Anion-Sensitive Mg$^{2+}$ ATP-Dependent Proton Pumping in Microsomal Membranes from Phycomyces blakesleeanus BGff.

Received for publication March 15, 1988 and in revised form July 6, 1988

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ABSTRACT

A light microsomal membrane fraction (collected on 28% weight/volume sucrose) from young mycelia of Phycomyces blakesleeanus BGff. stationary cultures exhibited Mg$^{2+}$ ATP-dependent proton pumping as monitored with the dye neutral red or by accumulation of $[^3]$H)methylamine. The substrate kinetics for ATP ($K_{m}$ATP: 1.1 millimolar in the presence of 2 millimolar Mg$^{2+}$), the competitive inhibition by ADP (LAGP: 0.8 millimolar), the anion sensitivity (stimulation by $Cl^-$, inhibition by NO$_3^-$ and SO$_4^{2-}$), the reversal of acidification by Ca$^{2+}$, the inhibition by diethylstilbestrol and dicyclohexylcarbodiimide, as well as the absence of any inhibition by azide, molybdate, and vanadate strongly suggest a tonoplast-type ATPase driven proton pumping. The same membrane fraction showed no pyrophosphate driven H$^+$-translocation. Electron microscopic examination of the fungal mycelium demonstrated the presence of vacuoles of different sizes and other nonidentified vesicles but no typical Golgi stacks.

Over the past few years an extensive amount of information has accumulated about the molecular structure and function of the higher plant tonoplast H$^+$ATPase (for a recent review, see Ref. 19). A comparison of the tonoplast H$^+$ATPase from Ascomycetes (Neurospora crassa [4, 5] and Saccharomycyes cerevisiae [27]) with the higher plant enzyme, including immunological cross-reaction for the 70 kD subunit (5), has revealed a conspicuous functional and structural similarity. Indeed, recent sequence analysis of plant and fungal H$^+$ATPase subunits showed a high degree of homology (L. Taiz, E. Bowman, personal communication). Furthermore, the tonoplast H$^+$ATPase of higher plants is similar if not identical with the H$^+$ATPase on the plant Golgi membrane (J. Fichmann, L. Taiz, personal communication).

Together with the higher plant and fungal enzymes the H$^+$ATPases on animal lysosomal membranes (23) and secretory granule membranes (8) are now believed to belong to a special group of H$^+$ATPases distinct from plasma membrane and mitochondrial H$^+$ATPases (19).

In contrast little is known about the H$^+$-pumping ATPases on vacuolar and/or Golgi membranes in lower fungi. Phycomyces blakesleeanus BGff, a member of the Zygomycetes was chosen as a model organism because of several obvious advantages such as a thoroughly studied genetic background (10), availability of extensive metabolic data (14), and, also, its being subject to regulation by plant hormones (3, 16). Apart from electron micrographs showing different degrees of vacuolization in different zones of the fungus (6, 20, 28) little information is available on the characteristics of acidic organelles in Phycomyces. Exposure to light induces rapid vacuolization in germings (11), and quinacrine which is known to accumulate in intracellular acidic organelles (where it acts as a buffer) stops growth of the mycelium in the light (18). Furthermore, thiamine pyrophosphate and nucleoside diphosphatase, two enzymes of the Golgi apparatus have been localized cytochemically on vesicles of Phycomyces, but no Golgi stacks have been found (2).

In an attempt to study primary and secondary active transport mechanisms at the vacuolar membrane of Phycomyces we have developed a method for the isolation of proton transport competent microsomal membrane vesicles presumably of tonoplast origin from young vegetatively growing mycelia. In this paper we demonstrate that a microsomal membrane fraction exhibits active H$^+$-transport having similar characteristics as described for tonoplast vesicles from higher plants and Ascomycetes.

MATERIALS AND METHODS

Electron Microscopy. Object slides covered with a 2 mm layer of nutrient solution (for composition, see Ref. 15) in 1% agar were inoculated with spores of Phycomyces blakesleeanus BGff. 1+ strain, Halbsguth collection (Botanical Inst., Frankfurt, F. R. G.). After development of the young mycelium in the dark at 25$^\circ$C in a moist chamber (but before beginning sporangiophore growth) small pieces of fungal material were chosen under light microscopic observation and subsequently fixed (17) and imbedded in low viscosity epoxyresin (25) according to standard procedures. Sections were analyzed with a Zeiss EM 9.

Growth of Fungal Material. Phycomyces was grown in the dark at 25$^\circ$C in stationary cultures in 1.5 L jars on d-glucose medium (15). After 72 h the fungus covered the nutrient solution as a dense mycelium and sporangiophore growth was about to start. Twenty cultures of this stage were harvested and the mycelia washed in deionized H$_2$O. After blotting on filter paper about 40 g fresh weight were obtained. The mycelia were cut into pieces of approximately 1 cm$^2$ size and collected on an ice-cooled glass plate.

Membrane Extraction. About 40 g fresh weight of cut mycelia were extracted in a precooled (ice-salt mixture) mortar at about -8$^\circ$C with 4 mL/g fresh weight extraction buffer of the following composition: 250 mM sucrose, 100 mM 3-[N-morpholino]propanesulfonic acid, 10 mM ascorbic acid, 10 mM EGTA, 2 mM MgSO$_4$, 2.5 mM DTT, 0.1% (w/v) BSA, adjusted to pH 7.2 with 1 N KOH. After straining the homogenate through four layers of cheesecloth the crude extract was centrifuged at 2°C for 20 min at 10,000g (Sorvall, SS 34 rotor). The supernatant was centrifuged for 60 min at 50,000g and the suspended pellet washed in the same buffer. Finally, the combined pellets of crude microsomal membranes were suspended in 4 mL extraction buffer and layered over a 10 mL 28% (w/v) sucrose cushion in the same buffer. After centrifugation for 90 min at 50,000g, the membranes at the interface were collected, diluted to 8.5% (w/v)
was not influenced by both inhibitors. Assay for Mg\(^{2+}\) ATP-Dependent Proton Pumping. The principle of monitoring H\(^+-\)pumping with neutral red has been described in detail by Hager et al. (13) and was performed according to Rausch et al. (21). Usually the test medium contained 50 mM KCl. For the assessment of Cl\(^{-}\)-dependence of H\(^+-\)pumping KCl was replaced by 50 mM HCl adjusted to pH 7.2 with Bis-Tris propane. The uptake of \(^{14}C\)methylamine by vesicles was determined using a membrane filtration assay as described earlier (21). Substrate kinetics for MgATP and inhibition kinetics for ADP were always determined in the presence of 2 mM MgSO\(_4\), NaCl (1 mM), sodium molybdate (1 mM), and sodium vanadate (0.2 mM) were routinely included in the test medium. In control experiments it was always confirmed that H\(^+-\)pumping activity was proportional to membrane concentration.

Assay for Pyrophosphate-Driven Proton Pumping. The procedure was the same as for the H\(^{+}\)ATPase-driven proton pumping, but NaCl, sodium molybdate and sodium vanadate were omitted from the test medium. The concentration of MgSO\(_4\) was always 2 mM.

Chemicals. ATP disodium salt and ADP sodium salt (both vanadate free), potassium pyrophosphate, from Sigma. Radiolabeled methylamine (specific activity 1.85 MBq/\(\mu\)mol) from Amersham. All other chemicals were of analytical grade.

RESULTS

Electron Microscopy of Fungal Mycelium. Young mycelia of Phycomyces before the beginning of sporangiophore growth were analyzed by electron microscopy (Fig. 1). The hyphae were densely packed with ribosomes, numerous mitochondria, and vesicles with diameters varying from 100 to 500 nm. Larger vacuoles, apparently free from cytoplasmic content, were surrounded by a single membrane (Fig. 1, right). The mycelia used for membrane isolation were in the same stage of development.

Extraction of Microsomal Membranes. Initially, mycelia were homogenized at 0 to 4\(^\circ\)C which consistently yielded membrane preparations with Mg\(^{2+}\) ATP-dependent proton pumping activity. For comparison, homogenization was performed at −8\(^\circ\)C which led to a significant increase (about twofold) in H\(^+-\)pumping activity. The Mg\(^{2+}\) ATP-dependent H\(^+-\)pumping of the crude microsomal fraction was stimulated considerably by sodium vanadate and sodium azide (Table I). Kinetic analysis revealed that in the absence of these inhibitors the apparent \(K_m\) for ATP was higher (2–3 mM) than in their presence, whereas in the further purified membranes (see below) the apparent \(K_m\) for ATP was not influenced by both inhibitors.

Light microsomal membranes were further enriched on a 28% (w/v) sucrose cushion. This membrane fraction contained no detectable Cyt c oxidase activity and its H\(^+-\)pumping activity was not influenced by sodium azide or sodium vanadate (Table I). Membranes could be kept on ice for 180 min without loss of H\(^+-\)pumping activity.

Characteristics of Mg\(^{2+}\) ATP-Dependent Proton Pumping. The Mg\(^{2+}\) ATP-dependent H\(^+-\)pumping was strictly dependent on Cl\(^{-}\) (Fig. 2). Cl Bis-Tris propane was even more effective than KCl in promoting H\(^+-\)pumping (+65%) and addition of 50 mM KCl after maximum acidification in the presence of 50 mM Cl

\(^1\) Abbreviations: Bis-Tris propane, 1,3-bis[(tris(hydroxymethyl)-methylamino)propane].
Fig. 1. Electron micrographs of young, dark grown Phycomyces hyphae in the developmental stage used for membrane extraction. V, vacuole; SV, small unidentified vesicles; M, mitochondria. Bar = 1 μm.

Table 1. Effects of NaN₃ and Na-Vanadate on Mg²⁺-ATP-Dependent H⁺-Pumping in Crude and Step-Gradient Purified Light Microsomal Membranes (from 8.5/30% [w/v] Sucrose Interface)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Crude microsomal membranes</th>
<th>Step-gradient purified microsomal membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔE₄₉₀nm × (min)⁻¹ × (g fr wt equivalent)⁻¹</td>
<td>jausexfrdttmfrwehivutbxdjg</td>
</tr>
<tr>
<td>Control</td>
<td>0.011 ± 0.001*</td>
<td>0.009 ± 0.001</td>
</tr>
<tr>
<td>1 mM NaN₃</td>
<td>0.029 ± 0.003</td>
<td>0.010 ± 0.001</td>
</tr>
<tr>
<td>0.2 mM Na-vanadate</td>
<td>0.013 ± 0.001</td>
<td>0.009 ± 0.001</td>
</tr>
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*Values ± sd from a representative experiment.

broken ends of the hyphae of this unicellular organism at temperatures above 0°C. The immediate sealing will result in a massive loss of subcellular vesicles (9, 11, 30). The lower temperature (−8°C) affects membrane fluidity and probably reduces the formation of small protoplasts otherwise lost during the initial centrifugation step. The reduction of fungal protease activity may also contribute to the increased yield of transport competent tonoplast-type vesicles; however, in view of, the long centrifugation times (as compared with the extraction which took little more than 5 min) this seems a less likely explanation.

The Cl⁻-stimulated Mg²⁺ ATP-driven H⁺-pumping activity characterized in this study shares several features with Mg²⁺ ATP-dependent H⁺-transport in tonoplast vesicles from higher plant and Ascomycetes. These include: (a) the stimulation by Cl⁻ (Fig. 2), (b) the inhibition by nitrate and sulfate, (c) the absence of vanadate, molybdate, and azide effects on H⁺-pumping in the step-gradient purified vesicles (Table I), (d) the strong inhibition by diethylstilbestrol and dicyclohexylcarbodiimide which are both effective inhibitors of higher plant and Ascomycete tonoplast H⁺ATPase, and (e) the substrate kinetics for ATP and competitive inhibition by ADP (Fig. 4)(1, 21). The relatively low inhibition by nitrate may indicate some remaining contamination with inside-out plasma membrane vesicles. However, the absence of any inhibition by vanadate rules out this interpretation. The apparent stimulation of H⁺-pumping in the crude microsomal fraction by sodium azide and sodium vanadate is difficult to explain at present. A massive mitochondrial and plasma membrane contamination might lead to rapid decrease of substrate (decrease of ATP, increase of the competitive inhib-
Fig. 3. Inhibition of Mg\textsuperscript{2+} ATP-dependent acidification of step-gradient purified membrane vesicles by CaCl\textsubscript{2} monitored with neutral red. EGTA was omitted from the test medium. Values give percent inhibition of \( v_{\text{max}} \).

Fig. 4. Eadie-Hofstee plot of substrate kinetics for Mg\textsuperscript{2+} ATP-dependent acidification, and its competitive inhibition by ADP, monitored with neutral red. Mg\textsuperscript{2+} concentration was kept constant at 2 mm. Values are calculated from \( v_{\text{max}} \) of acidification; \( k_{\text{M(ADP)}} \) 1.1 mm, \( k_{\text{M(ATP)}} \) 0.8 mm.

ator (ADP) without driving Cl\textsuperscript{-}-stimulated H\textsuperscript{+}-transport at a rate comparable to tonoplast vesicles (the higher \( k_{\text{M(ADP)}} \) supports this possibility).

Since ATP-dependent proton pumping in plant Golgi vesicles seems to be less sensitive towards nitrate than in tonoplast vesicles (7), this could suggest the presence of Golgi-type vesicles in our membrane preparation. However, in view of the poorly characterized Golgi system in Phycomyces it is too early to draw any firm conclusion.

The observation that Cl Bis-Tris propane promoted H\textsuperscript{+}-transport more strongly than KCl, as well as the partial reversal of Cl Bis-Tris propane driven maximum acidification by KCl suggest that like in plant tonoplast vesicles a K\textsuperscript{+}/H\textsuperscript{+} exchanger may be operative (12).

The absence of a detectable H\textsuperscript{+}-pumping pyrophosphatase in the same membrane fraction points to a dissimilarity with higher plant tonoplast and Golgi vesicles (22). Taiz (26) has recently proposed that in higher plants the pyrophosphatase pump may use the PPI formed during many biosynthetic reactions. Young growing Phycomyces mycelia are rapidly synthesizing chitin involving high concentration of UDP-N-acetylglucosamine, the formation of which yields PPI. Therefore, theoretically, a PPI driven pump would be of advantage to the fungus. Whether the failure to detect PPI driven H\textsuperscript{+}-pumping in Phycomyces was due to an experimental artifact, or really reflects its absence is still uncertain. However, as in the same assay system membranes from several higher plants showed PPI driven H\textsuperscript{+}-pumping the former possibility seems at least unlikely.

Preliminary studies (Western blots) with polyclonal antibodies against the 70 kD and 60 kD subunits of the Neurospora vacuole H\textsuperscript{+}-ATPase revealed crossreactivity with Phycomyces polypeptides of 71 kD and 57 kD, respectively, giving further support to the identification of a Phycomyces tonoplast-type H\textsuperscript{+}-ATPase (T Rausch, E Bowman, B Bowman, unpublished results).

With respect to the function of vacuoles and other acidic vesicles in Phycomyces it is tempting to speculate that like Neurospora and Saccharomyces high concentrations of basic amino acids may be stored therein (4). It is known that Phycomyces contains high concentrations of free arginine (14). The possible presence of basic amino acid/proton antiporter(s) is currently under investigation. This study add Phycomyces, as an otherwise thoroughly investigated lower fungus, to the extended list of organisms with an anion-sensitive Mg\textsuperscript{2+} ATP-dependent proton pump opening a range of transport studies in vitro at the membrane level.

Acknowledgments—Electron microscopy was done by Manfred Ruppel. Helpful discussions with Dr. Ralf Barckhausen and Thomas Botz are gratefully acknowledged.

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