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Alternative cyanide-sensitive oxidase interacting with photosynthesis in *Synechocystis* PCC6803. Ancestor of the terminal oxidase of chlororespiration?

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Abstract

Influence of respiration on photosynthesis in *Synechocystis* PCC6803 was studied by measuring the redox transients of cytochrome *f* (cyt *f*) upon excitation of the cells with repetitive single turnover flashes. Upon the addition of KCN the flash-induced oxidation of cyt *f* was increased and the rereduction of cyt f^+ was accelerated. Dependence of these effects on the concentration of KCN clearly demonstrated the existence of two cyanide-sensitive oxidases interacting with photosynthesis: cyt aa_3, which was sensitive to low concentrations of cyanide, and an alternative oxidase, which could be suppressed by using ≥1 mM KCN. The interaction between the photosynthetic and the respiratory electron transport chains was regulated mainly by the activity of the alternative cyanide-sensitive oxidase. The oxidative pathway involving the alternative cyanide-sensitive oxidase was insensitive to salicyl hydroxamic acid and azide. The close resemblance of the inhibition pattern reported here and that described for chlororespiration in algae and higher plants strongly suggest that an oxidase of the same type as the alternative cyanide-sensitive oxidase of cyanobacteria functions as a terminal oxidase in chloroplasts.

Additional key words: absorbance; azide; cyanobacteria; cytochromes; flash irradiation; KCN; NaN_3; phylogeny; respiration; salicyl hydroxamic acid.

Introduction

In cyanobacteria, the cytochrome (cyt) aa_3 type terminal oxidase of the respiratory electron transport and photosystem 1 (PS1) competes for electrons from the

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Abbreviations: cyt = cytochrome; C_{50} = concentration for half-maximal effect; PS = photosystem, SHAM = salicyl hydroxamic acid.
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plastoquinone pool (cf. Scherer 1990, Schmetterer 1994). This oxidase is similar to the mitochondrial terminal oxidase and should therefore be sensitive to low concentrations of KCN (Peschek *et al.* 1989). Using 1 μM KCN, the influence of this oxidase on PS1 activity could indeed be demonstrated for different species of cyanobacteria (Geerts *et al.* 1994, Schubert *et al.* 1995). However, using a cyt aa_3 deficient mutant of *Synechocystis* PCC6803, Schmetterer *et al.* (1994) found evidence for the existence of an alternative cyanide-sensitive oxidase, which was present after the deletion of the *cox*-genes; this oxidase was sensitive to high concentration (2 mM) of KCN.

High concentrations (≈1 mM) of KCN affect the activities of PS1 and cyt *f* in chloroplasts of some algae (Bennoun 1982, Büchel and Garab 1995a) and higher plants (Garab *et al.* 1989). This effect has been attributed to the inhibition of the terminal oxidase of the respiratory electron transport of chloroplasts, *i.e.*, of chlororespiration (Bennoun 1982; for recent review see Büchel and Garab 1997). Electrons can be donated to the plastoquinone pool from the NAD(P)H-plastoquinone oxidoreductase (Godde and Trebst 1980, Friedrich *et al.* 1995, Seidel-Guyenot *et al.* 1997). However, attempts to identify and isolate the terminal oxidase of chlororespiration have failed, thus the mechanism of oxidation of the plastoquinone pool in the dark is still unclear.

The interaction between the chlororespiratory and the photosynthetic electron transport systems in higher plants and some algae resembles the interaction between respiration and photosynthesis in cyanobacteria (Lajkó *et al.* 1997). However, systematic investigations in cyanobacteria under the conditions comparable to those done earlier in green algae, higher plant protoplasts, and chloroplasts have not been carried out. In this work, we studied the effects of respiratory inhibitors on the photosynthetic activity in a cyanobacterium, *Synechocystis* PCC6803, and investigated the possibility of involvement of an alternative cyanide-sensitive oxidase.

Materials and methods

Growth conditions: *Synechocystis* PCC6803 was grown photoautotrophically under "white light" of 70 μmol m^{-2} s^{-1} (PAR) at 35 °C in *BG 11* medium supplemented with 20 mM Hepes-NaOH (pH 7.5). Air-lift cultures were supplied with 1 % CO_2 in sterile air. Cells were harvested in the logarithmic growth phase by centrifugation at 1000×*g* for 5 min. Pelleted cells were diluted with the culture medium to A_{850nm} = 0.8 (measured in a *Shimadzu UV3000* spectrophotometer), and stored at 35 °C under "white light" of 70 μmol m^{-2} s^{-1} until the measurements.

Oxygen measurements: Uncoupled respiration of intact cells was measured in a Clark-type oxygen electrode (*Hansatech*) at 25 °C after 5 min of dark adaptation using 2 mM NH_4Cl. Inhibitors were added immediately before measurements.

Flash-induced absorbance changes induced by single turnover flashes at a repetition rate of 1 Hz were measured using an equipment described in Büchel and Garab

(1995b). If not stated otherwise, the cells were dark adapted for 2 min prior to the measurements, and 30 kinetic traces were averaged. Inhibitors were added immediately before the measurements. The amplitude of the flash-induced oxidation of cyt *f* was calculated from the absorbance change $\Delta A_{cyt\ f} = \Delta A_{(556\ nm\ -\ 545\ nm)}$ at 1 ms after the flash. The extent of the fast rereduction of cyt f^+ in the dark after the exciting flashes was characterised with the ratio $\Delta A(20\ ms)/\Delta A(1\ ms)$ of the same absorbance changes.

Results

In order to study the influence of respiration on the photosynthetic electron transport in *Synechocystis* PCC6803 we measured the effect of an oxidase inhibitor, KCN, on the flash-induced redox transients of the cyt b_6/f complex. The kinetics of absorbance changes of cyt *f* (Fig. 1*A*) show that the rereduction of cyt f^+ was strongly accelerated upon the addition of 2 mM KCN. This, similarly to the effect of 1-2 mM KCN in higher plant chloroplasts (Garab *et al.* 1989), can be accounted for by the inhibition of an oxidase competing with PS1 for electrons from the plastoquinone pool. This observation can hardly be accounted for by an inhibition of the Mehler reaction. Partial inhibition of the Mehler reaction by 1-2 mM KCN may lead to an accumulation of H_2O_2, possibly even under our conditions of flash excitation. This strong oxidant, if present at any sizeable concentration, is expected to decelerate the rereduction of cyt f^+. However, as shown by Fig. 1*A*, cyanide has an opposite effect: it significantly enhances the rereduction of cyt f^+.

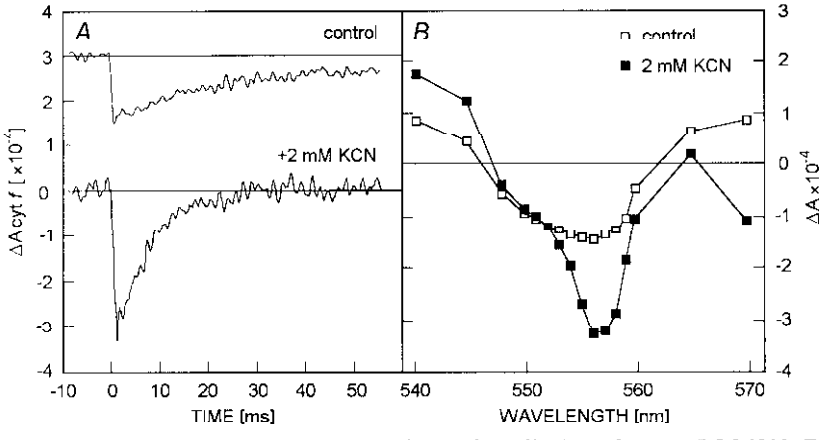

Fig. 1. *A*: Flash-induced absorbance transients of cyt *f* in *Synechocystis* PCC6803. Each kinetic trace represents the average of 120 transients. *Upper curve*, control, shifted by 0.0003; *lower curve*: upon addition of 2 mM KCN. *B*: Transient spectrum of absorbance changes recorded 1 ms after a single turnover flash in *Synechocystis* PCC6803. Points represent mean values of 10 independent measurements of untreated cells (□) and upon the addition of 2 mM KCN (■).

2 mM KCN also enhanced the extent of the flash-induced oxidation of cyt *f* in comparison to the control (Fig. 1*A*). This has also been reported for higher plants and

is most likely the consequence of the incomplete rereduction of cyt f^+ in the dark intervals between the flashes. These results also closely resemble those obtained using another strain of *Synechocystis* sp., PCC6714 (Matsuura *et al.* 1988), or a different cyanobacterium, *Synechococcus* PCC6301 (Lajkó *et al.* 1997).

The transient spectra recorded at 1 ms after the flashes (Fig. 1*B*) confirm our conclusion that cyanide indeed enhances the amplitude of the flash-induced oxidation of cyt *f*. The transient spectra also show that the cells contained mostly plastocyanin instead of cyt c_{553} and that KCN influenced only cyt *f*, whose α-absorption maximum is at 556 nm in cyanobacteria (cf. Kallas 1994). The shape of the spectra depended on the age of the culture (data not shown), reflecting an increasing amount of cyt c_{553} with the age, most probably due to a gradual copper-depletion of the culture medium. In contrast to *Synechococcus* (Lajkó *et al.* 1997), the cyanide-induced enhancement of the activity of cyt *f* did not depend on the age of the culture (data not shown).

To evaluate whether only one or more terminal oxidases compete for electrons with the photosynthetic electron transport chain, we titrated the cyt *f* signals against the enhancement of cyt *f* oxidation and the acceleration of rereduction of cyt f^+, respectively (Fig. 2). Low concentrations of KCN, which are sufficient to inhibit cyt aa_3, obviously did not have much influence on the redox state of cyt *f*, for which a concentration for half-maximal effect (C_{50}) of 200 μM could be calculated. Therefore, an additional oxidase with lower sensitivity to KCN must be present.

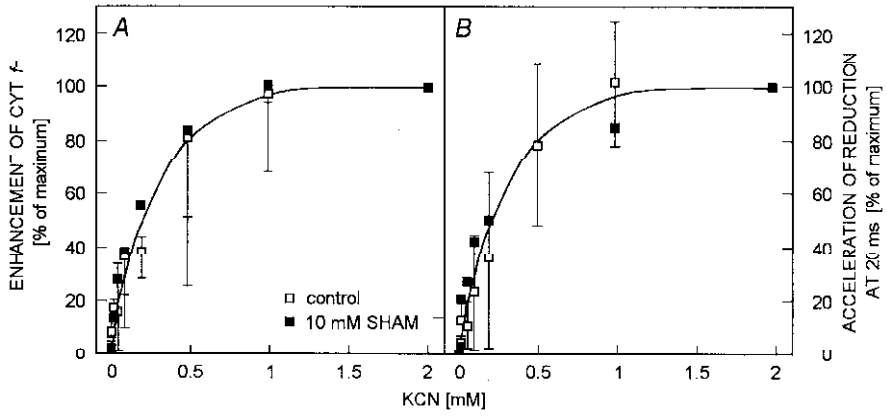

Fig. 2. Dependence of the enhancement of cyt *f* oxidation (*A*) and the acceleration of the rereduction of cyt f^+ (*B*) on the concentration of KCN in *Synechocystis* PCC6803 (for calculations see Materials and methods). Values were normalised to the effects observed at 2 mM KCN. Each point represents mean value and standard deviation of at least five independent experiments on different samples. For the experiments carried out in the presence of 10 mM SHAM (*closed symbols*) only the mean values are given.

The measurements were also carried out in the presence of 10 mM salicyl hydroxamic acid (SHAM), an inhibitor of plant mitochondrial alternative oxidase. SHAM did not affect noticeably the effect of cyanide on the flash-induced changes of cyt *f* (Fig. 2).

Fig. 3 shows dependence of the oxygen uptake and the flash-induced redox transients of cyt *f* on the concentration of KCN between 0 and 50 μM. As expected, the respiration of cells in the dark was inhibited efficiently with low concentrations of cyanide. In contrast, 50 μM KCN hardly affected the redox transients of cyt *f*. Taking into account that in cyanobacteria cyt aa_3 is on the thylakoid membranes (Nicholls *et al.* 1992) and 50 μM KCN is sufficient to suppress fully the activity of cyt aa_3, the effect of cyanide on cyt *f* is surprisingly low (less than 20 % of the maximal effect).

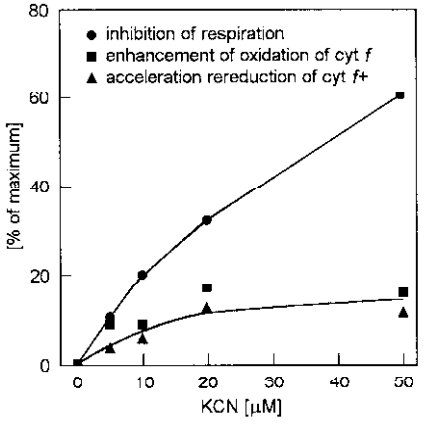

Fig. 3. Concentration dependencies of the effects of KCN on the acceleration of the rereduction of cyt f^+ (▲), the enhancement of the flash-induced oxidation of cyt *f* (■), and the inhibition of the dark respiration of whole cells (●). The points on the flash-induced absorbance changes of cyt *f* were obtained from experiments shown in Fig. 2.

We also tested the influence of azide on the flash-induced redox transients of cyt *f* and on the oxygen consumption in the dark. No significant effect of 100 μM azide could be detected on the redox transients of cyt *f* (Fig. 4*A*), which was insensitive even to 10 mM azide (values not shown). Nevertheless, respiration of whole cells, measured in the presence of 10 mM SHAM, could be inhibited with 50-100 μM

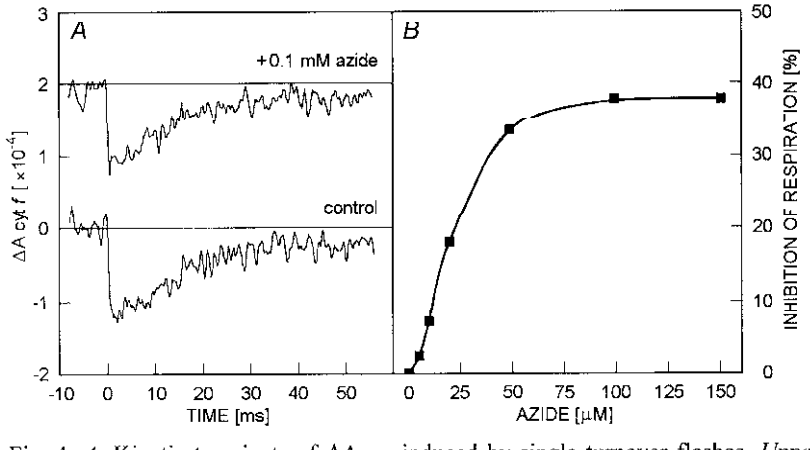

Fig. 4. *A*: Kinetic transients of $\Delta A_{cyt\ f}$ induced by single turnover flashes. *Upper trace*: upon the addition of 100 μM azide, trace shifted by 0.0002; *lower trace*: control. *B*: Dependence of the inhibition of dark respiration of the cells on the concentration of NaN_3. The measurements were performed in the presence of 10 mM SHAM.

NaN_3 (Fig. 4*B*). This latter inhibition pattern agrees well with those reported for the azide sensitivity of cyt aa_3 (Ikuma and Bonner 1967, Peschek *et al.* 1989).

The absorbance transients shown above were measured after a preirradiation with "white light" and 2 min relaxation in the dark. This might have induced an adaptation state favouring photosynthesis, and thus a decrease in the activity of cyt aa_3. To rule out this possibility, we measured both the flash spectroscopy and oxygen consumption on the same samples after 5 min dark adaptation. To avoid any influence of a SHAM-sensitive alternative oxidase, all measurements were done in the presence of SHAM. Under these conditions, 50 μM KCN should suffice to inhibit all oxygen consumption. Again, we found that 30 % of the respiratory activity was retained which could be suppressed with 2 mM KCN (Fig. 5). These results are in perfect agreement with those of Schmetterer *et al.* (1994) using a cyt aa_3-deficient mutant of *Synechocystis*. Further, we found that similarly to the results shown above (Fig. 2) low concentrations of cyanide did not affect the flash-induced transients of cyt *f*, which were very sensitive to 2 mM cyanide (Fig. 5). This shows that the effects of the oxidases on cyt *f* do not depend on the length of the dark interval before the measurements. They also allow to conclude that the alternative cyanide-sensitive oxidase, at least under our experimental conditions, is the main oxidase interacting with the photosynthetic electron transport in *Synechocystis* PCC6803.

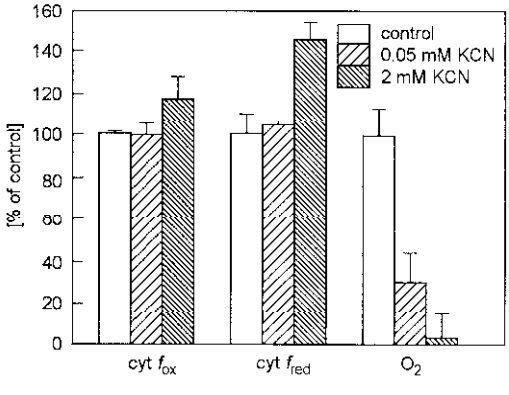

Fig. 5. Comparison of the effects of KCN on the flash-induced oxidation of cyt *f* (cyt f_{ox}), on the acceleration of the rereduction of cyt f^+ (cyt f_{red}), and on the rates of dark respiration (O_2). The measurements were performed after 5 min dark adaptation in the presence of 10 mM SHAM. KCN was added immediately prior to the measurements. Mean values and standard deviations of 5 independent measurements on different samples.

In summary, we show that (*1*) an alternative cyanide-sensitive oxidase interacts with photosynthesis in *Synechocystis* PCC6803; (*2*) this oxidase, under our experimental conditions, is the main oxidase competing with PS1 for electrons from the plastoquinone pool; (*3*) in contrast to the pathway involving the cyt aa_3, the respiratory activity associated with the cyanide-sensitive alternative oxidase is insensitive to both SHAM and azide.

Discussion

In cyanobacteria, the interaction between photosynthesis and respiration has mostly been studied using inhibitors of cyt aa_3 type oxidases. There is ample evidence in the literature for this type of interaction (cf. Scherer 1990, Schmetterer 1994, Schubert *et*

al. 1995). However, most studies were carried out only either in the micromolar or in the millimolar range of KCN.

Schmetterer *et al.* (1994) showed the existence of an alternative cyanide-sensitive oxidase in *Synechocystis* PCC6803, the activity of which could be blocked with 2 mM KCN. In order to discriminate between the effects of the activity of cyt aa_3 and of the alternative cyanide-sensitive oxidase on the photosynthetic electron transport, we measured the dependence of the flash-induced redox transients of cyt *f* on the concentration of KCN and compared these data with the cyanide-sensitivity of the oxygen uptake. Our results show the existence of two different oxidases with different sensitivities to KCN. This was demonstrated by the incomplete inhibition of oxygen consumption in the dark with low concentrations of KCN, which however block the activity of cyt aa_3 (Fig. 3). Further, we showed that the C_{50} of the flash-induced redox transients of cyt *f* was high (Figs. 2 and 5).

We also show that under our experimental conditions the interaction between the photosynthetic and the respiratory electron transport systems is regulated mainly by the activity of the alternative cyanide-sensitive oxidase, and cyt aa_3 exerts a much smaller influence (less than 20 % of the maximal effect). Peschek *et al.* (1989) suggest that in the logarithmic growth phase of the cells the cyt aa_3 content of the thylakoid membranes is high. Thus, one would expect that low concentrations of KCN very significantly affect the flash-induced redox transients of cyt *f*, but immunodetection revealed only low amounts of cyt aa_3 on the thylakoids (Sherman *et al.* 1994). Growing cells in high salt medium, which increases the total amount of cyt aa_3, Nicholls *et al.* (1992) calculated 0.02-0.05 cyt aa_3 per cyt *f* in *Anacystis nidulans*. Assuming a similar situation for *Synechocystis*, it explains that influence of the activity of cyt aa_3 on photosynthesis is small. In addition, our growth conditions favoured the synthesis of plastocyanin instead of cyt c_{553} (Briggs *et al.* 1992). Plastocyanin is a better donor to PS1 than to cyt aa_3 (Geerts *et al.* 1994).

The alternative cyanide-sensitive oxidase may be related to cyt b_0 of *E. coli* (Schmetterer *et al.* 1994). This cyt has high sequence homologies to cyt aa_3 (Chepuri *et al.* 1990), but it acts as a quinol-oxidase. Our results are consistent with the hypothesis that the alternative cyanide-sensitive oxidase is a quinol oxidase.

The C_{50} for KCN of the alternative oxidase reported here is very close to the values reported for the putative terminal oxidase of chlororespiration in higher plants (Garab *et al.* 1989) and in the xanthophyte alga *Pleurochloris meiringensis* (Büchel and Garab 1995a). Our results strongly suggest that the alternative cyanide-sensitive oxidase of *Synechocystis* is very similar to the chlororespiratory oxidase and, therefore, might be its ancestor. This testable hypothesis may be useful in identifying the terminal oxidase of chlororespiration in algae and higher plants.

Note added in proof: Very recently, Pils *et al.* (FEMS Microbiol. Lett. **152**: 83-88, 1997) studied different types of terminal oxidases in *Synechocystis* using a variety of deletion mutants. They proposed a scheme of the linked photosynthetic and respiratory electron transport chains which consists of three terminal oxidases: cyt *aa3*, a cyt *bd* quinol type oxidase, and a so-called alternative respiratory oxidase, respectively. All oxidases could be inhibited with 1.5 mM KCN, but only the cyt *bd*

type oxidase turned out to be insensitive against azide. Although the data presented in the above contribution do not allow to distinguish between the cyt *bd* type and the alternative oxidase, the insensivity of the signals to azide points to a more severe influence of the cyt *bd* quinol oxidase on the photosynthetic electron transport chain in *Synechocystis*.

References

Bennoun, P.: Evidence for a respiratory chain in the chloroplast. - Proc. nat. Acad. Sci. USA **79**: 4352-4356, 1982.

Briggs, L.M., Pecoraro, V.L., McIntosh, L.: Copper induced expression, cloning and regulatory studies of the plastocyanin gene from the cyanobacterium *Synechocystis* sp. PCC6803. - Plant mol. Biol. **15**: 633-642, 1992.

Büchel, C., Garab, G.: Evidence for the operation of a cyanide-sensitive oxidase in chlororespiration in the thylakoids of the chlorophyll *c*-containing alga *Pleurochloris meiringensis* (*Xanthophyceae*). - Planta **197**: 69-75, 1995a.

Büchel, C., Garab, G.: Electrochromic absorbance changes in the chlorophyll *c*-containing alga *Pleurochloris meiringensis* (*Xanthophyceae*). - Photosynth. Res. **43**: 49-56, 1995b.

Büchel, C., Garab, G.: Respiratory regulation of electron transport in chloroplasts: Chlororespiration. - In: Pessarakli, M. (ed.): Handbook of Photosynthesis. Pp. 83-93. Marcel Dekker, New York - Basel - Hong Kong 1996.

Chepuri, V., Lemieux, L., Au, D.C.T., Gennis, R.B.: The sequence of the cyo operon indicates substantial structural similarities between the cytochrome *o* ubiquinol oxidase of *Escherichia coli* and the aa_3-type family of cytochrome *c* oxidases. - J. biol. Chem. **265**: 11185-11192, 1990.

Friedrich, T., Steinmüller, K., Weiss, H.: The proton-pumping respiratory complex I of bacteria and mitochondria and its homologue in chloroplasts. - FEBS Lett. **367**: 107-111, 1995.

Garab, G., Lajkó, F., Mustárdy, L., Márton, L.: Respiratory control over photosynthetic electron transport in chloroplasts of higher-plant cells: evidence for chlororespiration. - Planta **179**: 349-358, 1989.

Geerts, D., Schubert, H., de Vrieze, G., Borrias, M., Matthijs, H.C.P., Weisbeek, P.J.: Expression of *Anabaena* PCC 7937 plastocyanin in *Synechococcus* PCC 7942 enhances photosynthetic electron transfer and alters the electron distribution between photosystem I and cytochrome-*c* oxidase. - J. biol. Chem. **269**: 28068-28075, 1994.

Godde, D., Trebst, A.: NADH as electron donor for the photosynthetic membrane of *Chlamydomonas reinhardii*. - Arch. Microbiol. **127**: 245-252, 1980.

Ikuma, H., Bonner, W.D.: Properties of higher plant mitochondria. III. Effects of respiratory inhibitors. - Plant Physiol. **42**: 1535-1544, 1967.

Kallas, T.: The cytochrome b_6-*f* complex. - In: Bryant, D.A. (ed.): The Molecular Biology of Cyanobacteria. Pp. 259-317. Kluwer Acad. Publ., Dordrecht - Boston - Lancaster 1994.

Lajkó, F., Kadioglu, A., Borbély, G., Garab, G.: Competition between the photosynthetic and the (chloro)respiratory electron transport chains in cyanobacteria, green algae and higher plants. Effect of heat stress. - Photosynthetica **33**: 217-226, 1997.

Matsuura, K., Murakami, A., Fujita, Y.: Changes in the light-induced electron transfer through cytochrome *b*-563 depending on the oxidation-reduction conditions in intact cells of the cyanobacterium *Synechocystis* PCC 6714. - Plant Cell Physiol. **29**: 1261-1268, 1988.

Nicholls, P., Obinger, C., Niederhauser, H., Peschek, G.A.: Cytochrome oxidase in *Anacystis nidulans*: stoichiometries and possible functions in the cytoplasmic and thylakoid membranes. - Biochim. biophys. Acta **1098**: 184-190, 1992.

Peschek, G.A., Wastyn, M., Trnka, M., Molitor, V., Fry, J.V., Packer, L.: Characterization of the cytochrome *c* oxidase in isolated and purified plasma membranes from the cyanobacterium *Anacystis nidulans*. - Biochemistry **28**: 3057-3063, 1989.

Scherer, S.: Do photosynthetic and respiratory electron transport chains share redox proteins? Trends biochem. Sci. **15**: 458-462, 1990.

Schmetterer, G.: Cyanobacterial respiration. - In: Bryant, D.A. (ed.): The Molecular Biology of Cyanobacteria. Pp. 409-435. Kluwer Academic Publishers, Dordrecht - Boston - Lancaster 1994.

Schmetterer, G., Alge, D., Gregor, W.: Deletion of cytochrome *c* oxidase genes from the cyanobacterium *Synechocystis* sp. PCC6803: Evidence for alternative respiratory pathways. - Photosynth. Res. **42**: 43-50, 1994.

Schubert, H., Matthijs, H.C.P., Mur, L.R.: *In vivo* assay of P700 redox changes in the cyanobacterium *Fremyella diplosiphon* and the role of cytochrome *c* oxidase in regulation of photosynthetic electron transfer. - Photosynthetica **31**: 517-257, 1995.

Seidel-Guyenot, W., Schwabe, C., Büchel, C.: Kinetic and functional characterization of a membrane-bound NAD(P)H dehydrogenase located in the chloroplasts of *Pleurochloris meiringensis* (*Xanthophyceae*). - Photosynth. Res. **49**:183-193, 1997.

Sherman, D.M., Troyan, T.A., Sherman, L.A.: Localization of membrane proteins in the cyanobacterium *Synechococcus* sp. PCC7942. - Plant Physiol. **106**: 251-262, 1994.