Ascorbate-Glutathione Cycle in Mitochondria and Peroxisomes of Pea Leaves: Changes Induced by Leaf Senescence

By

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Key words: Ascorbate, ascorbate-glutathione cycle, ascorbate peroxidase, dehydroascorbate reductase, glutathione, glutathione reductase, hydrogen peroxide, mitochondria, monodehydroascorbate reductase, peroxisomes, Pisum sativum, senescence.

Summary


Mitochondria and peroxisomes from pea (Pisum sativum L.) leaves were fractionated to determine the subcellular location of the enzymes of the ascorbate-glutathione (ASC-GSH) cycle. All four enzymes, ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1) and glutathione reductase (GR; EC 1.6.4.2) were present in the isolated mitochondria and peroxisomes, as well as the antioxidant compounds ascorbate and glutathione. By solubilization assays with 0.2 M KCl most of the APX and the MDHAR activities were found to be bound to the mitochondrial and peroxisomal membranes. The GR activity was shown to be a matrix-associated enzyme in mitochondria and peroxisomes and DHAR was also found mainly in the soluble peroxisomal and mitochondrial fractions.

In dark-induced senescent leaves, the activity of the mitochondrial and peroxisomal enzymes of the ASC-GSH cycle were assayed as well as the effect of leaf senescence on the ascorbate and glutathione content in mitochondria and peroxisomes. The results obtained suggest that in senescent pea leaves the ascorbate-glutathione cycle which is present in different cell compartments, appears to have a role in the oxidative mechanism of leaf senescence.

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Introduction

Activated oxygen species, like $O_2^{-}$ radicals, hydrogen peroxide ($H_2O_2$) and singlet oxygen ($'O_2'$), are produced in reactions taking place in different compartments of plant cells (ELSTNER 1991).

Scavenging of $H_2O_2$ by ascorbate peroxidase (APX) is the first step of the ascorbate-glutathione cycle which maintains the ascorbate pool in its reduced form (ASADA 1994, FOYER & HALLIWELL 1976). Dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR) also participate in this cycle, which has been clearly demonstrated in chloroplasts and cytosol (FOYER & al. 1994). However, there is scarce information on the mitochondrial hydrogen peroxide scavenging systems, although MDHAR and GR have been found in mitochondria from several plant species (EDWARDS & al. 1990, DALTON & al. 1993). Similarly, in peroxisomes the scavenging of $H_2O_2$ was thought to be carried out exclusively by catalase, and therefore the ascorbate glutathione cycle was not expected to be localized in these organelles. However one APX isoenzyme has been identified recently in membranes of pumpkin glyoxysomes and peroxisomes (YAMAGUCHI & al. 1995) and cotton Glyoxysomes (BUNKELMANN & TRELEASE 1996), and the presence of MDHAR activity was also detected in membranes of oilseed glyoxysomes (BOWDITCH & DONALDSON 1990, BUNKELMANN & TRELEASE 1996).

In very recent works carried out in our laboratory, glutathione and ascorbate were detected in mitochondria and peroxisomes from pea leaves (JIMENEZ & al. 1996).

Oxidative damage in plant tissues is especially important during senescence, which is characterized by a notable increase in the metabolism of the activated oxygen species. The possible involvement of peroxisomes and their activated oxygen metabolism in the oxidative mechanism of leaf senescence has recently been demonstrated in detached pea leaves (PASTORI & DEL RIO 1994), whereas mitochondria and chloroplasts may play an important role in the cellular toxicity mediated by activated oxygen species of NaCl in pea leaves (HERNANDEZ & al. 1993, 1995). Unfortunately, to date there is no precise information on the response of the ASC-GSH cycle components to induced senescence in mitochondria or peroxisomes.

In this work we have studied the presence of all the components of the ASC-GSH cycle in purified mitochondria and peroxisomes from pea ($Pisum sativum$) leaves. The changes induced by leaf senescence on the $H_2O_2$-scavenging cycle in mitochondria and peroxisomes have been also studied.
Materials and Methods

Plant material: Pea (Pisum sativum L., cv. Lincoln) plants were grown in pots containing aerated nutrient solutions in a growth chamber (ASL) under optimum conditions for 20 days, as described by HERNÁNDEZ & al. 1995.

Induction of senescence: Excised leaves (about 50-60 g fresh weight) from 15-17 d-old pea plants were placed in trays floating in air-saturated distilled water, and were incubated in permanent darkness at 28°C for up to 11 d, as described by PASTORI & DEL Río 1994. The leaves were then washed and used for the different assays.

Isolation of mitochondria and peroxisomes: Mitochondria and peroxisomes were isolated from pea leaves after 0 and 11 d in darkness by differential and density-gradient centrifugation. Two different isolation methods were used. For the purification of mitochondria, the washed 12000g particulated pellet resulting from crude extract centrifugation, which was rich in mitochondria and peroxisomes, was centrifuged in a self-generated Percoll gradient (28%; v/v) as previously described (JIMÉNEZ & al. 1996). Peroxisomes were isolated, as reported by SANDALIO & al. 1987. All operations were performed at 0-4°C. The purified mitochondria and peroxisomes had intactness percentages between 70-90% and were free of chloroplasts, as verified by marker enzyme evaluation (SANDALIO & al. 1987, JIMÉNEZ & al. 1996).

For studies of APX activity, an independent organelle isolation procedure was used, except that 20 mM of Na-ascorbate was added to the extraction medium and all the other solutions contained 1 mM ascorbate to prevent possible inactivation of APX.

Enzyme assays: Unless otherwise indicated, the activities of all enzymes were assayed in organelle samples diluted 2-5 fold with 50 mM K-phosphate buffer, pH 7.8, containing 0.1% (v/v) Triton X-100. Ascorbate peroxidase was determined at 25°C according to HOSSAIN & ASADA 1984. Monodehydroascorbate reductase was assayed by the decrease in absorbance at 340 nm due to the NADH oxidation (ε240 = 6.22 mM⁻¹ cm⁻¹) (ARRIGONI & al. 1981). Dehydroascorbate reductase was determined according to DALTON & al. 1993. Glutathione reductase was assayed by the decrease in absorbance at 340 nm due to NADPH oxidation (ε240 = 2.8 mM⁻¹ cm⁻¹), as described by EDWARDS & al. 1990. Superoxide dismutase (SOD; EC 1.15.1.1) was assayed by the ferricytochrome c method using xanthine/xanthine oxidase as the source of superoxide radicals as described MCCORD & FRIDOVICH 1969.

Solubilization of APX, MDHAR, DHAR and GR: Essentially the method of SANDALIO & DEL Río 1988 with some modifications was followed. Suspension of the purified mitochondria and peroxisomes were separated in two identical fractions each pelleted by centrifugation at 12000g for 10 min and suspended in 200 µl of 10 mM HEPES-KOH buffer (pH 7.2), 1 mM EDTA, 10% (v/v) glycerol, with 0.2 M KCl and without KCl (control), and maintained on ice for 30 min, with shaking every 10 min in a vortex mixer. The suspensions were centrifuged at 62000g for 30 min, and the pellets were resuspended in 200 µl of the same buffer with 0.2 M KCl and without KCl (control). For solubilization studies of APX activity, 1 mM ascorbate was included in the suspension buffers. The supernatants and resuspended pellets were assayed for APX, MDHAR, DHAR and GR activities and proteins.

Determination of glutathione and ascorbate. Both antioxidants were extracted at 0°C from leaf tissue, and from mitochondria and peroxisomes which were previously isolated using media without ascorbate and cysteine. For each antioxidant, mitochondria and peroxisomes obtained from two density-gradients were used. GSH and GSSG were measured by HPLC analysis according to FARISS & REED 1987. Ascorbate was determined by HPLC as previously described JIMÉNEZ & al. 1996. DHA was separated from ASC by incubating the samples for 24 h at room temperature with 1 mM dithiothreitol (FARBER & al. 1983). The DHA concentration was measured as ASC following rechromatography.
Results and Discussion

When the occurrence of the enzymes of the ASC-GSH cycle in the purified mitochondria and peroxisomes was examined, results showed that all the enzymes of the cycle were present in both cell compartments (Table 1).

Table 1. Specific activity of the ascorbate-glutathione cycle enzymes in mitochondria and peroxisomes purified from pea leaves.

Data are the means of at least four different experiments±SE.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>APX</th>
<th>GR</th>
<th>MDHAR</th>
<th>DHAR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol min⁻¹ mg⁻¹ protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>161±11</td>
<td>22.3±3.2</td>
<td>99±10</td>
<td>210±40</td>
</tr>
<tr>
<td>Peroxisomes</td>
<td>281±43</td>
<td>12.7±2.3</td>
<td>33±4</td>
<td>25±5</td>
</tr>
</tbody>
</table>

To examine further the suborganellar localization of the antioxidative enzymes in the purified mitochondria and peroxisomes, both intact organelles were subjected to hypotonic treatments in buffer with and without 0.2 M KCl. The results showed that when supernatants and resuspended pellets were assayed for APX and the percent solubilization was calculated, most of the ascorbate peroxidase activity in mitochondria and peroxisomes was in the KCl-insoluble fractions and only a small percentage of this activity was solubilized (8% and 4% respectively). This indicates that APX is bound to the mitochondrial and peroxisomal membranes (Table 2).

With regards to the other enzymes of the ASC-GSH cycle, the results obtained from the solubilization assays (Table 2) indicated that MDHAR, as APX, is also tightly associated with the mitochondrial and peroxisomal membranes with percentages of solubilization of about 30% and 22% respectively. However, GR and DHAR are matrix associated enzymes in mitochondria and peroxisomes, although the degree of association of DHAR activity to the membranes of both organelles after KCl treatment, open the question of whether a function of DHAR activity in mitochondria and peroxisomes is the result of non specific adsorption of cytosolic DHAR to the membranes or could indicate that a fraction of DHAR activity is an integral membrane protein in both organelles. This two possibilities remain to be seen.

For studying the effect of leaf senescence on the antioxidative enzymes, pea leaf peroxisomes and mitochondria were isolated from young and dark-induced senescent leaves at a well-defined stage of senescence (after 11 days of dark-incubation). At this stage, PASTORI & DEL RIO 1994 demonstrated that pea leaves have characteristic symptoms of senescence.
In dark-induced senescent leaves the mitochondrial and peroxisomal APX activity was notably decreased, by about 50% and 70% respectively. A similar inhibition effect by senescence was found for MDHAR activity in mitochondria and peroxisomes, which was up to 80% inhibited.

Table 2. Intraorganellar localization of the ascorbate-glutathione cycle enzymes in mitochondria and peroxisomes purified from pea leaves.

The percent solubilization was calculated by dividing the total activity in the supernatant by the sum of the total activities in the supernatant and in the pellet. Data are the means of two different experiments. nd, not detectable.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Hepes Buffer</th>
<th>Hepes Buffer plus 0.2 M KCl</th>
<th>Hepes Buffer</th>
<th>Hepes Buffer plus 0.2 M KCl</th>
</tr>
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<tbody>
<tr>
<td>APX</td>
<td>6</td>
<td>8</td>
<td>nd</td>
<td>4</td>
</tr>
<tr>
<td>MDHAR</td>
<td>36</td>
<td>30</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>DHAR</td>
<td>78</td>
<td>48</td>
<td>66</td>
<td>40</td>
</tr>
<tr>
<td>GR</td>
<td>98</td>
<td>98</td>
<td>100</td>
<td>40</td>
</tr>
</tbody>
</table>

With regard to GR and DHAR, after 11 days of senescence, the activity of the mitochondrial GR was substantially decreased, with inhibition percentage of about 65%, whereas in peroxisomes GR activity was maintained during leaf senescence. Interestingly, in peroxisomes the DHAR activity showed an inverse relationship, showing an increase of about two-fold in senescent pea leaves. In contrast, in mitochondria, the DHAR activity as the other enzymes of the cycle, was inhibited during leaf senescence (up to 80%).

The contents of the antioxidants ascorbate and glutathione were also measured after 11 days of leaf senescence (Table 3). A strong decrease in total ascorbate pool in the mitochondria and in crude extracts was found. The mitochondrial ASC/DHA (reduced/oxidized ascorbate) ratio was diminished mainly due to the important decrease in the reduced (ASC) form, from 90% of the total ascorbate pool in the young leaves (controls) to 20% at 11 days of senescence. In contrast, in senescent peroxisomes the ascorbate pool was slightly increased but no changes in the ASC/DHA ratio were found.

With respect to glutathione, dark-incubation of the leaves decreased the mitochondrial glutathione pool to about 40% the level in control mitochondria. This fall was due to loss of the reduced form, since oxidized glutathione levels were maintained. This pattern contrasts with that found in peroxisomes, in which
there was a large increase in the total glutathione content accompanied by a
decrease in the GSH/GSSG ratio.

Table 3. Ascorbate and glutathione contents in crude extracts and in intact mitochondria
and peroxisomes purified from pea leaves incubated in darkness for 11 days.
Values are the means of at least four different experiments ±SE. Differences from control
values were significant at p<0.001(a), p<0.01(b).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Senescence days</th>
<th>Total protein mg</th>
<th>ASC (µg (mg protein)^{-1})</th>
<th>DHA nmol. (mg protein)^{-1}</th>
<th>GSH nmol. (mg protein)^{-1}</th>
<th>GSSG nmol. (mg protein)^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extracts</td>
<td>0</td>
<td>1627±1.50</td>
<td>7.10±1.30</td>
<td>1.70±0.20</td>
<td>9.70±0.16</td>
<td>0.02±0.002</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>728±7.0</td>
<td>1.00±0.05 (b)</td>
<td>0.09±0.01 (a)</td>
<td>0.61±0.11 (a)</td>
<td>0.93±0.11 (a)</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0</td>
<td>1.03±0.41</td>
<td>3.81±0.52</td>
<td>0.41±0.10</td>
<td>5.92±0.12</td>
<td>0.21±0.05</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1.22±0.22</td>
<td>0.02±0.001 (a)</td>
<td>0.08±0.03 (b)</td>
<td>3.52±0.13 (a)</td>
<td>0.22±0.04 (ns)</td>
</tr>
<tr>
<td>Peroxisomes</td>
<td>0</td>
<td>0.15±0.05</td>
<td>0.65±0.12</td>
<td>0.94±0.015</td>
<td>4.15±0.80</td>
<td>0.15±0.03</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1.19±0.03</td>
<td>0.86±0.17 (ns)</td>
<td>1.26±0.22 (ns)</td>
<td>33.66±9.60 (b)</td>
<td>51.8±10.2 (a)</td>
</tr>
</tbody>
</table>

In summary it appears that the capacity of peroxisomes to maintain the
ascorbate pool at a controlled concentration depends on GR and DHAR activities
acting in a cyclic manner in the peroxisomal matrix. This and the enhanced
 glutathione pools could help to prevent oxidative injury in peroxisomes during leaf
senescence. In contrast, mitochondria under senescence conditions had lower
ascorbate and glutathione levels and less effective regeneration of their reduced
forms than control mitochondria. Thus mitochondria are more susceptible than
peroxisomes to oxidative damage during leaf senescence.

A c k n o w l e d g e m e n t s

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