ARTICLE

Gaining Insight into Mechanisms of Nonphotochemical Quenching of Chlorophyll Fluorescence in *Chlamydomonas reinhardtii* via the Observation of Dark-induced State Transitions

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ABSTRACT

Photosynthetic organisms are usually exposed to fluctuating light, and therefore they evolved mechanisms enabling fast acclimation to changing light conditions. Among them, two important ones are energy-dependent quenching of excited chlorophyll (qE) and state transitions (ST). qE is a photoprotective mechanism regulated by pH gradient across thylakoid membranes, in which excessive energy is dissipated as heat. ST are rearrangements of antenna systems regulated by the phosphorylation of LHC II complexes. Both of these mechanisms result in changes in NPQ parameter. In the present article, changes of NPQ in the green microalga *Chlamydomonas reinhardtii* were evaluated in the dark period, after various lengths of actinic light exposure, and after the application of the thiol reducing reagent dithiotreitol and the cyt b_6f inhibitor 2',4'-dinitrophenyl ether of 2-iodo-4-nitro-thymol. The impact of the length of actinic light exposure on xanthophyll cycle progression in *C. reinhardtii* was also analysed. The obtained results enabled us to gain more insight into the nonphotochemical quenching of chlorophyll fluorescence in model organism *C. reinhardtii*, i.e. the role of zeaxanthin-dependent quenching and chlororespiration-induced pH gradient, and the inhibitory action of tested compounds on state transitions in this species.

Keywords: Chlororespiration; Energy-dependent quenching; State transitions; Stt7 kinase; Xanthophyll cycle; Zeaxanthin-dependent quenching

1. Introduction

Photosynthetic organisms evolved to use light energy effectively. However, the enzymatic reactions of photosynthesis have their limitations and under high light photosynthesis becomes saturated. The light intensity that exceeds the threshold needed for saturation of photosynthesis is harmful to the organism due to unwanted side reactions leading to the generation of harmful compounds, such as reactive oxygen species [1]. Photosynthetic organisms are usually exposed to fluctuating light; therefore, there was a need to evolve mechanisms that enable acclimation to the changing light conditions [1]. Among them, two important ones, that enable a fast response to changes in light intensity, are energy-dependent quenching of chlorophyll fluorescence (qE) and state transitions (ST) [2]. qE is a photoprotective mechanism regulated by pH gradient across thylakoid membranes, in which excessive energy is dissipated as heat. In land plants, qE requires the presence of de-epoxidized xanthophylls, such as antheraxanthin and zeaxanthin, as well as PsbS protein, which does not contain strongly bound pigments and is considered to be a sensor of lumenal pH [3,4]. In *Chlamydomonas reinhardtii,* which belongs to green algae, the de-epoxidized xanthophylls are less important for qE, while the key elements are LHCSR proteins binding xanthophylls and chlorophylls *a* and *b* [1,5–9]. The efficiency of qE induction in this alga strongly depends on the amount of LHCSR3. It was shown that the expression of the gene encoding this protein is enhanced in stress conditions [1,6]. The LHCSR proteins function as both pH sensors and sites of excited chlorophyll quenching [1].

State transitions are rearrangements of antenna systems regulated by the phosphorylation of LHC II complexes [8]. In state 1, the LHC II antennae are associated with PS II and the linear photosynthetic electron transfer is favoured. The more intensive electron transfer results in a more reduced plastoquinone (PQ) pool. In such a case, serine/threonine kinase responsible for the phosphorylation of LHC II antennae is activated. The phosphorylated LHCs dissociate from PS II and associate with PS I; resulting in state 2 of photosynthetic apparatus [10]. The enhanced excitation of PS I protects from the overreduction of electron carriers functioning between PS II and PS I and favours cyclic electron transfer (CEF) around PS I [1,10]. In land plants, during ST, about 10–20% of the LHC II are relocated, while in *C. reinhardtii* up to 80% of LHC II were observed to migrate [1,11,12]. Interestingly, Ünlü et al. reported that in state 2 only about 10% of LHC II are associated with PS I, while the majority remain detached from any photosystem and quenched [12]. LHC II dephosphorylation triggers the transition back to state 1. In *C. reinhardtii* LHC II is phosphorylated by the kinase Stt7, while two phosphatases CrPPH1 and CrPBCP play a role in antennae dephosphorylation [13]. In higher plants, ST are considered to play a role in balancing of distribution of excitation energy between photosystems, while in *C. reinhardtii* the key function of this mechanism is to regulate the linear and cyclic electron transfer [1,10–12]. In the CEF the ATP is produced without the production of NADPH, therefore, the state 1 to 2 transition can be beneficial in the situation of limited CO_2 assimilation or increased ATP demand [10,14].

It was observed that the transition of *C. reinhardtii* from light to darkness may cause the state 1 to 2 transition [15]. This is an effect of induction of chlororespiration, a process of electron transfer from NADPH to O_2 occurring in thylakoid membranes in low light and darkness [16]. PQ participates both in photosynthetic and chlororespiratory electron transfer chains, therefore the activation of chlororespiration causes PQ reduction to plastoquinol (PQH_2) [17]. In the chlororespiratory electron transfer chain, PQH_2 is re-oxidized by plastid terminal oxidase (PTOX). If the reaction carried out by PTOX is limited, for example as a result of low oxygen level, PQH_2 can bind to cytochrome b_6f, which activates the Stt7 [1,18].

Nonphotochemical quenching of chlorophyll fluorescence embraces qE, ST, and photoinhibition (qI), the latter resulting from the PS II damage and the stable quenching in its antennae [9]. This phenomenon can be monitored by measuring NPQ parameter [19].

In the present paper, the author assessed the changes of NPQ in the dark period, following various lengths of actinic light (AL) exposure, and in the presence of thiol reducing reagent dithiotreitol (DTT) and cyt $b_6 f$ inhibitor 2',4'-dinitrophenyl ether of 2-iodo-4-nitro-thymol (DNP-INT). The impact of the length of AL treatment on xanthophyll cycle progression in *C. reinhardtii* was also analysed.

2. Materials and methods

2.1 Culture growth and chlorophyll fluorescence measurements

In this experiment, *C. reinhardtii* strain 11-32b (SAG collection*,* Goettingen, Germany) was used. The algae were cultivated on a shaker at 21 °C under the illumination of 35–40 µmol photons $m^{-2}s^{-1}$ for 3 weeks on the modified SG medium as described in the study by Nowicka [15].

Before each measurement, Chl $a + b$ content was assessed in cultures, according to the method described by Lichtenthaler [20]. Algal suspensions were centrifuged for 5 min × 600 g. The obtained pellets were resuspended in a certain volume of the growth medium to obtain the final Chl $a + b$ concentration of 15 µg/mL. Algae suspensions were then portioned into 48-well plastic plates (1 mL of algal suspension per well) and dark-adapted for 60 min. The measurements of chlorophyll fluorescence parameters were carried out using Open FluorCam FC 800-O (Photon Systems Instruments, Brno, Czech Republic). The following light conditions were applied: white saturating light of intensity 2700 µmol photons $m^{-2}s^{-1}$ and white AL of intensity 660 µmol photons $m^{-2}s^{-1}$. The efficiency of nonphotochemical quenching of chlorophyll fluorescence was measured as NPQ parameter calculated as $(F_m - F_{m'})/F_{m'}$ [19]. The duration of AL treatment and the following dark incubation period, as well as the timing of saturating pulses application, are given in the figures.

In the experiments with inhibitors, algae were measured in the same way as described above, the length of AL treatment was 10 min. In the experiment with DNP-INT, the inhibitor was added during AL exposure 3 min before the AL was turned off, to the final concentration of 10 µM. In the experiment with DTT, the compound was added 10 min before exposure to AL, to the final concentration of 10 mM.

2.2 Xanthophyll cycle pigments determination

Sample extraction was carried out as described in the study by Nowicka and Kruk [21]. The determination of xanthophyll cycle pigments by the RP-HPLC method was carried out using the Jasco LC-4000 system (Jasco Corporation, Tokyo, Japan). The separation was performed in the C_{18} reverse-phase column (Tracer Excel 120 ODS-A, 25 cm × 0.4 cm, grain diameter 5 µm, Teknokroma, Spain). Separation was carried out using two eluents, eluent A (pure methanol) and eluent B (methanol:hexan 340:40, v/v), and a Jasco quaternary gradient pump (PU-2089) that allows solvent mixing during separation. The program used is shown in **Table 1**, the flow rate applied was 1.6 mL/min, and absorbance was measured at $\lambda = 440$ nm. The content of certain xanthophylls was calculated on the basis of peak areas as described in the study by Nowicka et al. [22].

Table 1. Gradient separation of xanthophyll cycle pigments.

Time [min]	Eluents
0–15	only eluent A
15–17	change from eluent A to eluent B
17–31	only eluent B
31–32	change from eluent B to eluent A
32–35	only eluent A

3. Results and discussion

An example of the results of NPQ measurements in *C. reinhardtii* in conditions favouring dark ST is shown in **Figure 1**. The NPQ parameter is calculated according to the equation:

$$NPQ = (F_m - F_{m'})/F_{m'}$$

where F_m is the maximal chlorophyll fluorescence in a dark-adapted state and $F_{m'}$ is the maximal fluorescence measured during AL exposure and the following dark relaxation period. Therefore, a decrease in $F_{m'}$ would cause an increase in NPQ. If *C. reinhardtii*

are in state 1 at the beginning of the experiment, which can be achieved by exposing algae to weak far red light instead of adaptation in full darkness [15], exposure to AL results mainly in activation of qE. The observed decrease in $F_{m'}$ is due to the enhanced dissipation of absorbed light energy as heat. If the intensity of AL is high enough to cause degradation of the photosynthetic apparatus, photoinhibition can also occur, and another reason for the decrease of $F_{m'}$ would be damage to PS II and antennae [15]. Switching the AL off causes the relaxation of ΔpH between the lumen and stroma and, as a result, the decrease in qE and NPQ parameter. If the conditions favour the occurrence of ST in darkness, the relocation of LHC II antennae causes a decrease in the efficiency of energy transfer from LHC II to PS II and, as a result, the decrease in $F_{m'}$ and the increase in NPQ measured. The correlation between NPQ changes and ST was confirmed by measurements of low temperature (77 K) chlorophyll fluorescence spectra [15]. Therefore, monitoring of NPQ parameter may be a fast, simple, and non-invasive way to observe dark-induced ST. In the present experiments, during dark-induced ST NPQ parameter reached its maximum after 10 min of darkness, and then the process was slowly reverted (**Figure 1**).
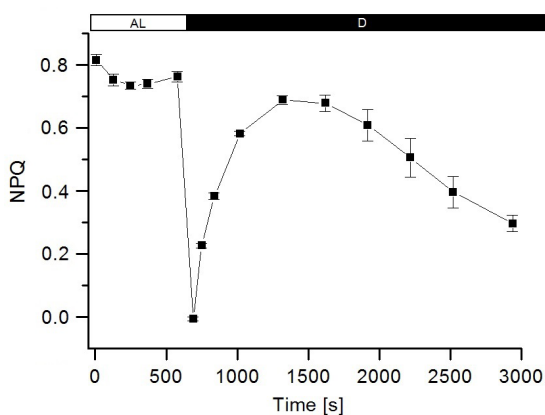

Figure 1. The changes of NPQ parameter in *C. reinhardtii* during 10 min of actinic light treatment (AL in the white bar) and subsequent 40 min of darkness (D in the black bar). Data are means *n* = 5 ± SD.

Measurements of the impact of the length of AL treatment on the course of the following dark ST were carried out (**Figure 2A**). It was expected that longer light exposure would result in a higher concentration of chlororespiration substrates and therefore shift of the ST induction/relaxation periods towards longer time. However, such an effect did not occur. In all examined cases, the highest NPQ was observed at 630 s after switching AL off (**Figure 2A**). An interesting observation was the dependence of the NPQ values reached on the length of the AL treatment. The longer the exposure to light, the higher NPQ was observed 30 s after the AL was turned off (**Figure 2A**). This trend was observed also for the longer times of dark incubation when ST occurred. It is known that in many algae, including those belonging to the green lineage, as well as in land plants, exposure to light causes the interconversion of xanthophyll cycle pigments and the accumulation of de-epoxidized xanthophylls [23]. *C. reinhardtii* contains a violaxanthin cycle, where violaxanthin is converted to antheraxanthin and then zeaxanthin. The role of de-epoxidized xanthophylls in nonphotochemical quenching of excited chlorophyll in this species is lesser than in higher plants, yet it is not marginal [1,9]. Troiano et al. [24] revealed that in *C. reinhardtii* zeaxanthin-dependent quenching is a process distinct from the quenching related to pH-induced conformation change in LHCSR proteins, and that it occurs also in the absence of pH gradient. In the present experiment, AL exposure led to the accumulation of de-epoxidized xanthophylls in *C. reinhardtii*, which were later slowly epoxidized in darkness (**Figure 2B**). Thus, the enhancement in dark-NPQ in algae exposed to AL for a longer time could be partly explained by the occurrence of ΔpH-independent component related to de-epoxidized xanthophylls formed. This component is responsible for "basal" NPQ present in darkness.

On the other hand, an amplitude of NPQ increase in darkness is also higher in algae exposed to AL for a longer time when compared to the series with shorter AL exposure times (**Table 2**). It is known that chlororespiration causes the formation of pH gradient across thylakoid membranes [25]. It can be therefore hypothesized that longer exposure to AL indeed led to the accumulation of bigger amounts of chlorores-

piration substrates. More abundant substrates enabled an enhanced activity of the chlororespiratory electron transfer chain, which in turn resulted in a more pronounced pH gradient across thylakoid membranes and more efficient ΔpH-dependent quenching at LHCSR proteins. The enhanced activity of the chlororespiratory electron transfer chain would also result in an increased concentration of PQH_2 in the thylakoid membranes. It is therefore possible, that in such a case more cyt b_6f were binding PQH_2 at the same time, which led to the enhanced activation of Stt7 and due to it more pronounced dissociation of phosphorylated antennae from PS II.
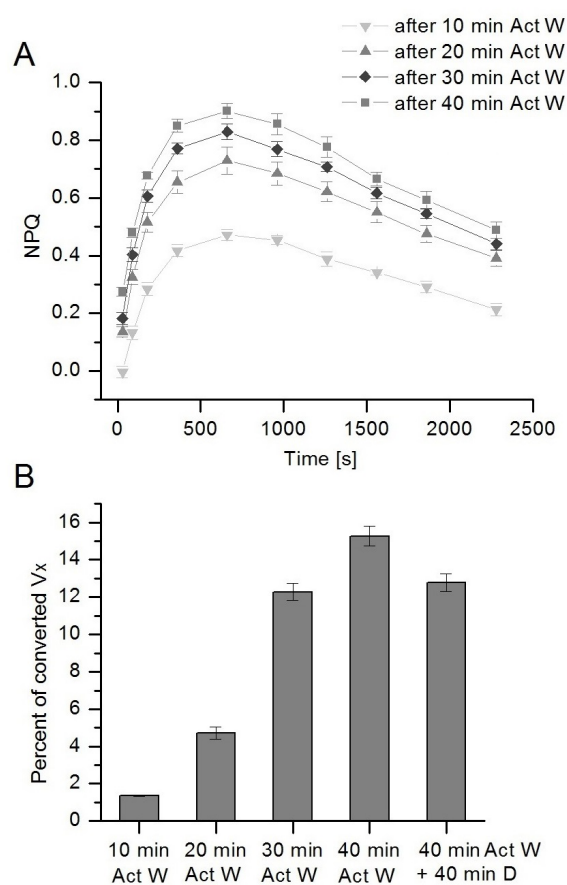

Figure 2. The impact of the length of white actinic light (Act W) treatment on the NPQ parameter measured during the following 40 min of dark incubation and on the xanthophyll cycle progression. (A) NPQ parameter, the data are means $n = 5 \pm SD$, (B) percentage of violaxanthin (Vx) converted to antheraxanthin and zeaxanthin, the data are means $n = 2 \pm SD$.

The addition of DTT caused partial inhibition of dark-induced ST in *C. reinhardtii* (**Figure 3**). This

kind of effect was reported to occur in land plants [26]. STN7 kinase responsible for LHC II phosphorylation in Arabidopsis contains in its lumenal domain two conserved Cys, crucial for its activity [27,28]. Reduction of these Cys makes the kinase inactive [29]. The Stt7 kinase of *C. reinhardtii* also contains two conserved Cys close to its N-terminal end exposed to the lumen. Mutants *Stt7* expressing transgenes coding Stt7 forms with any of above mentioned Cys replaced by Ala or Ser were deficient in ST [30]. The present results are therefore consistent with the ones reported by Lemeille et al. [30], suggesting that the role of lumen-exposed Cys in the regulation of LHC II kinases in higher plants and *C. reinhardtii* may be similar. During prolonged incubation in the dark under conditions that favour anaerobiosis, the Cys of Stt7 can become reduced [31]. This may cause inactivation of the kinase, which enables reverting to state 1.

Table 2. The difference between NPQ values recorded after 630 s (maximal NPQ) and after 30 s (initial NPQ) of dark incubation in *C. reinhardtii* pre-exposed to actinic light for different time periods.

Time of actinic light exposure [min]	ΔNPQ during 10 min of dark incubation after actinic light exposure
10	0.476
20	0.594
30	0.647
40	0.627

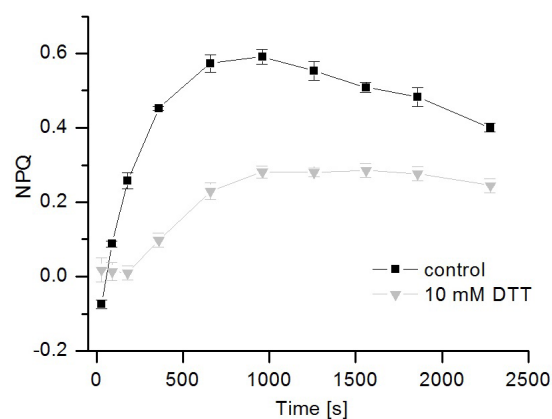Source: Calculated on the basis of the results presented in **Figure 2A**.

Figure 3. The changes of NPQ parameter in *C. reinhardtii* during 40 min of darkness following 10 min of white actinic light treatment in the presence of 10 mM dithiotreitol (DTT). Data are means $n = 5 \pm SD$.

Dark-induced ST was also partly inhibited by DNP-INT (**Figure 4**). The LHC II kinases STN7 and Stt7 need PQH$_2$ for their activation, but this dependence is not direct. These enzymes are known to associate with the cyt b_6f complex, in particular, it was shown that they interact with the Rieske protein [31]. Zito et al. [32] showed that the binding of PQH$_2$ to the Q$_o$ pocket in cyt b_6f is crucial for the activation of Stt7. A well-known inhibitor of cyt b_6f, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB), acts as a PQH$_2$ analogue and binds at the Q$_o$ site [33]. The application of DBMIB impaired ST both in *A. thaliana* and *C. reinhardtii* [34,35]. However, being a quinone, DBMIB may undergo redox reactions in cells, which may change its activity towards cyt b_6f [34]. Similarly to DBMIB, DNP-INT is a potent inhibitor of cyt b_6f, binding at the Q$_o$ site [36], but it does not contain ketone groups which could undergo reversible reduction. Due to this, in some applications, including ST monitoring, DNP-INT may be a better solution than DBMIB. However, one should remember that this compound does not provide complete inhibition of photosynthetic electron transfer [36]. The inhibitory action of DNP-INT on PQH$_2$ oxidation at cyt b_6f explains its ability to disturb dark-induced ST in *C. reinhardtii*.
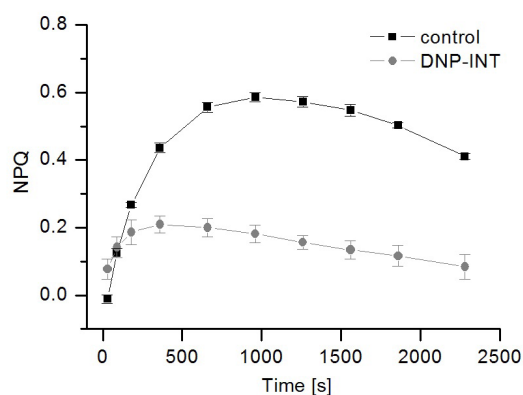

Figure 4. The changes of NPQ parameter in *C. reinhardtii* during 40 min of darkness following 10 min of white actinic light treatment in the presence of 10 μM 2',4'-dinitrophenyl ether of 2-iodo-4-nitro-thymol (DNP-INT). Data are means $n = 5 \pm$ SD.

4. Conclusions

The above-mentioned results show the usefulness of NPQ measurements for the research concerning ST in *C. reinhardtii*. They are relatively easy to conduct, non-invasive and fast. The method enabled us to gain more insight into the mechanisms of nonphotochemical quenching of chlorophyll fluorescence in above mentioned algal species, i.e. the role of zeaxanthin-dependent quenching and chlororespiration-induced ΔpH gradient, and the inhibitory action of DTT and DNP-INT on ST in this species.

Conflict of Interest

The author declares no conflict of interest.

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