Electron Transport Routes in Whole Cells of *Synechocystis* sp. Strain PCC 6803: The Role of the Cytochrome *bd*-Type Oxidase†

Stephan Berry,‡ Dirk Schneider,‡ Wim F. J. Vermaas,§ and Matthias Rögner*,‡

Lehrstuhl für Biochemie der Pflanzen, Fakultät für Biologie, Ruhr-Universität Bochum, D-44780 Bochum, Germany, and Department of Plant Biology and Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, Arizona 85287-1601

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ABSTRACT: The plastoquinone pool is the central switching point of both respiratory and photosynthetic electron transport in cyanobacteria. Its redox state can be monitored noninvasively in whole cells using chlorophyll fluorescence induction, avoiding possible artifacts associated with thylakoid membrane preparations. This method was applied to cells of *Synechocystis* sp. PCC 6803 to study respiratory reactions involving the plastoquinone pool. The role of the respiratory oxidases known from the genomic sequence of *Synechocystis* sp. PCC 6803 was investigated by a combined strategy using inhibitors and deletion strains that lack one or more of these oxidases. The putative quinol oxidase of the cytochrome *bd*-type was shown to participate in electron transport in thylakoid membranes. The activity of this enzyme in thylakoids was strongly dependent on culture conditions; it was increased under conditions where the activity of the cytochrome b_6f complex alone may be insufficient for preventing over-reduction of the PQ pool. In contrast, no indication of quinol oxidase activity in thylakoids was found for a second alternative oxidase encoded by the *ctaII* genes.

The cyanobacterium *Synechocystis* 6803[1] has become popular for genetic studies, as it is easily transformable and its whole genomic sequence is known (*1*). These features and the fact that the apparatus of oxygenic photosynthesis in cyanobacteria is homologous to the one in green algae and higher plants make *Synechocystis* 6803 an ideal model organism for the study of oxygenic photosynthesis.

Both thylakoid and cytoplasmic membranes of *Synechocystis* 6803 harbor a respiratory electron transport chain (*2*, *3*). The type-1 NADPH dehydrogenase (NDH-1) complex from cyanobacteria and higher plants (*4*, *5*) prefers NADPH over NADH as substrate. Additionally, the genome of *Synechocystis* 6803 contains genes for three proteins homologous to type-2 NADH dehydrogenase (NDH-2), which probably have a regulatory role (*6*). *Synechocystis* 6803 also has an active succinate dehydrogenase (*7*). The well-characterized cytochrome oxidase (COX = CtaI) (*8*) is a terminal oxidase found in both thylakoid and cytoplasmic membranes. Additionally, there are two alternative oxidases, CtaII and Cyd (*9*−*13*), the localization and function of which is still under debate (see Discussion). In contrast to the purely respiratory cytoplasmic membrane, the thylakoid membrane harbors the two photosystems and performs both respiration and photosynthesis.

As both the plastoquinone (PQ) pool and the cytochrome b_6f (cyt b_6f) complex are shared by the photosynthetic and respiratory pathways (*14*, *15*), plastoquinone is the acceptor of electrons from photosystem II (PS2) and the respiratory dehydrogenases and, on the other hand, the source of electrons for photosystem I (PS1), the terminal oxidase COX, and possibly alternative oxidases. Therefore, the PQ pool is important for balancing respiratory and photosynthetic electron transport and for regulation of the different enzymes.

For monitoring the PQ pool, chlorophyll fluorescence (*16*−*20*) is a valuable tool, because it is a noninvasive method that can be applied on the in vivo system, thus avoiding preparational artifacts. In particular, the respiratory reactions have often been studied in isolated systems (*3*), and the physiological relevance of in vitro results often remains unclear. The information gained from fluorescence induction depends on the timescale of the measurement: in the range of seconds to minutes, state transitions of the cyanobacterial thylakoid membrane correlate with fluorescence changes (*21*, *22*), while in the range of milliseconds to a few seconds, changes in fluorescence yield reflect the redox state of Q_A, the primary quinone-type electron acceptor of PS2. The redox state of Q_A, in turn, depends on the availability of PQ molecules, and fluorescence induction measurements can provide information regarding the redox state of the PQ pool.

The aim of the present study is to elucidate the processes around the PQ pool in vivo, in particular the contribution of various respiratory protein complexes, by means of fluores-

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* Corresponding author. Phone: +49-234-3223634. Fax: +49-234-3214322. E-mail: matthias.roegner@ruhr-uni-bochum.de.

‡ Ruhr-Universität Bochum.

§ Arizona State University.

[1] Abbreviations: COX, cytochrome *c* oxidase, also referred to as CtaI; CtaII, secondary terminal oxidase with limited, if any, activity; Cyd, putative cytochrome *bd*-type quinol oxidase; cyt, cytochrome; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; NDH, NAD(P)H dehydrogenase; PCP, pentachlorophenol; PPBQ, phenyl-*p*-benzoquinone; PS1, photosystem 1; PS2, photosystem 2; PQ, plastoquinone; PQH_2, plastoquinol; SDH, succinate dehydrogenase; *Synechocystis* 6803, *Synechocystis* sp. strain PCC 6803; WT, wild type.

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cence induction. To this end, we use inhibitors of the different enzymes and deletion strains lacking one or more of the terminal oxidases (*11*). One important result is that cyt *bd*-type oxidase activity can be detected in thylakoid membranes, particularly when cells are grown at a higher light intensity.

MATERIALS AND METHODS

Materials. Cells were cultivated at a light intensity of 20 to 100 μE $m^{-2}s^{-1}$ for 5 days in BG-11 medium at 30 °C under aerobic conditions in Erlenmeyer flasks, either photoautotrophically or with 10 mM glucose (see figure legends). The following WT strains were used: Amsterdam (from Dr. R. van Walraven); London (from Dr. P. Nixon); Moscow (from Dr. S. Shestakov); Osnabrück (from Dr. H. Lill); and St. Louis (from Dr. H. B. Pakrasi). Additionally, we used deletion strains lacking one or more of the terminal respiratory oxidases (*11*).

Measurements. Fluorescence induction was measured in a laboratory-built setup at room temperature (23 °C) using a 1 × 1 cm cuvette with 2 mL sample volume. Cells were incubated at a chlorophyll concentration of 2.5 μM in the dark under stirring for 2 min (with or without further additions), followed by illumination. The light source was a halogen lamp with a filter combination (3 mm Schott GG 400, Balzers DT Blue, Balzers Calflex-3000) to produce light with a center wavelength of 445 nm, 60 nm fwhm, and an intensity of 205 μE $m^{-2}s^{-1}$, except for the data in Figure 3E, where the intensity was varied. Illumination was controlled by an electronic shutter (opening time, 2 ms). Detection was done at 686 nm by a photomultiplier, protected by a combination of Schott DAL 686 + 4 mm Schott RG 665. The time course of fluorescence in the presence of 10 μM DCMU is shown in Figure 1A; the levels of minimal (F_0), variable (F_V) and maximal (F_M) fluorescence (*23*) are indicated. We take the fast phase of fluorescence rise, concomitant with the shutter opening, as F_0. The ratio F_M/F_0 was independent of the incident light intensity over the range used in this study (Figure 1B). This implies that, even at high light intensity, during the opening time of the shutter no significant Q_A reduction, which would induce an apparent increase of the F_0 level and a decrease of the F_M/F_0 ratio, occurred. Therefore, the F_0 level that was determined is indicative of the true (dark adapted) F_0 level under all conditions used in this paper.

The hatched complementary area above the curve reflects the electron capacity of the acceptor side of PS2. We define the area ratio R as the complementary area, normalized to the total area (which equals $F_M \times \Delta t$). R^* refers to signals in the presence of 10 μM DCMU, R^*_{PPBQ} refers to signals obtained with DCMU after preincubation with PPBQ. As oxidized PPBQ quenches chlorophyll fluorescence, the cells were incubated in darkness for 2 min in the presence of 0.5 mM PPBQ, then DCMU was added to interrupt electron exchange between Q_A and the PQ pool (or PPBQ), and finally 5 mM ascorbate was added to reduce PPBQ (quinols do not quench fluorescence (*24*)). This procedure restores the full F_M yield of untreated samples. The presence of 10 μM DCMU was found to be saturating for the complete exclusion of PPBQ from the Q_B site, because higher DCMU concentrations did not alter the resulting value of R^*_{PPBQ}. Ascorbate alone did not affect the signals.

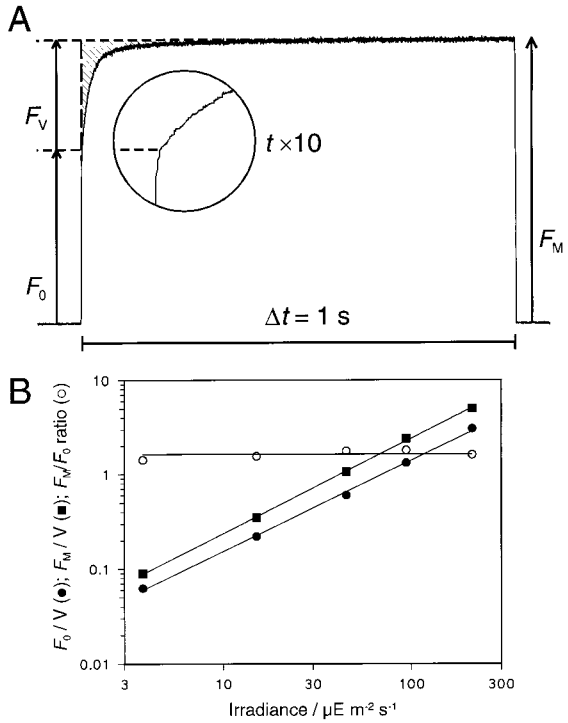

FIGURE 1: (A) Time course of fluorescence during illumination (205 μE $m^{-2}s^{-1}$) in the presence of 10 μM DCMU, supplemented by 10 μM DBMIB and 5 mM ascorbate. The insert has an expanded timescale. (B) Dependence of F_0, F_M, and the F_M/F_0 ratio on the light intensity.

As observed earlier (*15*) with cells of *Synechococcus* sp., when DCMU is used alone, the fluorescence yield continues to rise slowly after the F_M level has been reached. This slow component could be suppressed by using 10 μM DBMIB in all measurements with DCMU. DBMIB, in turn, was kept reduced by 5 mM sodium ascorbate in order to eliminate its effects as an electron acceptor or quencher of chlorophyll fluorescence (*15, 25*). Fluorescence induction curves, as shown in Figure 1A, were used to compare the various WT strains (see previous description). As no significant differences in fluorescence parameters such as the F_M/F_0 ratio or the rise time of F_V have been found, we conclude that the different laboratory strains are identical with respect to features relevant to the purposes of the present study.

Measurements of respiratory oxygen consumption of whole cells in darkness were done with a Clark-type oxygen electrode (Bachofer, Germany). Averages from multiple determinations are presented as "average ± standard deviation (n = number of experiments)".

RESULTS

Effects of the cyt b_6f Inhibitor DBMIB. When WT cells of *Synechocystis* 6803 are illuminated, a strong quenching of the variable fluorescence occurs (relative to the maximum fluorescence in the presence of DCMU), because plastoquinol is rapidly oxidized by PS1 via the cyt b_6f complex (Figure 2A). This is in striking contrast to the situation in higher plants, where PQH_2 oxidation at cyt b_6f is limiting and where, accordingly, the pool becomes completely reduced in the light. In *Synechocystis* 6803, a complete reduction of PQ during illumination can be observed only when the cyt b_6f complex is inhibited, for instance, by DBMIB (Figure 2B).

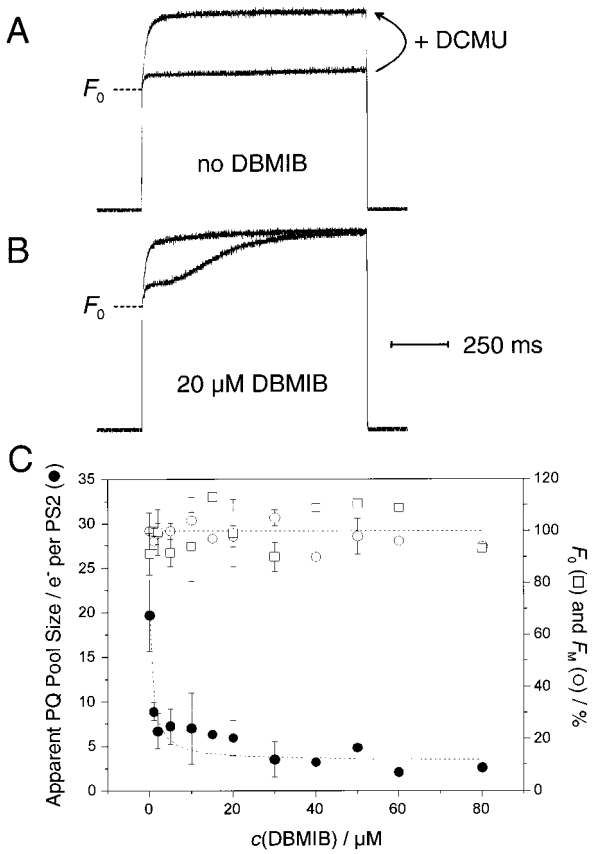

FIGURE 2: (A,B) Illumination of WT cells (grown in Erlenmeyer flasks at 20 μE m^{-2} s^{-1}) in the absence (A) or presence (B) of 20 μM DBMIB + 5 mM sodium ascorbate. To obtain the F_M level, the samples were illuminated a second time after the addition of 10 μM DCMU, as shown in (A). (C) Effect of the DBMIB concentration (in the presence of 5 mM ascorbate) on the apparent PQ pool size (closed circles, left ordinate) and on the size of the F_0 and F_M signal (measured in the presence of 10 μM DCMU; open symbols, right ordinate). The PQ pool size was calculated using eq 1; as this equation yields correct values only at saturating DBMIB concentration, the pool size is referred to as "apparent".

The number N of available oxidized equivalents in the pool per PS2 (i.e., twice the number of oxidized PQ molecules) can be determined from a comparison of the relative complementary areas R^* (which corresponds to one electron stored in Q_A) and R_{DBMIB} (which corresponds to the electron capacity of the whole PQ pool including Q_A)

$$N = \frac{R_{DBMIB} - R^*}{R^*} \quad (1)$$

However, this computation requires a saturating concentration of DBMIB; therefore, the effect of the DBMIB concentration on N was titrated (Figure 2C, solid circles, left ordinate). N declines rapidly, the inhibitor constant of DBMIB is about 1 μM, and concentrations above 20 μM are fully saturating. On the other hand, high concentrations of DBMIB may be deleterious, because oxidized DBMIB can also act as an electron acceptor or a quencher of chlorophyll fluorescence. To exclude such undesired side effects, we used DBMIB in combination with 5 mM ascorbate to keep it reduced. Under these conditions, no quenching of chlorophyll fluorescence by DBMIB occurs; the fluorescence intensity of the F_M signal is independent of the DBMIB concentration (Figure 2C, open circles, right ordinate). At saturating DBMIB concentration, the curve in Figure 2C levels off at about $N = 4$. Assuming a total number of 10 PQ molecules/PS2 (*26*), this suggests that 80% of the PQ pool is reduced before illumination.

Dark State of Q_A. Given this high degree of PQ reduction in darkness, and taking into account that the equilibrium constant of electron transfer between Q_A and PQ may be quite low (see Discussion), the redox state of Q_A in darkness needs to be considered; the determination of N according to eq 1 is correct only when Q_A is fully oxidized in darkness. Indeed, this appears to be the case, as the data in Figure 2C show that the F_0 level was independent of the DBMIB concentration. Moreover, as will be shown later, the F_0 level was independent of the incubation time with DBMIB (see Figure 5). To further check the redox state of Q_A in DBMIB-treated cells, we compared the F_0 and F_M levels with and without incubation with the electron acceptor PPBQ (0.5 mM), followed by addition of 10 μM DCMU (data not shown). These fluorescence levels were unchanged, ($F_{M(-PPBQ)}/F_{M(+PPBQ)} = 0.99 \pm 0.18$ ($n = 10$); $F_{0(-PPBQ)}/F_{0(+PPBQ)} = 1.04 \pm 0.16$ ($n = 10$)), indicating that Q_A is essentially fully oxidized in darkness under the conditions used. However, R^* (illumination after incubation with DCMU) was significantly smaller than R^*_{PPBQ} (incubation with PPBQ to fully oxidize Q_A prior to addition of DCMU); the ratio of R^*/R^*_{PPBQ} was 0.63 ± 0.14 ($n = 10$). In parallel, the rise of F_V during illumination was slowed (the half time increased from 13 to 17 ms after PPBQ treatment, and a slow phase in the kinetics became much more prominent than in untreated cells). We interpret these effects of preincubation with PPBQ to be due to PPBQ-induced oxidation of the non-heme iron, as reported earlier for artificial quinones (*27*, *28*).

Evidence for Activity of Cyd in the Thylakoid Membrane. Figure 3A shows an induction curve in the presence of 20 μM DBMIB, obtained with WT cells grown photoautotrophically at 20 μE m^{-2} s^{-1}. Within 750 ms, the fluorescence reached the same value as in a control experiment (in the presence of DCMU). However, when cells had been grown at higher light intensity (100 μE m^{-2} s^{-1}), a gap in the maximal fluorescence level remained between the curve of cells in the presence of DBMIB versus in the presence of DCMU (Figure 3B); despite a fully inhibitory concentration of DBMIB, a fraction of F_V remained quenched. Additionally, the half time of the F_V rise increased (from about 150 ± 50 ms to 220 ± 50 ms) under conditions where the full yield of F_M was not achieved.

Figure 3C shows that the F_V quenching was more pronounced for WT cells grown photomixotrophically, being already apparent in cells grown at 20 μE m^{-2} s^{-1}. The quenching of F_V was still observed when the illumination period was four times longer and the DBMIB concentration five times higher than before (Figure 3C). The dependence of this F_V quenching on the culture conditions is shown in Table 1. An increase in the DBMIB-insensitive quenching of F_V was induced by addition of glucose or increase of the light intensity during growth.

A possible explanation for quenching of F_V in the presence of DBMIB is a bypass around cyt b_6f via an alternative quinol oxidase. A candidate for such a reaction is the cyt *bd*-type complex known from the genomic sequence of *Synechocystis* 6803. The combined action of DBMIB and PCP, an inhibitor of cyt *bd* oxidases (*9*), in fact yielded the full amount of F_V

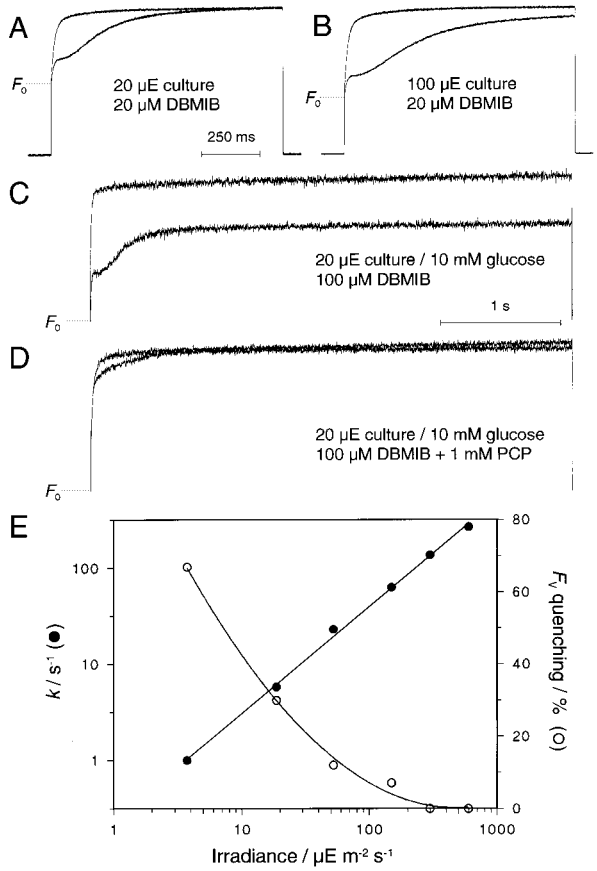

FIGURE 3: (A−D) Fluorescence induction curves for WT cells under different culture and measurement conditions. Illumination for 1 s (A,B) or 4 s (C,D). Each panel shows a pair of curves, of which the lower one was recorded in the presence of the indicated additions. The upper curve is the reference signal and shows the full yield of F_V in the presence of 10 μM DCMU. Parts C and D have an expanded scale at the ordinate to show the variation in F_V more clearly. (E) Effect of irradiance intensity during fluorescence induction in the presence of 50 μM DBMIB on the rate of Q_A reduction (solid circles, left ordinate; obtained from the half time of F_V rise as $k = \ln 2/t_{1,2}$) and the amount of F_V quenching (open circles, right ordinate). Cells in A and B have been grown without glucose in an airlift fermenter (300 mL) with air enriched in CO_2 (5%) and cells in C−E with 10 mM glucose in Erlenmeyer flasks at 20 (C,D) or 50 μE m^{-2} s^{-1} (E).

Table 1: Quenching of F_V in the Presence of 20 μM DBMIB for WT Cells Grown under Different Conditions[a]

culture conditions	quenching of F_V/%
air, no glucose, 20 μE m^{-2} s^{-1}	2 ± 0.5 (n = 8)
air, 10 mM glucose, 20 μE m^{-2} s^{-1}	21 ± 13 (n = 16)
air, no glucose, 50 μE m^{-2} s^{-1}	10 ± 9 (n = 14)
air, 10 mM glucose, 50 μE m^{-2} s^{-1}	28 ± 10 (n = 14)
air + 5% CO_2, no glucose, 20 μE m^{-2} s^{-1}	3 ± 0.2 (n = 4)
air + 5% CO_2, no glucose, 100 μE m^{-2} s^{-1}	22 ± 2 (n = 4)

[a] The illumination period was 1 s.

(compare parts C and D of Figure 3), while PCP alone had no significant effect (not shown). Figure 3E shows the effect of varying the irradiance during fluorescence induction of photomixotrophically grown cells in the presence of DBMIB. The rate of Q_A reduction (obtained from the rise time of F_V) correlated linearly with light intensity. At the same time, the amount of F_V quenching declined to zero at high irradiance. This agrees with the concept that the balance of two rates determines the steady-state level of F_V; a light-dependent PQ reduction via PS2 and a light-independent PQH_2 oxidation by a quinol oxidase.

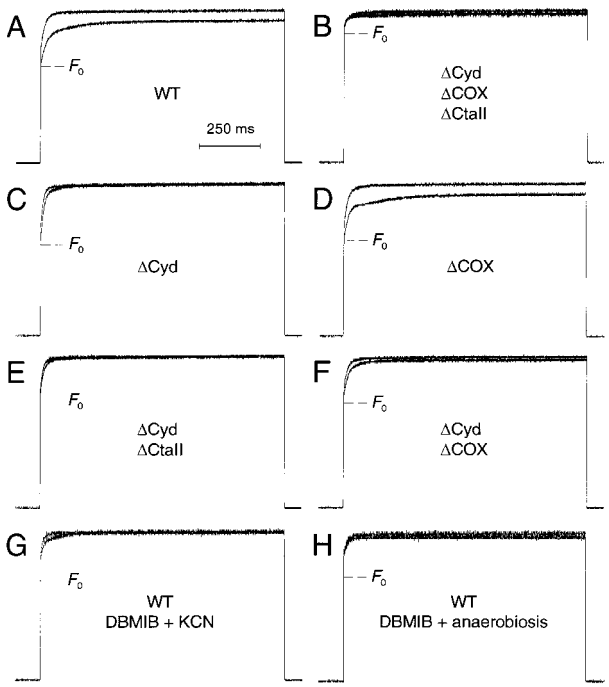

FIGURE 4: Fluorescence induction curves for WT and several mutant strains lacking one or more of the terminal oxidases (Cyd, cyt *bd*-type complex; COX, cyt *c* oxidase; CtaII, COX homologue of unknown function). All strains were cultured at 50 μE m^{-2} s^{-1} with 10 mM glucose; the illumination time was 1 s. Each panel shows a pair of induction curves, of which the lower one was recorded in the presence of 50 μM DBMIB. The upper curve is the reference signal and shows the full yield of F_V of the sample in the presence of 10 μM DCMU. Quenching of F_V was 28 ± 10% (n = 14) for WT (A) and 18 ± 6% (n = 8) for the Δ*C*OX strain (D). WT cells in the presence of 50 μM DBMIB + 1 mM KCN (G) or 50 μM DBMIB under anaerobic conditions (10 mM glucose, 400 U/mL catalase and 24 U/mL glucose oxidase) (H) lacked measurable F_V quenching under our experimental conditions.

We also investigated deletion strains (*11*) lacking one or more of the oxidases. The results (Figure 4) support the assumption that the F_V quenching can be attributed specifically to the cyt *bd*-type oxidase: No strain lacking this complex (parts B, C, E and F of Figure 4) showed the marked F_V quenching observed in WT, while deletion of COX did not remove the effect (Figure 4D). In additional support of our attribution of F_V quenching to quinol oxidase activity, in the presence of both DBMIB and KCN, which is an inhibitor of all respiratory oxidases in *Synechocystis* 6803, quenching was not observed (Figure 4G). F_V quenching also was absent in the presence of DBMIB under anaerobic conditions (Figure 4H).

The kinetics of PQ reduction in WT cells in darkness were investigated by variation of the DBMIB incubation time before the induction measurement (Figure 5). For cells grown photoautotrophically (50 μE m^{-2} s^{-1}, ambient CO_2 concentration, triangles in Figure 5), the PQ pool became transiently reduced within about 30 s and returned slowly to a more oxidized state at longer times. For cells cultured in the presence of 10 mM glucose (Figure 5, squares) or at elevated CO_2 concentration (Figure 5, circles) the PQ pool was less reduced. However, under these culture conditions, a significant contribution of Cyd occurred (see Figure 3 and Table

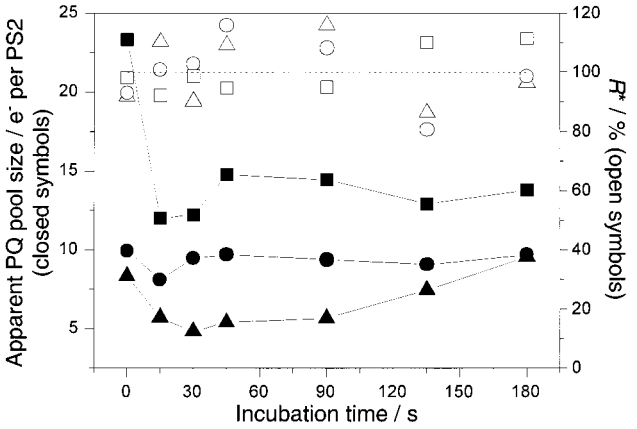

FIGURE 5: Determination of the apparent PQ pool size and redox state of Q_A as a function of the incubation time with 50 μM DBMIB and 5 mM ascorbate. The different symbols indicate the following growth conditions: (▲/△) Erlenmeyer culture without additions; (■/□) Erlenmeyer culture with 10 mM glucose; (●/○) airlift fermenter aerated with air + 5% CO_2. In all cases, the incident light intensity during growth was 50 μE m^{-2} s^{-1}. The PQ pool size was calculated using eq 1. *N* reflects the actual pool size only in the absence of PQH_2 oxidation not involving cyt b_6f (i.e., when no significant F_V quenching occurs); therefore, the pool size is referred to as "apparent". For clarity, no error bars are shown; the standard deviation of *N* is in the range of ± 2 e^- for all measurements and about ± 10% for *R**.

Table 2: Effects of Inhibitors on Oxygen Consumption of Whole Cells (at 15 μM chlorophyll) in Darkness[a]

strain	measurement conditions	rate of respiration (%)
WT	20 μM DBMIB + 5 mM ascorbate	18 ± 3
	1 mM PCP	89 ± 4
	5 mM KCN	2 ± 1
ΔCyd	20 μM DBMIB + 5 mM ascorbate	5 ± 2
	1 mM PCP	104 ± 6

[a] Cells were cultivated at 50 μE m^{-2} s^{-1} with 10 mM glucose. Each rate was determined using three independent cultures. The control rate for each strain (in the range of 30−40 μmol O_2 (mg Chl)$^{-1}$ h^{-1}) was set to 100%.

1); therefore, the PQ pool size cannot be precisely determined using eq 1.

The effects of DBMIB and PCP on respiration were tested in cells grown at 50 μE m^{-2} s^{-1} in the presence of glucose (Table 2). While, in the presence of 20 μM DBMIB, the respiratory rate of the WT declined to 18% of the control, it remained at 89% in the presence of 1 mM PCP. This shows that the cyt b_6f complex provided the major route for PQH_2 oxidation. Oxygen consumption in the ΔCyd strain was insensitive to PCP, indicating that this inhibitor acts specifically on the cyt *bd*-type complex. As expected, DBMIB suppressed virtually all oxygen uptake in the ΔCyd strain.

DISCUSSION

Fluorescence spectroscopy is a valuable method for studying photosynthesis, and its potential for investigating the PQ pool in cyanobacteria was demonstrated two decades ago in *Synechococcus* sp. (*14*, *15*). In this report, we used this method to investigate the redox reactions around the PQ pool of *Synechocystis* 6803, focusing on the contributions of respiratory reactions. Figure 6 shows a model of the electron transport routes and sites of inhibitor action, based on both the results of this report and published data.

Localization of Cyd in the Thylakoid Membrane. Several experiments showed a residual quenching of F_V (Figures 3 and 4 and Table 1), despite saturating concentrations of the cyt b_6f inhibitor DBMIB and a sufficiently long illumination period. Oxidized DBMIB is a quencher of PS2 fluorescence, but DBMIB was reduced by a large excess of ascorbate and, according to Figure 2C, the quenching of F_V was not caused by DBMIB itself. The most plausible explanation for the observed phenomenon is the inducible activity of a quinol oxidase near PS2 (i.e., presumably in thylakoids), providing an alternate route for electrons. The putative cyt *bd*-type quinol oxidase from *Synechocystis* 6803 is by far the best candidate for this activity, as PCP, which is an inhibitor of cyt *bd* oxidase (*9*), in combination with a cyt b_6f inhibitor could completely suppress PQH_2 oxidation and induce the full yield of F_V (Figure 3D). This was confirmed by the observation of a full F_V yield in the presence of both DBMIB and KCN (inhibiting all terminal oxidases) (Figure 4G) and in the presence of DBMIB under anaerobic conditions (Figure 4H). Moreover, studies on oxidase deletion strains (*11*) further supported our interpretation. The signals from Cyd-less strains in the presence of DBMIB resemble those from WT in the presence of DBMIB + PCP (Figure 4B−F). While expression and activity of the cyt *bd*-type complex have already been shown in *Synechocystis* 6803 (*9*, *11*−*13*), localization and function of this complex are still controversial. In ref *11*, a genetic approach was used, and the authors concluded that Cyd was located only in the cytoplasmic membrane. The data presented here, indicating a localization in the thylakoid membrane, are not necessarily in contradiction to ref *11*, because the activity level of Cyd strongly depends on culture conditions. Moreover, a crucial experiment in ref *11* was the measurement of the decay rate of Q_A^- in darkness after reduction of the PQ pool by light. This rate was not changed in the Cyd deletion strain compared to WT, and it was concluded that the cyt *bd*-type complex does not oxidize PQH_2 (and indirectly, Q_A^-) in thylakoids. However, the activity of the alternative oxidase was monitored against a high-background activity of COX, which could mask minor changes induced by deletion of Cyd, especially because a slight increase of cyt b_6f (or COX) activity in the deletion strain might compensate for the loss of the cyt *bd*-type complex.

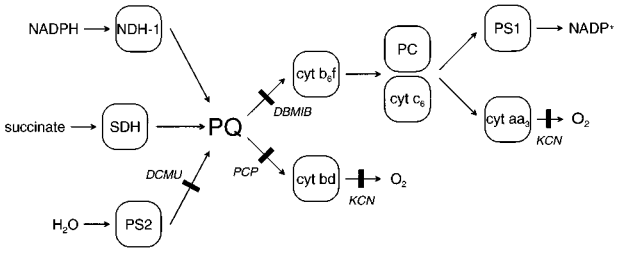

FIGURE 6: Routes of electron transfer and sites of action for different inhibitors in the thylakoid membrane of *Synechocystis* 6803. For details, see Discussion.

We recently analyzed light-induced proton translocation in whole cells of *Synechocystis* 6803 using fluorescent pH indicators (*29*). Acridine Yellow monitors ATP-driven proton extrusion at the cytoplasmic membrane; this process is indirectly light-dependent, because it consumes ATP generated at the thylakoid membrane. In the presence of DBMIB, a residual signal in the range of 20% of the control remained (*29*), which probably indicates ATP production utilizing the pH gradient formed across the thylakoid membrane by

electron transfer from PS2 to the quinol oxidase ($H_2O \rightarrow$ PS2 $\rightarrow$ PQ $\rightarrow$ Cyd $\rightarrow O_2$). Another dye, Acridine Orange, monitors directly the light-induced acidification of the lumen. Using this dye in the presence of DBMIB, a similar decline of the luminal signal to about 20% of the control value was observed, and as expected, for both dyes, the light-induced signals were completely absent upon combined use of DBMIB and PCP (Berry, S., Teuber, M., and Rögner, M., unpublished data; experimental details as in ref *29*). These data indicate that electron transport from PS2 to Cyd can make a small but, nevertheless, detectable contribution to proton pumping across the thylakoid membrane, and they support the conclusions based on chlorophyll fluorescence presented here. Data on the flash-induced turnover of cyt *f* also provide evidence for the presence of an oxidase in thylakoid membranes, which competes with the cyt b_6f complex for electrons from plastoquinol (*12*, *30*).

In addition to the strong dependence of Cyd activity on culture conditions, we have observed fluctuations in the apparent redox state of the PQ pool after different incubation intervals of the cells with DBMIB (Figure 5). Such fluctuations in the PQ redox state may be interpreted as temporal variations in the activity of Cyd or the dehydrogenases (*7*). According to Figure 5, the redox state of the PQ pool was most reduced 15−30 s after DBMIB/ascorbate addition but recovered thereafter. This may be explained by a rerouting of electrons to the cyt *bd*-type complex when COX is inhibited; this explanation is also supported by investigations of respiration using an oxygen electrode (Pils, D., and Schmetterer, G., personal communication). This short-term effect obviously involves activation of Cyd which is already present in the cells. The response at the level of de novo protein synthesis is expected to be much slower. Indeed, incubation of photoautotrophically grown cells with 10 mM glucose for up to 2 h did not increase the amount of F_V quenching (data not shown); the differences between cultures ± glucose, as presented in Table 1, became obvious only about 24 h after the addition of glucose.

In addition to COX and Cyd, the genome of *Synechocystis* 6803 contains the *ctaII* genes encoding a third oxidase. Presently, it is not yet clear whether this oxidase uses PQH_2 or cyt *c* as its direct electron donor. If it is active under the conditions of our experiments, it does not serve as a quinol oxidase in thylakoids; in a double mutant lacking both COX and Cyd (Figure 4F), there was no significant quenching of F_V, indicating that CtaII did not oxidize PQH_2 in thylakoids at a significant rate. This is in agreement with recently published results (*13*), which indicate that CtaII acts as a cyt *c* oxidase and is located exclusively in the cytoplasmic membrane.

Physiological Role of the Alternative Pathway for Plastoquinol Oxidation. As observed for other components of the cyanobacterial thylakoid membrane (*31*−*33*), the content of Cyd seems to be regulated in response to environmental factors, because the amount of F_V quenching depends on culture conditions (Figure 3 and Table 1). Presence of glucose in the growth medium, which should facilitate the reduction of the PQ pool via the respiratory dehydrogenases, increased the activity of the cyt *bd*-type complex in thylakoids: For this reason, it is plausible that one function of this complex is to prevent over-reduction of the pool. This, however, cannot be the only role of Cyd, because its activity was also increased at higher light intensity, which gives rise to a more oxidized PQ pool, due to the high PS1/PS2 stoichiometry in *Synechocystis* 6803.

The branching of electron transport chains via multiple oxidases is observed in many organisms. Cytochrome *bd* complexes are widespread among bacteria (*34*−*38*), and in light of the cyanobacterial ancestry of plastids, the presence of such a complex in chloroplasts would make sense (*12*), but no evidence for this has been presented so far. Mitochondria from protozoa, fungi, and plants contain a cyanide-insensitive alternative oxidase (AOX) with a di-iron center as cofactor (*39*−*45*). A homologue of AOX termed IMMUTANS has recently also been identified in plastids from *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* (*42*, *46*). Plastidal AOX is involved in the oxidation of plastoquinol in algae and higher plants (*46*, *47*) during chlororespiration (*48*). This pathway seems to provide an electron sink for carotenoid desaturation and is particularly active in growing cells and tissues (*49*, *50*). The cyt *bd*-type complex may fulfill an analogous role in cyanobacteria, and in this respect, it is interesting that Cyd activity increases when an external energy supply (glucose or increased light intensity) accelerates cell growth.

AOX from plant mitochondria is specifically activated by organic acids (*51*−*53*), and it seems that a major function of plant AOX is the adjustment of the metabolic turnover of carbon skeletons, in particular in the citric acid cycle (*43*, *44*). In line with this hypothesis, plant AOX can operate at a high rate, even when the cyt bc_1/cyt *c*/COX pathway is not saturated, indicating that the state of the ubiquinone pool alone is not the crucial regulating factor (*43*). Similarly, in *Synechocystis* 6803, the activity of the alternative pathway appears to be coupled to the metabolic activity of the cell, which may be increased by either high light intensity or the external supply of glucose.

Equilibrium between Q_A and PQ. In contrast to higher plants, the PQ pool of cyanobacteria is partially reduced in the dark by respiratory dehydrogenases. When the cyt b_6f complex was inhibited by a saturating concentration of DBMIB (Figure 2C), the number of oxidizing equivalents in the PQ pool after dark incubation was about 2 PQ/PS2 for cells grown photoautotrophically. This corresponds to about 80% pool reduction, assuming 10 PQ/PS2 (published data indicate about 8 ± 2 PQ/PS2 for cyanobacteria (*14*, *26*, *54*) and 9 ± 3 for higher plants (*55*−*62*)). A similar value of 3 oxidizing equivalents per PS2 was found for *Synechococcus* sp. (*15*). In this organism, however, the PQ pool was readily oxidized by aeration in the dark for less than 2 h; this treatment depleted the cell's storage of reducing equivalents. In contrast, *Synechocystis* 6803 shows much longer respiration in darkness; the yield of variable fluorescence upon illumination was higher after a 6-h dark incubation, and the value of R_{DBMIB} declined to 80%, as compared to light adapted cells (not shown). Both effects indicate a rather reduced state of PQ, even after 6 h in darkness. This coincides with results from other groups; in one study (*63*), it was necessary to dark incubate *Synechocystis* 6803 for 32 h to eliminate respiration. Likewise, the cellular ATP level remained at 90−100% of the light-adapted control after 6 h dark incubation and at 60−80% after 24 h dark incubation, indicating that respiration remained active (*25*).

Because there is evidence for a low equilibrium constant between PQ and Q_A in whole cells of *Synechocystis* 6803 (*7*) and in pea thylakoids (*64*), a high degree of PQ reduction might give rise to a concomitant reduction of Q_A. As shown in the Results section, no evidence for a partially reduced dark state of Q_A was found for DBMIB-inhibited cells using the artificial electron acceptor PPBQ. Furthermore, the redox state of Q_A was independent of the DBMIB concentration (at a constant incubation time of 2 min; Figure 2C) and of the incubation time (at a constant concentration of 50 μM; Figure 5). However, when both cyt b_6f and the cyt *bd*-type complex were blocked by a combination of DBMIB and PCP, the degree of PQ reduction increased even more (Figure 3D), and also, Q_A became partially reduced. R^* declined to $52 \pm 12\%$ ($n = 9$) as compared to the value without PCP, and N was 0.42 ± 0.22 ($n = 9$) (data not shown; the same effect occurred in the presence of DBMIB + KCN or in the presence of just DBMIB under anaerobic conditions). These figures indicate an approximate 50% reduction of Q_A at about 98% reduction of PQ, compatible with an equilibrium constant in the range of 50. The theoretical value can be calculated from the redox potentials. For Q_A, there is considerable variation in the published data, with the most reliable value seeming to be $E_m(Q_A) = -80$ mV (*65*). For PQ around neutral pH, the literature data are in the range of $E_m(PQ) = +80$ to $+110$ mV (*56*, *62*, *66*−*73*). On the basis of such a large potential difference, K_{eq} should be much higher than the empirical value estimated here. On the other hand, our results agree with experimental data that indicate a low K_{eq} (*7*, *64*). On the basis of our results, an operational ΔE_m of Q_A/PQ of about 100 mV is expected.

Conclusions. (1) By fluorescence induction measurements, the alternative quinol oxidase of the cyt *bd*-type was shown to participate in electron transport in thylakoid membranes of *Synechocystis* 6803, and we could show that the activity of this enzyme is dependent on the culture conditions. (2) A key function of this complex in the thylakoid may be to prevent overreduction of the PQ pool under stress conditions. Besides this, the cyt *bd*-type complex may also be involved in other physiological functions, similar to the alternative oxidase in fungal and plant mitochondria and the IMMUTANS protein in plastids from green algae and plants. (3) PQ can become significantly reduced in the dark; the equilibrium constant between Q_A and PQ was estimated to about 50, which is significantly lower than expected from published redox potentials.

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