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Activation of the *Dunaliella acidophila* Plasma Membrane H^+-ATPase by Trypsin Cleavage of a Fragment That Contains a Phosphorylation Site[1]

Israel Sekler[2], Meira Weiss, and Uri Pick*

Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

Trypsin treatment of purified H^+-ATPase from plasma membranes of the extreme acidophilic alga *Dunaliella acidophila* enhances ATP hydrolysis and H^+ pumping activities. The activation is associated with an alkaline pH shift, an increase in V_{max}, and a decrease in K_m(ATP). The activation is correlated with cleavage of the 100-kD ATPase polypeptide to a fragment of approximately 85 kD and the appearance of three minor hydrophobic fragments of 7 to 8 kD, which remain associated with the major 85-kD polypeptide. The N-terminal sequence of the small fragments has partial homology to residues 713 to 741 of *Arabidopsis thaliana* plasma membrane H^+-ATPases. Incubation of cells with ^{32}P-labeled orthophosphate (^{32}Pi) results in incorporation of ^{32}P into the ATPase 100-kD polypeptide. Trypsin treatment of the ^{32}Pi-labeled ATPase leads to complete elimination of label from the approximately 85-kD polypeptide. Cleavage of the phosphorylated enzyme with endoproteinase Glu-C (V-8) yields a phosphorylated 12-kD fragment. Peptide mapping comparison between the 100-kD and the trypsinized 85-kD polypeptides shows that the 12-kD fragment is derived from the trypsin-cleaved part of the enzyme. The N-terminal sequence of the 12-kD fragment closely resembles a C-terminal stretch of an ATPase from another *Dunaliella* species. It is suggested that trypsin activation of the *D. acidophila* plasma membrane H^+-ATPase results from elimination of an autoinhibitory domain at the C-terminal end of the enzyme that carries a vicinal phosphorylation site.

The H^+-ATPase from plasma membranes in plants is regulated at different levels by multiple internal and external factors. The plant hormone auxin was shown to induce de novo synthesis of a high-turnover pool of ATPase molecules (Hager et al., 1991); light induces activation of the H^+ pump in stomatal cells through a blue-light receptor (Assmann et al., 1985; Shimazaki et al., 1986); the fungus-derived phytotoxin FC activates H^+ pumping through a plasma membrane FC receptor that probably interacts with the ATPase (de Boer et al., 1989); and lysolecithin, possibly generated by a phospholipase (Palmgren et al., 1988), also activates the H^+-ATPase.

Several recent studies suggest that the regulatory domain of the enzyme is contained in the C-terminal end of the 100-kD H^+-ATPase polypeptide. Treatment of oat root plasma membranes with trypsin activates H^+ pumping, and the activation is associated with deletion of a 7- to 10-kD fragment from the C-terminal end of the ATPase (Palmgren et al., 1990, 1991). Also, lysolecithin activation (Palmgren et al., 1991) and FC activation (Johansson et al., 1993) seem to involve displacement of the C-terminal domain, since neither lysolecithin nor FC further activate a trypsin-treated enzyme. Similarly, deletion of the 11 C-terminal amino acids of the yeast PM-H^+-ATPase was reported to induce ATPase activation and to mimic Glc-induced activation of the yeast enzyme (Portillo et al., 1989). These and other studies led to the idea that an autoinhibitory domain at the C terminus is involved in regulation of PM-H^+-ATPases (Palmgren et al., 1990, 1991; Serrano et al., 1992).

A major drawback for in vitro studies of the mechanism of regulation of the plant PM-H^+-ATPase is the instability of the purified enzyme. We have recently demonstrated that the extreme acidophilic alga *Dunaliella acidophila* overproduces a vanadate-sensitive PM-H^+-ATPase (Sekler et al., 1991). We have also demonstrated that the enzyme is activated in vivo by illumination (Sekler et al., 1993) and in vitro by FC (Sekler et al., 1991), indicating that the mechanisms of regulation of the PM-H^+-ATPase in *D. acidophila* resemble those of higher plant enzymes. The enzyme retains high catalytic activity following purification and therefore is suitable for biochemical studies (Sekler and Pick, 1993). Here we show that the purified enzyme can be activated by trypsin treatment and that the trypsin-cleaved fragment carries a phosphorylation site.

MATERIALS AND METHODS

Growing of Cells

Dunaliella acidophila (Masyuk strain No. SAG 19.85, from the algal collection of the Institute of Plant Physiology, University of Göttingen, Germany) was a generous gift from Dr. Schlösser in Göttingen. The cells were grown in 15- to 30-L bath culture tanks as previously described (Sekler et al., 1991) to a cell density of 2×10^7 cells/mL. The pH of the growth medium was adjusted to 0.5 with concentrated sulfuric acid.

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[2] Present address: Department of Biological Sciences, Stanford University, Stanford, CA 94305–5020.

* Corresponding author; fax 972–8–344118.

Abbreviations: FC, fusicoccin; K_i, dissociation constant of the enzyme-inhibitor complex; PM-H^+-ATPase, plasma membrane proton ATPase.

Purification of PM-H$^+$-ATPase

Plasma membrane preparations were obtained from microsomal fractions of Yeda-Press lysed cells of *D. acidophila* as described previously (Sekler et al., 1991). Purification of the ATPase was carried out by sequential extractions of membranes with 2% Triton X-100 and a combination of 0.2% Mega-9 plus 0.2% Triton X-100 essentially as described before (Sekler and Pick, 1993).

Trypsin Treatment

Purified ATPase (1 mg/mL) was incubated at 21°C with 0.16 mg/mL 1-tosylamide-2-phenylethylchloromethyl ketone-treated trypsin (Worthington, Freehold, NJ) unless otherwise indicated. The incubation buffer contained 20 mM Tris-Hepes, pH 7, 20% glycerol, 2 mM EDTA, 50 mM KCl, and 1 mM DTT. The reaction was stopped after 30 min (or as indicated) by addition of 50 μg/mL soybean trypsin inhibitor. Samples of 1 to 2 μg or of 25 μg of protein were taken for ATPase activity assay (in the presence of 50 μg/mL soybean trypsin inhibitor) or for SDS-PAGE, respectively.

SDS-PAGE, Autoradiography, and Western Blot Analysis

Protease inhibitors leupeptin (10 μg/mL) and PMSF (1 mM) were added to trypsin-treated or untreated ATPase (0.5–1 mg/mL) followed by addition of sample buffer containing 1% SDS and 1% β-mercaptoethanol. Samples were either applied directly to SDS-PAGE after 5 min of incubation at 60°C or centrifuged (1 h at 150,000*g*) to eliminate soluble proteins as specified below. Either 10% SDS-PAGE (Laemmli, 1970) or 16.5% Tricine/SDS-PAGE (Capasso et al., 1992) was used.

The relative intensity of Coomassie blue-stained bands was determined by scanning with a Molecular Dynamics (Sunnyvale, CA) 300A computerized densitometer. For autoradiography of ^{32}P-labeled proteins, gels were dried (Bio-Rad model 583 gel dryer) at 65°C for 2 h and exposed to x-ray film (Agfa Curix RP2) for 1 to 2 d at −80°C.

For western blot analysis polypeptides were transferred to nitrocellulose membranes and reacted with rabbit antiserum against amino acid residues 340 to 650 of *Arabidopsis thaliana* plasma membrane H$^+$-ATPase as previously described (Sekler and Pick, 1993).

Peptide Mapping by V-8 Proteolysis

Limited proteolysis with *Staphylococcus aureus* endopeptidase V-8 was carried out according to a previously published procedure (Cleveland et al., 1977). In brief, gel slices containing the 100-kD ATPase polypeptide band (15–20 μg) were cut out of 10% SDS-PAGE and introduced into wells of a second gel (7.5→22% or 15% SDS-PAGE), overlaid with 0.05 to 5 μg of V-8, electrophoresed into the stacking gel, and incubated for 30 min with the protease. This was followed by electrophoresis and staining or autoradiography as described above.

N-Terminal Sequencing

Peptide bands were sliced out of 16.5% separating plus 4% stacking gels from Tricine/SDS-PAGE (Capasso et al., 1992), stained, transferred to polyvinylene difluoride paper, cut out, and sequenced in an Applied Biosystem (Foster City, CA) model 475A protein sequencer with an on-line model 120A phenylthiohydantoin analyzer.

Analytical Procedures

Protein concentration was determined by a modification of the Lowry procedure (Markwell et al., 1978) with BSA as standard. ATPase activity was determined by the release of Pi as previously described (Sekler and Pick, 1993). Measurement of pH gradient formation was performed following reconstitution of the enzyme into proteoliposomes and measurement of ATP-dependent fluorescence quenching with an acridine dye as described previously (Sekler and Pick, 1993).

RESULTS

Effect of Trypsin on H$^+$-ATPase and H$^+$ Pumping Activities

We have previously described the purification and reconstitution of a H$^+$-ATPase from plasma membranes of *D. acidophila*, which overproduces this enzyme. It was demonstrated that this preparation is free of any contaminating ATPases and consists of a major catalytic 100-kD polypeptide that constitutes 80% of the protein in the preparation (Sekler et al., 1991; Sekler and Pick, 1993). Treatment of this preparation with trypsin results in a 2- to 2.5-fold stimulation of ATPase activity (Fig. 1). It is noteworthy that fairly high concentrations of trypsin (1:6 to 1:3, trypsin:protein, w/w) and prolonged incubations (10–30 min, 21°C) are required for optimal activation, indicating that the ATPase is quite resistant to proteolytic inactivation. The presence of ATP, Mg ions, or KCl during trypsin treatment was found to have a negligible effect on ATPase activation (not shown).

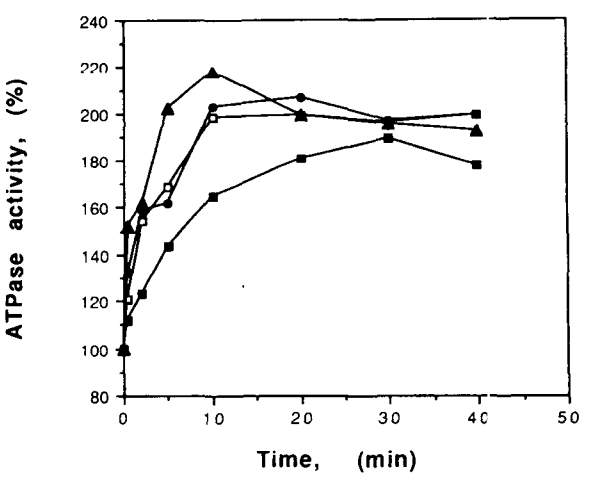

Figure 1. Time course of ATPase activation by trypsin. Purified *D. acidophila* ATPase (1 mg/mL) was incubated with 0.05 (■), 0.15 (□), 0.3 (●), or 0.6 (▲) mg/mL trypsin for the indicated time at 21°C and assayed for ATPase activity at pH 6.5.

The pH optimum of trypsin-treated enzyme is shifted from pH 6 to pH 6.5, and consequently the relative stimulation with respect to untreated enzyme increases from none at pH 5.5 to about 2-fold at pH 6 to 6.5 up to 3- to 4-fold at pH 7.5 to 8.0 (Fig. 2A). Estimation of ATP-dependent proton pumping, measured by fluorescence quenching of an acridine dye in reconstituted ATPase proteoliposomes (Sekler et al., 1991), reveals a similar pH-dependent activation by trypsin treatment (Fig. 2B).

A kinetic analysis of the control and trypsin-treated ATPase with respect to ATP concentration at pH 6 and pH 7 reveals that the activation is associated with a pH-dependent increase in V_{max} and a drop in K_m(ATP), (Fig. 3; Table I). The sensitivity to vanadate inhibition is hardly affected by trypsin treatment, as reflected by a similar K_i of about 1 μM in control and trypsin-treated enzyme (Table I).

A special feature of the *D. acidophila* PM-H^+-ATPase is the large stimulation by K^+ ions of 3- to 4-fold (Sekler et al., 1991). To find out whether the K^+ requirement is interrelated

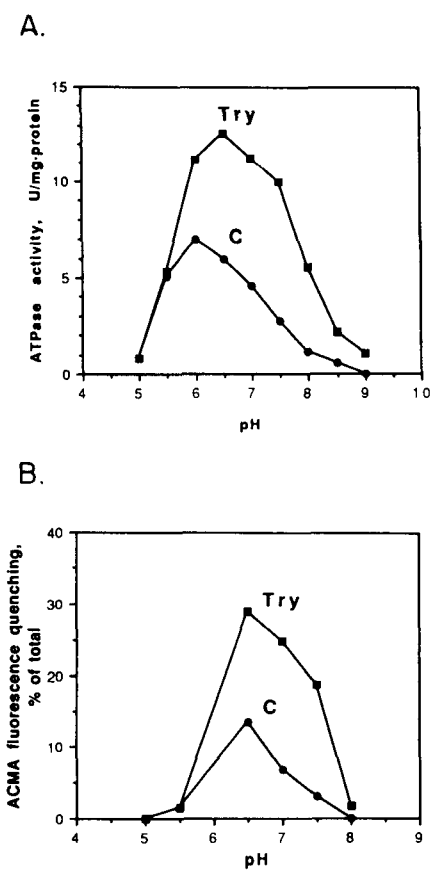

Figure 2. pH dependence of ATP hydrolysis and H^+ pumping activities in trypsin-treated and control ATPase. Purified ATPase was incubated with 0.2 mg/mL trypsin for 10 min and analyzed for ATPase activity (A) or reconstituted with phospholipids and assayed for H^+ pumping capacity, estimated by 9-aminochloro-2-methoxy-acridine bis-(hexachloroacetonyl)acetone (ACMA) fluorescence quenching (B) as described in "Materials and Methods." The assay medium contained 10 mM Tris-Mes (pH 5.0–6.5) or Tris-Hepes (pH 7.0–9.0).

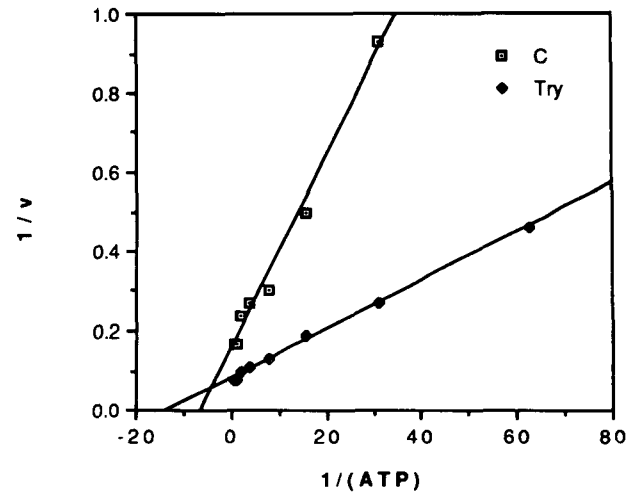

Figure 3. Effect of trypsin on apparent K_m and V_{max} for ATP hydrolysis. Trypsin-treated (Try) and control (C) ATPase was assayed for ATP hydrolysis activity at pH 7 in the presence of 16 μM to 2 mM ATP and an ATP-regenerating system (5 units of pyruvate kinase and 5 mM PEP). (ATP), ATP concentration in mM. v, Rate of ATP hydrolysis in μmol ATP hydrolyzed mg^{-1} protein min^{-1}.

with trypsin activation, we tested the effect of K^+ on ATPase activity in control and trypsin-activated preparations (Fig. 4). Both preparations exhibited a similar relative stimulation (about 3-fold) and similar concentration required for 50% stimulation (8 and 10 mM, respectively), indicating that the activation does not affect the interaction with K^+.

Identification of Trypsin Fragments and of the Trypsin Cleavage Site

The time course of digestion of the 100-kD H^+-ATPase polypeptide shows a rapid and complete disappearance of the 100-kD polypeptide (over 70% within 20 s), followed by a slower accumulation of a shorter polypeptide of approximately 85 kD, and a transient formation of an intermediate product of approximately 93 kD (Fig. 5A). All three polypeptides cross-react with antibodies directed against the conserved central catalytic domain of *A. thaliana* PM-H^+-ATPase (Fig. 5B).

Densitometric quantification of the relative amounts of each proteolytic product compared with ATPase activation during the course of trypsin treatment is shown in Figure 6. ATPase activation occurs subsequent to the disappearance of the 100-kD polypeptide and is best correlated with the appearance of the approximately 85-kD polypeptide. The latter suggests two consecutive fragmentations of the enzyme, the second of which causes ATPase activation.

Analysis of the trypsin-fragmented enzyme on 16.5% acrylamide Tricine/SDS gels revealed the appearance of three fragments of 7 to 8 kD (Fig. 7, arrows). Following high-speed centrifugation of the fragmented enzyme (1 h at 100,000*g*), the 85-kD and the three minor fragments were recovered in the pellet (not shown), indicating that they remain associated.

To identify the cleavage site we tried to sequence the N-

Table I. *Effect of trypsin treatment on V_{max}, K_m(ATP), and K_i (vanadate)*

ATP hydrolysis activity of untreated (control) or trypsin-treated (try) enzyme was assayed in the presence of 16 μM to 2 mM ATP, a constant $MgCl_2$ concentration of 5 mM, and an ATP-regenerating system [V_{max}, K_m(ATP)] or in the presence of 0.2 to 20 μM vanadate, 2 mM ATP, and 5 mM $MgCl_2$ [K_i(vanadate)]. Other details are as described in "Materials and Methods." V_{max}, K_m(ATP), and K_i (vanadate) were estimated from Lineweaver-Burk plots as in Figure 3.

pH	V_{max}		K_m(ATP)		K_i(vanadate)	
	Control	try	Control	try	Control	try
	μmol min^{-1} mg^{-1} protein		μM		μM	
6	9.1 ± 0.2	14.6 ± 0.3	87 ± 11	53 ± 7	1.2 ± 0.1	1.1 ± 0.1
7	6.1 ± 0.2	14.3 ± 0.36	147 ± 21	71.4 ± 12	1.0 ± 0.1	1.2 ± 0.2

terminal end of the trypsin fragments. Neither the approximately 85-kD fragment nor the intact 100-kD ATPase polypeptide could be sequenced, probably because of posttranslational modification (block) of the N-terminal amino acid of the protein. However, the smaller fragments were sequenced and found to have identical N-terminal sequences, indicating that they are derived from the same region in the enzyme. Comparison of the 29 N-terminal amino acids of these fragments with the deduced sequences of *A. thaliana* PM-H^+-ATPase (AHA-2, Harper et al., 1990) revealed a region of partial homology starting at amino acid 713, near the beginning of the putative transmembrane helix 6 (Fig. 8). Antibodies raised against the C-terminal domain of *A. thaliana* PM-H^+-ATPase, which weakly cross-react with the 100-kD *D. acidophila* ATPase, do not cross-react with the 85-kD fragment (not shown). Taken together, the results indicate that the trypsin cleavage site is located at the C-terminal end of the enzyme.

Identification of Phosphorylated Fragments of the ATPase

In preliminary in vivo phosphorylation experiments we observed that brief incubation of cells with ^{32}Pi results in incorporation of labeled phosphate into two major polypeptides of about 100 and 30 kD, which could be the PM-H^+-ATPase and the chloroplast light-harvesting Chl *a*/*b* binding proteins (not shown). Purification of the ATPase from plasma membranes of cells labeled for 30 min with ^{32}Pi revealed that the ATPase is indeed heavily labeled with ^{32}P (Fig. 9, lane 5). Treatment of the labeled enzyme with trypsin completely eliminates the labeling of the 85-kD polypeptide fragment (Fig. 9, lane 4). Also, the smaller 7- to 8-kD fragments, resolved on 16.5% Tricine/SDS gels, carry no ^{32}P label (not shown), indicating that the phosphorylation sites probably reside in very small hydrophilic cleavage products.

In an attempt to identify the phosphorylation site and to avoid contamination by other phosphorylated proteins, the phosphorylated 100-kD polypeptide was cut out of the gel and fragmented by different concentrations of the protease V-8 on denaturing SDS gels. As demonstrated in Figure 10A, V-8 treatment of 100-kD ATPase leads to a progressive

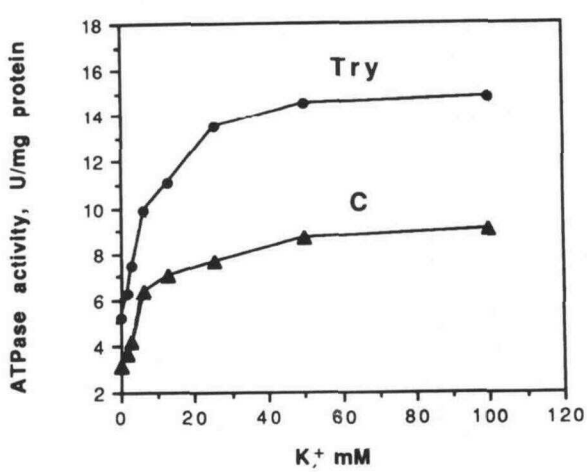

Figure 4. K^+ stimulation of ATPase activity of intact and trypsin-treated H^+-ATPase. ATPase activity of trypsin-treated (Try) and control (C) ATPase was assayed in the presence of the indicated KCl concentrations.

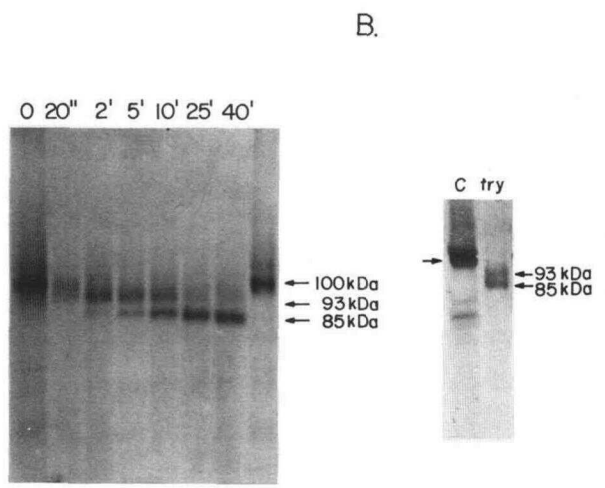

Figure 5. Time-dependent fragmentation of the H^+-ATPase polypeptides by trypsin. ATPase (1 mg/mL) was incubated with 0.16 mg/mL trypsin. After the indicated times (A) or 10 min (B), samples were centrifuged (60 min at 150,000*g*), the pellets were resuspended in original volumes of fresh buffer and analyzed on 10% SDS-PAGE, and either stained (A) or transferred to nitrocellulose paper and analyzed with antibodies against *A. thaliana* PM-H^+-ATPase (a generous gift from Ramon Serrano, Valencia, Spain) (B). Arrows indicate the uncut and two fragmentation states of the enzyme.

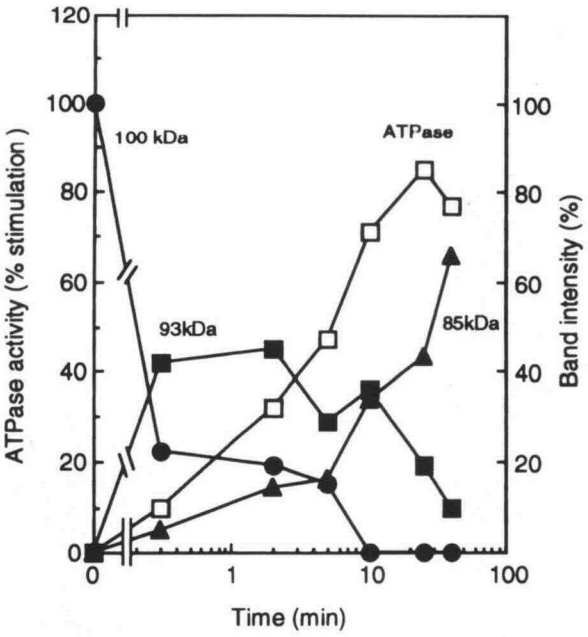

Figure 6. Correlation between ATPase fragmentation and ATPase activation. Densitometric quantitation of the uncut (100 kD) ATPase and two fragments (from Fig. 5), analyzed in parallel with ATPase activity measured on samples from the same preparations. Control ATPase activity = 9 μmol mg^{-1} min^{-1}. Band intensity, Relative numbers with reference to original uncut 100-kD polypeptide.

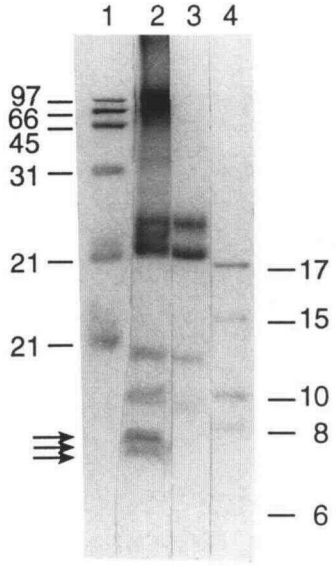

Figure 7. Detection of minor tryptic fragments on SDS-PAGE. ATPase (1 mg/mL) was treated with 160 μg/mL trypsin for 30 min and analyzed by 16.5% Tricine/SDS-PAGE as described in "Materials and Methods." Arrows indicate the three minor tryptic fragments. Lanes 1 and 4, Molecular mass markers; lane 2, trypsin-treated ATPase (40 μg); lane 3, trypsin + trypsin inhibitor.

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D. acidophila      ALHDTGAKIGMAVVLFLQLLGGVVVSMAF
                   ..   .|  .   |.|..|       . ..| .|
                   ..   .|  .   |.|..|       . ..| .|
                   ..   .|  .   |.|..|       . ..| .|
A.tha    AHA 2 713 GVVLGGYQAIMTVIFFWAAHKTDFFSDTF
         AHA 1 713 |I|||||Q|||S|I|||||H||||FS|K|
         AHA 3 714 |V|||||M|||T|V|||||Y||||PR|T|
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Figure 8. Sequence homology of N-terminal ends of the minor tryptic fragments with *A. thaliana* (*A.tha*) PM-H⁺-ATPase isoenzymes (according to Harper et al., 1990).

fragmentation, yielding an array of discrete fragments. Fragmentation of the ^{32}P-labeled ATPase yields two labeled polypeptides of apparent molecular mass of 12 and 15 kD (Fig. 10B). At the highest V-8 concentration most of the label was recovered in the 12-kD fragment.

The N-terminal ends of these fragments were sequenced and found to be identical, indicating that they are overlapping fragments derived from the same enzyme domain. The sequence of the first 15 amino acids has no homology to predicted sequences of PM-H⁺-ATPases from plants or yeast; however, it is almost identical to a region in a recently cloned PM-H⁺ ATPase from *Dunaliella bioculata*, located 110 to 125 amino acids from the C-terminal end (A. Wolf, personal communication; EMBL accession identifier DBPMA1):

D. acidophila (12 kD): F A G P S G M V P A N F S D P
D. bioculata: E F T G P S G M V P A N Y S N P

To find out if these fragments are contained within the trypsin-cleaved putative C-terminal part of the enzyme, the trypsin-generated approximately 85-kD fragment was cut out

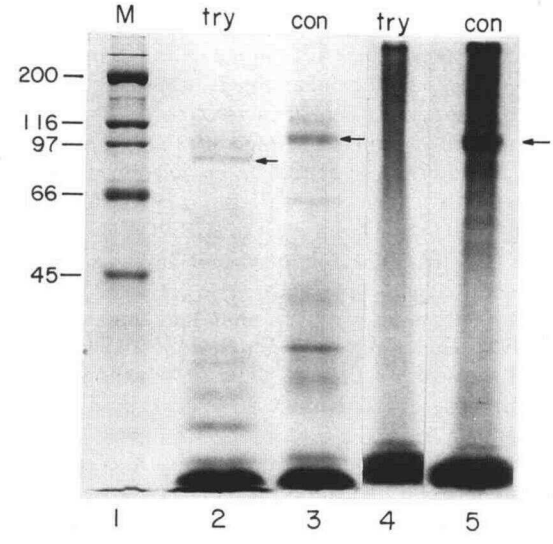

Figure 9. Effect of trypsin treatment on phosphorylated H⁺-ATPase. In vivo phosphorylation of the ATPase was carried out by incubation of algae (10^7 cells/mL) with 1 mCi/L carrier-free ^{32}Pi in the light for 30 min, followed by isolation and purification of the ATPase in the presence of 10 mM NaF. Trypsin treatment, SDS-PAGE (10%), and autoradiography were performed as described in "Materials and Methods." Lanes 1 to 3, Coomassie blue staining; lanes 4 and 5, autoradiographs. M, molecular mass markers; try, trypsin treated; con, control.

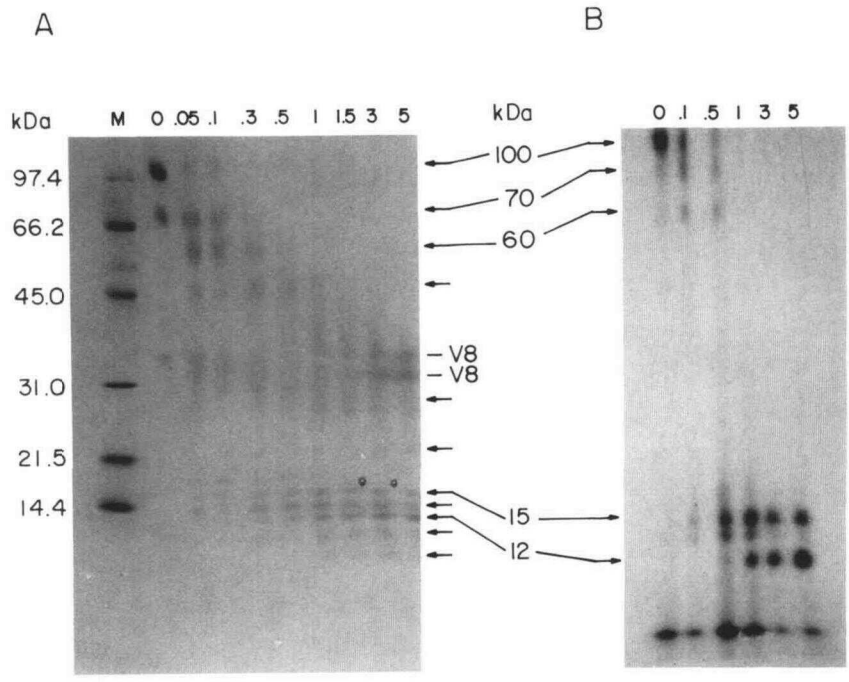

Figure 10. V-8 fragmentation of phosphorylated ATPase. Control (A) and ^{32}P-labeled (B) ATPase was treated for 30 min with the indicated amounts of V-8 (in μg), separated on 7 to 22% gradient (A) or 15% (B) SDS-PAGE, stained (A and B), and analyzed by autoradiography (B). Tryptic fragments are indicated by solid arrows. Molecular masses of the major phosphorylated peptide fragments are indicated by long arrows.

of the gels, exposed to extensive V-8 cleavage, and compared to the V-8-fragmented intact enzyme. As can be seen in Figure 11, the 12-kD V-8 fragment does not appear in the trypsin-derived 85-kD fragmented enzyme, indicating that it is contained in the domain that is cleaved off by trypsin.

DISCUSSION

The activation of the *D. acidophila* PM-H^+-ATPase by trypsin closely resembles activations of the plant and yeast PM-H^+-ATPases in the following features. (a) The kinetic characteristics, namely similar increase in V_{max} and decrease in K_m(ATP). (b) A similar alkaline shift in pH optimum was described following Glc activation or deletion of the 11 C-terminal amino acids in yeast (Serrano, 1983; Portillo et al., 1989). In plants, activation by lysolecithin (Palmgren et al., 1988) or by FC (Rasi-Caldogno et al., 1986) is also associated with a slight alkaline pH shift. Also in *D. acidophila*, FC activation is accompanied by a similar alkaline shift (Sekler et al., 1991). Therefore, it appears that the alkaline pH shift is a basic feature of PM-H^+-ATPase activation in plants, yeast, and algae. (c) Activation is obtained by deletion of a minor fragment from the C terminal end, which presumably carries the autoinhibitory domain. In yeast it consists of the 11 C-terminal amino acids (Portilla et al., 1989), and in plants it probably consists of residues 861 to 888 contained in the 7- to 10-kD C-terminal fragment (Palmgren et al., 1991). In *D. acidophila* the excised fragment is significantly larger, at least 15 kD in size. The observation that a larger fragment has to be eliminated to activate the ATPase suggests that the putative autoinhibitory domain in *D. acidophila* is located at a greater distance from the C terminus end compared to the plant enzyme. This may be due to the larger size of the C-terminal domain in *Dunaliella*, as deduced from the sequence of *D. bioculata* (A. Wolf, personal communication). Alternatively, ATPase activation may result from a gross conformational change induced by the fragmentation of a major portion of the ATPase between two transmembrane stretches (see below). The identification of the site of cleavage is not conclusive, because of the absence of the complete sequence of the ATPase from *D. acidophila*. However, the partial homology of the N-terminal sequence of the tryptic fragments

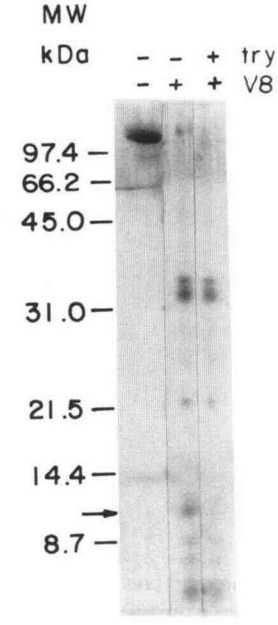

Figure 11. V-8 peptide mapping of control and trypsin-treated ATPase. Twenty-five micrograms of uncut or trypsin-treated ATPase were incubated with 5 μg of V-8 for 30 min.

with *A. thaliana* residues 713 to 741, antibody cross-reactivity studies, and the analogy to plants and yeast suggests that trypsin deletes the C-terminal end also in *D. acidophila*. The observation that the small tryptic fragments remain associated with the 85-kD catalytic fragment indicates that they are hydrophobic in nature. This is consistent with the predicted site of trypsin cleavage, just before transmembrane helix 6, since plants and yeast have at their C-terminal domains four adjacent transmembrane helixes, three of which may be contained in the hydrophobic fragments.

The observation that the *D. acidophila* PM-H^+-ATPase is phosphorylated in vivo is consistent with previous observations in plants and in yeast enzymes, which are phosphorylated at Ser and Thr residues (Schaller and Sussman, 1988; Chang and Slayman, 1991). The novel contribution of the present work is the localization of the phosphorylation site at the C-terminal end of the ATPase, which also contains the putative autoinhibitory domain. The coexistence of both sites at the C-terminal domain is supported by the elimination of the phosphorylation by trypsin, since the 12-kD phosphorylated fragment is not contained in the 85-kD catalytic fragment, and by the sequence homologies to other PM-H^+-ATPases. The results do not allow us to conclude whether the phosphorylation site is located within or vicinal to the putative autoinhibitory domain. However, we have observed by brief exposures to trypsin that dephosphorylation precedes ATPase activation, which suggests that the phosphorylation site does not overlap the autoinhibitory domain but may be located closer to the C terminus.

It is not clear whether phosphorylation plays a role in the physiological mechanism of regulation of PM-H^+-ATPases. It has been demonstrated that both the plant (Schaller and Sussman, 1988) and the yeast (Chang and Slayman, 1991) enzymes are phosphorylated by Ca^{2+}-stimulated protein kinases. The Ca^{2+}-stimulated protein kinase from plants has been cloned and sequenced and demonstrated to contain a calmodulin-like regulatory domain (Harper et al., 1991). In yeast, a correlation between Glc activation and phosphorylation of a distinct proteolytic fragment has been demonstrated (Chang and Slayman, 1991), suggesting that a Ca^{2+}-stimulated protein kinase may be involved in the regulation. No evidence for involvement of a protein kinase in regulation of the plant PM-H^+-ATPase has been demonstrated so far. In preliminary studies we observed that treatment of purified *D. acidophila* ATPase with a phosphatase leads to inhibition of ATP hydrolysis, suggesting that phosphorylation of the ATPase by a protein kinase may have a regulatory role also in *Dunaliella*.

The observations that purified *D. acidophila* PM-H^+-ATPase is phosphorylated and can be activated by trypsin make it an excellent biochemical system to study the mechanism of regulation of PM-H^+-ATPases and the involvement of phosphorylation in the process.

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LITERATURE CITED

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