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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.

Singlet oxygen was trapped using the water soluble spin-probe 2,2,6,6-tetramethyl-4-piperidone (TEMPD) hydrochloride [27] and measured with a Bruker e-scan™ (Bruker Biospin, Rheinstetten, Germany). Thylakoids (30 μg chl ml^{-1}) were incubated 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. 2 ml of the sample was exposed for 45 min to 250 μmol quanta m^{-2} s^{-1} of red (630 nm) LED light at 20 °C in the presence of 0.1 M TEMPD and 100 μM bromoxynil. Bromoxynil lowers the E_m of $Q_A/Q_A^{\bullet -}$ by 45 mV, thereby favouring charge recombination that leads to $^1O_2$ production [21]. Spectra were recorded using a flat cell containing 100 μl sample. The microwave power was 9.77 GHz and 14.07 mW with a modulation frequency of 86 kHz and amplitude of 1.01 G. Each spectrum is an average of 20 scans each with a sweep time of 10.5 s.

2.6. DanePy fluorescence

$^1O_2$ was detected in vivo using the hydrophilic oxalate salt of the $^1O_2$ trap 3-[N-(b-diethyl-aminoethyl)-N-dansyl]aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole (DanePy). It has been demonstrated previously that this dye enters into *Chlamydomonas* cells and stays in the cytoplasm [28]. DanePy oxalate was used at a concentration of 125 μM and the cells at a chl concentration of 20 μg ml^{-1}. Cells were illuminated for up to 15 min with white light (1000 μmol $quantam^{-2}s^{-1}$). DanePy fluorescence was measured using a CARY Eclipse fluorescence spectrophotometer (Varian); excitation at 330 nm (5 nm slit width).

2.7. Photoinhibition

Photoinhibition was induced by gently agitating samples under illumination (250 μmol quanta m^{-2} s^{-1}) provided by a compact fluorescent lamp in the presence of 5 mM lincomycin. Cultures were kept at constant temperature (21 °C) with fan-assisted cooling. The maximum quantum yield of PSII (Fv/Fm) was assayed by calculating the ratio of variable fluorescence, Fv, to maximal fluorescence, Fm, after 10 min dark adaptation.

2.8. Statistics

The data represent means or representative examples from measurements repeated 4–3 times. Standard errors are shown in Fig. 1, Fig. 2, Fig. 5, and Table 1.

3. Results

When *C. reinhardtii* cells grown at 50 μmol quanta m^{-2} s^{-1} were exposed to 250 μmol quanta m^{-2} s^{-1}, they suffered from photoinhibition.

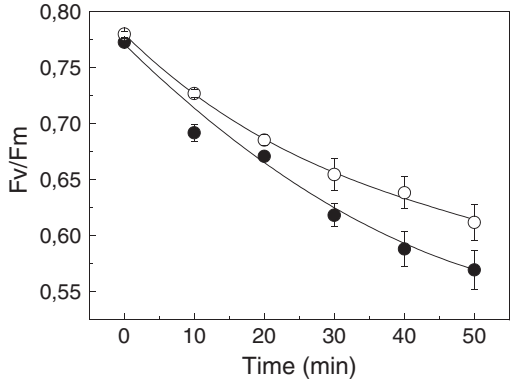

Fig. 1. Photoinhibition of PSII from *C. reinhardtii* during illumination at 250 μmol quanta m^{-2} s^{-1} in the presence of 5 mM lincomycin. PSII activity is represented by Fv/Fm of photoautotrophic (closed symbols) and mixotrophic (open symbols) cultures.

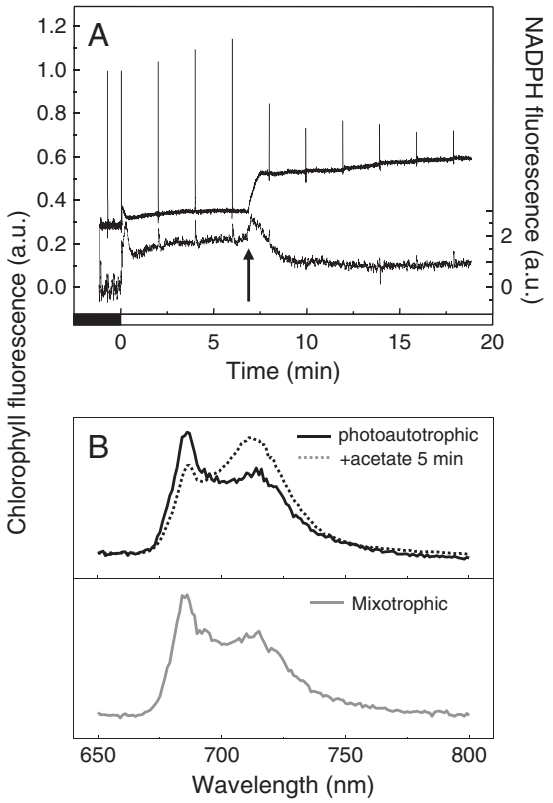

Fig. 2. Effect of acetate addition to photoautotrophic cells. [A] Chlorophyll and NADPH fluorescence before and after the addition of 17.5 mM acetate, as represented by the arrow. The actinic light at time 0 was at 70 μmol quanta m^{-2} s^{-1}. The upper trace shows chlorophyll fluorescence and the lower trace NADPH fluorescence. [B] 77 K chlorophyll fluorescence emission spectra of photoautotrophic cells before (solid line) and 5 min after (dotted line) the addition of 17.5 mM acetate (top), and of mixotrophic cells (bottom).

As shown in Fig. 1, the maximum quantum yield of PSII (Fv/Fm) decreased faster in photoautotrophically than in mixotrophically grown cultures. The same phenomenon was observed when O_2-evolution was measured instead of chlorophyll fluorescence. After 1 h of photoinhibition, O_2-evolution dropped to 80 ± 5% and 95 ± 2% of the controls for photoautotrophic and mixotrophic cultures, respectively (SI Fig. 1). These experiments were performed in the presence of protein synthesis inhibitor lincomycin to block synthesis of the D1 protein. The difference in the light-susceptibility of the two cultures may be due to several reasons: 1) differences in the efficiency of light absorption and excitation transfer to the PSII reaction centre; 2) differences in the reduction state of the photosynthetic electron transport chain; or 3) differences in the reaction centre itself caused by acetate. To investigate whether the lower susceptibility to light of mixotrophically grown cells was caused by difference in state transitions or in the reduction state of the chloroplast, we investigated changes in chlorophyll fluorescence at

Table 1
Maximum temperature of the Q-band in the presence of 10 μM DCMU in different samples, n = 3–4 ± SE. When indicated, PSII particles had been treated with 40 mM acetate.

Material	Q-band temperature (°C)		B-band temperature (°C)	
	Control	+Acetate	Control	+Acetate
Spinach PSII particles	6.5 ± 0.3	8.7 ± 1.0	28.6 ± 0.7	23.1 ± 0.3
	HSM	TAP	HSM	TAP
C. reinhardtii (+ nigericin)	3.6 ± 0.8	9.5 ± 1.0	32.0 ± 1.4	30.6 ± 1.7
C. reinhardtii (frozen)	4.5 ± 0.7	12.5 ± 0.8	–	–

room temperature and at 77 K. Furthermore, the NADPH fluorescence was measured to monitor the metabolic state of the chloroplast. Following the addition of acetate to a photoautotrophic culture the NADPH fluorescence transiently increased before falling below the level observed prior to the addition of acetate. This indicates that large changes in the metabolism of the chloroplast do occur upon acetate addition. At the same time, the level of variable chlorophyll fluorescence increased rapidly and the Fm dropped, indicating state transitions induced by an over reduced PQ pool (Fig. 2A), as also observed with 77 K chlorophyll fluorescence. Upon acetate addition, the fluorescence at 715 nm largely increased while the fluorescence at 685 nm decreased, showing the association of the mobile antenna with photosystem I (Fig. 2B). However, when comparing fully adapted mixotrophic cultures to photoautotrophic cultures, no major differences were seen in chlorophyll fluorescence at 77 K (Fig. 2B), at room temperature (Fig. 3), and in NADPH fluorescence over 25 min illumination when the cells were exposed to three non-saturating light intensities (Fig. 3). Therefore, we exclude that there are any alterations in the antenna composition or the redox state of the chloroplast in fully adapted cultures that may protect mixotrophic cultures against photoinhibition. Nonetheless, differences in the O_2-evolution activity (Fig. 4) indicated that acetate, even in fully adapted mixotrophic cultures, directly affects photosynthetic electron transport. The mixotrophic culture produced slightly less O_2. To test whether this difference was caused by a difference in PSII activity, O_2 evolution was measured in the presence of the artificial electron acceptor DCBQ and the uncoupler nigericin. In the presence of DCBQ, O_2 evolution at 250 μmol quanta m^{-2} s^{-1} increased to 133 ± 21 and 199 ± 31 μmol mg chl^{-1} h^{-1} for mixotrophic and photoautotrophic cultures, respectively, showing that the activity of PSII was altered in the presence of acetate.

Photoinhibition can be triggered by $^1O_2$ generation in the PSII reaction centre. Two methods were employed to detect differences in $^1O_2$ production between mixotrophic and photoautotrophic cells;

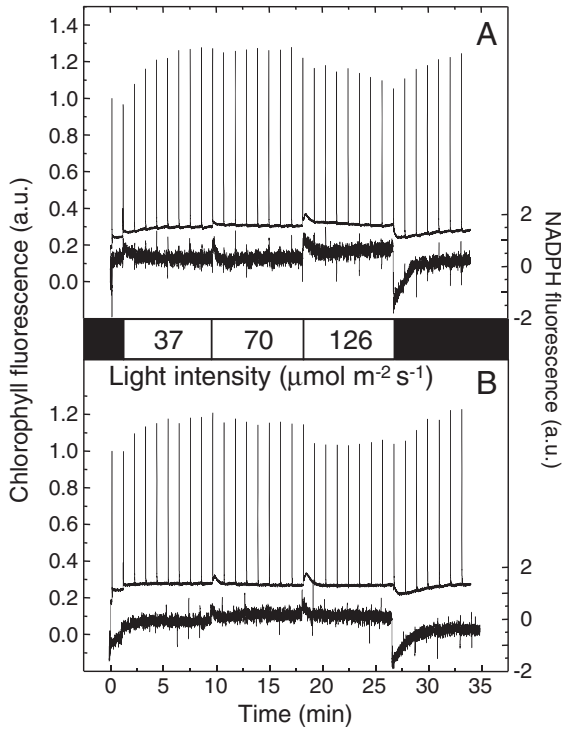

Fig. 3. Chlorophyll and NADPH fluorescence traces of *C. reinhardtii*, as represented in the same figure by the upper and lower traces respectively. The response of photoautotrophic [A] and mixotrophic [B] cultures to various light intensities (white bar) and after the actinic light is switched off (black bar). The cultures were concentrated to a chlorophyll concentration of 35 μg ml^{-1} and measured in the presence of 1 mM $NaHCO_3$ dark adapted for 10 min before measurement.

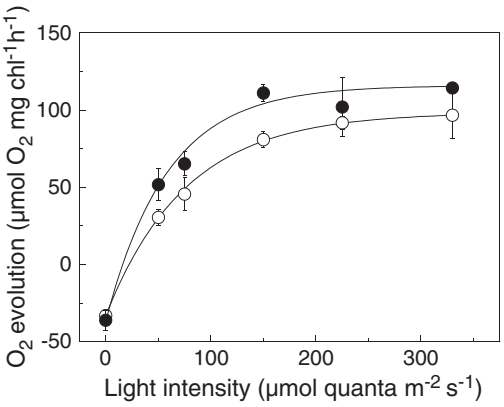

Fig. 4. Oxygen evolution by photoautotrophic (closed symbols) and mixotrophic (open symbols) *C. reinhardtii* cultures at various light intensities. Measurements were taken over 5 min in the presence of 1 mM $NaHCO_3$, n = 3–4 ± SE.

EPR spectroscopy with the spin probe TEMPD and the fluorescent dye DanePy-oxalate. While the DanePy assay could be performed in vivo, the spin probe TEMPD does not enter into intact cells and the measurements had to be performed on thylakoids. To generate a higher yield of $^1O_2$, the phenolic herbicide bromoxynil that binds to the Q_B-binding pocket was added for the TEMPD assay. Following high light treatment, the TEMPD signal of thylakoids from photoautotrophic cultures was significantly bigger than that from mixotrophic cultures (Fig. 5A) showing mixotrophic cultures were producing less light-induced $^1O_2$. Averaging the double integral of the total signal size showed that mixotrophic cells produced 66 ± 2% the amount of $^1O_2$ of photoautotrophic cells (100 ± 12%, n = 3 ± SE). To show that acetate neither perturbed the spin probe nor quenched $^1O_2$, we investigated the effect of 17.5 mM acetate on $^1O_2$ production by the photosensitizer methylene blue. The same TEMPD signal size was obtained when methylene blue was illuminated in the presence or absence of acetate (data not shown). Confirmation of an acetate-induced prevention of $^1O_2$ production was gained with the DanePy fluorescence assay. The fluorescence was quenched by about 10% in high-light treated photoautotrophic cells, whereas it barely changed in high-light treated mixotrophic cells (Fig. 5B).

The question arose as to whether TAP-grown cells produce less $^1O_2$ because of an acetate-induced change at the acceptor side of PSII. Acetate may affect the midpoint potential (E_m) of the redox couples Q_A/Q_A^- and Q_B/Q_B^-. A simple way to test for such alterations is thermoluminescence (TL) originating from the $S_2Q_A^-$ or $S_{2,3}Q_B^-$ charge recombination with $S_{2,3}$ being oxidation states of the water-splitting complex [29]. TL can be used as a probe of the behaviour of PSII reaction centres both in isolated systems and in whole *C. reinhardtii* cells [30,31].

Measurements in the presence of DCMU, a herbicide which binds to the Q_B binding pocket and blocks the electron transfer from Q_A^- to Q_B, revealed maximum TL emission (T_m) of the $S_2Q_A^-$ charge recombination (Q-band) to be 3.6–4.8 °C for photoautotrophic cultures and 9.5–12.5 °C for mixotrophic cultures (Fig. 6; Table 1). During mixotrophic growth the concentration of acetate decreases due to metabolism by the cells. Accordingly, saturated TAP grown cultures gave a TL curve with a lower T_m than TAP-grown cultures in the exponential phase (SI Fig. 2A). Changing the media of cells from TAP to acetate free media led to a shift in the T_m typical of autotrophic cells within 3 h (SI Fig. 2B). The higher T_m of mixotrophic cultures indicates a stabilisation of the charge pair $S_2Q_A^-$. This can either be caused by a less negative E_m of Q_A/Q_A^- or by a change of the E_m of the S_2 state. As the effect was observed in frozen as well as nigericin-treated samples, any differences between the cultures in the proton gradient or the electric field that may influence the recombination event can be discarded. Furthermore, cells had been resuspended in fresh media

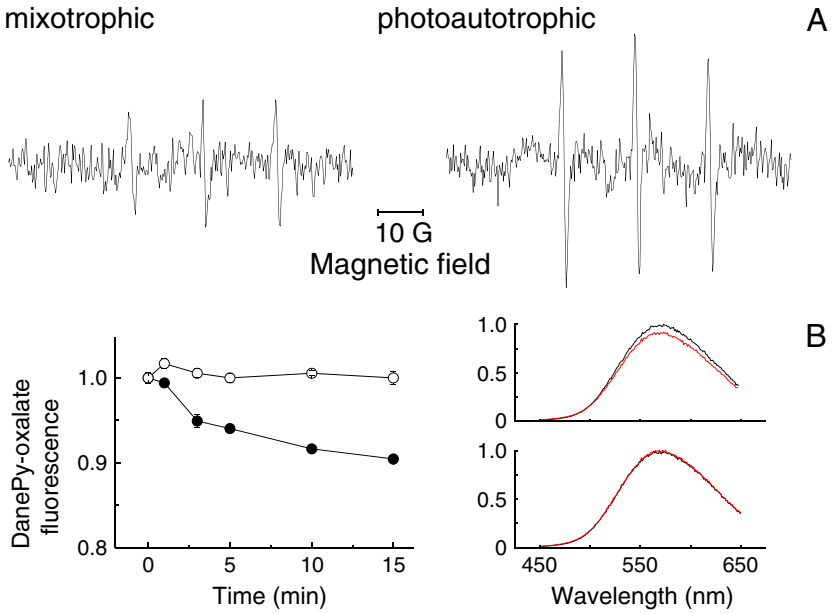

Fig. 5. Production of $^1O_2$ as measured by [A] spin-trapping EPR spectroscopy with TEMPD in thylakoids extracted from photoautotrophic or mixotrophic *C. reinhardtii* cultures, re-suspended in fresh media with acetate in case of thylakoids from mixotrophic culture and by [B] DanePy-oxalate fluorescence quenching in vivo. [A] Samples were illuminated in the presence of 100 μM bromoxynil with red light at 635 nm and 270 μmol quanta m^{-2} s^{-1} for 45 min; shown is the average of 3 measurements. [B] Samples were illuminated with white light (1000 μmol quanta m^{-2} s^{-1}) for up to 15 min in the absence of bromoxynil. The left figure shows the decrease in the fluorescence maximum at 570 nm of mixoautotrophic cultures (open symbols) and photoautotrophic cells (closed symbols). n = 3 ± SE. The right figure shows typical fluorescence traces of 0 (black) and 15 min (red) high light treated mixotrophic cultures (upper figure) or photoautotrophic cultures (lower figure).

at pH 7.0 so that differences in the pH of the medium can also be ruled out. In PSII particles from spinach, the same effect was observed: Treatment of the sample with acetate shifted the Q-band towards a higher temperature although the difference in T_m was smaller than observed for *C. reinhardtii* cells (Table 1).

Next, the effect of acetate on the $S_{2/3}Q_B^-$ recombination (B-band) with T_m values between 16 and 32 °C was investigated. The B-band is made of two components, the B1 band originating from $S_3Q_B^-$ recombination with a T_m at a lower temperature (16–21 °C in this study) and the B2 band originating from $S_2Q_B^-$ with a T_m at a higher temperature (26–32 °C in this study). As shown in Fig. 7A–C, mixotrophic cultures emitted luminescence at a lower temperature than photoautotrophic cultures. In addition, the intensity of the luminescence was slightly lower. The differences in T_m between mixotrophic and photoautotrophic growth were 3.4 ± 1.1 °C for the B1 band and 3.3 ± 1.1 °C for the B2 band when the T_m of both cultures for each number of saturating flashes (1–5) was compared. The overall pattern of the B-band TL intensity following 1–5 single turnover flashes was very similar for both mixotrophic and photoautotrophic cultures, although the oscillation pattern was slightly damped in mixotrophically grown cells (Fig. 7D). This can be explained by a small increase in the miss factors induced by acetate [14]. When photoautotrophic cells were placed in TAP media, rapid changes in T_m of the B-band and Q-band occurred that fully changed to mixotrophic values within 2 h (Fig. 8). The same effect of acetate was seen in isolated PSII-enriched membrane fragments from spinach. Treating dark-adapted PSII-enriched membrane fragments with acetate for 2 h led to a drop in the T_m of the B-band from 28.6 ± 0.7 °C to 23.1 ± 0.3 °C when TL was excited a 1 single turnover flash (Table 1).

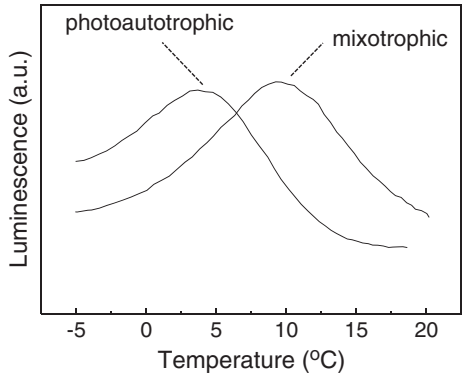

Fig. 6. Thermoluminescence glow curves of the Q-band of photoautotrophic and mixotrophic *C. reinhardtii* cultures in the presence of 10 μM DCMU. All samples were frozen before measurements and excited by a single turnover flash at −5 °C.

Low temperature EPR spectroscopy was employed to see any effects of acetate upon the non-heme iron at the acceptor side of PSII. The non-heme iron signal is best detectable in isolated and relatively pure samples of PSII-enriched membrane fragments, because in whole cells the signal is too small to be detectable. EPR spectra recorded at 8.5 K showed that the environment of the non-heme iron in PSII-enriched membrane fragments was modulated by acetate, with the most evident shift occurring at 1200 G (Fig. 9). The signal was not contaminated from overlapping g-values of the iron in the low spin state of Cyt b559 because it has no signal at 1200 G [32]. The main signals of Cyt b559 are at 2200 G and 3100 G (not shown in Fig. 9). The shape of the multiline signal, which reflects the S_2 state of the Mn_4Ca cluster, was unchanged in the presence of acetate (data not shown). This indicates that acetate did not exert an effect on the donor site in our conditions. Since the acetate-induced TL changes in *C. reinhardtii* cells were comparable to those in PSII-enriched membrane fragments, we assume that acetate also modifies the environment of the non-heme iron in *C. reinhardtii* in a similar way.

4. Discussion

In this paper we show that the acetate in TAP medium protects mixotrophic *C. reinhardtii* from photoinhibition by modulating PSII charge recombination reactions that lead to $^1O_2$ production. These findings provide an alternative explanation to the effects of acetate

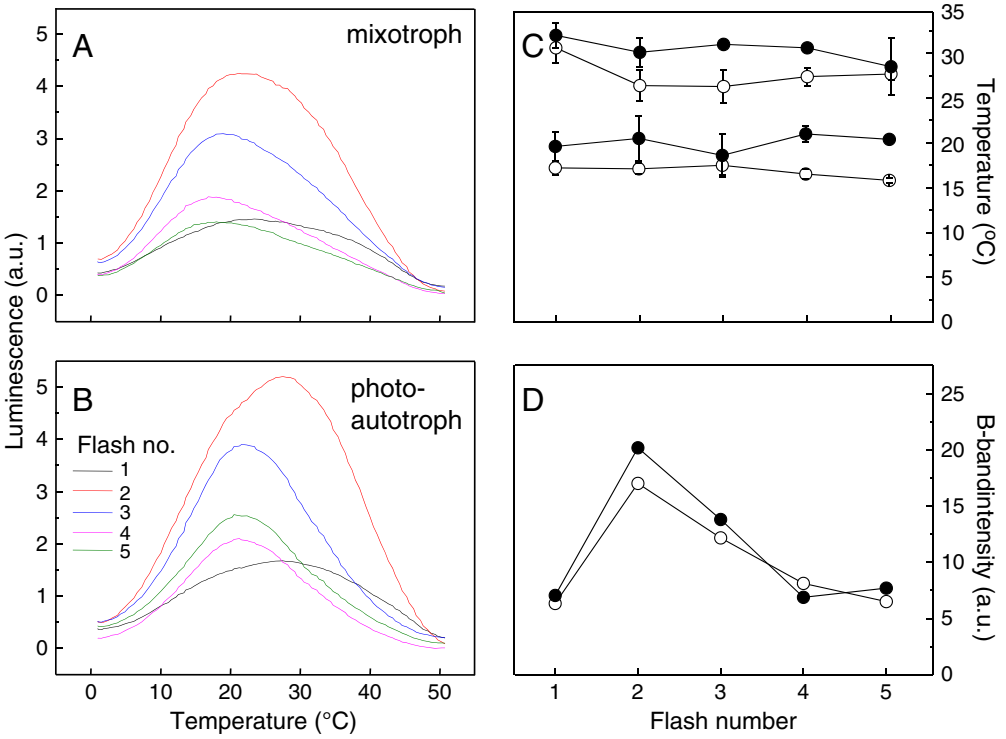

Fig. 7. Flash dependency of thermoluminescence measurements of the B-band. Thermoluminescence glow curve intensities of [A] mixotrophic and [B] photoautotrophic *C. reinhardtii* cultures following 1–5 single turnover flashes. Shown is the average of 3 measurements. [C] Dependence of the maximum temperatures of the B1- and B2- bands on the number of single turnover flashes. [D] Dependency of the total intensity of the B-band (B1 + B2) on the number of single turnover flashes. For C and D open symbols represent mixotrophic cells and closed symbols photoautotrophic cells. All cultures were previously incubated for 10 min in the presence of 10 μM nigericin in the dark before measurements.

on photosynthesis, separate to the previously published effects on chlorophyll fluorescence following the application of acetate to a photoautotrophic culture [8]. Similar to [8] we also observed rapid acetate-induced changes in room temperature and 77 K chlorophyll fluorescence, as well as in NADPH fluorescence, which taken together showed a severe reduction in the plastoquinone pool (Fig. 2). However, the same fluorescence measurements revealed that this particular 'acetate effect' was not maintained in fully adapted mixotrophic cultures. Hence, we found that the plastoquinone pool was not over-reduced after long-term growth in mixotrophic conditions. However, when photosynthetic activity was measured by following O_2-evolution in the presence of $NaHCO_3$ or DCBQ, mixotrophic cells produced slightly less O_2 than photoautotrophic cultures (Fig. 4), confirming data that has previously been published [11]. It was apparent that other aspects of PSII photochemistry had been altered by acetate, which we here show to have significantly modulated the response to high light. The yield of $^1O_2$ production was significantly lowered in TAP-grown cells (Fig. 5B) and thylakoids from mixotrophically grown cultures (Fig. 5A). $^1O_2$ can damage PSII leading to photoinhibition [33], as well as oxidise nearby membrane lipids [34] that can initiate signalling pathways for high light acclimation [2,35] or cell death [36,37]. Lipid peroxides are reactive and potentially damaging molecules that are broken down by glutathione peroxidases (GPX). The transcription of the homologue of glutathione peroxidase in *C. reinhardtii* (GPXH) is induced by high light and correlates to the amount of $^1O_2$ production [1]. A previous study comparing the high

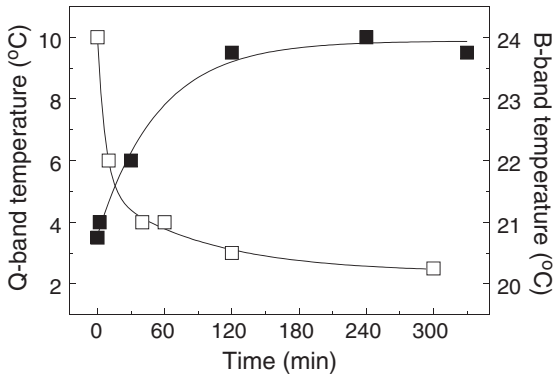

Fig. 8. Changes of the temperature of the thermoluminescence maximum of the $S_2Q_A^-$ (Q-band; black symbols) and the $S_{2/3}Q_B^-$ (B-band; white symbols) charge recombination event following the re-suspension of photoautotrophic *C. reinhardtii* cells into TAP media. Following the media change, cultures were placed back under growth light for the indicated time and frozen in liquid nitrogen. Before measurements, cells were thawed and dark adapted for 10 min. The Q-band was measured in the presence of 10 μM DCMU.

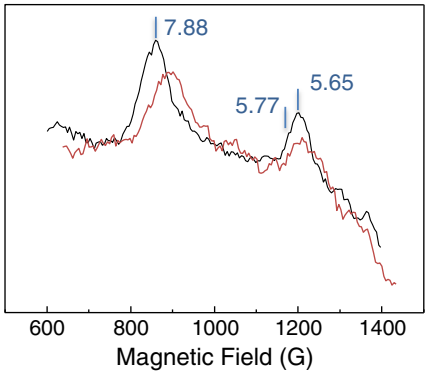

Fig. 9. X-band EPR spectra at 8.5 K of the non-heme iron of PSII following photoreduction at 200 K, in the presence (red) or absence (black) of 40 mM acetate. Before illumination the non-heme iron was oxidised by 2 mM ferricyanide. Each spectrum is the difference between the signal before and after 20 s of illumination. The g-values of the EPR signal are indicated in blue.

light response of mixotrophic and photoautotrophic *C. reinhardtii* cultures showed, on the one hand that the induction of GPXH expression was greater in mixotrophic cultures, while on the other hand that there was no difference in respect to photoinhibition between the cultures [38]. Both the results are inconclusive as to whether mixotrophic or photoautotrophic cultures produced more $^1O_2$. The very high light intensity (2500 μmol quanta m^{-2} s^{-1}) used [38] is 20 fold the growth light intensity and 10 fold more than the light intensity we used for measurements of photoinhibition (Fig. 1) and $^1O_2$ (Fig. 5). It has been argued that very high light intensities lead to such a severe over-reduction of PSII that subtle redox changes which influence $^1O_2$ production become overrun [39].

Thermoluminescence measurements showed a significant effect of acetate on the maximum temperatures of the Q-band and the B-band in vitro in PSII-enriched membrane fragments and in TAP-grown *C. reinhardtii* (Figs. 6–8, Table 1, SI Fig. 2). The Q-band was shifted by acetate to a higher temperature while the B-band was shifted to a lower temperature. We assign these shifts to a modification of the acceptor side since the EPR signal showed a modification of the environment of the non-heme iron (Fig. 9), while the multiline signal, indicative for the S_2 state of the water-splitting complex, was unchanged. Furthermore, the disassociation constant of Cl^- from the water-splitting complex is about 30 times lower than the disassociation constant of acetate [18]. Hence, TAP media that typically contains 7.7 mM Cl^- would protect the water-splitting complex from being affected by acetate at a concentration of 17.5 mM. In vitro acetate treatment was performed using a buffer that contained 10 mM (TL) or 30 mM (EPR) Cl^- so that also under these conditions, an out-competition of Cl^- by acetate at the water splitting complex can be neglected.

Carboxylates induce a significant shift in the E_m of the non-heme iron [20]. The ferrous state of the non-heme iron in PSII can be oxidised at an E_m of +400 mV [40], but if this occurs during electron transfer from Q_A^- to Q_B has been disputed [41]. X-ray absorption spectroscopy has recently provided further evidence that a temporary structural change actually occurs rather than an oxidation of the non-heme iron [42]. It was proposed that during electron transfer the bidendate ligand from the bicarbonate reversibly switches to a monodendate ligand, while the charge on the Q_B^- is stabilised by protonation of nearby amino acid residues. The similarity of carboxylates, such as acetate, allows substitution of the bicarbonate but is unlikely to provide the exact bidentate ligand. This infers that the modified activity of the non-heme iron and its influence upon Q_A and Q_B may be attributed to differences in polarity between the molecules that affect ligand formation. Therefore, different effects on the TL bands are to be expected when bicarbonate is replaced by different carboxylates. Effects of small carboxylate anions like formate on thermoluminescence signals have been described previously [43]. In this previous study, the B-band has been shifted after addition of formate towards a higher temperature opposite to the effect of acetate observed here. This discrepancy can either be due to the different nature of the carboxylate anion or to different treatment of the samples (for a detailed discussion see [17]).

It has been reported previously that mixotrophic growth lowers the T_m of the B-band [44,45]. Ducruet and co-workers [45] explained the downshift of the T_m by the presence of a larger proton gradient since it is known that the T_m is pH-dependent. However, we also see this downshift in the presence of the uncoupler nigericin. Nigericin is apparently needed in high concentrations for wild type *C. reinhardtii* because it does not easily enter the cells [45]. However, in the cell wall-less strain used here, 10 μM nigericin completely collapsed the proton gradient as seen by the rapid disappearance of the pH-dependent component (qE) of non-photochemical quenching (data not shown).

Previous studies have shown that acclimation of spinach and Arabidopsis to cold temperatures is accompanied by a decrease in the T_m of the B-band [46,47] and by a stabilisation of the Q-band [47]. Cold adapted plants suffered lower rates of PSII photoinhibition compared to non-adapted plants. During cold hardening, many changes on the metabolite level occur, including the accumulation of specific sugars, amino acids and small carboxylic acids, such as citric acid, succinic acid, oxalic acid and fumeric acid [48,49]. Similar to our observations, these carboxylates may compete with bicarbonate at the acceptor side of PSII and alter the E_m of the quinone acceptors, thereby protecting from $^1O_2$ production, as discussed below.

The up-shift of the T_m of the Q-band indicates a less negative E_m of Q_A/Q_A^-. A stabilisation of the Q-band is expected to lead to more direct charge recombination events between $P_{680}^+Q_A^-$ at the expense of Q_A^- re-reducing Phe [21]. As the direct recombination of $P_{680}^+Q_A^-$ bypasses the re-formation of $P_{680}^+Phe^-$, there is less chance of producing $^3P_{680}$, one of the major pathways of $^1O_2$ formation (reviewed in [22,50]). The relationship between the E_m of Q_A/Q_A^- and $^1O_2$ production has been supported by a study using herbicides that modify the E_m of Q_A/Q_A^- [23] and by site-directed mutagenesis of the Q_A pocket [51]. In agreement with these studies, thylakoids from mixotrophic *C. reinhardtii* cultures, with a stabilised Q-band (Fig. 6), produced less $^1O_2$ than thylakoids from photoautotrophic cultures (Fig. 5). A destabilisation of the B-band and a lowering of the E_m of Q_B/Q_B^- has the same effect on the energetics of charge recombination as a stabilisation of Q_A^-, since lower activation energy is needed to repopulate the Q_A^- state. Since the E_m of Q_A/Q_A^- is less negative in the presence of acetate, non-radiative dissipation by direct charge recombination to the ground state is favoured and thus the yield of $^1O_2$ formation is lowered. This is accompanied by a lower intensity of the B-band in mixotrophic cells compared with photoautotrophic cells (Fig. 7, best visible after 2 and 3 flashes). It is discussed in the literature [50] that Q_A may become double reduced during photoinhibition. When Q_A is double reduced, ^{3}Chl formation by charge recombination of the primary radical and hence $^1O_2$ generation is stimulated. One may speculate that the acetate-induced modification of the acceptor side of PSII makes a double reduction of Q_A more difficult and protects therefore against photoinhibition. However, in our hands, double reduction of Q_A does only occur when samples are illuminated in the presence of dithionite or under anaerobic conditions (data not shown). Therefore, we exclude inhibition of double reduction of Q_A to be responsible for the observed protective effects of acetate.

In conclusion, we demonstrate here that acetate significantly changes the energetics of PSII and that mixotrophic cells are less susceptible to photoinhibition. This should be taken into consideration when *Chlamydomonas* cells grown mixotrophically are used for studies on photosynthesis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2013.06.004.

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