Experimental Approach to Elucidating the Mechanism of Light-Independent Chlorophyll Biosynthesis in Greening Barley

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Etiolated seedlings of angiosperms illuminated for a few hours and then returned to darkness continue to accumulate chlorophyll (Chl) a (Popov and Dilova, 1969; Adamson et al., 1997, and refs. therein). In leaves of barley (Hordeum vulgare) seedlings and leaves of Tradescancia albiflora, Chl content was increased in darkness by 20% to 30% and by 38% to 69%, respectively (Adamson et al., 1980; Walmsley and Adamson, 1989). How do angiosperms make Chl in darkness without light-independent protoclorophyllide (Pchlide) reductase? This enigma has been waiting its resolution since 1938.

The reduction of Pchlide to chlorophyllide (Chlide) is a key step in the biosynthesis of Chl. There are two mechanisms via which the double bond in the fourth ring of Pchlide can be reduced: One is light independent, and the other is light dependent.

Light-independent Pchlide reductase in eukaryotes is a chloroplast-encoded enzyme comprised of three proteins—ChlB, ChlL, and ChlN. All organisms that contain Pchlide in the absence of light contain three genes—bchB, bchL, and bchN. These genes are absent in angiosperms (Suzuki et al., 1997; Wakasugi et al., 2001).

Light-dependent Pchlide to Chlide reduction proceeds in the catalytically active complex of NADPH: Pchlide oxidoreductase (POR; Beer and Griffiths, 1981). Light excitation of Pchlide triggers the dark reaction sequences of Pchlide reduction (Raskin and Schwartz, 2002, and refs. therein). POR is a nuclear-encoded, cytoplasmically synthesized protein that processed posttranslationally upon import into the plastid (Harpster and Apel, 1985). The existence of different POR enzymes (POR-B and POR-C) was discovered in recent years (Holtorf et al., 1995; Osawa et al., 2000). In contrast to POR-A, which is responsible for the initial Chl formation in illuminated etiolated tissues, POR-B is responsible for continued Chl formation until the full content of Chl is reached, and POR-C starts to accumulate only at the end of the rapid Chl accumulation phase (Su et al., 2001).

However, etiolated seedlings of angiosperms, illuminated for a few hours and then returned to darkness, continue to accumulate Chl a (Popov and Dilova, 1969; Adamson et al., 1997, and refs. therein). It was suggested that a light-independent enzyme was responsible for the dark Chl synthesis (Adamson et al., 1985), but the existence of such enzyme has not yet been found (Suzuki et al., 1997; Wakasugi et al., 2001).

We suggest that Pchlide reduction in angiosperms occurs in the dark by using the light-dependent POR, but instead of being by light, the excitation of Pchlide occurs via an enzymatically generated electronically excited state.

Here, we provide evidence that salicylic acid (SAc), an inhibitor of ascorbate peroxidase (APX; Durner and Klessig, 1995), inhibits dark Chl accumulation in greening barley seedlings at the Pchlide reduction step. APX scavenges hydrogen peroxide in chloroplasts, which do not contain catalase (Foyer et al., 1994; Noctor and Foyer, 1998). Our data show that SAc does not inhibit Pchlide accumulation in the dark or incorporation of the Glu l-[U-14C]Glu (14C-Glu) into Pchlide, but it does inhibit Chl accumulation and 14C-Glu incorporation into Chl. This means that in the absence of light-independent Pchlide reductase, Pchlide reduction in the dark can be achieved via POR by using the energy of electronically excited species generated in the enzymatic APX system.

THE INFLUENCE OF SAC ON CHL AND PCHLIDE CONTENTS IN GREENING BARLEY LEAVES

Barley seeds were germinated and grown in moist vermiculite in a darkroom at 23°C to 25°C for 5 to 6 d until the first leaf just started to break through the coleoptile. The seedlings were transferred to incandescent light at 20 to 30 μmol m⁻² s⁻¹ photosynthetically active radiation. Their roots were trimmed to about 0.5 to 1.0 cm and transferred to water. After 2 h, the seedlings were transferred to beakers containing a K-phosphate buffer solution (10 mM [pH 6.9]) containing 1 mM cold Glu, with or without 2 mM SAc (100 seedlings in 40 mL of buffer), for an additional 6 h under the same light conditions (a total of 8 h in the light). Leaves from one-half of the seedlings
from each treatment were extracted with acetone using a mortar and pestle. The other one-half were returned to the darkroom for 20 h in the same treatment solutions. After 20 h in the dark, the leaves from each treatment were harvested and homogenized in acetone in darkness. Chl \( \alpha \), Chl \( \beta \), total Chl, and Pchlide contents were measured spectrophotometrically (Shamay et al., 2001) and assayed according to Anderson and Boardman (1964; Table I).

Chl \( \alpha \) and total Chl contents of the seedlings increased during the 20-h dark period by 39\% and 29\%, respectively, in the absence of SAc, whereas in seedlings incubated in buffer containing SAc, the Chl \( \alpha \) content was unchanged, and total Chl content decreased by 12\%. The presence of SAc in the incubation medium did not affect the dark accumulation of Pchlide (Table I). The phototransformation of dark-accumulated Pchlide into Chlide was measured after the extraction of the pigments from dark-incubated leaves immediately after 1 min of illumination of the seedlings at a photosynthetic photon flux density of 400 \( \mu \)mol m\(^{-2}\) s\(^{-1}\). It was found that 85\% to 90\% of the Pchlide was phototransformed to Chlide in both the presence and absence of SAc. This means that accumulation of Pchlide in the dark and its incorporation into POR were not inhibited by SAc.

These results suggest that inhibition of APX activity by SAc (Durner and Klessig, 1995) significantly reduces the accumulation of Chl \( \alpha \) in the dark. APX uses two molecules of ascorbate to reduce one of hydrogen peroxide to water, with concomitant conversion of an enediol group of ascorbate to a diketo group of dehydroascorbate in the triplet excited state (Bogdanski and Grabiec, 1967; Villablanca and Cilento, 1985). Reduction of Pchlide to Chlide requires that the Pchlide be incorporated into POR in an excited state (Raskin and Schwartz, 2002, and refs. therein).

The data in Table I suggest that excitation of Pchlide proceeds in the dark by exchange resonance energy transfer from the excited triplet state of dehydroascorbic acid (Bogdanski and Grabiec, 1967; Villablanca and Cilento, 1985) to Pchlide. The increase in Chl \( \alpha \) and total Chl contents in the presence of SAc in the light could be explained by the increase in NADPH content in the chloroplast. Because less NADPH is consumed in the SAc-inhibited ascorbate-glutathione cycle (Foyer et al., 1994; Noctor and Foyer, 1998), some of it may be available for pigment biosynthesis.

### Table I

**The Influence of SAc on Chl \( \alpha \), Chl \( \beta \), and Pchlide Labeling in Greening Barley**

To support the quantitative pigment analysis, we also measured the effect of SAc on the incorporation of \(^{14}\)C-Glu into Chl \( \alpha \), Chl \( \beta \), and Pchlide in greening barley seedlings. Chl \( \alpha \) and Chl \( \beta \) were extracted and purified by thin-layer chromatography and HPLC analyses as described by Shamay et al. (2001), except that the flow rate of the mobile methanol phase was 0.5 mL min\(^{-1}\). Unesterified pigments were quantitatively transferred to diethyl ether and after evaporation under nitrogen redisolved in 1 mL of HPLC-grade methanol (Walmsey and Adamson, 1995). The purification of the pigments was performed by HPLC until a constant specific activity was obtained (Table II).

It is known that in darkness, \(^{14}\)C-Glu is incorporated into the Chl tetrapyrrole moiety (Tripathy and Rebeiz, 1987). In presence of a buffer solution containing SAc in the light, the seedlings increased \(^{14}\)C labeling in Chl \( \alpha \) and Chl \( \beta \) by 81\% and 54\%, respectively, whereas in the presence of Sac in the dark, Chl \( \alpha \) and Chl \( \beta \) labeling decreased by 85\% and 79\%, respectively (Table II). The data in Table II essentially support the results of quantitative pigment analysis presented in Table I.

### CONCLUSIONS

The enzymatic generation of electronically excited triplet states in vitro has been documented previously with the enzyme horseradish peroxidase (Bohne et al., 1986). The enzyme, acting as an oxidase, could generate carbonyl compounds from an appropriate substrate in the electronically excited triplet state. This reaction would proceed through dioxetane/dioxetanone intermediates. The enzymatically generated triplet acetone could efficiently elicit fluorescence of micelle-solubilized Chl (Bohne et al. 1986). It has been shown that APX can promote aer-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chl ( \alpha )</th>
<th>Chl ( \beta )</th>
<th>Chl ( \alpha + \beta )</th>
<th>Pchlide ( \times 10^{-3} ) nmol leaf(^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>2.86 ± 0.24</td>
<td>0.67 ± 0.07</td>
<td>3.54 ± 0.28</td>
<td>36.4 ± 8.76</td>
</tr>
<tr>
<td>Glu + D</td>
<td>3.98 ± 0.32**</td>
<td>0.58 ± 0.03 (NS)</td>
<td>4.56 ± 0.35**</td>
<td>418 ± 30</td>
</tr>
<tr>
<td>Δ (%)</td>
<td>+39</td>
<td>-13</td>
<td>+29</td>
<td>-</td>
</tr>
<tr>
<td>Glu + SAc</td>
<td>3.37 ± 0.22</td>
<td>0.82 ± 0.055</td>
<td>4.18 ± 0.2</td>
<td>55.1 ± 9.5</td>
</tr>
<tr>
<td>Glu + SAc + D</td>
<td>3.17 ± 0.2 (NS)</td>
<td>0.52 ± 0.1*</td>
<td>3.69 ± 0.24*</td>
<td>475 ± 75</td>
</tr>
<tr>
<td>Δ (%)</td>
<td>-6</td>
<td>-36</td>
<td>-12</td>
<td>-</td>
</tr>
</tbody>
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Each value is the mean of four replicates (±SD) containing 50 seedlings per replicate. LSD between illuminated plants and plants incubated in the darkness after illumination. *, \( P \leq 0.05 \); **, \( P \leq 0.005 \); NS, not significant.
obic oxidation of ascorbic acid to dehydroascorbic acid in excited triplet state (Bogdanski and Grabiec, 1967). In this case, an enediol group of ascorbate is converted to a diketo group of dehydroascorbate. Energy transfer from the electronically excited triplet state to Chl was observed in similar reactions, such as the oxidation of catechols to o-quinone by catechol oxidase and in the oxidation of dihydroxyfumaric acid to dihydroxytartrate (Villalbana and Cilento, 1985).

An attempt to use Chl as a sensitizer for the detection of the dehydroascorbate triplet state in the enzymatic system of APX in vitro was unsatisfactory (Villalbana and Cilento, 1985). This can be explained by a low efficiency of such energy transfer because intermolecular energy transfer from the triplet excited product to the singlet state of the acceptor molecule is considered an exchange resonance transfer (Vassil’ev, 1963). This mechanism of energy transfer differs markedly from those required by the theories of inductive resonance transfer because in the former, the energy transfer proceeds by diffusion-controlled collision. This appears to be the reason that initial attempts to use Chl as a sensitizer in the enzymatic system were unsatisfactory and only triplet acetone could be detected, with rather poor efficiency. Only when micelle-solubilized Chl was used as an acceptor of the enzyme-generated triplet acetone could fluorescence be efficiently elicited (Bohne et al., 1986).

The results of our experiments can be explained by the possibility of an energy transfer from triplet dehydroascorbate to Pchlide in vivo. We suggest that under appropriate conditions in vitro, it is possible to detect Chl-sensitized emission in the APX-promoted oxidation of ascorbate to dehydroascorbate. In leaves, APX is found in a thylakoid-bound form and in soluble cytosolic and stromal forms (Foyer et al., 1994).

In summary, it appears that dark Pchlide reduction in the absence of light-independent Pchlide reductase in angiosperms can be achieved in the light-dependent POR complex via the excitation of Pchlide by the enzymatically generated triplet excited state of dehydroascorbate. One can surmise that the interaction of POR (POR-A, POR-B, or POR-C?) with an enzymatically generated excited state can be effectively achieved mainly after the induction of structural changes in the etiochloroplast by illumination. The physiological significance of Chl synthesis in the dark is not yet fully understood.

ACKNOWLEDGMENTS

We would like to thank Dr. Alex Brandis and Dr. Dani Tal (The Weizmann Institute of Science, Rehovot, Israel) for helpful advice concerning the HPLC measurements.

Received April 19, 2003; returned for revision May 23, 2003; accepted May 23, 2003.

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