus (aha2Δ92). Some mutations could not be readily included in any of the groups. Thus, the growth phenotype of A907S and A907T was intermediate between that of A and B mutants, and growth of A910S and A910T was intermediate between that of B and C mutants.

Representatives of the three classes of mutant enzymes were tested for their proton pumping capabilities in intact cells. Yeast cells expressing wild-type PMA1 extrude protons at high rates to the extracellular medium, while cells expressing only AHA2 barely extrude protons (Figure 2; ref *5*). As seen in Figure 2 and Table 3, yeast cells expressing class A mutants did not have a higher ability to extrude protons than cells expressing AHA2. Cells expressing class B mutants extruded protons at a slightly higher rate than those expressing class A mutants. In general, class C mutants extruded protons with a rate comparable to the extrusion rate seen for yeast cells expressing aha2Δ92. One mutant, Y866A, showed higher proton extrusion rates than expected on the basis of its classification as a B mutant.

The increased efficiency of proton pumping was not due to an increased amount of H^+-ATPase in the yeast plasma membrane. Although there was some variation in the amount of mutant protein in the plasma membrane, there was no correlation between expression level and the growth phenotype of transgenic yeast cells (Figure 3; Table 4).

Table 3: Initial Rates of Medium Acidification by Transformed Yeast Cells

mutation	class	ΔpH/min[a]	mutation	class	ΔpH/min[a]
AHA2		0.10 ± 0.03	R912A	B	0.16 ± 0.03
aha2Δ40		0.14 ± 0.02	Y866A	B	0.22 ± 0.02
aha2Δ92		0.29 ± 0.09	A876S	B	0.19 ± 0.03
PMA1		0.45 ± 0.07	G867A	C	0.27 ± 0.04
E869A	A	0.08 ± 0.01	W875A	C	0.25 ± 0.03
K911A	B	0.12 ± 0.00[b]	R880A	C	0.27 ± 0.05
I916A	B	0.11 ± 0.01	L885A	C	0.22 ± 0.03
K868A	B	0.17 ± 0.03	R913A	C	0.25 ± 0.03

[a] Mean of 4 experiments. [b] Error was less than 0.005.

Kinetic Properties of Mutant aha2 Enzymes. The ATP affinity of wild-type AHA2 is relatively low ($K_m \approx 2$ mM ATP), whereas aha2Δ92 has a 10-fold higher affinity for ATP ($K_m \approx 0.3$ mM) (*20*). From Figure 4A, it appears that class A mutants possess the same ATP affinity as wild-type AHA2 while class C mutants have a comparable affinity to

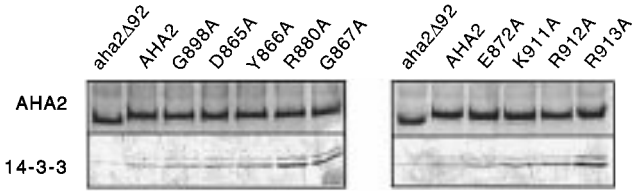

FIGURE 3: Protein gel blots showing plant AHA2 polypeptide and endogenous 14-3-3 protein in yeast plasma membranes. Plasma membranes were prepared from transgenic yeast expressing AHA2 or mutant aha2 proteins. Aliquots of plasma membrane protein were subjected to SDS−PAGE. After transfer of the protein to a Protran nitrocellulose transfer membrane (Schleicher & Schuell) nitrocellulose, the membrane was cut into halves. (Top) Upper part of the membrane that was probed with polyclonal antibody against the plant plasma membrane H+-ATPase. (Bottom) Lower half of membrane that was probed with an antiserum raised against a barley 14-3-3 protein. Immunodetectable protein is quantified in Table 4.

that of aha2Δ92. For class B mutants, the range of ATP affinities was generally intermediate between those of class A and C.

Figure 4B demonstrates that there was a positive correlation between the shift in pH optimum in the mutants and their ability to support yeast growth. In isolated class A mutants, ATP hydrolysis as a function of pH was the same as in wild-type AHA2 (optimum at pH 6.2). In class B mutants, a slight shift in the pH optimum toward neutral pH was observed. In general, the shift in pH optimum was more pronounced in class C mutants, which had a pH optimum of about 6.5.

Detection of 14-3-3 Protein Associated with the Plasma Membrane of Yeast Cells Expressing Plant H+-ATPase. *S. cerevisiae* possesses two 14-3-3 isoforms, BMH1 and BMH2, both of which are able to activate AHA2 provided their binding to the enzyme is facilitated, such as by the phytotoxin fusicoccin (*5*). It was therefore possible that alteration of residues in the C-terminus increased binding of yeast 14-3-3 protein to the H+-ATPase.

There was a clear correlation between the growth phenotype of transgenic yeast cells and the amount of 14-3-3 protein relative to the plant H+-ATPase associated with the yeast plasma membrane (Figure 3; Table 4). A significant portion of the 14-3-3 protein associated with the plasma membranes of yeast cells expressing AHA2 is attached to the C-terminus of the plant H+-ATPase (*5*). Yeast membranes harboring aha2Δ92, a mutant plant H+-ATPase devoid of its C-terminus, did bind only half the amount of 14-3-3 protein as those expressing full-length AHA2 (Figure 3; Table 4), in accordance with earlier observations (*5*). Strikingly, membranes harboring class C mutants bound three times more 14-3-3 protein than did membranes isolated from cells expressing wild-type AHA2 (Table 4).

DISCUSSION

Two Regulatory Regions Are Present in the C-terminal Domain of AHA2. In this study some insight was obtained into the mechanism of the autoinhibition of a plasma membrane H+-ATPase by its C-terminal domain. We performed alanine scanning mutagenesis throughout the C-terminal regulatory domain of the plant plasma membrane H+-ATPase AHA2 and examined the ability of the enzymes to rescue growth of a yeast mutant defective in endogenous H+-ATPase. As parameters for increased pump activity, the following were chosen: (i) increased growth of transformed yeast cells in acidic conditions; (ii) promotion of proton secretion from transformed yeast cells; and (iii) increased affinity of the isolated mutant enzymes for protons and ATP.

In this way, we identified 23 amino acid residues that when altered resulted in an increase in H+-ATPase activity. These mutants are clustered in two regions (Figure 5). Region I (amino acid residues K863 to L885) consists of amino acids conserved in plant H+-ATPases and with some homology to the putative autoinhibitory domain of the *S. cerevisiae* H+-ATPase PMA1. Region II (amino acid residues S904 to L919) is also conserved in plant H+-ATPases but is situated in an extension of the C-terminus not present in fungal H+-ATPases.

Mutations in either region I or region II resulted in a range of changes in the transport properties of the enzymes, depending on the particular altered amino acid. Three classes of mutants could be identified on the basis of their ability to complement the *pma1* mutant in *S. cerevisiae*. Transgenic yeast expressing class A mutants had the same phenotype as those expressing wild-type AHA2 (negligible growth at pH 5.5), whereas class C mutants were characterized by their ability to support growth of *pma1* mutants at pH 3.1. Class B mutants were intermediate and supported growth down to pH 4.1. There was a positive correlation between the ability of the various classes of mutant enzymes to support yeast growth and the following three parameters: (i) the rate of proton secretion from transformed yeast cells; (ii) an increased ATP affinity of the H+-ATPase; and (iii) a shift in pH optimum from slightly acidic toward neutral pH, which might reflect increased affinity for H+. These correlations support the notion (*20*) that the ability of the plant H+-ATPase to energize the plasma membrane in this system is dependent upon the proportion of enzymes in a state of high activity and high affinity for ATP and H+.

The mutation W874L in the related *Arabidopsis* H+-ATPase *AHA1* complements *pma1* and behaves as a class C mutant (*29*). This residue locates to region I. Several spontaneous mutants of tobacco *pma2* H+-ATPase that lead to complementation of yeast *pma1* have been isolated (*22*−*23*). Eighteen mutations are restricted to the C-terminus, whereas 24 point mutations are situated in other regions of the enzyme. Seventeen of the C-terminal mutants (localized in 10 codons) are situated in region I and a single mutant in region II.

Molecular Modeling of Regions I and II. According to a model (*30*) for the regulation of plasma membrane Ca^{2+}-ATPases, the C-terminus binds to a site close to the active site of the enzyme. If the affected amino acid residues in mutants belonging to classes B and C constitute the area in the C-terminal domain in contact with the rest of the pump molecule, then this interacting region is surprisingly large. Region I from K865 to L882 has a high probability of forming an α helix ($P > 0.8$; ref *31*). Outside these limits the conformation is less well predicted. The entire region II is also predicted to form an α helix with high probability ($P > 0.8$). None of the amino acid substitutions made resulted in changes in this predicted secondary structure. This might be an indication of a specific role for the activating amino acid residues, although changes in secondary structure that could not be predicted cannot be excluded. A three-

Table 4: Kinetic Parameters of ATP Hydrolysis, Amount of Plant H^+-ATPase, and Amount of 14-3-3 Bound to the Plasma Membrane of Selected Mutants

mutant	class	ATPase quantity (%)[a]	14-3-3 bound (%)[a]	14-3-3/ ATPase[b]	K_m[c] (mM)	V_{max}[c,d]	V_{max}/ ATPase[a]	pH optimum[c]	pK_1[e]	pK_2[e]	k[e]
AHA2		100	100	100	1.6 ± 0.3	0.6 ± 0.2	0.6	6.2 ± 0.2	6.0	6.0	4.7
aha2Δ922		81 ± 9	32 ± 12	43	0.3 ± 0.1	0.7 ± 0.5	0.9	6.7 ± 0.1	5.7	7.7	0.8
E872A	A	103 ± 11	112 ± 36	119	1.8 ± 0.4	0.8 ± 0.1	0.8	6.1 ± 0.0[f]	6.1	6.1	5.4
G898A	A	93 ± 1	150 ± 3	165	2.0 ± 0.2	0.7 ± 0.1	0.8	6.2 ± 0.1	6.1	6.1	5.2
K911A	B	97 ± 17	151 ± 9	138	1.9 ± 0.1	1.1 ± 0.5	1.1	6.3 ± 0.1	6.3	6.3	3.9
D865A	B	96 ± 13	146 ± 14	164	1.8 ± 0.2	0.7 ± 0.1	0.7	6.3 ± 0.0[f]	6.1	6.1	6.5
R912A	B	88 ± 2	177 ± 12	197	1.8 ± 0.1	1.2 ± 0.1	1.4	6.4 ± 0.2	6.3	6.3	4.4
Y866A	B	97 ± 5	148 ± 19	180	1.2 ± 0.4	1.5 ± 0.6	1.5	6.6 ± 0.1	6.3	6.3	5.1
R913A	C	93 ± 8	294 ± 125	369	0.4 ± 0.0[f]	0.6 ± 0.1	0.6	6.5 ± 0.2	5.6	7.0	1.0
R880A	C	94 ± 1	248 ± 53	323	0.6 ± 0.1	0.5 ± 0.0[f]	0.5	6.5 ± 0.2	5.7	6.9	1.0
G867A	C	103 ± 21	300 ± 12	367	0.8 ± 0.0[f]	1.1 ± 0.6	1.1	6.5 ± 0,1	5.9	6.7	2.5

[a] The amount of ATPase and 14-3-3 protein was estimated from Western blots using Scion Image software from Scion Corporation (*http://www.scioncorp.com*). Data are the mean of two experiments. [b] Data are calculated from the Western blot shown in Figure 3. [c] Data are the mean of at least 3 experiments from 2 different membrane preparations. [d] The units are μmol of ATP min^{-1} (mg of protein)$^{-1}$. [e] Fitted according to ref *38*, with $k = k_{deph,0}EP_0$. [f] Error was less than 0.05.

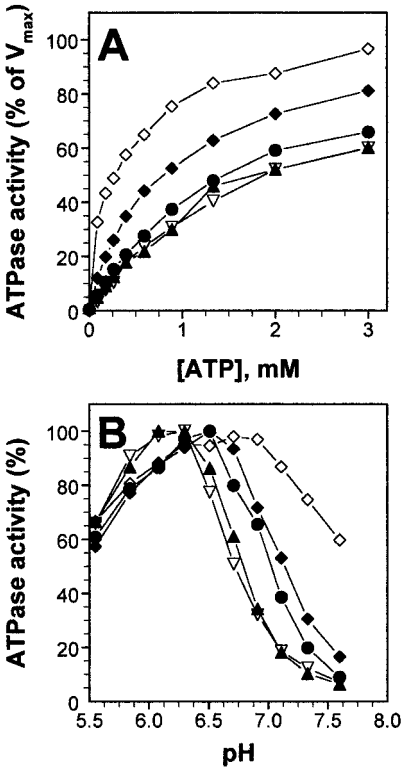

FIGURE 4: Effect of ATP concentration (A) and pH (B) on H^+-ATPase activity. (A) ATP dependence of ATPase activity at pH 7.0. Data were fit according to Michaelis−Menten kinetics. V_{max} values (100%) for each preparation were (units are μmol of P_i (mg of protein)$^{-1}$ min^{-1}): AHA2 = 0.74; aha2Δ92 = 1.06; G898A (a class A mutant) = 0.54; Y866A (a class B mutant) = 1.03; and G867A (a class C mutant) = 0.68. (B) pH dependence of H^+-ATPase activity measured with 3 mM ATP. The H^+-ATPase activities (100%) at optimal pH for each preparation used were as follows (units are μmol of P_i (mg protein)$^{-1}$ min^{-1}): AHA2 = 1.19; aha2Δ92 = 1.14; G898A = 1.21; Y866A = 1.31; G867A = 1.09. Symbols are the following: ▽, AHA2; ◇, aha2Δ92; ▲, G898A; ●, Y866A; ◆, G867A.

dimensional α helical wheel representation of the residues affected in class B and C mutants reveals that in both region I and region II these residues form a continuum, although not all residues are exposed to the same side of the helix (Figure 6). Interestingly, these regions are rich in positive charges. This site characteristic might allow for binding to a negatively charged region of the H^+-ATPase.

Direct or Indirect Inhibition by Regions I and II? A peptide of 32 amino acids (A861 to Q888) covering region I inhibits proton pumping (IC_{50} about 15 μM) by trypsinized H^+-ATPase devoid of its C-terminal domain (*19*). These results support the notion that region I interferes directly with another region of the H^+-ATPase. Region I might bind close to the active site of AHA2, or to another portion of the molecule, to stabilize the enzyme in a high-activity and high-affinity conformation.

However, it is intriguing that *aha2*R913A (a class C mutant) improves growth of transgenic yeast cells carrying a *pma1* deletion (Figure 1), since an AHA2 mutant carrying a deletion of 38 C-terminal amino acids (including R913; 36 amino acid residues away from the C-terminal end) fails to improve yeast growth significantly (*20*). Similarly, deletion mutants lacking 10, 20, 30, and 40 C-terminal amino acids fail to rescue *pma1* to the same degree as class C mutants (Anja T. Fuglsang; unpublished results). It is possible that alteration of residues in region II of the C-terminal domain of AHA2 directly or indirectly affects the position of other residues in contact with the rest of the H^+-ATPase. This would explain why single point mutations in region II affect the activity of AHA2, whereas deletion of the same residues in region II does not produce the same effect. A peptide (A912 to T943) covering several residues in region II does not affect proton pumping by the C-terminally truncated enzyme (*19*). This observation may also lead to the suggestion that region II might have a more indirect effect on H^+-ATPase activity.

Regulation by 14-3-3 Proteins. Interestingly, we observed a positive correlation between the ability of *aha2* mutants to rescue *pma1* and the amount of soluble 14-3-3 protein associated with the plasma membrane of transformed yeast cells (Figure 3; Table 4). 14-3-3 proteins have recently been identified as positive regulators of plant plasma membrane H^+-ATPase (*5*). Although the 14-3-3 binding site in the H^+-ATPase has not been identified, it is well-documented that 14-3-3 proteins bind to the C-terminus of the H^+-ATPase (*6*−*7*), and that the C-terminus of the plant H^+-ATPase is also recognized by yeast 14-3-3 proteins (*5*, *32*).

We still do not know whether (i) the increased activity of class C mutants is the result of the increased binding of 14-3-3 proteins to the C-terminus of the H^+-ATPase or

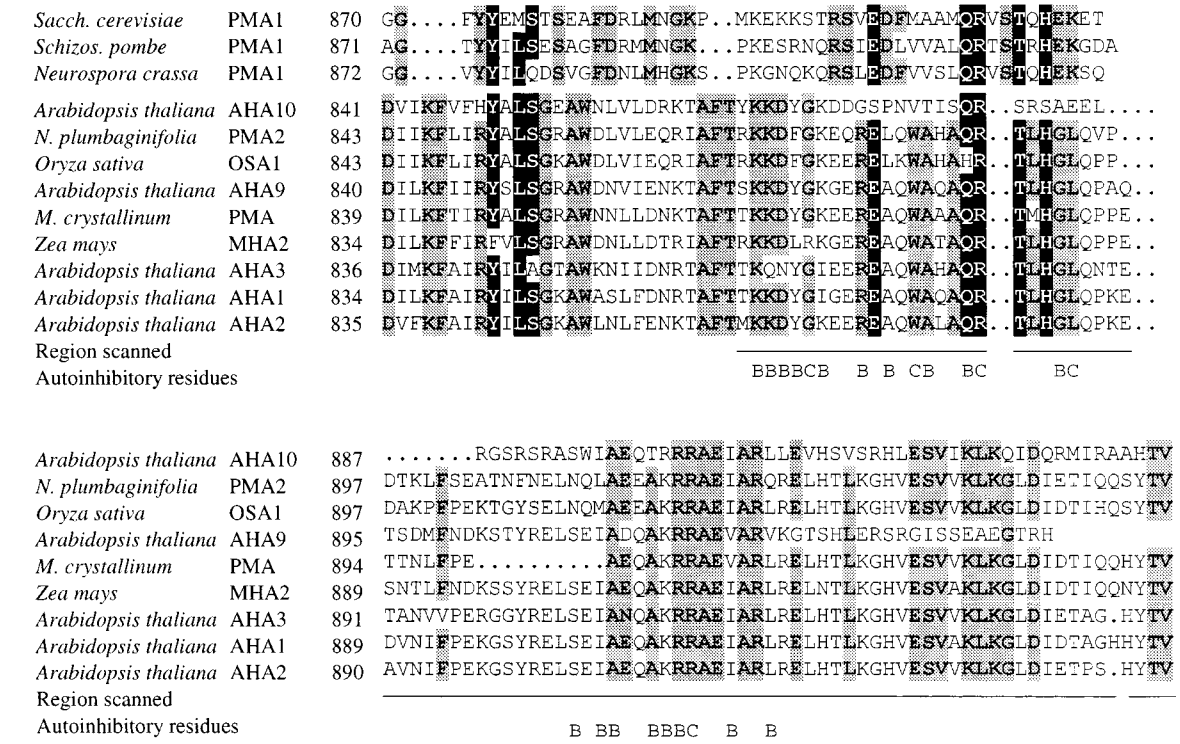

FIGURE 5: Alignment of C-termini of H+-ATPases from 12 different organisms with the region subjected to alanine scanning indicated. The alignment starts immediately after the last predicted transmembrane helix. The residue number of the first amino acid is indicated for each sequence. Putative autoinhibitory residues are indicated with letters according to the activity state of the mutant enzymes (B, class B; C, class C). Conserved residues from plant and fungal ATPases are indicated (in light gray). Conserved residues are present in 19 out of 21 plant H+-ATPase sequences, or in 9 out of 10 available fungal H+-ATPase sequences presently available in the databases. Residues conserved in both plants and fungi (black) are present in 26 of the 31 available sequences. Accession numbers are the following: *Saccharomyces cerevisiae* PMA1, P05030; *Schizosaccharomyces pombe* PMA1, P09627; *Neurospora crassa* PMA1, P07038; AHA10, S74033; AHA9, X73676; AHA3, P20431; AHA2, P19456; AHA1, P20649; *Nicotiana plumbaginifolia* PMA2, M27888; OSA1, D10207; MHA2, X85805; *Mesembryanthemum crystallinum PMA*, U84891. Fungal H+-ATPase sequences not shown in the alignment but used for identification of conserved residues: P24545, P28877, P49380, P19657, U65004, P28876, and Q07421. Plant H+-ATPase sequences not shown in the alignment: X76535, Q03194, U72148, S79323, D45189, D31843, U09989, P22180, Q08435, Q08436, X76536, and X85804.

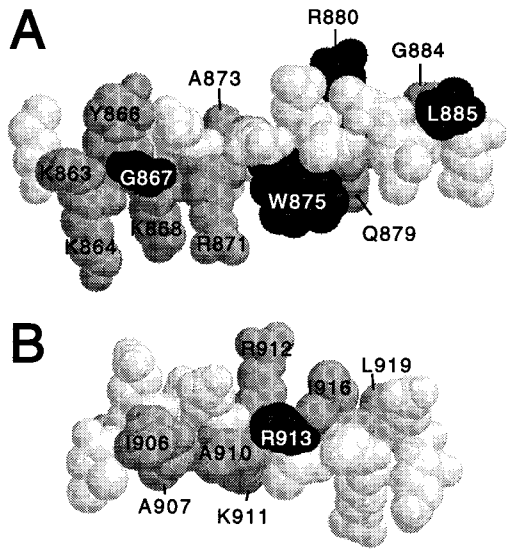

FIGURE 6: Three-dimensional model of region I (A) and region II (B) in the C-terminal part of *AHA2*. The regions are represented as α helices. Dark-colored amino acid residues: residues classified as class C. Gray-colored residues: classified as class B. White-colored residues: class A residues not involved in activation of AHA2. Visible amino acids of classes B and C are numbered according to the alignment of Figure 5.

whether (ii) mutation of class C residues directly results in activation of the H+-ATPase. Thus, the active conformation of H+-ATPase may be characterized by its ability to bind more 14-3-3 protein than the nonactivated form. One way to solve this problem may be to test whether class C mutants of AHA2 can rescue *pma1* in a (*bmh1 bmh2*) double knockout yeast strain.

Although there are exceptions, most 14-3-3 binding motifs involve a serine with an arginine at the −2, −3, or −4 position (*33*−*35*). In the crystal structure, this sequence motif binds in a groove of the 14-3-3 proteins conserved between fungi, animals, and plants (*34*, *36*). Why is it that single point mutations in the C-terminus result in increased binding of 14-3-3 proteins? One possibility is that new 14-3-3 binding sites are generated following the mutation of class C residues. Although this possibility cannot be ruled out, it seems unlikely since none of the class C sequences seems to contain a typical 14-3-3 binding motif. Another possibility is that mutations in the C-terminus change the conformation of the enzyme, making a pre-existing 14-3-3 binding site more accessible. There is only one sequence motif in the C-terminus of *AHA2* resembling the consensus 14-3-3 binding motif, namely R^{901}ELSE, located in the sequence between region I and region II. Another candidate target sequence, less related to the common 14-3-3 binding motif, is R^{920}ELHTL located just downstream of region II. Thus, it is possible that region I and region II act together to block access of 14-3-3 protein to a binding site that may involve R^{901}ELSE or R^{920}ELHTL.

A third possibility is that activation is more indirect and involves a protein kinase. Normally, 14-3-3 proteins only bind to sequence motifs containing a phosphorylated serine or threonine (*33*−*35*). Thus, in the R(x_{1-3})*S* motif, the italic serine would typically have to be phosphorylated. It can therefore not be ruled out that the effect of class C mutations is somehow to facilitate phosphorylation of a 14-3-3 binding motif in the H^+-ATPase sequence, in turn allowing for increased binding of 14-3-3 protein followed by enzyme activation. The elucidation of the mechanism by which class C mutants result in activation of H^+-ATPase will be much facilitated by the identification of the 14-3-3 binding site in the sequence.

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