Evidence for the operation of a cyanide-sensitive oxidase in chlororespiration in the thylakoids of the chlorophyll c-containing alga *Pleurochloris meiringensis* (Xanthophyceae)

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**Abstract.** For characterisation of chlororespiration in the chlorophyll c-containing alga *Pleurochloris meiringensis*, we measured the flash-induced electrochromic absorbance changes between 470 and 545 nm and the redox changes of cytochrome f and cytochrome c\(_{553}\). Cytochrome c\(_{553}\) was shown to be present in high amounts (1 mol cytochrome c\(_{553}\) per 300 mol chlorophyll) in this alga and to function as the obligatory electron donor for photosystem I instead of plastocyanin. Whereas salicylhydroxamic acid had no effect on the flash-induced absorbance transients, cyanide enhanced the slow-rising (\(t_{1/2} \approx 10\) ms) kinetic component of the electrochromic absorbance change. Cyanide also accelerated the re-reduction of the cytochrome F\(^+\)/C\(_{553}\) electron pool following the photooxidation by repetitive single-turnover flashes. These data suggest that an oxidase competes with the cytochromes for electrons. The KCN concentration needed to induce these effects was 0.25 mM at half-saturation, whereas mitochondrial respiration was completely blocked at 0.1 mM. Therefore, the oxidase cannot be identical to the cytochrome aa\(_3\)-oxidase of mitochondria and is most likely located in the chloroplast of *P. meiringensis*.

**Key words:** Chlororespiration – Cytochrome c\(_{553}\) – Electrochromism – Photosynthesis – Plastocyanin – *Pleurochloris* (1982, 1983) proposed the existence of a respiratory chain in the chloroplast and suggested that chlororespiration occurred in the green algae *Chlamydomonas* and *Chlorella*. The scheme consisted of a reduction of plastoquinone (PQ) via NAD(P)H followed by an oxygen-dependent oxidation, resulting in the formation of a pH gradient across the thylakoid membrane in the dark.

Changes in the redox level of the PQ pool due to an input of electrons in the dark were described earlier by Diner and Mauzerall (1973) and substantiated by the finding of an NAD(P)H-PQ oxidoreductase in the thylakoids of *Chlamydomonas reinhardtii* by Godde and Trebst (1980) and Godde (1982). This reducing activity seemed to be more pronounced in N-deficient cells (Peltier and Schmidt 1991). Chlororespiration was detected not only in green algae but also in prasinophytes (Wilhelm and Duval 1990), diatoms (Caron et al. 1987), xanthophyces (Büchel and Wilhelm 1990) and in higher plants (Garab et al. 1989). Seven chloroplastic genes of tobacco have been found to be homologous to mitochondrial genes coding for the NADH ubiquinone oxidoreductase (complex I; Shinozaki et al. 1986). The expression of one subunit was shown to be independent from, but stimulated by, light, and the preferential localisation of the gene product on stroma thylakoids of higher plants was demonstrated using antibodies (Berger et al. 1993). Recent reports confirm non-photochemical PQ reduction in higher-plants chloroplasts (Farineau 1993; Groom et al. 1993; Harris and Heber 1993).

Whereas evidence is growing for the reduction of PQ in the dark, its oxidation is much less clear. The oxygen dependency of chlororespiration led to the hypothesis of an oxidase in thylakoid membranes (Bennoun 1982). Garab et al. (1989) have shown that a cyanide- and CO-sensitive oxidase competes with PSI for electrons from the PQ pool in higher plants. This competition resembled that between photosynthesis and respiration in purple bacteria (Verméglio and Joliot 1984) and in heterocysts (Houchins and Hind 1983). However, the biochemical identification of an oxidase in chloroplasts has not been successful.

The function of chlororespiration is not clear. Its contribution to total respiration was found to be about 10%.

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**Introduction**

Over the last few years many reports have dealt with the interaction between the respiratory and the photosynthetic electron-transport chains in prokaryotic and eukaryotic cells (for review, see Scherer 1990). Bennoun (1982, 1983) proposed the existence of a respiratory chain in the chloroplast and suggested that chlororespiration occurred in the green algae *Chlamydomonas* and *Chlorella*. The scheme consisted of a reduction of plastoquinone (PQ) via NAD(P)H followed by an oxygen-dependent oxidation, resulting in the formation of a pH gradient across the thylakoid membrane in the dark.
The formation of a pH gradient across the thylakoid membrane was suggested to maintain ATPases in a more functional state during short periods of darkness (Peltier et al. 1987). Additionally, chlororespiration may serve to poise the redox level of the photosynthetic electron-transport chain for more efficient photosynthesis after re-illumination (Maione and Gibbs 1986). The poised of redox-levels further supports the idea of chlororespiration as a regulatory tool under environmental stress conditions (Garab et al. 1989) and for balancing excitation-energy transfer between the two photosystems (Büchel and Wilhelm 1990; Wilhelm and Duval 1990; Harrison and Allen 1993).

In Pleurochloris meiringensis, a xanthophyte alga, fluorescence induction kinetics showed that a pH gradient across the thylakoid membrane was established in the dark. This membrane energization without photosynthetically driven electron fluxes was explained by the activity of chlororespiration in the chloroplasts (Büchel and Wilhelm 1990).

In a previous paper we reported the existence of an electrochromic shift in *P. meiringensis* (Büchel and Garab 1995). In this work we used this method, along with flash-induced transients of cytochrome *(cyt)* ‐ and cyt *c553* to further characterise the chlororespiratory activity in this alga.

### Materials and methods

*Pleurochloris meiringensis* Vischer (no. 860-3, Culture collection Göttingen, Germany) was grown as batch cultures in a nutrient medium according to Büger (1969) under white light of 15 μmol photons m ‐2 s ‐1. Media were supplemented one week before measurement with 1 μM Cu 2+ or 10 μM Cu 2+. Cells were harvested after three weeks of total culturing at the end of the logarithmic growth phase by centrifugation. Homogenisation of the cells was carried out in a cell mill at 4°C in a buffer containing 0.05 M N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine), 0.15 M KCl and 0.35 M sucrose (pH 7.2).

For the determination of the cytochrome content the homogenate was frozen and thawed three times and subsequently centrifuged for 2 h at 50 000 × g. The cyt *c553* content was determined from the supernatant spectroscopically at 533 nm by measuring the reduced (ascorbate) minus oxidised (ferricyanide) absorbance change in the sample using a differential extinction coefficient of 17.3 mM ‐1 cm ‐1 (Sandmann and Büger 1980). Baseline correction was carried out by interpolation from the values at the isosbestic points, at 559 nm and 543.5 nm.

The pellet obtained after the high-speed centrifugation was solubilized in Tris-HCl buffer (pH 7.5) containing 1% Triton. The concentration of cyt *f* was determined from the suspension at 533 nm in the same way as the soluble cyt *c553* with a differential extinction coefficient of 20 mM ‐1 cm ‐1 (Sandmann and Büger 1980).

Because plastocyanin shows a very broad absorption maximum, a purification procedure was necessary to determine the content spectroscopically. The homogenate was solubilized for 3 h in Tricine buffer containing 2% Triton and subsequently centrifuged for 10 min at 30 000 × g. The supernatant was purified with 25% ammonium sulfate and again spun at 30 000 × g for 10 min. The remaining supernatant was further precipitated by adding ammonium sulfate up to 55% and a further centrifugation step. The supernatant was then measured by differential absorbance spectroscopy between 700 and 400 nm to check the plastocyanin content at 597 nm (Sandmann and Büger 1980). As a control, the same procedure was carried out with *Chlorella* cells which are known to contain plastocyanin.

Flash-induced absorption changes were measured as previously described (Büchel and Garab 1995). Cells grown in the original medium were harvested in the logarithmic growth phase and concentrated by sedimentation. Chlorophyll (Chl) content was measured after homogenisation of the cells in 90% acetone according to Jeffrey and Humphrey (1975). For absorption transients the cells were used at a Chl content of 70–100 μg ml ‐1 and dark-adapted for at least 20 min. The frequency of the exciting flashes was 1 Hz and 30 traces were averaged following 30 preillumination flashes of the same frequency. Inhibitors were added prior to measurement in the concentration given in the figure legends. The ethanol content did not exceed 1%, which had no effect on the transients.

The flash-induced electrochromic absorbance shift was measured in the maximum at 520 nm. Therefore, it will be referred to as ΔAA520. The flash-induced absorbance changes due to redox changes of cyt *f* and cyt *c553*, ΔAA533–545 nm, were corrected for the contribution of the electrochromic absorbance change in *P. meiringensis* by a method similar to that described by Bouges-Bocquet (1977). By using the fitted values of the 520-nm electrochromic band (Büchel and Garab 1995), the contribution of ΔAA520 in ΔAA533–545 nm was estimated to be 8.6%. Due to the similar absorption maxima of cyt *f* and cyt *c553* in vivo, we could not discriminate between the transients of these two cytochromes. Therefore, the corrected flash-induced absorbance transients due to the redox changes of cyt *(f + c553)* will be referred to as ΔAA520 *(f + c553)*.

Oxygen uptake in the dark in the presence of 5 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and 5 mM salicylhydroxamic acid (SHAM) was measured with an oxygen electrode (Hansatech, Kings’ Lynn, Norfolk, UK).

### Results

The contents of cyt *f* and cyt *c553* in *P. meiringensis* are shown in Table 1. Cells grown in the original medium containing 0.23 μM Cu 2+ did not contain any plastocyanin. Because copper is known to induce plastocyanin synthesis and to suppress cyt *c553* in some algae (Sandmann and Büger 1980), the same experiments were carried out after one week of culturing with increased Cu 2+ supply. Even then no plastocyanin was detectable and the cyt *c553* content did not change significantly. To check whether the copper concentration was sufficient for the induction of plastocyanin, the concentration of Cu 2+ was further increased. This culture condition, however, led to a decreased growth followed by a decay of the cultures in 3 d.

Figure 1 shows the effect of 2 mM KCN on ΔAA520 and ΔAA533 *(f + c553)*. The ΔAA520 could be attributed to flash-induced electrochromic absorbance changes, and

### Table 1. Cytochrome contents of *P. meiringensis* grown under different culture conditions. All data are given in mol chlorophyll per mol cytochromes as mean values ± SD; of at least five independent measurements.

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>cyt <em>f</em></th>
<th>cyt <em>c553</em></th>
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<tbody>
<tr>
<td>0.23 μM Cu 2+</td>
<td>1767.4 ± 165</td>
<td>297.7 ± 73</td>
</tr>
<tr>
<td>1.23 μM Cu 2+</td>
<td>1499.6 ± 253</td>
<td>285.9 ± 33</td>
</tr>
</tbody>
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Peltier et al. 1987. The formation of a pH gradient across the thylakoid membrane was suggested to maintain ATPases in a more functional state during short periods of darkness (Peltier et al. 1987). Additionally, chlororespiration may serve to poise the redox level of the photosynthetic electron-transport chain for more efficient photosynthesis after re-illumination (Maione and Gibbs 1986). The poised of redox-levels further supports the idea of chlororespiration as a regulatory tool under environmental stress conditions (Garab et al. 1989) and for balancing excitation-energy transfer between the two photosystems (Büchel and Wilhelm 1990; Wilhelm and Duval 1990; Harrison and Allen 1993).

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