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Controlled Proteolysis Mimics the Effect of Fusicoccin on the Plasma Membrane H^+-ATPase[1]

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We analyzed the effects of controlled treatments with trypsin of plasma membrane (PM) isolated from radish (*Raphanus sativus* L.) seedlings on the activity of the PM H^+-ATPase, and we compared them with those of fusicoccin (FC). Mild treatments of the PM with trypsin, which led to a decrease of the molecular mass of the peptide of about 10 kD, markedly increased the H^+-ATPase activity. The effect strongly increased with the increase of pH of the assay medium from 6.1 to 7.5, so the pH optimum of the enzyme activity shifted from 6.8 in untreated PM to 7.1 in trypsin-treated PM. The proteolytic treatment activated only the portion of PM H^+-ATPase activity that is stable to preincubation in assay medium in the absence of ATP and determined a strong increase of V_{max} and a less marked decrease of the apparent K_m for Mg-ATP. All of these effects were very similar to those determined by FC, which activated the PM H^+-ATPase without promoting its proteolytic cleavage. FC did not further activate the H^+-ATPase activity of trypsin-treated PM under conditions in which the FC receptor was protected from the attack of trypsin. Conversely, trypsin treatment had little effect on the PM H^+-ATPase preactivated with FC. Moreover, the activity of the PM H^+-ATPase preactivated with FC was not further activated by lysolecithin. These results indicate that the modification of the PM H^+-ATPase of higher plants triggered by the FC-receptor complex hinders the inhibitory interaction of the regulatory C-terminal domain with the active site.

The PM H^+-ATPase plays a crucial role in several physiological functions in higher plants because it generates an electrochemical proton gradient that drives the transport of several solutes and controls both intra- and extracellular pH values. Physiological studies based on measurements of membrane potentials and of fluxes of protons and other ions have provided evidence that its activity is regulated in vivo by several endogenous and environmental factors (for review, see Marrè, 1979; Marrè and Ballarin-Denti, 1985; Serrano, 1989; Palmgren, 1991). Among these factors, FC has received the most attention since in vivo it has the most dramatic activating effect on the PM H^+-ATPase (Marrè, 1979). During the last few years, studies on isolated PM vesicles and on proteoliposomes reconstituted with solubilized and partially purified H^+-ATPase and FC receptor have shown that binding of FC to its PM receptor protein determines the activation of the PM H^+-ATPase (Rasi-Caldogno and Pugliarello, 1985; Rasi-Caldogno et al., 1986, 1989; Aducci et al., 1988; De Michelis et al., 1988, 1989, 1991; Marra et al., 1992; Olivari et al., 1993).

The FC-stimulated activity has been characterized in some detail in PM isolated from radish (*Raphanus sativus* L.) and other plant materials. In native PM vesicles, FC-induced stimulation of the H^+-ATPase is much stronger at the slightly alkaline pH values typical of the cytoplasm of plant cells than at the relatively acidic pH optimum of the enzyme, and thus shifts the pH optimum of the activity from pH 6.6 to around pH 7.0. Activation of the PM H^+-ATPase by FC leads to a marked increase of V_{max} accompanied by a less marked decrease of the K_m of the enzyme for Mg-ATP (Rasi-Caldogno and Pugliarello, 1985; Rasi-Caldogno et al., 1986, 1989; De Michelis et al., 1988, 1989, 1991; Olivari et al., 1993). Moreover, FC stimulates only the portion of PM H^+-ATPase activity that is stable to preincubation in assay medium without ATP (De Michelis et al., 1988; Olivari et al., 1993). The mechanism through which the FC-receptor complex triggers the activation of the PM H^+-ATPase is unknown.

In yeast, a qualitatively similar although more dramatic modification of the kinetic parameters of the PM H^+-ATPase is triggered by Glc fed in vivo (Serrano, 1983). The mechanism through which Glc triggers the activation of the yeast PM H^+-ATPase is also unknown, although a covalent modification of the enzyme seems to be involved (Serrano, 1983). In mutants of *Saccharomyces cerevisiae* lacking the last 11 amino acids of the PM H^+-ATPase, the enzyme had high activity independent of Glc activation, indicating that Glc triggers a modification of the enzyme, or of associated components, which abolishes the inhibitory interaction of the C-terminal domain with the active site (Portillo et al., 1989). Recently, Palmgren and co-workers (1990, 1991) have shown that the PM H^+-ATPase of higher plants contains a C-terminal inhibitory domain that can be cleaved by controlled proteolytic treatments.

In this work we characterized the activity of the H^+-ATPase in PM from radish seedlings subjected to controlled treatments with trypsin. We show that controlled proteolytic treatments that led to a decrease of the molecular mass of the PM H^+-ATPase peptide of about 10 kD activated the PM

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Abbreviations: Brij 58, polyoxyethylene-20-cetyl ether; BTP, bis-tris propane (1,3-bis[tris(hydroxymethyl)methylamino]-propane); FC, fusicoccin; PM, plasma membrane.

H^+-ATPase activity in a manner very similar to that of FC. Moreover, we show that the effects of FC, which does not induce any proteolytic cleavage of the PM H^+-ATPase, and of controlled proteolysis were not additive in conditions in which the proteolytic treatment did not affect the FC receptor. These results indicate that the modification of the PM H^+-ATPase of higher plants triggered by the FC-receptor complex hindered the inhibitory interaction of the regulatory C-terminal domain with the active site.

MATERIALS AND METHODS

Preparation of PM Vesicles

Radish seeds (*Raphanus sativus* L. cv Tondo Rosso Quarantino, Ingegnoli, Milano, Italy) were germinated for 24 h and stored at −80°C as previously described (De Michelis et al., 1991). Microsomal membranes were prepared as described (De Michelis et al., 1989) with slight modifications. Briefly, frozen seedlings were homogenized in ice-cold, freshly prepared extraction medium containing 0.3 M Suc, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 5 mM DTT, 0.5% BSA, 0.2% casein hydrolysate, 3 mM *p*-aminobenzamidine, 1 mM PMSF from a freshly prepared 100 mM stock in methanol, and 25 mM Mes adjusted to pH 7.2 with Tris. The homogenate, filtered through four layers of cheesecloth, was centrifuged at 8,000*g* for 10 min and the resulting supernatant was centrifuged at 48,000*g* for 35 min. When microsomes were to be prepared, the pellet was resuspended in 0.25 M Suc, 0.1 mM EGTA, 3 mM DTT, 1 mM PMSF, 0.3% BSA, 1 mM BTP adjusted to pH 7.0 with Hepes and collected by centrifugation at 48,000*g* for 30 min. PM was purified by partitioning the unwashed microsomes in 6.2% (w/w) Dextran T500 (Pharmacia, Uppsala, Sweden) PEG 3350 (Sigma) containing 5 mM KCl and 5 mM potassium phosphate buffer (pH 7.8) as described (De Michelis et al., 1991). The third upper phase was diluted at least 5-fold with 10% glycerol, 0.1 mM EGTA, 3 mM DTT, 1 mM PMSF, 0.1 mg mL^{-1} Brij 58, 1 mM BTP-Hepes, pH 7.0, and collected by centrifugation at 48,000*g* for 35 min; this washing procedure yielded a PM fraction containing 50 to 80% vesicles in the inside-out configuration, as estimated from the increase of PM H^+-ATPase activity induced by the detergent Brij 58 (see, for example, Fig. 8). Aliquots of microsomes or of PM suspensions (1–2 mg protein mL^{-1} in 10% glycerol, 0.5 mM DTT, 1 mM BTP-Hepes, pH 7.0) were immediately frozen and kept at −80°C until use. Membrane proteins were assayed according to Markwell et al. (1978).

Treatment of the Membranes with Trypsin

Membranes (0.5–1.2 mg protein mL^{-1}) were incubated for 0.5 to 10 min on ice in 0.75 mM DTT, 5% glycerol, 1 mM ATP, 0.25 mM $CaCl_2$, 375 μg mL^{-1} Brij 58, 2.5 mM BTP-Hepes (pH 7.5) in the presence of 5 to 100 μg mL^{-1} trypsin (Boehringer catalog No. 109819). The reaction was blocked by addition of an at least 80-fold excess of soybean trypsin inhibitor (Sigma catalog No. T9003). Controls run in the absence of trypsin or by adding the trypsin inhibitor prior to trypsin gave identical results (see Fig. 2).

Measurements of the PM H^+-ATPase Activity

Unless otherwise specified, membranes (3–10 μg membrane protein per sample) were preincubated for 30 min at 33°C in 125 μL of 50 mM KNO_3, 5 mM $MgSO_4$, 0.1 mM ammonium molybdate, 0.2 mM EGTA, 1 μg mL^{-1} oligomycin, 100 μg mL^{-1} Brij 58, 40 mM BTP-Mes or BTP-Hepes adjusted to the specified pH values. The reaction was started by diluting the samples 1:1 with the above medium supplemented with 6 mM ATP plus or minus 200 μM vanadate, and the assay was performed at 25°C over 1 h of incubation. The PM H^+-ATPase activity was evaluated as the activity inhibited by 100 μM vanadate. Released Pi was assayed as described (De Michelis and Spanswick, 1986). All the experiments were performed at least two times, each with three replicates, on independent membrane preparations. Unless otherwise specified, results are from one representative experiment.

Measurements of FC Binding

FC binding was assayed by measuring the radioactivity associated with membrane samples (30–130 μg protein per sample) incubated in 0.25 M Suc, 5 mM $MgSO_4$, 100 μg mL^{-1} Brij 58, 1 mM BTP-Mes, pH 6.0 (500 μL final volume) in the presence of 1 nM [^{3}H]dihydrofusicoccin (1.3 KBq $pmol^{-1}$, kindly supplied by Prof. G. Randazzo, Naples University, Italy) and 1 nM FC, plus or minus 10 μM FC. Incubation was at 25°C for 3 h in glass tubes. The samples were then processed by a rapid filtration technique (Bruns et al., 1983) using GF/B Whatman filters (2.5 cm diameter) soaked for at least 2 h in 1% polyethylenimine (Sigma) at pH 8. Each sample, diluted with 5 mL of 25 mM Gly-KOH, pH 9.5, containing 1 mM $MgCl_2$, was poured onto a filter under strong vacuum; the tube was washed once and the filter was washed three more times with 5 mL of the above medium. To filters placed in scintillation vials, 10 mL of Pico Aqua (Packard) were added and the samples were counted in a Packard Tricarb 1500 scintillation counter. Nonspecific binding, measured from radioactivity associated with samples incubated in the presence of 10 μM unlabeled FC, was subtracted from all the binding values to evaluate specific binding. The reported results are from one experiment with three replicates, representative of at least two, and performed on different membrane preparations.

SDS-PAGE

Membranes were incubated in 700 μg mL^{-1} tosyl-L-Lys, 140 μg mL^{-1} leupeptin, 2.8 mM *p*-aminobenzamidine, 3.5 μg mL^{-1} soybean trypsin inhibitor for 5 min on ice and then solubilized for 1 h at room temperature in 4% SDS, 20% glycerol, 3% β-mercaptoethanol, 2 mM EDTA, 20 mM H_3PO_4 adjusted to pH 2.4 with Tris. Twenty to 60 μg of membrane proteins per lane were subjected to slab-gel electrophoresis essentially according to Laemmli (1970). Total monomer concentration was 7%; cross-linking was 2.7%.

Western Blot Analysis

After SDS-PAGE, the polypeptides were electrophoretically transferred to an Immobilon NC/HA transfer membrane

(Millipore Corp.) and reacted with a rabbit antiserum against the central domain (amino acids 340–630) of the *Arabidopsis thaliana* PM H+-ATPase (Altabella et al., 1990; generous gift of Prof. R. Serrano, Universidad Politecnica de Valencia, Spain). Immunodetection was performed with a second antibody coupled to alkaline phosphatase (Sigma catalog No. A9919).

RESULTS

Effect of Controlled Proteolysis on the Characteristics of the PM H+-ATPase Activity of Radish

Similar to what has been observed in PM from other plant materials (Palmgren et al., 1990, 1991), mild treatments with trypsin of PM isolated from radish determined an increase of the activity of the PM H+-ATPase. Preliminary experiments indicated that optimal activation could be obtained by treating the PM with trypsin on ice in the presence of 0.25 mM $CaCl_2$, 1 mM ATP, 0.75 mM DTT, and 375 μg mL^{-1} of the detergent Brij 58. We selected to treat the PM on ice because we were interested in comparing the effect of controlled proteolysis on the PM H+-ATPase with that of FC, and it is known that the FC receptor is very labile (Pesci et al., 1979; Aducci et al., 1984; De Michelis et al., 1991). The effect of trypsin on the activity of the PM H+-ATPase depends on its concentration as well as on the concentration of membrane proteins (Fig. 1) and on the length of incubation (see Fig. 5). Maximal activation of the H+-ATPase was induced by treating the PM for 5 to 10 min with 50 μg trypsin mg^{-1} PM protein.

Western blot analysis using antibodies generated against the central domain of the *A. thaliana* PM H+-ATPase (Altabella et al., 1990) showed that the antibody recognized a doublet of about 100 kD and that maximal activation of the H+-ATPase by trypsin treatment of the PM was associated with a decrease of the molecular mass of the enzyme to about 90 kD (Fig. 2). The decrease in molecular mass of the enzyme from radish seeds induced by trypsin treatment was very similar to that observed by Palmgren et al. (1990, 1991) in PM from other plant materials. Thus, it is likely that in PM from radish, mild treatment with trypsin in the presence of ATP preferentially cleaved a fragment from the C-terminal domain of the H+-ATPase (Palmgren et al., 1991).

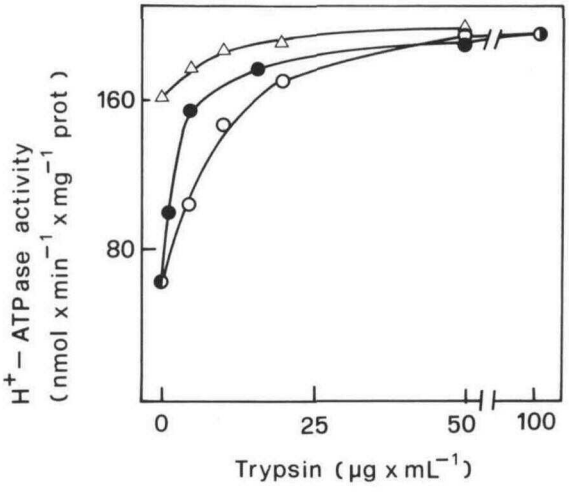

Figure 1. Effect of trypsin treatment of the PM on the H+-ATPase activity. PMs were treated for 5 min with the specified trypsin concentrations as described in "Materials and Methods." PM concentration during the treatment was 0.5 mg protein mL^{-1} (closed symbols) or 1.2 mg protein mL^{-1} (open symbols). PM H+-ATPase activity was assayed at pH 6.6 (△) or at pH 7.5 (○, ●).

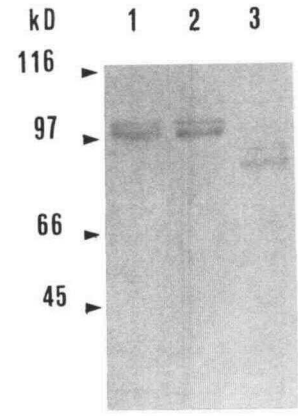

Figure 2. Western blot analysis of tryptic cleavage products from the PM H+-ATPase. Plasma membranes (1 mg protein mL^{-1}) were treated with or without 50 μg trypsin mL^{-1} as described in "Materials and Methods." Lane 1, No trypsin added; lane 2, trypsin inhibitor added prior to trypsin; lane 3, incubation with trypsin for 5 min. Twenty-five micrograms of PM proteins were applied per lane. The activity of the PM H+-ATPase assayed at pH 7.5 was 61 (lane 1), 60 (lane 2), and 182 (lane 3) nmol min^{-1} mg^{-1} protein.

Figure 1 also shows that the increase of H+-ATPase activity induced by trypsin treatment of the PM was much stronger (up to 200%) on the activity assayed at pH 7.5 than on the activity assayed at pH 6.6. So the ratio between the activity at pH 6.6 and that at pH 7.5 decreased from about 2.5 in untreated PM to about 1 in PM subjected to optimal trypsin treatment. This result prompted us to analyze the effect of trypsin treatment on the activity of the PM H+-ATPase as a function of the pH of the assay medium. Figure 3 shows that under these experimental conditions (50 mM KNO_3, 100 μg mL^{-1} Brij 58), the pH optimum for the H+-ATPase activity of untreated PM was around pH 6.8. Trypsin treatment of the PM had little if any effect on the H+-ATPase activity assayed in the acidic side of the pH curve; the trypsin-induced increase of activity became evident around pH 6.6 and increased as the pH was increased to pH 7.5. Thus, the H+-ATPase activity of trypsin-treated PM showed an optimum pH of about pH 7.1. The shift of pH optimum for H+-ATPase activity induced by trypsin treatment of the PM was very similar to that induced by FC (Rasi-Caldogno et al., 1986; De Michelis et al., 1991).

We have shown previously that preincubation of PM in assay medium devoid of ATP at 33°C led to a decrease of H+-ATPase activity by about 20 to 30%, which was completed within 30 to 45 min, after which the activity remained stable for at least 1 h. Only the portion of activity stable to

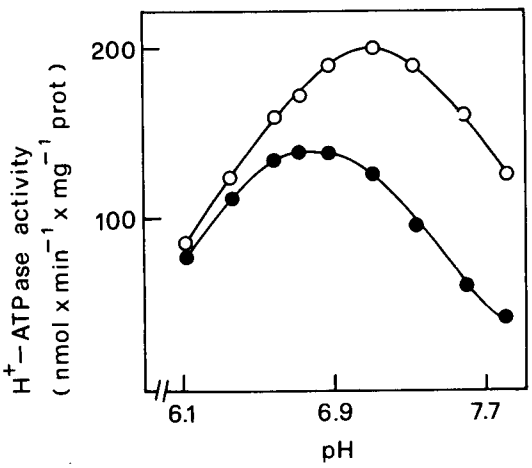

Figure 3. Effect of pH on the activity of the H^+-ATPase in PM pretreated with (open symbols) or without (closed symbols) trypsin. Trypsin treatment was performed for 5 min with 50 μg trypsin mL^{-1} in the presence of 1.2 mg PM protein mL^{-1}.

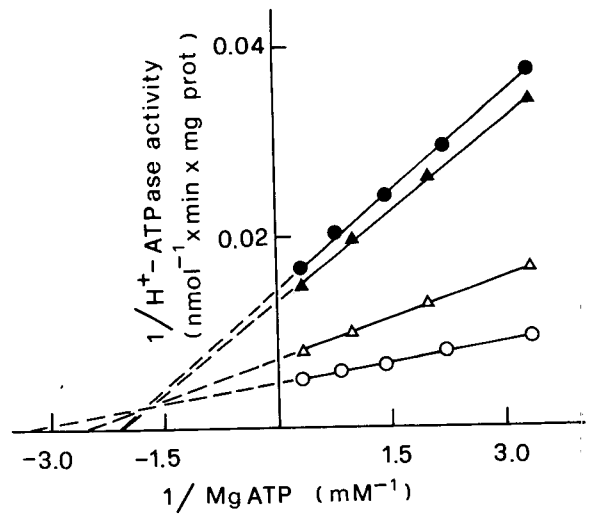

Figure 4. Effects of trypsin treatment of the PM and of FC on the H^+-ATPase activity as a function of the concentration of Mg-ATP. Data are plotted according to Lineweaver-Burk; the straight lines were drawn by linear regression analysis ($r > 0.99$). Trypsin treatment was performed for 5 min with (○) or without (●) 50 μg trypsin mL^{-1} in the presence of 1.2 mg PM protein mL^{-1}. The effect of FC (△) was measured on untreated PM (▲); FC was added to the preincubation medium to give a final concentration during the assay of 10^{-5} M. Assays were performed at pH 7.5. After 30 min of preincubation at 33°C as described in "Materials and Methods," the reaction was started by diluting the samples 1:1 with assay medium containing stoichiometric $MgSO_4$ and ATP to give the specified final concentrations. Excess $MgSO_4$ was 2.5 mM in all cases.

this preincubation was stimulated by FC (De Michelis et al., 1988; Olivari et al., 1993). Table I also shows that trypsin treatment of the PM affects only the portion of H^+-ATPase activity stable to preincubation in assay medium in the absence of ATP.

The increases of H^+-ATPase activity induced by trypsin treatment of the PM and by FC similarly depend on the concentration of Mg-ATP in the assay medium: in both cases, stimulation was maximal at the lowest Mg-ATP concentrations tested and slightly decreased with the increase of Mg-ATP concentration. Kinetic analysis of the data according to Lineweaver and Burk (Fig. 4) indicated that both trypsin treatment and FC markedly increased V_{max} (by 210% and by 85%, respectively) and decreased to a lower extent the apparent K_m of the enzyme for Mg-ATP (from 0.48 mM of untreated PM to 0.40 mM in the presence of FC and 0.31 mM in trypsin-treated PM).

Table I. *Effect of trypsin treatment of the PM on the H^+-ATPase activity assayed before or after preincubation in assay medium without ATP*

Trypsin treatment was performed for 5 min in the presence of 1.1 mg of PM protein × mL^{-1}. H^+-ATPase activity was assayed at pH 7.5 before (a) and after (b) 30 min of preincubation at 33°C in assay medium without ATP (see "Materials and Methods"). Values in parentheses are the increases of activity induced by trypsin treatment.

Trypsin Treatment	H^+-ATPase Activity		(a-b)
	No preincubation[a]	30 min of preincubation[b]	
	nmol min^{-1} mg^{-1} protein		
None	76	60	16
5 μg mL^{-1}	116 (+40)	101 (+41)	15
10 μg mL^{-1}	150 (+74)	136 (+76)	16
50 μg mL^{-1}	196 (+120)	180 (+120)	14

The Activation of the PM H^+-ATPase by Controlled Proteolysis and That by FC Were Not Additive

Treatment of the PM with trypsin made the H^+-ATPase insensitive to further activation by FC (Fig. 5). However, in the conditions of this experiment, trypsin also drastically decreased FC binding to its receptor (Fig. 5), which was necessary for activation of the PM H^+-ATPase (Aducci et al., 1988; De Michelis et al., 1989, 1991; Rasi-Caldogno et al., 1989). So the lack of additivity between the effects of trypsin treatment and of FC might simply have reflected a trypsin-induced inactivation of the receptor. The FC receptor was exposed to the outer surface of the PM and was protected from tryptic cleavage in sealed inside-out vesicles (Aducci et al., 1980; Feyerabend and Weiler, 1988), which exposed to trypsin the C-terminal domain of the H^+-ATPase (Palmgren, 1991). Thus, we performed a very mild trypsin treatment of microsomes in the absence of detergent and subsequently assayed FC binding and the nonlatent PM H^+-ATPase activity (Table II). Under these conditions, trypsin treatment only very slightly decreased FC binding, and the effects of trypsin treatment and of FC on the PM H^+-ATPase were still not additive.

Addition of FC to isolated PM stimulated the H^+-ATPase activity always less than optimal treatment of the PM with trypsin (see, for example, Fig. 4). Stimulation by FC of the

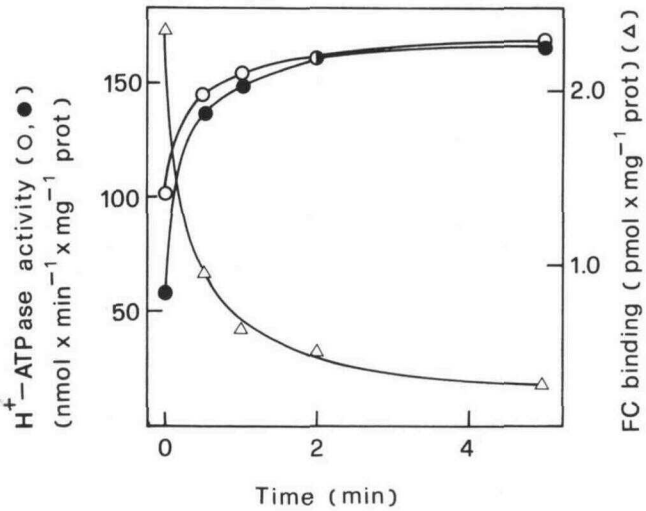

Figure 5. FC binding and FC effect on the H^+-ATPase activity in trypsin-treated PM. Trypsin treatment of PM was performed for the specified times in the presence of 0.6 mg PM protein mL^{-1} and of 10 μg trypsin mL^{-1}. The PM H^+-ATPase activity was assayed at pH 7.5 in the presence (○) or absence (●) of 10 μM FC. FC binding (△) was assayed as described in "Materials and Methods."

PM H^+-ATPase activity was much higher when the toxin was applied in vivo or during membrane isolation, possibly because FC binding protected the receptor from degradation during PM extraction and purification (Rasi-Caldogno et al., 1989; Schulz et al., 1990). Table III shows that treatment of radish seedlings with 10 μM FC for 2 h prior to harvesting induced an activation of the PM H^+-ATPase similar to that induced by trypsin treatment of the PM isolated from control seedlings. In fact, the activity assayed at pH 7.5 was about 150% higher than that of control membranes, whereas that assayed at pH 6.6 was only scarcely higher than that of control membranes, so the ratio between the activity measured at pH 6.6 and that measured at pH 7.5 decreased from 2.5 in control membranes to about 1 in membranes from FC-treated seedlings.

Table II. *Effect of a mild trypsin treatment in the absence of detergent on the activity of the PM H^+-ATPase assayed in the presence and absence of FC and on FC binding*

Microsomes (0.4 mg protein mL^{-1}) were treated for 3 min on ice with or without 15 μg trypsin mL^{-1} in the presence of 40 mM BTP-Hepes, pH 7.5, 0.3 mg mL^{-1} BSA, 40 mM Suc, 1 mM DTT, 5 mM EDTA. The PM H^+-ATPase activity was assayed at pH 7.5 in the presence or absence of 5 μM FC, as described in "Materials and Methods," but in the absence of Brij 58 and in the presence of 5 mM $(NH_4)_2SO_4$ and 5 μM carbonylcyanide *p*-trifluoro-methoxyphenylhydrazone.

Membrane Treatment	H^+-ATPase Activity		FC Binding
	Control	FC	
	nmol min^{-1} mg^{-1} protein		*pmol mg^{-1} protein*
None	21	34	1.3
Trypsin	36	37	1.1

Table III. *Effect of in vivo treatment with FC on the activity of the PM H^+-ATPase*

Microsomes were extracted as described in "Materials and Methods" from frozen seedlings treated with or without 10 μM FC for 2 h before harvesting. PM H^+-ATPase activity was assayed at the specified pH values as described in "Materials and Methods." Results are from two independent experiments, performed on different microsomal preparations, plus or minus SE ($n = 6$).

Membranes	H^+-ATPase Activity	
	pH 6.6	pH 7.5
	nmol min^{-1} mg^{-1} protein	
From control seedlings	76 ± 2	31 ± 1
From FC-treated seedlings	83 ± 3	79 ± 4

The activation of the PM H^+-ATPase by FC did not depend on proteolytic cleavage of the enzyme. In fact, western blot analysis of membranes extracted from FC-treated seedlings (Fig. 6) showed that the molecular mass of the PM H^+-ATPase was identical to that of the enzyme in membranes extracted from control seedlings.

Figure 7 shows that trypsin treatment of the membranes from FC-treated seedlings had very little effect on the PM H^+-ATPase activity. In the presence of suboptimal trypsin concentrations, the activity was much higher in membranes from FC-treated seedlings than in control membranes; at optimal trypsin concentration, the activities of PM from control and FC-treated seedlings were virtually identical. These results confirm that the effects of controlled proteolysis and of FC on the activity of the PM H^+-ATPase were not additive.

The observation that the PM H^+-ATPase activity of membranes extracted from FC-treated seedlings was higher than that of the controls also in PM treated with trypsin under conditions in which the free FC receptor would be extensively degraded (compare Fig. 7 and Fig. 5) may indicate that FC protected its receptor from the proteolytic attack of trypsin. Alternatively, it may suggest that activation of the PM H^+-ATPase by FC involved a covalent modification of the en-

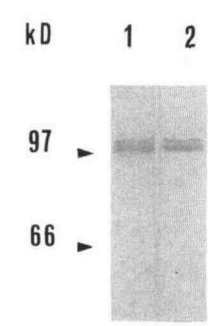

Figure 6. Western blot analysis of the PM H^+-ATPase from seedlings treated with FC in vivo. Microsomes were extracted from seedlings pretreated with (lane 2) or without (lane 1) 10 μM FC as described in the legend to Table III. Fifty micrograms of microsomal proteins were applied per lane.

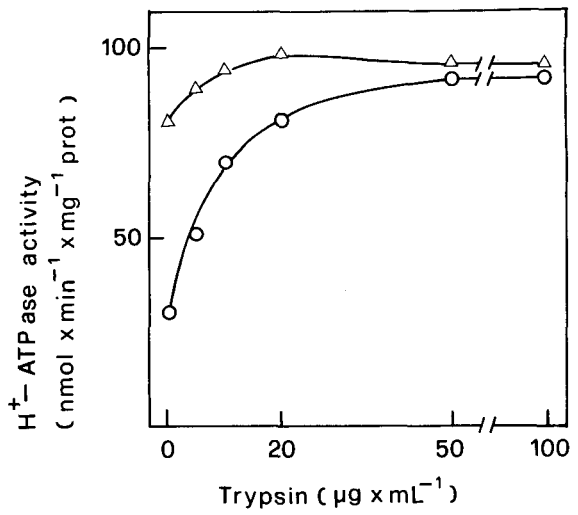

Figure 7. Effect of trypsin treatment on the PM H^+-ATPase of seedlings treated with FC in vivo. Microsomes were extracted from seedlings pretreated with (Δ) or without (O) 10 µM FC as described in the legend to Table III. Trypsin treatment was performed for 8 min in the presence of 1.1 mg microsomal protein mL^{-1}. PM H^+-ATPase activity was assayed at pH 7.5.

zyme, similar to the action of Glc fed in vivo to yeast (Serrano, 1983).

The PM H^+-ATPase Activated by FC Was Insensitive to Lysolecithin

Palmgren et al. (1991) showed that tryptic cleavage of the C-terminal regulatory domain of the PM H^+-ATPase did not further increase the activity stimulated by lysolecithin. We checked whether the sensitivity of the PM H^+-ATPase to lysolecithin was affected by FC. Data in Figure 8 show that lysolecithin stimulated the H^+-ATPase activity of PM from control seedlings about 3-fold and only by 30% that of PM from FC-treated seedlings. The effect of lysolecithin on the H^+-ATPase activated by FC was similar in extent to that of Brij 58 on both basal and FC-activated H^+-ATPase (Fig. 8): thus, it mainly reflected the unmasking of the activity of the minority of right-side-out vesicles.

DISCUSSION

The results reported in this paper confirm and extend the previous observations that controlled proteolytic treatment of the PM determines an activation of the H^+-ATPase by preferentially cleaving an inhibitory fragment of about 10 kD from the C-terminal domain (Palmgren et al., 1990, 1991; Olivari et al., 1993).

The analysis of the biochemical characteristics of the H^+-ATPase in trypsin-treated PM showed that controlled proteolysis increased the activity assayed at neutral to alkaline pH values much more than that assayed at or below the pH optimum of the native enzyme, so the ratio between the activity assayed at pH 6.6 and that at pH 7.5 decreased from about 2.5 in control PM to about 1 in trypsin-treated PM, and the pH optimum shifted from pH 6.8 to pH 7.1. Controlled proteolysis strongly increased the V_{max} of the enzyme and, less markedly, decreased its apparent K_m for Mg-ATP. Moreover, it activated only the portion of activity that was stable to preincubation in assay medium devoid of ATP (see also Olivari et al., 1993). All of these changes of the biochemical characteristics of the PM H^+-ATPase are very similar to those induced by FC (Rasi-Caldogno et al., 1986, 1989; De Michelis et al., 1988, 1991).

The effects of FC and of controlled proteolysis are not additive even under conditions in which the FC receptor is protected from the attack of trypsin, thus suggesting a common step in the mechanism through which the two treatments activate the PM H^+-ATPase. The observation that the molecular mass of the FC-activated PM H^+-ATPase was identical to that of the control enzyme (Fig. 6) rules out the possibility that the FC-receptor complex triggers the activation of the H^+-ATPase by stimulating the proteolytic cleavage of its C-terminal regulatory domain. It is worth noting that activation of the PM H^+-ATPase by lysolecithin is not additive with that of controlled proteolysis (Palmgren et al., 1991), and lysolecithin does not further stimulate the activity of the FC-induced PM H^+-ATPase.

These results suggest that FC, similar to lysolecithin and to Glc fed in vivo to yeast (Portillo et al., 1989; Palmgren et al., 1991), activates the PM H^+-ATPase by somehow modifying the enzyme conformation and thus hindering the interaction between the inhibitory C-terminal domain and the catalytic site.

FC-induced activation of the PM H^+-ATPase might involve a modification of the phosphorylation state of the enzyme.

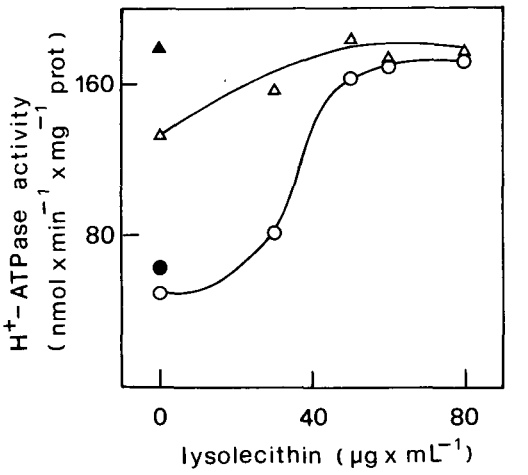

Figure 8. Effect of lysolecithin on the PM H^+-ATPase of seedlings treated with FC in vivo. PMs were isolated from seedlings pretreated with (Δ) or without (O) 10 µM FC as described in the legend to Table III. Plasma membrane H^+-ATPase activity was assayed at pH 7.5 in the presence of the specified concentrations of lysolecithin as described in "Materials and Methods," but in the absence of Brij 58 and in the presence of 5 µM carbonylcyanide *p*-trifluoro-methoxyphenylhydrazone and 5 mM $(NH_4)_2SO_4$. Closed symbols represent the activity measured in the presence of Brij 58 (100 µg mL^{-1}).

The PM H+-ATPase contains several potential sites for phosphorylation (Serrano, 1989; Palmgren, 1991), and its phosphorylation has been demonstrated in different conditions (Schaller and Sussman, 1988; Suzuki et al., 1992), but the effects of phosphorylation on the enzyme activity have not yet been unequivocally demonstrated (Zocchi et al., 1985; Suzuki et al., 1992).

It is also possible that the action of the FC-receptor complex involves a modification of the lipidic environment of the enzyme, which has important influence on its activity (Serrano, 1989; Palmgren, 1991). The lack of additivity between the effect of lysolecithin and those of FC (this paper) and of proteolytic cleavage of the C-terminal domain (Palmgren et al., 1991) is particularly interesting from this point of view.

Finally, from a physiological point of view, it is important to note that activation of the PM H+-ATPase by different treatments, such as Glc fed in vivo to yeast or FC in higher plants, involves a shift of the pH optimum of the enzyme activity toward more alkaline values. Such a shift makes the PM H+-ATPase much less sensitive to variations of pH in the physiological range of cytoplasmic pH values, and thus allows sustained stimulation of proton extrusion under conditions in which this leads to an increase of the cytosolic pH value (Marrè et al., 1993; Ullrich and Novacky, 1993).

The lack of additivity between the effect of FC and those of controlled proteolysis and of lysolecithin have been reported also in a paper by Johansson et al. (1993), published after completion of this paper.

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