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In Vivo Manipulation of the Xanthophyll Cycle and the Role of Zeaxanthin in the Protection against Photodamage in the Green Alga Chlorella pyrenoidosa*

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Chlorella pyrenoidosa was grown in steady-state continuous cultures in either high or low light. Samples of these cultures were incubated in darkness (violaxanthin state) or in saturating light (zeaxanthin state). These samples were kept in the respective preadapted states throughout the entire photodamage treatment. Photodamage involved exposure to single-turnover flashes fired at a low (non-actinic) frequency. The damage caused by the light stress thus applied was monitored by changes in photosynthetic properties and pigment composition. Cells preadapted in the light resisted photodamage better than those kept in darkness. The low light grown cells were more vulnerable to photodamage than the high light grown cells. Our experimental approach permitted the equilibria between the components that participate in the xanthophyll cycle to be set without addition of inhibitors. Regardless of the total amount of violaxanthin being present, its conversion to antheraxanthin and zeaxanthin is a prerequisite for protection. The protection is most effective for photosystem II. It appeared that antheraxanthin accumulates as a result of photodamaging flashes provided that these are fired in the presence of background light, i.e., with zeaxanthin present. From this, it is newly derived that the xanthophyll cycle operates in full in the light, including epoxidation of zeaxanthin. The latter conversion was also demonstrated in vitro, via nonenzymatic oxygen-dependent turnover of zeaxanthin into violaxanthin.

Dynamic conversion of violaxanthin, antheraxanthin, and zeaxanthin in the so-called xanthophyll cycle has been documented in higher plants and algae (reviewed by Hager (1981)). The xanthophyll cycle was first described by Sapozhnikov et al. (1957). Its function was originally suggested to be linked to oxygen evolution (Sapozhnikov, 1972). Alternatively, a role in the electron transfer activity of PS II was proposed (reviewed by Hager (1981)). Another role for the xanthophyll cycle as a protection mechanism against photodamage was first suggested by Krinsky (1971). Chl triplet states can relax via triplet energy transfer to zeaxanthin followed by dissipation of the excited triplet via the trans-cis isomerization of zeaxanthin, the latter reaction is exothermic. More recently, another energy dissipating process (non-photochemical quenching, qNP) connected with the xanthophyll cycle was introduced by Demmig-Adams (1990). Contrary to the relaxation of Chl triplet states in the former process the qNP has been proposed to be a singlet-singlet exchange between chlorophyll and carotenoids (Owens et al., 1992).

The scheme of reactions that take place in the light involves two de-epoxidation steps through which violaxanthin via the intermediate antheraxanthin becomes zeaxanthin (Hager and Stransky, 1970). This way the latter compound accumulates in the light. In darkness the reactions are reversed to violaxanthin. All reaction steps have been well characterized, except for the epoxidizing step from zeaxanthin to antheraxanthin and in which a "mixed-function oxygenase" (Hager, 1981) was suggested to be involved. The different pH ranges at which the respective enzymes operate give rise to a scheme in which the steady-state concentrations of the components of the xanthophyll cycle are determined by the pH of the lumen. The de-epoxidation reactions yielding the final product zeaxanthin rely on enzymes that become activated at a thylakoid lumen pH of 5.2 and thus operate in the light. The backreactions involve enzymatically catalyzed epoxidation steps that rely on a higher pH of the thylakoid lumen and by consequence operate in darkness (Pfundel and Dilley, 1992; Gilmore and Yamamoto, 1993). Thus, according to these observations, pH transitions between light and dark effect the differences in the presence of violaxanthin and zeaxanthin relative to one another.

Dithiothreitol has been applied as a successful inhibitor of the violaxanthin de-epoxidation steps (Yamamoto and Kamite, 1972). However, additional effects of dithiothreitol under in vivo conditions on several other thioreredoxin-regulated reactions, such as carbon metabolism enzymes (Rowell et al., 1986) or the ATP synthase (Mills, 1986) may obscure the answer to the question whether in addition to the decreased availability of zeaxanthin other inhibitory effects of dithiothreitol are responsible for the observed increased sensitivity to photodamage in the presence of dithiothreitol. In addition, the use of an inhibitor in the study of a cyclic process, excludes the possibility to retrieve information about the dynamic properties of such a cycle.

The aim of the present study was to evaluate the photoprotective potential of the xanthophyll cycle with different steady-state contents of violaxanthin and zeaxanthin generated in vivo without disturbance of the cellular metabolism by external additions other than light. The data presented indicate that epoxidation of zeaxanthin also proceeds in the light as a result of the photoprotective (excited oxygen quencher activity) proc-
essing of zeaxanthin. This observation reveals that the dy-
namic function of the xanthophyll cycle in vivo is larger than
would be predictable from existing data. Our approach to as-
sess photodamage in a constant background of photoprotection,
established by introducing continuous background illumina-
tion, may be useful in other areas of photosynthesis research.

MATERIALS AND METHODS

Culture—Two types of steady-state continuous cultures of Chlorella
pyrenoidosa were used, both were grown in 2-liter chemostats in BG-11
medium (Rippka et al., 1979) at 20 °C. One was grown at 30 μE·m⁻²·s⁻¹
(low light, LL) the other one at 240 μE·m⁻²·s⁻¹ (high light, HL). Circular
fluorescent tubes (Philips TLX 12W/37) were used for continuous illu-
sination. The set up of the culture system was as in Van Liere and Mur
(1978). Aeration at 60 liters/min provided adequate mixing and CO₂
supply. The cultures were maintained at an A₀ of 0.18–0.20.

Preadaptation and Flash Experiments—Samples from the HL and
LL cultures were preadapted during 30 min at 30 °C in either darkness
or in the presence of actinic (background) light. The actinic light inten-
sities for the LL and HL samples were 430 and 600 μE·m⁻²·s⁻¹, respec-
tively. These light conditions were arranged to be saturating from the
photosynthesis versus irradiance (P/I) curves (see Fig. 1). Preadaptation
proceeded directly in the 12-ml oxygen electrode measuring chamber.
This device has been described elsewhere (Dubinsky et al., 1987). The
samples were bubbled with air to ensure a constant partial oxygen
pressure. Next, while maintaining the conditions of preadaptation (i.e.
background light or darkness), one group of samples was exposed to one
thousand supersaturating flashes (see below) in order to incite photo-
photodamaging flashes. In this case, no appreciable photodamage (as judged from changes in the pigment con-
tent and physiological activity presented), while avoiding lethality. All
flash-treated samples used for the photosynthesis activity assays were
allowed recovery during 1 min in darkness to equalize the metabolic
states. The samples were then frozen and stored at -80 °C until used. The zeaxanthin samples used in the in vitro degradation studies (cf. Fig. 2) were from Chlorella and were purified by HPLC.

RESULTS

Changes in photosynthetic activity (O₂ production) and pho-
tochemical quenching (qP) after exposure of C. pyrenoidosa
cells to control or photodamaging conditions are shown in Fig. 1.
Control samples of LL and HL Chlorella cells behave differ-
ently. The LL cells have a lower maximal photosynthetic activity/Chl than the HL ones. The LL cells show a stronger qP decrease than the HL cells. The rate of O₂ evolution decreases at higher irradiance of the LL cells. Using preadaptation to the light and darkness did not change the activities in both HL and LL cells retain comparable activities. Following exposure to the photodamaging flash treatment in the continued presence of actinic background light gave rise to relatively minor losses of activity through photodamage, both in the LL and HL cells. In contrast, clear photodamage is obvious in the samples that were kept in darkness during preadaptation and while being exposed to the photodamaging flashes. Especially the LL cells show an appreciable loss of oxygen evolution and qP at increasing actinic light intensities over the course of the P/I curve determination.

The observed differences of the photosynthetic activities were related to changes in the pigment composition of the samples. Table I depicts the pigment analysis of the HL and LL cultured cells. The data reflect that in the LL cells the total Chl to carotenoid ratio is at least twice that of the HL cells, the Chl to summed xanthophyll cycle components ratio is 7.6-fold higher. The dark or light preadaptation condi-
tions are mainly restricted to the three xanthophyll cycle pig-
ments. The violaxanthin content decreases in the light and the
dezaxanthin content increases. This way, variable pool sizes of
the xanthophyll cycle components were established before ex-
posure to potentially photodamaging conditions.

The exposure to photodamaging flashes induced extensive

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changes in the pigment composition (Table II). Relative to the data displayed in Table I, the overall picture depicts photodamage of most pigments, including Chl a, lutein, and β-carotene, with the marked exception of the antheraxanthin content in the light preadapted samples. The neoxanthin content decreases in the LL cells only. In general, the damage is small in the light-preadapted HL cells and somewhat more pronounced in the dark-preadapted HL cells. Noticeable damage is induced in the dark preadapted cells of the LL culture. As opposed to the HL grown cells in which the total of xanthophyll cycle components becomes reduced by 17% in the dark-flashed group, the loss in the analogous LL experiment amounts to 66% (Table II). Zeaxanthin is the predominantly disappearing compound in the dark-flashed HL cells with reference to the just dark-incubated HL control cells. In the absence of zeaxanthin, β-carotene is a target for breakdown, as can be seen most clearly in a comparison of the LL dark-adapted and dark-flashed samples. Lutein appears to be relatively little involved in the protection.

To define the site where the actual photodamaging process occurs and especially to locate the site at which the xanthophyll cycle provides protection against photodamage, the electron transfer capacity of the total electron transfer chain (PS II and PS I) was compared to the capacity of PS I alone (Table III). Full chain electron transfer rates in the samples that had received the strong flashes in the presence of background light appeared to remain nearly unaltered. The samples that were exposed to the flashes in the absence of background light displayed more than 20% photodamage (both HL and LL), comparable to the data given in Fig. 1. As opposed to the full chain data, PS I capacity appeared to diminish even when the strong flashes were administered in the presence of background light. The inhibition was stronger in the LL samples. However, in the dark-flashed samples and in comparison to the full chain, the damage to PS I appeared relatively low. Compared to the full chain electron transfer rate numbers, the PS I change in the light-flashed samples is already big. The increased damage observed for the full chain rates in the dark-flashed sample does not correspond to a similar decrease in the PS I sample. The protective function of the xanthophyll cycle therefore ap-
The effect of photodamaging conditions on the electron transfer capacity of PS II plus PS I and of PS I measured separately

Results are given in μmol of oxygen per mg of Chl a and hour. Assay conditions are given under "Materials and Methods." The control cells were preadapted in light, otherwise sample preparation and exposure to photodamaging conditions were as in Fig. 1. Experiments were performed three times with independently flamed samples, standard deviations are indicated by ±.

<table>
<thead>
<tr>
<th>Growth</th>
<th>PS I + PS II</th>
<th>PS I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.6 ± 0.9</td>
<td>25.5 ± 1.9</td>
</tr>
<tr>
<td>LL</td>
<td>9.6 ± 0.7</td>
<td>20.1 ± 1.2</td>
</tr>
<tr>
<td>Light flashed</td>
<td>11.5 ± 0.5 (99.2%)</td>
<td>24.2 ± 0.9 (94.9%)</td>
</tr>
<tr>
<td>HL</td>
<td>9.5 ± 0.5 (98.6%)</td>
<td>17.7 ± 1.2 (88.1%)</td>
</tr>
<tr>
<td>LL</td>
<td>9.2 ± 0.5 (79.3%)</td>
<td>23.1 ± 0.5 (90.6%)</td>
</tr>
<tr>
<td>Dark flashed</td>
<td>7.1 ± 0.8 (73.9%)</td>
<td>16.1 ± 2.6 (80.1%)</td>
</tr>
</tbody>
</table>

The observed changes in the relative abundance of the carotenoids may exert effects on the light energy transfer efficiency of PS II. If so, a lower light energy conductance would give rise to a lesser fluorescence output from PS II in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethyleurea. To eliminate effects of sample geometry, fluorescence excitation was done with broad blue or orange light. The latter excites chlorophylls only, the former both chlorophylls and carotenoids. Normalizing on fluorescence yield in the orange by using ratio's equalizes any changes in fluorescence yield of Chl related, for example, to qNP. In HL and LL cultures, the lower fluorescence ratio observed in the dark-preadapted samples indicates that the light energy transfer efficiency from carotenoids to chlorophyll remains higher in darkness than following preadaptation in the light (Table IV). The difference in the efficiency of energy transfer carotenoids → chlorophyll between the dark and light adapted samples is more pronounced in the HL cells.

The results presented in Table II indicate that the formation of the monoeoxide antheraxanthin can only in part be accounted for by conversion of violaxanthin, in addition, the disappearance of zeaxanthin appears to add to antheraxanthin formation as well. The apparent two reactions by which antheraxanthin can be formed addresses the question on the nature of the molecular conversions of zeaxanthin that happen as a part of the reductive function. In order to investigate the involvement of nonenzymatic processes, the breakdown of zeaxanthin under in vitro conditions was examined. The HPLC chromatograms shown in Fig. 2 indicate that when isolated zeaxanthin (retention time 13.4 min) was exposed to damaging conditions (10,000 μE·m⁻²·s⁻¹) during 20 min at 50 °C in the presence of air; B, as A but in the presence of nitrogen gas instead of air, and C, rechromatographed after storage on ice in the dark in the presence of air. From the ratios of the emission from orange excited samples divided by blue emission of the same sample. Experiments were performed 14 times with independent samples, numbers in parentheses are standard deviations.

The two different types of cultures (i.e. LL and HL grown) allowed assays with different contents of xanthophyll cycle pigments present, i.e., relatively abundant in HL cells and low in LL cells as in Thayer and Björkman (1990). Applying or omitting actinic background light appeared to be a useful approach to allow or avoid the conversion of violaxanthin to zeaxanthin (Blas et al. 1989 and Yamamoto et al. 1989). In earlier studies dimethoate was used to study the role of zeaxanthin in the prevention of photodamage. Those experiments precluded the possibility of studies on a dynamically operating cycle. Our approach involved preadaptation of the cells in either darkness or light to install a stable pH in the thylakoid lumen. To this end, the light intensity was chosen to 0.5 μE·m⁻²·s⁻¹ during 20 min at 50 °C in the presence of air; B, as A but in the presence of nitrogen gas instead of air, and C, rechromatographed after storage on ice in the dark in the presence of air. Other details are given under "Materials and Methods."
pigments, be it to different extents. A clear exception is the increase for antheraxanthin in the samples that were flashed in the presence of actinic background light. This increase is of great interest for the understanding of the physiological function of the xanthophyll cycle. Comparison of the pigment distribution in between HL with background light only (Table I) and HL flashed with background light present (Table II), shows that the decrease of the violaxanthin content is less than the actual increase of antheraxanthin. The only feasible explanation for this observation is epoxidation of zeaxanthin. We conclude that epoxidation of zeaxanthin under photodamaging conditions in the light also contributes to antheraxanthin formation. Interestingly, earlier work (Hager, 1981; Pfündel and Dilley, 1993) established the regulatory function of the light-dependent proton gradient formation for the xanthophyll cycle.

From that work can be concluded that epoxidation occurs only after relaxation of the proton gradient, i.e. in darkness. Given the conditions in our experiment, changes of the content of antheraxanthin, other than at the expense of violaxanthin, would not be expected (see above). It is concluded that in addition to the "mixed oxidase" function operating in high lumen pH, i.e. darkness (Hager, 1981), a nonenzymatic epoxidation reaction occurs in the light as well, in accordance with Fig. 2.

Control experiments in which purified zeaxanthin was treated with light plus heat in the presence of air indeed gave rise to the formation of the (di-)epoxy compound violaxanthin. This is comparable to the earlier report on the oxidative degradation of antheraxanthin for which in vitro treatment with heat and oxygen has been shown to facilitate the formation of violaxanthin (Thomas and Goodwin, 1965). A recent report describes that oxidative degradation of β-carotene yields monooximes and dioxides (Lieberl and Kennedy, 1992). This explains our observation that, regardless of the continued presence of a stable proton gradient, formation of antheraxanthin in the light is possible via a nonenzymatic epoxidation of zeaxanthin. The nonenzymatic epoxidation of zeaxanthin results from its function as a photoprotective pigment, i.e. in quenching of singlet oxygen in this particular case. Thus in the light a complete cycle is active. This includes enzymatic reutilization of nonenzymatically epoxidized zeaxanthin (i.e. recycled violaxanthin).

Our work shows that the quenching of excited oxygen by zeaxanthin involves an epoxidation reaction which effectively results in recycling to antheraxanthin and probably violaxanthin as made likely in the in vitro assay. This means that after reaction of zeaxanthin with singlet oxygen, the zeaxanthin is not lost from the cycle but is actually converted into the epoxy compounds antheraxanthin and violaxanthin, through which in the presence of the appropriate acidification of the lumen in the light zeaxanthin can be made again. Table III showed that the xanthophyll cycle was most effective in relation to PSII, the site at which singlet oxygen generation is most likely to occur.

The position of the steady state of all the processes involved determine the actual distribution of viola-, anthera-, and zeaxanthin in a given sample. This way, the xanthophyll cycle has a real dynamic function in the photoprotective process (Fig. 3).

The net decrease of xanthophyll cycle components over the course of exposure to photodamaging conditions is due to the limited number of times that zeaxanthin, in its function as quencher of excited chlorophyll triplet states, is able to withstand trans-cis-trans transitions. In this, according to Krinsky (1971), zeaxanthin has to become damaged during the quenching at a statistical rate of 1000 quenching events per degradation.

In addition to the chemical modifications associated with the operation of the xanthophyll cycle, a change of the energy transfer efficiency in the carotenoid absorbance region related to the state of the xanthophyll cycle and the amount of the xanthophyll cycle pigments as well, was observed (Table IV). The difference in the molecular absorbance coefficient between zeaxanthin and violaxanthin cannot be the only reason for this appreciable change. This points to differences in the transfer efficiency between violaxanthin and zeaxanthin to Chl. An explanation for these differences is the number of conjugated double bonds: 9 in violaxanthin and 11 in zeaxanthin. With an increasing number of conjugated double bonds the energy level of the excited states becomes lower, i.e. the zeaxanthin excited states (S1, S2) lie below that of violaxanthin by which the possibility of an energy transfer to the S1 of Chl a from zeaxanthin becomes increasingly unfavorable (Owens et al., 1992). Violaxanthin has been shown to act as light-harvesting pigment (Owens et al., 1987). This implies that the energy level of the first excited state of violaxanthin is higher than the one of the final Chl acceptor. The three ways in which the xanthophyll cycle provides protection against photodamage are qNP (singlet transfer), decreased light harvesting capacity (singlet transfer), and photosensitizer-quenching reactions (triplet related). These processes are cooperative: if a carotenoid has a protective func-

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2 A. Friedman and H. Schubert, unpublished results.
In conclusion, the xanthophyll cycle provides a dynamic tool for Chl a and b containing organisms and possibly also for brown algae with the diatoxanthin/diadinoxanthin conversion: tailor made photosensitizer quenching without loss of light harvesting efficiency under changing light conditions.

REFERENCES