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Ultrafast fluorescence study on the location and mechanism of non-photochemical quenching in diatoms

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ABSTRACT

The diatom algae, responsible for at least a quarter of the global photosynthetic carbon assimilation in the oceans, are capable of switching on rapid and efficient photoprotection, which helps them cope with the large fluctuations of light intensity in the moving waters. The enhanced dissipation of excess excitation energy becomes visible as non-photochemical quenching (NPQ) of chlorophyll *a* fluorescence. Intact cells of the diatoms *Cyclotella meneghiniana* and *Phaeodactylum tricornutum*, which show different NPQ induction kinetics under high light illumination, were investigated by picosecond time-resolved fluorescence under dark and NPQ-inducing high light conditions. The fluorescence kinetics revealed that there are two independent sites responsible for NPQ. The first quenching site is located in an FCP antenna system that is functionally detached from both photosystems, while the second quenching site is located in the PSII-attached antenna. Notwithstanding their different npq induction and reversal kinetics, both diatoms showed identical NPQ via both mechanisms in the steady-state. Their fluorescence decays in the dark-adapted states were different, however. A detailed quenching model is proposed for NPQ in diatoms.

1. Introduction

Diatoms (Bacillariophyceae) are unicellular photosynthetic organisms that play a key role in the biochemical cycles of carbon, nitrogen, phosphorus and silica. They have a strong impact on the global climate not only in the ocean, where they are responsible for at least a quarter of global carbon fixation [1], but also in the freshwater environment. Diatoms experience large fluctuations in light intensity due to unpredictable water motions that can vary over several orders of magnitude on a time-scale of minutes [1–3]. In order to survive the periodic exposure to high excess light intensities, they developed mechanisms to dissipate the excess energy by non-photochemical processes, presumably by some modification/reorganization in their antenna complexes attached to photosystem (PS) II.

The organization of the thylakoid membrane, the light-harvesting complexes and the pigmentation in diatoms differ from vascular plants and green algae. Diatoms do not contain granal and stromal thylakoids as the vascular plants do. Their chloroplasts rather contain one homogeneous thylakoid membrane organized in bands of three [4]. According to present knowledge, the antenna proteins of PSI and PSII in diatoms are similar or even identical and they do not differentiate into PSI and PSII antennae nor into minor and major complexes as it is the case in vascular plants [5]. Besides chlorophyll (Chl) *a*, diatoms contain various Chl *c* species as antenna chromophores, and fucoxanthin as the major light-harvesting xanthophyll, which is also responsible for their brown colour. The proteins of the light-harvesting complexes in diatoms are thus called fucoxanthin-chlorophyll-binding proteins (FCPs). They are encoded by a conserved family of *fcp* genes of more than 5–6 members [6]. Evidence accumulated on the structural organization of FCPs, showing them to form higher oligomeric states in the species *Cyclotella meneghiniana* and *Phaeodactylum tricornutum* [7–10]. Two different FCP fractions can be isolated by sucrose density gradient centrifugation showing distinct oligomerization and pigmentation patterns. A recent study presented evidence that fluorescence quenching in one population of *C. meneghiniana* FCPs depended on the presence of diatoxanthin (Dtx) while in another fraction it was not affected by Dtx accumulation [8]. Regarding the distribution of xanthophyll cycle (XC) pigments, Lepetit et al. [11] could show in *P. tricornutum* that apart from the highly enriched FCPs, a population of diadinoxanthin (Ddx) and Dtx is tightly associated with the core complex of both PSI and PSII, and that de-epoxidation takes place in PSI as well. The main xanthophyll cycle of diatoms is the diadinoxanthin cycle (DdC) but the situation in diatoms

Abbreviations: Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorphenyl)-1,1-dimethylurea; DdC, diadinoxanthin cycle; Ddx, diadinoxanthin; DAS, decay associated spectra; DES, de-epoxidation state; Dtx, diatoxanthin; FCPs, fucoxanthin-chlorophyll-binding proteins; HL, high light; LHC, light-harvesting complex; NPQ, non-photochemical quenching; ΔpH, transthylakoid proton gradient; PS, photosystem; RC, reaction centre; RP, radical pair; XC, xanthophyll cycle; Zx, zeaxanthin

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is more complex than in vascular plants because diatoms contain both the DdC and the violaxanthin cycles [12]. In the DdC the xanthophyll Ddx is de-epoxidized into Dtx [13] in a one-step reaction.

In the diatom *P. tricornutum* triggering of NPQ is assigned to the DdC [14,15]. A strict correlation between Dtx content and NPQ was found under most natural conditions [16] and it could not be shown that a ΔpH alone induces NPQ [17]. However, the proton gradient is needed during the development of Dtx-dependent NPQ, both for the de-epoxidation of Ddx to Dtx and for the activation of the Dtx-dependent NPQ. Once the Dtx-dependent NPQ has been established, it remains stable in darkness after a high light period even in the absence of a proton gradient and only relaxes with the epoxidation of Dtx [18]. These differences suggest a different mechanism of NPQ in diatoms as compared to vascular plants [for a recent review see 19,20], because in vascular plants the main part of NPQ (the so-called qE-quenching) is quickly abolished upon the collapse of ΔpH. A zeaxanthin (Zx)-dependent NPQ without a proton gradient is observed in vascular plants if intact leaves are illuminated with high light for longer time periods [21]. Recently, evidence for an additional, faster, NPQ mechanism, that is not directly related to the DdC and relaxes rapidly in darkness, was found in the diatom *C. meneghiniana* [22]. This component seems to be missing, however, in *P. tricornutum*. An equivalent to the PsbS protein, which in vascular plants is crucial for NPQ generation and ΔpH sensing [23], has not been found in the genome of the diatoms *Thalassiosira pseudonana* [6] or *P. tricornutum*. However, the LI818-like protein has been suggested to play the role of PsbS in diatoms [24]. The search for a functional counterpart of PsbS still remains an open question in the context of NPQ formation in diatoms.

Until now, only fluorescence induction kinetics has been used to study NPQ in diatoms. The information gained from such studies is limited as far as the exact location of NPQ within PSII or PSI is concerned. Thus, the main goal of our study is to gain insight into the NPQ characteristics and in particular the locations of NPQ in the photosynthetic complexes of diatoms based on ultrafast fluorescence decay measurements. The fluorescence decay kinetics was measured for intact cells of both *C. meneghiniana* and *P. tricornutum* in order to understand the differences in NPQ observed for these two diatoms. The time-resolved fluorescence method combined with target analysis of the data allows distinguishing between different locations of quenching, like e.g. quenching occurring in a PSII-attached or a PSII-detached antenna part.

2. Materials and methods

2.1. Material and growth conditions

C. meneghiniana and *P. tricornutum* cells were grown at 20 °C with a light to dark cycle of 14:10 h at a light intensity of 40 μmol photons m^{-2} s^{-1}. Algal cultures were obtained from the Culture Collection of Algae from the University of Göttingen (SAG). *P. tricornutum* was grown in ASP medium according to [25] with modifications introduced by [26]. *C. meneghiniana* was grown in silica-enriched f/2 medium according to [27]. Immediately before measurements cells were concentrated by centrifugation (3500 × *g*) at 4 °C to a Chl *a* content of 10 mg l^{-1}. For all measurements performed in the present study, 10 mM $NaHCO_3$ was added to the algal cultures to ensure sufficient CO_2-supply during the periods of actinic high light illumination. To ensure that the physiological state of the cells was not affected by the centrifugation procedure, F_v/F_m values were calculated from measurements of fast fluorescence rise performed by means of a handy plant efficiency analyser (PEA Hansatech, Norfolk, England) before and after centrifugation.

FCPs were isolated from dark-adapted cells of *C. meneghiniana* and *P. tricornutum* as described in [7] and [9], respectively. Thylakoid membranes, suspended in isolation buffer containing 10 mM MES (pH 6.5), 2 mM KCl, 5 mM EDTA, were solubilized with 2% β-dodecylmaltoside (corresponding to a detergent: Chl ratio of 20) and separated by density gradient centrifugation on a buffered 0–0.7 M linear sucrose gradient containing 0.03% β-dodecylmaltoside. The two fractions of FCP from *C. meneghiniana*, FCP1 and FCP2 [7] were collected and analysed separately. Aggregated FCPs were prepared by incubation of the FCP suspension diluted to 10–15 μg Chl *a* ml^{-1} with 200 mg ml^{-1} Bio-beads SM-2 (Bio-RAD) for 2 h at room temperature.

2.2. Fluorescence kinetics

Fluorescence kinetics was measured by a single-photon timing apparatus at several wavelengths on a total volume of 500 ml alga suspension at a Chl *a* concentration in the range of approximately 5–10 μg ml^{-1}. Excitation (662 nm) was provided by a synchronously pumped cavity-dumped dye laser system with a mode-locked argon ion laser (Spectra Physics) as a pumping source [28,29]. A fresh alga suspension was used for each measurement. The cells were pumped through a flow-through cuvette (cross-section of 1.5 × 1.5 mm, see Fig. S1 in Supplemental Material (SM)). The alga suspension was constantly stirred in the reservoir during measurements in order to ensure a homogeneous distribution and illumination. Time-resolved fluorescence decays were measured at several wavelengths under three different conditions: 1) unquenched dark-adapted cells with open PSII reaction centres (RCs) (F_0). The pumping speed was around 500 ml min^{-1}. 2) Unquenched dark-adapted cells with PSII RCs closed by addition of 100 μM DCMU (F_{max}). The pumping speed was around 50 ml min^{-1}. To ensure that the RCs are closed, low light (100 μmol photons m^{-2} s^{-1} for 1 s) was applied just before the algal suspension entered the measuring cuvette. 3) Quenched light-adapted cells with closed PSII RCs (F_{NPQ}). Quenching was induced by irradiation of the reservoirs with an intensity of ~600 μmol photons m^{-2} s^{-1}. Closure of the PSII RCs was achieved by applying additional strong light of ~1000 μmol photons m^{-2} s^{-1} focused onto the flowing suspension immediately before it entered the measuring laser beam. The cells were subjected to this additional light for at least 200 ms. The intensity was sufficient to saturate the induced variable fluorescence. The pumping speed was the same as for measuring F_0. Cells were light-adapted for at least 30 min prior to data recording in order to reach a steady-state quenched fluorescence signal. One measurement took two to 3 h in order to achieve desired signal-to-noise ratio and high spectral resolution. In order to test if the physiological state of the cells remained unchanged during the light treatment, HPLC and oxygen measurements were carried out on the samples. The fluorescence decays were analysed by global target analysis [30]. Fluorescence induction measurements were performed independently on cultures taken from their growth conditions to observe the induction of NPQ.

For *in vitro* measurements of the fluorescence kinetics, the isolated FCPs were diluted to $A_{660} = 0.3$ with isolation buffer supplemented by an oxygen-scavenging system (65 μg/ml glucose-oxidase (SIGMA), 65 μg catalase (SIGMA), 1.3 mg/ml glucose) and 0.03% μ-dodecylmaltoside. For aggregated FCP the detergent was omitted. The sample was placed in a 1 mm cuvette, which was translated during the measurement to avoid bleaching of the illuminated small volume by the laser beam.

2.3. Fluorescence induction measurements

Fluorescence induction kinetics of variable Chl fluorescence and the development of NPQ were monitored with a PAM fluorometer (Walz GmbH, Effeltrich) on cells adjusted to a Chl *a* content of 2 μg ml^{-1}. Saturating light flashes (3500 μmol photons m^{-2} s^{-1}) with a duration of 800 ms were applied every 10 s. The cells were dark-adapted for 2 min before F_{max} was determined.