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Nitrogen deprivation strongly affects Photosystem II but not phycoerythrin level in the divinyl-chlorophyll *b*-containing cyanobacterium *Prochlorococcus marinus*

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Abstract

Effects of nitrogen limitation on Photosystem II (PSII) activities and on phycoerythrin were studied in batch cultures of the marine oxyphotobacterium *Prochlorococcus marinus*. Dramatic decreases in photochemical quantum yields (F_V/F_M), the amplitude of thermoluminescence (TL) B-band, and the rate of Q_A reoxidation were observed within 12 h of growth in nitrogen-limited conditions. The decline in F_V/F_M paralleled changes in the TL B-band amplitude, indicative of losses in PSII activities and formation of non-functional PSII centers. These changes were accompanied by a continuous reduction in D1 protein content. In contrast, nitrogen deprivation did not cause any significant reduction in phycoerythrin content. Our results refute phycoerythrin as a nitrogen storage complex in *Prochlorococcus*. Regulation of phycoerythrin gene expression in *Prochlorococcus* is different from that in typical phycobilisome-containing cyanobacteria and eukaryotic algae investigated so far.

Keywords: Cyanobacteria; Phycoerythrin; Photosynthesis; Light-harvesting complex; Variable fluorescence; Nitrogen deprivation

1. Introduction

Prochlorococcus marinus is the only known prokaryote containing phycoerythrin (PE) in the presence of divinyl-chlorophyll (DV-Chl) *a* and *b* as the major light-harvesting pigments [1]. This remarkable pigment complement is specific of several low-light adapted *Prochlorococcus* strains [2–4].

In contrast, *Prochlorococcus* strains adapted to high light (e.g., MED4) have a much lower DV-Chl *b* content [2,3,5] and do not possess the minimal set

Abbreviations: Chl, chlorophyll; DV, divinyl; F_0, minimum fluorescence yield; F_M, maximum fluorescence yield; F_V, variable fluorescence; F_V/F_M, yield of Photosystem II photochemistry; FRRf, fast repetition rate fluorometer; N, nitrogen; PE, phycoerythrin; P.S.I., Photon Systems Instruments; PSII, Photosystem II; Q_A, the primary quinone electron acceptor in Photosystem II; σ_{PSII}, effective absorption cross-sections of Photosystem II; TL, thermoluminescence

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of genes to synthesize PE-III as it is present in the low-light strains [1,4,6]. The functional *cpeB-cpeA* operon (encoding the α- and β-PE subunits) and its associated gene cluster which has been extensively studied in SS120 [1,7] is reduced in MED4 to a highly mutated residual *cpeB*-like gene (Hess et al., in preparation).

In the central part of inter-tropical oceans, high-light adapted ecotypes are typically found from the surface down to the deep Chl maximum (approx. 100–140 m). In contrast, low-light adapted populations colonize the light niche corresponding to approx. 0.1–6% of incident surface irradiance (approx. 80–200 m) [3,8–10]. Differences in pigmentation, such as genotypic variations in the Chl *b* to *a* ratio or the presence/absence of PE, may be important factors for the different ecotypes to be competitive within their respective light niches. Function and regulation of PE expression in low-light adapted *Prochlorococcus* strains have remained unresolved. *Prochlorococcus* PE is not associated with classical phycobilisomes, as is C-PE in common cyanobacteria [7]. However, a light-harvesting function for this PE was previously suggested by its (1) association with thylakoid membranes [7] and (2) apparent energy transfer to Photosystem II (PSII) [11]. The small amount of PE within SS120 cells argues against a high relevance for photosynthetic light-harvesting.

Within the cyanobacterial radiation, *Prochlorococcus* is most closely related to the marine *Synechococcus* cluster A [3,12]. In one representative strain of this cluster, *Synechococcus* DC2 (=WH7803), PE reportedly functions as a dynamic N storage pool that is rapidly degraded when N becomes limiting [13]. Indeed, N deprivation in cyanobacteria causes rapid proteolytic degradation of major light-harvesting pigments (PE and other phycobiliproteins), inevitably coincident with a loss in photosynthetic capacity [14–16].

Since *Prochlorococcus* is the most abundant phototroph in the chronically low-N tropical and subtropical oceans [17], its adaptive responses to N stress are of particular interest. Effects of N limitation on the photosynthetic apparatus of *Prochlorococcus* in general, and with particular focus on its PE content, have not been studied. We therefore evaluated photosynthetic performance during the time course of N stress. Variable fluorescence (F_V) and thermoluminescence (TL) techniques were used to measure initial Chl fluorescence emission (F_0), effective absorption cross-sections of PSII (σ_{PSII}), photochemical quantum yields (F_V/F_M), Q_A reoxidation rates, and the fraction of functional PSII reaction centers. These photosynthetic parameters were correlated with changes in PE content and D1 protein concentration.

2. Material and methods

2.1. Culture and growth conditions

P. marinus clone SS120 (=CCMP 1375) (courtesy of Prof. S.W. Chisholm and Dr. L.R. Moore) was grown at 21±1°C in PCR-S11 medium [6,18] under 20 μmol quanta m^{-2} s^{-1} continuous blue light. Prior to the N starvation experiment, a 0.7 l aliquot was withdrawn from the 5.5 l preculture at time zero to determine initial values for measured physiological variables. The preculture was then divided into four batches of 1.2 l and independently harvested by centrifugation at 10 000 rpm for 10 min in a Beckman Avanti J25. Two batches of pelleted cells were then separately resuspended in 3.6 l of PCR-S11 medium minus $(NH_4)_2SO_4$ (i.e., −N experimental treatment). The other two pelleted samples were resuspended in 3.6 l of standard PCR-S11 medium (i.e., +N control treatment). The replicate samples for each treatment were then incubated at the light and temperature conditions described above and 0.7 l aliquots collected over the subsequent 48 h for analysis of protein content, photosynthetic performance and cell concentration. For the latter analysis, 1 ml samples were fixed with 10% paraformaldehyde and 0.02% glutaraldehyde, frozen in liquid nitrogen and kept at −80°C. After thawing, analysis was made using a FacSort flow cytometer (Becton Dickinson), as detailed elsewhere [19].

2.2. Immunology

For protein extraction, cells were collected by centrifugation and disrupted by adding 0.1% SDS sonicating them for 10 s each at 4°C with three strokes of a Sonopuls HD 60 set at 50% of maximum power. Protein concentrations were measured using the Bio-

Rad protein assay. The generation of a polyclonal antiserum specific of recombinant *P. marinus* SS120 α-PE was described previously [7]. An antiserum against D1 from pea chloroplasts (courtesy of Dr. P.J. Nixon) was used for comparison. Western blots were prepared from total protein samples separated on 12% SDS-polyacrylamide gels (normalized to 5 μg per lane) and blotted on Hybond-C extra membrane (Amersham). Incubation with antisera was performed at titers of 1:600 (α-PE antibody) and 1:1000 (D1 antibody), respectively. Each blot was developed by both antisera first employing a chemiluminescence substrate and then using a chromogenic substrate. Secondary antisera were conjugated with alkaline phosphatase or horseradish peroxidase and blots developed using the chromogenic substrates nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate or the chemiluminescence substrate SuperSignal (Pierce). All Western blots were repeated 7 times.

2.3. Fluorescence emission spectra

Fluorescence emission spectra were determined with a LS50 spectrofluorometer (Perkin-Elmer) equipped with a red-sensitive photomultiplier. For these measurements, 250 ml samples of control and experimental cultures of the last time point were centrifuged and resuspended in 5 ml PCR-S11 +N or PCR-S11 −N medium. Fluorescence was excited at 495 nm (absorption and fluorescence excitation maximum of phycourobilin) in the presence or absence of glycerol, which detaches PE from thylakoid membranes thus enhancing PE fluorescence emission [11,13]. To obtain comparable Chl concentrations, cell suspensions from both +N and −N cultures were either mixed with glycerol to a final concentration of 50% or with the same volume of culture medium.

2.4. Variable fluorescence measurements

F_V measurements were conducted using a benchtop fast-repetition-rate fluorometer (FRRf) [20–22]. The FRRf exposes cells to a 60–80 μs series of subsaturating excitation flashes (blue LEDs, λ = 450 nm) and detects changes of Chl fluorescence yield from the initial dark-adapted state (F_0), when all functional PSII reaction centers are oxidized, to the light-saturated state (F_M), when all PSII reaction centers are photochemically reduced. Photochemical quantum yields (F_V/F_M) were calculated as a ratio of variable to maximum fluorescence (i.e., $F_V/F_M = (F_M - F_0)/F_M$). The FRRf measurement permits calculation of effective absorption cross-sections for PSII (σ_{PSII} in Å^2), based on kinetics analysis of fluorescence induction from F_0 to F_M [20,22] and subsequent kinetics of Q_A^- reoxidation. The samples were briefly dark adapted prior to measurements to ensure complete opening of PSII reaction centers. Values of F_V/F_M, σ_{PSII}, and Q_A^- reoxidation rate were calculated as averages from five standard FRR flash sequences [20], each separated by 1 min.

F_V measurements were also conducted using a Dual-Modulation Kinetic Fluorometer (FL-100, Photon Systems Instruments (P.S.I.), Brno, Czech Republic) following Trtilek et al. [23] and Dijkman et al. [24] with the following modifications [23,24]. The standard PIN detector was replaced by a photomultiplier (R2228, Hamamatsu, Japan) protected by a 700 nm interference filter. Measuring pulses (2.5 μs) were provided by a set of seven blue LEDs (λ = 450 nm) filtered through a 650 nm short-pass dichroic filter. Samples (1.5 ml) were placed in 10 × 10 mm quartz cuvette mirrored on the side opposite the detector. F_0 values were determined in the dark by four weak measuring flashes. PSII centers were then transiently closed by applying a single turnover pulse of light. F_M was measured by probing 45 μs after the ST pulse and Q_A reoxidation kinetics determined by following F_V decay. Chl fluorescence induction in the presence of DCMU was elicited by a train of 1000 subsaturating flashes spaced every 100 μs. The resultant induction curve was then fitted to the cumulative one-hit Poisson function, $F = F_0 + F_V e^{-\sigma_{PSII} t}$, where F = fluorescence measured during induction, F_V = variable fluorescence, and t = time.

2.5. Thermoluminescence measurements

TL was measured with a computerized, laboratory-built apparatus [25]. Samples were gently collected on a support filter (Millipore HA, pore size 0.4 μm, 25 mm diameter) and placed on the sample holder. The sample was kept in the dark at laboratory tem-

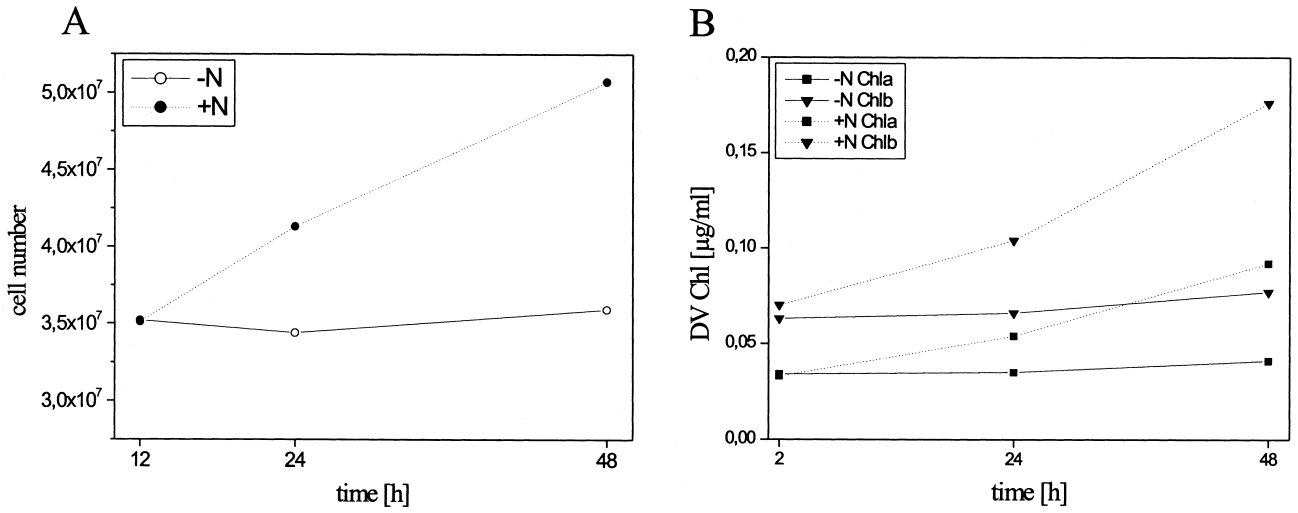

Fig. 1. Content in DV-Chl *a* and *b* (B) during one of each experimental series (−N and +N, respectively) and cell concentration (A).

perature and then rapidly cooled (10°C s^{-1}) down to 5°C. The sample was then illuminated by two single turnover flashes from a Xe flash lamp (EG and G). TL was then recorded during a linear heating treatment (0.5°C s^{-1}) from +5 to +70°C.

2.6. Pigment analyses

15–50 ml (depending on cell density) samples were filtered onto Whatman GF/F glass fiber filters, flash frozen in liquid N_2 and then stored at −80°C. Pigment analysis was performed within 1 month after sample collection according to a modification of the protocol described by Vidussi et al. [26]: the column internal diameter was 3 mm (instead of 4.6 mm), the flow rate was set at 0.5 ml min^{-1} (instead of 1 ml min^{-1}) while the solvent gradient was varying as follows: (*min*; % solvent A; % solvent B): (*0*; 80; 20), (*4*; 50; 50), (*18*; 0; 100), (*22*; 0; 100).

3. Results

3.1. Cell growth and changes in pigmentation

Following an initial increase in both treatments, cell division ceased after 12 h in the −N treatment, but continued unabated for the duration of the experiment in the +N treatment (Fig. 1A). Similarly, DV-Chl *a* and *b* and zeaxanthin concentrations steadily increased in the +N cultures, but remained essentially constant under −N conditions (Table 1, Fig. 1B).

Initial (F_0) and maximum (F_M) fluorescence yields in the control culture increased 260% (±15%) during the 48 h experiment, entirely accounted for by corresponding increases in DV-Chl *a* and *b* (Table 1, Figs. 1 and 4). Under N deplete conditions, F_M exhibited a transient increase within 12 h, followed by a monotonic decrease to 58% (±1%) of the initial

Table 1
Pigment content and pigment ratios

	Zea (µg/ml)	α-Car (µg/ml)	DV-Chl *b*/*a*	α-Car/DV-Chl *a*	Zea/DV-Chl *a*
Preculture	0.064	0.049	2.010	0.264	0.349
2 h −N	0.013	0.008	1.865	0.251	0.375
24 h −N	0.015	0.010	1.903	0.273	0.433
48 h −N	0.018	0.010	1.889	0.244	0.436
2 h +N	0.013	0.009	2.104	0.271	0.392
24 h +N	0.022	0.014	1.903	0.263	0.409
48 h +N	0.037	0.024	1.901	0.263	0.401

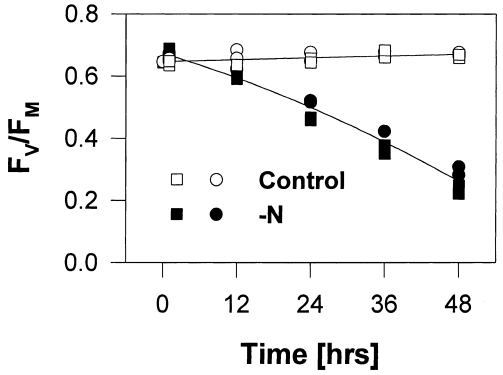

Fig. 2. Changes in F_V/F_M ratio. Empty symbols signalize control, the closed symbols the N deprived culture (both treatments in duplicate). Photochemical quantum yields (F_V/F_M) were calculated as a ratio of variable and maximum fluorescence (i.e., $F_V/F_M = (F_M - F_0)/F_M$) obtained either by rapid induction FRR technique or by standard pump-probe method (P.S.I.) fluorometer. The data points shown are means of five FRR measurements (squares) or three P.S.I. fluorometer measurements (circles) separated by 1 min recovery.

value. F_0 likewise increased slowly during the first 24 h in the −N treatment and then plateaued at a value of 30% (±17%) above the initial level (Fig. 4). This increase of F_0 was markedly higher than the coincident increase in Chl concentration (approx. 15%).

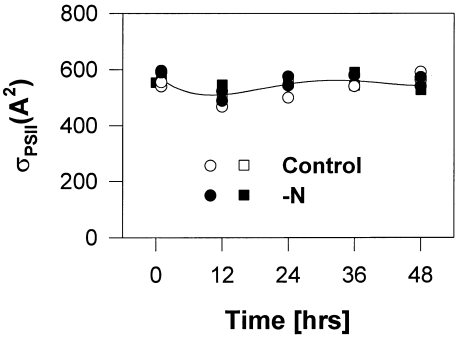

Fig. 3. Time course of the effective absorption cross-section of PSII (σ_{PSII}). Empty symbols signalize control, the closed symbols the N deprived culture. Effective absorption cross-section was determined from analysis of FRR Chl fluorescence induction [20] or alternatively by a standard slow induction in the presence of DCMU (P.S.I. fluorometer). The data points shown are means of five FRR measurements separated by 1 min recovery (squares) or two DCMU induction measurements (P.S.I.) separated by 5 min recovery (circles).

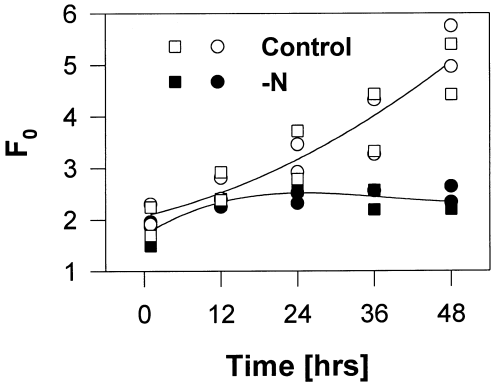

Fig. 4. Time course of F_0 – minimum fluorescence of PSII Chl emission. Empty symbols signalize control, the closed symbols the N deprived culture. The data points shown are means of five FRR measurements (squares) or three measurements by dim probing flashes (P.S.I.) separated by 5 min recovery (circles).

3.2. Photosynthetic measurements

A consistent value for F_V/F_M (0.66 ± 0.02) was observed in all cultures during the first 2 h of the N limitation experiment (Fig. 2). F_V/F_M decreased monotonically during the subsequent 46 h in the −N treatment, while no change was observed in the +N treatment (Fig. 2).

Fluorescence-based measurements of PSII effective absorption cross-sections (σ_{PSII}) provide an index of the average functional size of light-harvesting antennae for all functional PSII reaction centers. We as-

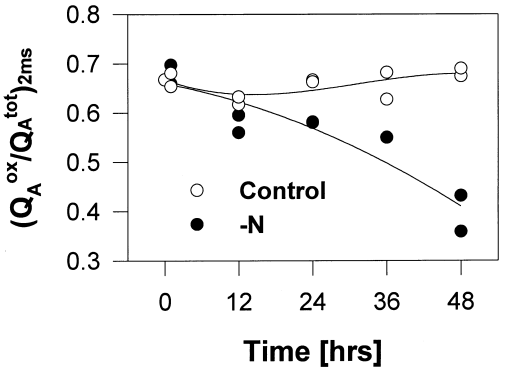

Fig. 5. Time course of Q_A reoxidation (PSII turnover) rate. The Q_A reoxidation rate was determined as a fraction of Q_A which reoxidizes within 2 ms after the single turnover flash. The data points shown are means of three pump-and-probe measurements (P.S.I.) separated by 1 min recovery (circles).

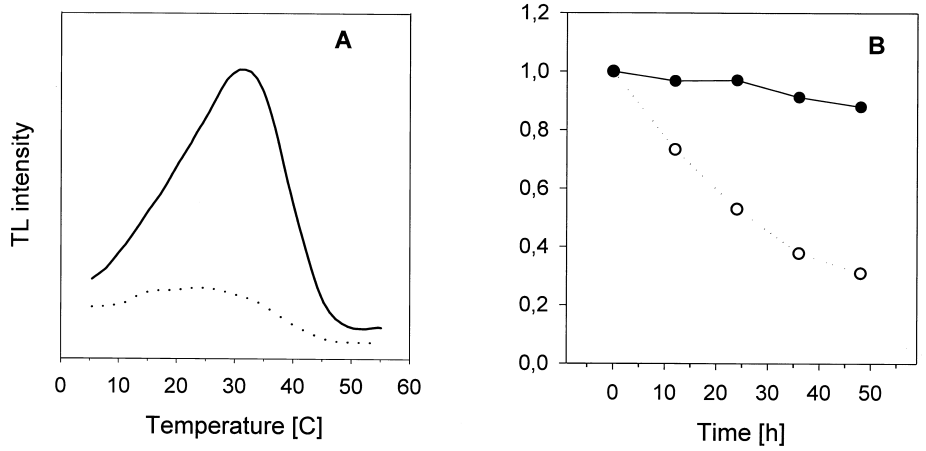

Fig. 6. TL glow curves of intact control cells (time = 0 h, full line) and N depleted cultures (time = 48 h, dotted line) (B). Fig. A shows the changes of the overall TL signal (the area below the glow curve from +5 to +55°C, normalized to Chl content) during the course of the N deprivation experiment.

sessed variability in σ_{PSII} using both single-turnover FRR induction techniques [20] and standard slow fluorescence induction in the presence of DCMU (P.S.I. fluorometer). Both measurement techniques clearly documented little change in σ_{PSII} in either the +N or −N treatments throughout the experimental time course (Fig. 3).

The rate of Q_A reoxidation provides an index of electron transfer kinetics between the primary electron acceptor (Q_A) and the secondary acceptor (Q_B) of PSII. Q_A reoxidation rate was constant in the +N treatment for the duration of the experiment. In contrast, −N cultures exhibited a continuous decrease in Q_A reoxidation rate to a final value only 60% of that in the control treatment (Fig. 5).

TL emission curves for intact +N cells peaked in the B-band at 32°(Fig. 6A), corresponding to the $S_{2/3}Q_B^-$ recombination [27]. No corresponding TL maximum was observed in −N cultures, but rather a broad plateau of increased TL emission occurred between +15 and +30°C. Normalized TL intensity also decreased significantly in the −N cultures (Fig. 6B), while remaining nearly constant in control cells. The intensity of TL emission in N deprived cells

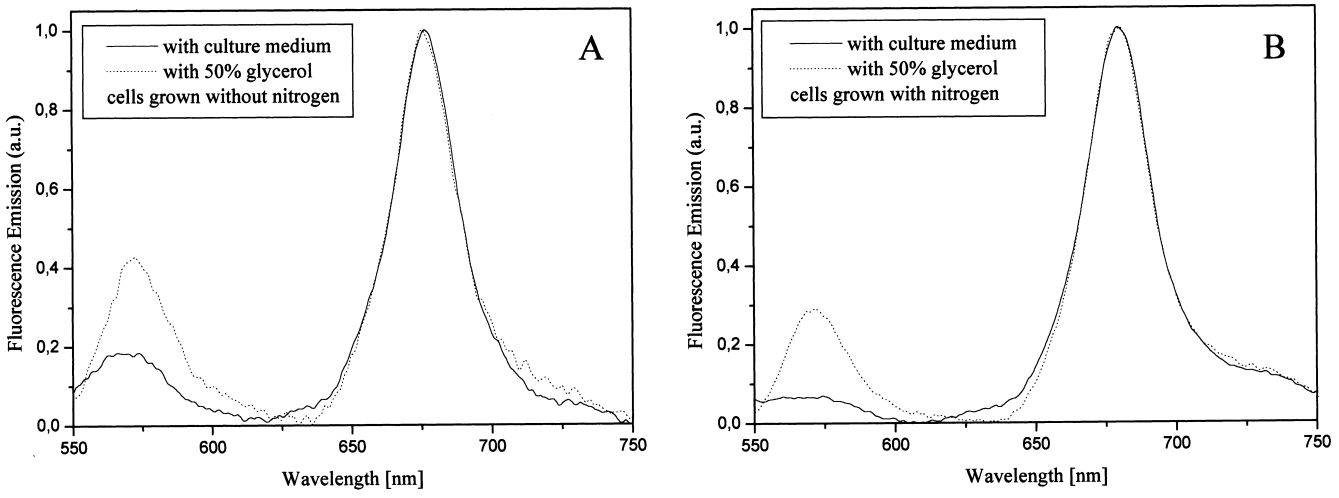

Fig. 7. Fluorescence emission spectra of cell suspensions of *Prochlorococcus* grown under N-depleted (A) or N sufficient (B) conditions. Cells were treated with 50% glycerol (dashed lines) enhancing the otherwise very weak PE signal. To obtain the same cell density untreated cell suspensions (solid lines) were diluted with the same volume of culture medium. Samples were excited at 495 nm and fluorescence emission detected at 572 nm. Spectra were normalized to the DV-Chl *a* peak at around 675 nm.

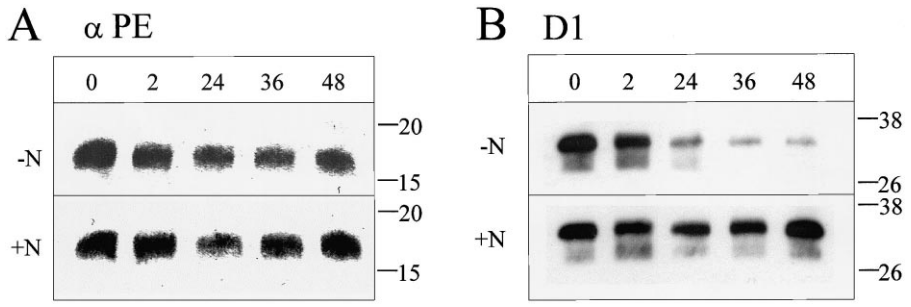

Fig. 8. Western blot analysis of amounts of α-PE (A) and D1 protein (B), respectively, under N starvation (−N) and under N sufficient conditions (+N). Each lane was loaded with 5 μg of total protein. Values on top correspond to hours after cells were transferred to PCR-S11 −N or PCR-S11 medium. Molecular masses (in kDa) of selected marker bands are given for the respective size range on the right. Blots were developed using the chromogenic substrates nitroblue tetrazolium and bichlorindophenol (A) or the chemiluminescence substrate SuperSignal (Pierce) (B).

dropped to 30% of control values by the end of the experiment.

3.3. Detection of α-phycoerythrin and D1 protein

Fluorescence emission spectra (495 nm excitation) were measured at the cessation of the 48 h experiment. Glycerol addition provoked a strong increase in fluorescence at 572 nm in both +N and −N samples, relative to samples without glycerol (Fig. 7). We attribute this fluorescence increase to the detachment of PE from thylakoid membranes and consequential interruption of energy transfer from PE to Chl [11,13]. For the +N treatment, compared to Chl fluorescence (peaking at around 675 nm), PE fluorescence was weak prior to glycerol addition (Fig. 7B), whereas −N cells exhibited pronounced emission prior to glycerol treatment (Fig. 7A).

Western blots were performed to assess whether N limitation altered the relative abundance of PE or the reaction center core protein, D1. Neither +N or −N treatment invoked significant changes of PE level during the time course of the experiment (Fig. 8A). However, N limitation did induce severe reductions in D1 content (Fig. 8B) content with regard to the total protein pool. Samples from the duplicate culture showed identical trends (not shown).

4. Discussion

Chl fluorescence data did not indicate any significant changes in F_V/F_M, σ_{PSII}, Q_A^- reoxidation rate, or the TL emission in the +N control treatment (Figs. 2,3,5 and 6A). The rise of absolute values of F_0 and F_M fluorescence yields is caused solely by the increase in Chl content. In contrast, already within the first 12 h of the treatment, N limitation caused a dramatic decrease in F_V/F_M as well as in the relative amplitude of TL B-band, and in the rate of Q_A reoxidation. Thus, the rapid onset of N limitation in the −N cultures and stable growth in the control, +N treatment is clearly illustrated. The decline in F_V/F_M paralleled decreases in the relative amplitude of the TL B-band, indicating an increase in non-functional PSII centers. In addition, disappearance of a distinct B-band and co-occurrence of broad TL emission at lower temperatures suggests a possible functional modification on the PSII acceptor side in the Q_A-Q_B region. Acceptor side electron transfer efficiencies decreased in the remaining functional PSII centers, as evidenced by decreased Q_A^- reoxidation rates. Increases in F_0 (approx. 30%) were higher than coincident increases in Chl content (approx. 15%) in the −N treatment, suggesting an alteration in the photosynthetic apparatus. An increase in F_0 can result from the decoupling of light-harvesting complexes. However, the constant σ_{PSII} observed in the −N cells throughout the experiment contradicts this potential mechanism. An alternative mechanism for increases in F_0 is the formation of inactive reaction centers with fluorescence emission above the F_0 level of active PSII centers. This possibility is consistent with observed decreases in F_V/F_M (Fig. 2) and D1 protein content (Fig. 8). D1 protein is the most rapidly turned over component of the thylakoid membrane

[28] and its continuous recycling is critical for maintaining PSII activity [29]. In the case of *P. marinus*, N limitation quite probably blocked de novo protein synthesis and consequently inhibited PSII repair, leading to the progressive inactivation of PSII reaction centers despite the low incident growth irradiance. The strong decrease of D1 protein levels under N deprivation in *P. marinus* is consistent with results for the eukaryotic alga, *Phaeodactylum tricornutum* [30]. In contrast, no significant changes in D1 content was reported for the cyanobacterium *Synechococcus* PCC 6301 grown under comparable N limiting conditions [31]. Fluorescence emission spectra showed a pronounced PE signal under N deprivation. This might be interpreted as a functional decoupling of PE from the thylakoid membranes.

In contrast to what has been observed in several *Synechococcus* strains [13,15,32,33] and some cryptophyceae [34], N deprivation did not significantly reduce PE content in *P. marinus*. Consequently, utilization as a N storage complex, as previously suggested for *Synechococcus* DC2 PE [13], can unambiguously be excluded for the *Prochlorococcus* PE. In a previous study, we showed that growth irradiances from 8–38 μmol photons m^{-2} s^{-1} did not induce any change in PE content as well [7]. Thus, PE gene expression in *Prochlorococcus* is different from what has been observed for all other classical phycobilisome-containing cyanobacteria investigated so far.

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