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Acetate in mixotrophic growth medium affects photosystem II in *Chlamydomonas reinhardtii* and protects against photoinhibition

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ABSTRACT

Chlamydomonas reinhardtii is a photoautotrophic green alga, which can be grown mixotrophically in acetate-supplemented media (Tris–acetate–phosphate). We show that acetate has a direct effect on photosystem II (PSII). As a consequence, Tris–acetate–phosphate-grown mixotrophic *C. reinhardtii* cultures are less susceptible to photoinhibition than photoautotrophic cultures when subjected to high light. Spin-trapping electron paramagnetic resonance spectroscopy showed that thylakoids from mixotrophic *C. reinhardtii* produced less $^1O_2$ than those from photoautotrophic cultures. The same was observed in vivo by measuring DanePy oxalate fluorescence quenching. Photoinhibition can be induced by the production of $^1O_2$ originating from charge recombination events in photosystem II, which are governed by the midpoint potentials (E_m) of the quinone electron acceptors. Thermoluminescence indicated that the E_m of the primary quinone acceptor (Q_A/Q_A^-) of mixotrophic cells was stabilised while the E_m of the secondary quinone acceptor (Q_B/Q_B^-) was destabilised, therefore favouring direct non-radiative charge recombination events that do not lead to $^1O_2$ production. Acetate treatment of photosystem II-enriched membrane fragments from spinach led to the same thermoluminescence shifts as observed in *C. reinhardtii*, showing that acetate exhibits a direct effect on photosystem II independent from the metabolic state of a cell. A change in the environment of the non-heme iron of acetate-treated photosystem II particles was detected by low temperature electron paramagnetic resonance spectroscopy. We hypothesise that acetate replaces the bicarbonate associated to the non-heme iron and changes the environment of Q_A and Q_B affecting photosystem II charge recombination events and photoinhibition.

1. Introduction

The green alga *Chlamydomonas reinhardtii* is a model organism that has been used to establish many fundamental aspects of photosynthesis, including responses to high light (e.g. [1–3]). *C. reinhardtii* is a photoautotrophic organism that can be grown mixotrophically in a Tris–acetate–phosphate (TAP) media. Acetate can be metabolised to triose by an ATP-dependent entry into the glyoxylate or citric acid cycle to produce reducing equivalents [4], which can be used by the NAD(P)H dehydrogenase to reduce the plastoquinone pool [5]. The movement of light harvesting complex II (LHCII) between PSII and PSI, the so-called state transitions, is under governance of the redox state of the plastoquinone pool [6,7]. Hence, the addition of acetate to a dark-adapted photoautotrophic culture induces a transient migration of LHCII from PSII to PSI [8], until cells adapt to the new metabolic state and the redox of the plastoquinone pool readjusts back. Furthermore, the ratio of NADPH and ATP produced from photosynthesis can be modulated under different metabolic conditions by switching between linear and cyclic electron flows. Linear electron flow produces a fixed ratio of NADPH:ATP, whereas cyclic flow around PSI enhances ATP synthesis without reducing $NADP^+$ [9,10].

Beside changes in the metabolic state of the cell and the reduction state of the photosynthetic electron transport chain, acetate in TAP media may also directly affect photosystem II (PSII). It was reported that net photosynthesis was reduced in TAP-grown *C. reinhardtii* compared to photoautrophic cultures, even in the presence of 5% CO_2 [11]. Small carboxylate anions, like formate and acetate, can substitute the bicarbonate/carbonate associated to PSII [12–14]. The non-heme iron at the acceptor side of PSII is coordinated by four

Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-1,4-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; E_m, midpoint redox potential; Fm, maximum chlorophyll fluorescence signal; Fv, variable chlorophyll fluorescence signal; HSM, high salt medium; LHC, light harvesting complex; P_{680}, primary electron donor in PSII; Pheo, pheophytin, primary electron acceptor in PSII; PS, photosystem; Q_A, the primary quinone acceptor of PSII; Q_B, the secondary quinone acceptor of PSII; $S_{2/3}$, oxidation states of the oxygen evolving complex of PSII; TAP, Tris–acetate–phosphate; TEMPD, 2,2,6,6-tetramethyl-4-piperidone; TL, thermoluminescence, T_m TL emission maximum; Y_z, redox active tyrosine residue of PSII

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histidine residues and an exchangeable bidentate ligand to bicarbonate [15]. Low temperature electron paramagnetic resonance (EPR) measurements have shown that small carboxylate anions influence the environment of the non-heme iron [16,17]. Carboxylate anions do not only affect the acceptor side of PSII, but also the donor side. Binding of acetate close to the Mn_4Ca cluster was reported to occur in competition with chloride, which is an obligatory cofactor for an active water-splitting complex [18]. As a consequence, the reduction kinetics of the redox active tyrosine residue (Y_Z) may be slowed down [19].

Acetate-induced changes of PSII photochemistry may substantially influence the effect of high light exposure on *C. reinhardtii*. Carboxylate anions affect the midpoint potential (E_m) of the non-heme iron [20] and the electron transfer rates between the primary quinone acceptor Q_A and the secondary quinone acceptor Q_B [13]. Changes in the E_m of the iron are expected to induce changes of the E_m of the quinone acceptors since all three are connected via the histidine ligands. It is known that binding of different herbicides to the Q_B-binding pocket shifts the E_m of the redox couple (Q_A/Q_A^-), thereby affecting the charge recombination reaction between P_{680}^+ (the oxidised primary donor) and Q_A^- [21,22]. When the midpoint potential of Q_A is shifted to a more negative value, the probability of charge recombination via the primary radical pair ($P_{680}^+Pheo^-$) is increased. This enhances the formation of the triplet state of P_{680} ($^3P_{680}$) that can react with $^3O_2$ (O_2 in its ground state) to the highly toxic singlet oxygen ($^1O_2$). When the midpoint potential of Q_A is shifted to a less negative value, the probability of a direct recombination of $P_{680}^+Q_A^-$ to the ground state is increased and the yield of $^3P_{680}$ and $^1O_2$ generation is lowered [21,23]. Therefore, we propose that acetate may influence the energetics of the acceptor side of PSII, which influences the susceptibility of TAP-grown *C. reinhardtii* to high light-induced $^1O_2$-mediated damage (photoinhibition).

In order to address this question, we used a combined in vivo and in vitro approach using photoautotrophic and mixotrophic (TAP-grown) *C. reinhardtii* cells, and PSII-enriched membrane fragments from spinach. We used low temperature EPR spectroscopy to demonstrate that the environment of the non-heme iron was indeed affected by acetate treatment of PSII-enriched membrane fragments. Thermoluminescence (TL) curves showed that Q_A^- of TAP-grown *C. reinhardtii* or acetate-treated PSII from spinach was stabilised, indicating that safe direct charge recombination events are more likely to happen in the presence of acetate. TAP-grown *C. reinhardtii* generated less $^1O_2$ than photoautotrophically grown *C. reinhardtii*, as observed in thylakoids with spin-trapping EPR and in vivo by quenching of the fluorescence of DanePy oxalate. Ultimately, less inhibition of PSII activity observed in mixotrophically grown cultures exposed to light stress can be attributed to the influence of acetate on the acceptor side of PSII affecting charge recombination events.

2. Materials and methods

2.1. Material

Cell wall-less green algae *C. reinhardtii* (strain D66) were grown in either acetate-free medium (HSM) or acetate-supplemented medium (TAP) containing 17.5 mM acetate, by agitation under continuous illumination at 50 μmol quanta m^{-2} s^{-1}. Cultures were kept in the exponential growth phase below 5×10^6 cells ml^{-1}. All measurements were performed with cells in the exponential growth phase.

Thylakoids were isolated from mixotrophic and photoautotrophic *C. reinhardtii* cells by two consecutive freezing and thawing cycles in liquid nitrogen. Thylakoids were collected by centrifugation at 5000 × g for 5 min and resuspended to 30 μg chl ml^{-1} in 0.3 M sorbitol, 50 mM KCl, 1 mM $MgCl_2$, 25 mM HEPES at pH 7.6, with or without 17.5 mM acetate, respectively.

PSII-enriched membrane fragments (PSII particles) were isolated from spinach according to [24]. The presence of Q_B in this preparation was verified by thermoluminescence. For thermoluminescence the PSII particles, at a concentration of 100 μg chl ml^{-1}, were suspended in 0.3 M sucrose, 10 mM NaCl, 20 mM MES/NaOH at pH 6.5, for 2 h at 10 °C in the presence or absence of 40 mM acetate.

2.2. Measurements of photosynthetic O_2 evolution

The measurement of O_2 evolution was performed in a Liquid-Phase Oxygen Electrode Chamber (Hansatech Instruments, Norfolk, England). *C. reinhardtii* cultures at a concentration of 5–7 μg chl ml^{-1} were illuminated at various light intensities in the presence of 1 mM $NaHCO_3$ or 0.5 mM 2.6-dichloro-1,4-benzoquinone (DCBQ) and 10 μM nigericin.

2.3. Chlorophyll and NADPH fluorescence

Room temperature chlorophyll and NADPH fluorescence were measured simultaneously using a pulse-amplitude modulation fluorometer (DUAL-PAM, Walz, Effeltrich, Germany). NADPH fluorescence was measured by the photomultiplier unit DUAL-DNADPH/ENADPH, at excitation 365 nm; emission 420–580 nm. Red light at 635 nm was used for actinic light. All cultures were dark-adapted for 10–15 min prior to the measurement to allow most of the reversible quenching to relax.

Chlorophyll fluorescence at 77 K was performed with a CARY Eclipse fluorescence spectrophotometer (Varian) at an excitation of 440 nm (5 nm slit width for both monochromators) using samples at a chlorophyll content of 10 μg chl ml^{-1}.

2.4. Thermoluminescence

Thermoluminescence (TL) was measured with a homebuilt apparatus [25]. TL was charged by single turnover flashes with a xenon flash lamp at −5 °C in the presence of 10 μM DCMU for the Q-band ($S_2Q_A^-$ recombination) and at 1 °C without DCMU for the B-band ($S_{2/3}Q_B^-$ recombination). The TL signal was recorded during warming to 70 °C at a heating rate of 0.4 °C s^{-1}. The material was either previously frozen in liquid nitrogen or incubated with 10 μM nigericin for 10 min, as indicated in the figure legends. Both treatments dissipate the proton-motive force that can influence the maximum temperature of the TL bands. Cells were resuspended in fresh media prior to the measurements. The B-band and Q-band were measured with a chlorophyll concentration of 40 μg chl ml^{-1} and 100 μg chl ml^{-1}, respectively. Data analysis was performed according to [26].

2.5. Electron paramagnetic resonance spectroscopy

We used PSII particles to determine the acetate-induced change in the environment of the non-heme iron. Treatment of PSII particles was conducted according to [14] by gently agitating samples in 400 mM mannitol, 20 mM $CaCl_2$, 10 mM $MgCl_2$, and 50 mM MES/NaOH at pH 6.5, for 2 h at 10 °C in the presence or absence of 40 mM acetate. Spectra were recorded using a Bruker Elexsys 500 X-band spectrometer (Bruker, Rheinstetten, Germany) equipped with standard ER 4102 resonator and Oxford Instruments ESR 900 cryostat. Instrument settings were as follows: microwave frequency at 9.4 GHz, modulation frequency at 100 kHz. All stages were completed in the dark. 120 μl of PSII particles (4 mg chl ml^{-1}) was loaded into 4 mm outer diameter quartz EPR tubes and oxidised with 2 mM $K_3[Fe(CN)_6]$ added directly to the EPR tube and incubated for 1 h on ice in the dark. The samples were then frozen in a dry-ice/ethanol bath at 200 K, degassed and then filled with helium gas. EPR tubes were then transferred to liquid nitrogen. Low-temperature red-light illuminations were performed for 20 s in a dry-ice/ethanol bath at 200 K.