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Characterization of a Plasma Membrane H^+-ATPase from the Extremely Acidophilic Alga *Dunaliella acidophila*

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Summary. *Dunaliella acidophila* is an unicellular green alga which grows optimally at pH 0–1 while maintaining neutral internal pH. A plasma membrane preparation of this algae has been purified on sucrose density gradients. The preparation exhibits vanadate-sensitive ATPase activity of 2 μmol P_i/mg protein/min, an activity 15 to 30-fold higher than that in the related neutrophilic species *D. salina*. The following properties suggest that the ATPase is an electrogenic plasma membrane H^+ pump. (*i*) ATP induces proton uptake and generates a positive-inside membrane potential as demonstrated with optical probes. (*ii*) ATP hydrolysis and proton uptake are inhibited by vanadate, diethylstilbestrol, dicyclohexylcarbodiimide and erythrosine but not by molybdate, azide or nitrate. (*iii*) ATP hydrolysis and proton uptake are stimulated by fussicoccin in a pH-dependent manner as found for plants plasma membrane H^+-ATPase. Unusual properties of this enzyme are: (*i*) the K_m for ATP is around 60 μM, considerably lower than in other plasma membrane H^+-ATPases, and (*ii*) the ATPase activity and proton uptake are stimulated three to fourfold by K^+ and to a smaller extent by other monovalent cations. These results suggest that *D. acidophila* possesses a vanadate-sensitive H^+-ATPase with unusual features enabling it to maintain the large transmembrane pH gradient.

Key Words H^+ · ATPase · plasma membrane · acidophile · fusicoccin

Introduction

Vanadate-sensitive plasma membrane proton pumps are characteristic of plants, yeast, fungi and related organisms. These H^+-ATPases play a major role in the growth and development of cells, in generating an electrical gradient across the plasma membrane, that provide the driving force for nutrient and ion uptake, regulation of intracellular pH, osmoregulation and several other essential functions (Sussman & Surowy, 1987). In plants, the activity is regulated by the phytohormones auxin and cytokinin and is activated by the fungal toxin fusicoccine and the bacterial toxin syringomycin. However, the significance and the regulatory mechanism of these factors are largely unknown (Serrano, 1988).

The H^+-ATPase has been characterized and purified from plasma membrane of several plants. The enzyme is composed of a single polypeptide of about 100 kDa. Recently the gene of this enzyme has been cloned from several plants, and the deduced sequence closely resembles that of plasma membrane H^+-ATPase from fungi and yeast (Serrano, 1988, 1989; Harper, Surowy & Sussman, 1989; Pardo & Serrano, 1989).

The existence of a plasma membrane H^+-ATPase in microalgae has been suggested on the basis of several physiological observations although very limited biochemical characterization of plasma membrane H^+-ATPase from microalgae has been reported. The group of Gimmler (Gilmour et al., 1985) and our group (Weiss, Sekler & Pick, 1989) have demonstrated that plasma membrane preparations from the halotolerant algae *Dunaliella* possess a vanadate-sensitive ATPase activity and suggested that it is a proton pump although we were unable to demonstrate ATP-dependent H^+ uptake in these preparations. Recently an acidophilic strain of *Dunaliella, D. acidophila*, has been described which grows optimally at pH 1 while maintaining an intracellular pH of 7 (Albertano et al., 1982; Gimmler et al., 1989*b*). Since *D. acidophila* sustains a pH gradient of 6 pH units across its plasma membrane, it should possess an extremely efficient proton pump and might well be a richer source for a plasma membrane H^+-ATPase.

In this paper we describe the characterization of vanadate-sensitive ATPase from plasma membrane vesicles of *D. acidophila* and demonstrate that it is an electrogenic proton pump.

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Materials and Methods

Growing of Cells

Dunalliella acidophila (Masyuk strain no. SAG 19.85, algal collection of the Institute of Plant Physiology, University of Göttingen, FRG) was a generous gift from Dr Schlösser in Göttingen. The cells were grown in 5–20 liter bath cultures as previously described (Weiss et al., 1989). The growth medium contained 2 mM $MgSO_4$, 2 mM K_2HPO_4, 5 mM $(NH_4)_2SO_4$, 1.5 μM EDTA, 0.3 μM $FeCl_3$, 250 mM of Na_2SO_4, and the micro elements: 0.185 mM H_3BO_3, 7 μM $MnCl_2$, 0.8 μM $ZnCl_2$, 20 nM $CoCl_2$ and 0.2 nM $CuCl_2$. The pH of the growth medium was adjusted to 1 with concentrated sulphuric acid.

Isolation of Plasma Membranes

Two to fifteen liters of algae, 10^7 cells/ml, were collected by centrifugation (500 × *g*, 10 min) and washed once in 1 liter of glycerol buffer containing 0.7 M glycerol, 30 mM Tris-MES, pH 6.5, and 2 mM $MgCl_2$. From this stage all purification steps were performed at 0–4C°. The cells were resuspended in 30–40 ml of glycerol buffer and osmotically ruptured by dilution to a final volume of 270–350 ml in bursting buffer, containing 5 mM EDTA, 5 mM β mercaptoethanol, 20 mM Tris-MES, pH 6.5, 1% polyvinylpyrrolidone (PVP-40), 5 mM γ aminocaproic acid and 1 mM benzamidine. Following 10-min incubation the cells were passed twice through a Yeda press apparatus (pressure of 1000 atm, flow rate of approximately 100 ml/min). Two hundred mM ethanolaminechloride, 100 mM cholinechloride and 50 mM KCl were added to the ruptured cells. Unbroken cells and cell debris were removed by a brief centrifugation (5000 × *g*, 10 min) and the resulting supernatant was supplemented with 10% glycerol. Fractionation of plasma membranes was obtained by layering 30 ml of this mixture onto 30/43% sucrose, 18 ml each, containing also 2 mM EDTA, 20 mM Tris-MES, 0.5% PVP-40, 50 mM KCl and 200 mM ethanolamine chloride. The gradients were centrifuged at 25,0000 × *g* for 120 min and the interphase between the 30 and 43% sucrose was carefully collected with a syringe, diluted to 300 ml with suspension buffer containing 1 mM EDTA, 10 mM Tris-MES, pH 6, 10 mM KCl, 250 mM sucrose and 1 mM β mercaptoethanol and centrifuged again. The pellet was resuspended in 4–10 ml suspension buffer, homogenized with a potter homogenizer, centrifuged at 500 × *g* for 10 min to remove residual aggregates and kept at −196°C.

ATPase Assay

Enzyme samples (2–10 μg protein) were incubated in 200 μl of buffer containing 10 mM Tris-MES, pH 6, 5 mM $MgCl_2$ and 2 mM Tris-ATP. Unless otherwise indicated, the released P_i was determined as described before (Ames, 1966). One unit is the enzyme activity which hydrolyses 1 μmol P_i per 1 min at 37°C. The K_m for ATP was determined in the absence or presence of an ATP-regenerating system that included 5 units of pyruvate kinase and 5 mM of phosphoenolpyruvate.

Measurements of ΔpH and Δψ in Plasma Membrane Vesicles

ATP-dependent internal acidification of plasma membrane vesicles was followed by measuring the fluorescence quenching of the acridine dye 9-aminochloro-2-metoxyacridine *bis*(hexafluoracetonyl)aceton (ACMA)[1]. Plasma membrane vesicles (30–60 μg protein) were suspended in buffer containing Tris-MES at pH 6.0, 10 mM $MgSO_4$, 4 μM ACMA and 12–25 mM alkali salt. The reaction was started by addition of ATP (0.5–1 mM). Fluorescence changes were measured at 25°C in a Perkin-Elmer MPF-44A spectrofluorimeter at 412 nm (excitation) and 480 nm (emission). Generation of positive-inside transmembrane electrical potential (Δψ) was measured by following oxonol VI absorbance changes at 603 and 590 nm in an Aminco DW 2a dual-wavelength spectrophotometer (Silver-Springs, MD). The reaction mixture contained 10 mM Tris-MES, pH 6.0, 5 mM $MgSO_4$, 1.2–2.4 μM oxonol VI and 20–40 μg plasma membrane protein vesicles. Absorbance changes were initiated by addition of 0.5 mM ATP.

Analytical Procedures

Protein was determined by a modification of the Lowry procedure (Markwell et al., 1978) with bovine serum albumin as a standard.

Results

Purification of Plasma Membrane Vesicles from *Dunaliella acidophila*

In a previous paper we have reported a technique for isolation of plasma membrane vesicles from *D. salina* by a mild osmotic shock (Weiss et al., 1989). This technique turned out to be inefficient for lysing of *D. acidophila* since even a 10-fold dilution in water resulted in a poor yield of plasma membranes. A harsher procedure was developed by transferring of hypotonically treated cells twice through a Yeda press apparatus. This resulted in a high yield of plasma membrane vesicles as indicated by the increase of vanadate-sensitive ATPase activity. Several other changes which have been introduced are as follows. (*i*) The pH of the lysis buffer was changed from 1 in the growth medium to 6, in order to avoid inactivation upon bursting of the cells. (*ii*) Inclusion of polyvinylpyrrolidone and of phospholipase inhibitors EDTA, choline chloride and ethanolamine re-

[1] *Abbreviations:* ACMA: 9-aminochloro-2-metoxyacridine *bis*(hexafluoracetonyl) acetone; oxonol VI: *bis*-(3-propyl-5-oxois-oxazol-4-yl) pentamethine oxonol; SF-6847: 3,5-di(tert-buthyl)-4-hydroxybenzylidene malonitrile; DCCD: dicylohexylcarbodiimide; DES: diethylstibestrol; NEM: N-ethylmaleiimide; pCMBS: *p*-chlorobezenesulphonate; MES: 4-morpholinoethanesulphonate.

Table 1. Isolation of vanadate-sensitive plasma membrane ATPase fraction[a]

Purification stage	Total protein (mg)	Total activity (μmol P_i/min)	Specific activity (U)	Fold purification	Yield (% total)
Total cell lysate	4560	19	4.2×10^{-3}	—	100
Sup of low speed centrifugation	450	14.4	3.2×10^{-2}	7.6	75
30/43% sucrose interphase	6	7.8	1.33	326	41

[a] The data correspond to a typical preparation starting with 3 liters of algae that were harvested as described in Materials and Methods.

Table 2. Characterization of the inhibitor sensitivity of *D. acidophilla* plasma membrane ATPase[a]

Inhibitor	Concentration	ATPase activity (% of control)	IC_{50}
Vanadate	25 μM	33	12.5 μM
DCCD	33 μM	44	20 μM
DES	120 μM	43	40 μM
Erythrosine	10 μM	38	3 μM
NaN_3	2 mM	93	—
NO_3	80 mM	100	—
NH_4MO_4	100 μM	87	—
$HgCl_2$	10 μM	21	4 μM
PCMBS	7.5 μM	30	10 μM
NEM	10 μM	20	2 mM

[a] Isolated plasma membrane vesicles (5 μg protein) were preincubated with the indicated inhibitors for 10 min at 0°C before the ATPase assay. IC_{50} is the inhibitor concentration required for 50% inhibition.

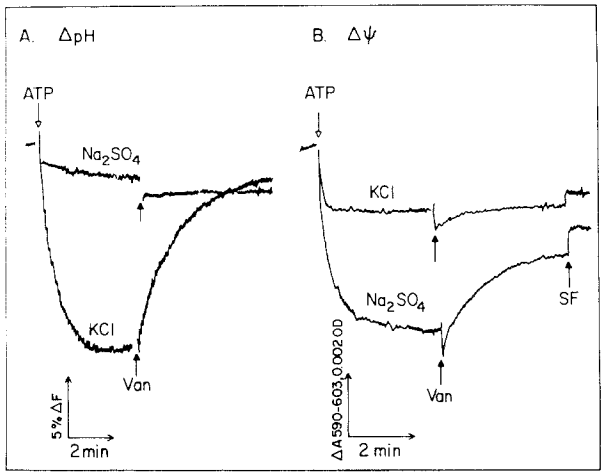

Fig. 1. (*A*) ATP-dependent ΔpH formation across plasma membrane vesicles. ACMA fluorescence quenching was measured in 2 ml containing plasma membrane vesicles (25 μg protein) 5 mM $MgSO_4$ and 25 mM Na_2SO_4 or 5 mM $MgCl_2$ and 25 mM KCl, 5 mM Tris-MES, pH 6, and 1 μM ACMA. ATP (0.5 mM) and vanadate (200 μM) were added as indicated. (*B*) ATP-dependent $\Delta\psi$ formation across plasma membrane vesicles. Assay conditions were as in Fig. 1*A* except that 2 μM oxonol VI replaced ACMA. SF-6847 (0.2 μM) was added when indicated

sulted in a significant stabilization of the vanadate-sensitive ATPase activity. (*iii*) The two-step sucrose gradient of 30 and 43% turned out to be optimal for separation of the vanadate-sensitive ATPase activity from thylakoid membranes, which are recovered in the pellet, and from two lighter membrane fractions which are retained above the 30% sucrose layer. As seen from the summary of the protocol in Table 1, this procedure results in a 300-fold purification of ATPase activity which is inhibited 80–90% by 100 μM orthovanadate.

In order to evaluate possible contamination of the preparation by other ATPase-containing membranes, the sensitivity to inhibitors of different classes of ATPase has been tested, (*see* Table 2). Four typical inhibitors of plasma membrane H^+ ATPases, orthovanadate, DCCD, DES and erythrosine, all inhibit ATP hydrolysis at micromolar concentrations. In contrast inhibitors of mitochondrial and vacuolar ATPase, and of alkaline phosphatase, such as azide, nitrate or molybdate ions, had little or no effect on the activity. These results indicate that the preparation is essentially free of contaminating ATPases. The ATPase activity is also particularly sensitive to thiol reagents such as mercurials and maleimides similar to other plasma membrane H^+-ATPase.

ATP-Dependent Proton Uptake

Conclusive identification of the electrogenic H^+-ATPase requires demonstration of ATP-dependent H^+ uptake. Figure 1 demonstrates that addition of ATP to the enriched plasma membrane preparation results in generation of a pH (Fig. 1*A*) and electrical (Fig. 1*B*) gradients. Internal acidification (ΔpH formation) measured by the fluorescence quenching of an acridine dye (ACMA), requires the presence of permeable ions, probably for charge neutralization, and was found to be maximal in KCl medium and

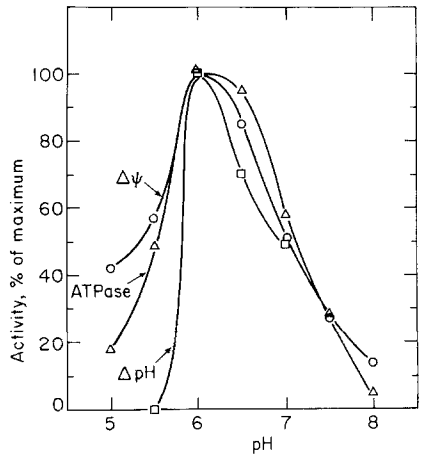

Fig. 2. pH dependence of plasma membrane H^+ ATPase. Sample of plasma membrane vesicles were assayed for ATPase activity (5 μg protein), ΔpH formation (20 μg protein) and $\Delta\psi$ formation (20 μg protein) in the presence of the following buffers: Tris-MES (pH 5.0–6.5) or Tris-HEPES (pH 7.0–9.0)

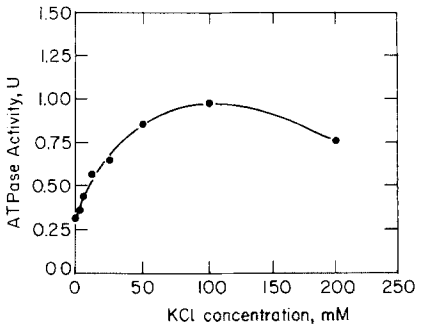

Fig. 3. Stimulation of ATPase activity by K^+. Plasma membrane vesicles were dialyzed twice for 2 hr against 500 ml of 10 mM Tris-MES buffer, pH 6, containing 200 mM sucrose and 2 mM $MgCl_2$. ATPase activity was assayed immediately after dialysis in the presence of the indicated concentration of KCl

Table 3. Stimulation of ATPase activity and proton transport by monovalent cations[a]

Cation	Activity, % of maximum	
	ATPase	ΔpH
—	29	10
Li^+	44	29
Na^+	63	66
K^+	100	100
Rb^+	58	—
Cs^+	51	—

[a] Plasma membrane vesicles were dialyzed as described in Fig. 3. The ATPase activity and ACMA fluorescence quenching were measured in the presence of 50 mM of the indicated cation chloride salts as described in Materials and Methods. Control ATPase activity was 0.9 U/mg protein.

minimal in Na_2SO_4 medium (Fig. 1*A*). Conversely, formation of a positive inside transmembrane electrical potential ($\Delta\psi$), measured from absorbance changes of oxonol VI, is maximal in Na_2SO_4 and is significantly reduced in a KCl medium (Fig. 1*B*). The latter effect is probably due to the rapid charge neutralization by Cl^- influx or K^+ efflux. Both signals are sensitive to the ATPase inhibitor vanadate and to protonophores such as SF-6847. These results suggest that the *D. acidophila* plasma membrane ATPase is an electrogenic H^+ pump.

pH Optimum and Dependence on K^+ Ions

Two characteristic properties of the plasma membrane H^+-ATPase from plants and yeast are a pH optimum around 6.5 and stimulation by K^+ ions (Goffeau & Slayman, 1981). As shown in Fig. 2 the pH optimum for ATP hydrolysis, ΔpH or $\Delta\psi$ formation by the *D. acidophila* enzyme is exceptionally sharp around 6. The activity of the pump is almost completely inhibited at pH 8. This pH dependence apparently reflects the cytoplasmic pH optimum of the ATPase and not the external pH, consistent with an inside-out orientation.

A stimulation by K^+, does not usually exceed twofold in plants and fungi, but is exceptionally prominent in *D. acidophila*, reaching three to fourfold stimulation at 100 mM (Fig. 3). The relative specificity for K^+ in comparison to other monovalent cations is summarized in Table 3. Clearly K^+ stimulates both ATP hydrolysis and H^+ uptake more than other cations. It may be noted that this comparison was made with chloride salts (presumably permeable) in order to avoid possible constrains due to limited charge compensation in the event that K^+ must permeate in order to activate. In fact it is likely that the effect of K^+ is at the cytoplasmic surface since ATPase activity was hardly affected by valinomycin or by replacing chloride with different less permeable anions including sulfate or nitrate (*not shown*). These results suggest a general resemblance to plasma membrane H^+-ATPase from plants and fungi, with the difference of a slightly more acidic pH optimum and a high K^+ stimulation.

Substrate Specificity of the ATPase

The plasma membrane H^+-ATPase activity is highly specific for ATP in both ATPase activity and in ΔpH formation by comparison with other nucleotides like

Table 4. Substrate specificity of *D. acidophila* ATPase[a]

Nucleotide	Activity, % of control	
	P_i released	ΔpH
ATP	100	100
GTP	4	0
ITP	1	0
UTP	1	0
CTP	9	5
ADP	4	2

[a] The release of inorganic phosphate and the H^- transport of the plasma membrane vesicles was measured as described in Materials and Methods with 2 and 0.5 mM of the indicated nucleotide for the ATPase and H^+ transport assays, respectively. Control ATPase activity was 1.1 U/mg protein. Control ΔpH was 18% ACMA fluorescence quenching.

Table 5. Effect of divalent cations on ATPase activity[a]

Addition	ATPase activity (% of control)
Mg	100
EDTA	3
Mn	34
Co	90
Zn	1

[a] ATPase activity was measured in the presence of 2 mM ATP and 3 mM of the indicated divalent cations or with 1 mM EDTA as described in Materials and Methods. Control ATPase activity with Mg was 1.1 U/mg protein.

GTP, UTP, ITP, CTP and ADP (Table 4). The only notable exception, in comparison to other H^+-ATPases, is the partial activity with CTP (reviewed by Goffeau & Slayman, 1981). The specificity for Mg^{2+} is less striking, with Co^{2+} and to a lesser extent Mn^{2+}, but not Zn^{2+}, substituting for Mg^{2+} in catalyzing ATP hydrolysis (Table 5). This specificity resembles that found for *Neurospora* and *Sch. pombe* H^+-ATPase at metal/ATP ratio of 1.5 (Dufour & Goffeau, 1980; Bowman, Blasco & Slayman, 1981). Ca^{2+} is a competitive inhibitor of ATP hydrolysis with respect to Mg^{2+}, having an apparent K_i of 20 μM (Fig. 4). The K_m value for ATP of plasma membrane H^+-ATPase is controversial, values ranging from 0.1 to 3 mM have been reported (reviewed by Goffeau & Slayman, 1981). The K_m values for ATP hydrolysis, ΔpH or $\Delta\psi$ at saturating Mg^{2+} concentration (5 mM) for *D. acidophila* are 59, 70 and 57 μM, respectively (Fig. 5, ΔpH *not shown*). It may be noted that ATP hydrolysis was measured in the presence of an ATP-regenerating system to avoid substrate depletion. These results demonstrate that *D. acidophila* plasma membrane H^+-ATPase has an exceptionally high affinity for ATP.

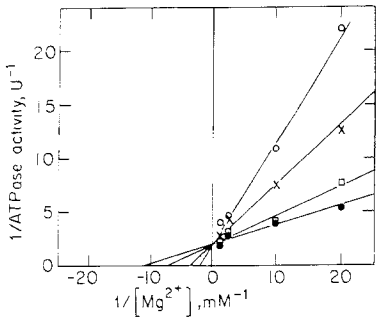

Fig. 4. Lineweaver-Burk analysis of the inhibition of Mg-dependent ATPase activity by Ca. The reaction buffer contained 50 mM KCl, 10 mM Tris-MES, and 2 mM ATP, the indicated concentration of Mg and 0, 50, 100, or 200 μM of Ca. The calculated K_i for Ca is 20 ± 3 μM

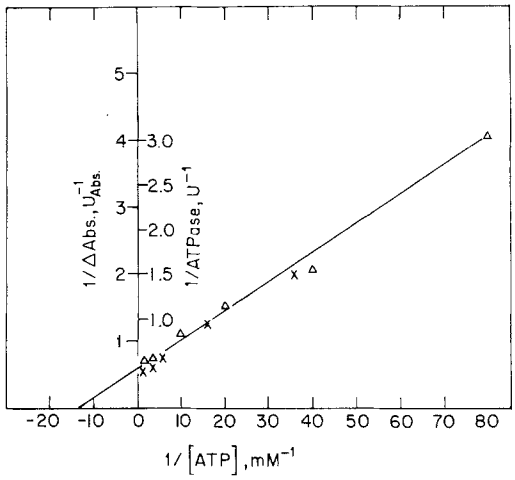

Fig. 5. Lineweaver-Burk analysis of the ATP concentration dependence for the ATPase activity and $\Delta\psi$ formation. ATPase activity or $\Delta\psi$ formation assays were performed as described in Materials and Methods

Stimulation of the *D. acidophila* ATPase by Fussicoccin

Fussicoccin was previously demonstrated to stimulate the plasma membrane H^+-ATPase from plants, both in vivo and in purified plasma membrane (reviewed by Serrano, 1989) but not that from yeast or fungi. Figure 6 demonstrates that fusicoccin also stimulates ATPase activity and proton uptake (*inset*) in a pH-dependent fashion, similar to previous observations in plants (Rasi-Caldogno et al., 1986; Serrano, 1989). We have observed that the effectiveness of fusicoccin depends on a fairly prolonged preincu-

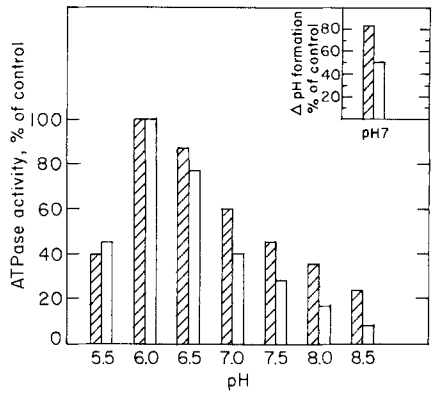

Fig. 6. pH-dependent stimulation of H^+-ATPase activity by fusicoccin. Plasma membrane vesicles were incubated at 0°C for 2 hr in a medium containing 200 mM sucrose, 10 mM Tris-HEPES, 2 mM $MgCl_2$ and with (striated columns) or without (blank columns) 10 μM fusicoccin followed by ATPase assay as described in Fig. 4. Insert shows ATP-dependent ΔpH formation (at pH 7) in plasma membrane vesicles incubated in the presence (striated column) or absence (blank column) of 10 μM fussicoccin

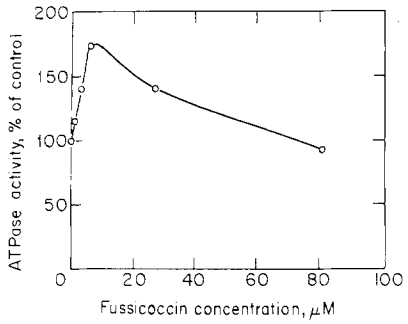

Fig. 7. Stimulation of ATPase activity by fusicoccin. Plasma membrane vesicles were incubated in the presence of the indicated fusicoccin concentrations at pH 7 as described in Fig. 6, and ATPase activity was determined as described in Materials and Methods

bation with the membrane vesicles (2 hr on ice). The maximal stimulation is obtained at alkaline pH (over twofold above pH 8) whereas no stimulation is obtained below pH 6. Surprisingly we find that excess of fusicoccin (above 10 μM) inhibits ATPase activity (Fig. 7) unlike the situation in plants. These results suggest an unexpected similarity between the *D. acidophila* and plant plasma membrane H^+-ATPases, since fussicoccin has been considered to be unique effector of higher plants.

Discussion

Plasma membrane H^+-ATPases have been purified and characterized from plants, fungi and yeast which grow at either neutral or moderately acidic pH (Sussman & Surowy, 1987; Serrano, 1988, 1989), but this enzyme has never been characterized in plasma membranes of an extreme acidophile which grows at pH 0–1. Since the H^+ pump is likely to be the major (if not the only) mechanism to sustain a pH gradient of 6–7 pH units in *D. acidophila*, the enzyme could be expected either to be overproduced and/or exceptional in its kinetic properties, in order to cope with large pH stresses.

The K_m value for ATP of *D. acidophila* plasma membrane H^+-ATPase is about an order of magnitude lower than that of previously described plasma membrane H^+-ATPases (Goffeau & Slayman, 1981; Serrano, 1988), and its pH optimum is slightly more acidic. These properties may have an important physiological significance in enabling *D. acidophila* to withstand acid stresses which may be encountered upon exposure to weak acids, high CO_2 or transient permeabilization of the plasma membrane. We have previously observed that acid stress, induced by addition of acetic acid, leads to a rapid drop of about 95% in the cellular ATP level (Gimmler et al., 1989*a*). A high affinity for ATP can enable the H^+ pump to function at maximal rate under such conditions in order to relieve the acid stress. The sharp acidic pH optimum as well as the high internal buffering capacity and positive membrane potential (Gimmler et al., 1989) also seem to reflect adaptations to resist cytoplasmic acidification in *D. acidophila*.

The large stimulation of ATP hydrolysis and H^+ uptake by K^+ is exceptional for plasma membrane H^+-ATPases. Interestingly, the only report of a similar large and relatively specific stimulation by K^+ is for an acidophilic yeast *Metschnikowia reukaufii* (Gläser & Höfer, 1987). The mechanism of K^+ stimulation is not clear and has been attributed to stimulation of the hydrolysis of the phosphorylated intermediate E_2-*P*, to a shift of the equilibrium between the two enzyme conformers (Briskin, 1988), or a primary H^+/K^+ antiport activity of the pump (Briskin, 1986). We have observed that in *D. acidophila* plasma membranes valinomycin does not stimulate ATP hydrolysis (I. Sekler, *unpublished observations*). In another report we will provide conclusive evidence that the *D. acidophila* ATPase is not a primary H^+/K^+ pump from studies of ^{86}Rb transport in a reconstituted plasma membrane system (Gläser, Sekler & Pick, 1990). These results suggest that K^+ is not a substrate but an activator of the plasma membrane H^+-ATPase in *D. acidophila* at the cytoplasmic surface.

The observation that fussicoccin stimulates the *D. acidophila* ATPase is of particular interest since this fungicide is considered to be a specific activator in plants, and supposedly mimics effects of plant

hormones. It has been demonstrated that fussicoccin does not bind to the 100-kDa ATPase protein itself but to a different polypeptide (Stout & Cleland, 1980; Feyerbend & Weiler, 1988) which may be a regulatory subunit of the H^+-ATPase. The mechanism of action of fusicoccin becomes even more perplexing considering recent studies which suggest that the primary target of fusicoccin is a K^+ transport system and not the H^+-ATPase (Blatt & Clint, 1989). In any event the presence of a fusicoccin-stimulated H^+-ATPase in plasma membrane of a unicellular algae raises interesting questions concerning the function and evolutionary origin of this regulatory mechanism.

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