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# Genes Encoding A-Type Flavoproteins Are Essential for Photoreduction of $O_2$ in Cyanobacteria

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## Summary

**$O_2$ photoreduction by photosynthetic electron transfer, the Mehler reaction [1], was observed in all groups of oxygenic photosynthetic organisms [2–4], but the electron transport chain mediating this reaction remains unidentified. We provide the first evidence for the involvement of A-type flavoproteins that reduce $O_2$ directly to water in vitro. *Synechocystis* sp. strain PCC 6803 mutants defective in *flv1* and *flv3*, encoding A-type flavoproteins, failed to exhibit $O_2$ photoreduction but performed normal photosynthesis and respiration. We show that the light-enhanced $O_2$ uptake was not due to respiration or photorespiration. After dark acclimation, photooxidation of $P_{700}$ was severely depressed in mutants Δ*flv1* and Δ*flv3* but recovered after light activation of $CO_2$ fixation, which gives $P_{700}$ an additional electron acceptor. Inhibition of $CO_2$ fixation prevented recovery but scarcely affected $P_{700}$ oxidation in the wild-type, where the Mehler reaction provides an alternative route for electrons. We conclude that the source of electrons for $O_2$ photoreduction is PSI and that the highly conserved A-type flavoproteins Flv1 and Flv3 are essential for this process in vivo. We propose that in cyanobacteria, contrary to eukaryotes, the Mehler reaction produces no reactive oxygen species and may be evolutionarily related to the response of anaerobic bacteria to $O_2$.**

## Results

Information in the cyanobase (http://www.kazusa.or.jp/cyano/cyano.html) and our own sequence analysis suggested that the genome of *Synechocystis* sp. strain PCC 6803 (hereafter *Synechocystis* PCC 6803) contains several genes encoding putative flavoproteins including *sll1521*, *sll0219*, *sll0550*, and *sll0217* (designated *flv1*, *flv2*, *flv3*, and *flv4*, respectively). In vitro biochemical and biophysical analyses of expressed proteins confirmed that they belong to the A-type flavoproteins, exhibiting typical spectral and FMN/FAD binding properties [5, 6]. Their role, in vivo, is not known but the presence of the metal β-lactamase, flavodoxin, and flavin reductase domains (from the N to the C terminus) suggest participation in electron transfer processes.

On the basis of their sequence similarity, the *flv* genes can be subdivided into two pairs, *flv1/flv2* and *flv3/flv4,* with a high degree of homology within each pair. Mutants Δ*flv1*, Δ*flv2*, Δ*flv1*/Δ*flv2*, Δ*flv3*, Δ*flv4*, and Δ*flv3*/Δ*flv4* were constructed (see Experimental Procedures in Supplementary Material available with this article online). Under the conditions used in our studies, the growth rates of the mutants were similar to that of the wild-type (not shown). Further, the steady-state rates of net photosynthesis and net dark respiration (calculated from the rate of $O_2$ evolution in the light or $O_2$ uptake in the dark, respectively) did not differ markedly between the wild-type and the mutants (see Table S1 in Supplementary Material).

### Light-Dependent $O_2$ Uptake Is Inhibited in Δ*flv1* and Δ*flv3* Mutants

In oxygenic photosynthetic organisms, including cyanobacteria, the Flv proteins might conceivably be involved in various electron transport processes including $O_2$ evolution due to cleavage of water molecules in photosynthesis and/or concomitant $O_2$ consumption in respiration, photorespiration, and the Mehler reaction. The latter can best be followed as $O_2$ uptake upon illumination of dark-adapted cells. Simultaneous measurements of $^{18}O$-$^{18}O$ uptake (provided as a tracer to the cell suspension) and net $^{16}O$-$^{16}O$ evolution by dark-adapted cells [7] using a membrane inlet mass spectrometer (MIMS) enabled assessment of $O_2$ consumption concomitantly with $O_2$ formation due to cleavage of water molecules. Illumination strongly stimulated $O_2$ uptake by dark-adapted wild-type (Figure 1A) and mutants Δ*flv2* (Figure 1C) and Δ*flv4* (Figure 1F). In contrast, mutants where either *flv1* or *flv3* was inactivated, i.e., Δ*flv1* (Figure 1B), Δ*flv3* (Figure 1E), Δ*flv1*/Δ*flv2* (Figure 1D), and Δ*flv3*/Δ*flv4* (Figure 1G), did not exhibit the light-enhanced $O_2$ uptake.

In cyanobacteria, the respiratory and photosynthetic electron transport pathways share components including the plastoquinone pool and cytochrome *b6f* [8]. Further, in addition to the photoreduction of $O_2$, the light-enhanced $O_2$ uptake could conceivably stem from respiratory (involving cytochrome- and/or quinone-dependent oxidases) or photorespiratory (ribulose bisphosphate oxygenase and glycolate oxidase) $O_2$ consuming processes. Mutant Δ*ctaDIEI*/*ctaDIIEII*/*cydAB* of *Synechocystis* PCC 6803, defective in genes encoding the cytochrome oxidases and quinol oxidase, does not exhibit respiratory $O_2$ uptake in the dark [9, 10]. Nevertheless, it displays normal light-enhanced $O_2$ uptake (Figure 2A), indicating that this process is not due to

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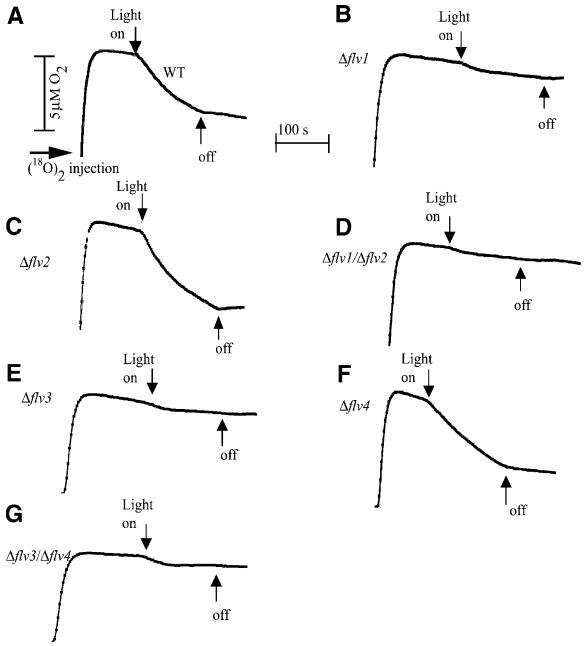


Figure 1. Time Course for Light-Dependent $^{18}O$-$^{18}O$ Uptake by Dark-Adapted *Synechocystis* PCC 6803 and *flv* Mutants

(A) Wild-type; (B) mutant Δ*flv1*; (C) mutant Δ*flv2*; (D) mutant Δ*flv1*/Δ*flv2*; (E) mutant Δ*flv3*; (F) mutant Δ*flv4*; and (G) mutant Δ*flv3*/Δ*flv4*. $O_2$ exchange was measured by means of membrane inlet mass spectrometer (see Supplementary Material). The density of the cell suspensions corresponded to 5 μg chlorophyll $ml^{-1}$, light intensity was 50 μmol photons $m^{-2}$ $s^{-1}$, and temperature was 30°C. Note that the slope of the curves depicted here does not provide a precise quantitative measure of the rate of $O_2$ consumption (see Supplementary Material).

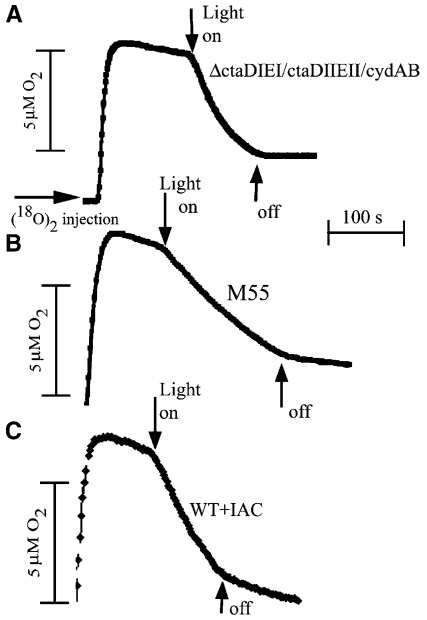


Figure 2. Time Course for Light-Dependent $^{18}O$-$^{18}O$ Uptake by Mutants and by Wild-Type Cells Treated with Iodoacetamide (IAC)

(A) Mutant Δ*ctaDIEI/ctaDIIEII/cydAB* defective in genes encoding terminal oxidases.

(B) Mutant M55 where *ndhB* encoding subunit II of NAD(P) dehydrogenase (NDH-1) was inactivated.

(C) *Synechocystis* PCC 6803 treated with 8 mM IAC 10 min before illumination.

The density of the cell suspensions corresponded to 7.1 μg Chl $ml^{-1}$ in (A), 3.3 μg Chl $ml^{-1}$ in (B), and 7.3 μg Chl $ml^{-1}$ in (C). Other conditions as in Figure 1.

the activity of terminal respiratory oxidases. Further, the *flv1* and *flv3* gene products are probably not directly involved in dark respiration, the rate of which is similar in the Δ*flv* mutants and the wild-type (see Supplementary Table S1). The Flv1 and Flv3 proteins are also not directly involved in electron flow from the NADH dehydrogenase complex (NDH-1) to the plastoquinone pool since inhibition of this path in mutant M55 of *Synechocystis* PCC 6803, by inactivation of *ndhB* [11], scarcely affected the light-enhanced $O_2$ uptake (Figure 2B).

The light-enhanced $O_2$ uptake was not due to photorespiration, as indicated by the fact that wild-type cells treated with iodoacetamide (IAC), which completely blocks $CO_2$ fixation in *Synechocystis* PCC 6803 [12] and consequently the photorespiratory $O_2$ uptake activity, exhibited normal light-dependent $O_2$ uptake (Figure 2C). Further, since the $CO_2$ concentration was saturating for $CO_2$ fixation, the contribution of photorespiration to $O_2$ uptake in the above experiments was probably small. Moreover, when dark-adapted cells are illuminated, they show a lag phase in $CO_2$ fixation since several of the enzymes involved in the Calvin cycle are not active in darkness and must undergo activation by light-driven electron transport [13]. Nevertheless, the light-enhanced $O_2$ uptake was already observed during the lag phase in $CO_2$ fixation (and net $O_2$ evolution). We conclude that *flv1* and *flv3* are not involved in photorespiration but probably take part in electron transport to $O_2$, i.e., in the Mehler reaction. Both Flv1 and Flv3 are essential since inactivation of either *flv1* or *flv3* abolished this activity. On the other hand, *flv2* and *flv4*, though highly homologous to *flv1* and *flv3*, are not required.

### Flv1 and Flv3 Participate in the Oxidation of $P_{700}$ by Oxygen

If Flv1 and Flv3 are essential for photoreduction of $O_2$ by electron transfer from PSI, inactivation of these genes might alter the steady-state oxidation level of $P_{700}$ in illuminated mutant cells. We therefore measured the kinetics of light-driven oxidation of $P_{700}$ following illumination of dark-adapted wild-type and mutant cells (Figure 3). When wild-type cells were illuminated for 1 s, the level of $P_{700}^+$ rose transiently but fell immediately after the light was turned off. When exposed to longer illumination, the $P_{700}$ oxidation signal reached a maximal steady-state value (within about 1 s), which persisted as long as the illumination continued (Figure 3A). This indicates that $P_{700}$ was able to transfer electrons and that a carrier capable of accepting these electrons was available during the entire light period (even prior to the activation of carbon fixation). Identical results were obtained with mutants Δ*flv4* (Figure 3B) and Δ*flv2* (not shown). In contrast, in mutants where either *flv1* or *flv3* was inactivated, illumination for either 1 or 10 s resulted in only a brief (1 s) transient rise in the level of $P_{700}^+$

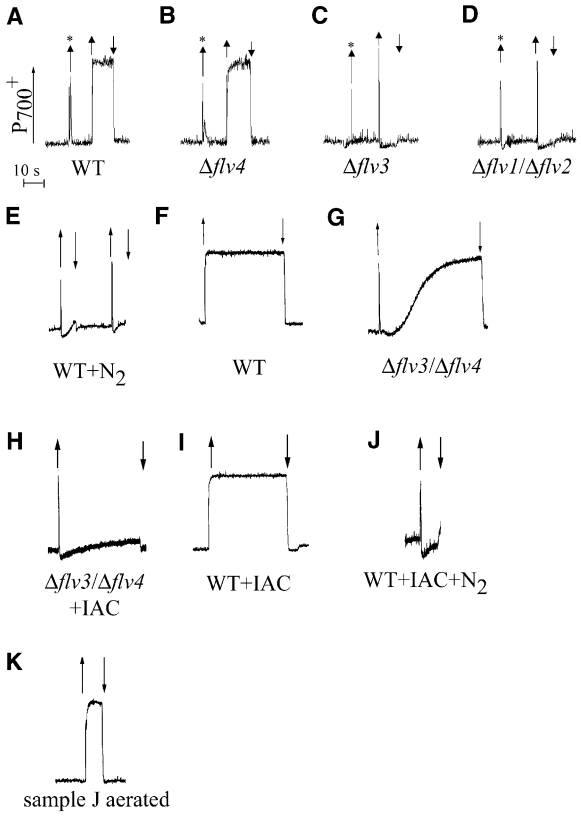


Figure 3. Oxidation Level of $P_{700}$, Reflecting the Availability of Electron Acceptors, as Affected by the Presence of $O_2$ and Various Illumination Regimes

$P_{700}$ oxidation was induced by exposure to actinic light (650 nm, 40 μmol photons $m^{-2}$ $s^{-1}$) for various durations at room temperature. Arrow with asterisk, a 1 s pulse of light; upward and downward arrows, actinic light on and off, respectively.

(A) Wild-type cells; (B) Δ*flv4*; (C) Δ*flv3*; (D) Δ*flv1*/Δ*flv2*; (E) wild-type cells under anaerobic conditions; (F and G) wild-type and mutant Δ*flv3*/Δ*flv4*, respectively, illuminated for 50 s period; (H) mutant Δ*flv3*/Δ*flv4* preincubated, in the dark for 10 min, with 10 mM of iodoacetamide (IAC); (I) wild-type cells treated with IAC as in (H); (J) wild-type treated with IAC as in (I) but under anaerobic conditions; and (K) sample (J) after reaeration. Cell densities corresponded to 20 μg Chl $ml^{-1}$.

followed by a rapid fall to the dark level (shown for mutants Δ*flv3* and Δ*flv1*/Δ*flv2*; Figures 3C and 3D, respectively). The results obtained for mutants Δ*flv3*/Δ*flv4* and Δ*flv1*, not shown, closely resembled those in Figures 3C and 3D. Apparently, a renewable acceptor of electrons from $P_{700}$ was lacking in these mutants. Clearly, prior to the activation of $CO_2$ fixation, those Δ*flv* mutants that were impaired in light-dependent $O_2$ uptake (Figure 1) were also impaired in $P_{700}$ oxidation, suggesting that the *flv* gene products participate in the electron transfer from $P_{700}$ to $O_2$. Depriving dark-adapted wild-type cells of $O_2$ resulted in inability to maintain $P_{700}$ oxidation during the illumination period (Figure 3E, see also Figure 3J). Essentially, under low $O_2$ levels, the $P_{700}$ oxidation pattern in the wild-type resembled that exhibited by the single or double Δ*flv1* or Δ*flv3* mutants (Figures 3C and 3D). $O_2$ evidently served as the main electron acceptor during oxidation of $P_{700}$, immediately upon illumination, for dark-adapted wild-type cells but not for mutants where either *flv1* or *flv3* was inactivated. As indicated in Figure 3F, a steady-state level of $P_{700}^+$ was reached about 1 s after illumination of dark-adapted wild-type cells and persisted during the light period. When mutants Δ*flv1* or Δ*flv3* were exposed to light for longer than 20 s, $P_{700}$ oxidation gradually increased (cf. Figure 3G) and the steady-state level was reached within about 40 s. Apparently an efficient acceptor of electrons from PSI became available during illumination of the mutants. Since the activation of photosynthetic $CO_2$ fixation requires light [13], the rising level of $P_{700}^+$ in mutant cells could stem from the increasing flow of electrons to $NADP^+$ during $CO_2$ fixation. To examine this possibility, we inhibited $CO_2$ fixation with IAC. This treatment resulted in total suppression of the rise in $P_{700}^+$ level in the mutant (Figure 3H) but not in the wild-type (Figure 3I). Apparently, flow of electrons to $O_2$ in the wild-type enabled their sustained transfer from $P_{700}$ even in IAC-inhibited cells. This is also indicated by the fact that removal of $O_2$ from suspensions of IAC-treated wild-type cells led to complete arrest of $P_{700}$ oxidation (Figure 3J), and aeration of this sample resulted in full recovery of the $P_{700}^+$ signal (Figure 3K). The results in Figure 3 show that $O_2$ can serve as an efficient electron acceptor from PSI in the wild-type and that inactivation of either *flv1* or *flv3* in the mutants abolishes this electron flux.

### Analysis of Electron Flow by Pulse-Modulated Fluorimetry

Depressed ability to oxidize $P_{700}$ in single or double mutants defective in either *flv1* or *flv3* (Figure 3) might result in slower oxidation of plastoquinol. The presence of the Fm and Fs signals in pulse-modulated fluorimetry experiments (see Supplementary Figure S1) showed that, as expected from the photosynthetic (see Supplementary Table S1) and growth capabilities, photosystem II was active in both the wild-type and the Δ*flv* mutants. Figure 4 provides the fluorescence parameters deduced from typical time course experiments (see Supplementary Figure S1). The PSII activity, measured as variable fluorescence (Fv/Fm = (Fm − Fo)/Fm), did not differ significantly between the wild-type and the mutants (Figure 4). However, the increase in level of fluorescence emitted during continuous illumination (Fs) was considerably larger in single or double mutants defective in either *flv3* or *flv1* than in wild-type (Figure 4). Consequently, (Fm − Fs)/Fm (indicative of oxidation/reduction state of the plastoquinone pool) was significantly lower in these mutants (Figure 4), suggesting that their capacity to oxidize the plastoquinol pool was impaired. This could result from the lower ability of the mutants to oxidize $P_{700}$ (Figure 3). Decreased ability to oxidize plastoquinol could also theoretically be due to participation of Flv1 and Flv3 in respiratory plastoquinol oxidation in the pathway to cytochrome/quinol oxidase and $O_2$. However, this suggestion contrasts with the observation that mutant Δ*ctaDIEI/ctaDIIEII/cydAB*, where all known terminal oxidases were inactivated, leading to complete inhibition of respiratory $O_2$ uptake [10], exhibited normal light-enhanced $O_2$ uptake (Figure 2). Notably, during longer exposure to light, the Fs emitted by the Δ*flv3*

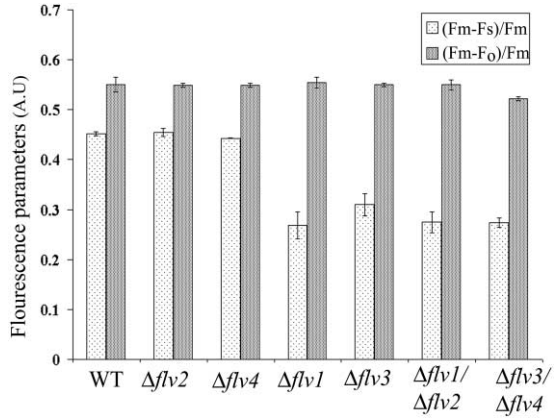


Figure 4. The Fluorescence Parameters (Fm − Fs)/Fm and (Fm − Fo)/Fm for the Wild-Type and Δ*flv* Mutants

The values shown were obtained in experiments such as presented in Supplementary Figure S1. The Fs value used in these calculations was the maximum obtained during illumination with the actinic light. Fm, maximal fluorescence level during 1 s exposure white light, 3000 μmol photons $m^{-2}$ $s^{-1}$; Fo, intrinsic fluorescence level while the cells were exposed to the modulated measuring beam; Fs, fluorescence level under continuous illumination. The intensity of the actinic light was 50 μmol photons $m^{-2}$ $s^{-1}$. The cells were preincubated in the dark, their densities corresponded to 10 μg Chl $ml^{-1}$.

mutants declined to a level close to that of the wild-type (see Supplementary Figure S1). Inhibition of $CO_2$ fixation by IAC abolished this decline in Fs (see Supplementary Figure S1), supporting the notion that activation of $CO_2$ fixation by illumination enabled electron transfer to $NADP^+$ and thus oxidation of plastoquinol, leading to the decline in Fs.

## Discussion

Our study provides the first evidence for the involvement of A-type flavoproteins in the ubiquitous process of photoreduction of $O_2$ in a photosynthetic oxygenic organism. Further, the Δ*flv1* and Δ*flv3* mutants are the first mutants to show complete inhibition of $O_2$ photoreduction by PSI while otherwise exhibiting normal photosynthesis. The A-type flavoproteins inactivated in the Δ*flv* mutants are characterized by their ability to reduce molecular $O_2$ to water [14]. In vitro analysis of the activity of Flv3 from *Synechocystis* PCC 6803 showed NAD(P)H-dependent $O_2$ uptake, which was not affected by the presence of superoxide dismutase and catalase [6], confirming its dioxygen reductase activity. The inference is that, in cyanobacteria, the Mehler reaction operates via direct reduction of $O_2$ to water without production of significant amounts of reactive oxygen species. Thus, photoreduction of $O_2$ in these organisms differs from that in eukaryotes where the Mehler reaction leads to the formation of free $O_2$ radicals. Our study establishes that the source of electrons for the A-type flavoprotein-dependent $O_2$ reduction in cyanobacteria is electron transfer from PSI (Figure 3). This light-dependent $O_2$ reduction is not attributable to respiration, photorespiration, or cyclic electron transfer around PSI (Figure 2).

As pointed out above, Flv1 and Flv3 contain conserved domains of β-lactamase, flavodoxin, and flavin reductase. These proteins are highly conserved in all the cyanobacterial genomes that we have examined (see Supplementary Figure S2). Some of the residues conserved in Flv1 and Flv3 from various cyanobacteria are not conserved in Flv2 or Flv4 (marked by an asterisk in Supplementary Figure S2). These residues are thought to be involved in the stabilization of the NAD(P)H binding site [6]; their substitution may account for the fact that Flv2 and Flv4 apparently do not function in the photoreduction of $O_2$ under the conditions of our experiments.

Interestingly, A-type flavoproteins homologous to Flv1 and Flv3 are found in certain anaerobic organisms, including the $N_2$-fixing photosynthetic bacterium *Rhodobacter capsulatus* (P18607; see Supplementary Figure S2) and the nonphotosynthetic anaerobe *Desulfovibrio gigas* (Q9F0J6) [14]. However, the A-type flavoproteins from anaerobic bacteria do not possess the flavin reductase domain at the C terminus. In *D. gigas*, an A-type flavoprotein serves as the terminal electron acceptor leading to the reduction of $O_2$ to water when this organism is exposed to $O_2$ [14]. Ability to perform the dioxygen reductase activity in anaerobic bacteria such as *D. gigas* involves several entities, whereas in cyanobacteria these three entities are combined in a single polypeptide. It is intriguing to speculate that the Mehler reaction in cyanobacteria is evolutionarily related to the response of anaerobic bacteria, including $N_2$-fixing anaerobic photosynthetic organisms, to $O_2$. One might recall that certain cyanobacteria can perform anoxygenic photosynthesis when exposed to anaerobic conditions in the presence of $H_2S$ [15].

Clearly, reduction of $O_2$ to the level of $H_2O$ would involve transfer of four electrons per water molecule, whereas one molecule of NADPH can provide only two. Both Flv1 and Flv3 are essential for photoreduction of $O_2$ in *Synechocystis* PCC 6803, suggesting that they are either part of the same complex or work consecutively. Flv1 and Flv3 may possibly form a heterodimer that mediates the transfer of four electrons from two molecules of NADPH. The observation that Flv3 alone suffices in the in vitro reaction [6] suggests that a homodimer, too, might be able to function in vitro.

As deduced from the in vitro experiments [6], NAD(P)H probably serves as the source of electrons for the A-type flavoprotein in *Synechocystis* PCC 6803. Formation of NADPH appears to be normal in the Δ*flv* mutants, as their photosynthesis and growth were similar to that of the wild-type. The flux of electrons to $O_2$ in the cyanobacterial Mehler reaction is likely to be influenced by the level of NADPH, but may also be affected by kinetic constraints. Intense controversy prevails as to the extent to which the Mehler reaction operates in vivo, its role in the generation of reactive oxygen species such as hydrogen peroxide, and its significance for the dissipation of excess light energy [16–19]. Under the experimental conditions used here, the rate of light-enhanced $^{18}O$-$^{18}O$ uptake by the wild-type was 15% to 30% that of gross $O_2$ evolution. This was deduced from the rate of $O_2$ uptake (calculated from the rate of $^{18}O$-$^{18}O$ uptake and the isotopic ratio of the dissolved $O_2$) and the rate of gross $O_2$ evolution (calculated from the rise in $^{16}O$-$^{16}O$ concentration) at steady-state photosynthesis. That

light-dependent electron flow to $O_2$ occurs at significant rates in *Synechocystis* PCC 6803 was also deduced from the fact that $O_2$-dependent quenching of chlorophyll *a* fluorescence, in the absence of $CO_2$ fixation, was substantial [19]. By contrast, a very low rate of electron transfer from PSI to $O_2$ has been deduced from the observation that in mutant Δ*KatG* of *Synechocystis* PCC 6803, where decomposition of $H_2O_2$ is greatly reduced due to inactivation of the gene encoding catalase-peroxidase, the extent of electron flow to $H_2O_2$ formation was only 1% the rate of photosynthetic electron transport [20]. This apparent contradiction is probably attributable to the fact that, as concluded here, the Mehler reaction in this organism leads to reduction of $O_2$ to water without $H_2O_2$ formation. Interestingly, there was no detectable difference in the rate of net $O_2$ evolution between the Mehler-deficient and the Mehler-performing mutants (see Supplementary Table S1). In cyanobacteria, several routes compete for the available NADPH, including cyclic electron transfer around PSI [21, 22] and nitrate reduction. Further, NADP formation by oxidation of NADPH in the Mehler reaction may stimulate water splitting in PSII. These various processes may be compensating one another and may well be under homeostatic control, accounting for the lack of differences in net $O_2$ evolution under the conditions used here.

Finally, there is a large unexplained difference, known as the Dole effect [23, 24], between the isotopic composition ($^{16}O/^{18}O$) of the oxygen atoms of water molecules and that of atmospheric $O_2$ generated from water. Earlier assessments of the effect of the Mehler reaction on the composition of stable oxygen isotopes in the atmosphere rested on studies with isolated thylakoids from higher plants [24] where superoxide dismutase and catalase are engaged in the water-water cycle [2]. The degree of discrimination by dioxygen reductase, which converts $O_2$ to water in cyanobacteria, is not known; its analysis, with the aid of the Δ*flv* mutants, should clarify whether it contributes to the Dole effect.

### Supplementary Material

Supplementary Material including the Experimental Procedures, a table showing the rate of photosynthesis and respiration in the wild-type and the mutants, and figures showing the fluorescence kinetics and the alignment of the A-type flavoproteins is available at http://images.cellpress.com/supmat/supmatin.htm.

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