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Immunological cross-reactivity and inhibitor sensitivities of the plasma membrane H^+-ATPase from plants and fungi

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Properties of the plasma membrane proton pump (H^+-ATPase) isolated from several species of higher plants were compared to those isolated from the mycelial fungus, *Neurospora crassa*. Under identical experimental conditions, differences were observed in the vanadate concentrations required for half-maximal inhibition (1 μM and 10 μM, respectively, for fungal and plant enzymes) and in the stability towards treatment with detergents at 30°C. Similarities were noted in the reactivity to *N,N'*-dicyclohexylcarbodiimide, an irreversible inhibitor that reacts with an essential amino acid in the putative proton-transport site (Sussman, M.R. and Slayman, C.W. (1983) J. Biol. Chem. 258, 1839–1843). A structural comparison was performed using immunoblot analysis with specific polyclonal antibodies directed towards the M_r = 100 000 polypeptide of the enzyme isolated from hyphal cells of *N. crassa* and from root cells of oat. Weak cross-reactivity was observed between the fungal and plant enzymes. Strong cross-reactivity was observed between the M_r = 100 000 H^+-ATPases of oat and tomato or potato roots, providing evidence for structural homology between the enzymes isolated from phylogenetically diverse species of higher plant.

Introduction

An electrogenic proton pump (H^+-ATPase) has been identified in the plasma membrane of both fungi and higher plants [1,2]. The enzyme converts chemical energy obtained from hydrolysis of ATP into a proton-motive force. This protonmotive force in turn is used to drive the transport of solutes across the membrane and in plants may also be involved in regulating growth [2]. The plasma membrane H^+-ATPase has a catalytic polypeptide of $M_r = 100\,000$ that is phosphorylated during the reaction cycle. These structural properties relegate this protein to a group of M_r = 100 000 cation-translocating ATPases including the $(Na^+ + K^+)$-ATPase of animal cell membranes, the Ca^{2+}-ATPase of sarcoplasmic reticulum, the $(H^+ + K^+)$-ATPase of gastric mucosa and the K^+-ATPase of *E. coli*. At present the fungal H^+-ATPase is much better characterized than the higher plant H^+-ATPase and, in fact, purification to homogeneity and reconstitution into synthetic liposomes without loss of catalytic activity has been achieved [3,4]. The higher plant H^+-ATPase has been purified to near homogeneity only recently [5,6].

Since the electrogenic plasma membrane H^+-ATPase appears to be found only in fungi and plants, there is a need for direct comparison of the enzyme from these two types of organism. We have compared inhibitor sensitivities of the plasma

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Abbreviations: P_i, inorganic phosphate; EDAC, 1-ethyl-3-(dimethylaminopropyl)carbodiimide; DCCD, *N,N*-dicyclohexylcarbodiimide.

membrane H^+-ATPases prepared from the mycelial fungus *N. crassa* and two higher plants, oat (a monocot) and tomato (a dicot). Specifically, the characteristics of inhibition by vanadate and by *N,N'*-dicyclohexylcarbodiimide (DCCD) were examined. Vanadate has been shown to inhibit the ion-translocating ATPases by interfering with formation of the phosphorylated intermediate [7]. DCCD has been shown to inhibit several cation-translocating ATPases including the F_0F_1 H^+-ATPase [8], the plasma membrane H^+-ATPase from *N. crassa* [9] and the Ca^{2+}-ATPase of sarcoplasmic reticulum [10]. Inhibition occurs through covalent modification of a single essential residue involved in cation translocation [10,11]. We also compared detergent sensitivities of these enzymes in order to gain information on the lipid requirements of the H^+-ATPases.

Very little structural information is available on the $M_r = 100000$ H^+-ATPases, chiefly because of difficulties in sequencing hydrophobic proteins. The recent observation that two disparate $M_r = 100000$ cation-translocating ATPases (the K^+-ATPase of *E. coli* and the Ca^{2+}-ATPase of muscle cells) share sequence homology [12] raises the possibility of conserved sequences in all such proteins. Structural relatedness of the plasma membrane H^+-ATPases from fungi and plants was investigated using immunoblot analysis [13,14]. Specific polyclonal antibodies directed to the $M_r = 100000$ plasma membrane H^+-ATPases from *N. crassa* and oat roots were generated and used to investigate immunological cross-reactivities of the plasma membrane H^+-ATPases from *N. crassa* and from oat, tomato and potato roots.

Materials and Methods

Plasma membrane preparation. Plasma membranes were isolated from roots of 5–6-day-old oat seedlings (Olds Seed Co., Madison, WI) grown under a 24 h dark regime at 28°C in flats containing moist vermiculite, from roots of tomato plants grown hydroponically in the greenhouse for approx. 1 month, and from 14-day-old potato plants grown from meristem cuttings in tissue culture. Membrane isolation was based on the procedure of Vara and Serrano [5] with some modification. All solutions were at 2°C and contained 1 mM PMSF. Roots were homogenized in a medium consisting of 0.25 M Tris-HCl/25 mM EDTA/35 mM 2-mercaptoethanol/1.15 M sucrose (pH 8.5) at 0.5 ml/g roots. After an initial centrifugation at $8000 \times g$ for 15 min, the microsomal fraction was pelleted by centrifugation at $48000 \times g$ for 1.5 h. It was resuspended in 20% (v/v) glycerol/10 mM Tris-HCl (pH 7.5), containing 1 mM dithiothreitol and 1 mM EDTA, and fractionated by centrifugation at $200000 \times g$ for 1.5 h on a discontinuous sucrose gradient (1 ml of 46% (w/w) sucrose and 1.5 ml of 33% (w/w) sucrose in the same buffer). The plasma membrane fraction was isolated from the lower interface.

Plasma membranes were isolated from wild type strain RL21a of *N. crassa* by sonication of enzyme-treated mycelial pads according to the procedure of Bowman et al. [15], as modified by Sussman and Slayman [9].

Removal of protein from plasma membranes by treatment with low concentrations of deoxycholate. Deoxycholate treatment of plasma membranes was used to remove approx. half of the membrane protein without solubilizing the $M_r = 100000$ H^+-ATPase. The procedure of Bowman et al. [4] was followed, except when deoxycholate-treated membranes were subjected to HPLC, in which case the modification of Sussman [16] was used. Specific details of protein and deoxycholate concentrations are given in Table I.

Purification of the H^+-ATPase. Plasma membranes prepared as described above were washed with Triton X-100 instead of deoxycholate, solubilized with lysolecithin, and run on a glycerol density gradient according to Serrano [6].

ATPase assay, detergents and inhibitors. ATPase activity was assayed colormetrically, by release of P_i from ATP. The standard reaction mixture contained an ATP-regenerating system and consisted of 5 mM Na_2ATP/5 mM $MgCl_2$/5 mM phospho*enol*pyruvate/50 μg per ml pyruvate kinase/11 mM $(NH_4)_2SO_4$/50 mM KCl/5 mM NaN_3 (to inhibit mitochondrial ATPase) and 10 mM 1,4-piperazinediethanesulfonic acid, adjusted to pH 6.7 with Tris base. For plant plasma membrane H^+-ATPase, the mixture contained 100 mM KNO_3 (to inhibit vacuolar ATPase) instead of 50 mM KCl, and in addition, 0.1 mM ammonium molybdate (to inhibit non-specific phosphatases). Reactions were initiated by addition of plasma mem-

brane to 0.5 ml reaction mixture. After 15–20 min at 30°C, reaction was stopped by addition of 0.1 ml 50% (w/v) trichloroacetic acid and P_i assayed for by the method of Dryer et al. [17]. Detergents and inhibitors were made up as concentrated (at least 100-fold) stock solutions.

Sodium orthovanadate (Sigma Chemical Co., St. Louis, MO) was used to prepare a 100 mM stock solution of vanadate. After dissolving in water, the pH of the solution was brought to 9.5 with HCl and the solution was boiled until it turned colorless. The pH of the solution was adjusted further with HCl, followed by boiling, until a pH of 7.5 was obtained. At this pH, and at the concentration of vanadate used in the assay (not more than 0.1 mM), the most predominant vanadate species are $H_2VO_4^-$ and HVO_4^{2-} [18]. Prior to assay, plant plasma membranes were washed extensively by centrifugation and resuspension in 10% (v/v) glycerol/1 mM EGTA, adjusted to pH 7.5 with Tris base. This procedure was used to remove EDTA, which complexes vanadate [19]. For vanadate concentrations above 10 μM, phosphate standard controls containing the appropriate concentration of vanadate were included in the assay to correct for the decrease in absorbance at 710 nm caused by vanadate interference [19].

N,N'-dicyclohexylcarbodiimide (DCCD) and 1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDAC) were made up as concentrated stock solutions in ethanol. Assays were carried out using glassware only, since DCCD binds to plastic [9]. For the experiment of Fig. 3, membranes were suspended in reaction mix lacking ATP, $MgCl_2$, phospho*enol*pyruvate and pyruvate kinase (in $(NH_4)_2SO_4$). Carbodiimide was added and incubation carried out as indicated. ATP, $MgCl_2$, phospho*enol*pyruvate and pyruvate kinase were then added from a stock solution to standard concentrations (see above) and ATPase activity was measured. For the experiment of Fig. 4, membranes were suspended in 1 mM EGTA, adjusted to pH 7.5 with Tris base, DCCD was added and the suspension incubated as indicated. The DCCD reaction was stopped by 20-fold dilution into standard plant ATPase reaction mixture and ATPase activity measured directly.

Protein determination. Plasma membrane protein was determined by the method of Lowry [20] and purified ATPase was determined by the method of Bradford [21].

SDS polyacrylamide gel electrophoresis. Discontinuous SDS polyacrylamide gel electrophoresis was carried out according to Laemmli [22]. Reagents were from BioRad Labs., Richmond, CA, U.S.A.

Antibody generation. New Zealand white rabbits were used to generate polyclonal antibodies against the $M_r = 100000$ plasma membrane H^+-ATPases of *N. crassa* and oat roots. For the primary immunization, the *N. crassa* enzyme was purified by HPLC as described by Sussman [16], concentrated and then emulsified with an equal volume of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO, U.S.A.) for injection. For subsequent immunizations and for all immunizations carried out to generate antibody directed toward the oat root plasma membrane $M_r = 100000$ H^+-ATPase, the $M_r = 100000$ band was excised from SDS polyacrylamide gels of deoxycholate-washed membranes. Deoxycholate washing was carried out as described under Materials and Methods and in the legend to Table I. Gels were 1.5 mm thick with 5% (w/v) acrylamide stacking gels and 8% (w/v) acrylamide running gels. Gel slices were homogenized and injected at multiple intradermal sites. Approx. 0.1 mg of $M_r = 100000$ H^+-ATPase was used for each immunization.

Immunoblotting and dot-blots. Following SDS polyacrylamide gel electrophoresis, proteins were transferred electrophoretically onto nitrocellulose paper (HAHY 304 FO, Millipore Corp., Bedford, MA, U.S.A.) at 200 mA for 2.5 h in 25 mM Tris/192 mM glycine/20% methanol (pH 8.3) buffer containing 0.02% (w/v) SDS (Transblot cell, BioRad Labs., Richmond, CA, U.S.A.) [13,14].

Visualization of protein electroblotted onto nitrocellulose was with 0.1% (w/v) aniline blue in isopropanol/acetic acid/water (25 : 10 : 65, v/v) and destaining was done in the same solvent mixture.

Immunological detection was based on the method of Knecht and Dimond [23], as modified by Vierstra et al. [24]. The nitrocellulose paper was incubated overnight at room temperature in a blocking solution consisting of 10 mM Tris-HCl, 150 mM NaCl (Tris-saline), 1% (w/v) bovine serum

TABLE I

REMOVAL OF PROTEIN FROM PLASMA MEMBRANES BY TREATMENT WITH LOW CONCENTRATIONS OF DEOXYCHOLATE

Plasma membranes were suspended at 2 mg/ml (*N. crassa*, oat) or 1 mg/ml (tomato) protein concentration, in buffer containing 0.2 M KCl, 2 mM EDTA and 25 mM Tris, adjusted to pH 7.5 with HCl. The appropriate volume of a 10% (w/v) sodium deoxycholate solution (pH 8.0) was added (final concentrations: 0.2% deoxycholate for *N. crassa* and oat, 0.05% for tomato) and the membranes centrifuged at 200000 × *g* for 1 h. The pellet was resuspended at 5 mg protein/ml in 45% (v/v) glycerol/10 mM Tris-2 mM EGTA, adjusted to pH 7.5 with HCl. All solutions were kept at 2°C. Protein and ATPase activities were determined as described under Materials and Methods.

Plasma membrane		ATPase activity		Total protein	
		specific activity (μmol/min per mg protein)	yield (%)	amount (mg)	yield (%)
N. crassa	unwashed	4.9	100	1.4	100
	washed	9.6	84	0.6	43
Oat root	unwashed	2.0	100	1.2	100
	washed	3.9	81	0.5	42
Tomato root	unwashed	1.2	100	0.76	100
	washed	2.2	89	0.37	33

albumin and 0.02% (w/v) NaN_3, pH 7.3 (25°C). Primary antibody was either *N. crassa* M_r = 100000 H^+-ATPase serum (preimmune or immune) diluted 1 : 1000 or oat root M_r = 100000 H^+-ATPase serum (preimmune or immune) diluted 1 : 1000 in blocking buffer. The nitrocellulose paper was incubated with primary antibody for 2 h at room temperature. It was then washed for 1 h at room temperature with four changes of Tris-saline containing 0.1% (w/v) bovine serum albumin and 0.1% (v/v) Triton X-100, pH 7.3 (25°C). Second antibody was affinity-purified alkaline phosphatase-conjugated goat antibody to rabbit IgG (Kirkegaard and Perry Labs., Gaithersburg, MD, U.S.A.) diluted to 1 μg/ml with blocking buffer. The nitrocellulose paper was incubated with this antibody for 1 h at room temperature and then washed for 1 h at room temperature with four changes of Tris-saline containing 0.1 (w/v) bovine serum albumin, 0.1% (v/v) Triton X-100 and 0.05% (w/v) SDS (pH 7.3) (25°C).

Visualization involved a modification of the alkaline phosphatase-staining procedure developed by Knecht and Dimond [23] based on a staining method described by Leary et al. [25] (Dimond, R., personal communication). For 60 ml of staining solution, 20 mg of nitroblue-tetrazolium (Sigma Chemical Co., St. Louis, MO, U.S.A.) was suspended in 1 ml of 0.1 M Tris/0.1 M NaCl/5 mM $MgCl_2$ buffer adjusted to pH 8.8 with HCl, warmed, vortexed, centrifuged briefly in a microfuge and the pellet extracted further, as such, 4–5 times. The volume was made up to 60 ml with the above buffer and 30 mg of 5-bromo, 4-chloro, 3-indolyl phosphate (Sigma Chemical Co., St. Louis, MO, U.S.A.), dissolved in 0.2 ml dimethylsulfoxide, added. The nitrocellulose paper was placed upside down in this solution and the purple color of immunoreactive bands was allowed to develop for 0.5–1.0 h. Reaction was terminated by washing in water. This method is approx. 10-times more sensitive than that described in ref. 23.

For dot blots, application of antigen and pretreatment of nitrocellulose paper prior to the blocking step was carried out as described by Hawkes et al. [26]. Subsequent incubations and staining were as described above.

Results

Plasma membrane H^+-ATPase activity

Specific activity of the H^+-ATPase in *N. crassa* plasma membranes used in the experiments described below varied from 2.5 to 5.5 μmol/min per mg protein. Values for the H^+-ATPase of oat, tomato and potato root plasma membranes were 0.6–2.3, 0.5–1.2 and 1.0 μmol/min per mg protein, respectively, in the presence of 0.1 mg/ml

lysolecithin. Lysolecithin increased the specific activity of the plant plasma membrane H^+-ATPase at least 2-fold.

Specific activity of the H^+-ATPase could be further increased by using low concentrations of deoxycholate to remove loosely bound membrane proteins, leaving the integral H^+-ATPase intact. This was used successfully by Bowman et al. [4] to remove approximately half of the membrane proteins of *N. crassa* plasma membranes, as a first step in the purification of the H^+-ATPase in an active form. Results shown in Table I indicate that the plant plasma membrane H^+-ATPases are also stable to this low concentration-deoxycholate wash procedure. Specific activities of the oat and tomato plasma membrane H^+-ATPases were doubled by using conditions very similar to those used for the *N. crassa* enzyme.

Effect of detergents

The effect of detergents on the ATPase activity of the plasma membrane H^+-ATPases from *N. crassa*, oat roots and tomato roots was investigated. Three different detergents, lysolecithin (a zwitterionic detergent), deoxycholate (an anionic bile salt) and Triton X-100 (a nonionic detergent) were tested. Results are shown in Fig. 1. There was a striking difference in the sensitivity of the plant and fungal H^+-ATPases to the presence of detergents in the assay at 30°C. All three detergents increased activity of the plant plasma membrane H^+-ATPases, but inactivated the fungal enzyme. Maximal activation (at least 2-fold) of oat and tomato root enzymes occurred at 0.1 mg/ml lysolecithin and 0.03 mg/ml Triton X-100. Activation of the plant enzymes with deoxycholate was less pronounced (1.25-fold at 0.3 mg/ml). Complete inactivation of the *N. crassa* plasma membrane H^+-ATPase was observed in the presence of as little as 0.03 mg/ml lysolecithin or Triton X-100 and 75% inactivation occurred in the presence of 0.3 mg/ml deoxycholate.

Effect of vanadate

Vanadate inhibits ion-translocating ATPases which form a phosphorylated intermediate [27,28]. Both the *N. crassa* and plant plasma membrane H^+-ATPases have been shown to form phosphorylated intermediates [29–31]. Vanadate inhibition of the *N. crassa* and plant H^+-ATPases has been demonstrated [19,28,32], although published results for the half-maximal inhibition obtained for the plant enzyme have been variable.

We compared the inhibition of the fungal and plant plasma membrane H^+-ATPases by vanadate, using identical assay conditions and the same vanadate stock solutions (Fig. 2). Half-maximal inhibition by vanadate was obtained at 1.25 and at

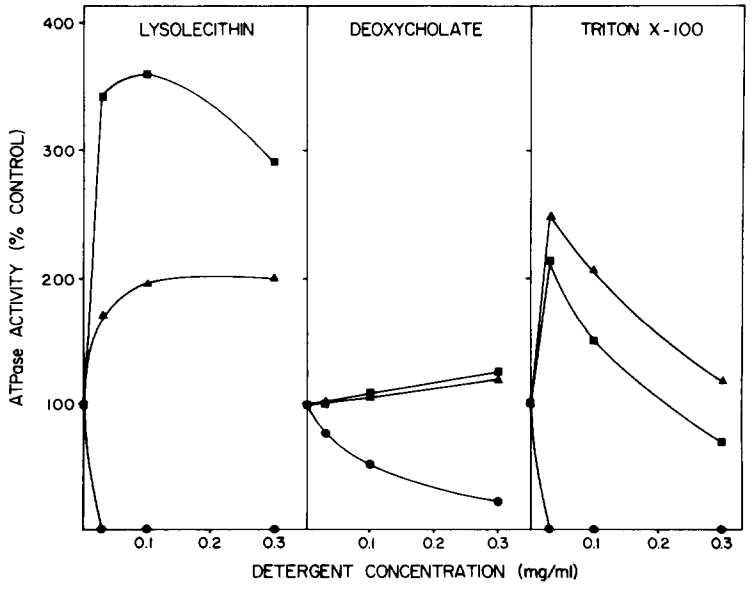

Fig. 1. Effect of detergents on plasma membrane H^+-ATPase activity. Membranes were assayed for ATPase activity as described under Materials and Methods in the presence of increasing concentrations of detergent. Control activities were 2.5 μmol/min per mg protein (10 μg protein/0.5 ml) for the *N. crassa* enzyme (●), 0.6 μmol/min per mg protein (20 μg protein/0.5 ml) for the oat enzyme (▲) and 0.5 μmol/min per mg protein (20 μg protein/0.5 ml) for the tomato enzyme (■).

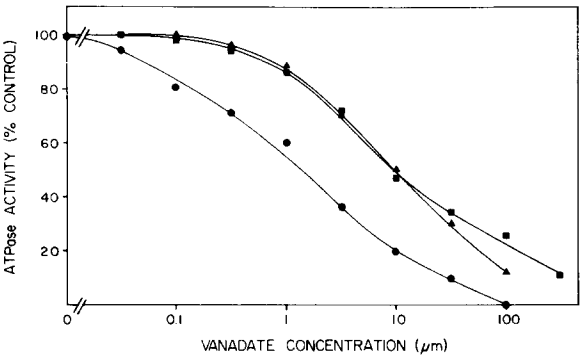

Fig. 2. Effect of vanadate on plasma membrane H^+-ATPase activity. Membranes were assayed for ATPase activity as described under Materials and Methods in the presence of increasing concentrations of vanadate. Control activities were 5.5 μmol/min per mg protein (3 μg protein/0.5 ml) for the *N. crassa* enzyme (●), 0.75 μmol/min per mg protein (25 μg protein/0.5 ml) for the oat enzyme (▲) and 0.65 μmol/min per mg protein (36 μg protein/0.5 ml) for the tomato enzyme (■).

10 μM for the fungal and plant enzymes, respectively. A reproducible 10-fold difference in sensitivity of the fungal and plant plasma membrane H^+-ATPases was always observed. This was not due to incomplete removal of EDTA (which prevents vanadate inhibition [19]) from the plant membranes nor to the higher protein concentration used in the assays of the plant enzymes, since there was no effect on vanadate inhibition of the *N. crassa* H^+-ATPase when washed oat plasma membranes were boiled and added to the fungal membranes before assay.

Vanadate also inhibits the molybdate-sensitive non-specific phosphatase activity in plants [32]. We found no such activity in the oat root plasma membranes, but approx. 7% of the non-mitochondrial ATPase activity was sensitive to molybdate in the tomato root plasma membranes. No difference in vanadate inhibition was observed for the plant plasma membrane H^+-ATPases when assays were carried out in the presence or absence of 0.1 mM ammonium molybdate, used to inhibit non-specific phosphatase activity [32]. This suggests an insignificant contribution of non-specific phosphatase activity to the vanadate inhibition curves obtained for the plant H^+-ATPases and supports the argument that an observed ten-fold difference in vanadate sensitivities reflects a real difference between plant and *N. crassa* plasma membrane H^+-ATPases.

Effect of DCCD

N,N'-Dicyclohexylcarbodiimide (DCCD) has been found to be a potent irreversible inhibitor of enzymes involved in cation translocation across membranes [33]. This includes the F_0F_1 H^+-ATPase [11], the plasma membrane H^+-ATPase of *N. crassa* [9] and the Ca^{2+}-ATPase of sarcoplasmic reticulum [10].

In the experiment of Fig. 3, plasma membranes of *N. crassa*, oat roots and tomato roots were partially delipidated by treatment with deoxycholate as described in the legend to Table I and under 'Materials and Methods', incubated in the presence of various concentrations of the hydrophobic carbodiimide DCCD or of the hydrophilic analogue 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDAC) and then assayed for ATPase activity. The inhibition by DCCD was very similar in all three cases. DCCD gave half-maximal inhibition of the *N. crassa*, oat root and tomato root H^+-ATPases at 17, 22 and 30 μM, respectively. In contrast, the hydrophilic EDAC gave no inhibition, even at concentrations of 1 mM, suggesting a lipophilic location of the DCCD reactive site. Similar results were obtained with non-deoxycholate-treated plasma membranes. In this case, half-maximal inhibition was obtained at slightly higher concentrations, 63, 30 and 100 μM for DCCD inhibition of *N. crassa*, oat root and tomato root plasma membrane H^+-ATPases, respectively.

The kinetics of inactivation of the plant plasma membrane H^+-ATPases by DCCD was examined by incubating oat root and tomato root plasma membranes with various concentrations of DCCD, removing aliquots at timed intervals and assaying for ATPase activity. The results are shown in Fig. 4. Similar experiments by Sussman and Slayman [9] on the *N. crassa* enzyme suggested that DCCD inhibition was pseudo-first order with respect to time and that inactivation of the *N. crassa* H^+-ATPase was caused by reaction of DCCD with a single site on the ATPase molecule. For both the oat root and tomato root plasma membrane H^+-ATPases, plots of the logarithm of enzymic activity as a function of time of DCCD inactivation were linear for several different DCCD concentra-

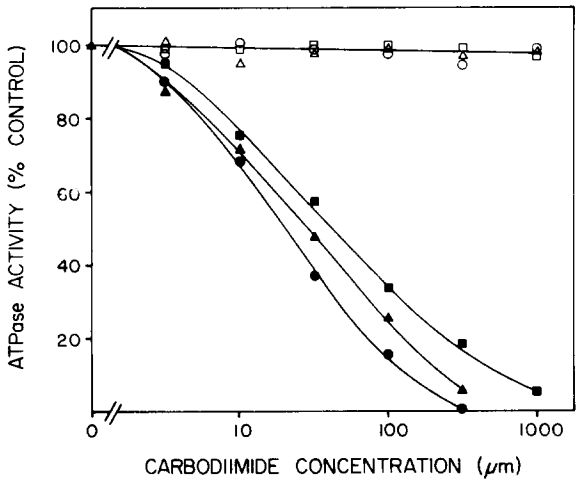

Fig. 3. Comparison of the effects of DCCD and EDAC on plasma membrane H^+-ATPase activity. Membrane-bound ATPase was partially delipidated by treatment with deoxycholate prior to assay as described under Materials and Methods and in the legend to Table I. ATPase was incubated for 30 min at 30°C with the carbodiimide as described under Materials and Methods. ATP was then added and ATPase activity was assayed. Control activities were 9.6 μmol/min per mg protein (5 μg protein/0.5 ml) for the *N. crassa* enzyme (●, ○), 2.2 μmol/min per mg protein (24 μg protein/0.5 ml) for the oat enzyme (▲, △) and 1.1 μmol/min per mg protein (18 μg protein/0.5 ml) for the tomato enzyme (■, □).

tions (Fig. 4A). This suggests that the inhibition at any fixed concentration of DCCD exhibits pseudo-first-order kinetics with respect to time. The same data were replotted so that the logarithm of the rate of inactivation, expressed as the logarithm of the pseudo-first-order rate constant k (min^{-1}), was plotted as a function of the logarithm of the DCCD concentration. Straight-line graphs were obtained for both the oat root and tomato root H^+-ATPases with slopes of 1.12 and 0.91, respectively (Fig. 4B). Since the slopes approximate 1, this suggests that DCCD inhibition of the plant H^+-ATPases is also caused by reaction of one molecule of DCCD with a single site on the ATPase molecule.

Immunological cross-reactivity

In order to investigate structural relatedness between the plasma membrane H^+-ATPases from plants and fungi, we generated specific polyclonal antibodies to the *N. crassa* and oat root M_r = 100 000 H^+-ATPases.

Antibodies from rabbits were obtained as described in 'Materials and Methods' and their specificity tested by immunoblot analysis of the respective plasma membrane preparations. Fig. 5A

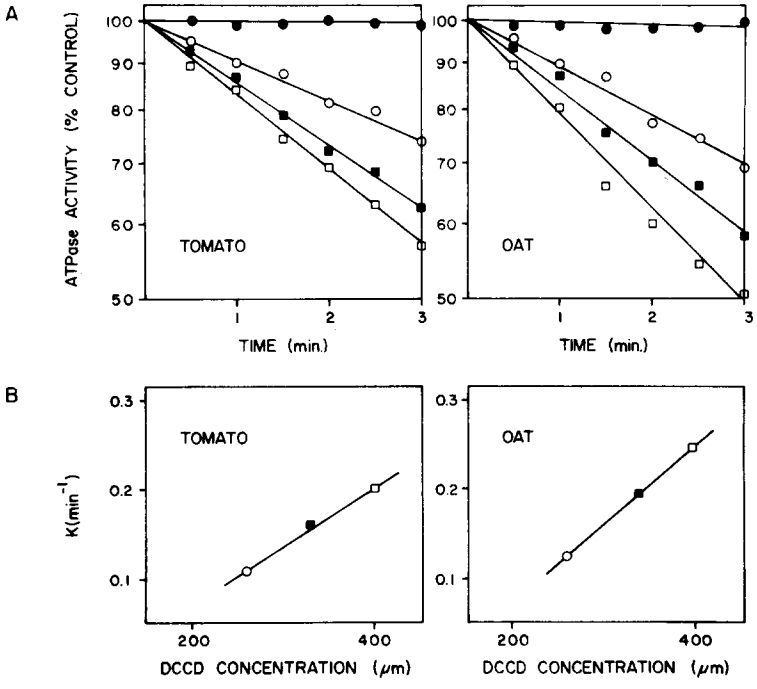

Fig. 4. Kinetics of inactivation of the plant plasma membrane H^+-ATPases by DCCD. Membrane-bound ATPase was incubated with DCCD at 30°C as described under Materials and Methods. Aliquots were removed at timed intervals and assayed for ATPase activity (Panel A). Control activities were 0.8 μmol/min per mg protein (48 μg protein/0.5 ml) for the oat enzyme and 0.65 μmol/min per mg protein (36 μg protein/0.5 ml) for the tomato enzyme. Panel B shows the logarithm of the pseudo-first-order rate constants, determined by exponential regression, plotted as a function of the logarithm of DCCD concentration.

shows Coomassie-stained plasma membrane proteins of *N. crassa* and oat roots, separated by SDS polyacrylamide gel electrophoresis. A clear M_r = 100 000 band (arrow) can be seen in both preparations. Fig. 5B shows the immunoblot analysis results, indicating that specific antibody was obtained for the M_r = 100 000 band of both *N. crassa* and oat root plasma membranes. Bands were not observed when preimmune serum was used. The H^+-ATPase represents approx. 5% of the *N. crassa* plasma membrane protein [16] and, based on comparison with *N. crassa*, approx. 2% of the oat root plasma membrane protein. On this basis, the sensitivities of immunological detection of both M_r = 100 000 H^+-ATPases were greater than 0.1 μg.

It is well established that the prominent M_r = 100 000 polypeptide seen in SDS gel electrophoresis patterns of *N. crassa* plasma membranes is the H^+-ATPase [4]. In order to confirm that the oat root plasma membrane M_r = 100 000 polypeptide we had used to generate antibodies was indeed the H^+-ATPase for this species we followed the lysolecithin solubilization procedure of Serrano [6] to obtain purified, biologically active enzyme, as described under 'Materials and Methods'. Fractions were collected from the glycerol gradient and protein and ATPase activities were determined. Results are shown in Fig. 6. The ATPase moved ahead of most of the solubilized protein. Also shown is the reactivity of the protein in each fraction with the antibody directed toward the oat root plasma membrane M_r = 100 000 polypeptide. Clearly the purified ATPase activity coincided directly with those fractions (fractions 7–9) highly reactive with antibody.

Using the specific antibodies directed toward the M_r = 100 000 H^+-ATPases of *N. crassa* and oat root plasma membranes, we tested the cross-reactivity of these enzymes using immunoblot analysis as described under Materials and Methods. Cross-reactivities of the M_r = 100 000 plasma membrane H^+-ATPases from tomato and potato roots were also determined. Fig. 5B shows the results obtained. Cross-reactivity between the plant plasma membrane H^+-ATPases was clearly apparent, suggesting structural homology between these enzymes. Cross-reactivity between the fungal and plant enzymes was weak. However, very faint staining was apparent in some experiments with

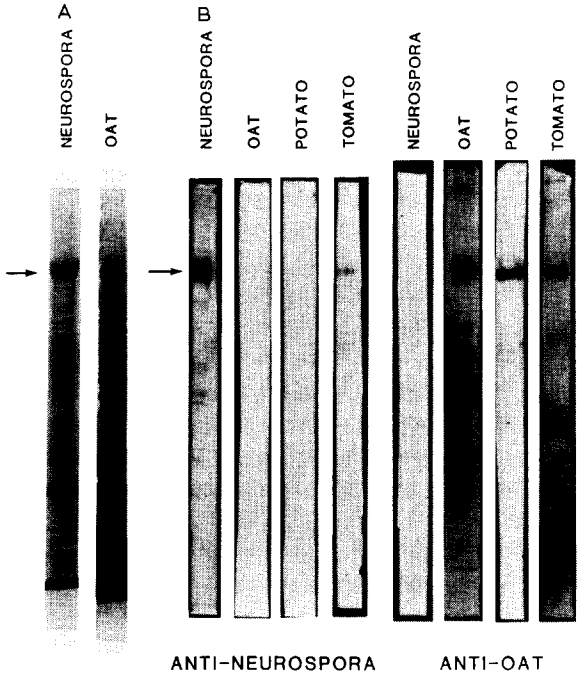

Fig. 5. (A) SDS polyacrylamide gel electrophoresis of plasma membrane proteins. Plasma membrane proteins from *N. crassa* (50 μg) and oat roots (50 μg) were separated by SDS polyacrylamide gel electrophoresis according to Laemmli [22]. The stacking and running gels were 4% (w/v) and 8% (w/v) acrylamide, respectively. Gels were Coomassie-stained. Arrow indicates the M_r = 100 000 position. (B) Immunoblot analysis of cross-reactivities between the M_r = 100 000 H^+-ATPases of fungal and plant plasma membranes. Membrane proteins were separated by SDS polyacrylamide gel electrophoresis using an 8% (w/v) acrylamide running gel and electroblotted onto nitrocellulose as described under Materials and Methods. Polypeptides were visualized using antibody directed toward the M_r = 100 000 *N. crassa* H^+-ATPase or using antibody directed toward the M_r = 100 000 oat H^+-ATPase. Amounts of membrane protein applied to each gel lane were, from left to right, 4, 40, 40, 40, 40, 4, 16 and 16 μg.

N. crassa antibody and tomato membranes (Fig. 5B). We have observed this weak cross-reactivity reproducibly in immunoblots. No bands were detected when preimmune serum was used.

Discussion

In order to clarify the relatedness of the M_r = 100 000 plasma membrane H^+-ATPases from fungi and higher plants, we compared functional and structural properties under the same experimental conditions.

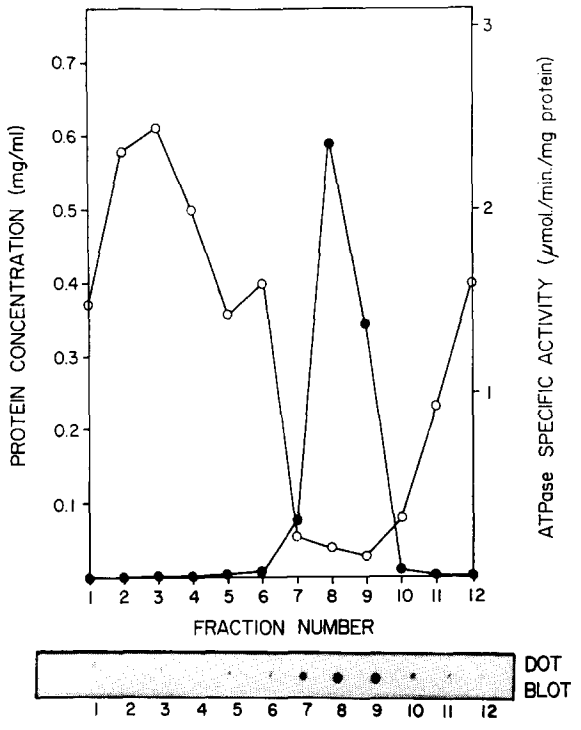

Fig. 6. Dot immuno-blot analysis of antibody directed toward the $M_r = 100000$ H^+-ATPase of oat root plasma membranes. Oat root H^+-ATPase was purified from plasma membrane protein by the procedure of Serrano [6]. 3 ml fractions were collected from the glycerol gradient and protein (○) and ATPase activities (●) were determined as described under Materials and Methods. Protein (0.2 μg) from each fraction was dot-blotted onto nitrocellulose and protein reactive with antibody directed toward the $M_r = 100000$ oat H^+-ATPase visualized as described under Materials and Methods.

The plasma membrane H^+-ATPases are integral membrane proteins which require high concentrations of detergent for solubilization and added phospholipids to maintain activity of the purified enzyme [3–6,34]. In order to gain information on the lipid requirements of the membrane-bound H^+-ATPases, we investigated the effects of low concentrations of detergent included during assay and found a striking difference in the sensitivities of the fungal and plant enzymes. At 30°C the *N. crassa* enzyme was inactivated by even the lowest concentrations of the three detergents tested, suggesting a strict dependence of activity on lipid environment. In contrast, both plant H^+-ATPases were stimulated, up to 3-fold. This stimulation may reflect a direct activation, perhaps through conformational change, or a breaking down of permeability barriers between enzyme and substrate. It suggests that the *N. crassa* and plant H^+-ATPases differ markedly in their dependence on protein-lipid interactions in the plasma membrane and probably reflects, at least in part, different lipid compositions of the fungal and plant plasma membranes. Other investigators have also reported detergent activation of the plant H^+-ATPase, from oat root [5] and corn root [35] plasma membranes. However, since different conditions were used, it is difficult to compare results directly. Activation of the yeast plasma membrane H^+-ATPase by detergents has also been reported [36,37], although, again, different conditions were used.

Vanadate characteristically inhibits the ion-translocating plasma membrane ATPases by interfering with the formation of the phosphorylated intermediate [7] and has been used to study the reaction mechanisms of these enzymes [7,19,38]. The plasma membrane ATPases of animal and fungal cells appear to be similarly sensitive to this inhibitor, half-maximal inhibition requiring 10 μM vanadate or less [27]. Similar sensitivities have been reported by some investigators for plant plasma membrane H^+-ATPases [5,28], but other studies have suggested that the plant enzyme is much less sensitive, requiring 35–233 μM vanadate for half-maximal inhibition [32]. When we compared the fungal and plant enzymes using identical assay conditions and the same vanadate stock solutions, we observed half-maximal inhibition of the *N. crassa* enzyme at 1.25 μM vanadate and half-maximal inhibition of both oat and tomato root enzymes at 10 μM vanadate. These sensitivities agree with those obtained by Bowman and Slayman [18] for the *N. crassa* enzyme and by Vara and Serrano [6] and O'Neill and Spanswick [28] for the plant H^+-ATPase. The lower sensitivity to vanadate observed for the plant H^+-ATPase by some investigators may be because no precautions were taken to remove EDTA, which complexes vanadate [19]. The 10-fold difference in vanadate sensitivities that we observed between the *N. crassa* and oat and tomato root H^+-ATPases was reproducible and appeared to reflect a real difference between the enzymes. In this respect, it is interesting to note that where the kinetics of

vanadate inhibition have been studied, they have suggested a difference in the kinetics of the ATPase reaction between the plant and *N. crassa* H^+-ATPases. The plant enzyme shows strict Michaelis-Menten kinetics [28,32]. A sigmoidal dependence of ATPase activity on ATP concentration was found for the *N. crassa* enzyme [9,38], suggesting two ATP binding sites; vanadate appears to reduce the cooperativity between the sites. The 10-fold difference in vanadate sensitivities that we have observed for the *N. crassa* and plant H^+-ATPases may reflect this difference in kinetics.

DCCD has been used as a potent inhibitor of enzymes involved in cation translocation across membranes [33]. Sussman and Slayman [9] have carried out a detailed analysis of DCCD inhibition of the *N. crassa* plasma membrane H^+-ATPase. DCCD inhibition of the plant plasma membranes H^+-ATPase has also been reported [5]. When we measured DCCD inhibition under the same experimental conditions, we found that the DCCD sensitivities of the *N. crassa* and plant H^+-ATPases were very similar. For deoxycholate-treated membranes, half-maximal inhibition was obtained at 17, 22 and 30 μM DCCD for *N. crassa*, oat and tomato root enzymes, respectively. Furthermore, the hydrophilic EDAC did not inhibit ATPase activities. The DCCD sensitivities agree with those found by Sussman and Slayman [9] and Vara and Serrano [5]. The results suggest that the DCCD sensitive site in the plant H^+-ATPase is essential for ATPase activity, and like that of the *N. crassa* enzyme, is located in a hydrophobic environment. For the *N. crassa* enzyme, it has been shown that DCCD inhibition results from covalent modification of the $M_r = 100\,000$ polypeptide [9] which possesses the ATP hydrolysing activity [29]. Further studies, using radiolabelled DCCD, are required to substantiate whether this is true for the plant H^+-ATPase. Kinetic analysis of DCCD inhibition was used to investigate the stoichiometry of DCCD reaction with the plasma membrane H^+-ATPases of higher plants. Inhibition was found to be pseudo-first-order. When the data were replotted, they suggested that inactivation involved reaction of DCCD with a single essential residue on the ATPase molecule. These results are very similar to those obtained with the *N. crassa* enzyme [9]. Further studies using radiolabelled inhibitor are needed to substantiate the stoichiometry.

Although it is established that an $M_r = 100\,000$ polypeptide is a catalytic subunit of the plasma membrane H^+-ATPases of both plants and fungi [4,6,29], very little other structural information is known. We have investigated the structural relatedness of the plasma membrane H^+-ATPases from *N. crassa*, oat, potato and tomato roots, using specific antibodies directed toward the *N. crassa* and oat enzymes in immunoblot analysis. We found that there was significant cross-reactivity between the different plant H^+-ATPases, but only weak cross-reactivity between the fungal and plant enzymes. Since both $M_r = 100\,000$ polypeptides used for antibody generation were isolated in the presence of SDS, cross-reactivity probably demonstrates sequence homologies between the ATPases. Thus our results demonstrate, for the first time, sequence homologies between the plasma membrane H^+-ATPases for monocotyledenous and dicotyledenous plants. The slight cross-reactivity observed between the plant and fungal enzymes suggests minimal homology at this level, but does not necessarily exclude homologies between sequences not recognized by antibody. Our demonstration of conserved structure between higher plant species is not surprising in light of the recent discovery of sequence homology between the $M_r = 100\,000$ subunits of the Ca^{2+}-ATPase isolated from muscle cells and the K^+-ATPase of *E. coli* [12]. Further determination of structural homologies must await sequencing of the plant/fungal H^+-ATPases.

The above-mentioned results stress the importance of a comparison between the fungal and plant plasma membrane H^+-ATPases under identical experimental conditions. Similarities in inhibitor sensitivities and molecular masses have suggested that the fungal and plant plasma membrane H^+-ATPases are structurally and functionally related. Both enzymes have been purified and reconstituted into liposomes as integral membrane polypeptides of $M_r = 100\,000$ [3,6]. Our results demonstrate that the plasma membrane H^+-ATPases from these two phylogenetic groups are similar. However, there are structural differences, differences in the dependence of activity on protein-lipid interactions in the membrane and possi-

bly differences in reaction mechanism. These presumably reflect different needs of the organisms with respect to transport of solutes across the plasma membrane. Further work, on sequence and reaction mechanism, will help clarify the relatedness of these plasma membrane H^+-ATPases.

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