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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.

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 Stränzky, 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. The scheme of reactions presented here has been proposed to be a single-singlet exchange between chlorophyll and carotenoids, with the xanthophyll cycle being 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 our observations, pH transitions between light and dark affect 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 thioredoxin-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 enhanced zeaxanthin content in the presence of dithiothreitol is 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 illu-mination, 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 TLE 32W/33) were used for continuous illu-mination. The set up of the culture system was as in Van Liere and Mur (1978). Aeration at 60 liter/min provided adequate mixing and CO₂ supply. The cultures were maintained at an A₅₇₀ of 0.18–0.20.

Preladapation 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 intensities for the LL and HL samples were 430 and 600 μE·m⁻²·s⁻¹, respectively. These light conditions were arrived at to be saturating from the photosynthesis versus irradiance (PI) curves (see Fig. 1). Preladaption protocol (preadaptation at 120 °C and 30 min) was as described elsewhere (Dubinsky et al., 1987). 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 photodamage. The other group was not exposed to flashes and remained in the preincubation conditions during this time.

A delay between the flashes of 3 s was chosen in the samples without background light. At this frequency, controls demonstrated that the oxygen consumption rate (dark respiration) remained identical, i.e. no oxygen production was revealed, with or without flashes. The light preadapted samples (which receive the flashes in the continued pres-ence of saturating background light), already perform photosynthesis at a maximal rate. This allowed a faster flashing regime with 300–ms intervals. A General Electric FT 230 flash tube was used at a discharge voltage of 1.3 kV, which provided flashes of 5 μs half-width with an energy output of 2 J/flash in the forward direction. Calculated by the surface of the incubation chamber this amounts to the supersaturating photonflux of approximately 10,000 μE·m⁻²·flash. The flash tube was connected directly to the incubation chamber (i.e. the one used for the oxygen and fluorescence measurements, cf. below). During the flashes the aeration was continued. The number of flashes was selected to yield 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 15 min in darkness to equalize the metabolic conditions of the samples. Samples for pigment analysis were taken immediately removed from the reaction chamber and frozen for pigment content estimation. This involved immediate centrifugation during precisely 1 min and mixing of the pellet with ice-cold acetone. Before the actual HPLC analysis, the samples were stored at -18 °C until used. The xanthophyll samples used in the in vitro degradation studies (cf. Fig. 2) were from Chlorella and were purified by HPLC. A₅₇₀ and Chl Estimation—A₅₇₀ was measured on a Pharmacia Nova-spec II photometer. Chlorophyll was measured in acetone extracts (Jeffrey and Humphrey 1975).

RESULTS

Changes in photosynthetic activity (O₂ production) and photo-chemical 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 photosynthesis 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 irradiances of the LL cells. Using preadaptation, the light of darkness did not change the overall pigment content of 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 PI 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 at least 3-fold higher. The dark or light preadaptation condi-tions are mainly restricted to the three xanthophyll cycle pigments. The violaxanthin content decreases in the light and the zeaxanthin 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
changes in the pigment composition (Table I). 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.

Table I

<table>
<thead>
<tr>
<th>Pigment content of Chlorella cells from LL and HL cultures prior to exposure to photodamage in the presence or absence of background light</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preadaptation conditions</strong></td>
</tr>
<tr>
<td><strong>Darkness</strong></td>
</tr>
<tr>
<td>Neoxanthin</td>
</tr>
<tr>
<td>Violaxanthin</td>
</tr>
<tr>
<td>Antheraxanthin</td>
</tr>
<tr>
<td>Lutein</td>
</tr>
<tr>
<td>Chl b</td>
</tr>
<tr>
<td>Chl a</td>
</tr>
<tr>
<td>β-Carotene</td>
</tr>
</tbody>
</table>

Calculated ratios and sums

- Chl a/Chl b: 2.92
- ΣXanthophyll cycle pigments: 43.3
- ΣCarotenoids: 122.1
- Chl/carotenoids: 1.10
- Chl/xanthophyll cycle pigments: 3.10

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 I and PS II) 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-
pears to be predominantly effective for PS II.

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 as well as 628-nm 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 chlorophylls 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 monoepoxide 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 enzymatically catalyzed reactions of the xanthophyll cycle in the light.

**DISCUSSION**

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 (Blaas et al. (1959) and Yamamoto et al. (1962)). In earlier studies dimethylsulfoxide 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 allow pE.m-2.s-1 white light, 50 °C, air) degradation occurred. With oxygen present, formation of violaxanthin (retention time of 8.6 min) became evident. The identity of the other "breakdown products" with retention times between 16 and 17 min has not yet been extensively determined. The cis peak in the UV region in the absorbance spectra (data not shown) indicated that these may be the different cis-isomers of zeaxanthin. A similar experiment with zeaxanthin was performed in the presence of the singlet oxygen-generating agent cosine, in just room light and at room temperature. Violaxanthin was formed, other zeaxanthin conversion products were nearly absent (data not shown). The in vitro conversion reactions of zeaxanthin support our view on the actual process of singlet oxygen quenching as part of a dynamic xanthophyll cycle in the light: zeaxanthin is recycled into violaxanthin in the light. In vivo, antheraxanthin is formed this way as well, either by monoeoxipation of zeaxanthin or by the normal viola-
Fig. 3. A scheme depicting the interactions between the various singlet and triplet states of chlorophyll, oxygen, and carotenoids and the role of the xanthophyll cycle in these processes. Reactions indicated with numbers are: 1, excitation of ground state chlorophyll; 2, direct singlet ground state relaxation of excited chlorophyll by photobleaching; 3, triplet state relaxation of excited chlorophyll by photo-oxidation, singlet oxygen quenching or singlet quenching via zeaxanthin (Demmig-Adams, 1990; Owens et al., 1992); 4, chlorophyll triplet quenching by ground state oxygen which produces (via spin reversal) singlet excited oxygen or by ground state carotenoid which produces triplet excited carotenoid; 5, reaction of excited singlet oxygen with ground state non- or monoepoxy carotenoids resulting in the epoxidation of the epoxidized compounds; 6, singlet energy transfer of violaxanthin absorbed light to chlorophyll; 7, enoloid; 8, energy conversion operating in the xanthophyll cycle.

Further details are presented in the text.

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 importance 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; Pfendel and Dilley, 1993) established the regulatory function of the light-dependent proton gradient formation for the xanthophyll cycle. From this 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 monoo- and diepoxides (Liebler 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 excited oxygen in this particular case. Thus in the light a complete cycle is active. This includes enzymatic reutilization of non-enzymatically 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 violoxanthin 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 violoxanthin, 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 PSI, 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-, anther-, 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 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 violoxanthin cannot be the only reason for this appreciable change. This points to differences in the transfer efficiency between violoxanthin and zeaxanthin to Chl. An explanation for these differences is the number of conjugated double bonds: 9 in violoxanthin 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 (1Ag*, 1Bg*) lies below that of violoxanthin by which the possibility of an energy transfer to the S1 of Chl a from zeaxanthin becomes increasingly unfavorable (Owens et al., 1992). Violoxanthin 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 violoxanthin 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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...tion it also has a shadowing effect in the blue region of Chl absorbance and the possibility to quench excited chlorophylls (both singlet and triplet). This effect is important, not only with reference to the mole % numbers presented in Tables I and II, but more so because of the about 3.5 times higher molar absorbance coefficient of a carotenoid in comparison to Chl. In other words, in cases of excessive irradiation the shadowing effect is useful, but it should be reversed at less than optimal irradiance, which indeed occurs through the enzymatic epoxidation steps in darkness.

The advantages of the xanthophyll cycle are clear, its dynamically adjustable sun/shade function excludes the need for a constantly present shadowing pool of carotenoids, the chemical trans-cis-trans heat release involved in triplet Chl a photosensitizer quenching strongly reduces the need for de novo synthesis to replace photodamaged molecules. To this, the observation in the present study that singlet oxygen quenching provides a means for recycling of zeaxanthin to violaxanthin in the light further extends the functional role of the xanthophyll cycle. The equilibria of the system can rapidly switch from a protective (shadowing, chl triplet, and singlet quenching (Demmig-Adams, 1990)) to a light harvesting (singlet transfer from diatoxanthin to diadinoxanthin) conversion:...