Plasma membrane isolation from freshwater and salt-tolerant species of Chara: antibody cross-reactions and phosphohydrolase activities

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Abstract
Plasma membranes were isolated using the aqueous polymer two-phase partition method from the algae Chara corallina and Chara longifolia, algae which differ in their ability to grow in saline environments. Enrichment of plasma membrane and depletion of tonoplast relative to the microsomal fraction was monitored using phosphohydrolase assays and cross-reactions to antibodies raised against higher plant transporters. Antibodies to the vacuolar ATPase and pyrophosphatase cross-reacted with epitopes in the microsomal fraction, but showed little affinity for the plasma membrane fraction. Pyrophosphatase activity also declined in the plasma membrane fraction relative to the microsomal fraction. The V-type H⁺-ATPase activity, sensitive to nitrate or bafilomycin, was low in both fractions, though the cross-reaction to the antibody was reduced in the plasma membrane fraction. By contrast, the antibody recognition of a P-type H⁺-ATPase amino acid sequence from Arabidopsis did not occur strongly in the anticipated 90–100 kDa range. While there was enhanced recognition of a polypeptide at around 140 kDa in the plasma membrane fraction, salt treatment of Chara longifolia resulted in plasma membrane fractions with reduced amounts of this epitope, but no change in vanadate-sensitive ATPase activity, suggesting that it does not represent the only P-type ATPase. Microsomal membranes from salt-adapted C. longifolia have higher reactivity with the antibody to the tonoplast ATPase.

Key words: Chara, plasma membrane, salt tolerance, ATPase.

Introduction
The giant internodal cells of the Characeae have been of considerable utility as a model system for elucidating the transport properties of plant cells due to the relative ease with which it is possible to measure electrophysiological properties such as membrane potential (Beilby, 1989), fluxes of transported substrates and their concentrations in cytoplasmic compartments (Hope and Walker, 1975). However, the very property that makes these advances possible, the large cell size and its concomitant low surface area to volume ratio, presents a serious obstacle if the biochemical properties of the plasma membrane, particularly the ATPase activity associated with the electrogenic pump (Spanswick, 1981), are to be investigated.

Early work by Atkinson and Polya (1967) demonstrated the presence of ATPase activity in Chara. Cytochemical studies showed the association of ATPase activity with several types of cellular and organelar membranes (Price and Whitecross, 1983). The only published account of the isolation of plasma membranes from Chara appears to be that of Andjus and Beljanski (1992). Using the aqueous polymer two-phase partitioning technique with 6.7% polymer, they were able to obtain a fraction in which the ATPase activity was 0.02 μmol Pi mg⁻¹ protein min⁻¹. The activity was inhibited by up to 48% by vanadate, but retained some sensitivity to NO₃⁻ (17%), which may indicate some residual tonoplast contamination.

The separation of plasma membranes and tonoplast from Chara corallina, a freshwater species, and from Chara longifolia, a salt-tolerant species is described here. This project was initiated as a biochemical complement...
to electrophysiological and flux studies on the sodium tolerance of *C. longifolia* (Hoffmann and Bisson, 1987). These indicated that H\(^+\) pumping is enhanced under salt conditions (Yao et al., 1992) and that transport processes which depend on the activity of the ATPase, namely the Na\(^+\)/H\(^+\) antiport (Whittington and Bisson, 1994), are important for survival under saline conditions. The primary objective was to obtain a plasma membrane fraction relatively free of tonoplast contamination as the basis for future studies on the enzyme composition of the membrane in relation to salt tolerance.

**Materials and methods**

**Plant material**

Microsomal and plasma membrane-enriched fractions were isolated from *Chara corallina* and *C. longifolia*. *C. corallina* was cultured in a series of pond water (APW), which contains low salt levels (e.g. 1 mM Na\(^+\)). *C. longifolia* (previously identified as *C. buckellii*) was cultured under identical low-salt conditions or saline culture with 130 mM Na\(^+\) (Hoffmann and Bisson, 1986).

**Membrane isolation**

Plants were cut from culture tanks, rinsed in distilled water, and homogenized at 4°C using a food processor. The homogenization buffer consisted of 250 mol m\(^{-3}\) sucrose; 2 mol m\(^{-3}\) Na\(_2\)EDTA; 10% glycerol (w/v); 0.5% protease-free BSA (w/v); 2 mol m\(^{-3}\) DTT; 1 mol m\(^{-3}\) PMSF; and 50 mol m\(^{-3}\) BTP adjusted to pH 8.0 with dry MES. The breakage of nuclei, which results in gel-like pellets, was minimized by adding the plant material to the food processor first and then starting homogenization immediately upon addition of the homogenization buffer. Homogenates were filtered through four layers of cheesecloth centrifuged at 10,000 x g for 10 min. The supernatants were filtered through nylon mesh (149 μm spaces) and centrifuged at 85,000 x g for 35 min. The resulting microsomal pellets were resuspended in buffer (250 mol m\(^{-3}\) sucrose, 3 mol m\(^{-3}\) KCl, 5 mol m\(^{-3}\) potassium phosphate, pH 7.8, 1 mol m\(^{-3}\) PMSF, 0.2 mol m\(^{-3}\) EGTA) for aqueous polymer two-phase partitioning.

**Aqueous polymer two-phase partitioning**

Phase partitioning in a range of polymer concentrations was accomplished by application of 0.5 g aliquots (1.8 mg membrane protein each) of divided microsomal resuspension to each of a series of 3.5 g phase systems that ranged from 6.1% to 7.3% final polymer concentration (polyethylene glycol 3350, Sigma, and dextran T 500, Pharmacia, w/w) in phase buffer. The phase systems were mixed, centrifuged at 500 x g for 12 min at 4°C. After one such partition, the upper phase was designated U\(_1\); after repeated extractions, it was designated U\(_n\) (e.g. U\(_2\) after three extractions). The upper phases were removed for protein assay and SDS-PAGE. Phase separations were also carried out by obtaining U\(_3\) fractions using 10 g phase systems (Faraday and Spanswick, 1992) that contained 7.0% polymer in phase buffer. Generally, 70 g of starting material yielded 6 mg of microsomal protein which, in turn, would yield 300 μg of U\(_3\) plasma membrane protein.

**Results and discussion**

To determine the optimal polymer concentration for separation of membrane fractions, microsomal fractions membranes from *C. corallina* were subject to aqueous polymer two-phase partitioning with varying concentrations of polymer. Immunoblotting was used for preliminary characterization of the resulting membrane fractions. Figure 1 shows typical SDS-PAGE and marker phosphohydrolase immunoblots obtained with microsomal and U\(_1\) fractions obtained by aqueous polymer two-phase partitioning with polymer concentrations that ranged from 6.1% to 7.3%. At a polymer concentration of 7% there was a diminished reaction with tonoplast marker antibodies (Fig. 1B, C) relative to the reaction with an antibody to the plasma membrane ATPase (Fig. 1D). The silver-stained gel is shown in Fig. 1A. The mixed

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anti-V-ATPase antibodies recognized an epitope at about 56 kDa in both maize and Chara (Fig. 1B), while the epitope at about 70 kDa was recognized in maize but was barely detectable in Chara. The anti-V-PPase antibodies recognized an appropriate polypeptide in maize microsomal membranes at about 60 kDa and in the Chara microsomal fraction at a slightly lower mobility (Fig. 1C). The P-ATPase antibodies recognized the appropriate

![Image of SDS-PAGE and marker phosphohydrolase immunoblots](image_url)

Fig. 1. SDS-PAGE and marker phosphohydrolase immunoblots of Chara corallina microsomal (m) fraction and upper phases after aqueous polymer separation in 6.1% to 7.3% polymer. Maize microsomal and U₄ fractions (where shown) are presented for comparison and positive controls. (A) Silver-stained gel. The amount of protein applied to each lane was 5 μg. (B–D) Marker phosphohydrolase immunoblots. Polypeptides from gels equivalent to that shown in (A) were electrotransferred to nitrocellulose, and probed with antibodies raised against (B) subunits of the vacuolar H⁺-translocating ATPase (V-ATPase); (C) the vacuolar H⁺-translocating pyrophosphatase (V-PPase); (D) the plasma membrane H⁺-translocating ATPase (P-ATPase). The relative molecular mass (kDa) of prestained markers is shown on the left. The amount of protein applied to each lane was 20 μg.
polypeptide at about 100 kDa in maize, but the reactions in the Chara fractions were stronger with polypeptides that ran above the 125 kDa marker and in the low molecular mass (30 kDa) range. The tonoplast marker epitopes were enhanced in the 6.1% phase-separated material relative to the microsomal fraction lanes, but diminished as the polymer concentration increased. A polymer concentration of 7.0% was used for further work. Marker phosphohydrolase enzyme assays were conducted on microsomal and U₃ phase partitioned membrane fractions from Chara corallina (Table 1). Pyrophosphatase specific activity in the microsomal fraction was nearly four times higher than in the U₃ fraction. Nitrate- and bafilomycin-sensitive ATPase activities at pH 8, both characteristic of the V-type ATPase, were close to the limits of detection. Thus the enzyme assays support the results obtained with the antibodies in suggesting that the U₃ fraction obtained using 7.0% polymer for phase partitioning had low tonoplast contamination. An unusual property of the ATPase activity in the U₃ fraction was the relatively high proportion of the ATPase activity measured at pH 8 that was sensitive to vanadate (Table 1).

A silver-stained gel (SDS–PAGE) and immunoblots of microsomal and phase-partitioned membrane fractions from C. longifolia subjected to low- and high-salt treatments during growth are shown in Fig. 2. While the microsomal fractions appear similar in the number and position of bands, the U₃ fractions show a number of marked differences, including bands more marked in the freshwater cultures around 90, 67, and 30 kDa, and more marked in the salt around 88, 65, 50, 36, and 32 kDa. This is consistent with the microsomal fraction having a large percentage of endomembranes, which would be protected from changes in salinity in the external medium, and the U₃ fraction being enhanced in plasma membrane, which is most exposed to the changing external medium.

Table 1. ATPase and pyrophosphatase specific activities associated with microsomal and phase-partitioned U₃ (7% polymer) plasma membrane vesicles from Chara corallina; reactions included azide and molybdate

<table>
<thead>
<tr>
<th>ATPase specific activity (µmol Pi mg⁻¹ protein min⁻¹)</th>
<th>Microsomal fraction</th>
<th>U₃ fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control pH 6.5</td>
<td>0.137</td>
<td>0.225</td>
</tr>
<tr>
<td>d NO₃</td>
<td>0.039</td>
<td>0.027</td>
</tr>
<tr>
<td>d Vanadate</td>
<td>0.147</td>
<td>0.233</td>
</tr>
<tr>
<td>Control pH 8.0</td>
<td>0.164</td>
<td>0.272</td>
</tr>
<tr>
<td>d NO₃</td>
<td>0.164</td>
<td>0.002</td>
</tr>
<tr>
<td>d Bafilomycin</td>
<td>0.001</td>
<td>0.003</td>
</tr>
<tr>
<td>Control pH 8.0p</td>
<td>0.145</td>
<td>0.210</td>
</tr>
<tr>
<td>d Vanadate</td>
<td>0.068</td>
<td>0.118</td>
</tr>
<tr>
<td>Pyrophosphatase specific activity (µmol PiP mg⁻¹ protein min⁻¹)</td>
<td>0.174</td>
<td>0.046</td>
</tr>
</tbody>
</table>

a,b Independent assays

The V-ATPase antibodies recognized an epitope in the 50 kDa range, as in C. corallina, that was enhanced in the microsomal fraction of the salt-grown compared with the freshwater-grown plants (Fig. 2B). The epitope was nearly eliminated by two-phase partitioning. There have been reports of enhancement of the vacuolar ATPase in higher plants on salt treatment (Klink et al., 1990; Reuveni et al., 1990). The V-ATPase antibody recognized epitopes at the appropriate relative mobilities in the microsomal fractions. No significant difference was seen between fresh- and salt-cultured plants. As expected, the reaction was greatly reduced in the U₃ phase-partitioned fractions (Fig. 2C). The P-ATPase antibody reactions were greatest in the low-salt-treated U₃ phase-partitioned fractions (Fig. 2D). This was unexpected, since many electrophysiological experiments performed previously indicated that the proton pumping activity was enhanced in the salt-cultured C. longifolia (Yao et al., 1992), and since enhancements of the plasma membrane ATPase or proton pumping capacity have been shown in a number of higher plants (Braun et al. 1986; Niu et al., 1993; Watad et al., 1986) challenged by salt. While enhanced pumping can be the result of physiological control on the ATPase, rather than on increased amounts of ATPase, it was not expected that the amount of ATPase would decrease. Vanadate-sensitive ATPase specific activity in the U₃ plasma membrane fractions from low- and high-salt-grown C. longifolia were similar (Table 2). The higher proton pumping activity in vivo in the salt-cultured plants could represent physiological control on a similar amount of transport enzyme present. Since this physiological control would be lost in the in vitro enzyme assays, no differences would be seen. The fact that antibody reaction decreases in the salt-cultured alga despite the fact that vanadate-sensitive ATPase remains similar suggests that the antigen recognized is not the P-type ATPase responsible for the enzymatic activity or proton-pumping measured. The molecular weight is higher than that measured for most P-type ATPases. However, Wada et al. (1989) have identified two polypeptides in a marine alga, Heterosigma akashiwo, which are like P-type ATPases in that they are phosphorylated by ATP in the presence of Mg²⁺ alone, and dephosphorylated in the presence of monovalent ions. One has a typical molecular weight of 95 kDa, but the other is much larger, around 150 kDa. They suggest that the latter is a Na-ATPase, based on the fact that its activity is enhanced by Na⁺, although this would have to be confirmed by measuring actual transport activity. They also show high vanadate-sensitive ATPase activity at pH 8.0, like the vanadate-sensitive ATPase activity reported here.

Conclusions

The aqueous polymer two-phase partitioning method appears to be useful for separating plasma membranes
Fig. 2. SDS–PAGE and marker phosphohydrolase immunoblots of Chara longifolia microsomal and U₃ phase-partitioned (7% polymer) membrane fractions. Plants were cultured under high-salt (+) or low-salt (−) conditions. Relative molecular mass of prestained markers are shown on the left. (A–D) are as in Fig. 1.

Table 2. ATPase and vanadate-sensitive specific activities at pH 6.5 associated with microsomal and phase-partitioned U₃ (7% polymer) plasma membrane vesicles from Chara longifolia grown in high and low salt. Assays included azide and molybdate.

<table>
<thead>
<tr>
<th>ATPase specific activity (μmol Pi mg⁻¹ protein min⁻¹)</th>
<th>Microsomal fraction</th>
<th>U₃ fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-salt control</td>
<td>0.087</td>
<td>0.150</td>
</tr>
<tr>
<td>Δ Vanadate</td>
<td>0.055</td>
<td>0.077</td>
</tr>
<tr>
<td>High-salt control</td>
<td>0.145</td>
<td>0.105</td>
</tr>
<tr>
<td>Δ Vanadate</td>
<td>0.082</td>
<td>0.075</td>
</tr>
</tbody>
</table>

from tonoplast in Chara as judged both by enzyme assays and by immunoblots using antibodies to plasma membrane and tonoplast proteins. The ATPase specific activity of the U₃ fraction obtained from C. corallina (Table 1) was approximately 10 times that of the phase-partitioned preparation obtained by Andjus and Beljanski (1992) even though it was assayed at 30°C instead of 38°C. This may partly be the result of the inclusion of detergent in the ATPase assays reported in the present work to include the latent activity present in right-side-out membrane vesicles. In any case, the activities reported in the present work approach the range reported for preparations from higher plants and should make it feasible to characterize the activity associated with the plasma membrane. Salt culture enhances the expression of an apparent V-type ATPase. However, the expression of a polypeptide which is recognized by an antibody to a P-type ATPase is reduced. Further work is needed to assess the physiological significance of these changes for salt adaptation.

Acknowledgements

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