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Lipid composition and proton transport in *Penicillium cyclopium* and *Ustilago maydis* plasma membrane vesicles isolated by two-phase partitioning

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Abstract

Plasma membranes have been isolated and purified from two species of fungi, *Penicillium cyclopium* and *Ustilago maydis*, using a two-phase aqueous polymer technique. The membranes were characterised using marker enzyme assays (e.g., vanadate-sensitive (Mg^{2+}-K^+)-ATPase and glucan synthetase II) and lipid composition (sterol enrichment, increased phosphatidylethanolamine/phosphatidylcholine ratio, and the absence of diphosphatidylglycerol). The proton-pumping activities of the plasma membrane-bound H^+-ATPases from these species were compared. H^+-ATPase activity was found to be greater in *U. maydis* than in *P. cyclopium*, which was attributed to differences in orientation of the plasma membrane vesicles. There was evidence to suggest the presence of redox chain activity in the plasma membranes of both species.

Keywords: Plasma membrane purification; Inside-out vesicle; Sterol; Phospholipid; ATPase, H^+-

1. Introduction

Sealed plasma membrane vesicles of known orientation are a useful tool to study cell transport processes. A number of methods have been used to separate the plasma membrane from the intracellular membranes. However, fungal plasma membrane vesicle isolation has proved to be a difficult task, since many of the preparation methods yield open or relatively impure vesicles. For example, with *Neurospora crassa* and using concanavalin A, plasma membrane sheets which could be vesiculated were produced, however, these preparations were only 2-fold enriched in marker enzymes compared with microsomes [1]. Plasma membranes were purified from *Saccharomyces* [2] and *Metschnikowia reukaufii* [3] protoplasts, using cationic silica microbeads. Apart from the disadvantage of having to start from protoplasts, this technique only produced sheets which could not be vesiculated. Plasma membrane vesicles were obtained from *Saccharomyces* by a series of pH changes [4]. Unfortunately, these vesicles proved to be leaky and it was necessary to add lipids to seal them. Sucrose density gradients have been used to isolate plasma membrane vesicles from yeasts and fungi. For example, sealed plasma membrane vesicles were obtained from *Phytophtora megasperma* [5] and from *Saccharomyces* [6]. In the last 10 years, plasma membrane vesicle isolation by aqueous two-phase system partitioning [7] has become the technique of choice for plant material, as this method produces pure preparations with a good yield of mostly right side-out vesicles [8]. Nevertheless, only recently has this method been applied to fungal systems [9].

The plasma membrane H^+-ATPase in fungi is responsible for the maintenance of an electrochemical gradient necessary for the transport of ions and nutrients. The activity of this enzyme is known to be af-

Abbreviations: CMH, cerebroside monohexose; DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilboestrol; DMSO, dimethylsulphoxide; DPG, diphosphatidylglycerol (cardiolipin); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol *bis*-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PM, plasma membrane; PMSF, phenylmethylsulphonyl fluoride; TP, tonoplast; UDP[^{14}C]G, uridine 5'-diphospho[^{14}C]glucose.

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fected by metabolic factors, such as the availability of fermentable sugars [10,11] or nitrogen starvation [12]. Such studies have almost always involved the characterization of changes in the hydrolytic activity. Because of the difficulties in isolating functional membrane vesicles, the study of proton pumping-activity has very often involved the purification and reconstitution of the H^+-ATPase into liposomes, with the consequent change in the enzyme's natural environment.

Lipids modulate the activity of membrane-bound ATPases, although the mechanism is still controversial [13]. For example, the plasma membrane H^+-ATPase partially loses its activity following delipidation [14]; lysophospholipids have been shown to activate ATP hydrolysis in plant plasma membrane vesicles [15] and the alteration of the normal sterol profile in plant plasma membrane vesicles was associated with changes in the ATPase activity [16]. Therefore, a regulatory role for lipids in the modulation of membrane-bound ATPase activity has been postulated by a number of workers [17,18,13].

In the present work, we report a simplified method for the isolation of sealed plasma membrane vesicle-enriched fractions by aqueous two-phase partitioning from two different fungal species: a filamentous organism (*Penicillium cyclopium*, Deuteromycotina) and a yeast-like Basidiomycotina (*Ustilago maydis*). We have characterised the lipid composition and marker activities associated with these preparations and examined their proton-transporting activities.

2. Materials and methods

2.1. Strains and culture conditions

Penicillium cyclopium Westling (IMI 29034) was maintained in malt extract agar slopes at 25°C. Liquid cultures were inoculated as described in [19] and cultured for 24 h in 10 l fermenters containing 'F' medium [20] with an aeration rate of 6 l min^{-1}.

Ustilago maydis (IMI 103761) was maintained in frozen aliquots with 9% DMSO at −70°C. Liquid cultures were inoculated with 3 mg (fresh weight) of cells per 400 ml flask and cultured for 48 h in YEPD medium (Gifco) on a rotatory shaker at 25°C.

2.2. Plasma membrane purification

Penicillium cyclopium mycelia were harvested by filtering through cheese cloth (typical harvest, 40 g fresh weight). *U. maydis* cells were collected by centrifuging at $6000 \times g$ for 10 min (typical harvest, 20 g fresh weight). The biomass was mixed with 50 ml of homogenization buffer (50 mM Hepes adjusted to pH 7.5 with KOH, 330 mM sucrose, 5 mM EDTA, 5 mM EGTA, 0.2% BSA, 0.2% casein hydrolysate, 1 mM PMSF, 2% choline, 5 mM DTT) and 75 g of glass beads (0.125 mm diameter). Cells were homogenized in a Bead-Beater (Biospect Products), the homogenate filtered through nylon cloth (240 μm) and centrifuged at $10000 \times g$ for 15 min. The pellet (unbroken cells, cell debris and intact mitochondria) was discarded and the supernatant centrifuged at $100000 \times g$ for 30 min to produce a microsomal pellet which was resuspended in 5 mM phosphate buffer (pH 7.8), 330 mM sucrose. Plasma membranes were isolated and purified using the aqueous two-phase polymer technique described by Larsson et al. [7], with the following modifications. Microsomal fraction (200–300 mg protein) was loaded on to six 20 g two-phase systems (typical load, 30 mg protein per system) to give a final composition of 7.0% Dextran T500, 7.0% PEG, 5 mM phosphate buffer (pH 7.8), 330 mM sucrose and 3 mM KCl. After centrifuging at $3000 \times g$ for 3 min to aid partitioning, the top phase was carefully removed, diluted three times with dilution medium (DM – 10 mM Mops-BTP (pH 7.5), 330 mM sucrose) and centrifuged at $100000 \times g$ for 30 min. The resulting pellet, which constituted the plasma membrane fraction, was resuspended in DM. Where appropriate, the bottom phase of the two-phase system was diluted and centrifuged as described for the top phase; this fraction is referred to as intracellular membranes.

2.3. ATPase assays

ATP hydrolytic assays were done using the procedure of Widell and Larsson [21]. The assay medium contained 10–25 μg of membrane protein, 330 mM sucrose, 4 mM magnesium sulfate, 4 mM sodium ATP, 100 μM sodium molybdate, 1 mM sodium azide, 50 mM potasium nitrate and 0.0125% Triton X-100 made to a final volume of 120 μl in the presence of 40 mM Mes-Tris (pH 6.7). The reaction was started by the addition of ATP, incubated for 30 min at 25°C and terminated by the addition of 0.120 ml of a 'stopping' reagent which consisted of 2% hydroquinone in 2 N sulphuric acid. Immediately after, 120 μl of 5% ammonium molybdate was added and the colour developed by the addition of 625 μl of a mixture of 0.3 M sodium sulphite and 20% sodium carbonate. The colour, which remained stable for up to 2 h, was allowed to develop for at least 10 min and the absorbance read at 750 nm.

Vanadate sensitivity was determined at pH 6.7 in the presence and absence of 0.1 mM sodium orthovanadate.

Nitrate sensitivity was assayed at pH 7.5 in the presence and absence of 50 mM sodium nitrate. Both 0.1 mM vanadate and 20 mM KCl were included in the reaction mixture.

2.4. Latency tests

To ascertain the sidedness of the vesicles, the latency of the ATPase activity at pH 6.7 was determined by assaying in the presence and absence of 0.0125% (v/v) Triton X-100 [22].

2.5. Glucan synthetase II assay

This was done according to the method of Ray [23], modified as follows. Glucan synthetase II (GS-II) activity was measured in the presence of 50 mM Hepes-KOH (pH 7.25), 800 μM spermine (pH 7.25), 4 mM $CaCl_2$, 4 mM EGTA, 16 mM cellobiose and 0.15% digitonin in Eppendorf tubes. Membrane fraction (20 μl, 10–20 μg protein) was added to sample tubes, while 20 μl of buffer was added to blanks and standards to give a total volume of 140 μl. After adding the reagents and samples to the Eppendorf tubes, they were warmed for 2 min at 25°C in a waterbath. Uridine 5′-diphospho[^{14}C]glucose (UDP[^{14}C]G, 1 μCi ml^{-1}), 20 μl, was added to the samples and blanks to give a concentration of 750 μM and the mixture incubated for 60 min at 25°C. The reaction was terminated by the addition of 200 μl magnesium chloride (100 mM), 0.5 mg excess protein and 0.7 ml ethanol. The contents of the tubes were filtered through GF/F glass fibre filters (Whatman) and washed four times with equal volumes (approx. 1 ml) of 70% ethanol. The filters were transferred to scintillation vials with plastic inserts. UDP [^{14}C]G (5 μl) was added to the standards, followed by 3 ml of scintillant to standards, blanks and samples, and counted for 2 min.

2.6. Cytochrome-c oxidase

The oxidation of cytochrome *c* was assayed as described by Appelmans et al. [24]. A mixture of 50 μM cytochrome *c*, 50 mM Tris-acetate buffer (pH 7.4) and 0.025% Triton X-100 was reduced by the addition of 20 mg of dithionite and allowed to stand for at least 30 min in a water bath at 25°C to evaporate excess reducing agent. The reaction mixture (0.7 ml) was placed in a 1.5 ml spectrophotometer cell and 15–25 μg of membrane protein added. The reaction was recorded as the decrease in absorbance at 550 nm over 30 s and the activity calculated from the extinction coeficient of cytochrome *c* (19.2 mM^{-1} cm^{-1}).

2.7. NADH–cytochrome-c oxidoreductase

This was determined essentially as described by Schekman [25]. Assay medium (50 μM cytochrome *c*, 50 mM Tris-acetate (pH 7.0), 1 mM KCN and 0.2 NADH), 0.7 ml, was placed in a 1.5 ml spectrophotometric cuvette and the reaction started by the addition of 30–40 μg of membrane protein. The reaction was recorded as the change in absorbance at 550 nm over 30 s with 19.2 mM^{-1} cm^{-1} as extinction coeficient.

2.8. Measurements of proton-pumping in PM vesicles

The generation of a MgATP-dependent ΔpH was assayed by monitoring the change in fluorescence emission of the fluorescent probe 9-amino-6-chloro-2-methoxyacridine (ACMA) (Molecular Probes, USA), as described in Coupland et al. [26]. Approx. 250 μl of vesicle suspension (enough to contain at least 100 μg of protein and which had undergone one freeze-thaw cycle), was added to a cuvette containing 20 mM BTP/Mes (pH 6.5), 5 mM ATP, 25 mM KNO_3, 2 μM ACMA and water to 1.980 ml. The cuvette was placed in a luminiscence spectrophotometer and maintained at 30°C with constant stirring. The reaction was started by the addition of 20 μl $MgCl_2$ (0.5 M). The change in fluorescence emission was measured at 485 nm with excitation at 415 nm and recorded. The rate of proton-pumping was calculated from the initial slope of the trace.

2.9. Lipid analysis

In an Eppendorf tube, chloroform/methanol (0.75 ml, 1:2, v/v) was added to resuspended membranes (0.5 ml) along with β-cholestanol (20 μl, 0.1 mg ml^{-1}), as an internal standard for sterol analysis. Chloroform (0.25 ml) was added, the mixture shaken and centrifuged at 10000 × *g* for 6 min. The chloroform layer was retained, evaporated to dryness under N_2 and made up to 100 μl with chloroform.

For sterol analysis, 20 μl of the chloroform extract was placed in a glass vial (2 ml), evaporated to dryness under N_2 and ethyl acetate (20 μl) added. The sterols were analysed by GC using an SE52-bonded capillary column coupled to FID, with H_2 as carrier (1 ml min^{-1}) and a temperature programme of 120–265°C at 10°C min^{-1}. The injector and detector temperatures were 250 and 320°C, respectively.

Phospholipids were analysed by HPLC, using the method described by Christie [27], with minor modifications. The chloroform extract (20 μl) was injected into an Econosphere silica 3 μm column (150 mm × 4.6 mm) and its components detected with an evaporative light-scattering detector. The detector conditions were as follows: N_2 flow, 20 ml min^{-1}; detector response, 900 V; temperature 50°C. A three-solvent gradient system was used, comprising: (A) hexane/2,2′-dimethoxypropane (99:1, v/v), (B) isopropanol/chloroform (4:1, v/v) and (C) isopropanol/water (1:1, v/v); the flow rate was 2 ml min^{-1} throughout. The solvent programme was as follows: 100% A at time 0 and 1 min. At 5 min, A was 80% and B 20%. A, B and C

were 42, 52 and 6%, respectively, at 5.1 min and at 15 min they were 35, 49 and 16%. At 20 min, A was 42%, B 52% and C 6% and at 25 min A was 30% and B 70%. Finally, A was 100% at 30 min. Phospholipids were quantified by comparing peak areas with those of known standards.

2.10. Determination of protein

Protein concentration was determined by the method of Bradford [28] using Bio-Rad reagent and thyroglobulin as the standard.

Except where indicated all experiments were done at least in triplicate.

3. Results

The degree of purification of the plasma membranes was assessed by the distribution of different enzyme activities. Marker assays, which were performed on the different fractions obtained after aqueous two-phase partition in *P. cyclopium* and *U. maydis* (Tables 1 and 2) indicated a 5-fold enrichment of both the vanadate-sensitive (Mg^{2+}-K^+)-ATPase (PM-ATPase) and GS-II for *P. cyclopium*, while for *U. maydis* the values were 2.5-fold, in the case of the PM-ATPase, and 7.5-fold for GS-II. In *P. cyclopium*, the percentage inhibition of the PM-ATPase, by vanadate, was increased from 57% in the microsomes, to 63% in the plasma membrane fraction, with 38% inhibition in the intracellular membranes (Table 1). For *U. maydis*, the inhibition increased from 47% in microsomes, to 60% in the plasma membrane fraction (Table 2). A comparision of vanadate inhibition with DES in proton pumping assays (Fig. 2) showed that the H^+-ATPase was more sensitive to the latter compound. Therefore, the use of DES may be a good alternative to vanadate as an inhibitor of ATPases in fungi.

Table 1
Total protein and specific activities of markers for the different fractions obtained by two-phase partioning in *P. cyclopium*

	Microsomes	Intracellular membranes	Plasma membrane fraction
Protein (mg)	220.78 ±22.42	90.05 ±4.41	0.64 ±0.07
Glucan synthetase	0.067± 0.029	0.121±0.029	0.322±0.025
ATPase (PM)			
Normal (pH 6.7)	0.037± 0.001	0.037±0.003	0.175±0.009
+ vanadate	0.016± 0.001	0.023±0.002	0.064±0.009
Latency	52.0 ± 4.4	44.6 ±1.1	51.2 ±3.6
Cytochrome-*c* oxidase	0.540± 0.062	0.610±0.039	0.107±0.019
NADH–cytochrome-*c* oxidoreductase	0.036± 0.010	0.022±0.010	0.071±0.006
ATPase (TP)			
Normal (pH 7.5)	0.010± 0.001	0.013±0.001	0.029±0.005
+ nitrate	0.012± 0.001	0.024±0.003	0.020±0.004

Activities given in μmol/min per mg protein, except for glucan synthetase where they are in nmol/min per mg protein. Latency is given as a percentage of right-side-out vesicles (±S.E.).

Table 2
Total protein and specific activities of markers for the different fractions obtained by two-phase partitioning in *U. maydis*

	Microsomes	Intracellular membranes	Plasma membrane fraction
Protein (mg)	321.01 ±43.31	118.47 ±2.25	1.00 ±0.21
Glucan synthetase	0.179± 0.006	0.226±0.005	1.344±0.299
ATPase (PM)			
Normal (pH 6.7)	0.132± 0.010	0.164±0.017	0.368±0.029
+ vanadate	0.070± 0.005	0.102±0.005	0.149±0.013
Latency	7.6 ± 3.2	12.4 ±2.3	10.3 ±3.0
Cytochrome-*c* oxidase	0.650± 0.202	0.586±0.138	0.154±0.064
NADH–cytochrome-*c* oxidoreductase	0.129± 0.012	0.125±0.010	0.100±0.019
ATPase (TP)			
Normal (pH 7.5)	0.044± 0.004	0.061±0.006	0.115±0.027
+ nitrate	0.063± 0.012	0.099±0.022	0.116±0.022

Activities are given in μmol/min per mg protein, except for glucan synthetase where they are given in nmol/min per mg. Latency is given as a percentage of right-side-out vesicles (±S.E.).

With *P. cyclopium*, repeated extraction of the upper phase with fresh lower phase did not improve purity, but simply reduced the yield. Cytochrome-*c* oxidase activity remained constant at 0.026 μmol/min per mg, whilst total protein was reduced from 536.4 to 393.6 μg. Similarly, with *U. maydis*, repeated extractions of the upper phase reduced protein content from 410.4 to 216.6 μg, whereas cytochrome-*c* oxidase remained at 0.240 μmol/min per mg.

The sidedness of vesicles was assessed using latency assays for the ATPase activity at pH 6.7. The proportion of right-side out vesicles differed in the two species. In *P. cyclopium*, approx. 50% of the vesicles in the plasma membrane fraction were right-side out (Table 1), while for *U. maydis* the majority of the vesicles were inside out (approx. 10% right-side out) (Table 2). However, there is some controversy concerning the use of Triton X-100 to determine latency, as it has been shown that the concentration we used (0.0125%) may have been inhibitory [29]. Thus, the proportion of inside-out vesicles we found may be an underestimation.

In both organisms, the activity of the markers used to trace intracellular membranes was mostly distributed in the bottom phase. Thus, cytochrome-*c* oxidase activity was found mainly in the intracellular membrane fraction, with less than a quarter of the

Table 3
Major complex lipids and sterols (μg/mg protein) in the different fractions obtained by two-phase partition in *P. cyclopium*, (±S.E.)

	Microsomes membranes	Intracellular fraction	Plasma membrane
CMH	4.96±0.26	6.25±1.53	12.99±0.69
PG	0.33±0.08	0.51±0.34	ND [a]
DPG	5.28±0.30	8.40±1.05	ND [a]
PE	15.03±0.97	20.00±0.56	74.07±3.93
PC	27.58±0.93	35.93±0.78	26.85±0.31
Ergosterol	6.25±0.60	5.86±0.41	84.37±4.13

[a] ND = not detected (limit of detection of DPG and PG = 0.05 μg).

specific activity being present in the top phase; this residual activity may be accounted for by mitochondrial contamination or by redox chains in the plasma membrane, as previously suggested [30] (Tables 1 and 2). The relatively high specific activity found for NADH–cytochrome-*c* oxidoreductase in the plasma membrane fraction may be explained by the presence of endoplasmic reticulum. No nitrate-sensitive ATPase activity (pH 7.5) was found in any of the fractions assayed (Tables 1 and 2).

Lipid analysis of the fractions obtained before and after partitioning showed marked differences (Tables 3 and 4). Cerebrosides were nearly 3-fold more abundant in the plasma membrane fraction than in microsomes, suggesting that they are true components of these membranes. Neither phosphatidylglycerol nor cardiolipin (a lipid associated with the mitochondria) were found in the plasma membrane fractions of either species, whilst they were relatively abundant in the microsomal and intracellular membrane fractions. Interestingly, the PE/PC ratio of plasma membranes was very different from that of microsomes and endomembranes, increasing from 0.5 to 1 in *U. maydis* and 0.5 to 2.7 in *P. cyclopium*, in microsomes and plasma membranes, respectively (Tables 3 and 4).

Ergosterol was the only sterol found in significant amounts in *P. cyclopium* membranes (Table 3). The plasma membrane fraction of this organism contained a much greater proportion of sterols than the other fractions. In *U. maydis*, the sterol profile was more

Table 4
Major complex lipids and sterols (μg/mg protein) in the different fractions obtained by two-phase partition in *U. maydis*, (±S.E.)

	Microsomes membranes	Intracellular fraction	Plasma membrane
CMH	1.76 ±0.24	0.91±0.19	3.75±0.23
DPG	3.49 ±0.16	5.34±0.33	ND [a]
PE	13.04 ±0.236	22.06±2.89	43.92±5.18
PC	27.33 ±1.50	46.69±6.20	40.08±7.19
Ergosterol	4.29 ±0.09	6.62±1.20	73.82±7.00
Ergosta-5,7-dien-3β-ol	0.900±0.09	1.19±0.16	13.60±3.18
Ergosta-7-en-3β-ol	0.26 ±0.01	0.38±0.06	1.75±0.10

[a] ND, not detected (limit of detection of DPG = 0.05 μg).

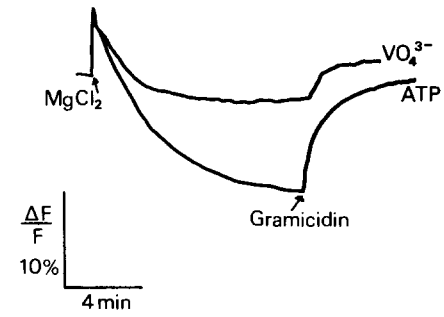

Fig. 1. Proton-pumping activity in *P. cyclopium* plasma membrane vesicles measured as fluorescence quenching of ACMA. The reaction (at pH 6.5 with 50 μg protein ml^{-1}) was started by adding 20 μl $MgCl_2$ (0.5 M) and stopped with 5 μl gramicidin (2 mg ml^{-1}). Effect of 300 μM vanadate is also shown. Data are from a representative experiment.

complex. In addition to ergosterol, ergosta-5,7-dien-3β-ol and ergosta-5-en-3β-ol were also found. The plasma membrane fraction from both species was greatly enriched in sterols (Table 4).

The plasma membrane proton-pumping activity (Figs. 1 and 2) of both species was Mg-ATP dependent. When GTP was used instead of ATP, no activity was found in *U. maydis* (Fig. 2). Vanadate severely inhibited the activity in both organisms, indicating a P-type ATPase. In *U. maydis*, diethylstilboestrol (DES) and vanadate inhibited activity by 90% and 73%, respectively, whereas nitrate, azide and DCCD had no effect (Table 5). Addition of 400 μM $CaCl_2$ resulted in a 46% inhibition of the proton transport in *U. maydis*.

4. Discussion

The upper fractions obtained by aqueous two-phase partitioning were greatly enriched in plasma membrane vesicles as assessed by marker enzymes. The apparent

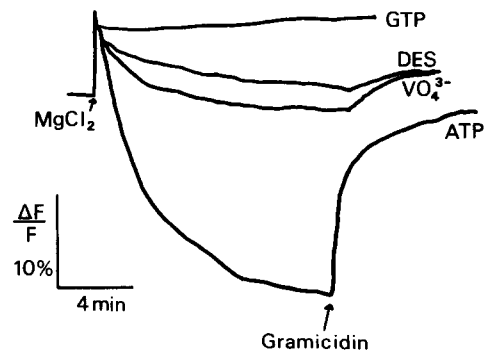

Fig. 2. Proton-pumping activity in *U. maydis* plasma membrane vesicles measured as fluorescence quenching of ACMA. The reaction (at pH 6.5 with 50 μg protein ml^{-1}) was started by adding 20 μl $MgCl_2$ (0.5 M) and stopped with 5 μl gramicidin (2 mg ml^{-1}). Effects of 300 μM vanadate, 100 μM DES and GTP are also shown. Data are from a representative experiment.

Table 5
Effect of different inhibitors on the rate of proton-pumping activity (AU [a] per min per mg protein) at pH 6.5 in *U. maydis* plasma membrane, (±S.E.)

Treatment	Rate	% of control
Control (25 mM KNO_3)	112.3±3.0	100.0
DES (100 μM)	11.6±2.2	10.3
Vanadate		
(100 μM)	81.5±3.5	72.6
(300 μM)	25.7±0.8	22.9
$CaCl_2$ (400 μM)	59.8±9.6	53.3
KCl (25 mM, no KNO_3)	112.8±3.5	100.4
N_3Na (1 mM)	113.1±3.9	100.7
DCCD (10 μM)	122.1±0.8	108.7
Ethanol (1%)	113.1±3.9	100.7

[a] AU = Arbitrary units.

discrepancy between the PM-ATPase activity and the GS II in *U. maydis* may be due to the presence of other P-type ATPases in the cell. Glucan synthetase activity is considered a more reliable marker for plasma membrane than ATPase activity, which is present in other organelles [21,31].

The *P. cyclopium* PM fraction was isolated as a mixture of inside- and right-side out vesicles, while in *U. maydis* the majority of vesicles were inside-out. The proton-pumping activity indicated that these vesicles were sealed and transport competent.

Cardiolipin (diphosphatidylglycerol) is considered a typical mitochondrial lipid [32]. The absence of this lipid in the plasma membrane fraction points to the absence of mitochondrial contamination. Although the remaining cytochrome-*c* oxidase activity in the plasma membrane fraction (less than 25% of the microsomal activity), might suggest contamination by mitochondrial membranes, the absence of cardiolipin indicates that this is not the source of cytochrome-*c* oxidase activity. From studies in plants, the existence of a redox chain at the plasma membrane has been postulated for some time [30,33]. The presence of relatively high specific activities of NAD(P)H–cytochrome-*c* oxidoreductase in plant plasma membranes has been taken as an indication of the presence of such a redox chain [30], although it may also be indicative of contamination by endoplasmic reticulum. However, it seems logical that if a redox chain exists in the plasma membrane, a part of which is capable of reducing cytochrome *c*, there should be a similar enzyme capable of oxidising it. Therefore, it is possible that the cytochrome-*c* oxidase and -oxidoreductase activity we found in the fungal plasma membrane might have been due to the presence of a redox chain, rather than mitochondrial, or endoplasmic reticulum, contamination.

The predominance of PE over PC in plasma membrane vesicles has already been reported [34]. The results found in this study confirm that this is a common characteristic of fungal plasma membranes. Also, our plasma membrane preparations were sterol-enriched, which has been reported as a general feature of plasma membranes from different organisms [35,32]. Therefore, we propose that sterol enrichment, together with a high PE/PC ratio and the absence of cardiolipin, may be used as alternative markers to determine the purity of fungal plasma membranes.

Proton transport in these vesicles was magnesium dependent and, in the case of *U. maydis*, ATP specific. The lower transport activity found in *P. cyclopium* was consistent with decreased hydrolytic activity and a lesser proportion of inside-out vesicles compared with *U. maydis*. The sensitivity of proton-pumping activity to vanadate and DES indicated that the enzyme was a P-type ATPase. Moreover, the absence of inhibition by nitrate and azide pointed to the absence of V- and F-type ATPases in these fractions. Futhermore, Ca^{2+}-inhibition confirmed that the ATPase activity was associated with the plasma membrane, as calcium has been postulated as a specific inhibitor of the H^+-ATPase in plants [21]. DCCD had no effect on *U. maydis* proton transport, in contrast to its effect in *Neurospora crassa*, where the apparent K_i was found to be 3 μM [36]. However, at the concentration we used (10 μM), DCCD may only inhibit mitochondrial ATPases. It is probable that proton transport activity in *U. maydis* is inhibited by greater concentrations (approx. 100 μM), indicating that the H^+-ATPase in *Neurospora* was more sensitive to DCCD.

In this work, we have reported a simplified method for the preparation of enriched plasma membrane vesicles from two different species of fungi. These preparations gave a reasonable yield without compromising the purity of the fractions. The vesicles had a number of differences between them and those from intracellular sources, in terms of lipid composition, which may be used for future characterisations. Proton-pumping studies confirmed the presence of a P-type ATPase in these vesicles. Lack of directly comparable data make comparisions between different plasma membrane isolation techniques difficult. Nevertheless, compared with sucrose density gradient preparations, the two-phase technique has a number of advantages and disadvantages. The latter is certainly quicker, simpler and produces purer vesicles. For example, compared with the data of Weete et al. [34] where a less than 2-fold increase in purity was found using a series of sucrose density gradients, we found a five-fold enrichment in plasma membrane markers with the two-phase system. However, the yield is less than that obtained with sucrose density gradients (about 0.3% of microsomal protein and about 0.03% of total cellular protein) and which are, of course, cheaper. Future work will involve the characterisation of the proton pump and calcium transporter, as well as the use of sterol deficient mutants to study the influence of changes on the lipid

composition of these membranes on the activity of the proton and calcium pumps.

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