Determination of the Inorganic Pyrophosphate Level and Its Subcellular Localization in Chara corallina*

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In order to determine the concentration of pyrophosphate (PPi) and its subcellular distribution in Chara corallina, a new method to concentrate PPi from cell extracts was developed. PPi was extracted and concentrated as Ca$_2$P$_2$O$_7$ under alkaline conditions. The amount of PPi in the precipitate was measured using an enzyme system containing pyrophosphate:fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.80) coupled to NADH oxidation in the presence of [ethylenebis(oxyethylenenitrilo)tetraacetic acid].

The subcellular localization of PPi, and inorganic phosphate (Pi) was studied using the intracellular perfusion technique. The relative volumes of the cytoplasm (6.4%) and the vacuole (93.6%) were determined by perfusing Lucifer Yellow CH into the vacuole and by assuming that the Lucifer Yellow CH dead space represented the cytoplasmic volume. The volume of the chloroplast layer was determined microscopically, and it was found that it occupied 10% of the Chara cytoplasm. PPi was present predominantly in the cytosol at a level of 193 μM, while it existed in the vacuole at a level of only 2.20 μM and less than 1 μM in chloroplasts.

By contrast, Pi was distributed almost equally in the cytosol (12.0 mM), chloroplasts (16.2 mM), and the vacuole (6.70 mM). The electrochemical potential gradient across the tonoplast for H$^+$ ($\Delta\mu$ H$_2$O = -11.6 to -18.0 KJ/mol) was nearly equal to the free energy release from the hydrolysis of PPi in cytoplasm ($\Delta G_{\mu}$ = -18.9 KJ/mol), indicating that the H$^+$-translocating inorganic pyrophosphatase can work as a H$^+$ pump in C. corallina.

Many biological reactions, including the synthesis of acyl-CoA during $\beta$-oxidation, the activation of amino acids, DNA and RNA polymerization, and polysaccharide synthesis, yield high levels of PPi. For example, 9.4 nmol/g fresh weight tissues in pea seedlings (Edwards et al., 1984), 5-39 nmol/g fresh weight tissues in corn seedling (Smyth and Black, 1984), and 3.8-4.7 nmol/mg chlorophyll in spinach leaves (Weiner et al., 1987). The existence of a significant amount of PPi in plant tissues indicates that PPi can serve as an energy source for PPi-consuming reactions. Pyrophosphatase:fructose-6-phosphate 1-phosphotransferase is ubiquitous enzyme that is found in bacteria, algae, and higher plants (Carnal and Black, 1983). Also, the recent discovery of H$^+$-translocating inorganic pyrophosphatase (H$^+$-PPase) in the vacuolar membrane of higher plants (Rea and Sanders, 1987, and references therein) and Characeae (Takeshige et al., 1988) suggests that the H$^+$-PPase together with the H$^+$-ATPase functions to maintain a steep H$^+$ gradient across the tonoplast and may in turn control the transport of inorganic ions and organic solutes via secondary active transporters in the vacular membrane.

To elucidate these possibilities, we have to first determine the subcellular localization of PPi, or its contents in the cytosol, chloroplasts, and vacuole. Therefore, this kind of work has not been done largely due to the difficulties in separating the various cell components and determining their relative volumes.

Internodal cells of Characeae have been used widely for studies on physiological activities such as cell motility, membrane excitation, and membrane transport (Tazawa et al., 1987; Tazawa and Shimmen, 1987). The cells have several advantages. Using the intracellular perfusion technique (Tazawa, 1984; Tazawa et al., 1976), subcellular organelles can easily be fractionated (Takeshige et al., 1988). Furthermore, the relative volumes of the organelles has been determined (Kishimoto and Tazawa, 1965; Sakano and Tazawa, 1984; Saltman and Christensen, 1961; and Tazawa et al., 1974). In the present study, we developed a new method to concentrate PPi from a cell extract. Using this method and the perfusion technique, we obtained quantitative data on the subcellular localization of PPi in Chara corallina. Based on these results, a possible role of the tonoplast H$^+$-PPase is discussed.

**EXPERIMENTAL PROCEDURES**

Plant Material—C. corallina was cultured as described before (Takeshige et al., 1985).

**Measurement of the Cytoplasmic Volume**—Taking advantage of the intracellular perfusion technique, the cytoplasmic volume of Chara cells was measured. A membrane-impermeable dye, Lucifer Yellow CH (Stewart, 1978), was used as an internal standard. The intracellular perfusion was performed according to Tazawa (1964). The composition of the artificial vacuolar sap (AVS) was 80 mM KCl, 90 mM Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), bovine serum albumin (BSA), and 1 mM EGTA. The AVS composition was 1% per HzO, 10% per AVS, 10% per HzO, 10% per AVS, 10% per HzO, 10% per AVS, 10% per HzO, and 10% per AVS. The intracellular perfusion technique was used to determine the cytoplasmic volume of Chara cells. A membrane-impermeable dye, Lucifer Yellow CH, was used as an internal standard.

**Intracellular Perfusion Technique**—The intracellular perfusion technique was used to determine the cytoplasmic volume of Chara cells. A membrane-impermeable dye, Lucifer Yellow CH, was used as an internal standard.

**Measurement of the Cytoplasmic Volume**—Taking advantage of the intracellular perfusion technique, the cytoplasmic volume of Chara cells was measured. A membrane-impermeable dye, Lucifer Yellow CH (Stewart, 1978), was used as an internal standard. The intracellular perfusion was performed according to Tazawa (1964). The composition of the artificial vacuolar sap (AVS) was 80 mM KCl, 90 mM Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), bovine serum albumin (BSA), and 1 mM EGTA. The AVS composition was 1% per HzO, 10% per AVS, 10% per HzO, 10% per AVS, 10% per HzO, 10% per AVS, 10% per HzO, and 10% per AVS. The intracellular perfusion technique was used to determine the cytoplasmic volume of Chara cells. A membrane-impermeable dye, Lucifer Yellow CH, was used as an internal standard.
The volume of a single chloroplast layer.

$$V_{\text{ch}} = \frac{4}{3} \pi abc, \quad V_{\text{p}} = \frac{8}{3} abc$$

where $a$, $b$, and $c$ represent half of three axial lengths of a chloroplast, respectively. Then, the volume ratio between $V_{\text{ch}}$ and $V_{\text{p}}$ is calculated to be $\frac{1}{6}$. This ratio is immutable for any combination of length, width, and thickness of chloroplasts, and can be applicable for calculation of the volume ratio between the chloroplasts and the chloroplast layer.

Using this value, the volume ratio between the chloroplasts and the whole cell was calculated. In this study, the cell diameter ranged from 0.7 to 0.9 mm and the chloroplast layer occupied 0.63-0.88% of the cell volume. On the average, chloroplasts were assumed to occupy 0.61% of the cell volume. In the following, we call the cytoplasm excluding chloroplasts the cytosol.

**Extraction and Concentration of PP, from Cells**—The process of extracting and concentrating PP, is summarized in Fig. 2. Fifteen internodal cells (0.6-0.8 g) were frozen in liquid nitrogen and ground into a powder in a mortar. The powder was transferred into 200 $\mu$l of 20% (w/v) trichloroacetic acid and was allowed to stand for 15-30 min on ice to denature the enzymes. The extract was then centrifuged for 10 min at 15,000 $\times$ g at 0 °C. The pellet was resuspended by adding 150 $\mu$l of water and centrifuged again for 10 min at 15,000 $\times$ g.

The first and second supernatants were combined and neutralized to pH 7-8 with 4 M Tris.

To study the subcellular localization of PP, the vacuolar sap and the cytoplasm were collected separately by using the intracellular perfusion technique (Takeshige et al., 1988). The vacuolar sap was collected directly by internally perfusing the cells with AVS. It was mixed with 200 $\mu$l of 20% trichloroacetic acid and the mixture was placed on ice for 15-30 min. The portion of the perfused cells that remained, including cytoplasm, plasma membrane, and cell wall, were

15 internodes

Freeze in liquid N$_2$

Grind into powder in a mortar

Crude homogenate (~800 $\mu$l)

$\sim$-Add 20% (v/v) trichloroacetic acid, 206 $\mu$l (final concentration = 5% trichloroacetic acid)

15-30 min. 0 °C

Centrifuge (15,000 $\times$ g, 10 min) once

$\sim$-Pellet, resuspend in H$_2$O

Supernatants

$\sim$-Add 1 M Tris (100 $\mu$l)

$\sim$-pH 10 with KOH

$\sim$-Add 1 M CaCl$_2$ (40 $\mu$l), 0.25 M K$_2$CO$_3$ (40 $\mu$l)

15-30 min at 0 °C

Centrifuge (15,000 $\times$ g, 10 min)

Pellet, wash twice with $\text{H}_2\text{O}$

Resuspend in 1 N HCl

**Fig. 2.** Schematic diagram of the procedure used for extracting and concentrating inorganic pyrophosphate from the cells of *C. corallina.*
directly dropped into liquid nitrogens and ground into a powder in a mortar. The extraction of PPi was performed as described above.

For the concentration of PPi, the following procedure was performed. The pH of the solution was carefully adjusted to 10.0 and 10.5 with 0.05 M KOH. Then 40 μl of 1 M CaCl₂ and subsequently 40 μl of 0.25 M K₂CO₃ were added to the solution. The resulting precipitate of CaCO₃ acts as a co-precipitant for Ca₃P₂O₇. The solution was kept on ice for 15–30 min and centrifuged for 10 min at 15,000 x g at 0 °C. The supernatant was discarded and the pellet was washed twice with water and dissolved into a small amount of 1 N HCl.

**Determination of the Amount of P** and **PPi**—The PPi present in the samples was determined enzymatically using a commercial pyrophosphate assay kit (Sigma, P7257). In addition, 400 μM Hepes-KOH (pH 7.5), 15 μM ascorbate, and 25 mM EGTA were added to the assay mixture. The reaction was initiated by adding an aliquot of sample and the oxidation of NADH was measured spectrophotometrically at 340 nm using a Hitachi Model 220A spectrophotometer.

The amount of P, was determined as described before (Takebishi et al., 1988).

**Relationship between Cell Volume and Fresh Weight**—In some experiments, we only measured the fresh weight of cells. To convert it into the cell volume, the fresh weight and the cell volume of Chara were measured and compared. For the calculation of the cell volume, the shape of Chara internodes was approximated to be a cylinder. The diameter was measured microscopically and the length was measured with a ruler. The fresh weight of Chara cells was measured using an electronic reading balance (Shimadzu Libror EB-280) after blotting the surface water. The density of the Chara cells was calculated to be 1.15 mg fresh weight/pl cell volume. A relatively high density was due to the surface CaCO₃ precipitate, that has a density of 2.7–2.9 g/ml (Raven, 1984).

**Chemicals**—Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.11) from baker’s yeast and Lucifer Yellow CH were purchased from Sigma. Percoll was purchased from Pharmacia (Uppsala, Sweden). All chemicals used were of analytical grade.

**RESULTS**

**Determination of the Relative Volumes of the Vacuole, Chloroplasts, and Cytosol**—Table I shows the results of the measurements. Because the squeezed-out cell sample contained components from both the AVS and the cytoplasm, concentrations of Lucifer Yellow CH in this sample should be lower than that of the AVS alone. The decrease is proportional to the relative volume of cytoplasm. From the calculation, the vacuole and the cytoplasm occupied 93.6 and 6.4% of the cell volume, respectively. Since the chloroplasts occupy 0.61% of the cell volume, the relative volume of the cytosol is 5.8%.

We used these values for the calculation of the PPi; concentrations in the vacuole and the cytoplasm.

**Extraction of Pyrophosphate**—We first investigated the reliability of the assay method. Because the final sample was dissolved in 1 M HCl, this acidic sample might have some effects on the assay system. To check this, 22.5 nmol of PPi was dissolved in 1 M HCl and was added to the sample. Although the pH of the assay mixture decreased from 7.41 to 7.16 despite the existence of 400 nM Hepes, this pH shift had no effect on the enzyme assay, EGTA, which is necessary to prevent the formation of Ca₃P₂O₇, precipitates at neutral pH and had no effect on the assay (data not shown).

To confirm the reliability of the extraction method, the recovery of PPi, was examined. The cell extract was divided into two parts. Five nanomoles of Na₄P₂O₇ was added to one sample, and 5 nmol of K₂HPO₄ was added to a control sample. Following extraction and assay, the difference in the amount of PPi recovered in two samples was 5.12 ± 0.57 nmol (mean ± S.E. of four experiments); that is, the added PPi was completely recovered.

We also checked whether the measured molecule was truly PPi. After the samples were neutralized with Tris, they were divided into two parts. Either PPase or BSA was added to each sample. The samples were incubated for 30 min at 37 °C and assayed for PPi. The amount of PPi, recovered from the samples treated with PPase decreased more than 85% compared with that of the sample treated with BSA. The extract did not contain any substances that interfered with the assay, because a known amount of PPi, was recovered without any loss in the presence of cell extracts.

One novel point in our method consists of concentrating PPi, as a Ca₃P₂O₇ precipitate at an alkaline pH. As is well known, several phosphate compounds like ATP are unstable in alkaline solutions, so we checked the possibility that PPi, was generated by the alkaline conditions (Table II). In addition to 8.75 nmol of Na₄P₂O₇, 50 nmol of either KH₂PO₄, KADP, or K₂ATP was added to the samples, and the pH was raised to various values. The pH of samples should be more than 9.8 in order to obtain a complete recovery of PPi, (Table II). Almost no PPi, formation from ADP occurred in the pH range tested. In the case of ATP, PPi, recovery had already exceeded 100% at pH 10.5 and reached 165% at pH 11.5. This means that the PPi, formation from ATP occurred especially at pH values above 11.0. In conclusion, the pH of samples should be adjusted between 9.8 to 10.5 to obtain complete recovery of PPi, and to avoid the formation of PPi, from the nucleotides.

**Subcellular Localization of PPi**—Concentrations of PPi, in the total cytoplasm and vacuole were calculated (Table III). The total cytoplasm contained 193 μM PPi, and the vacuole

### TABLE I

**Measurement of the absorbance of Lucifer Yellow CH in AVS and samples**

<table>
<thead>
<tr>
<th>Absorbance %</th>
<th>AVS</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.467 ± 0.002 (100) (n = 4)</td>
<td>0.437 ± 0.012 (93.6) (n = 10)</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE II**

**PPi, recovery from samples containing P₄, ADP, or ATP at various pH values**

<table>
<thead>
<tr>
<th>pH</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.7</td>
<td>1.1</td>
</tr>
<tr>
<td>9.0</td>
<td>82.9</td>
</tr>
<tr>
<td>9.5</td>
<td>88.7</td>
</tr>
<tr>
<td>9.8</td>
<td>93.7</td>
</tr>
<tr>
<td>10.0</td>
<td>111</td>
</tr>
<tr>
<td>10.5</td>
<td>165</td>
</tr>
<tr>
<td>11.0</td>
<td>152</td>
</tr>
</tbody>
</table>

**TABLE III**

**Subcellular localization of PPi, and P₄ in Chara corallina**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>[PPi]</th>
<th>[P₄]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasm</td>
<td>182 ± 12.0 (n = 4)</td>
<td>12.4 ± 3.69 (n = 14)</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>&lt;1</td>
<td>16.2 ± 4.90 (n = 2)</td>
</tr>
<tr>
<td>Cytosol*</td>
<td>193</td>
<td>12.0</td>
</tr>
<tr>
<td>Vacuole</td>
<td>2.20 ± 0.53 (n = 9)</td>
<td>6.70 ± 0.76 (n = 7)</td>
</tr>
</tbody>
</table>

*Concentrations of PPi, and P₄, in the cytosol were calculated as described under "Experimental Procedures."
had 2.19 \mu M P_{i}. The isolated chloroplasts contained very little P_{i} (less than 1 \mu M). Considering that the chloroplasts occupy approximately 10\% of the cytoplasm, the concentration of P_{i} in the cytoplasm was calculated to be 215 \mu M. When P_{i} was extracted from whole cells, the level of P_{i} was 14.4 \mu M, which was equal to the sum of P_{i}. measured separately in the vacuolar sap and cytoplasm (14.4 \mu M). This indicates that P_{i} was not lost during the separation of the vacuolar sap.

In contrast to P_{i}, P was distributed almost equally between all compartments. Similar P distribution in the vacuole and cytoplasm is equal to the sum of P_{i} measured separately.

In the internodal cells of Nitella opaca, the chloroplasts were measured directly under a microscope. An additional advantage of our method in estimating the relative volume of the chloroplasts had been overestimated in former works, where the chloroplasts were packed by centrifuging the cells. The packed chloroplasts should have the inter-chloroplastic space occupied with cytoplasm. The real volume of the chloroplasts would have been obtained by reducing this interchloroplastic space. Such an error can be eliminated by using our present method, since the volume of chloroplasts was measured directly under a microscope. Another advantage of our method in estimating the relative volume of the vacule and cytoplasm is that we do not need to know the absolute cell volume but only the magnitude of the dilution of the Lucifer Yellow CH in the samples.

**Discussion**

**Determination of Relative Vacuolar and Cytoplasmic Volume**—To obtain accurate concentrations of substances in Chara cells, it is necessary to determine exactly the relative volume of the cytosol, chloroplasts, and vacuole. Saltman and Christensen (1961) first studied this problem by centrifuging the internodal cells of Nitella opaca. The three cellular fractions, chloroplast fraction, cytosolic fraction, and vacuolar fraction, were separated distinctly, and each fraction occupied 3.8, 4.9, and 91.3\%, respectively, of the cell volume. Kishimoto and Tazawa (1965), applied a similar centrifugation method to Nitella flexilis and determined the relative volume of the chloroplast layer to be 4.63–5.84\% and that of the cytosol to be 3.15–3.37\%, respectively.

In our present report, we determined the relative volume of the cytosol, chloroplasts, and vacuole to be 5.8, 0.6, and 93.6\%. The relative volume of the chloroplasts had been overestimated in former works, where the chloroplasts were packed by centrifuging the cells. The packed chloroplasts should have the inter-chloroplast space occupied with cytoplasm. The real volume of the chloroplasts would have been obtained by reducing this interchloroplastic space. Such an error can be eliminated by using our present method, since the volume of chloroplasts was measured directly under a microscope. Another advantage of our method in estimating the relative volume of the vacule and cytoplasm is that we do not need to know the absolute cell volume but only the magnitude of the dilution of the Lucifer Yellow CH in the samples.

**Inorganic Pyrophosphate Extraction**—The method to measure the PP_{i} content in tissues of Pisum sativum was developed using fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90) coupled with the oxidation of NADH (Edwards et al., 1984; Smyth and Black, 1984). Although the specificity of the method was sufficient, the recovery of PP_{i} from the tissues during extraction deviated from sample to sample as described below. Smyth and Black (1984) reported that less than 60\% of added PP_{i} was recovered during their extraction process, while Edwards et al. (1984) reported a recovery of 91.4\% using almost the same extraction procedure as that of Smyth and Black (1984). Our preliminary experiments revealed that there are several disadvantages when using HClO_{4}, for PP_{i} extraction. When HClO_{4}, was neutralized with KOH, a certain amount of PP_{i} was found to co-precipitate with the KClO_{4} (data not shown). Moreover, the treatment of spinach leaves with 20\% (v/v) HClO_{4} for 3 h on ice was not sufficient to inactivate the endogenous pyrophosphatase, while extraction with trichloroacetic acid led to complete inactivation (Weiner et al., 1987). For this reason we used trichloroacetic acid instead of perchloric acid to extract PP_{i} from Chara. Incubation of cell extracts in 5\% (w/v) trichloroacetic acid for 15 to 30 min on ice was enough to inactivate the endogenous phosphatase, as evidenced by the fact that exogenous PP_{i} was not hydrolyzed by the enzyme after this treatment.

The concentration of PP_{i} in the initial cell extract was not high enough to measure the PP_{i} content quantitatively. Our attempt to concentrate PP_{i} with CaCl_{2} and KF, as reported by Heinonen et al. (1981) was unsuccessful, partly because we could not find a good method to dissolve the formed PP_{i} precipitates in our assay medium. Instead, we developed a new method to co-precipitate PP_{i} with Ca^{2+} as Ca_{2}P_{2}O_{7} in alkaline conditions. In this method the pH during the concentration step was most critical, because pH values lower than 9.0 decreased the recovery of PP_{i}, while pH values higher than 11.0 led to the formation of PP_{i}, from ATP. By contrast, no generation of PP_{i} from ADP occurred during this process. Since the concentration of phosphate compounds in a cell extract was usually not high enough, the addition of CaCO_{3} as a co-precipitant of Ca_{2}P_{2}O_{7} was essential. The precipitate was easily dissolved into a small amount of 1 N HCl (usually 50 \mu l). This final sample was directly added to the assay mixture, which contained a high buffer capacity (400 mM Hepes). The presence of 25 mM EGTA in the assay mixture prevented the re-precipitation of Ca_{2}P_{2}O_{7} at a neutral pH by removing free Ca^{2+}.

The subcellular localization of PP_{i}, was determined using the intracellular perfusion technique. As shown in Table III, most of the PP_{i} was located in the cytosol (193 \mu M). The low PP_{i} concentration in the vacuolar fraction may be explained either by the occurrence of a high acid phosphatase activity in the Chara vacuolar sap (Takeshige et al., 1988) or the absence of a PP transporter in the vacuolar membrane. Also, the absence of PP in the chloroplasts was consistent with the finding that chloroplasts contain an alkaline pyrophosphatase (Weiner et al., 1987).

**Function of the Tonoplast H^{+} Pumps**—A new type H^{+}-translocating Pase, was found in the Chara vacuolar membrane (Takeshige et al., 1988). Both pumps were located in the same vacuolar membrane (Rea and Sanders, 1987; Shimmen and MacRobbie, 1987). This raises a new interest in the physiological roles of two functional H^{+} pumps existing in the same vacuolar membrane. One possibility is that these pumps may control the level of either ATP or PP_{i} in the cytosol by switching the direction of the pumps from pumping H^{+} into the vacuolar sap to synthesizing ATP or PP_{i}, using the electrochemical H^{+} gradient (\Delta \phi_{H}) across the tonoplast. The answer to this question can be obtained from

<table>
<thead>
<tr>
<th>Table IV</th>
<th>References</th>
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<tbody>
<tr>
<td>[ATP]_{tot} (mM) *</td>
<td>1.17</td>
</tr>
<tr>
<td>[ADP]_{tot} (mM) *</td>
<td>0.547</td>
</tr>
<tr>
<td>[Pi]_{cyt} (mM)</td>
<td>12.0</td>
</tr>
<tr>
<td>[Pi]_{cytosol} (mM)</td>
<td>0.193</td>
</tr>
<tr>
<td>pH</td>
<td>7.09, 7.82</td>
</tr>
<tr>
<td>\Delta \phi_{H}, (mV)</td>
<td>5.19</td>
</tr>
<tr>
<td>\Delta G_{PP} (KJ/mol)</td>
<td>-30.7</td>
</tr>
<tr>
<td>\Delta G_{PP} (KJ/mol)</td>
<td>-18.2</td>
</tr>
</tbody>
</table>

* [ATP]_{tot}, [ADP]_{tot} were recalculated from the data in Takeuchi and Kishimoto (1983) under the assumption that the volumes of the cytoplasm (cyt) and vacuole were 6.4 and 93.6\%, respectively.
a thermodynamic treatment by comparing the free energy change during the hydrolysis of the substrate and the $\Delta \mu_{H^+}$ across the vacuolar membrane. The free energy change during the hydrolysis of ATP ($\Delta G_{\text{ATP}}$) and PPi ($\Delta G_{\text{PP}}$) was calculated from Equations 1 and 2, respectively,

$$\Delta G_{\text{ATP}} = \Delta G'_{\text{ADP}} + R T \ln \frac{[\text{P}_i]}{[\text{ATP}]}$$

$$\Delta G_{\text{PP}} = \Delta G'_{\text{PP}} + R T \ln \frac{[\text{P}_i]}{[\text{PP}]}$$

where $\Delta G'_{\text{ATP}}$ and $\Delta G'_{\text{PP}}$ are the standard free energy changes of ATP and PPi, hydrolysis, respectively, $R$ is the gas constant (8.314 J/mol K), $T$ is the absolute temperature (293 K), and $[\text{P}_i]$ is, for example, the concentration of inorganic phosphate in the cytoplasm. All the parameters, necessary for the calculations are now reported in C. corallina, and they are summarized in Table IV. From these values, $\Delta G_{\text{ATP}}$ and $\Delta G_{\text{PP}}$ are calculated to be $-43.3$ KJ/mol and $-18.9$ KJ/mol, respectively. $\Delta \mu_{H^+}$ across the vacuolar membrane can also be calculated from Equation (3).

$$\Delta \mu_{H^+} = R T \ln \frac{[H^+]_v}{[H^+]_c} + F \Delta \phi_v$$

where $[H^+]_v$ and $[H^+]_c$ are the proton activities in the cytoplasm and the vacuole, respectively, $F$ is Faraday constant, and $\Delta \phi_v$ is the vacuolar membrane potential between the cytoplasm and the vacuole. From the values in Table IV, $\Delta \mu_{H^+}$ is calculated to be between $-11.6$ and $-18.0$ KJ/mol. In order for the $H^+$-translocating ATPase and PPase to work as $H^+$ extruding pumps, the conditions, where $\Delta G_{\text{ATP}} < n\Delta \mu_{H^+}$ and $\Delta G_{\text{PP}} < n\Delta \mu_{H^+}$, should be fulfilled, where $n$ is the pump stoichiometry. In Characeae we do not know the stoichiometry of these pumps, but for the tonoplast-type $H^+$-ATPase from storage tissues of Beta vulgaris a $H^+$/ATP stoichiometry of 2 was reported (Bennett and Spanswick, 1984). Even in the case where the $H^+/ATP$ stoichiometry is 2, the tonoplast $H^+$-ATPase in Chara is still capable of pumping $H^+$ into the vacuolar sap. When the $H^+/PP$, stoichiometry is 1, $\Delta \mu_{H^+}$ is nearly equilibrated with $\Delta G_{\text{PP}}$ and the $H^+$-PPase can work as a $H^+$-extruding pump. If the $H^+/PP$, stoichiometry is greater than 2, the $H^+$-PPase may work as PP, synthesizing enzyme by utilizing the $\Delta \mu_{H^+}$ established by the $H^+$-ATPase.

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REFERENCES


