Changes in Properties of Barley Leaf Mitochondria Isolated from NaCl-Treated Plants

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ABSTRACT

Treatment of barley (Hordeum vulgare) seedlings with 400 millimolar NaCl for 3 days resulted in a reduction in plant growth and an increase in the leaf content in ions (K⁺ + Na⁺) and proline. Purified mitochondria were successfully isolated from barley leaves. Good oxidative and phosphorylative properties were observed with malate as substrate. Malate-dependent electron transport was found to be only partly inhibited by cyanide, the remaining oxygen uptake being SHAM sensitive. The properties of mitochondria from NaCl-treated barley were modified. The efficiency of phosphorylation was diminished with only a slight decrease in the oxidation rates. In both isolated mitochondria and whole leaf tissue of treated plants, the lower respiration rate was due to a lower cytochrome pathway activity. In mitochondria, the activity of the alternative pathway was not modified by salt treatment, whereas this activity was increased in whole leaf tissue. The possible participation of the alternative pathway in response to salt stress will be discussed.

An increase in the salt concentration of the rhizosphere must be accompanied by an osmotic readjustment in plant cells to avoid water outflow and cell dehydration. This can be accomplished by absorption of ions in plants showing a tolerance to high salt concentrations (13). In cells of halophytes, salts fill the vacules while compatible solutes accumulate in the cytoplasm to avoid osmotic desiccation (29). With salt-sensitive species, such events do not, or only partly, take place. Thus, in crop plants, resistance to low salinities could be explained by the lack of accumulation of toxic ions such as sodium in photosynthetic tissues (34). Furthermore, in response to a severe saline stress, plants have to cope with a high influx of ions in all the organs, and generally show a growth reduction.

Uptake and compartmentation of ions in whole plant organs require energy mainly in the form of ATP generated from respiration (32). The production of compatible solutes is linked not only to the supply of reducing power and carbon skeletons, but also to energy production which depends on the efficiency of the phosphorylation process. Knowledge about respiratory metabolism during saline stress conditions is still lacking. In this context, the role of the nonphosphorylating cyanide-resistant pathway, which is a common feature of higher plant respiration (18, 28), is not fully elucidated. Some reports show that the activity of this alternative pathway could be modified in stress situations (17, 25).

Among crop plants, barley seems to be rather resistant to deleterious effects of NaCl salinity. However, under conditions of high saline environment barley plants do show growth reduction. In this context, changes in ion uptake (2), proline levels (33), gene expression (24), or more recently, changes in metabolic rates in roots (8) have been investigated. However, no work has been done on the role of mitochondria in leaves of barley plants submitted to high NaCl stress.

The isolation and purification of green leaf mitochondria from barley was performed, and the oxidative and phosphorylative properties of these mitochondria were compared between control plant and the treated with 400 mM NaCl. The effects of salt on cyanide resistance were also investigated in both isolated mitochondria and whole leaf tissue to look for a role of the alternative pathway in salt-treated barley leaves.

MATERIALS AND METHODS

Plant Material

Barley (Hordeum vulgare L., var Triumph) seeds were germinated on vermiculite soaked with Hoagland solution. For treated plants, the nutrient solution was supplemented with 400 mM NaCl, 72 h before harvesting. The plants were grown in a controlled chamber with a light intensity of 30 W·m⁻² at leaf level during a 15 h photoperiod, temperatures of 20°C (day) and 18°C (night), and 70 to 80% RH. Mitochondria were isolated from the primary leaves of 7-d-old plants.

Preparation of Mitochondria

Primary leaves (20 g) were cut into 150 mL of cold extraction medium containing 0.35 M mannitol, 30 mM MoPS buffer, 7 mM DTT, 2 mM EGTA, 2.5 mM MgCl₂, 0.2% BSA (w/v), 0.3% PVP (w/v), 0.3% PVPP¹ (w/v), the pH being adjusted to 7.4. The leaves were homogenized 4 × 1 s at full speed in a mixer (Moulinex). The homogenate was filtered through a 60 μm nylon net. The leaf fragments retained in the net were blended again in the mixer with 50 mL of the extraction medium and homogenizing and filtration repeated as described above. The two successive filtrates were pooled and submitted to differential centrifugation according to Gerard and Dizengremel (15). The washing medium was 0.35 M

¹ Abbreviations: PVPP, polyvinylpolypyrrolidone; SHAM, salicylhydroxamic acid; Mw, washed mitochondria; Mp, purified mitochondria.
mannitol, 10 mM MoPS buffer (pH 7.2), and 0.1% BSA (w/v).

Purification of Washed Mitochondria

The purification was performed using a self-generated Percoll (Pharmacia Fine Chemicals) gradient, according to Neuburger (21) and Gerard and Dizengremel (15), with some modifications. Washed mitochondria were suspended in a 28% (v/v) Percoll medium containing 0.25 M sucrose, 20 mM MoPS buffer, 0.1% BSA (w/v), 100 mM propane-1,2-diol, and 1% PVP-25 (w/v). After 40 min centrifugation at 40,000 g (Rotors 30, Beckman centrifuge L-5-50) the mitochondrial band was collected, diluted 10 times with washing medium, and centrifuged at 13,000 g for 15 min. The pellet was resuspended in a minimum volume of washing medium.

For analytical studies, 1 mL fractions of the Percoll gradient were collected using a capillary connected to a peristaltic pump. The density of the Percoll fractions was determined by refractometry.

Mitochondrial Respiration

Oxygen uptake was followed polarographically with a Clark electrode (Hansatech) at 25°C. Respiratory studies were performed in 1 mL of the reaction medium containing 0.35 M mannitol, 5 mM MgCl$_2$, 10 mM KCl, 0.1% BSA (w/v), and 10 mM phosphate buffer (pH 7.2).

The oxidation of succinate (20 mM) was measured in the presence of 200 $\mu$M ATP. Glutamate (2 mM) and NAD (400 $\mu$M) were added to follow the oxidation of malate (30 mM). Protein concentrations were determined by the procedure of Bradford (6). KCN in a water solution and SHAM in ethanol were used as inhibitors, respectively, of the Cyt and alternative pathways. During glycine oxidation, antimycin A (dissolved in ethanol) was used instead of KCN.

Leaf Slice Respiration

The primary leaves were harvested near the middle of the photoperiod. Leaf segments (0.8 cm long) were rinsed and infiltrated with a reaction medium containing 100 mM mannitol, 10 mM Heps, 10 mM Mes (pH 6.6), 0.2 mM CaCl$_2$. The leaf fragments were transferred in a Rank O$_2$ electrode and the O$_2$ uptake was measured at 25°C in 4 mL of the air-saturated reaction medium. The combination of inhibitors (10 mM SHAM, 5 mM NaN$_3$, 1 mM KCN) indicated that side effects, caused by peroxidases, were minimized (31). Therefore, the determination of respiratory parameters were made with single concentrations of SHAM (10 mM) and KCN (1 mM). SHAM was dissolved in 2-methoxyethanol and 6 to 8 min were necessary to obtain a stabilized inhibition rate.

Enzyme Assays and Analytical Methods

Succinate Cyt c oxidoreductase (EC 1.6.99.1) activity was assayed by following the reduction of Cyt c at 550 nm at 25°C. In the high tonicity medium (respiration medium) the activity is low when outer mitochondrial membranes are intact. In hypotonic medium (10 mOsm phosphate buffer [pH 7.2]), the activity is maximal as a result of disruption of the outer membranes. A comparison of the activities in the two media allowed assessment of the relative intactness of the outer mitochondrial membranes. The inner mitochondrial membrane integrity was assessed using the spectrophotometric assay for ferricyanide reduction (11). Intact inner mitochondrial membranes are impermeable to ferricyanide. When the electron transfer chain is blocked by antimycin A, electron transfer from succinate to ferricyanide cannot proceed. The presence of damaged inner mitochondrial membranes results in ferricyanide reduction after antimycin addition.

Hydroxypyruvate reductase (EC 1.1.1.29) activity was measured as described by Schwitzguebel and Siegenthaler (27).

Na$^+$ and K$^+$ were determined by flame photometry and proline was estimated spectrophotometrically using the ninhydrin method. Chl was determined according to Arnon (1).

RESULTS

Growth and Solute Contents

After a 72-h salt treatment, the growth of barley plants was severely inhibited. A decrease in dry matter was observed, more marked for the shoots (46%) than for the roots (33%) (Fig. 1). This decrease in dry matter production was accompanied by a slight water deficit in the two organs (~3%), the water content staying around 90% (fresh weight basis). There was only a minor decrease in the Chl content of the treated shoots (Fig. 1).

The NaCl enrichment in the culture medium induced an increase in Na$^+$ content of the tissues (Table I), more marked in the shoots. By contrast, the decreased K$^+$ content in treated plants was more pronounced in the roots (Table I). These changes found expression in a fall in the K$^+$/Na$^+$ ratio with a value less than 1 for both organs of the treated plants (Table I). Concerning the contribution of these electrolytes to the osmotic potential, it was interesting to note an increase in the

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**Figure 1.** Effect of NaCl treatment on the growth of 7-d-old barley plants. NaCl (400 mM) was applied 72 h before harvesting. Control (○); treated (●).
The electrolytes and proline contents were expressed in meq·g⁻¹ dry weight and μmol·g⁻¹ dry weight, respectively. The values in parentheses were expressed in mM, on a tissue water basis, considering that ions are sequestered in the vacuole and most of the proline in the cytoplasm. Each value is the mean of two independent experiments.

<table>
<thead>
<tr>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td>Shoot</td>
</tr>
<tr>
<td>[Na⁺]</td>
<td>0.21 (13)</td>
</tr>
<tr>
<td>[K⁺]</td>
<td>0.71 (43)</td>
</tr>
<tr>
<td>[Na⁺ + K⁺]</td>
<td>0.92</td>
</tr>
<tr>
<td>K⁺/Na⁺</td>
<td>3.4</td>
</tr>
<tr>
<td>Proline</td>
<td>ND*</td>
</tr>
</tbody>
</table>

*Not detected.

ADP severely inhibited the rate of O₂ consumption. Furthermore, succinate oxidation was poorly coupled, as shown by the low observed ADP/O and RC ratios (Fig. 3D). Glycine was oxidized more slowly than the other substrates, but with a good phosphorylative efficiency. The rates of glycine oxidation were increased by exogenous NAD (Fig. 3E), as were the rates of malate oxidation (Fig. 3C). The purified mitochondria showed a time-dependent loss of phosphorylation ability, with succinate and glycine as substrates.

Succinate and glycine oxidation were totally blocked by KCN or antimycin A, showing that, with these substrates, the electron flow was mediated only by the Cyt pathway (Figs. 3, D and E). On the other hand, KCN only partially inhibited malate oxidation and the cyanide-insensitive respiration (20%) was fully inhibited by SHAM, an inhibitor of the alternative pathway (26) (Fig. 3, A and B). SHAM added first inhibited 40% of the malate oxidation rate (Fig. 3C).

### Changes in Mitochondrial Properties Upon Salt Treatment

Based on the properties of mitochondria isolated from nontreated plants, malate seemed the most suitable substrate for comparison with the respiratory activities of mitochondria isolated from treated plants. Purified mitochondria isolated from treated plants oxidized malate and maintained phosphorylation (Fig. 3F). Cyanide partially inhibited malate oxidation; the cyanide-insensitive respiration being fully inhibited by SHAM (Fig. 3F).

Table II summarizes the parameters used to compare the oxidative and phosphorylative mitochondrial properties of both treated and nontreated plants with malate as substrate. In mitochondria from treated plants, the state 3 rate was slightly lower, while ADP/O and RC ratios were significantly diminished and accounted for a less efficient oxidative phosphorylation. Moreover, the sensitivity of the oxygen uptake to KCN and SHAM differed for treated and control plants. KCN inhibited 67% malate oxidation by mitochondria isolated from control plants compared to only 38% with mito-

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**Table I. Effect of NaCl Treatment on Solutes Content of Barley Plants**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Nontreated</th>
<th>Treated</th>
<th>Salt Stress</th>
<th>Salt Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Root</td>
<td>Shoot</td>
<td>Root</td>
<td>Shoot</td>
<td>Root</td>
</tr>
<tr>
<td>[Na⁺]</td>
<td>0.21 (13)</td>
<td>0.04 (4)</td>
<td>0.94 (93)</td>
<td>1.21 (153)</td>
<td></td>
</tr>
<tr>
<td>[K⁺]</td>
<td>0.71 (43)</td>
<td>1.40 (128)</td>
<td>0.22 (22)</td>
<td>0.94 (119)</td>
<td></td>
</tr>
<tr>
<td>[Na⁺ + K⁺]</td>
<td>0.92</td>
<td>1.44</td>
<td>1.16</td>
<td>2.15</td>
<td></td>
</tr>
<tr>
<td>K⁺/Na⁺</td>
<td>3.4</td>
<td>32.0</td>
<td>0.2</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>62 (85)</td>
<td></td>
</tr>
</tbody>
</table>

*Not detected.*

**Figure 2.** Purification on a self-generated Percoll gradient of barley leaf mitochondria isolated from control plants. Distribution of markers in separated fractions 1 mL. Fraction density (○), Chl (■), succinate Cyt c oxidoreductase (□).
Mitochondria isolated from treated plants. With SHAM, inhibition was slightly higher with treated mitochondria.

In order to appreciate changes in the distribution of electron flow along the Cyt and alternative pathways upon salt treatment, their capacities and activities were determined (Table III). According to Bahr and Bonner (4) who postulated that the Cyt pathway operates at the highest possible rate under the given experimental conditions \((V_{\text{Cyt}} = V_{\text{Cyt}}^*)\), the activity of the alternative pathway was directly determined as the fraction of the respiration rate inhibited by SHAM. In isolated mitochondia, the ratio of the activity and capacity gives the engagement \((p')\) of the alternative pathway. This ratio value was slightly higher than the engagement value \((p)\) determined by titration experiments. Thus, whatever the means of \(p\) determination, the alternative pathway functions near to maximal capacity in control plants and the engagement of this pathway was lower in treated plants (Table III). However, the alternative pathway activity \((v_{\text{alt}})\) was poorly modified upon salt treatment, while the total oxygen uptake decreased. Consequently, \(P(v_{\text{alt}}/V_1)\), which represents the participation of the alternative pathway to the total electron flow, was higher in treated plants.

Respiratory parameters were also determined in whole tissues (Table III). Upon salt treatment, the total respiration rate decreased as well as in isolated mitochondria. By contrast, the capacity of the alternative pathway decreased, while its activity was higher. Consequently, both the engagement of the alternative pathway and the \(P\) ratio increased in whole leaf tissue in response to the salt stress.

Finally, the lower rates of total O2 uptake in leaves of treated plants found mainly expression in a lesser Cyt pathway activity when the alternative pathway activity did not change or increased according to the leaves or mitochondria isolated therefrom are considered. It should be noted that changes in mitochondrial respiratory parameters upon salt treatment accompanied a decrease in the integrity of the outer mitochondrial membrane (from 82–72%) and a less pronounced decrease in the inner membrane integrity (from 73–69%).

**DISCUSSION**

Purified Chl-free mitochondria were successfully isolated for the first time from barley leaves. Many experiments have used C4 leaf mitochondria (Zea mays, Panicum) but very few isolations have been performed on C3 gramineous plants: wheat (3) and oat (16). The purified barley leaf mitochondria showed normal oxidative properties with succinate and malate as substrates, comparable to those observed with mitochondria from other gramineous leaves (14, 16, 22). A high degree of intactness of the purified mitochondria was demonstrated by determining the level of outer (82%) and inner (73%) membrane integrity. Moreover, the rate of glycine oxidation was similar to that of other C3 plant mitochondria (12), with rather good respiratory control and ADP/O ratios. Succinate oxidation displayed a progressive inhibition as oxidation proceeded. This inhibition could result from an accumulation of oxaloacetic acid (10). But the high succinate concentration (20 mM), used to minimize this effect (12), did not improve the oxidative processes. Consequently, the optimal conditions for succinate oxidation in purified barley leaf mitochondria were only partly met, as is also observed with other leaf mitochondria (22). Malate was the best oxidized

<table>
<thead>
<tr>
<th>Table II. Changes in Respiratory Activities of Purified Barley Leaf Mitochondria Isolated from Control and Treated Plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>The oxidation rates in the presence of ADP (state 3) are expressed as nmol O2·min⁻¹·mg⁻¹ protein. The inhibition of malate oxidation (µM of state 3 rate) were determined in the presence of 800 µM KCN and 750 µM SHAM. Values are means from five independent experiments ± SD.</td>
</tr>
<tr>
<td>Malate Oxidation</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>State 3 rate</td>
</tr>
<tr>
<td>Respiratory control</td>
</tr>
<tr>
<td>ADP/O ratio</td>
</tr>
<tr>
<td>KCN inhibition (%)</td>
</tr>
<tr>
<td>SHAM inhibition (%)</td>
</tr>
</tbody>
</table>

Figure 3. Respiratory activities of washed (Mw) and purified (Mp) leaf mitochondria from nontreated (A, B, C, D, E) and treated (F) barley seedlings. Thirty mm malate (Mal) was oxidized in the presence of 2 mm Glutamate (Glu) and 400 µM NAD (NAD). ATP (200 µM) was added to the medium prior to the addition of 20 mm Succinate (Succc.). The glycine (Glyc.) concentration was 10 mm. Mitochondrial protein amounts were: A, 340 µg; B–C, 150 µg; D, 85 µg; E, 200 µg; F, 150 µg. The oxidation rates are expressed in nmol O2·min⁻¹·mg⁻¹ protein.
During succinate induced changes a severe production of oxygen uptake was detected in barley leaves and in mitochondria isolated therefrom. However, to a lesser extent, even if the alternative path was impaired, the sum of activities of both the Cyt pathway (Vcyt) and the ADP/O ratio (Vad) was determined, in mitochondria and whole tissues, in the presence of 0.75 mM SHAM and 10 mM KCN, respectively. Concentrations of 0.8 mM KCN and 1 mM KCN were used to determine the alternative pathway capacity in mitochondria and in whole tissues, respectively. The residual respiration (Vres) is the portion of oxygen uptake resistant to a combination of KCN and SHAM. The degree of the engagement of the alternative pathway (ρ') is the ratio: Vres/Vad. The activity of the alternative pathway (Vres) was the fraction of Vr inhibited by SHAM. Values in parentheses (ρ') were determined according to the Bahr and Bonner's method (1973). P is the ratio of Vme/VT and expresses the effective participation of the alternative pathway. Values are the mean of three to five independent experiments ± SD.

<table>
<thead>
<tr>
<th>Mitochondria</th>
<th>Vr</th>
<th>Vcyt</th>
<th>Vad</th>
<th>Vad</th>
<th>Vres</th>
<th>ρ'</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>85 ± 29</td>
<td>57 ± 7</td>
<td>28 ± 9</td>
<td>28 ± 9</td>
<td>0</td>
<td>1 (0.67)</td>
<td>0.34</td>
</tr>
<tr>
<td>Treated</td>
<td>63 ± 10</td>
<td>37 ± 7</td>
<td>39 ± 6</td>
<td>26 ± 7</td>
<td>0</td>
<td>0.66 (0.49)</td>
<td>0.41</td>
</tr>
<tr>
<td>Whole tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>120 ± 38</td>
<td>89 ± 3</td>
<td>49 ± 3</td>
<td>20 ± 5</td>
<td>11 ± 6</td>
<td>0.41</td>
<td>0.17</td>
</tr>
<tr>
<td>Treated</td>
<td>95 ± 15</td>
<td>53 ± 8</td>
<td>38 ± 3</td>
<td>31 ± 8</td>
<td>11 ± 2</td>
<td>0.82</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Preliminary experiments showed that rotenone inhibition of malate oxidation was only slightly lower in mitochondria from salt treated barley. The control at the first phosphorylating site is probably the major constraint from electron flow in mitochondria of both tissues, the further preferred distribution of electrons into the alternative pathway of mitochondria from salt treated plants being due to a restricted Cyt phosphorylating pathway.

In vivo, other regulatory mechanisms should be expected. The lower rates of respiration could be a consequence of a low supply of carbohydrates (23). The nature of the Cyt impairment at the whole leaf tissue level may be a consequence of a direct control of respiration by the turnover of ATP: a reduced ATP utilization in shoots of treated plants could explain the lower rate of respiration. Such an hypothesis has been already proposed in barley root (5). However, it must be admitted that the energy cost for the Na+ influx in shoots of treated plants has to be low to avoid a rise in ATP demand.

Furthermore, in whole leaf tissue, the activity and the engagement of the alternative pathway increased in response to salt treatment. In these conditions, the alternative pathway may have a particular role. In our experiment the NaCl stress was applied when the primary leaf was in full expansion (4 d old). The NaCl treatment involves a break in the shoot growth, with probable diversions in metabolism of products. Thus, sugars would not be used for growth, but in condition where the Cyt path is reduced by ATP (17), the alternative pathway could act in a way to removing respiratory substrates to produce carbon intermediates. These intermediates could be the precursors of solutes such as proline, used as a compatible osmoticum or known to have some specific roles in stress conditions (30).

Finally, mitochondria isolated from salt-stressed leaves and saturated with a particular substrate showed a clear tendency to keep a correct alternative pathway activity. At the whole leaf tissue level, this corresponds to an increased role of this pathway in providing the minimal energy needed, the Cyt substrate, with a good efficiency of oxidative phosphorylation. However, a cyanide resistance (30%) was observed in this substrate whereas no alternative electron transport occurred during succinate and glycine oxidation. Consequently, the changes induced by the salt treatment were looked at the oxidation of malate.

Treatment with 400 mM NaCl for 72 h was sufficient to produce a severe reduction in barley growth. Shoot growth was more affected than the root growth and it is tempting to correlate this result with the high content of Na+ in the shoot even if the toxicity of this cation is debatable (20). In response to salt treatment, the total rate of oxygen uptake declined in leaves and in mitochondria isolated therefrom. Moreover, respiratory parameters as the RC and ADP/O ratios showed lower values in mitochondria isolated from treated plants. Lower rates of oxygen uptake were determined in pea leaves when saline environment was imposed (7). By contrast, a rise in respiration was detected in whole leaf tissue and in mitochondria of pea seedlings submitted to 77 mM NaCl (19). However, in this case the reduction of the plant growth was minor and no change in ADP/O ratio was achieved, which accounted for a slight saline stress.

Considering the membranes integrity poorly modified upon salt treatment, the changes in mitochondrial respiratory parameters could be induced from changes in the electron fluxes of the respiratory chain. In plant mitochondria, electron transport mainly occurs via a cyanide-sensitive, phosphorylating pathway and occasionally via an alternative, SHAM sensitive, and nonphosphorylating pathway. In isolated mitochondria of treated plants oxidizing malate, the activity of the Cyt pathway declined. In the same time, the activity of the alternative pathway was unchanged. Therefore, the lower rates of oxygen uptake in mitochondria only involved a lower Cyt pathway activity. Moreover, the contribution of the alternative pathway in the total electron flux (P ratio) increased. Consequently, both the variations of the Cyt and alternative pathways activities may partly explain the decrease in the phosphorylation process.
pathway being limited by the diversion of respiratory substrates for the accumulation of organic solutes combined with adenylate constraint. Probably, it could be useful to examine the response of isolated mitochondria to a cocktail of substrates (9), not necessarily furnished at saturating levels, to get a better understanding of the respiratory process in salt-stressed barley leaves.

LITERATURE CITED