The manganese stabilizing protein (MSP) and the control of O₂ evolution in the unicellular, diazotrophic cyanobacterium, *Cyanothece* sp. ATCC 51142

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Abstract

The unicellular diazotrophic cyanobacterium, *Cyanothece* sp. ATCC 51142 temporally separates N₂ fixation from photosynthesis. To better understand the processes by which photosynthesis is regulated, we have analyzed Photosystem (PS) II O₂ evolution and the PSII luminal proteins, especially the Mn stabilizing protein (MSP). We describe a procedure using glycine betaine to isolate photosynthetic membranes from *Cyanothece* sp. that have high rates of PSI and PSII activity. Analysis with these membranes demonstrated similar patterns of O₂ evolution in vivo and in vitro, with a trough at the time of maximal N₂ fixation and with a peak in the late light period. The pattern of PSI activity was also similar in vivo and in vitro. We cloned the genes for MSP (*psbO*) and the 12 kDa protein (*psbU*) and analyzed their transcriptional properties throughout the diurnal cycle. We suggest that the changes in PSII activity in *Cyanothece* sp. were due to conformational changes in a highly flexible MSP, a suggestion which can now be studied in a chimera. The *Cyanothece* sp. *psbO* gene has been transformed into *Synechocystis* sp. PCC 6803; MSP and O₂ evolution in the resulting transformant had properties that were similar to those in *Cyanothece* sp., providing additional confirmation for the properties of *Cyanothece* sp. MSP. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

*Cyanothece* sp. strain ATCC 51142 is a unicellular, aerobic, diazotrophic cyanobacterium that maintains a temporal separation of N₂ fixation and photosynthetic O₂ evolution during its diurnal cycle [1] in order to protect the O₂ labile nitrogenase enzyme. The peak of N₂ fixation occurs approx. 4 h into the dark period, when cultures were grown under 12 h light-12 h dark (LD) conditions, and is repeated at 24 h intervals. Photosynthetic O₂ evolution varies throughout the day, with a peak of activity at about L8 and a minimum of activity at D4. These rhythms of O₂ evolution and N₂ fixation occur with peaks of activity approx. 12 h apart in LD, continuous light (LL), and continuous dark (DD) grown cultures [2-4].

At the peak of N₂ fixation, O₂ evolution is markedly decreased in *Cyanothece* sp. by a mechanism or
mechanisms for the downregulation of oxygenic photosynthesis [5–7]. Fluorescence analysis of the Cyanothece sp. photosynthetic reaction centers by 77 K and pulse amplitude modulated (PAM) fluorescence has revealed a cyclic heterogeneity of the Photosystem (PS) II reaction centers [6,7], which are the sites of water oxidation and the concomitant evolution of O2. This analysis revealed state transitions and changes in the oligomerization state of PSII during the Cyanothece sp. diurnal cycle [6]. State transitions represent the way that cyanobacteria and plants regulate the transfer of excitation energy between PSI and PSII. In cyanobacteria, this regulation of excitation energy transfer involves the association or dissociation of the light harvesting phycobilisomes with PSII [8]. The oligomerization state of PSII (monomers or dimers) may also affect phycobilisome association with PSII and PSII electron transport efficiency [9]. Transcriptional and translational analysis of photosynthetic reaction center proteins has also revealed a strong regulation of protein production during the diurnal cycle of Cyanothece sp. [5]. These results have led to our interest in the mechanisms for downregulating Photosystem II and O2 evolution at the peak of N2 fixation in this organism.

In both prokaryotic and eukaryotic O2 evolving organisms, three extrinsic luminal proteins of PSII are involved in stabilizing O2 evolution. The manganese stabilizing protein (MSP, coded for by the psbO gene) is found in all photosynthetic organisms at the PSII O2 evolving complex, but the other two proteins differ [10]. Prokaryotes contain a 12 kDa protein (coded for by psbU) and cytochrome c550 (psbV) in place of the eukaryotic 23 kDa protein (psbP) and the 16 kDa protein (psbQ). Even though the 12 kDa protein and cytochrome c550 are distinctly different in sequence from the 23 kDa and 16 kDa proteins, they appear to share common binding and functional properties [10,11]. Our interest in the regulation of O2 evolution has led us to study psbO and psbU transcription and MSP accumulation in Cyanothece sp. As the name suggests, the MSP stabilizes the PSII Mn center, which is the site of water oxidation. Removal of MSP from the PSII Mn center by ion extraction or deletion mutagenesis greatly inhibited O2 evolution in cyanobacteria [12,13]. The suggestion that the MSP is involved in O2 evolution regulation stemmed from the observation that Cyanothece sp. O2 evolution, during discrete periods of its diurnal cycle, resembled the O2 evolution characteristics of a Synechocystis sp. PCC 6803 MSP deletion mutant [7,12]. This hypothesis was based on O2 evolution stability results on a bare platinum electrode, 77 K fluorescence analysis, and net O2 evolution yield on a Clark-type electrode [6,7]. This regulation would be timed to insure the production of reducing equivalents and fixed carbohydrate during the light period for later use in N2 fixation and the prevention of oxygen accumulation during nitrogen fixation in the dark or subjective dark period.

Our previous results indicated that short-term (e.g., state transitions) and long-term (e.g., biosynthetic alterations) occurred in the photosynthetic mechanism during the Cyanothece sp. ATCC 51142 diurnal cycle. We were interested in understanding the changes occurring in PSII in greater detail. For example, are the alterations in PSII permanent, such that O2 evolution in vitro will be downregulated as in vivo? Similarly, what happens to PSI when measured in isolated membranes? Is MSP responsible for these changes in O2 evolution, and can we detect changes in MSP accumulations as a function of time? We have attempted to answer some of these questions by developing a procedure to study PSII O2 evolving activity in vitro. We have also analyzed MSP and PsbU in some detail, including the cloning of their respective genes in Cyanothece sp. We will describe an excellent procedure for the isolation of membranes active in O2 evolution and describe how in vitro and in vivo photosynthetic activities compare and contrast. We will also discuss the nature of MSP accumulation and the transcriptional regulation of psbO and psbU. We will suggest that the structure of MSP is somewhat different in cyanobacteria than in plants [14] and that Cyanothece sp. MSP has properties that are distinct from other cyanobacteria. To test this contention, we have successfully transformed the Cyanothece sp. ATCC 51142 psbO gene into Synechocystis sp. PCC 6803, a unicellular, non-diazotrophic, transformable strain that is an important cyanobacterial model system. We will provide a preliminary characterization of this chimeric transformant.
2. Materials and methods

2.1. Growth conditions, nitrogenase activity, and chlorophyll determination

*Cyanothece* sp. strain ATCC 51142 (formerly *Cyanothece* strain BH68K) was cultured as previously described in a modified minimal salt medium (ASP2) with or without 1.5 g NaNO₃ per liter [1,3]. Nitrogenase activity was measured by employing a modified acetylene reduction method [1,15,16] and is described in detail by Colón-López et al. [2]. Cell counting and chlorophyll determination [17] have been described previously [2,6,16]. These experiments were performed under LD or LL growth conditions.

2.2. Measurement of O₂ evolution, PSI activity, and respiration

Photosynthetic and respiratory rates were determined by measuring oxygen production and consumption on a Clark-type electrode (YSI Model 5300 Biological Oxygen Monitor and Oxygen Probe Model 5331, Yellow Springs Instruments, Yellow Springs, OH) in a water jacketed 3 ml stirred cuvette (maintained at 30°C) under heat filtered illumination (light intensity of 1800 μE m⁻² s⁻¹) [6]. The rate of O₂ evolution was determined as previously described [6]. PSI activity was measured by observing light activated O₂ consumption in cell extracts with the following additives: 10 μM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), 0.1 mM methyl viologen, 5 mM Na azide, 5 mM ascorbate; 0.2 mM DCPIP (2,6-dichlorophenolindophenol), and 10 mM KCN. For maximum PSI activity, 25 μM cytochrome c₆ (isolated from *Arthrospira maxima*, and a kind gift of Dr. David Krogmann [18]) was added to the PSI assay buffer in place of DCPIP. Whole cell respiration was determined from the rate of O₂ consumption in the dark. The signal from the Clark electrode was digitized and a linear O₂ regression analysis was performed using LabView 4.0.1 (National Instruments, Austin, TX; programmed by Dr. Pascal Meunier) on a Macintosh computer [6]. Rates of O₂ evolution, PSI activity, and respiration are reported in μM O₂ (mg Chl)⁻¹ h⁻¹.

2.3. Isolation of O₂ evolving extracts

Highly active and stable O₂ evolving extracts of *Cyanothece* sp. ATCC 51142 were prepared by slightly modifying the protocol of Mamedov et al. [19]. This protocol is based on the observation that glycine betaine (betaine) stabilizes cyanobacterial and plant O₂ evolution [19-21]. Two buffers were used to prepare the O₂ evolving membranes: (1) cell wash buffer (Wash, 50 mM MES pH 6.5 and 30 mM CaCl₂); (2) cell breakage/extraction buffer (Breakage, 50 mM MES pH 6.5, 20 mM CaCl₂, 0.8 M sorbitol (Sigma), 1.0 M betaine (Sigma), and 1 mM ε-amino-n-caproic acid). Both buffers were filter-sterilized and stored at 4°C.

N₂ fixing cultures of *Cyanothece* sp. were harvested during logarithmic growth (3-8×10⁶ cells ml⁻¹). For time course experiments, cultures were harvested at 2 h intervals or occasionally as bulk harvests at specific times of the diurnal cycle. Cells were maintained at 4°C or on ice unless otherwise noted. The cells were pelleted from the medium and washed twice in 20 ml of Wash buffer. The washed cells were then resuspended in 10-20 ml of Breakage buffer (volume of resuspension dependent on original cell density). The cells were broken by 1–3 passages through a precooled French Pressure Cell (Model No. FA-073) at 20 000 psi. Unbroken cells and debris were removed by centrifugation at 8000×g for 10 min. The chlorophyll concentration of the extract was determined and Breakage buffer was used to dilute the extracts to 3 μg Chl ml⁻¹. Highly active and stable O₂ evolving extracts of *Cyanothece* sp. ATCC 51142 were prepared by slightly modifying the protocol of Mamedov et al. [19]. This protocol is based on the observation that glycine betaine (betaine) stabilizes cyanobacterial and plant O₂ evolution [19-21]. Two buffers were used to prepare the O₂ evolving membranes: (1) cell wash buffer (Wash, 50 mM MES pH 6.5 and 30 mM CaCl₂); (2) cell breakage/extraction buffer (Breakage, 50 mM MES pH 6.5, 20 mM CaCl₂, 0.8 M sorbitol (Sigma), 1.0 M betaine (Sigma), and 1 mM ε-amino-n-caproic acid). Both buffers were filter-sterilized and stored at 4°C.

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2.4. Western blot analysis and antibody probes

Whole cell protein extracts were prepared as described previously [2,16]. Protein concentration was determined with Bradford reagent (0.1 mg ml⁻¹ Coomassie blue G-250 (Bio-Rad, Hercules, CA), 5% ethanol, 8.5% phosphoric acid) with bovine serum albumin (Sigma, St. Louis, MO) as the standard
2.5. RNA isolation, Northern blot analysis, and DNA probes

Total RNA from LD grown Cyanothece sp. cultures was extracted at 2 h intervals for 24 h. RNA isolation and Northern blot analysis procedures were performed as described by Reddy et al. [23] and Colón-López et al. [2] with only slight modifications. To increase cell breakage efficiency, sample vortexing with glass beads and phenol was increased from 5 to 7 min. During ultracentrifugation for collection of RNA, samples were spun at 264,000 × g for 90 min. Nitrogenase activity was measured concurrently with RNA extraction to verify that the peak of nitrogenase occurred near D4 as seen previously in Cyanothece sp. The following homologous DNA probes were used for Northern blot analysis: (1) a 600 bp fragment containing psbO (accession No. AF201467) and (2) a 400 bp fragment containing psbU (accession No. AF36250 [24]). Densitometric band analysis of Northern blots was performed by digitizing the blots with a Sharp JX-610 Scanner and Power Macintosh computer with Adobe Photoshop 3.0 software. Band density, from the Northern blot scans, was analyzed with IPLab Gel 1.5c (Signal Analytics, Fairfax, VA).

2.6. Cloning, sequencing and analysis of psbO

Labeling and psbO probe purification were performed as previously described [2,25,26]. A number of putative positive clones were identified during primary library screening with the homologous 600 bp psbO fragment. Three of these clones provided the N- and C-terminal psbO coding regions not present in the PCR produced 600 bp internal psbO fragment. Sequencing was performed at the Purdue DNA Sequencing Center. DNA sequences were aligned with AssemblyLign 1.0.7 software (IBI, New Haven, CT) and analyzed with MacVector 5.0 and GCG (Genetics Computer Group, Madison, WI). Homology searches were performed in GenBank using the Entrez Browser (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/Entrez/index.html). Prettybox analysis was performed on GCG and visualized with MacBoxshade 2.15E (Shareware, M.D. Baron, Perbright, UK). Secondary structure analysis was performed with ClustalW (Network Protein Sequence Analysis, Lyon, France).

The psbO gene from Cyanothece sp. ATCC 51142 was transformed into Synechocystis sp. PCC 6803 using standard techniques. The construct included a gene for spectinomycin resistance, the full psbO gene and flanking regions on both sides of the Synechocystis sp. PCC 6803 psbO gene. PCR and Southern blot analysis of the transformant, Synechocystis Cy1, indicated that all copies of the wild-type gene had been eliminated (H. Li, B. Boardman and L.A. Sherman, unpublished observations).

3. Results

3.1. Rhythms of N2 fixation and O2 evolution in Cyanothece sp.

Diurnal rhythms of N2 fixation and O2 evolution occur in Cyanothece sp. with a peak of nitrogenase activity approx. 4 h into the dark (or subjective dark) period (Fig. 1A). The capacity to evolve oxygen was greatest during the mid-light period and at a minimum during the peak of nitrogenase activity (Fig. 1A) [2,6]. These rhythms, which were seen in LD, LL and DD grown cultures, appear to be temporally separated for the protection of the oxygen-labile nitrogenase enzyme [2,3]. The change in O2 evolution capacity under all growth conditions was an indication that the regulation of photosynthesis was independent of light [16]. A further understanding of this regulation required the analysis of photosynthesis in vitro, and led to the development of a procedure to isolate cell extracts from Cyanothece sp. ATCC 51142 that were highly active in O2 evolution.

3.2. Isolation of oxygen evolving extracts

Early attempts at developing O2 evolving extracts using a variety of protocols met with little success in
Cyanothece sp. ATCC 51142. This led to the use of glycine betaine to stabilize the Mn center of PSII and in vitro O₂ evolution [19–21], an approach that was immediately successful. The initial experiment, which employed a 1 M betaine buffer for Cyanothece sp. cell breakage and extract dilution, produced highly active and stable O₂ evolving extracts. The protocol employed in these experiments was based on Mamedov et al. [19] with only slight modifications. The major modification involved a decrease in the cell wash and cell breakage buffers from pH 7.5 [19] to pH 6.5. This change increased the overall net yield of Cyanothece sp. extract O₂ evolution (data not shown). Lowering the pH required a change in the buffer used in the cell wash and breakage buffer solutions, and MES was chosen because of its strong buffering capacity at pH 6.5. We also found that two passages through the French pressure cell at 20,000 psi provided the most highly active O₂ evolving extracts while maintaining good cell breakage. The chlorophyll concentration of the cell extracts were routinely 60–80% of the initial whole cell concentration. Extract O₂ evolution rates routinely ranged from 50 to 105% of the whole cell O₂ evolution that was measured prior to cell breakage on a per chlorophyll basis (data not shown).

The effect of high ion concentrations on in vitro O₂ evolution has been studied extensively in plant chloroplasts [27,28]. 1 M MgCl₂ and 1 M CaCl₂ have been reported to remove all extrinsic proteins from PSII and greatly inhibit O₂ evolution [27]. Similar O₂ evolution results have been observed in Cyanothece sp. (data not shown). 1 M NaCl removes the 24 kDa protein in eukaryotes, thereby reducing in vitro O₂ evolution [28]. Although cyanobacteria do not possess the 24 kDa protein, 1 M NaCl reduced the amount of in vitro O₂ evolution in Cyanothece sp. by 35% (data not shown). This has been shown previously to be due to partial removal of the cyanobacterial 12 kDa protein and cytochrome c₅₅₀ [11,29]. The standard betaine O₂ assay buffer used in these experiments contained 20 mM CaCl₂, which is the control concentration. The use of 250 mM CaCl₂ also inhibited O₂ evolution, but to a much lesser extent than did the addition of 1 M CaCl₂ (data not shown).
3.3. Rhythms of Cyanothece sp. in vitro photosynthetic activity

The isolation of in vitro $O_2$ evolving extracts has allowed for a more detailed analysis of the photosynthetic activities occurring during the Cyanothece sp. diurnal cycle. $O_2$ evolving extracts isolated from the light period of a LD time course consistently showed higher rates of $O_2$ evolution than samples isolated from the dark period (Fig. 1B), with a peak-to-trough difference of approx. 47%. The in vitro rate of $O_2$ evolution decreased during the peak of $N_2$ fixation (Fig. 1B), in agreement with in vivo results (Fig. 1A). This significant reduction in in vitro $O_2$ evolution during $N_2$ fixation was not always as apparent as the decrease seen in vivo (compare $O_2$ evolution in Fig. 1A and B), but the rate of $O_2$ evolution was always lower during the period of $N_2$ fixation. It is interesting to note that the peak of in vitro $O_2$ evolution typically occurred at L6.

To ensure that the maximum possible PSI activity was measured in the Cyanothece sp. time course samples, the PSI electron donor cytochrome c$_6$ (Cyt c$_6$), from _A. maxima_ [18], was utilized (Fig. 1B). The peak of PSI activity with Cyt c$_6$ (2462 $\mu$M $O_2$ consumed (mg Chl)$^{-1}$ h$^{-1}$) occurred just prior to the peak of nitrogenase activity and was approx. 8 times greater than the $O_2$ consumption seen with the standard PSI additives. The peak of activity with Cyt c$_6$ as the electron donor was 16% higher than the Cyt c$_6$ PSI activity seen during the remainder of the Cyanothece sp. diurnal cycle (Fig. 1B). Notably, we found that addition of 25 $\mu$M Cyt c$_6$ to the standard additives with 0.2 mM DCPIP present actually reduced the amount of oxygen consumption compared to Cyt c$_6$ used alone (data not shown).

$O_2$ evolving extracts from a Cyanothece sp. LL time course were prepared for comparison to the LD extracts and with the previously published in vivo $O_2$ evolution results [2,5]. A similar pattern of in vitro $O_2$ evolution was identified in the LL samples compared to the LD extracts with in vitro $O_2$ evolving activity decreasing during the peak of nitrogenase activity (data not shown). These data are also consistent with the previous in vivo Cyanothece sp. LL results. The peak-to-trough difference in $O_2$ evolution is only 17% in LL cultures, compared to 47% in LD cultures, indicative of the loss of synchrony in cells grown in continuous light.

3.4. Identification of the manganese stabilizing protein

MSP is important for $O_2$ evolution, and as a membrane associated protein, may be involved in regulation of this process. We have demonstrated that the presence of MSP is not essential for $O_2$ evolution in the cyanobacterium _Synechocystis_ sp. PCC 6803, since removal or mutation of MSP greatly reduces, but does not abolish, the $O_2$ evolving capacity of PSII [30]. Under these conditions, PsbU and cytochrome $c_{550}$ bind to PSII and permit activity to proceed at about 50% the normal, wild-type level [12,30,31]. With this in mind, Western blot analysis was performed to determine if any change in the...
MSP occurred during the *Cyanothece* sp. diurnal cycle and if this change could be related to the rhythms of O$_2$ evolution identified in *Cyanothece* sp. [1–3]. The presence of the MSP was verified by Western blots for both nitrogen replete (+NO$_3$ grown) and N$_2$ fixing (−NO$_3$ grown) *Cyanothece* sp. cultures (Fig. 2A). Interestingly, the MSP of *Cyanothece* sp. migrated with a larger apparent molecular mass (33 kDa) than that seen for *Synechococcus* sp. PCC 7942 (28 kDa) (Fig. 2A). The reason that *Cyanothece* sp. MSP has a larger apparent molecular mass will be discussed later.

3.5. Translational analysis of the PSII MSP in LD and LL grown *Cyanothece* sp. cultures

Western blot analysis of *Cyanothece* sp. samples, isolated at 2 h intervals during 24 h LD and LL time courses, was performed to determine if MSP concentration or migration varied during the diurnal cycle. The *Cyanothece* sp. MSP cross-reacting material remained relatively constant, regardless of the growth light regime (LD or LL) throughout the diurnal cycle (Fig. 2B and C, respectively). Western analysis of β-ATPase was performed concurrently with many of the MSP Westerns to verify that similar protein concentrations exist between the individual sample lanes. β-ATPase is constitutively transcribed and translated and does not vary in concentration during the diurnal cycle [5], as shown in Fig. 2B. Similar results were obtained when isolated O$_2$ extracting extracts were used in the Western blot experiments (data not shown). From these results, it appears that the changes in net O$_2$ evolution occurring in *Cyanothece* sp. during its diurnal cycle were not related to changes in overall MSP accumulation.

The migration rate of the *Cyanothece* sp. MSP on polyacrylamide gels changes in response to cell extract solubilization with or without β-mercaptoethanol (βME). βME is normally present in the solubilization solution to prevent disulfide linkages between cysteine residues. The mature MSP from both prokaryotic and eukaryotic organisms possess a pair of cysteine residues near the N-terminus and these cysteines form a disulfide linkage in mature MSP [32]. Solubilization (in the presence or absence of βME) and gel electrophoresis have revealed that the *Cyanothece* sp. MSP migrated at approx. 33 kDa in the presence of βME, but migrated faster (31 kDa) in the absence of βME (Fig. 3). Thus, the absence of βME in the solubilization buffer for *Cyanothece* sp. ATCC 51142 proteins appears to alter folding which yielded a more compact conformation and a lower apparent molecular mass, although this has no visible effect on protein concentration. As a control, we analyzed *Synechococcus* sp. PCC 7942 MSP and no change in mobility was identifiable when the sample was solubilized in the presence or absence of βME (Fig. 3).

3.6. Transcriptional analysis of *psbO* and *psbU*

Northern blot analysis was performed to determine if *psbO* transcript accumulation levels differed during the *Cyanothece* sp. ATCC 51142 diurnal cycle.
cycle. RNA samples were extracted at 2 h intervals for 24 h from a LD time course. This analysis revealed changes in the expression of at least two of the three PSII lumenal extrinsic proteins in Cyanothece sp. An approx. 1 kb transcript, corresponding to the psbO (which codes for MSP) message, was detected with a homologous DNA probe. A 0.52 kb transcript, corresponding to psbU (codes for the prokaryotic, lumenal, extrinsic 12 kDa protein) was detected with a homologous 0.4 kb probe. The relative rhythmic changes in concentration of the two lumenal protein transcripts is almost identical when compared throughout the Cyanothece sp. diurnal cycle (Fig. 4).

Both transcripts accumulate during the early light and reach a peak concentration at L2. Transcript accumulation then began to decrease until, at D0 and D2, very little or no transcript was present. The transcripts reappear at D4, at a high concentration, and remain high through D10 (Fig. 4). This variation in the transcription levels of psbO and psbU correlates strongly with the significant changes in O2 evolution seen in Cyanothece sp. in vivo and in vitro during its diurnal cycle [2].

3.7. Cloning and sequencing of psbO from Cyanothece sp.

The cloning and sequencing of psbO from Cyanothece sp. became important once it was determined that Cyanothece sp. ATCC 51142 MSP had a larger apparent mass on Western blots compared to other cyanobacterial MSPs (Fig. 2A). PCR primers, developed for studying mutated psbO in Synechocystis sp. PCC 6803, produced an internal 600 bp psbO fragment from Cyanothece sp. This homologous psbO fragment was used to probe a 2–3 kb Cyanothece sp. library as well as being used as a probe for the psbO Northern blot analysis mentioned above.

Alignment of N- and C-terminal clones with the internal 600 bp psbO PCR fragment produced a 831 bp Cyanothece sp. psbO open reading frame. This open reading frame is highly homologous to other known psbO sequences, both prokaryotic and eukaryotic, with many of the sequence differences occurring as third base degeneracy in the codons. A relatively small number of amino acid changes in the protein sequence were identified (Fig. 5). The Cyanothece sp. psbO transcript, when translated, produced a 277 amino acid MSP. The N-terminal 30 amino acids serve as a signal sequence for translocation across the thylakoid membrane, leaving a mature Cyanothece sp. MSP protein of 247 amino acids. This is the same number of amino acids identified in the mature MSP from Anacystis nidulans R2 [33]. The location of the actual start codon (ATG) remains unclear in the Cyanothece sp. psbO gene because two ATG sequences are present at the beginning of the psbO coding region, such that the actual number of amino acids in the translated and mature MSP may actually be 275 and 245, respectively (Fig. 5). The possible addition of two N-terminal amino acids to Cyanothece sp. MSP does not account for the difference in migration seen on Western blots of
the MSP between *Cyanothece* sp. and *Synechococcus* sp. (Fig. 2). In Fig. 5, stars denote the beginning of the mature MSP protein (star 1 for plant MSP, star 2 for cyanobacterial MSP). Arrowheads denote lysines present in plants but absent in cyanobacteria. Diamonds denote the two conserved cysteine residues.

The *Cyanothece* sp. MSP appeared to be most closely related to the *Synechocystis* sp. PCC 6803 protein, but there are distinct differences (especially in the N-terminus; see Fig. 5). Secondary structure analysis indicated three regions of structural difference at the N-terminus of MSP between plants and cyanobacteria (see Section 4). Conversely, the C-terminal 150 amino acids reveal an almost identical secondary structure between plants and cyanobacteria (data not shown). MSP sequence comparison has revealed that cyanobacteria lack all four lysines at the N-terminus of the mature MSP protein and, in turn, have more negatively charged amino acids (arrowheads, Fig. 5). This is particularly true of *Cyano-
Thece sp., which differs most substantially from all other MSP sequences precisely in this region.

Sequencing of the Cyanothece sp. psbO C-terminal clone also identified a heme oxygenase gene 357 bp downstream from the psbO gene. Heme oxygenase is the initial enzyme in a heme-dependent pathway dedicated to the production of phycobiliprotein chromophores [34,35]. Translation of the heme oxygenase coding region would produce a 239 amino acid protein. The size and sequence of this protein are highly comparable to heme oxygenases from other cyanobacteria (data not shown).

3.8. Construction of a Synechocystis strain containing the psbO gene from Cyanothece

The striking features of the Cyanothece sp. MSP led us to construct a strain of Synechocystis sp. PCC 6803 that contained the Cyanothece sp. psbO gene. This strain, referred to as Synechocystis Cy1, segregated completely, such that all copies of the original psbO gene had been eliminated (data not shown). We have demonstrated that MSP in Synechocystis Cy1 and in Cyanothece had virtually identical physical properties. As shown in Fig. 3B, MSP from Synechocystis Cy1 (lane 3) migrated as a 33 kDa protein when the solubilization buffer contained β-mercaptoethanol. This is similar to Cyanothece (lane 4), but distinct from Synechocystis sp. PCC 6803 MSP (lane 1) which migrated at 28 kDa. Lanes 2 and 7 contained extracts from Synechocystis sp. PCC 6803 ΔpsbO which lacks MSP and which gave no signal on this Western blot. Furthermore, MSP from Synechocystis Cy1 responded similarly to Cyanothece MSP when β-mercaptoethanol was excluded from the solubilization buffer. In this case, MSP in Synechocystis Cy1 (lane 8) again migrated as an approx. 31.5 kDa protein, whereas MSP from Synechocystis sp. PCC 6803 still migrated as a 28 kDa protein. The extract in lane 5 was solubilized without β-mercaptoethanol; however, bleed over from the adjoining lane, which did contain β-mercaptoethanol, resulted in MSP displaying two bands (at 33 kDa and 31.5 kDa) indicating that Cy1 MSP is very sensitive to the presence of thiols. Therefore, the physical property of Cyanothece MSP in response to reducing agents was retained in the chimeric strain of Synechocystis.

Similarly, Synechocystis Cy1 demonstrated functional properties that are more typical of Cyanothece than of Synechocystis. One of the more striking features of O2 evolution in Cyanothece was the stability of the process after dark incubation [6,7]. This feature was maintained in Synechocystis Cy1 as shown in Fig. 6. O2 evolution was measured in three strains of Synechocystis sp. PCC 6803: wild type, ΔpsbO, and Cy1. The data are stated as per cent control for each strain. Maximum rates (100%) for wild type, ΔpsbO, and Cy1 were (in μmoles O2 evolved (mg Chl)–1 h–1): 290±25, 210±20, and 270±25, respectively. The results were plotted with standard deviations for n=5.

Fig. 6. Oxygen evolution measurements in three strains of Synechocystis sp. PCC 6803. Cultures were grown to a cell density of approx. 1×10^6 cells/ml and suspended in assay buffer at approx. 3 μg Chl ml–1. The samples were kept in the light prior to the O2 evolution assay or incubated in the dark for 20, 40 and 60 min prior to the assay. The data are stated as per cent control for each strain. Maximum rates (100%) for wild type, ΔpsbO, and Cy1 were (in μmoles O2 evolved (mg Chl)–1 h–1): 290±25, 210±20, and 270±25, respectively. The results were plotted with standard deviations for n=5. ○, Synechocystis sp. PCC 6803 wild type; □, Synechocystis ΔpsbO; Δ, Synechocystis Cy1.
4. Discussion

We describe a procedure for the isolation of highly active, O2 evolving cellular extracts from Cyanothece sp. ATCC 51142 utilizing glycine betaine. This procedure has permitted us to analyze the in vitro photosynthetic properties of whole cell extracts as a function of the Cyanothece sp. diurnal cycle and emphasized the value of glycine betaine for PSII protection. The O2 evolving capacity of the extracts could be maintained for extended periods of time and O2 evolving activity responded to inhibitory ion concentrations in the expected manner. It was evident that the in vivo and in vitro O2 evolving activity of N2 fixing Cyanothece sp. showed similar patterns with a trough at the peak of N2 fixation (~D4) and with a peak in the late light period (~L6 to L8). Therefore, the changes in PSII activity identified during the diurnal cycle were retained to some extent in the isolated O2 evolving extracts.

Previous work has demonstrated that the photoactivation of the O2 evolving mechanism changes substantially in Cyanothece sp. throughout the diurnal cycle [6,7], a feature that implicated MSP and the other extrinsic luminal proteins associated with the O2 evolving complex of PSII. Analysis of Cyanothece sp. protein patterns showed no obvious differences in PSII or PSI in regard to the known proteins (data not shown), except for the larger apparent molecular mass of the Cyanothece sp. MSP (33 kDa) compared to other cyanobacterial MSPs (28 kDa) (Fig. 2). We devoted particular effort to analyzing potential alterations in the concentration and conformation of MSP, but found no significant changes during the diurnal cycle. It was evident that there was substantial transcriptional control on psbO and psbU, and this pattern strongly resembled transcriptional control of psbA, and, to a lesser extent, psbB, psbC and psbD [5]. All of these PSII proteins demonstrated very little transcript accumulation during the first ~6 h of the dark period. In addition, protein analysis indicated very little change in levels of accumulation for these PSII proteins during this period (except for a shift to more D1 form 2 near the end of the light period [5]). The synthesis of new MSP was studied extensively with pulse-chase experiments; however, the pattern of new MSP synthesis was similar to the transcriptional pattern and there was little correlation to the periodicity of PSII O2 evolution (data not shown). Western analysis with and without β-mercaptoethanol has indicated that Cyanothece sp. MSP can readily form intramolecular disulfide bridges and these bridges may be important in regulating O2 evolution in Cyanothece sp.

An intriguing feature of Cyanothece sp. is the increases in PSI at the end of the light period and the beginning of the dark period. The transcription of psaAB, the translation of PsaAB [5] and PSI activity also increased around L10 to D4 (Fig. 1). Specifically, this means that the capacity for PSI activity is enhanced, although it is uncertain how this capability is utilized. When light is available, this might result in enhanced cyclic photophosphorylation and ATP synthesis. However, it is also possible that the PSI is involved with larger complexes in the membrane and that there is some interrelation with the respiratory apparatus.

The amino acid sequence of Cyanothece sp. MSP has provided important information for the analysis of the structure of the cyanobacterial MSP. The model postulated by Bricker and Frankel [14] that outlined a hypothetical structure of spinach MSP substantially aided this analysis. Based on both sequence and functional analysis, we believe that cyanobacterial and Cyanothece sp. MSP differ from the spinach model in the following ways. (i) The cyanobacterial and chloroplast proteins are somewhat distinct in the N-terminal 100 amino acids. (ii) Cyanothece sp. MSP is most closely related to the Synechocystis sp. PCC 6803 protein, but there are distinct differences, especially in the N-terminal 30–50 amino acids of the mature protein. (iii) Secondary structural analysis has indicated that the cyanobacterial MSP has a β-sheet structure in place of the helical α1 (as shown in the Bricker and Frankel model [14]), an α-helical region in place of part of β4, and an additional β-sheet in place of part of the large loop between amino acids ~90 and 110. On the other hand, the C-terminal 150 amino acids give rise to identical structures in both cyanobacteria and chloroplasts. Most importantly, the loop between the β3 and α1 regions is substantially different in cyanobacteria relative to chloroplasts. (iv) Cyanobacteria lack all four lysines in the 40–50 amino acid region of the N-terminus and, in turn, have more negatively charged amino acids. This is particularly
true of *Cyanothece* sp. MSP, which differs most substantially from all other MSPs precisely in this region and has many more negatively charged amino acids. We postulate that this region, which is close to the MSP binding region near the N-terminus [14], is of importance in the overall regulation of O\(_2\) evolution. This region may also be directly involved in MSP conformational changes and consequently in the regulation of O\(_2\) evolving activity throughout the *Cyanothece* sp. diurnal cycle.

Previous site directed mutagenesis studies on *Synechocystis* sp. PCC 6803 MSP demonstrated the importance of the Cys residue at position 20 in the mature protein [32]. The MSP C20S mutant had a phenotype similar to that of the strain in which the *psbO* gene had been deleted. Strikingly, MSP was completely degraded in C20S, implying that the Cys was important for transport of the nascent protein into the thylakoid lumen or for proper folding and stability [32]. As shown in Fig. 5, all MSPs have two conserved Cys near the N-terminus and the current consensus is that these residues likely form a disulfide bond [14,36]. Our initial results with *Cyanothece* sp. MSP (Fig. 3A) indicated that disulfide bonds were important to structural stability and this property has now been transferred to *Synechocystis* Cy1. However, the amino acid comparison in Fig. 5 provides an additional clue to the interesting properties of *Cyanothece* MSP. All cyanobacterial MSPs sequenced to date have a Cys at position 16 in the signal sequence [10]. However, only *Cyanothece* and *Anabaena* sp. PCC 7120 MSPs have an additional Cys in the signal sequence (at position 23 in the *Cyanothece* sequence). Furthermore, these are the only cyanobacterial MSPs to migrate at 33 kDa on SDS acrylamide gels. One interpretation for the results shown in Fig. 3A,B is that the *Cyanothece* MSP (and possibly the *Anabaena* MSP) form an additional disulfide bond during transport of MSP into the thylakoid lumen. This disulfide bond then prevents cleavage of the signal sequence, resulting in a protein with some 277 amino acids. This protein would migrate closer to 33 kDa, would be more sensitive to sulphydryls in solution and may have additional structural and functional attributes. All of these suggestions are testable in the chimeric *Synechocystis* Cy1 strain. Furthermore, these results emphasize the need to better understand the importance of disulfide bond formation and redox regulation in the thylakoid lumen (and the periplasm) of cyanobacteria.

Our analysis complements that of Bricker and Frankel [14] and that of De Las Rivas and Heredia [36]. De Las Rivas and Heredia [36] aligned 14 PsbO sequences from cyanobacteria, algae, and plants. Their conclusions are similar to those presented herein, in that they obtained a somewhat different secondary structure for the consensus MSP than Bricker and Frankel [14] did for the spinach MSP. Specifically, their model also suggested more \(\beta\)-strands in MSP than did Bricker and Frankel [14]. However, the use of a consensus sequence glosses over the real secondary structure differences that certainly exist among the MSP proteins. It should be noted that the MSP from *Cyanothece* sp. ATCC 51142 and *Anabaena* sp. PCC 7120 (data not shown) are the only cyanobacterial MSP studied to date that migrate at 33 kDa, the same as the plant and algal MSPs. In addition, computer alignments always place *Cyanothece* sp. MSP closest to the algae and plant proteins relative to other cyanobacterial MSPs. Thus, the MSP from *Cyanothece* sp. ATCC 51142 is most plant-like in its structural and physical properties. De Las Rivas and Heredia [36] also emphasized the flexibility inherent in a protein like MSP, which has a high proportion of \(\beta\)-strand, loop, and undefined structure. It is this flexibility that provides the structural basis for the MSP conformational changes that occur upon binding to PSII [37], and that represents the key regulatory feature of this protein.

Finally, Shutova et al. [38] have argued that MSP in solution has the characteristics of a ‘molten globule’ structure. They base this assignment primarily on the comparison of the far-UV CD spectra of MSP with that of \(\alpha\)-lactalbumin [39]. One important feature of this concept is that the flexibility of MSP will aid in optimal binding to the PSII complex. It is also possible that this flexibility leads to the ability of MSP to rapidly move from a bound state (with a more open structure) to an unbound state (with a more ‘molten globule’ structure). We and others [10,12] have shown that removing MSP from PSII leads to an enhanced requirement for Ca\(^{2+}\). Thus, Shutova et al. [38] speculate that the unbound form of MSP may not bind Ca\(^{2+}\). The role played by the additional ~30 amino acids at the N-terminus is
also of interest, since this disulfide-bonded domain might provide the protein with more flexibility. Significantly, Shutova et al. [38] propose that the molten globule forms a hydrophobic core of β-sheets that is stabilized by the disulfide bond. Reduction of this bond by sulphydryls would then give rise to major structural changes. If MSP from *Cyanothece* (and *Anabaena*) had a second set of disulfide bonds, this could generate more degrees of freedom. This structure could provide the type of regulation needed by organisms that must quickly change from high to low rates of O$_2$ evolution as they prepare for N$_2$ fixation.

All of these considerations are of interest, but a true understanding of MSP structure will require a 3-D structure by X-ray crystallography. Nonetheless, it is evident that MSP is a highly flexible molecule, capable of significant conformational changes. It may be able to shift from a bound to an unbound state, a change that is characterized by a shift in conformation. This type of switch is more rapid than new protein synthesis and can help the cell to quickly adapt to altered environmental conditions. We suggest that the regulation of PSII O$_2$ evolution in *Cyanothece* sp. ATCC 51142 is mediated by such an MSP switch. The ability of MSP to undergo such a bound/unbound transition, coupled with changes in lumenal ion concentrations, can be used to explain the complex characteristics of O$_2$ evolution stability and photoreactivation [40].

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