Nitrogen Starvation-Induced Chlorosis in *Synechococcus* PCC 7942. Low-Level Photosynthesis As a Mechanism of Long-Term Survival[1]

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Cells of the non-diazotrophic cyanobacterium *Synechococcus* sp. strain PCC 7942 acclimate to nitrogen deprivation by differentiating into non-pigmented resting cells, which are able to survive prolonged periods of starvation. In this study, the physiological properties of the long-term nitrogen-starved cells are investigated in an attempt to elucidate the mechanisms of maintenance of viability. Preservation of energetic homeostasis is based on a low level of residual photosynthesis; activities of photosystem II and photosystem I were approximately 0.1% of activities of vegetatively growing cells. The low levels of photosystem I activity were measured by a novel colorimetric assay developed from the activity staining of ferredoxin:$NADP^+$ oxidoreductase. Photosystem II reaction centers, as determined by chlorophyll fluorescence measurements, exhibited normal properties, although the efficiency of light harvesting was significantly reduced compared with that of control cells. Long-term chlorotic cells carried out protein synthesis at a very low, but detectable level, as revealed by in vivo [^{35}S]methionine labeling and two-dimensional gel electrophoresis. In conjunction with the very low levels of total cellular protein contents, this implies a continuous protein turnover during chlorosis. Synthesis of components of the photosynthetic apparatus could be detected, whereas factors of the translational machinery were stringently down-regulated. Beyond the massive loss of protein during acclimation to nitrogen deprivation, two proteins that were identified as SomA and SomB accumulated due to an induced expression following nitrogen reduction.

The lifestyle of most cyanobacteria is photoautotrophic growth, performing oxygenic photosynthesis, and assimilating simple inorganic nutrients. Since water and light are ubiquitous electron and energy sources, respectively, these organisms are able to grow in almost all illuminated environments provided inorganic nutrients are available. In a variety of ecosystems the combined nitrogen supply limits growth, and cyanobacteria have developed different strategies to cope with this stressful condition. The diazotrophic strains are capable of fixing molecular dinitrogen, thereby escaping nitrogen depletion. In contrast, non-diazotrophic cyanobacteria respond to the lack of combined nitrogen sources by bleaching, a process since known as chlorosis (Allen and Smith, 1969). We recently demonstrated that nitrogen chlorosis in the non-diazotrophic cyanobacterium *Synechococcus* sp. strain PCC7942 was not accompanied by the loss of cell viability, but is a specific acclimation process enabling the cells to survive prolonged periods of nitrogen depletion (Görl et al., 1998; Sauer et al., 1999). This acclimation process involves three phases. The first response to nitrogen deprivation is the trimming of the phycobilisomes due to proteolytic degradation of phycoyanin (CPC) and allophycocyanin (APC; Görl et al., 1998; Collier and Grossman, 1992, 1994). In the second phase, a gradual loss of chlorophyll *a* occurs, and in the third phase the cells become almost completely depigmented and reside in a dormant-like state from which they are able to reinitiate growth within a few days following the addition of a combined nitrogen source.

A previous physiological study revealed that the experimental conditions for the initiation of nitrogen depletion had a strong influence on the chlorosis process (Görl et al., 1998). When cells were shifted to nitrogen-deprived medium by filtration, a decline of photosynthetic oxygen evolution (V_{max} and quantum efficiency) was observed within a few hours. Photosystem II (PSII) activity decreased to undetectable values (as measured by the Hill reaction) within the first 120 h of nitrogen deprivation, whereas photosystem I (PSI) activity declined much more slowly and reached undetectable values (as measured by the Mehler reaction) only after about 350 h. Protein synthesis was drastically reduced at that point, but traces of de novo synthesized proteins could still be detected 15 d after nitrogen deprivation (Görl et al., 1998). We recently found that the alternative Gln

[1] This work was supported by the Deutsche Forschungsgemeinschaft (grant no. Fo195/2–3).

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synthetase GlnN, which in cyanobacteria is only present in non-diazotrophic strains, aids the recovery from prolonged periods of nitrogen starvation (Sauer et al., 2000). However, it is presently not clear by which means these non-growing cells retain energetic homeostasis that allows them to reinitiate growth soon after the addition of a combined nitrogen source.

The present investigation aimed to characterize biochemical and physiological features of long-term nitrogen-starved *Synechococcus* PCC 7942 cells to understand the molecular mechanism underlying the ability of this cyanobacterium to survive periods of prolonged starvation.

RESULTS

Nitrogen chlorosis was initiated by diluting cells from stock cultures 1:200 into a medium containing a limited quantity of nitrate (1 mM). Chlorosis started as soon as the nitrogen source was exhausted, and typically the cultures reached an optical density at 750 nm (OD_{750}) of approximately 0.6. Because this method of nitrogen step-down avoids cell harvest, it excludes the risk of contaminating the cultures and, therefore, was used to study prolonged nitrogen deprivation. In contrast to long-term starved cells in which chlorosis was initiated by a sudden transfer to nitrogen-deprived medium (Görl et al., 1998), the cells that entered chlorosis gradually reinitiated growth only 2 to 3 d after the addition of nitrate (Sauer et al., 2000), rather than after 4 to 5 d as described by Görl et al. (1998).

Characterization of the Outer Membrane Proteins in Chlorotic *Synechococcus* PCC 7942 Cells

As a first step in analyzing the molecular adaptations of the cells to prolonged nitrogen starvation, protein extracts from cells of different phases of chlorosis were separated by one-dimensional SDS-PAGE and were visualized by silver staining. As shown in Figure 1A, the abundance of most of the cellular proteins decreased strongly, whereas two bands at approximately 50 kD (designated A and B) accumulated during chlorosis. No further change in the one-dimensional protein pattern of cells starved for 19 d (phase 3) could be observed in cells starved for even longer periods (data not shown). To identify the two abundant proteins in chlorotic cells, the corresponding bands were isolated and the material was subjected to N-terminal sequencing. Because no result could be obtained due to a blockage of the N terminus, the eluted proteins were digested with V8 protease, and peptides were separated by gel electrophoresis according to Schägger and von Jagow (1987). From isolated peptides of band "B", N-terminal sequences could be obtained (see "Materials and Methods"), which by comparison with the

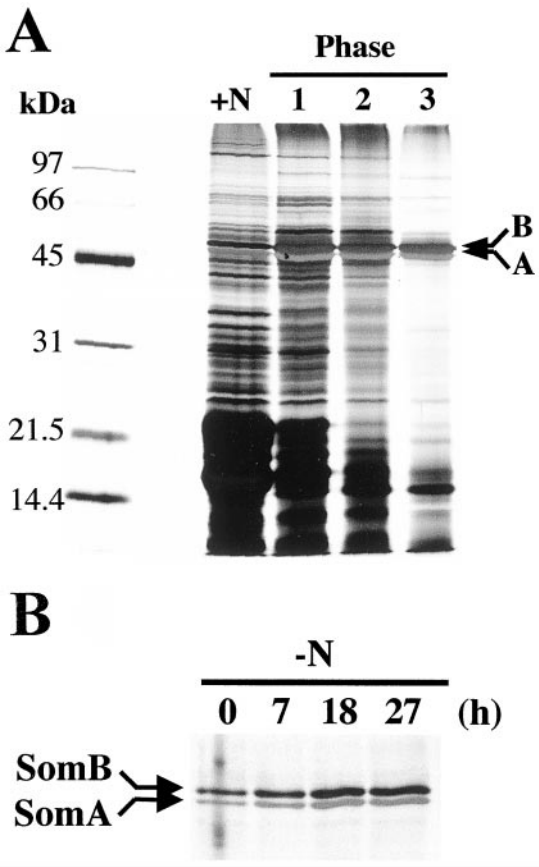

Figure 1. Total protein of *Synechococcus* PCC 7942 cells during chlorosis (A) and accumulation of the porins SomA and SomB following nitrogen step-down (B). A, SDS-lysates of 0.3 OD cells (1 OD cells is defined as the amount of cells of 1 mL of culture at an optical density at 750 nm of 1) from exponentially growing cells (+N) and from cells harvested after 6 d (Phase 1), 9 d (Phase 2), or 19 d (Phase 3) following inoculation in low $BG11^N$ medium were separated by 12.5% (w/v) SDS-PAGE (Laemmli, 1970) and silver stained (Blum et al., 1987). B, SDS-PAGE analysis of outer membrane proteins following nitrogen step-down. Outer membrane proteins were prepared from cells harvested directly (0 h) and 7, 18, and 27 h after the cells had been transferred into nitrogen-free medium (see "Materials and Methods").

databank, revealed the *somB* gene product from *Synechococcus* PCC 6301 (Hansel at al., 1998). The protein corresponding to band "A" was identified by peptide mass fingerprinting and could be assigned as the *somA* product from *Synechococcus* PCC 7942 (Umeda et al., 1996). SomA and SomB had previously been identified as the major outer membrane porins in this organism (Hansel and Tadros, 1998).

To confirm the accumulation of these proteins following nitrogen deprivation, outer membranes were prepared from *Synechococcus* PCC 7942 cells that had been shifted to nitrogen-deprived medium by filtration. The preparations were solubilized in SDS-lysis buffer and were analyzed by SDS-PAGE (Fig. 1B). Two proteins with identical migrations in SDS-PAGE and silver staining properties to those of SomA and

SomB could be identified and their amount accumulated during nitrogen starvation. As a final proof that the synthesis of *somA* and *somB* is stimulated by nitrogen starvation, northern-blot experiments were performed. RNA was prepared from cells after different times of nitrogen deprivation and it was subjected to RNA/DNA hybridization analysis employing a *somA*- or *somB*-specific probe. As shown in Figure 2, the intensity of the *somA*- and *somB*-specific bands increased almost immediately following nitrogen step-down. Densitometric quantification of the blot revealed that transcript levels increased about 3.5-fold, as compared with nitrogen-replete cells, within 40 min. To compare this result with the repression of *apc* expression following nitrogen deprivation (Sauer et al., 1999), the same blot was rehybridized with an *apcAB*-specific probe. Figure 2 shows that the increase in *somA* and *somB* mRNA levels slightly precedes the reduction of the *apcAB* mRNA level. Equal total RNA load per lane was confirmed by hybridization with a 16S rRNA gene probe. (In this experiment, to determine exactly the onset of nitrogen deprivation, cells were transferred to nitrogen-deprived medium by filtration.)

De Novo Protein Synthesis in Long-Term Chlorotic Cells

To investigate residual protein synthesis in nitrogen-starved cells, in vivo protein labeling experiments were performed with [^{35}S]Met. To obtain sufficient incorporation of radioactivity into proteins for subsequent analysis, cells had to be labeled for 5 d with 10 μCi/mL [^{35}S]Met. Cell extracts were then separated by two-dimensional gel electrophoresis, silver stained, and then autoradiographed (Fig. 3). The porins SomA and SomB were not resolved by this analysis due to their low solubility in the first-dimension buffer. As already shown in the one-dimensional SDS-PAGE analysis, chlorotic cells contain only low amounts of cellular proteins. Quantification of the soluble protein contents in chlorotic cells yielded levels of 0.2 μg of protein per OD_{750} cells, which corresponds to 0.4% of control cells. In contrast to this low level of soluble proteins, they displayed a remarkable complex protein synthesis pattern, indicating a continuous turnover of the newly synthesized proteins, which prevents their accumulation to detectable amounts (compare Fig. 3, B with D).

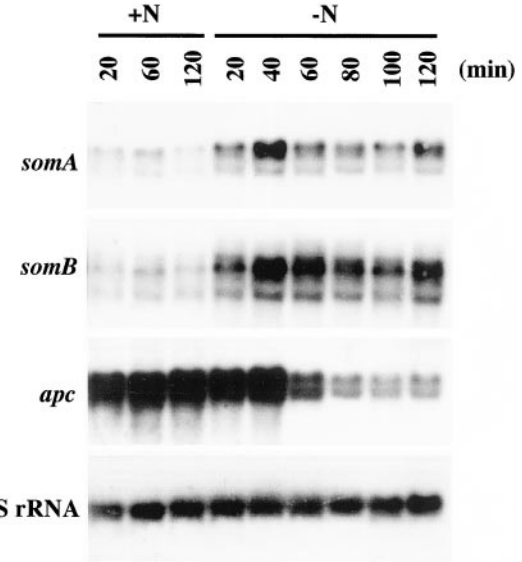

Figure 2. Northern-blot analysis of *somA*, *somB*, *apc*, and 16S rRNA transcripts following nitrogen step-down. *Synechococcus* PCC 7942 cells were grown in BG11^{N} medium and transferred in BG11^{N} medium (+N) as a control or in BG11^{O} medium (-N) by filtration; total RNA was isolated from cells harvested at the indicated time points after cell transfer, and 12 μg of total RNA was loaded per lane. The same RNA blot was used for all four hybridizations.

Most of the labeled spots from chlorotic cells can be matched to those of ammonium-replete control cells, although the relative labeling intensities can differ significantly. Among those proteins for which the synthesis continues in chlorotic cells, we identified plastocyanin and thioredoxin M by N-terminal sequence analysis (see "Materials and Methods"). Various proteins produced in the control cells were not synthesized to detectable levels in chlorotic cells. Among these we identified a protein homologous to ribosomal protein S6 (Rps6) and the small subunit of Rubisco (RbcS) by N-terminal sequence analysis and the protein synthesis elongation factor Tu by peptide mass fingerprinting (see "Materials and Methods"). To our surprise we detected a substantial signal in the autoradiogram of chlorotic cells that matched with the phycobiliproteins CPC and APC. To confirm this observation and to exclude the possibility that the labeling agent [^{35}S]Met (used at a concentration of 10 nM) had an effect as a nitrogen source that caused the synthesis of phycobiliproteins, the abundance of CPC and APC was quantified by immunoblot analysis from long-term nitrogen-deprived cells, and as control from cells that were supplied with 10 nM or 100 nM Met for a period of 5 d prior to the analysis.

As a reference for quantification, different dilutions of extracts from nitrate-replete cells were applied to the same analysis (Fig. 4A). The experiment showed that the addition of Met had no effect on the quantity of the phycobiliproteins. Densitometric analysis of the blots gave an estimate of 0.018% for the CPC level and of 0.042% for the APC level, as compared with an equivalent OD_{750} of nitrate-replete cells. Assuming that the CPC to APC ratio is 2.5:1 in nitrate-replete cells (Collier and Grossman, 1992), the ratio would drop to 1.1:1 in long-term nitrogen-starved chlorotic cells; this value is very similar to the value reported for 2-d chlorotic cells (Collier and Grossman, 1992).

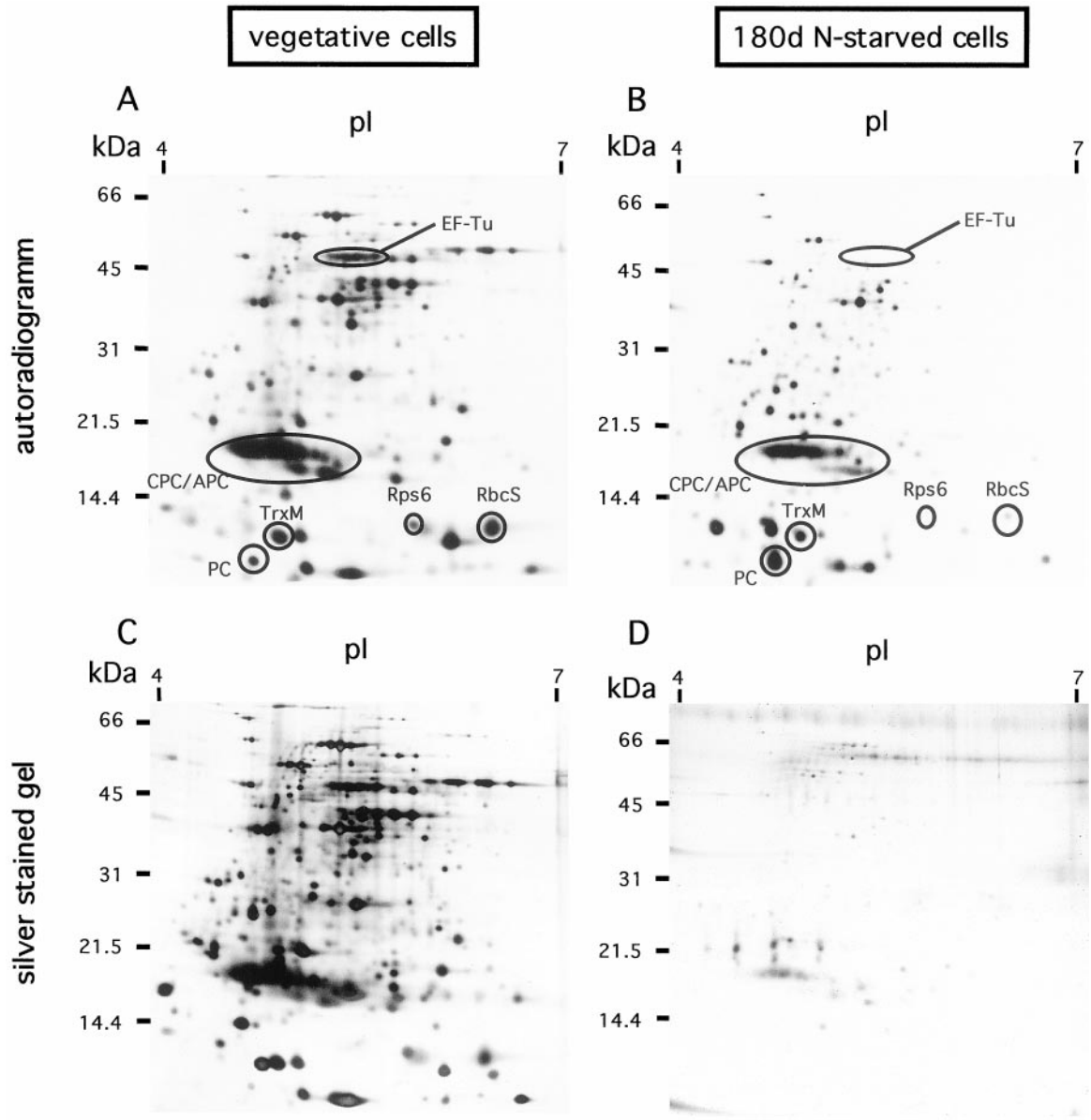

Figure 3. Protein synthesis patterns (autoradiograms, A and B) and total protein (silver-stained gels, C and D) of *Synechococcus* PCC 7942 cells during exponential growth (vegetative cells) and in long-term chlorotic cells (180d nitrogen-starved cells). Vegetative cells were labeled for 2 h and chlorotic cells were labeled for 5 d with L-[^{35}S]Met and sample volumes of 1.1 OD cells (vegetative cells) and 0.9 OD cells (chlorotic cells) were separated by two-dimensional PAGE. TrxM, Thioredoxin M; PC, plastocyanin.

Photosynthetic Activities in Long-Term Nitrogen-Depleted *Synechococcus* PCC 7942 Cells

The results described above revealed that energy-consuming reactions such as protein synthesis take place in chlorotic cells. This raised the question of how the cells maintain energetic homeostasis. In a previous study we could not detect measurable photosynthetic activities in prolonged nitrogen-starved cells. However, based on the delayed decay of PSI activity we suggested that residual cyclic electron transport around PSI may be responsible for the energy supply of the cells (Görl et al., 1998). A simple confirmation for some low-level residual photosynthetic activity came from the observation that prolonged nitrogen-starved cells still depend on light since they started to loose viability after 14 d incubation in darkness (Table I). Moreover, incubation of chlorotic cells for 48 h with metronidazol in the light poisoned the cells, whereas incubation in the dark for the same period of time did not do any harm, as determined by the ability to reinitiate growth following nitrate supplementation. Metronidazol is an artificial electron acceptor of PSI-reduced ferredoxin (Joset, 1988), and it is converted to a toxic compound

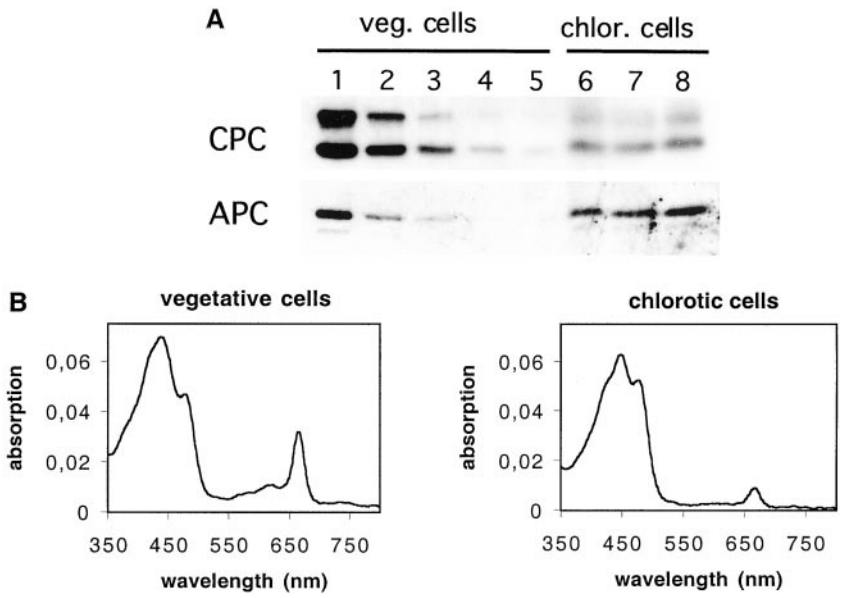

Figure 4. Quantification of phycobiliproteins (A) and chlorophyll *a* (B) in long-term chlorotic cells. A, Long-term chlorotic cells (85 d in low $BG11^N$) were incubated for 5 d in the absence (lane 6) or in the presence of 10 nM (lane 7) or of 100 nM L-Met (lane 8) and SDS lysates of 1.25 OD cells (lanes 6–8) were loaded per lane. To quantify CPC and APC by immunoblotting, SDS lysates of vegetative cells equivalent to 7.8×10^{-4} OD cells (lane 1), 3.9×10^{-4} OD cells (lane 2), 1.95×10^{-4} OD cells (lane 3), 9.8×10^{-5} OD cells (lane 4), and 4.9×10^{-5} OD cells (lane 5) were loaded. B, Absorbance spectra of methanol-extracted chlorophyll from 0.06 OD cells of a nitrate-replete culture and 10.7 OD cells of chlorotic culture (120 d in low $BG11^N$).

upon reduction. Therefore, the light-dependent metronidazol sensitivity of chlorotic cells is an indication of PSI activity. Furthermore, a methanol extraction was performed to detect and quantify traces of chlorophyll *a* and carotenoids. As shown in Figure 4B, chlorotic cells indeed contain traces of chlorophyll *a*, which amounts to approximately 0.16% as compared with vegetative cells and carotenoid levels of approximately 0.6%.

A direct measurement of PSI activity through the Mehler reaction failed, since this method is not sensitive enough to determine activities that are below 1% of the activity in vegetative cells (Görl et al., 1998). Therefore, we developed a much more sensitive method to measure the activity of PSI. This determination is based on the reduction of nitroblue tetrazolium (NBT) by PSI via ferredoxin:$NADP^+$ oxidoreductase (Swegle and Mattoo, 1996). NBT reduction by respiration was almost completely inhibited by the addition of azide. Reduced NBT precipitates to form an insoluble blue formazan dye that is usually used for activity staining of enzymes (Auclair and Voisin, 1985; Swegle and Mattoo, 1996). We modified this procedure by solubilizing the formazan in dimethyl sulfoxide for subsequent photometric quantification (see "Materials and Methods"). Figure 5 shows that in vegetative control cells, a red light-dependent linear increase in formazan formation could be detected. In dark incubated cells, only minor formazan formation was measured, and in cells inactivated with 0.5% (w/v) formaldehyde, no NBT reduction occurred. 3-(3,4-dichlorophenyl)-1,1-Dimethylurea treatment slowed down NBT reduction by 50%, due to a limitation of electron supply to the donor site of PSI (not shown). These results indicate that in the vegetative control cells, the observed NBT reduction is mediated by PSI activity and that about 50% of the electrons are provided by PSII.

With the same experimental procedure, light-dependent NBT reduction was measured in 70-d chlorotic cells (Fig. 5). By comparing the slopes of NBT reduction (corrected for dark formazan formation), it appears that the chlorotic cells exhibit approximately 0.1% PSI activity as compared with the vegetative control cells.

To clarify the question of whether the chlorotic cells have also retained some PSII activity, chlorophyll fluorescence measurements were performed using a special pulse-amplitude-modulation (PAM) fluorometer in conjunction with saturation pulse quenching analysis (Schreiber et al., 1994). This fluorometer allows assessment of photosynthetic activity at chlorophyll concentrations below 1 μg of chlo-

Table I. *Delay of growth of vegetative cells and delay of recovery of chlorotic cells after incubation in the dark for various times*

Aliquots of a vegetative growing culture with an optical density of $OD_{750} = 0.13$ and of a chlorotic culture (70 d in low $BG11^N$) with an optical density of $OD_{750} = 0.2$ were incubated in the dark, and at the indicated time points, the chlorotic cultures were supplemented with 20 mM $NaNO_3$ and afterwards vegetative and chlorotic cultures were incubated at a photosynthetic photon flux density (PPFD) of 10 μmol m^{-2} s^{-1}. To determine a delay in growth the optical density of the vegetative cultures was measured. Recovery of chlorotic cultures was estimated by newly synthesized pigments (phycobiliproteins and chlorophyll *a*), which was determined as previously described in Görl et al. (1998); in control cultures of chlorotic cells that were incubated under these conditions the levels of chl *a* and phycobiliproteins increased concomitantly and were detectable 3 d after addition of nitrogen.

Cells	Delay of Growth/Recovery after Dark Incubation		
	4	8	14
		d	
Vegetative	0	3–4	5–6
Chlorotic	0	0	6–8

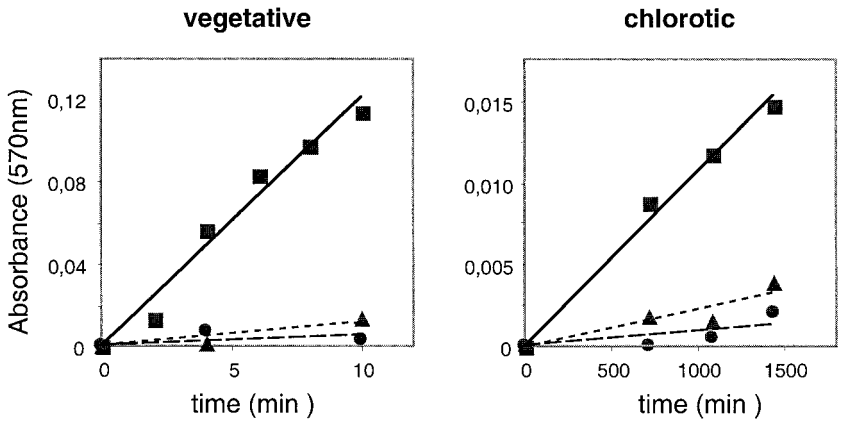

Figure 5. PSI activity determined as the formation of formazan in vegetative and chlorotic cells. Equivalent amounts of vegetative cells and chlorotic cells (70 d in low $BG11^N$) were incubated with NBT in the presence (■) or in the absence (▲) of red light for the indicated times; as a control, formaldehyde-treated cells were incubated with NBT in red light (○).

rophyll L^{-1} (Schreiber 1998). The fluorescence responses of control cells and chlorotic cells are compared in Figure 6. To obtain similar signal levels the control cells (OD_{750} = 0.41) were diluted by a factor of 100, whereas the chlorotic cells (OD_{750} = 0.82) were used undiluted. The dark-adapted cells were first illuminated by a single pulse of saturating light (at 45 s after start of recording) to assess F_v/F_m, which is a relative measure of the maximum quantum yield of energy conversion at PSII reaction centers. Continuous actinic light was then switched on (at 90 s), giving rise to dark-light induction kinetics (Kautsky effect). When a quasi-stationary fluorescence level was reached (at 180 s), another saturation pulse was applied to assess $\Delta F/F_{m'}$, the effective quantum yield of PSII photochemistry in the illuminated state, which provides a relative measure of ETR (Genty et al., 1989). After an approximate 3.5-min illumination, the actinic light was switched off and 30 s thereafter another saturation pulse was applied to obtain information on the reversibility of the light-induced fluorescence changes. The control and chlorotic cells qualitatively display the same responses, indicating that the chlorotic cells not only have retained some PSII activity, but also that the remaining PSII units are exhibiting nearly normal properties. It should be noted that the relatively dense suspension of chlorotic cells, in contrast to the highly diluted control cells, displayed strong scattering of the measuring light, which may result in an increased background signal, such that the measured values of F_v/F_m and $\Delta F/F_{m'}$ could be underestimated. Considering the different cell densities and signal amplifications, on the basis of variable fluorescence it can be estimated that the PSII content of the chlorotic cells was approximately 0.1% of the control cells.

Upon the onset of actinic illumination, control and chlorotic cells show a rapid fluorescence rise close to F_m reached in the preceding saturation pulse, followed by a slow-rise phase to a level distinctly higher than $F_{m'}$, which is more pronounced in the control cells (Fig. 6). The rapid rise reflects reduction of the PSII acceptor side by electrons derived from water-splitting, whereas the slow rise is due to a state 2-state 1 transition, characteristic of blue-green algae (Allen, 1992; Schreiber et al., 1995a). The values of $\Delta F/F_{m'}$ observed during illumination were very similar in control and chlorotic cells, suggesting that the photosynthetic units retained in the chlorotic cells perform efficient and similar electron transport. As expected, this electron transport could be completely inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (data not shown).

The light response curves of control and chlorotic cells are shown in Figure 7. The apparent relative electron transport rate (ETR) was calculated on the basis of $\Delta F/F_{m'}$ values measured at step-wise increasing photosynthetically active radiation (PAR; Schreiber et al., 1994). From the difference in the initial slopes it may be concluded that the PSII an-

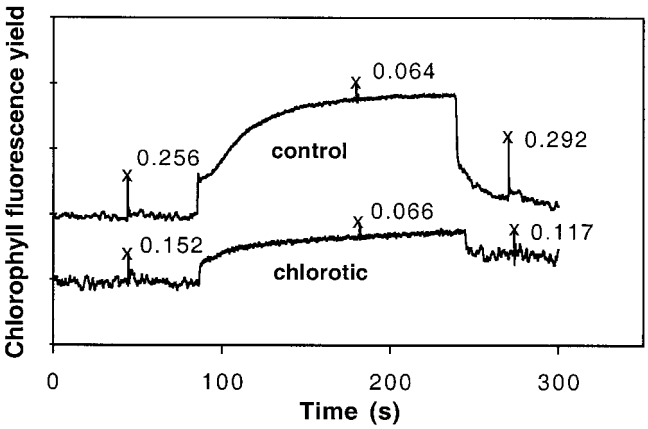

Figure 6. Comparison of chlorophyll fluorescence dark-light induction kinetics of chlorotic (120 d in low $BG11^N$) and control cells of *Synechococcus* PCC 7942 as measured with a WATER-PAM chlorophyll fluorometer. Red actinic illumination at 750 μmol quanta m^{-2} s^{-1} was turned on at 90 s for approximately 3.5 min. The "X" symbols denote the F_m and $F_{m'}$ values reached during 0.2-s pulses of saturating red light (7,000 μmol quanta m^{-2} s^{-1}). The numbers represent the calculated values of F_v/F_m and $\Delta F/F_{m'}$. For the sake of presentation, the signal of the chlorotic cells, which was amplified by a factor of 3 with respect to that of the control cells, is shifted downwards. The base line belongs to the control signal.

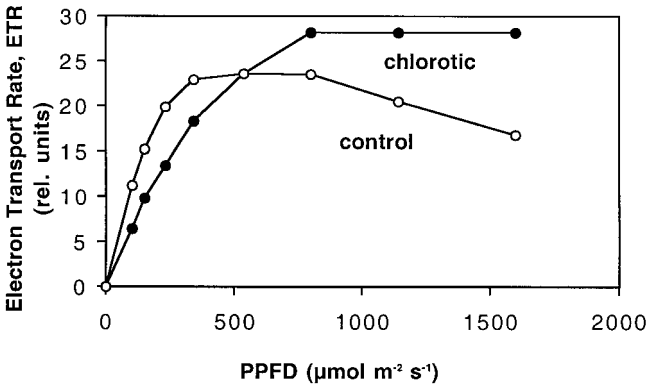

Figure 7. Comparison of light response curves of chlorotic and control cells as assessed by chlorophyll fluorescence measurements. The relative ETR was determined at step-wise increasing PAR levels with 1-min illumination periods at each step (see also "Materials and Methods"). For other conditions, see legend of Figure 6.

tenna size in the chlorotic cells is smaller than in the control cells (approximately 55% of control). This difference also explains the higher PAR value required for light saturation, as well as the apparently higher maximal electron transport capacity in the chlorotic cells. Furthermore, the chlorotic cells, in contrast to the control cells, do not show a decline of ETR at PAR values above 1,000 μmol m^{-2} s^{-1}, which suggests that they are better protected against photoinhibition.

DISCUSSION

The present investigation has provided an answer to the question of how cells can survive in a nongrowing state of starvation for periods of months. In the case of nitrogen chlorosis we were able to recover cultures after 12 months of starvation without any increase in the time period required for pigment resynthesis and reinitiation of growth (J. Sauer and K. Forchhammer, unpublished data). In the final chlorotic state the cells maintain photosynthetic activities at a very low basal level, which was approximately 0.1% compared with vegetatively growing cells, as measured by two independent methods. This value is in good agreement with the residual 0.16% chlorophyll *a*, which was detected in the chlorotic cells. From these observations we conclude that the ratio of chlorophyll *a* to photosynthetic reaction centers remains almost constant. By contrast, the ratio of phycobiliproteins to photosynthetic activity is significantly reduced. Relative to PSII activity, the APC and CPC quantities are 2.4- or 5.5-fold reduced, respectively. This decrease in light-harvesting pigments per PSII reaction center explains why PSII activity in chlorotic cells saturates at significantly higher light intensities. As a result, chlorotic cells are less susceptible to photoinhibition by irradiation. The fact that chlorotic cells respond to a dark-to-light shift by a state 2-to-state 1 transition, as vegetatively growing cells do (Schreiber et al., 1995a), shows that regulation of quanta distribution between the two photosystems is occurring. The residual photosynthetic activity provides the cells with ATP and reducing equivalents. Because no net CO_2 fixation takes place in chlorotic cells, the reducing equivalents have to be removed by alternative reactions. A possible valve for the electrons in PSI-reduced ferredoxin is the Mehler-ascorbate-peroxidase or water-to-water cycle (Schreiber et al., 1995b; Asada, 1999), which is characterized by zero net oxygen evolution. In an alternate manner, or in addition, the cells could perform photorespiration (Wu et al., 1991) through Rubisco activity. However, two-dimensional gel electrophoretic analysis showed that the synthesis of RbcS is strongly reduced in chlorotic cells, favoring the first alternative.

The residual photosynthetic activity is apparently sufficient to drive protein synthesis at a very low level. The amino acids required for this process can be provided by continuous protein turnover. In the stationary state of chlorosis (phase 3), no net changes in protein composition can be observed. This implies that there is an equilibrium between protein synthesis and degradation. Proteins that belong to the photosynthetic apparatus are permanently turned over, whereas proteins of the translational apparatus are stringently down-regulated in the stationary chlorotic state, as shown by the hardly detectable levels in the synthesis of elongation factor Tu and rps6. Since the synthesis of the ribosomal proteins is strictly coregulated (Keener and Nomura, 1996), we can safely assume that the synthesis of new ribosomes is correspondingly reduced. Northern-blot analysis revealed that ribosomal RNA disappears in chlorotic cells to levels beyond the limits of detection (data not shown). This implies that cyanobacteria are able to adjust the synthesis of the translational machinery according to the demand, at least under these extreme conditions. The coregulation of ribosomal synthesis with growth rate or amino acid deprivation is long known from other bacteria as growth rate control and stringent control (Cashel et al., 1996). Since a RelA/SpoT homolog is encoded in the genome of the cyanobacterium *Synechocystis* PCC6803 (Kaneko et al., 1996), a similar control mechanism may operate in cyanobacteria.

The outer membrane porins SomA and SomB are resistant to proteolytic degradation during the induction of chlorosis, in contrast to the intracellular proteins. A similar accumulation of SomA and B to that for nitrogen starvation was observed under conditions of sulfur starvation, but not for phosphate starvation (data not shown). Starvation-regulated synthesis of the porins OmpC and OmpF has recently been reported for *Escherichia coli* (Liu and Ferenci, 1998). In *Pseudomonas fluorescens* cells the accumulation of a specific porin (OprP) has been demonstrated under conditions of phosphatate deprivation

(Leopold et al., 1997). It is apparent that the promotor of the *som* genes is highly susceptible to nitrogen down-shift. The increase in *somA* and *somB* mRNA levels was significantly faster than other responses to nitrogen deprivation such as the decrease of *apc* mRNA levels or the increase of *glnN* mRNA levels, which encodes an alternative Gln synthetase specifically induced under conditions of nitrogen deprivation (García-Domínguez et al., 1997; Reyes et al., 1997; Sauer et al., 2000). We suspect that the accumulation of the porins SomA and SomB enhances the permeability of the cells for combined nitrogen or sulfur molecules, which would cause a selective advantage in the ability to utilize traces of these nutrients.

The preservation of a permeable outer membrane, of basal levels of the entire photosynthetic apparatus, and of a low level of gene expression provides a simple explanation for the exceptional ability of the cells to survive starvation for extended periods of time and to rapidly reinitiate growth following the addition of nitrate. When all components that are required to assimilate nitrate are present at basal levels, the cells are immediately able to utilize this nitrogen source. They could instantly initiate de novo amino acid synthesis and thereby could gradually increase de novo protein synthesis to produce new enzymes that, by positive feedback, would accelerate nitrogen assimilation and other metabolic activities. As a result, the completely chlorotic cells recover pigmentation within 3 d after nitrate supplementation and resume growth. This strategy of adapting to prolonged starvation differs from the formation of spores since viability is maintained by a continuous energy supply (light). The evolution of this capacity emphasizes the selective pressure of nutrient deprivation in natural ecosystems.

MATERIALS AND METHODS

Strain and Culture Conditions

Synechococcus sp. strain PCC 7942 (Kuhlemeier et al., 1983; hereafter designated *Synechococcus* PCC 7942) was grown in $BG11^N$ medium (Rippka, 1988) supplemented with 5 mM $NaHCO_3$ and ferric ammonium citrate was replaced by ferric citrate. For experiments during short-term nitrogen starvation, exponentially growing cells were transferred by filtration into a medium lacking combined nitrogen ($BG11^O$) as described previously (Görl et al., 1998). For experiments with long-term nitrogen-starved cells, *Synechococcus* PCC 7942 was diluted from stock cultures 1:200 into a nitrate-poor BG11 medium containing only 1 mM $NaNO_3$ (low $BG11^N$). All cultures were grown photoautotrophically at 30°C under continuous illumination of 50 μmol m^{-2} s^{-1} from white fluorescent tubes (LUMILUX deLuxe, Osram, Munich). To maintain constant aeration and reduce evaporation, cultures were stirred in baffled Erlenmeyer flasks capped with silicone sponge closures (Bellco Glass, Vineland, NJ). Dark conditions were obtained by wrapping culture flasks in aluminum foil.

Identification of SomB

To identify the proteins corresponding to SomB, an SDS lysate of 1.5 OD from a long-term chlorotic culture was separated by SDS-PAGE (12.5% [w/v] acrylamide; Laemmli, 1970), was then stained by an inverse protein staining method (Roti-White, Roth), and gel slices containing protein B (compare Fig. 1A) were cut out of the gel. To obtain peptides from protein B for N-terminal sequencing, eight gel slices were crushed and mixed with 300 μL of modified gel slice overlay solution (0.125 M Tris-HCl, pH 6.8, 0.1% [w/v] SDS, 1 mM EDTA, and 43 mM 2-mercaptoethanol) as described by Cleveland (1983). *Staphylococcus aureus* V8 protease (1.4 μg) was added and this was incubated for 7 h at 30°C. Afterward, the supernatant was removed and ethanol was added to the gel slices for extraction of soluble peptides out of the polyacrylamide. The peptides were concentrated by lyophilization, separated on a Tricine {*N*-[2-hydroxy-1,1-Bis(hydroxymethyl)ethyl]glycine} 16.5% (w/v) SDS acrylamide gel (Schägger and von Jagow, 1987), and transferred to a 0.2-μm polyvinylidene difluoride membrane. Peptides were visualized by amido black, cut out of the membrane, and subjected to N-terminal sequencing as described by Schmid et al. (1997). The following N-terminal sequences were obtained: peptide 1: LSYFFNNPVPGIYXDAD; peptide 2: LEATQFSTTTKLQGDVLF; and peptide 3: NFTISGSYG ISFL.

Preparation of Outer Membrane Proteins

Preparation of outer membrane proteins was performed principally according to the method of Weckesser and Jürgens (1988) with some modifications. Exponentially growing cells were transferred into $BG11^O$ medium by filtration and 8-mL aliquots were harvested directly and at various times after the shift. The cells were resuspended in 400 μL of 20 mM Tris-HCl (pH 7.4), 0.5 mM $CaCl_2$, and 0.5 mM $MgCl_2$, and were broken by sonication. Cell debris were removed by centrifugation (3 min, 4,000*g*, 4°C) and the protein concentration was determined by the method of Bradford (1976). During the first 2 d following nitrogen step-down, total protein remained constant per culture volume. Crude extracts containing 70 μg of total protein were centrifuged for 30 min at 18,000*g* and 4°C, the pellets were washed in 400 μL of 20 mM Tris-HCl (pH 7.4), 0.5 mM $CaCl_2$, and 0.5 mM $MgCl_2$, and recentrifuged. The pellets were subsequently resuspended respectively in 1 mL of three different buffers, incubated on a rocking platform, and after each step the outer membrane proteins were pelleted by centrifugation (30 min, 18,000*g*, 4°C). Buffer 1 was 20 mM ammonium acetate buffer (pH 6.5) and was incubated for 5 min; buffer 2 was 10 mM Tris-HCl (pH 7.4), 2% (v/v) Triton X-100 and was incubated for 45 min; and buffer 3 was 20 mM Tris-HCl (pH 7.4), 0.5 mM NaCl, 10 mM $MgCl_2$, 10% (v/v) glycerol, and 1% (w/v) *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate and was incu-

bated for 80 min. The resulting pellets were dried, resuspended in 70 μL of SDS sample buffer, boiled, and 15 μL of each sample was separated by SDS-PAGE using 12.5% (w/v) acrylamide gels (Laemmli, 1970). The gels were silver stained (Blum et al., 1987) and dried.

Analytical and Preparative Two-Dimensional PAGE

For analytical two-dimensional PAGE, 1.5 mL of bacterial cultures of exponentially grown cells or chlorotic cells (180 d low $BG11^N$) at an OD_{750} of 0.5 were labeled with 10 μCi L-[^{35}S]Met (370 kBq) in 50-mL Erlenmeyer flasks and were incubated as before for 2 h (exponentially grown cells) or for 5 d (chlorotic cells). Cell harvesting, sample preparation, protein separation, and spot visualization were performed as previously described (Görl et al., 1998) with the following modifications. For isoelectric focusing in the first dimension, 18-cm immobilized pH gradients (IPG dry strips, pH 4–7, Pharmacia) were used, and in the second dimension, gels were run on 18 × 18 × 0.1-cm Tricine-SDS 10% (w/v) acrylamide gels (Schägger and von Jagow, 1987). For preparative two-dimensional gel electrophoresis, a number of cells equivalent to 25 mL of culture with an optical density (OD_{750}) of 1 were washed five times with ice-cold 80% (v/v) acetone, resuspended in 120 μL of lysis buffer (Görl et al., 1998), and the proteins were separated by using the electrophoretic system as for analytical two-dimensional PAGE (see above).

Identification of Proteins from Two-Dimensional Gels by N-Terminal Sequencing

To identify proteins from two-dimensional gels by N-terminal sequence analysis, Coomassie-stained protein spots were collected from several two-dimensional gels and were concentrated, blotted, and sequenced as described by Schmid et al. (1997). The proteins Rps6, RbcS, plastocyanin, and thioredoxin M could be identified by the following N-terminal amino acid sequences: Rps6: MKDFYYETMYILLADLTEEQV; RbcS: SMKTLPKERRFETFSY; plastocyanin: QTVAIKTGADNGMLAFEPXTIEIQA; and thioredoxin M: VAAAVTDATFKQEVLE.

Identification of SomA and Elongation Factor-Tu (EF-Tu) by Peptide Mass Fingerprinting

Proteins spots cut from Coomassie Blue-stained two-dimensional gels (EF-Tu) or Roti-White-stained SDS-PAGE gels (ProteinA/SomA) were destained, digested with trypsin (Promega, Madison, WI), and peptides were extracted according to Otto et al. (1996). Peptides were dissolved in 30% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid, and were mixed with an equal volume of saturated α-cyano-3-hydroxycinnamic acid solution in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid and than applied to a sample template for a MALDI-TOF mass spectrometer. Peptide masses were determined in the positive ion reflector mode in a Voyager DE RP mass spectrometer (PerSeptive Biosystems) with internal calibration. Peptide mass fingerprints were compared with databases using the program MS-Fit (http://prospector.ucsf.edu). The searches considered oxidation of Met, pyro-Glu formation at an N-terminal Gln, and modification of Cys by acrylamide, as well partial cleavage leaving one internal cleavage site. The mass accuracy was in the range of 50 ppm and SomA and EF-Tu were identified with a peptide coverage of 23% and 50%, respectively.

RNA Isolation and Northern-Blot Analysis

Initiation of nitrogen deprivation, isolation of total RNA, separation of RNA in 1.2% (w/v) agarose gels containing 2.2 M formaldehyde, transfer of RNA to nylon membranes, and RNA-DNA hybridization experiments were performed as described previously (Sauer et al., 1999). For rehybridization experiments, blots were stripped by incubation in 50% (v/v) formamide, 2 × SSPE (sodium chloride/sodium phosphate/EDTA) for 1 h at 65°C (Sambrook et al., 1989). To detect levels of *apc* mRNA and 16S rRNA, radiolabeled DNA fragments were used as probes, as described elsewhere (Sauer et al., 1999). The *somB* probe was a 985-bp, PCR-amplified fragment containing a 5′ region of the *somB* gene and the 5′ one-half of the *somB*-coding region. The *somA* probe was a 1,390-bp, PCR-amplified fragment containing the coding region of the *somA* gene (Hansel et al., 1998).

Analysis of Phycobiliproteins, Chlorophyll *a* and Carotenoid Contents, and of Soluble Protein

To estimate the amount of phycobiliproteins in long-term chlorotic cells, samples of cell extracts were subjected to SDS-PAGE on 15% (w/v) polyacrylamide (Laemmli, 1970), proteins were blotted on nylon-supported nitrocellulose, and APC and CPC was detected immunologically using antibodies that were raised against purified *Synechococcus* strain PCC 7942 APC or CPC. The cross-reactive primary antibodies were detected using peroxidase-linked anti-rabbit antibodies and an enhanced chemiluminescent antibody detection system (Roche Diagnostics, Basel). Chlorophyll *a* was determined as previously described (Forchhammer and Tandeau de Marsac, 1995) and total carotenoids were estimated as described by Chamovitz et al. (1993).

To determine the amount of total soluble cellular protein, 100 OD of exponentially growing cells or chlorotic cells (70 d low $BG11^N$) were harvested by centrifugation, resuspended in 1 mL of 20 mM Tris-HCl (pH 7.4), 0.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$, and were then broken in the presence of glass beads using the HYBAID RiboLyser Cell Disrupter (HYBAID Ltd., Ashford, Middlesex, UK). Cell debris and unsoluble material was removed by centrifugation (18,000*g*, 30 min, 4°C) and the amount of soluble protein was determined according to Bradford (1976).

Measurement of Photosynthetic Activities

To estimate PSI activity, 0.25 OD cells of exponentially growing or chlorotic cultures were resuspended in 90 μL of

10 mM potassium phosphate buffer (pH 7.8), 30 mM NaN_3; as a control, the same amount of cells was resuspended in 90 μL of 10 mM potassium phosphate buffer (pH 7.8), 30 mM NaN_3, and 0.5% (v/v) formaldehyde. The samples were pre-incubated for 15 min in 1.5-mL reaction tubes at 25°C in red light (cut-off filter > 650 nm, PPFD 20 μmol m^{-2} s^{-1}) or in the dark and the reaction was started by addition of 10 μL of 10 mg mL^{-1} NBT in 10 mM potassium phosphate buffer (pH 7.8). Oxidized NBT is a yellow compound soluble in aqueous solutions. Its reduced form, formazan, appears dark blue and is insoluble in water. Incubation in red light or in the dark was stopped by the addition of 0.9 mL of dimethyl sulfoxide, thereby solubilizing the formazan dye. The absorbance of formazan was recorded at 570 nm immediately following its solubilization.

Modulated chlorophyll fluorescence was measured with a WATER-PAM chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) consisting of the WATER-ED emitter-detector unit, the PAM-CONTROL unit, and the WinControl Data Acquisition software. The sample (3 mL), which was contained in a 15-mm diameter quartz cuvette, was illuminated by a circular array of 14 red light emitting diodes peaking at 655 nm, three of which served for pulse modulated measuring light, whereas the rest provided actinic light and saturation pulses. A miniature photomultiplier module (type H-6779–01, Hamamatsu, Hamamatsu City, Japan) served as fluorescence detector at wavelengths above 700 nm. The experiments were carried out at room temperature (24°C). The effective quantum yield of PSII photochemistry was determined by the saturation pulse method (Genty et al., 1989; Schreiber et al., 1994). The relative ETR was calculated according to the equation: $ETR = c \times Y \times PAR$, with c being a proportionality factor and Y corresponding to the effective quantum yield. ETR was measured as a function of PPFD, making use of the data acquisition software WinControl provided with the WATER-PAM chlorophyll fluorometer. The sample was illuminated at step-wise increasing PPFD with 1-min illumination periods at each (Schreiber et al., 1994). The resulting light response curves provide information on the antenna size and photosynthetic capacity of a sample.

Received October 11, 2000; returned for revision November 29, 2000; accepted January 19, 2001.

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