In vivo manipulation of the xanthophyll cycle and the role of zeaxanthin in the protection against photodamage in the green alga Chlorella pyrenoidosa
Schubert, H.; Kroon, B.M.A.; Matthijs, J.C.P.

Published in:
The Journal of Biological Chemistry

Citation for published version (APA):
Schubert, H., Kroon, B. M. A., & Matthijs, H. C. P. (1994). In vivo manipulation of the xanthophyll cycle and the role of zeaxanthin in the protection against photodamage in the green alga Chlorella pyrenoidosa. The Journal of Biological Chemistry, 269, 7267-7272.
In Vivo Manipulation of the Xanthophyll Cycle and the Role of Zeaxanthin in the Protection against Photodamage in the Green Alga Chlorella pyrenoidosa*

(Received for publication, June 28, 1993, and in revised form, November 22, 1993)

Hendrik Schubert†, Bernd M. A. Kroon§, and Hans C. P. Matthijs‡

From the †E. C. Slater Institute and the ‡Amsterdam Research Institute for Substances in Ecosystems (ARISE), Laboratory of Microbiology, University of Amsterdam, Nieuwe Achtergracht 127, 1018 WS Amsterdam, The Netherlands

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 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 in which a "mixed-function oxygenase" (Hager, 1981) was suggested to be involved. 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 (Pfündel 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 thioeductin-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 dynamic function of the xanthophyll cycle in vivo is larger than would be predictable from existing data. Our approach to assess photodamage in a constant background of photoprotection, established by introducing continuous background illumination, 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 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) (low light, LL), the other one at 240 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) (high light, HL). Circular fluorescent tubes (Philips TL-L 12W 32) were used for continuous illumination. The set up of the culture system was as in Van Liere and Mur (1978). Aeration at 60 l/min provided adequate mixing and \( \text{CO}_2 \) supply. The cultures were maintained at an \( A_r^{\text{total}} \) 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 intensities for the LL and HL samples were 430 and 600 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \), respectively. These light conditions were arrived at to be saturating from the \( A_o \) measurements. 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 samples were 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 presence 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 ms 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 photon flux of approximately 10,000 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{flash} \). 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 content 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 samples. Flash-treated samples were exposed to flashes at different light intensities (15–240 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \)) in LL and HL cells. To observe loss of oxygen evolution and \( q_P \) at increasing light intensities, the HL and LL cells were preincubated with dimethylurea (10 mM) and 1 mM sodium azide (Allen and Holmes, 1986b).

RESULTS

Changes in photosynthetic activity (O₂ production) and photochemical 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 differently. The LL cells have a lower maximal photosynthetic activity/\( A_o \) 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 dependence of the LL cells is higher than the HL cells. The LL cells exhibit a higher \( q_P \) in the dark than the HL cells do. The HL 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 HL cells show an appreciable loss of oxygen evolution and qP at increasing actinic light intensities over the course of the P/IV 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 cultures. 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 3.6 times higher. The dark or light preadaptation conditions 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 exposure to potentially photodamaging conditions.

The exposure to photodamaging flashes induced extensive
Fig. 1. Samples of cells from the HL (left) and LL (right) cultures were monitored for oxygen production activity and photochemical quenching in a range of actinic light intensities. The two top frames (A and B) display oxygen production versus light intensity profiles (P1 curves). The two lower frames show the changes in the relative photochemical quenching. Details on these measurements and preadaptation procedures are provided under “Materials and Methods.” Activities are expressed as milligrams of oxygen/mg of Chl and hour or relative units for qP. Four different incubation conditions were chosen: +, control cells incubated in the presence of actinic background light; (C), control cells preincubated in darkness; A, light-preadapted cells exposed to flashes with background light present; B, dark preadapted cells exposed to flashes without background light (lowest line).

The different incubation conditions are equivalent to the ones used in Fig. 1. Pigment contents were estimated by HPLC. Results are expressed in mol % of Chl a. Details are given under “Materials and Methods” and in the text. Chl a content (used as 100% values in Tables I and II) were: 1.08 nm/ml and 1.21 nm/ml, for LL and HL cells respectively.

| Pigment content of Chlorella cells from LL and HL cultures prior to exposure to photodamage in the presence or absence of background light |
|---|
| Pigments | High light grown | Low light grown |
| Neoxanthin | 18.2 | 17.1 |
| Violaxanthin | 34.4 | 17.5 |
| Antheraxanthin | 2.6 | 4.2 |
| Zeaxanthin | 6.5 | 21.2 |
| Lutein | 37.5 | 38.1 |
| Chl b | 34.2 | 33.9 |
| Chl a | 100.0 | 100.0 |
| β-Carotene | 20.1 | 22.8 |

| Calculated ratios and sums |
|---|
| Chl α/Chl β | 2.92 | 2.95 |
| ΣXanthophyll cycle pigments | 43.3 | 42.9 |
| ΣCarotenoids | 122.1 | 120.9 |
| Chl/Carotenoids | 1.10 | 1.11 |
| Chl/xanthophyll cycle pigments | 3.10 | 3.12 |

Table II shows the changes in the relative photoactivity and photochemical quenching monitored for oxygen production activity and photochemical quenching. The two top frames (A and B) display oxygen production versus light intensity profiles (P1 curves). The two lower frames show the changes in the relative photochemical quenching. Details on these measurements and preadaptation procedures are provided under “Materials and Methods.” Activities are expressed as milligrams of oxygen/mg of Chl and hour or relative units for qP. Four different incubation conditions were chosen: +, control cells incubated in the presence of actinic background light; (C), control cells preincubated in darkness; A, light-preadapted cells exposed to flashes with background light present; B, dark preadapted cells exposed to flashes without background light (lowest line).

| Pigment content of Chlorella cells from LL and HL cultures, preadapted as in Table I after exposure to photodamaging strong light flashes |
|---|
| Pigments | High light grown | Low light grown |
| Neoxanthin | 19.2 | 17.0 |
| Violaxanthin | 32.1 | 16.8 |
| Antheraxanthin | 9.5 | 5.9 |
| Zeaxanthin | 3.2 | 18.2 |
| Lutein | 36.7 | 34.3 |
| Chl b | 32.2 | 32.9 |
| Chl a | 93.2 | 98.8 |
| β-Carotene | 19.2 | 21.8 |

| Calculated ratios and sums |
|---|
| Chl α/Chl β | 2.89 | 3.00 |
| ΣXanthophyll cycle pigments | 35.8 | 40.9 |
| ΣCarotenoids | 110.9 | 114.0 |
| Chl/Carotenoids | 1.13 | 1.15 |
| Chl/xanthophyll cycle pigments | 3.50 | 3.22 |

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 flashed samples, standard deviations are indicated by ±.

| Growth | PS I + PS II | PS I |
|--------|-------------|------|
| Control | 11.6 ± 0.9 | 25.5 ± 1.9 |
| HL | 9.6 ± 0.7 | 20.1 ± 1.2 |
| LL | Light flashed | |
| HL | 11.5 ± 0.5 (99.2) | 24.2 ± 0.9 (94.9) |
| LL | 9.5 ± 0.5 (98.6) | 17.7 ± 1.2 (88.1) |
| Dark flashed | |
| HL | 9.2 ± 0.5 (79.3) | 23.1 ± 0.5 (90.6) |
| LL | 7.1 ± 0.8 (73.9) | 16.1 ± 2.6 (80.1) |

In Vivo Manipulation of the Xanthophyll Cycle

| Table IV Light energy transfer efficiency |
|-----------------------------------------|
| High light grown | Low light grown |
| Light incubated | 0.96 (±0.07) | 1.06 (±0.06) |
| Dark incubated | 0.62 (±0.04) | 0.78 (±0.05) |
| Difference light/dark | 34.8% | 26.5% |

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. (1959) and Yamamoto et al. (1962)).

FIG. 2. Effects of in vitro damaging treatments on zeaxanthin. HPLC purified and collected samples of zeaxanthin were: A, exposed to light (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. Other details are given under "Materials and Methods."

DISCUSSION

Regardless of the growth conditions and the preincubations, the flashed light induces general photodamage of nearly all
The xanthophyll cycle is active. This includes enzymatic reutilization of nonenzymatic epoxidation of zeaxanthin under photodamaging conditions 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 mono- 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 oxygen. The position of the steady 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 (1Aγ, 1Bγ) lies below that of violaxanthin by which the possibility of an energy transfer to the Si 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-

---

2 A Friedman and H. Schubert, unpublished results.
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 the light further extends the functional role of the xanthophyll cycle.

As stated by Hager (1981), the xanthophyll cycle is present in higher plants and green algae but is absent in phycoobiliprotein containing organisms. This remarkable difference may be related to another way of discarding excess excitation energy in cyanobacteria via decoupling of the phycobilisome antennae (Mullineaux et al., 1990). Otherwise, the spectral region of light harvesting in phycoobiliprotein containing organisms is largely shifted outside the carotenoid region. This way, light harvesting in cyanobacteria in the blue spectral region is circumvented through which the capacity losses by shadowing carotenoids are in principle negligible in comparison to Chl a and b containing organisms. Cyanobacteria indeed contain a high carotenoid over Chl ratio, to provide for a shading and a photosensitizer quenching function. Likely, these two functions are confined to the cytoplasmic and thylakoid membranes, respectively. The apparent need for an appreciably higher poolsize of carotenoids acting as photosensitizer quenching pigments may be explained by the lack of a recycling system.

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/diadinaxanthin conversion: tailor made photosensitizer quenching without loss of light harvesting efficiency under changing light conditions.

REFERENCES
Allen, J. F., and Holmes, N. G. (1986) FEBS Lett. 202, 175–181
Allen, J. F., and Holmes, N. G. (1986) in Photosynthesis Energy Transduction (Hipkins, M. F., and Baker, N. R., eds) pp. 103–142, IRL Press, Oxford
Blass U., Anderson J. M., and Calvin, M. (1959) Plant Physiol. 34, 329–333
Demmig-Adams, B. (1990) Biochim. Biophys. Acta 1020, 1–24
Dubinsky, Z., Falkowski, P. G., Post, A. F., and van Hees, U. M. (1987) J. Plankton Res. 9, 607–612
Gilmore, A. M., and Yamamoto, H. Y. (1995) Photosynth. Res. 45, 67–78
Hager, A. (1991) in Pigments in Plants (Oxseyan F. C., ed) pp. 57–80, Akademie Verlag, Berlin
Hager, A., and Stransky, H. (1970) Arch. Mikrobiol. 72, 68–83
Jeffrey, S. W., and Humphrey, G. F. (1975) Biochem. Physiol. Pflanzen. (BPP) 187, 191–194
Krinsky, N. I. (1971) in Carotenoids (Iaier, O., ed.) pp. 569–716, Birkhäuser-Verlag, Basel
Läbler, D. C., and Kennedy, T. A. (1992) Methods Enzymol. 213, 472–479
Mantoura, R. F. C., and Llewellyn, C. A. (1983) Anal. Chim. Acta 151, 297–314
Mills, J. D. (1986) in Photosynthesis Energy Transduction (Hipkins, M. F., and Baker, N. R., eds) pp. 143–187, IRL Press, Oxford
Mullineaux, C. W., Bittsann, R., Allen, J. F., and Holsworth, A. R. (1990) Biochim. Biophys. Acta 1015, 231–242
Owens, T. G., Gallagher, J. C., and Alberto, R. S. (1987) J. Phycol. 23, 79–85
Owens, T. G., Shreve, A. P., and Albrecht, A. C. (1992) in Research in Photosynthesis (Murata, N., ed) Vol. I, pp. 179–186, Kluwer Academic Publishers, Dordrecht
Pfundel, E. E., and Dilley, R. A. (1995) Plant Physiol. 101, 65–71
Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y. (1979) J. Gen. Microbiol. 111, 1–61
Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y. (1979) J. Gen. Microbiol. 111, 1–61
Rowell, P., Darling, A. J., Amla, D. V., and Stewart, W. D. P. (1986) in Biochemistry of the Algae and Cyanobacteria (Logers, L. J., and Gallon, J. R., eds) pp. 201–217, Clarendon Press, Oxford
Sapozhnikov, D. J., Kraskovskaya, T. A., and Mayevskaya, A. N. (1957) Dokl. Akad. Nauk SSSR 113, 465–467
Schreiber, U., Sliwa, U., and Bilger, B. (1986) Photosynth. Res. 10, 51–62
Thayer, S. S., and Björkman, O. (1990) Photosynth. Res. 23, 331–334
Thomas, D. M., and Goodwin, T. W. (1963) J. Physiol. 1, 118–121
Van der Staay, G. M. W., Brouwer, A., Raadt, R. L., Van Mourik, F., and Matthijs, H. C. P. (1992) Biochim. Biophys. Acta 1102, 220–229
Van Liere, E., and Mur, L. R. (1978) Mitt. Int. Ver. Limnol. 21, 158–167
Yamamoto, H. Y., and Kamite, L. (1978) Biochim. Biophys. Acta 202, 175–181
Yamamoto, H. Y., Nakayama, T. O. M., and Chichester, C. O. (1962) Arch. Biochem. 97, 168–173