Mechanism of the Decline in Vacuolar H+-ATPase Activity in Mung Bean Hypocotyls during Chilling

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ABSTRACT

The mechanism responsible for the decrease in the activity of vacuolar H+-ATPase during chilling was investigated in seedlings of mung bean (Vigna radiata). After chilling at 0°C for 3 d, the activity of vacuolar H+-ATPase, calculated on the basis of membrane protein, decreased to 47% of the original value. Of the nine subunits of the ATPase, the specific contents of at least six subunits, of 68, 57, 44, 38, 37, and 32 kD, decreased in vacuolar membranes after chilling, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These subunits were released by treatment with chaotropic anions such as thiocyanate. The level of the 16-kD subunit did not change. Immunoblot analyses showed the decrease in the levels of the subunits of 68, 57, and 32 kD. Furthermore, the specific activity of the ATPase purified from chilled hypocotyls was two-thirds of that of the enzyme from nonchilled seedlings, and the enzyme from chilled tissue retained only a small amount of the 32-kD subunit. These results suggest that a selective release of the peripheral subunits of the ATPase from the membrane and a partial degradation of the ATPase complex may occur in vivo during chilling.

An H+-ATPase acidifies the vacuolar lumen of plant cells together with an H+-pyrophosphatase (2, 6, 22). A pH gradient induced across vesicle membranes by the proton pumps is used as an energy source for the secondary transport systems of vacuolar membrane (2, 22). Vacuolar H+-ATPase is known to be sensitive to cold temperatures (1, 2, 16, 19). Among several enzymes in mung bean (Vigna radiata) hypocotyls, the activity of vacuolar H+-ATPase preferentially decreased during chilling of etiolated seedlings (27). A nearly identical phenomenon has been demonstrated in suspension cultures of rice (9). In consideration of cellular function of the ATPase, the decline in vacuolar ATPase activity during chilling may cause physiological damage to cells. Therefore, we are conducting a series of experiments to investigate the effect of chilling treatment on the vacuolar ATPase in seedlings of mung bean, a chilling-sensitive plant.

Vacuolar-type ATPases are widely distributed in a variety of acidic endomembranes of eucaryotes (5, 13, 14, 17-20, 25). These ATPases are composed of a hydrophilic catalytic part and a hydrophobic membrane part, similar to the F0F1-type ATPases (3, 8, 11, 17, 21, 23). The "ball and stalk" structures of the ATPases were detected by EM on negatively stained vacuolar membranes (3, 23). Vacuolar H+-ATPases are large molecular complexes consisting of several subunits (1, 3, 4, 8, 11, 16, 17, 19, 26). Mung bean enzyme appears to be composed of nine subunits with molecular masses of 68, 57, 44, 38, 37, 32, 16, 13, and 12 kD (15). Vacuolar H+-ATPases are rapidly inactivated by cold treatment in the presence of Mg-ATP and chaotropic anions such as nitrate (1, 3, 11, 16, 19). Cold inactivation in vitro results from the detachment from the vacuolar membranes of the peripheral catalytic complex consisting of the catalytic A subunit, noncatalytic B subunit, and several minor subunits.

We focused our attention on the mechanism of decrease in the activity of vacuolar H+-ATPase during chilling in vivo. In the present study, antibodies against the two major subunits (A and B subunits) and the 32-kD subunit of the enzyme were prepared and used for the determination of levels of subunits in the vacuolar membranes. Differences in the specific activity of ATPase and levels of ATPase subunits between chilled and nonchilled seedlings are described. A change in the subunit profile of the ATPase during chilling is also described.

MATERIALS AND METHODS

Plant Material

Seeds of mung bean (Vigna radiata L. Wilczek) were allowed to imbibe 1 mM CaSO4 and germinated in the dark at 26°C for 3.5 d (27). The 3.5-d-old seedlings were wrapped in moist paper towels and chilled at 0°C in the dark for 1 to 3 d (27). Seedlings did not grow at 0°C. The 3.5-d-old seedlings without chilling treatment were used as nonchilled seedlings. Hypocotyls were excised from seedlings and used for the preparation of vacuolar membranes.

Membrane Preparation and Enzyme Purification

Vacuolar membranes were prepared from tissue homogenate of hypocotyl by differential and floating centrifugation as reported previously (15). The vacuolar membranes were treated with 5% (w/v) Triton X-100 in 0.1 M KCl prior to solubilization of H+-ATPase with 2 mg/mL lysophosphatidylcholine and purification by column chromatography on QAE-Toyopearl (15).

Preparation of Antibodies and Immunoblot Analysis

Three subunits of 68, 57, and 32 kD of the ATPase were isolated by SDS-PAGE and electroelution. Antibodies against

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MECHANISM OF DECLINE IN VACUOLAR ATPase ACTIVITY

Table 1. Decrease in Activity of Vacuolar H+-ATPase during Chilling of Seedlings

<table>
<thead>
<tr>
<th>Days at 0°C</th>
<th>Protein</th>
<th>Hydrolysis of ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>µmol·h⁻¹</td>
</tr>
<tr>
<td>0</td>
<td>14.1 ± 0.2</td>
<td>272 ± 30</td>
</tr>
<tr>
<td>1</td>
<td>13.1 ± 1.6</td>
<td>182 ± 28</td>
</tr>
<tr>
<td>3</td>
<td>7.6 ± 0.3</td>
<td>69.2 ± 0.2</td>
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these subunits were raised in rabbits by injection of the isolated subunits (300 µg each), and then immunoglobulin G fractions were prepared from the sera by fractionation with ammonium sulfate and column chromatography on DEAE-cellulose. Electrophoretic blotting was performed by the method of Towbin et al. (24). The antibodies that reacted to the corresponding antigens on nitrocellulose membranes were visualized with horseradish peroxidase-linked protein A and 4-chloro-1-naphthol. Levels of antigens on the nitrocellulose filter were quantified by densitometric scanning.

Treatment with Chaotropic Anions

The vacuolar membranes (2 mg/mL) were treated with 5 mM EDTA and centrifuged at 170,000g for 40 min. The precipitates were suspended in 20 mM Tris-acetate, pH 7.5, 10% (w/v) glycerol, 1 mM DTT, and 1 mM EGTA, then incubated with 0.5 M KCl, KSCN, or NaClO₄ at 4°C for 30 min. After dilution with 20 mM Tris-acetate, pH 7.5, the suspension was centrifuged at 170,000g for 40 min. The polypeptides in the supernatants were precipitated by cold 10% TCA and subjected to SDS-PAGE.

Analytical Measurements

ATPase activity of the purified enzyme was assayed in 250 µL containing 3 mM ATP, 3 mM MgSO₄, 50 mM KCl, 1 mM sodium molybdate, 20 µg of phospholipid, and 30 mM Tris-Mes, pH 7.0 (15). When the vacuolar membranes were assayed for ATPase activity, Triton X-100 (0.03%, w/v) was added and phospholipid was omitted (15). Activity of ATP-dependent H⁺ transport across membrane vesicles was measured as the rate of fluorescence quenching of acridine orange and expressed as the relative fluorescence (%ΔQ) as described previously (27). The reaction medium (2.0 mL) contained 0.25 M sorbitol, 3 mM ATP, 3 mM MgCl₂, 50 mM KCl, 3 µM acridine orange, 25 mM Hepes-Bis Tris propane, pH 7.5, and membrane vesicles (100 µg). Protein content was determined by the method of Lowry et al. (12). SDS-PAGE was performed using 13.5% polyacrylamide gels by the method of Laemmli (10). After Coomassie blue staining, the intensity of staining of protein bands was quantified by densitometric scanning.

RESULTS

Decrease in Vacuolar H+-ATPase Activity during Chilling in Vivo

Mung bean seeds germinated for 3.5 d at 26°C were chilled at 0°C, then the vacuolar membrane fractions were prepared from 300 g of the hypocotyls and assayed for ATPase activity (Table I). The total activity of ATPase markedly decreased from 272 to 69 µmol·h⁻¹ during chilling for 3 d. The amount of membrane protein decreased to 54% of the control value after chilling for 3 d, suggesting that degradation of the membrane occurred in the hypocotyls in the late period of chilling treatment. It may be due to a decrease in biosynthesis and/or an increase in degradation of membrane proteins during chilling in vivo. The specific activity of ATPase, calculated on the basis of the protein amount in the membranes, decreased to 72 and 47% of the nonchilled control value during chilling for 1 and 3 d, respectively. This may be a reflection of either a decrease in the amount of ATPase and/or a partial inactivation of the enzyme.

Changes in Levels of H+-ATPase Subunits during Chilling in Vivo

To determine the amounts of ATPase subunits, we prepared antibodies against the subunits of 68, 57, and 32 kD. As shown by the immunoblot test in Figure 1, each antibody reacted specifically with the corresponding subunit. The effects of the three different antibodies on vacuolar H+-ATPase were tested (Table II). All three antibodies inhibited both ATP hydrolysis and H⁺ translocation, although the latter activity was more severely inhibited by every antibody. The...
Table II. Antibody Inhibition of ATPase Activity

Vacuolar membranes (12.4 μg) prepared from nonchilled seedlings were incubated with the anti-68, anti-57, or anti-32 (immunoglobulin G fraction, 480 μg) at 20°C for 30 min, then assayed for ATPase activity. The activity of ATP-dependent H⁺ translocation was assayed after preincubation of the membranes (62 μg) with antibody (1.4 mg) at 20°C for 20 min. The control immunoglobulin G fraction was prepared from nonimmune serum. Values are expressed as mean ± se calculated for three individual measurements.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>ATP-Hydrolyzing Activity</th>
<th>H⁺-Translocating Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol·h⁻¹·mg⁻¹</td>
<td>%ΔQ·min⁻¹·mg⁻¹</td>
</tr>
<tr>
<td>None</td>
<td>12.9 ± 0.1</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td>12.3 ± 0.3</td>
<td>95</td>
</tr>
<tr>
<td>Anti-68</td>
<td>6.1 ± 0.9</td>
<td>47</td>
</tr>
<tr>
<td>Anti-57</td>
<td>10.3 ± 0.9</td>
<td>80</td>
</tr>
<tr>
<td>Anti-32</td>
<td>10.1 ± 0.3</td>
<td>78</td>
</tr>
</tbody>
</table>

Inhibitory effect of anti-32² provides further evidence that the 32-kD subunit is an essential component of the enzyme. The subunits with molecular masses of 32 to 33 kD were detected in the vacuolar-type ATPases from plants (4, 19, 26), yeast (8), and mammals (17).

Polypeptide profiles of the vacuolar membrane were compared among the three purified preparations by SDS-PAGE (Fig. 2). The membrane fractions were treated with Triton X-100 to remove the proteins weakly associated with the membrane. By this treatment, none of the ATPase subunits were released from the membranes (15) and the resolution of separation in the gel was improved. The nine subunits of ATPase were distinguished on the stained gel. The intensity of staining of the subunits of 68, 57, 44, 38, 37, and 32 kD decreased during chilling of seedlings, whereas the band at 16 kD showed no change even after chilling for 3 d (Fig. 2, lanes 1–3). The subunits of 13 and 12 kD slightly decreased. On the other hand, the intensity of staining of the polypeptides of 50, 33, 27, 23, and 17 kD increased.

The decrease in the relative levels of the subunits was substantiated by the immunoblot analysis (Fig. 2, lanes 4–12). In these experiments, the vacuolar membranes were not treated with Triton X-100. Assayed by densitometric scan of the immunoblots, the levels of the 68-, 57-, and 32-kD subunits decreased to about 50% of the control values after chilling for 3 d. These results mean that selective degradation of the ATPase took place in the chilled tissue.

A band was observed at 33 kD in the immunoblot, and its amount increased during chilling (Fig. 2). The 33-kD polypeptide was not detected in the ATPase purified from the nonchilled seedlings (15). The polypeptide reacted with the anti-32, suggesting that it is immunologically analogous to the 32-kD subunit. The 33-kD polypeptide is not a proteolytic product of the 68- or 57-kD subunit, because neither the anti-68 nor the anti-57 reacted with the polypeptide (data not shown).

² Abbreviation: anti-32, anti-57, and anti-68, antibodies against the 32-, 57-, and 68-kD subunits, respectively.

Properties of the Purified Enzyme from Chilled Seedlings

To examine the changes in the molecular and functional properties of the enzyme during chilling of seedlings, the ATPase was purified from the chilled hypocotyls. The specific activity of the purified ATPase decreased to 75 and 66% of the control value after chilling for 1 and 3 d, respectively (Table III). To compare the subunits, the purified ATPases from three different seedlings were subjected to SDS-PAGE (Fig. 3). There was no difference during electrophoresis in the mobility of either subunit of the enzyme, i.e. their molecular masses did not change during chilling. These results mean that there is a partial dysfunction of the enzyme.

The level of the 32-kD subunit associated with the ATPase complex decreased after chilling in vivo for 3 d (Fig. 3). The

Table III. Changes in the ATP-Hydrolyzing Activity of the Purified Enzyme during Chilling of Seedlings

Mung bean seedlings (3.5-d-old) were placed at 0°C for the time indicated. Vacuolar membranes were prepared from 300 g of hypocotyls. The vesicles were treated with 5% Triton X-100, then the enzyme was solubilized and purified. Values are presented as mean ± se calculated for three independent experiments.

<table>
<thead>
<tr>
<th>Days at 0°C</th>
<th>Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>μmol·h⁻¹·mg⁻¹</td>
<td>μmol·h⁻¹·mg⁻¹</td>
</tr>
<tr>
<td>0</td>
<td>0.213 ± 0.016</td>
<td>36.4 ± 1.8</td>
<td>171 ± 15</td>
</tr>
<tr>
<td>1</td>
<td>0.238 ± 0.019</td>
<td>30.5 ± 0.6</td>
<td>128 ± 11</td>
</tr>
<tr>
<td>3</td>
<td>0.099 ± 0.004</td>
<td>11.3 ± 0.4</td>
<td>114 ± 6</td>
</tr>
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</table>
levels of the 13- and 12-kD subunits also slightly decreased. Densitometry of the stained gel revealed that the level of the 32-kD subunit in the purified ATPase decreased to 73 and 31% of the control value after chilling for 1 and 3 d, respectively. The 33-kD polypeptide that reacted with anti-32 was detected in the purified enzyme from the seedlings chilled for 3 d.

Effects of Chaotropic Anions on ATPase Subunits

The vacuolar membranes were treated with chaotropic anions to estimate the topology of ATPase subunits. Figure 4 shows the SDS-PAGE and immunoblot analysis of the supernatant fractions after treatment with 0.5 M KCl, KSCN, or NaClO₄. The subunits of 68, 57, and 32 kD were detected in extracts with KSCN and NaClO₄ but not in the extract with KCl. In addition to these three subunits, the subunits of 44, 38, 37, 13, and 12 kD were released from the membranes by treatment with KSCN or NaClO₄. The 16-kD subunit was retained in the membranes after treatment. The result is consistent with the previous reports (8, 16, 17, 19, 21, 26). The eight subunits extracted with chaotropic anions may make up the cytoplasmic catalytic complex of the ATPase.

DISCUSSION

The present work has shown three different effects of chilling treatment on the vacuolar H⁺-ATPase in mung bean seedlings: a partial degradation of vacuolar membranes, release of ATPase subunits, and inactivation of the enzyme. The decrease in the ATPase activity during chilling can be explained in part by the partial degradation of vacuolar membranes. In this study, we directed our attention to the decrease in the specific activity and specific content of the ATPase on the basis of membrane protein during chilling.

Mung bean H⁺-ATPase consists of nine different polypeptides, which include two major subunits of 68 and 57 kD, a N,N'-dicyclohexylcarbodiimide-binding subunit of 16 kD, and six other minor subunits (15). The subunits of 68, 57, 44, 38, 37, 32, 13, and 12 kD were released from the membranes by treatment with chaotropic anion at a concentration of 0.5 M (Fig. 4). These eight subunits may be components of the peripheral part of the ATPase. In this study, we found that the amounts of the eight subunits of ATPase, but not the 16-kD subunit, decreased during chilling in vivo. The 16-kD subunit is an integral membrane protein, and the interaction between the subunit and membrane lipids was not broken during chilling. Immunoblot analysis also showed the decrease in the amounts of the subunits of 68, 57, and 32 kD. Thus, it was concluded that chilling treatment of seedlings caused a selective release of the peripheral subunits of the ATPase from the vacuolar membrane.

The decline in ATPase activity during chilling in vivo seems to be related to the structural instability of the enzyme. It has been shown that chaotropic ions release the peripheral subunits of vacuolar ATPase from the membrane (1, 3, 8, 11, 16, 21). A similar mechanism was proposed for cold inactivation of vacuolar H⁺-ATPase. Parry et al. (19) reported that incubation of the vacuolar membranes from red beet with Mg-ATP and NaCl at 4°C caused a remarkable reduction in ATPase activity and detachment of five subunits of 67, 55, 52, 44, and 32 kD from the membrane. From the results of in vitro cold inactivation of the ATPase and the present experiments, we think that a partial degradation of the pe-
The purified H\textsuperscript+-ATPase from seedlings chilled for 3 d retained a small amount of the 32-kD subunit (Fig. 3), and ATPase activity of this preparation was 67\% of that of the intact, nine-subunit ATPase. This abnormal ATPase complex is a possible intermediate in the degradation of the enzyme. Horak et al. (7) obtained a mitochondrial F\textsubscript{1}-ATPase that lacked the 26.5-kD subunit by treatment of the enzyme with ammonium sulfate and deoxycholate. The resulting F\textsubscript{1} complex was considerably less stable at 0\textdegree C than was the intact F\textsubscript{1} complex with six subunits (7). Release of the component from the multimeric enzymes probably accelerates breakdown of the enzyme complex. In contrast with the 32-kD subunit, the 33-kD polypeptide that reacted with the anti-32 increased during chilling. The present results suggest that the low activity of the ATPase purified from chilled seedlings is due to a loss of the 32-kD subunit and/or an appearance of the 33-kD polypeptide. The immunoreactivity of this polypeptide with anti-32 suggests that the 33-kD polypeptide may be derived from the 32-kD subunit. Further characterization of the polypeptide is necessary to understand the relationship between the 32-kD subunit and 33-kD polypeptide.

LITERATURE CITED