In diatoms, a transthylakoid proton gradient alone is not sufficient to induce a non-photochemical fluorescence quenching

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Abstract Non-photochemical fluorescence quenching (NPQ) in diatoms is associated with a xanthophyll cycle involving diadinoxanthin (DD) and its de-epoxidized form, diatoxanthin (DT). In higher plants, an obligatory role of de-epoxidized xanthophylls in NPQ remains controversial and the presence of a transthylakoid proton gradient (ΔpH) alone may induce NPQ. We used inhibitors to alter the amplitude of ΔpH and/or DD de-epoxidation, and coupled NPQ. No ΔpH-dependent quenching was detected in the absence of DT. In diatoms, both ΔpH and DT are required for NPQ. The binding of DT to protonated antenna sites could be obligatory for energy dissipation.

Key words: Diatom; Diadinoxanthin; Diatoxanthin; Non-photochemical quenching; Xanthophyll cycle; *Phaeodactylum tricornutum*

1. Introduction

The dissipation of excess excitation energy and the related operation of a xanthophyll cycle are important for the photoprotection of photosystem (PS) II against light-induced damage. It is of particular relevance in diatoms which experience large fluctuations of light intensity in turbulent waters where they are the dominant species [1]. In diatoms, the photosynthetic apparatus organization differs in many respects from that of higher plants. The thylakoids are loosely appressed and organized in extended bands of three without grana stacking and PS I and II are not segregated [2]. The light-harvesting complexes (LHCs), which contain chlorophyll (Chl) *a*, Chl *c*, fucoxanthin and the xanthophyll cycle pigments [3], are equally distributed among appressed and non-appressed regions [2] and there is no evidence of any state transitions [4]. The LHC subunits are made of several highly homologous proteins encoded by a multigene family (fucoxanthin chlorophyll proteins, FCPs [5]). Some of these subunits appear to be PS I specific, although their pigment content is similar to the other LHC subunits [6]. Some of the minor subunits (CP26 and CP29) specific to PS II in higher plants have not been found in diatoms [7] and no PsbS-like protein has yet been identified. Only two species (instead of three in higher plants), diadinoxanthin (DD) and its de-epoxidized form diatoxanthin (DT) are involved in the xanthophyll cycle. The xanthophyll concentration relative to Chl can be much larger in the LHCs of diatoms than in higher plant LHCs [8]. Upon exposure to high light intensity, DT and non-photochemical fluorescence quenching (NPQ) are formed rapidly [9].

We have previously shown that the DD pool size could be doubled under intermittent light regime (IL) (5 min light per hour) while the size and pigment composition of the PS II antenna were otherwise unchanged. The relative amount of DD of 9 mol/100 mol Chl *a* in cells grown under a 16/8 h light–dark cycle, CL (continuous light regime) cells, could be increased to 18 mol/100 mol Chl *a* in IL cells, grown with a regime of 5 min light per hour [10]. Upon exposure to high light intensity, the formation of a transthylakoid proton gradient (ΔpH) and de-epoxidation of DD to DT rapidly caused a NPQ that was fully reversible in the dark. In diatoms with the larger DD pool size, NPQ could reach values up to 12, well above those found in higher plants (at most 4). In cells with different DD pool size, the NPQ expressed by the Stern–Volmer equation was always proportional to the DT concentration [10]. Our previous results did not rule out the possibility of a DT-independent, ΔpH-dependent NPQ as observed in higher plants, where a ΔpH-dependent quenching has been detected in the absence of de-epoxidized xanthophylls [7,11]. In this report, specific inhibitors were used to alter the amplitude of the ΔpH and/or DD de-epoxidation reaction. Their effects on the relationship between NPQ and DT were studied. No conditions could be found where part of NPQ was independent of DT formation and only dependent on ΔpH. We propose that, in diatoms, both protonation of the antenna complexes and binding of DT to specific protonated sites are required for NPQ.

2. Materials and methods

2.1. Culture

Phaeodactylum tricornutum Böhlin cells were grown photoautotrophically in sterile natural seawater F/2 medium [12]. Cultures of 300 ml were incubated at 18°C in airlifts continuously flushed with sterile air. They were illuminated at a light intensity of 40 μE m^{-2} s^{-1} with white fluorescent tubes (Claude, Blanc Industrie, France). Two light regimes were used: a classical 16 h light/8 h dark photoperiod (CL cells) and an intermittent light regime of 5 min illumination per

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Abbreviations: Chl, chlorophyll; CL, continuous light regime (16 h light/8 h dark per day); DCCD, *N*,*N*′-dicyclohexyl-carbodiimide; DD, diadinoxanthin; DT, diatoxanthin; DTT, dithiothreitol; IL, intermittent light regime (5 min light per hour); LHC, light-harvesting complex; NPQ, non-photochemical fluorescence quenching; PS I and II, photosystem I and II; ΔpH, transthylakoid proton gradient

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hour (IL cells). Cells were harvested during the exponential phase of growth, centrifuged at 3000×*g* for 10 min and resuspended in their culture medium to a final concentration of 10 μg Chl *a* ml^{-1}. This concentrated suspension was slowly stirred at 18°C under low continuous light for CL cells and intermittent light for IL cells until use.

2.2. Xanthophyll contents

Pigment analyses were performed by high-performance liquid chromatography (HPLC) as previously described [9]. Cells collected from the PAM fluorometer (see below) were frozen in liquid nitrogen. Pigments were extracted with a methanol:acetone (70:30, v/v) solution. Published extinction coefficients were used for Chl [6] and for DD and DT [13].

2.3. Chl fluorescence yield and NPQ

Standard modulated Chl fluorescence measurements were performed with a PAM-101 fluorometer (Walz, Effeltrich, Germany) as described previously [10]. After a dark adaptation of 20 min, cells were subjected to a saturating light pulse and then illuminated with an actinic light of adjustable intensity. The average fluorescence measured during the last 400 ms of the saturating pulse was taken as F_m or F_m'. Data were recorded with a microcomputer through a 12-bit analogue digital interface and the system was driven by home-made software [14]. For each experiment, 2 ml of cell suspension was used. Sodium bicarbonate was added at a concentration of 4 mM from a freshly prepared 0.2 M stock water solution to prevent any limitation of the photosynthetic rate by carbon supply. When appropriate, NH_4Cl (ammonium chloride), DCCD (*N*,*N*′-dicyclohexyl-carbodiimide) or DTT (dithiothreitol), were added at the start of dark incubation. Stock solutions of NH_4Cl (Prolabo, 0.1 or 1 M in distilled water), DCCD (Sigma, 1 mM in absolute ethanol) and DTT (Sigma, 20 mM in distilled water) were freshly prepared.

NPQ was calculated as $F_m/F_m'-1$ [15], where F_m' is the maximum PS II fluorescence in the light-adapted state and F_m that in the dark-adapted state. This Stern–Volmer expression of NPQ is proportional to the concentration of the quencher state [10,11].

3. Results and discussion

The ΔpH value required for de-epoxidase activation was not reached under low light, and no de-epoxidation can occur during growth (at 40 μE m^{-2} s^{-1}). Thus, the cells did not contain any DT before the shift to high light. On exposure to high light (450 μE m^{-2} s^{-1}), the de-epoxidation of DD to DT occurred very rapidly in CL and IL cells with biphasic kinetics (Fig. 1). The fast phase was completed within a few minutes. The de-epoxidation is rather fast in diatoms in contrast to higher plants grown in the same light conditions, either CL or IL [16]. It could be due to the fact that the DD de-epoxidase is activated at lower ΔpH than the violaxanthin de-epoxidase of higher plants [17] and/or that in diatoms, the thylakoids are loosely appressed [2]. In higher plants, de-epoxidation is faster in unstacked than in stacked membranes [16].

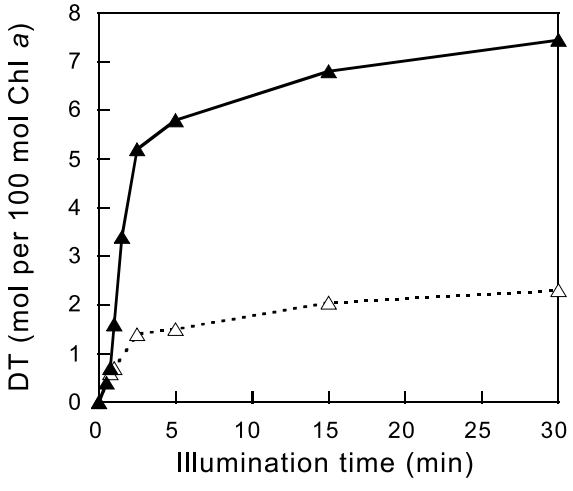

Fig. 1. De-epoxidation kinetics in *P. tricornutum* cells with a low (CL cells, open triangles) or with a large (IL cells, solid triangles) DD pool size during an illumination of 450 μE m^{-2} s^{-1}.

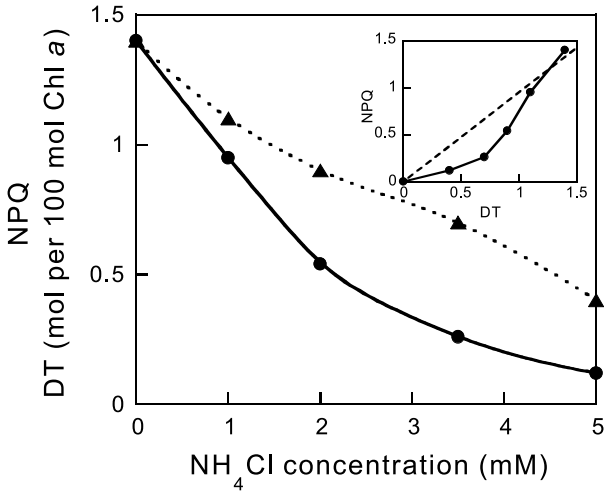

Fig. 2. Effect of increasing concentrations of NH_4Cl on NPQ (circles) and DT concentration (triangles) in *P. tricornutum* CL cells (low DD pool size). Cells were incubated in the dark for 20 min with different concentrations of NH_4Cl prior to illumination (5 min at 350 μE m^{-2} s^{-1}). Inset: Corresponding correlation of NPQ and DT concentration. The dotted linear regression (NPQ = 0.95 DT) illustrates the correlation between NPQ and DT concentration in the absence of NH_4Cl [10].

During the high light illumination, NPQ was always proportional to the concentration of DT (dotted line in inset to Fig. 2, [10]). However, this proportionality broke down when cells were dark incubated with increasing concentrations of NH_4Cl. This reagent is a well-known uncoupler that decreases the ΔpH formed during illumination. The 5 min illumination at 350 μE m^{-2} s^{-1} led to lower values of NPQ and DT for increasing concentrations of NH_4Cl but the decrease in NPQ was larger than that of DT (Fig. 2). As a consequence the linear relationship between NPQ and DT was replaced by an S-shaped one (solid circles, inset Fig. 2). Since NPQ can be fully suppressed this way while some DT accumulation persists, we must conclude that the ΔpH affects not only the de-epoxidation of DD but also the effectiveness of DT in causing NPQ.

A divergence from a linear relationship between NPQ and DT was also observed during the dark decay of NPQ and epoxidation of DT after a high light illumination. It was best observed with IL cells where a 5 min illumination at 450 μE m^{-2} s^{-1} induced an NPQ of 6, much larger than the values attained in CL cells due to the smaller DD pool. NPQ was proportional to the DT concentration during illumination for both types of cells (see dotted line, inset to Fig. 3, [10]). During the dark period following the illumination, epoxidation of DT and relaxation of NPQ occurred (Fig. 3). NPQ decreased faster than the DT content and their relationship became sigmoidal, similar to that observed in the presence of NH_4Cl (Fig. 3, inset). In cells with a small DD pool, the deviation from linearity also existed but was less pronounced (Fig. 3, inset), possibly because of the faster epox-

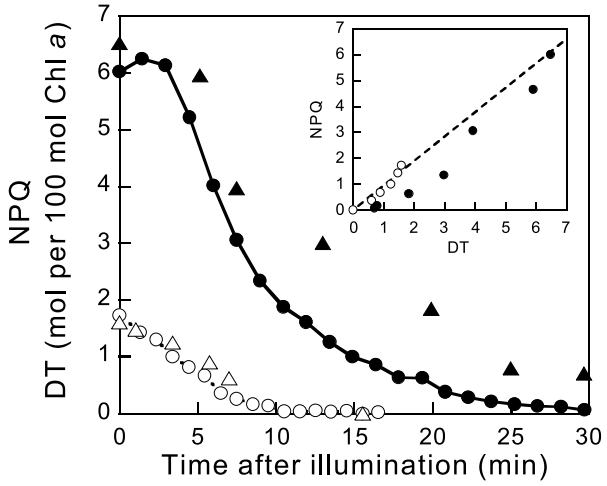

Fig. 3. Decay of NPQ (circles) and epoxidation of DT (triangles) in darkness following an illumination of 5 min at 450 μE m^{-2} s^{-1} for *P. tricornutum* cells with a low (open symbols) or with a large (solid symbols) DD pool. Inset: Corresponding correlation of NPQ and DT concentration for cells with a low (open circles) or with a large (solid circles) DD pool. The dotted linear regression (NPQ = 0.95 DT) illustrates the correlation of NPQ and DT concentration during illumination.

idation in CL cells. The epoxidation is faster in diatoms in contrast to higher plants grown in the same light conditions, either CL or IL [16]. Darkness following high light was the second situation where some DT remained while NPQ was fully suppressed. The reason might be similar to the situation observed in the presence of an uncoupler: DT cannot cause NPQ after ΔpH dissipation. If for NPQ a ΔpH is required in addition to the presence of DT, one possible explanation is that protonation of light-harvesting protein(s) is necessary for the binding of DT. The p*K* of the protonation could be higher than the p*K* of DD de-epoxidase so that on illumination protonation precedes de-epoxidation and NPQ is strictly proportional to DT. Epoxidation of DT to DD is pH independent and was apparently slower than deprotonation of the light-harvesting protein(s) after illumination, so that some DT remained while NPQ was completely suppressed.

On the other hand, a strict proportionality between NPQ and DT was maintained under conditions designed to selectively increase the ΔpH or decrease DT accumulation. At low concentrations, DCCD is an inhibitor of ATP synthase [18]. Its presence should therefore increase the ΔpH formed during illumination. Cells were exposed to a 5 min illumination at 100 μE m^{-2} s^{-1}. The intensity used for this experiment was chosen low enough not to induce any NPQ or DT in the absence of added DCCD. When dark-adapted cells were pre-incubated with a low concentration of DCCD (10 μM), the effects of DCCD on DT and NPQ were dependent on the incubation time. In the presence of DCCD (5 or 10 min incubation in the dark) both DT and NPQ could be detected at the end of the 5 min illumination at 100 μE m^{-2} s^{-1} as shown in Table 1. The relationship between NPQ and DT remained linear as under higher illumination in the absence of DCCD.

Table 1
Effect of dark incubation of *P. tricornutum* CL cells (low DD pool size) with DCCD on NPQ and DT accumulation after a 5 min illumination at 100 μE m^{-2} s^{-1}

DCCD (μM)	Incubation time (min)	NPQ	DT (mol/100 mol Chl *a*)
0	0	0.06	0.0
10	5	1.73	1.7
10	10	1.83	2.3

DTT is a well-known inhibitor of de-epoxidase and has been used to detect zeaxanthin-independent fluorescence quenching in higher plants [11]. DTT acts on the de-epoxidase without affecting lumen acidification [19]. DTT also inhibits the DD de-epoxidase in diatoms [20]. NPQ coupled to DT was formed by a 5 min illumination at 450 μE m^{-2} s^{-1} in cells with different DD pool sizes (CL and IL cells). Cells were pre-incubated with increasing concentrations of DTT (up to 150 μM) and the DT content and NPQ were determined at the end of the illumination. As shown in Fig. 4, both DT and NPQ were decreased by increasing DTT concentrations. NPQ remained proportional to DT and their relationship followed the same linear regression curve as in the absence of DTT. If DTT does not affect the ΔpH, the results of Fig. 4 clearly demonstrate the obligatory presence of DT for NPQ to exist in diatoms. This is different from higher plants where DTT inhibits the accumulation of de-epoxidized xanthophylls without totally suppressing NPQ [11]. An analogous situation was reported for *Chlamydomonas* and *Arabidopsis* mutants with defective xanthophyll cycling that continues to exhibit a small zeaxanthin-independent NPQ under high light [21,22]. DTT has been shown to have many other effects including the inhibition of ΔpH. In the present experiment such effects cannot be completely ruled out, but the concentrations used are well below those required for the suppression of ΔpH [23].

In conclusion, we propose that, in diatoms, upon acidification of the lumen, LHC subunits become protonated, de-epoxidase is rapidly activated and DT is formed. The binding of DT to the LHCs is influenced by protonation. Protonation alone is not sufficient to modify the dissipative function of the

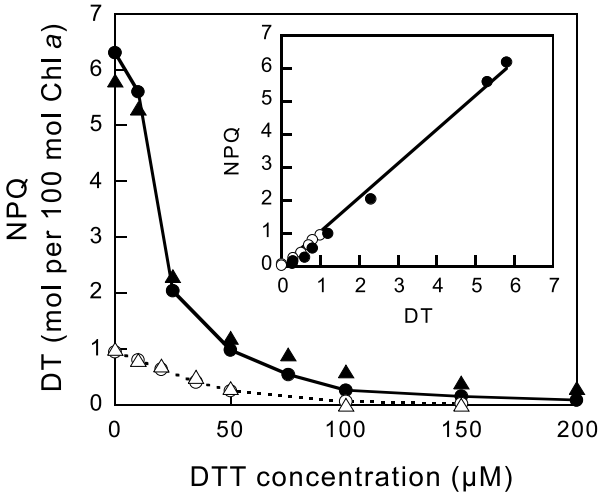

Fig. 4. Effect of increasing concentration of DTT on NPQ (circles) and DT (triangles) for *P. tricornutum* cells with a low (open symbols) or with a large (solid symbols) DD pool. Cells were incubated in the dark for 20 min with DTT prior to the illumination (5 min at 350 μE m^{-2} s^{-1}). Inset: Corresponding correlation of NPQ and DT concentration. The linear regression was NPQ = 0.93 DT with $R^2 = 0.98$ for the cells with a low DD (open circles) and NPQ = 1.04 DT with $R^2 = 0.99$ for cells with a large DT (solid circles).

antenna complexes in contrast to higher plants [24]. It is the presence of DT bound to the protonated LHCs which induces NPQ. The exact role of DT remains to be elucidated. It is still unclear if it can play a direct role in energy dissipation processes; alternatively, its presence could cause association of other pigments leading to the formation of quenchers (carotenoid, carotenoid–Chl or Chl–carotenoid–Chl, Chl–Chl associations) [25,26].

The large concentrations of DT, the high values reached by NPQ, the absence of photosystem segregation and of state transitions have to be taken into account when comparing the LHCs of diatoms and higher plants. The DD de-epoxidase is activated by a low ΔpH value and the xanthophyll cycle kinetics are faster than in higher plants [16,17]. A ΔpH alone is not sufficient to induce NPQ. In diatoms, the molecular mechanisms involved in the ΔpH-dependent photoprotective dissipation of excess energy are partly distinct from those occurring in higher plants. Some properties of diatoms and especially the rapidity of the xanthophyll cycle kinetics are important for a rapid switch of the LHC function from the dissipation of excess energy to the efficient transfer of energy to the reaction centers.

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References

[1] Harris, G.P. (1986) Phytoplankton Ecology: Structure, Function and Fluctuation, Chapman and Hall, London.
[2] Pyszniak, A.M. and Gibbs, S.P. (1992) Protoplasma 166, 208–217.
[3] Brown, J.S. (1988) J. Phycol. 24, 96–102.
[4] Owens, T.G. and Evarina, R.W. (1986) Plant Physiol. 80, 732–738.
[5] Bhaya, D. and Grossman, A.R. (1993) Nucleic Acids Res. 21, 4458–4466.
[6] Berkaloff, C., Caron, L. and Rousseau, B. (1990) Photosynth. Res. 23, 181–193.
[7] Müller, P., Li, X.-P. and Niyogi, K.K. (2001) Plant Physiol. 125, 1558–1566.
[8] Wilhelm, C. (1990) Plant Physiol. Biochem. 28, 293–306.
[9] Arsalane, W., Rousseau, B. and Duval, J.-C. (1994) Photochem. Photobiol. 60, 237–243.
[10] Lavaud, J., Rousseau, B., van Gorkom, H.J. and Etienne, A.-L. (2002) Plant Physiol. 129 (3), in press.
[11] Gilmore, A.M. and Yamamoto, H.Y. (1991) Plant Physiol. 96, 635–643.
[12] Guillard, R.R.R. and Ryther, J.H. (1962) Gran. Can. J. Microbiol. 8, 229–238.
[13] Johansen, J.E., Wa, S., Liaaen-Jensen, S. and Haxo, F.T. (1974) Phytochemistry 13, 2261–2271.
[14] Arsalane, W., Paresys, G., Duval, J.-C., Wilhelm, C., Conrad, R. and Büchel, C. (1993) Eur. J. Phycol. 28, 247–252.
[15] Krause, G.H. and Weis, E. (1991) Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 313–349.
[16] Färber, A. and Jahns, P. (1998) Biochim. Biophys. Acta 1363, 47–58.
[17] Jakob, T., Goss, R. and Wilhelm, C. (2001) J. Plant Physiol. 158, 383–390.
[18] McCarty, R.E. and Racker, E. (1967) J. Biol. Chem. 242, 3435–3439.
[19] Sokolove, P.M. and Marsho, T.V. (1976) Biochim. Biophys. Acta 430, 321–326.
[20] Olaizola, M., Laroche, J., Kolber, Z. and Falkowski, P.G. (1994) Photosynth. Res. 41, 357–370.
[21] Niyogi, K.K., Björkman, O. and Grossman, A.R. (1997) Plant Cell 9, 1369–1380.
[22] Niyogi, K.K., Grossman, A.R. and Björkman, O. (1998) Plant Cell 10, 1121–1134.
[23] Pfündel, E.E. and Bilger, W. (1994) Photosynth. Res. 42, 89–109.
[24] Horton, P., Ruban, A.V. and Walters, R.G. (1996) Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 655–684.
[25] Frank, H.A., Cua, A., Chynwat, V., Young, A., Gosztola, D. and Wasielewski, M.R. (1996) Biochim. Biophys. Acta 1277, 243–252.
[26] Frank, H.A., Bautista, J.A., Josue, J.S. and Young, A.J. (2000) Biochemistry 39, 2831–2837.