SODORIDE REDUCTION AS A MECHANISM OF ASCORBATE-
STIMULATED OXYGEN UPTAKE BY ISOLATED CHLOROPLASTS

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SUMMARY: Addition of 1 mM ascorbate to isolated chloroplasts with methyl
viologen (MV) as electron acceptor trebled the rate of oxygen uptake and
decreased the ADP/O ratio to a third of that with no ascorbate present.
These effects of ascorbate were reversed by superoxide dismutase (SOD),
which in the absence of ascorbate had little effect on O₂ uptake or ADP/O
ratio. A chloroplast-associated SOD activity equivalent to 500 units/mg
chlorophyll was detected. The effects of ascorbate and SOD on O₂ uptake
were similar in both coupled and uncoupled chloroplasts. The results are
consistent with the hypothesis that ascorbate stimulates O₂ uptake by
reduction of superoxide, which is formed by autoxidation of the added
electron acceptor (MV), and which dismutates in the absence of ascorbate.
Ascorbate does not seem to stimulate O₂ uptake by replacing water as the
photosystem II donor.

Ascorbate has been reported to act as a photosystem (PS) II donor in
chloroplasts with water-oxidation inactivated by heating¹,² or tris-
treatment³. Bohme and Trebst² have suggested that ascorbate-stimulated O₂
uptake by isolated chloroplasts - which are still able to perform the Hill
reaction in the absence of ascorbate - may be explained by ascorbate
replacing water as the PSII electron donor. They suggest that electron
transport in coupled chloroplasts is accelerated by ascorbate, since
electron donation by ascorbate bypasses a normally rate-limiting PSII phos-
phorylation site.

Epel and Neumann⁴ have, however, proposed an alternative mechanism of
ascorbate-stimulated O₂ uptake in the presence of a low potential PSI
acceptor. In this scheme superoxide, produced by autoxidation of the
acceptor, is reduced by ascorbate to peroxide (as has also been proposed
by Klutner et al⁵), rather than the superoxide dismutating to peroxide
and oxygen, which occurs in the absence of ascorbate. Aerobic oxidation
of the semiquinone of low potential dyes, such as methyl viologen, has been
reported to involve superoxide formation.6,7

The results reported here support Epel and Neumann's scheme in three ways: 1) the observed ascorbate stimulation of O₂ uptake and depression of the ADP/O ratio require only that ascorbate trebles the net O₂ uptake per pair of electrons transferred from water, by reducing the superoxide formed to H₂O₂ rather than the superoxide dismutating to H₂O₂ and oxygen; 2) the addition of superoxide dismutase reverses the effects of ascorbate; 3) the effects of ascorbate and SOD on O₂ uptake are similar for both coupled and NH₄Cl-uncoupled chloroplasts.

METHODS: Chloroplasts (unbroken; type B of Hall's classification) were isolated from greenhouse spinach by a method based on that of Hall et al., using a Polytron homogenizer, with ascorbate omitted from the grinding medium. Broken, washed chloroplasts (type C) were prepared by resuspending the chloroplasts in 50 ml of tenfold diluted resuspending medium; centrifuging for 4 minutes at 4000g and resuspending in undiluted medium. Chlorophyll estimation was performed as described by Arnon. O₂ uptake was measured in a Rank O₂ electrode. Illumination by two 300W slide projectors gave an intensity of 8.8 x 10⁶ erg cm⁻² sec⁻¹, with Cinemoid 5A filters transmitting light of wavelengths between 540 nm and 740 nm. ADP/O ratios were calculated by the method of Hall et al. SOD, isolated as bovine erythrocuprein by the method of Weser et al., was kindly supplied by U. Weser and was added to the reaction vessel at 8000 units/ml.

RESULTS AND DISCUSSION: Addition of ascorbate to both coupled and NH₄Cl-uncoupled broken, washed (type C) chloroplasts gave a three-fold stimulation of O₂ uptake at concentrations greater than 0.5 mM (Figure 1). With unbroken type B chloroplasts (which were swollen in the hypotonic reaction medium) maximal ascorbate stimulation was only 250% of the rate without ascorbate, but there was still no significant difference between ascorbate stimulation of the coupled and uncoupled rates.
Figure 1  O₂ uptake as % of the minus-ascorbate rate versus ascorbate concentration in the reaction medium, which also contained 0.1M sorbitol, 5mM MgCl₂, 20mM NaCl, 2mM EDTA, 50mM HEPES pH 7.5, 3mM NaH₂PO₄, 50μM MV, and chloroplasts equivalent to 100 μg of chlorophyll, in a final volume of 2 ml. ○—○; coupled rate, ×—×; uncoupled rate (+5mM NH₄Cl).

Figure 2  The effects of ascorbate (1mM), SOD (400 units), and KCN (10mM) on O₂ uptake by broken chloroplasts in the electrode. Other conditions as in Figure 1. Bracketed figures are the rates of O₂ uptake in μmoles/mg chl/hr.

The inhibitory effect of SOD was completely reversed by 10mM KCN, which caused up to a trebling of the SOD-inhibited rate, and which caused only a 20% stimulation of the rate of O₂ uptake in the absence of SOD (Table 1). The effects on chloroplast O₂ uptake of addition of ascorbate, SOD and KCN, are shown in the trace reproduced in Figure 2.

The effect of SOD on O₂ uptake stimulated by 1mM ascorbate is shown for coupled and uncoupled chloroplasts in Figure 3. In both cases the
Coupled chloroplasts Uncoupled chloroplasts

Figure 3  O₂ uptake with 1mM ascorbate, as % of the rate without ascorbate, versus SOD in the 2 ml reaction medium. Other conditions as in Figure 1.
- - - - o; unbroken (type B) chloroplasts;  x-x-x; broken (type C) chloroplasts.

difference between unbroken (type B) and broken (type C) chloroplasts corresponds to a chloroplast-associated SOD activity of about 50 units in chloroplasts containing 100 µg of chlorophyll. From this one can calculate that there are 5-10 SOD molecules per electron transport chain.

Ascorbate and SOD had effects on the ADP/O ratios of both unbroken and broken, washed chloroplasts (Table 1), analogous to their effects on O₂ uptake. Figure 4 shows the ADP/O ratio of the chloroplasts plotted against the concentration of ascorbate in the reaction medium. For broken chloroplasts, the ADP/O ratio is depressed by ascorbate to about a third of its original value, and to slightly more than a third for unbroken chloroplasts.

If the oxygen taken up per electron pair is trebled by the addition of ascorbate, as suggested by Figure 1, the decrease in the observed ADP/O ratio (Figure 4) does not require the assumption that the ATP/2e⁻ has decreased. Hence these results do not require that electrons from ascorbate pass through only one of the two phosphorylation sites that are now thought to be involved in non-cyclic photophosphorylation⁹,12-16.
Ascorbate µM

Figure 4  ADP/O versus ascorbate concentration in reaction medium. Conditions as for Figure 1, with 10mM K₂HPO₄ and 0.125 mM ADP added. ○-○; unbroken (type B) chloroplasts. ×-×; broken, (type C) chloroplasts.

Table 1  The effects of ascorbate (1mM), SOD (400 units), and KCN (10mM) on coupled O₂ uptake and the ADP/O ratio for broken (type C) chloroplasts. Reaction conditions as in Figure 4, i.e. all rates are with methyl viologen as electron acceptor.

<table>
<thead>
<tr>
<th>Additions</th>
<th>O₂ uptake (µmoles/mg chl/hr)</th>
<th>ADP/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>33</td>
<td>1.44</td>
</tr>
<tr>
<td>KCN alone</td>
<td>38</td>
<td>1.49</td>
</tr>
<tr>
<td>SOD alone</td>
<td>29</td>
<td>1.67</td>
</tr>
<tr>
<td>Ascorbate alone</td>
<td>86</td>
<td>0.63</td>
</tr>
<tr>
<td>Ascorbate + KCN</td>
<td>84</td>
<td>0.67</td>
</tr>
<tr>
<td>Ascorbate + SOD</td>
<td>20</td>
<td>1.44</td>
</tr>
<tr>
<td>Ascorbate + SOD + KCN</td>
<td>72</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Photosynthetic transport of a pair of electrons from water to a low potential dye such as methyl viologen is summarized in equation 1. Aerobic autoxidation of the dye may produce superoxide ions, (equation 2), and spontaneous superoxide dismutation may then occur (equation 3). If catalase is inhibited by azide so that no breakdown of H₂O₂ occurs, the net O₂ uptake per pair of electrons (O₂+/2e⁻) may be deduced from the summation of equations 1-3 to give equation 4, the Mehler reaction:

\[
\begin{align*}
\text{H₂O} + 2 \text{MV}_{\text{ox}} & \rightarrow \frac{1}{2} \text{O}_2 \uparrow + 2 \text{MV}_{\text{red}} + 2\text{H}^+ \quad \text{................. 1} \\
2 \text{MV}_{\text{red}} + 2 \text{O}_2^- & \rightarrow 2 \text{MV}_{\text{ox}} + 2\text{O}_2^- \quad \text{................. 2} \\
2\text{O}_2^- + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \uparrow \quad \text{................. 3} \\
\text{H}_2\text{O} + \frac{1}{2} \text{O}_2 \uparrow & \rightarrow \text{H}_2\text{O}_2 \quad \text{................. 4}
\end{align*}
\]
Hence $O_2^{\dagger}/2e^- = \frac{3}{8}$ in this system; i.e., $O_2$ uptake proceeds at the same rate as $O_2$ evolution in a Hill reaction with ferricyanide.

If ascorbate replaces water as the PSI donor, producing dehydroascorbate (DHA), equation 5 replaces equation 1, and the net reaction becomes that shown in equation 6, the same as that proposed by Elstner et al.

$$
\text{Ascorbate} + 2MV_{\text{ox}} \rightarrow \text{DHA} + 2MV_{\text{red}} + 2H^+ \quad \text{(5)}
$$

$$
2MV_{\text{red}} + 2O_2 \rightarrow 2MV_{\text{ox}} + 2O_2^- \quad \text{(2)}
$$

$$
2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(3)}
$$

$$
\text{Ascorbate} + O_2 \rightarrow \text{DHA} + H_2O_2 \quad \text{(6)}
$$

In this case $O_2^{\dagger}/2e^- = 1$. Hence the effect of ascorbate addition would be to double the rate of $O_2$ uptake for any given rate of electron transport, as proposed by Bohme and Trebst.

If, however, ascorbate exerts its effect by reducing to $H_2O_2$ the superoxide formed in reaction 2 (as suggested by Epel and Neumann), equation 7 replaces equation 3, and the overall reaction becomes that expressed in equation 8.

$$
H_2O + 2MV_{\text{ox}} \rightarrow \frac{3}{8}O_2^{\dagger} + 2MV_{\text{red}} + 2H^+ \quad \text{(1)}
$$

$$
2MV_{\text{red}} + 2O_2 \rightarrow 2MV_{\text{ox}} + 2O_2^- \quad \text{(2)}
$$

$$
2O_2^- + \text{Ascorbate} + 2H^+ \rightarrow \text{DHA} + 2H_2O_2 \quad \text{(7)}
$$

$$
H_2O + \text{Ascorbate} + 1\frac{1}{3}O_2 \rightarrow \text{DHA} + 2H_2O_2 \quad \text{(8)}
$$

Here $O_2^{\dagger}/2e^- = 1\frac{1}{2}$; thus the ratio is trebled (from $\frac{3}{8}$ to $1\frac{1}{2}$) by the addition of ascorbate. SOD catalyzes reaction 3 and may interact with $O_2^-$ before ascorbate can, so the observed reversal of ascorbate's effect is predicted by the scheme summarized in equation 8.

The data presented here also show the predicted trebling of the rate of $O_2$ uptake on addition of ascorbate (Figure 1), which occurs in both coupled and uncoupled chloroplasts. Since the trebling occurs in uncoupled chloroplasts it cannot be a result of electrons from ascorbate by-passing a rate-limiting PSII phosphorylation site. Bohme and Trebst report a constant rate of ATP formation in chloroplasts with and without ascorbate, which in
our view may be adequately explained by an unchanged rate of electron transport through both phosphorylation sites. This conclusion is supported by the reversal by added SOD of ascorbate-depression of the ADP/O ratio (Table 1) and of ascorbate-stimulation of \( O_2 \) uptake (Figure 3).

The mechanism of ascorbate-stimulated \( O_2 \) uptake, proposed by Epel and Neumann\(^4\), has the added advantage that it predicts an absence of stimulation by ascorbate of NADP\(^+\) reduction in untreated chloroplasts\(^2\), since it does not require the ad hoc assumption that the effectiveness of a PSII electron donor depends directly on the chemical nature of the terminal acceptor.

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REFERENCES: