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Inactivation of the water-oxidizing enzyme in manganese stabilizing protein-free mutant cells of the cyanobacteria *Synechococcus* PCC7942 and *Synechocystis* PCC6803 during dark incubation and conditions leading to photoactivation *

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Abstract

The previously constructed MSP (manganese stabilizing protein–*psb*O gene product)-free mutant of *Synechococcus* PCC7942 (Bockholt R, Masepohl B and Pistorius E K (1991) FEBS Lett 294: 59–63) and a newly constructed MSP-free mutant of *Synechocystis* PCC6803 were investigated with respect to the inactivation of the water-oxidizing enzyme during dark incubation. O_2 evolution in the MSP-free mutant cells, when measured with a sequence of short saturating light flashes, was practically zero after an extended dark adaptation, while O_2 evolution in the corresponding wild type cells remained nearly constant. It could be shown that this inactivation could be reversed by photoactivation. With isolated thylakoid membranes from the MSP-free mutant of PCC7942, it could be demonstrated that photoactivation required illumination in the presence of Mn^{2+} and Ca^{2+}, while Cl^- addition was not required under our experimental conditions. Moreover, an extended analysis of the kinetic properties of the water-oxidizing enzyme (kinetics of the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition, S-state distribution, deactivation kinetics) in wild type and mutant cells of *Synechococcus* PCC7942 and *Synechocystis* PCC6803 was performed, and the events possibly leading to the reversible inactivation of the water-oxidizing enzyme in the mutant cells are discussed. We could also show that the water-oxidizing enzyme in the MSP-free mutant cells is more sensitive to inhibition by added NH_4Cl – suggesting that NH_3 might be a physiological inhibitor of the water oxidizing enzyme in the absence of MSP.

Abbreviations: Chl – chlorophyll; DCBQ – 2,6-Dichloro-p-benzoquinone; MSP – manganese stabilizing protein (*psb*O gene product); PS II – Photosystem II; WOE – water oxidizing enzyme; WT – wild type

Introduction

The thylakoid embedded PS II complex catalyzes the process of photochemically charge separation and water oxidation. The overall reaction is the photoinduced transfer of electrons from water to plastoquinone with release of molecular O_2 as a by-product. The process of photochemical charge separation is catalyzed by the D1/D2 heterodimer, while the identity of the WOE requiring Mn, Ca^{2+} and Cl^- for activity remains controversial (for recent reviews see Hansson and Wydrzynski 1990; Rutherford et al. 1992; Debus 1992; Pistorius 1993). The manganese stabilizing protein (MSP – *psb*O gene product) is present in PS II complexes of all oxygenic organisms. It is located at the lumenal side of PS II, and two molecules seem to be

* This paper is dedicated to Prof. Dr. Bernard Axelrod on the occasion of his 80th birthday

present per reaction center (Xu and Bricker 1992). The MSP has been shown to be very closely associated with the water-oxidation process (Miyao and Murata 1984). However, in vitro experiments (removal of MSP from PS II complexes by $CaCl_2$ or urea-NaCl washing) as well as in vivo experiments (construction of cyanobacterial mutants lacking MSP) have provided evidence that this protein is not a major site of Mn coordination, since O_2 evolution can occur in its absence. Thus, MSP does not have a catalytic but rather a regulatory, stabilizing and/or protecting role in the process of water-oxidation. However, the precise function has remained uncertain (see reviews: Rutherford et al. 1992; Debus 1992).

Recently, three groups have constructed MSP-free mutants of the cyanobacterium *Synechocystis* PCC6803 (Burnap and Sherman 1991; Mayes et al. 1991; Philbrick et al. 1991). These mutants can grow photoautotrophically – although at a reduced rate (about 70% of WT rate). They assemble photochemically active PS II reaction centers at concentrations comparable to WT, but the measured O_2 evolving activity with bicarbonate as electron acceptor only corresponds to about 70% of the WT rate. It was shown that the characteristics of the O_2 evolution in the MSP-free mutants was distinct from that in WT in several aspects (Philbrick et al. 1991; Burnap et al 1992; Vass et al. 1992). In the mutants, O_2 evolution was more sensitive to high light intensities than in WT cells. This enhanced sensitivity of the mutants to photoinhibition was suggested to be possibly due to a limited rate of electron donation from the MSP-free water-oxidizing complex to the PS II reaction center resulting in accumulation of highly oxidizing species (such as $P680^+$) which may damage the pigment and protein surroundings. It was also shown by Philbrick et al. (1991) that under conditions of Ca^{2+} depletion where WT growth was unaffected, the MSP-free mutant was unable to grow. A comparable differential effect on growth was not obtained under conditions of Cl^- depletion. On the basis of these results it was concluded that MSP enhances Ca^{2+} binding or protects the reaction center at low Ca^{2+} concentrations. Moreover, investigations of flash-induced O_2 evolution were performed by Burnap et al. (1992) and Vass et al. (1992). Those experiments gave evidence that a significantly decreased and greatly variable O_2 yield as well as a largely damped oscillation pattern was obtained with the mutants.

In our group a MSP-free mutant of the cyanobacterium *Synechococcus* PCC7942 was constructed which could also grow photoautotrophically with a rate approximately 70% of WT (Bockholt et al. 1991) – comparable to the growth rate of the *Synechocystis* mutants. We could demonstrate that this mutant had a reduced manganese binding capacity which is in agreement with the suggested manganese stabilizing role of the *psb*O gene product. This conclusion was based on O_2 evolution measurements with potassium ferricyanide as electron acceptor in a Clark electrode using isolated thylakoid membranes. We were able to show that isolated thylakoid membranes of the mutant required addition of $CaCl_2$ and $MnCl_2$ for O_2 evolution, while thylakoid membranes from WT only required addition of $CaCl_2$. Since the MSP-free mutant of *Synechococcus* PCC7942 could rapidly lose manganese, we thought that this mutant might be suited for studying the light-driven incorporation of manganese into the WOE and for examining the role which MSP might have in this process. In addition, we were interested in investigating whether the effect of inhibitory amines on the WOE might be more pronounced in the absence than in the presence of MSP. A positive result would be in line with a protecting and regulatory role of MSP besides its cofactor stabilizing function.

For better generalization of our results we utilized the previously constructed MSP-free mutant of *Synechococcus* PCC7942 (Bockholt et al. 1991) and in addition the more recently constructed MSP-free mutant of *Synechocystis* PCCC6803 (Lott and Bockholt, unpublished results). Now having two MSP-free cyanobacterial mutants available in our group allowed a comparison of the characteristics of the WOE in the two cyanobacterial species (WT als well as MSP-free mutants) and also proved to be advantageous because the two cyanobacteria were differently suited for the experiments which we performed in order to answer the above raised questions.

Materials and methods

Strains and growth conditions

WT strains of *Synechococcus* PCC7942 and *Synechocystis* PCC6803 were obtained from the Collection Nationale de Cultures de Microorganismes, Institute Pasteur, Paris, France. The MSP-free mutants of *Synechococcus* PCC7942 and *Synechocystis* PCC6803, referred here to as A5 and M7.5, respectively, both bear an insertionally inactivated *psb*O gene. *Synechococcus* PCC7942 A5 was constructed by insertion of a chloramphenicol interposon into the *psb*O gene as

described by Bockholt et al. (1991). The construction of *Synechocystis* PCC6803 was performed in a similar way by insertion of a kanamycin resistance gene into the *psb*O gene and is described below.

All four strains were grown in BG11 medium according to Rippka et al. (1979) essentially under the same culture conditions as described previously (Engels et al. 1992: growth conditions of *Synechococcus* PCC7942 used for the isolation of PS II-complexes).

The MSP-free mutants *Synechococcus* PCC7942 A5 and *Synechocystis* PCC6803 M7.5 were selected for their antibiotic resistance by adding 10 μg chloramphenicol per ml BG11 for A5 and 7.5 μg kanamycin per ml BG11 for M7.5.

For growth under Ca^{2+} or Cl^- deficiency, cells were collected by centrifugation, washed once with distilled water and resuspended in depleted or regular BG11 medium under aseptic conditons. The depleted BG11 media were prepared by replacing $CaCl_2$ (regular concentration in BG11 medium: 0.24 mM) with 0.48 mM NaCl for Ca^{2+} deficiency or with 0.24 mM $Ca(NO_3)_2$ for Cl^- deficiency.

Construction of a MSP-free mutant of the cyanobacterium Synechocystis *PCC6803*

The protocol for construction of the MSP-free mutant of *Synechocystis* PCC6803 was similar to the one published previously for construction of the MSP-free mutant of *Synechococcus* PCC7942 (Bockholt et al. 1991). The DNA sequence of a 5.5 kb *Hin*dIII fragment encoding the *Synechocystis* PCC6803 *psb*O gene has been published previously (Philbrick and Zilinskas 1988). In order to inactivate the *psb*O gene, a 2 kb *Hin*dIII/*Nde*I fragment was chosen as a basis for insertional mutagenesis. An *Escherichia coli* clone carrying the corresponding 2 kb fragment from PCC6803 was used in this study. This clone was isolated from a partial PCC6803 gene bank consisting of size-fractionated *Hin*dIII/*Nde*I fragments cloned into the vector plasmid pUC19 (Vieira and Messing 1982). A clone carrying the *psb*O gene was identified by southern blotting using a synthetic 24mer oligonucleotide (5′-CCCGGTGGTGAAGAAGTACCCTTC-3′) as a probe. The resulting recombinant plasmid was called pAL2 and was shown to contain the DNA fragment in the direction opposite to the ampicillin resistance gene of pUC19. The lac promotor and the multiple cloning site had been removed by the *Hin*dIII/*Nde*I digestion and are therefore missing in pAL2. The size of pAL2 was 4.4 kb (2. 4 kb of the residual pUC 19 plasmid and 2 kb insert). The hybrid plasmid pAL2 contained a single *Bam*HI site approximately 600 bp downstream the starting codon of the structural *psb*O gene. This *Bam*HI site was used to clone a 1800 bp *Bam*HI interposon carrying a neomycin/kanamycin resistance gene from Tn5 (Jorgensen et al. 1979). The kanamycin resistance gene in the resulting plasmid pAL2/a was orientated in the opposite direction to the *psb*O gene. Plasmid pAL2/a was transformed into *Synechocystis* PCC6803. Selection for kanamycin resistance almost exclusively resulted in double recombination events because single cross-over events are very seldom in PCC6803 and because plasmid pAL2/a cannot replicate in PCC6803. Correct insertion of the kanamycin interposon into the chromosomal *psb*O gene by marker rescue was proven by Southern analysis (not shown). In the corresponding PCC6803 strain carrying a mutated *psb*O gene no MSP was detectable in immunoblot experiments with an antiserum raised against MSP from oat (Specht et al. 1990), and this mutant was called M7.5.

Preparation of cell suspensions and of thylakoid membranes

For experiments with whole cells WT and MSP-free mutant cells from *Synechococcus* PCC7942 and *Synechocystis* PCC6803 were harvested by centrifugation (WT cells after 2 days and MSP-free mutant cells after 3 days of growth having optical densities of approximately 2.0 at 750 nm in case of both strains). Cells were then washed with 20 mM Hepes-NaOH, pH 6.5, containing 20 mM $CaCl_2$ and resuspended in the same buffer to a final Chl concentration of 0.5–1.0 mg/ml.

Thylakoid membranes from *Synechococcus* PCC7942 WT and mutant A5 were isolated using the method previously described (Pistorius and Gau 1986) with the following modifications: the French press extract was centrifuged for 10 min at 2500 × *g*, the resulting supernatant was then centrifuged for 45 min at 90,000 × *g* (Sorvall centrifuge RC 28 S, rotor F 28/36) and the thylakoid membranes were once washed in 50 mM Hepes-NaOH, pH 6.5, containing 400 mM sucrose and 50 mM $CaCl_2$ and resuspended in the same buffer. When the effect of $Ca^{2+}$and Cl^- on photoactivation was examined, the thylakoid membranes were washed and suspended in the above buffer from which $CaCl_2$ was omitted.

Measurements of oxygen evolved by whole cells as a consequence of short saturating light flashes

O_2 evolution of whole cells under flash light was measured with the 'Three Electrode System' described in detail earlier by Schmid and Thibault (1979) at room temperature using a polarization voltage of 680 mV. The electrode system was interfaced with an Atari Mega ST 4 computer which was used for digital recording and kinetic analysis of the signals. Flash illumination was provided by the Xenon stroboscope 1539-A of General Radio with a flash duration of 5 μs. The assay mixture of 1 ml contained 20 mM Hepes-NaOH, pH 6.5, 20 mM $CaCl_2$, 0.1 mM $MnCl_2$, 5 mM $NaHCO_3$ and the cell suspension sample containing 20 μg Chl/ml. In some experiments DCBQ, NH_2OH-HCl or NH_4Cl were added to the mixture as indicated in the legends to the figures and tables. After layering the complete reaction mixture on the platinum surface of the electrode system, cells were usually allowed to sedimentate for 30–45 min before an analyzing train of 15 flashes spaced 300 ms apart was given. In case of pre-illumination the reaction mixture was either pretreated with 800 flashes (the flash interval was either 300 ms or as indicated) or pre-illuminated with continuous red light. Continuous light was provided by a halogen lamp (24 V, 250 W from Spindler and Hoyer, Göttingen) and was filtered through a glass cuvette containing 2% $CuSO_4$ and through a red plexiglass filter (RG 1 (610) from Schott, Mainz) before being collected and transmitted to the electrode surface through a light conductor. For experiments in which comparative measurements of signal amplitudes were performed, the total sedimentation time before the measurement was exactly the same for all samples. If steady-state amplitudes were determined from the measured sequences, this was done by calculating the average amplitude under the last five flashes.

Calculation of S-state distributions

Oxygen signal amplitude sequences were fitted with the Kok application of the 'Voyons' general modelling software from Thiéry for IBM-compatible computers (Thiéry 1991). Specific algorithms for the modelling of oxygen evolution have been described previously by Thibault and Thiéry (1981) and Thibault (1982).

Oxygen evolution measurements under continuous light

O_2 evolution under continuous light in experiments with *Synechocystis* PCC6803 WT and M7.5 cells and with isolated thylakoid membranes from *Synechococcus* PCC7942 WT and A5 was measured in a Clark-type electrode (Rank Brothers, Bottisham Cambridge, England) at 20 °C at a polarization voltage of 600 mV. Red continuous light was provided by the halogen lamp described above. For flash pretreatments the Xenon lamp from the flash light electrode (see above) was placed over the top of the reaction chamber and the reaction mixture was illuminated with 800 (whole cells) or 1000 (thylakoid membranes) light flashes spaced 300 ms apart.

O_2 evolution under continuous light in experiments with *Synechocystis* PCC6803 WT and M7.5 cells was measured in a reaction mixture containing 20 mM Hepes-NaOH, pH 6.5, 20 mM $CaCl_2$, 0.1 mM $MnCl_2$ and either 5 mM $NaHCO_3$ or 0.6 mM DCBQ/1.7 mM potassium ferricyanide.

The reaction mixtures for the measurements with isolated thylakoid membranes of *Synechococcus* PCC7942 WT and A5 are given in the legends to the respective tables.

Results

In the experiments presented here four cyanobacterial cell types were investigated: WT and MSP-free mutant (called A5) of *Synechococcus* PCC7942 and WT and MSP-free mutant (called M7.5) of *Synechocystis* PCC6803. The MSP-free mutant of PCC7942 had previously been constructed (Bockholt et al. 1991), and the details for construction of the MSP-free mutant of PCC6803 are given in Materials and methods. Almost all experiments were done with intact cells with one exception: The influence of Mn^{2+}, Ca^{2+} and Cl^- addition on photoactivation of the WOE was investigated with isolated thylakoid membranes from *Synechococcus* PCC7942 (WT and mutant).

Growth characteristics and influence of Mn^{2+}, Ca^{2+} and Cl^- depletion on growth rates

The growth rates of the two MSP-free mutants under photoautotrophic conditions were about 70% of the corresponding WT rates (not shown, but see Bockholt et al. 1991 for the MSP-free PCC7942). In the

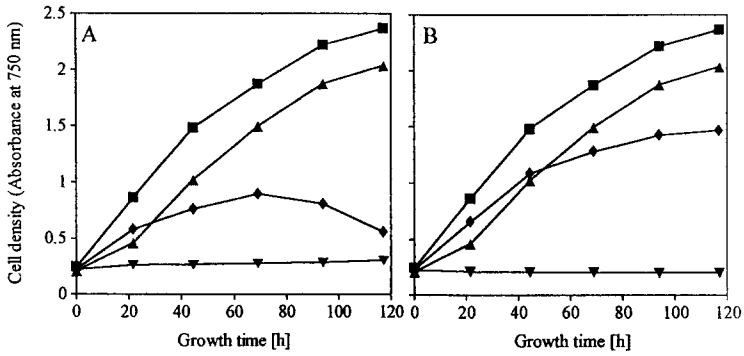

Fig. 1. Influence of Ca^{2+} and Cl^- depletion on growth of *Synechocystis* PCC6803 WT and MSP-free mutant M7.5. (A) Growth in BG11 medium depleted of Ca^{2+} and (B) Growth in BG11 medium depleted of Cl^-. -■-: WT in regular BG11 medium, -◆-: WT in depleted BG11 medium, -▲-: M7.5 in regular BG11 medium, -▼-: M7.5 in depleted BG11 medium.

absence of Ca^{2+} or in the absence of Cl^- in the medium the growth of the MSP-free PCC6803 was totally inhibited. Under our growth conditions Cl^- deficiency in the light caused an irreversible inactivation of the WOE, while Cl^- deficiency in the dark caused an inactivation of the WOE which could be reversed by readdition of Cl^- (results not shown). These observations suggest that Ca^{2+} deficiency (Philbrick et al. 1991) as well as Cl^- deficiency can cause growth inhibition due to a specific lesion in the WOE of the MSP-free PCC6803 mutant. Under our growth conditions Ca^{2+} or Cl^- depletion also reduced the growth rate of PCC6803 WT, but the reduction was much lower than that observed for the MSP-free mutant (Fig. 1A and B). The observation of Philbrick et al. (1991) that Cl^- deficiency had no effect on the growth rate of their MSP-free mutant and corresponding WT may be due to different growth conditions. We did not observe a significant reduction of the growth rate for the MSP-free PCC7942 under Ca^{2+} or Cl^- depletion over a growth period of 5 days (not shown). The most likely explanation of this difference is that removal of Ca^{2+} and Cl^- from the medium does not cause a rapid depletion of these ions from *Synechococcus* cells, while such a depletion occurs in *Synechocystis* cells. Over a growth period of 5 days no effect of Mn^{2+} omission from the growth medium was observed on growth for both mutants (not shown), suggesting that the minor residual Mn concentration was sufficient to maintain regular growth.

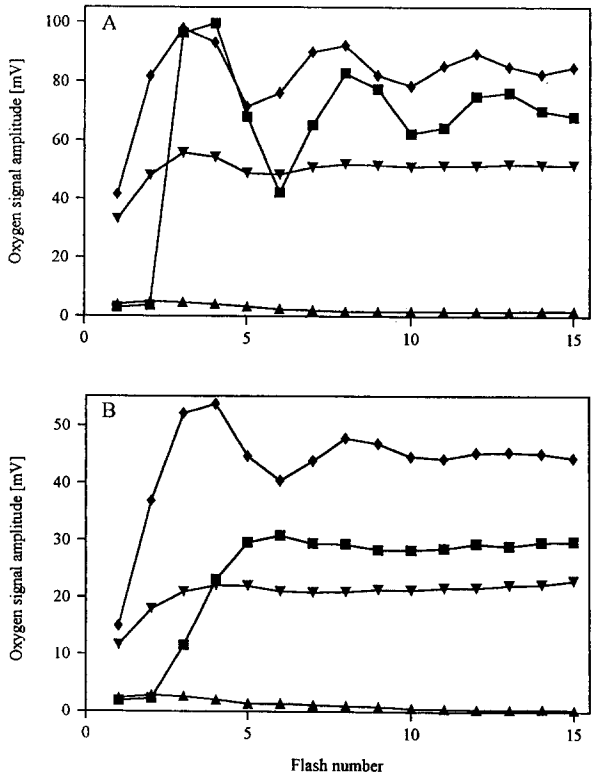

Fig. 2. Oxygen amplitudes of dark adapted and flash pre-illuminated *Synechocystis* PCC6803 and *Synechococcus* PCC7942 WT and MSP-free mutant cells. (A) *Synechocystis* PCC6803 WT and MSP-free mutant M7.5 and (B) *Synechococcus* PCC7942 WT and MSP-free mutant A5. -■-: O_2 yield of WT cells after 5 h dark adaptation under flash illumination without pre-illumination, -◆-: O_2 yield of WT cells dark adapted for 5 h under flash illumination measured 10 s after pre-illumination with 800 flashes.-▲-: O_2 yield of mutant M7.5 and A5 cells after 5 h dark adaptation under flash illumination without pre-illumination, -▼-. O_2 yield of mutant M7.5 and A5 cells after 5 h dark adaptation under flash illumination measured 10 s after pre-illumination with 800 flashes.

Photosynthetic O_2 yield in WT and MSP free mutant cells of Synechococcus *PCC7942 and* Synechocystis *PCC6803 under flash light conditions*

O_2 evolution of WTs and MSP-free mutants as a consequence of short saturating light flashes (5 μs duration, spaced 300 ms apart) was measured on a bare platinum electrode of the 'Three Electrode System' previously described (Schmid and Thibault 1979). The results of Fig. 2A and B show that the MSP-free mutant cells of PCC6803 and PCC7942 had a very low O_2 yield practically approaching zero, when measured after a dark adaptation of about 5 h. In contrast, WT cells retained their O_2 evolving activity after such a dark adaptation (Fig. 2A and B). More thorough investigations of this phenomenon led to the discovery that the O_2 yield of the mutant cells greatly varied depending on the length of dark-adaptation and that the O_2 signal of dark adapted mutant cells greatly increased when cells were pre-illuminated with about 600 light flashes. In contrast, there was no significant reduction of O_2 yield during prolonged dark adaptation when WT cells of both cyanobacteria species immediately after harvesting were compared with harvested cells after several hours of dark incubation (reduction of the O_2 yield never exceeded 20%). Pre-illumination of extensively dark-adapted WT cells only caused a minor increase of O_2 yield (Fig. 2A and B) – especially in case of PCC6803 WT, where the increase of O_2 yield by pre-illumination never exceeded 20%. However PCC7942 WT cells grown under our standard conditions sometimes possessed a WOE which after harvesting of cells was partially inactive. Thus, occasionally O_2 evolution could approximately be doubled by pre-illumination.

When mutant cells which practically showed no O_2 evolution after dark adaptation were photoactivated, the O_2 yield in the mutant cells greatly increased and now on chlorophyll basis corresponded to 50–75% or to about 50% of the corresponding WT yield in case of the MSP-free PCC6803 or MSP-free PCC7942, respectively, when O_2 signal amplitudes were compared (Fig. 2A and B). The photoactivation actually achieved was even higher, when the O_2 yield was determined from integrated O_2 signals. On this basis, the O_2 yield of pre-illuminated mutants and WT were approximately equal (not shown). The difference between the O_2 yield determined from signal amplitudes or integrated signals is due to a slower kinetic of O_2 release in the mutants as compared to WT (see later). In conclusion of these experiments, it can be said that in contrast to the WOE in WT the WOE in both mutants becomes completely inactivated after a prolonged dark adaptation but that it can readily be photoactivated.

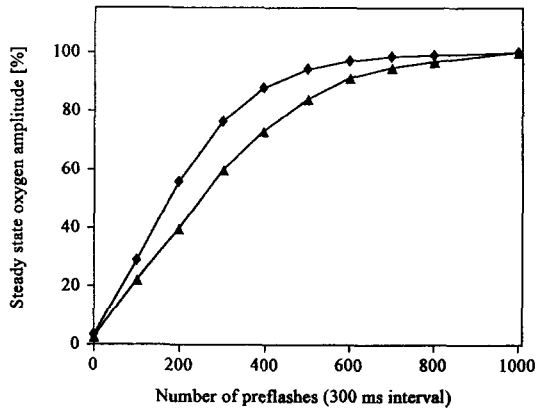

Fig. 3. Influence of the number of preflashes on the O_2 yield. Dependence of the steady-state oxygen amplitude of cells from the MSP-free mutants *Synechocystis* PCC6803 M7.5 (-◆-) and *Synechococcus* PCC7942 A5 (-▲-) on preflashing treatment. Prior to the preflash treatment the cells were dark adapted for 5 h. Steady-state amplitudes were determined in a train of 15 saturating light flashes given 10 s after preflash treatment.

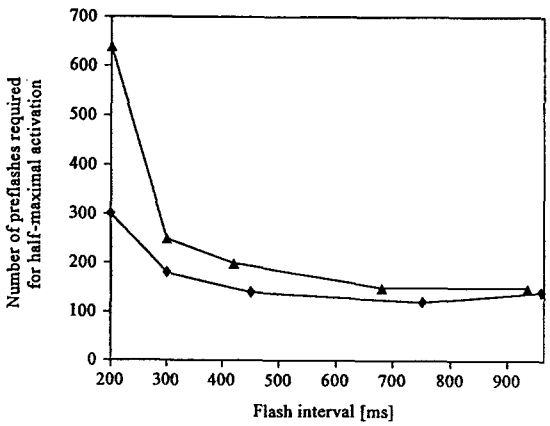

Fig. 4. Influence of the dark time between the preflashes on the O_2 yield. The flash interval of preflashes was varied as indicated in the figure and the numbers of preflashes leading to 50% of the maximal O_2 yield were estimated graphically from diagrams as shown in Fig. 3 for a flash interval of 300 ms. The cells of *Synechocystis* PCC6803 M7.5 (-◆-) and *Synechococcus* PCC7942 A5 (-▲-) were dark adapted for 5 h prior to the experiments.

Conditions leading to photoactivation of the WOE in the MSP-free mutants

The requirements leading to photoactivation of the WOE in the mutant cells were examined in detail,

Table 1. Influence of different pre-illumination conditions on the oxygen yield of the MSP-free mutant cells of *Synechocystis* PCC6803 (M7.5) and *Synechococcus* PCC7942 (A5). Cell suspensions were dark adapted for 5 h, and the steady-state oxygen signal amplitude was determined with the 'Three Electrode System' – either without or with pre-illumination as indicated in the table. The analyzing flash train was given exactly 30 s after the respective pre-illumination procedure when performed

	Pre-illumination	Relative oxygen evolution under flash light [%]	
	Pre-illumination procedure	*Synechocystis* PCC6803 M7.5	*Synechococcus* PCC7942 A5
I	No pre-illumination	< 2	< 2
II	240 s red continuous light	71.0	46.7
III	800 preflashes	100.0	100.0
IV	800 preflashes given simultaneously with red continuous light	71.5	43.6
V	200 additional preflashes given 20 s after pre-illumination procedure IV	92.4	68.1

and the results are given in Table 1 and Figs. 3 and 4. In Table 1 the influence of continuous light and flash light on photoactivation was studied. The results show that flash light proved to be superior – especially for the MSP-free PCC7942 mutant. Moreover, it became obvious that continuous light given in addition to flash light reduced the degree of photoactivation reached with flash light alone – implying that a dark period between flashes is essential for reaching maximal photoactivation. Thus, the results indicate that photoactivation of the WOE in the MSP-free mutants can be achieved with flash light as well as with continuous light but that the latter results in a lower quantum efficiency for the photoactivation process.

At least 600 flashes were required for obtaining maximal O_2 evolution (Fig. 3). More flashes increased photoactivation only insignificantly. Figure 4 shows the effect of the dark time between flashes on the photoactivation efficiency of the preflash treatment. A minimal dark interval of about 300 ms was required for optimal activation. Shorter dark intervals significantly reduced the quantum yield resulting in a requirement for an increased number of light flashes to obtain maximal activation. The low quantum yield obtained with light flashes spaced close together is most likely due to a slow rate limiting dark step in the process of photoactivation. This rate limiting step seems to be slower in the MSP-free mutant of PCC7942 than of PCC6803.

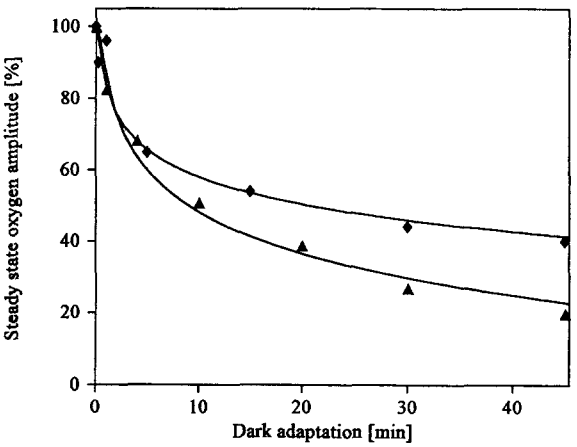

Fig. 5. Influence of the length of dark incubation after preflash treatment on the O_2 yield. Cells were pre-illuminated with 800 flashes and then dark incubated for the times given in the figure. After the respective dark period, the steady-state O_2 amplitude was determined. -◆-: *Synechocystis* PCC6803 M7.5, -▲-: *Synechococcus* PCC7942 A5.

For both mutants the dark interval between flashes can at least be extended to 950 ms without diminishing photoactivation efficiency. The conditions leading to photoactivation here reported agree quite well with those reported in the literature (e.g. Radmer and Cheniae 1977; Tamura and Cheniae 1987). Having now optimized conditions for photoactivation, the length of the dark period required for obtaining 50% reduction of the

maximal possible O_2 yield was determined (maximal O_2 yield was the one obtained after a pre-illumination with 800 flashes). As the results in Fig. 5 show, 50% reduction of the O_2 yield was observed after approximately 10 or 20 min dark adaptation of the MSP-free mutant of PCC7942 or PCC6803, respectively.

When performing the experiments related to photoactivation of the WOE, we made the observation that mutant cells which were dark adapted until no 'regular' oscillating O_2 evolution could be detected, showed a low non-oscillating amperometric signal (maximally corresponding up to about 5 – 10% of the maximal signal – see Fig. 2) with a slow kinetic (not shown). This slow O_2 evolution of the mutants was completely exhausted after about 10 flashes (in extensively dark adapted mutant cells regular O_2 evolution could not be detected until at least 10 – 20 pre-illuminating flashes were given; therefore, the slow signals could be separated from regular O_2 evolution). The slow signals seemed to be unaffected by the photoactivation process, since they could still be detected in photoactivated, 20 min dark adapted mutant cells under the first two flashes. In relation to the steady state amplitude, the first two signals in mutant cells were usually higher than the signals obtained under the first two flashes in the corresponding WTs. The slow signals are most likely due to a decomposition of H_2O_2 accumulated during the dark period. H_2O_2 degradation by PS II is a well documented process especially for preparations which had been chemically stressed or depleted of extrinsic polypeptides (Schröder and Åkerlund 1986; Berg and Seibert 1987; Bader und Schmid 1988; Mano et al. 1993; Taoka et al. 1993).

Measurements of O_2 evolution in a Clark electrode and investigations about photoinhibition of the water-oxidizing process

Since the absolute O_2 yield of the mutant cells detectable with the 'Three Electrode System' greatly depended on the pretreatment, we have also reinvestigated the O_2 evolution rates obtained in a Clark-type electrode under continuous light using either sodium bicarbonate or DCBQ/potassium ferricyanide as electron acceptor. Moreover, the degree of photoinhibition of the water oxidizing process was investigated for WT and MSP-free mutant cells before and after photoactivation of the WOE. The results obtained for PCC6803 are given in Table 2. For these experiments, cells were dark adapted for 5 h and either used without any further treatment or after pre-illumination with 800 light flashes. O_2 evolution of such cells was measured in continuous red light in a Clark electrode for a period of 15 min (for standardization measurements started exactly 90 s after the end of the preflash illumination). The results show that the O_2 evolving activities in the dark adapted mutant cells could be substantially increased (about 3 fold) and reached values comparable to WT rates when the mutant cells were pre-illuminated. These results confirm the results obtained with the 'Three Electrode System' and leave no doubt that the detectable O_2 evolving activities in the mutants are strongly dependent on the pretreatment of cells.

In the experiments under continuous light it could also be shown that the process of O_2 evolution in the mutant cells became less sensitive to photoinhibition when the WOE was photoactivated prior to the measurements. Using bicarbonate or DCBQ/ferricyanide as electron acceptor total inhibition of O_2 evolution occurred substantially later in mutant cells with pre-illumination by 800 flashes as compared to mutant cells without pre-illumination (Table 2). The high degree of photoinhibition in mutant cells without pre-illumination is most likely due to the accumulation of highly oxidizing species which are produced by the PS II reaction center when the WOE is in the dark inactivated state. The degree of photoinhibition decreases in flash pre-illuminated mutant cells but is still higher than in WT cells (Table 2). This might be partly due to the slower kinetic of O_2 release in the MSP-free mutant cells and partly due to endogenous reductants which are produced during continuous illumination and which can interact more readily with the S-state system when MSP is absent (see later).

Influence of Mn^{2+}, Ca^{2+} and Cl^- on photoactivation of the WOE

Since under our experimental conditions, depletion as well as uptake of Mn, Ca^{2+} and Cl^- by whole cells was relatively slow, it was not possible to show an effect of these ions on photoactivation of the WOE in intact cells under assay conditions as described above. Therefore, we used isolated thylakoid membranes from the MSP-free mutant of *Synechococcus* PCC7942 for these experiments. The O_2 evolution of the isolated thylakoid membranes was determined in a Clark electrode with DCBQ as electron acceptor under continuous illumination using thylakoid membranes either having no pretreatment or being pretreated with 1000 light flashes in the presence or absence of ions as indicated in Tables 3 and 4. As the results show, the O_2

Table 2. Influence of flash pre-illumination on the O_2 evolving activity determined under continuous light and on the degree of photoinhibition of the WOE in *Synechocystis* WT and mutant cells. Cell suspensions of *Synechocystis* PCC6803 WT and MSP-free mutant M7.5 were dark adapted for 5 h, and the O_2 evolving activity was determined in a Clark electrode under red continuous light in a reaction mixture containing either $NaHCO_3$ or DCBQ/potassium ferricyanide as electron acceptor. The samples (containing 30 μg Chl) were used without pretreatment or after pre-illumination with 800 flashes. Measurements of pre-illuminated samples were performed exactly 90 s after the pretreatment. The O_2 evolving rates were calculated on the basis of O_2 evolved in the first 60 s of measurement. The degree of photoinhibition was estimated by comparing this initial O_2 evolving activity with the activity after 15 min illumination and is given in parenthesis as percent residual O_2 evolving activity after 15 min or, if total inhibition occurred within 15 min, the time is given until total inactivation of O_2 evolution was observed

Synechocystis PCC6803	Pretreatment of cells	Photosynthetic oxygen evolution [μmol O_2/h × mg Chl]	
		Bicarbonate as electron acceptor	DCBQ/ferricyanide as electron acceptor
M7.5	Dark adapted for 5h	56.6 (totally inactivated after 6 min)	52.5 (totally inactivated after 8 min)
	1.5 min after 800 flashes	166.2 (totally inactivated after 12 min)	155.8 (totally inactivated after 15 min)
WT	Dark adapted for 5h	142.4 (15% activity after 15 min)	204.3 (82% activity after 15 min)
	1.5 min after 800 flashes	157.9 (10% activity after 15 min)	131.4 (66% activity after 15 min)

evolution rates approximately doubled when thylakoid membranes were photoactivated by preflashing, but this enhancement of O_2 evolution only occurred when $MnCl_2$ was added before preflashing. To obtain 50% activation, a concentration of 5 to 10 μM $MnCl_2$ was required (not shown). Under these conditions besides Mn^{2+} addition of Ca^{2+} was necessary for obtaining maximal photoactivation of the WOE, while Cl^- addition was not required. In contrast to the results obtained with intact mutant cells, no significant dark inactivation was observed for isolated thylakoid membranes of the MSP-free mutant during a dark period of 15 min. Most probably, this lack of dark inactivation is due to depletion of endogenous reductants in isolated thylakoid membranes (see 'Discussion').

S-state distribution, deactivation kinetics and O_2 release kinetics

The O_2 evolution patterns of WT and mutants were analyzed according to the five-state Kok model, because this model provided a better fit for the measured sequences than the four state Kok model. In Fig. 6A and B, the O_2 evolving pattern of WT and mutant cells which were photoactivated (800 light flashes) and then exactly 20 min dark adapted, are given (pattern of PCC6803 WT and M7.5 without added DCBQ and pattern of PCC7942 WT and A5 with added DCBQ). The corresponding calculated S-state distribution values are given in Table 5 (for PCC7942) and Table 6 (for PCC6803), and the deactivation kinetics for PCC6803 are given in Fig. 7. In general, it can be concluded that in all four cell types O_2 yield oscillated as a function of flash number with a periodicity of four. Comparison of the two WTs shows that the flash pattern of PCC6803 WT basically resembles a normal *Chlorella* sequence (Cheniae 1970), while the pattern of PCC7942 WT showed some deviations from the usual shape (see Fig. 2). The latter was much more damped than the pattern in PCC6803 WT – suggesting that the positive charges generated in the reaction center of PCC7942 WT are less efficiently utilized for the advancement of the WOE through the sequence of S-states leading to O_2 evolution. This is confirmed by the calculated average miss factor which was significantly higher in PCC7942 WT (especially without added DCBQ – not

Table 3. Influence of $MnCl_2$ addition on the photoactivation process in isolated thylakoid membranes of *Synechococcus* PCC7942 WT and MSP-free mutant A5. Thylakoid membranes were isolated as deseribed in Materials and methods, and O_2 evolving activity was determined in a Clark electrode. For photoactivation the samples (40 μg Chl) were in a reaction mixture containing 50 mM Hepes-NaOH, pH 6.5, 60 μM DCBQ, 400 mM sucrose, 100 mM NaCl, 17 mM $CaCl_2$ and, when indicated, 0.1 mM $MnCl_2$. After preflash treatment with 1000 flashes, O_2 evolution was determined in the same reaction mixture in which the DCBQ concentration was increased to 600 μM DCBQ and to which 0.1 mM $MnCl_2$ was added if omitted during preflash treatment. O_2 evolution rates were calculated from the O_2 evolved in the first 60 s. To show the relative degree of photoinhibition, the total oxygen evolved within the first 5 min of measurement is also given in parenthesis

Conditions	Oxygen evolution of isolated thylakoid membranes after different pretreatments [μmol O_2/h × mg Chl] *Synechococcus* PCC7942	
	A5	WT
No preflashing, $MnCl_2$ omitted	48.3 (74nmol)	208.9 (430 nmol)
1 min after 1000 preflashes, $MnCl_2$ omitted	39.8 (57 nmol)	213.4 (450 nmol)
No preflashing, $MnCl_2$ added	56.8 (123 nmol)	205.6 (507 nmol)
1 min after 1000 preflashes, $MnCl_2$ added after preflashes	59.6 (129 nmol)	235.3 (512 nmol)
1 min after 1000 preflashes, $MnCl_2$ added before preflashes	103.7 (199 nmol)	240.0 (522 nmol)
15 min after 1000 preflashes, $MnCl_2$ added before preflashes	100.0 (182 nmol)	

shown) than in PCC6803 WT. Another significant difference was found in the relative population of the more reduced states S_0 and S_{-1} which after dark adaptation were populated to a higher degree in PCC7942 WT than in PCC6803 WT when no DCBQ was added (not shown for PCC7942). In this respect the WOE in PCC7942 WT shows a great similarity to the WOE in *Euglena gracilis* which was assumed to be in a more reduced state than e.g. the WOE of *Chlorella* (Schmid and Thibault 1982). We also observed that the relative S_0 and S_{-1} population as well as the miss factor of PCC7942 WT greatly decreased in the presence of the artificial electron acceptor DCBQ. A similar behaviour has been published previously for *Euglena gracilis* by Schmid and Thibault (1982) and recently for *Synechocystis* PCC6714 by Etienne and Kirilovsky (1993). In the presence of DCBQ, the relative population of the more reduced S-states in PCC7942 WT dropped from up to 90% (in the absence of DCBQ) to about 30% (sum of S_0 and S_{-1}) and the miss factor decreased from about 40% to 25–30% (not shown). Because the S-state distribution of PCC7942 was greatly variable with different cell preparations in the absence of DCBQ, we only give the values in the presence of DCBQ for PCC7942 (Table 5), since under such conditions a better reproducibility was achieved. However, even in the presence of DCBQ the miss factor of PCC7942 WT was still higher than the miss factor of PCC6803 WT in the absence of DCBQ. In PCC6803 WT, DCBQ only had a minor effect on the S-state distribution and the miss factor (not shown).

These observations suggest that in PCC7942 a component on the acceptor side of PS II significantly influences the S-state distribution of the WOE shifting the WOE to a more reduced state. Interestingly, Styring and Rutherford (1988) also observed a strong influence of the PS II acceptor side on the S-state system. They reported an increased stability of the oxidized S-states S_2 and S_3 when the PS II acceptor side was oxidized by exogenous electron acceptors (see also Schmid et al. 1994). Our results suggest that in PCC7942 the redox state of the acceptor side also had a great influence on the advancement of the WOE through the sequence of S-states leading to O_2 evolution. The influence of the

Table 4. Effect of Ca^{2+} and Cl^- on photoactivation of thylakoid membranes of the MSP-free mutant *Synechococcus* PCC7942 A5. The experimental procedure was basically the same as in Table 3. The following changes were made: Isolated thylakoid membranes were washed and suspended in buffer from which $CaCl_2$ was omitted and ions were added to the reaction mixture as indicated in the table (concentrations: $MnCl_2$, 0.1 mM; NaCl, 100 mM; $CaCl_2$, 10 mM; $Ca(NO_3)_2$, 17 mM). In the experiments without pre-illumination the samples were pre-incubated in the reaction mixture for 15 min to allow maximal equilibration with Ca^{2+} and Cl^-

Conditions	Oxygen evolution of isolated thylakoid membranes after different pretreatments [μmol O_2/h × mg Chl] *Synechococcus* PCC7942 A5
$MnCl_2$ and NaCl present:	
No preflashing, $CaCl_2$ added	64.1 (162 nmol)
15 min after 1000 preflashes, $CaCl_2$ added before preflashing	99.3 (237 nmol)
15 min after 1000 preflashes, $CaCl_2$ added after preflashing	71.1 (187 nmol)
$MnCl_2$ and $Ca(NO_3)_2$ present:	
No preflashing, NaCl added	41.9 (106 nmol)
15 min after 1000 preflashes, NaCl added before preflashing	68.9 (167 nmol)
15 min after 1000 preflashes, NaCl added after preflashing	63.9(173 nmol)

Table 5. S-state distribution of *Synechococcus* PCC7942 WT and MSP-free mutant A5 in the presence of DCBQ calculated in the five-state Kok model. *Synechococcus* PCC7942 WT cells were pre-illuminated to steady-state with 100 flashes and *Synechococcus* PCC7942 A5 cells were pre-illuminated with 800 flashes for maximal photoactivation. After a dark adaptation of exactly 20 min or 10 s, respectively, the measurements were performed and the given values were calculated from the amplitudes under a train of 15 flashes as described under 'Materials and methods'. QD/Y_{max} is the quadratic deviation in relation to the amplitude maximum of the respective sequence. Fits in the four-state Kok model were in all cases less significant than in the five-state Kok model. Occasional negative values for S_2 are caused by the fitting system when normalizing the sum of S-states to 100%

Strain	Conditions	S-states [%]					Transition probability [%]			
		S_{-1}	S_0	S_1	S_2	S_3	Misses	Successes	Double hits	QD/Y_{max} [%]
Synechococcus PCC7942 wild type	With 0.6 mM DCBQ after 20 min dark adaption	15.2	31.5	58.7	-6.8	1.4	28.5	66.8	4.5	0.95
	With 0.6 mM DCBQ after 10 s dark adaption	2.5	33.3	28.7	24.0	11.5	24.9	69.4	5.5	0.82
Synechococcus PCC7942 A5	With 0.6 mM DCBQ after 20 min dark adaption	37.6	15.3	51.1	-9.7	5.7	39.3	55.6	6.0	1.02
	With 0.6 mM DCBQ after 10 s dark adaption	3.7	24.5	31.0	20.2	20.6	35.0	60.0	5.1	0.76

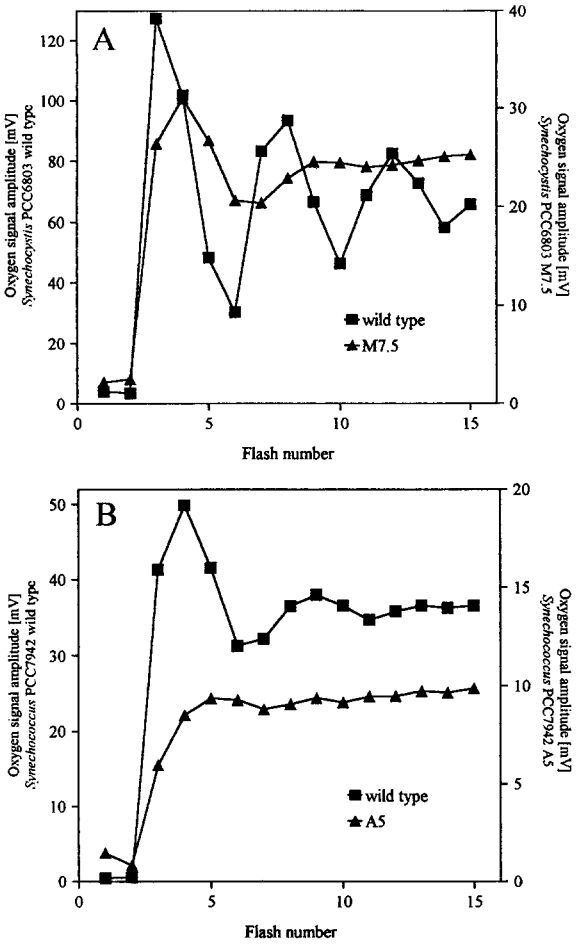

Fig. 6. Oxygen evolution pattern of *Synechocystis* PCC6803 and *Synechococcus* PCC7942 WT and MSP-free mutant cells. Cell suspensions of *Synechocystis* PCC6803 and *Synechococcus* PCC7942 WT and MSP-free mutant were pre-illuminated with 800 flashes. After pre-illumination the cells were exactly 20 min dark adapted and then the O_2 amplitudes obtained under flash light were determined. In case of PCC7942 0.6 mM DCBQ was present in the cell suspensions, while DCBQ was absent in case of PCC6803. The corresponding S-state distributions of these patterns are given in Table 5 (for *Synechococcus* PCC7942) and 6 (for *Synechocystis* PCC6803). (A) *Synechocystis* PCC6803 WT and MSP-free mutant M7.5 and (B) *Synechococcus* PCC7942 WT and MSP-free mutant A5.

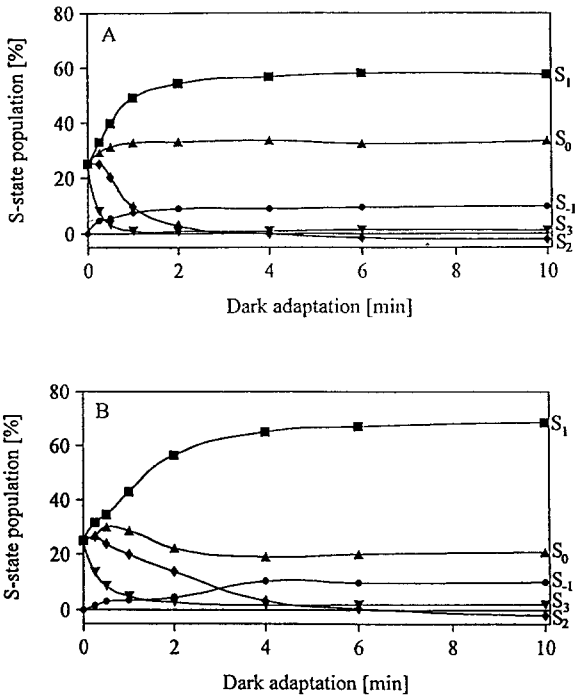

Fig. 7. S-state deactivation kinetics of *Synechocystis* PCC6803 WT (A) and MSP-free mutant M7.5 (B). WT cells were pre-illuminated with 100 flashes and MSP-free mutant cells with 800 flashes. The S-state distributions after different dark periods were calculated from the O_2 amplitude under a sequence of 15 saturating light flashes.

PS II acceptor side on the WOE possibly might occur via a pathway very similar to the one used in cyclic electron flow around PSII (Falkowski et al. 1986). An alternative or additional explanation could be that the acceptor side is more completely reduced in PCC7942 than in PCC6803.

Besides comparison of the two WTs, the two MSP-free mutants were compared with their corresponding WT. As the results in Fig. 6 show, for 20 min dark adapted mutant cells a much more damped pattern was observed for both mutants relative to the pattern obtained for the corresponding WT, clearly demonstrating that the misses were higher in both mutants than in the corresponding WT (Tables 5 and 6). On absolute basis the calculated miss factor was higher in the MSP-free mutant of PCC7942 than in the MSP-free mutant of PCC6803 (especially in the absence of DCBQ when values up to 50% were obtained for A5, not shown). This is due to the fact that the misses in PCC7942 WT already reached values of 40% in the absence of DCBQ (not shown). Another striking difference between 20 min dark adapted cells of the MSP-free mutants and the corresponding WTs was the higher population of the S_{-1} state in the mutant cells – most pronounced in PCC7942. Usually, the S_{-1}-populations in 20 min dark-adapted MSP-free mutant cells and WT cells of PCC6803 were about 15% and 5%, respectively (Table 6). The corresponding values for PCC7942 were approximately 40% and 15% when DCBQ was present (Table 5). In the absence of DCBQ, the calculated S_{-1}-population of the MSP-free PCC7942 mutant increased and reached values up

Table 6. S-state distribution of *Synechocystis* PCC6803 WT and MSP-free mutant M7.5 in the absence or presence of hydroxylamine in the five- or six-state Kok model. *Synechocystis* PCC6803 WT cells were pre-illuminated to steady-state with 100 flashes and *Synechocystis* PCC6803 M7.5 cells were pre-illuminated with 800 flashes for maximal photoactivation. The preflash treatment was performed after pre-incubating the samples with the hydroxylamine concentrations given in the table for 25 min and the analyzing flash train was given after 10 min dark adaptation except for the experiments marked with (*). In those experiments one preflash was given before hydroxylamine was added to the samples. Then the samples were incubated for 25 min, pre-illuminated as above and dark adapted for 10 min before the analyzing flash train was given. In cases where fits were obtained in the five- as well as in the six-state Kok model the S-state distribution with the best fit is given. For none of the data useful fits were obtained in the four-state Kok model

Hydroxyl-amine [μM]	S-states [%]						Transition probability [%]			
	S_{-2}	S_{-1}	S_0	S_1	S_2	S_3	Misses	Successes	Double hits	QD/Y_{max} [%]
					Synechocystis PCC6803 wild type					
0	-	4.3	32.8	62.0	-1.0	1.9	12.5	84.6	2.9	0.51
5	-	19.2	39.8	42.0	-1.9	1.0	14.0	82.9	3.6	0.39
10	3.4	29.0	39.9	27.4	-1.1	1.4	13.4	83.7	3.7	0.53
25	22.9	75.0	-5.9	3.7	1.8	2.5	15.1	82.0	5.0	0.56
100	40.5	47.4	-1.1	4.3	3.7	5.4	24.0	77.2	2.9	4.47
100 (*)	31.6	71.1	-15.3	5.6	3.2	3.8	18.6	80.6	5.4	5.09
					Synechocystis PCC6803 M7.5					
0	-	13.5	17.9	68.5	-2.2	2.4	34.4	63.8	2.5	1.01
5	-	32.5	23.8	36.2	0.8	6.8	33.1	60.7	7.9	1.22
10	-	60.1	7.8	20.3	4.6	7.2	30.9	66.9	4.9	2.53
25	-	72.4	-10.3	11.6	12.6	13.7	30.3	68.2	4.4	3.16
25 (*)	-	20.7	20.3	46.0	5.8	7.1	26.4	69.6	5.0	0.56

to 60% (not shown) while it was less affected in WT cells (not shown).

In order to obtain further information on the characteristics of the WOE in WT and mutant cells, we followed the deactivation of the S-states in the dark. The results which we obtained for PCC6803 are given in Fig. 7. The results are based on the assumption that under steady-state conditions the S-state distribution is $S_0=S_1=S_2=S_3=25\%$ and no S_{-1} is present. As the results show, the deactivation of WT could be considered normal and was comparable to deactivation kinetics, e.g. observed for *Chlorella* (Cheniae 1970). In the MSP-free PCC6803 the S-state deactivation was slowed down compared to WT. This difference seems to be mainly due to an increased stability of the S_2 and S_3 states in the mutant – an observation already published by Burnap et al. (1992). Moreover, a slight shift from S_0 to S_{-1} in the M7.5 deactivation observed after 1–2 min suggests the presence of additional kinetic phases in the mutant, since this shift is invisible in the WT kinetics. Because of the relatively damped oscillation pattern obtained with PCC7942 WT which made an evaluation of such experiments rather difficult, the corresponding results are not presented for this cyanobacterium.

Kinetically resolved oxygen signals typical of mutant and WT cells are given in Fig. 8. Since the rise kinetic depends on the O_2 diffusion rate, only WT with its corresponding mutant can be compared. This comparison shows that the rise kinetic of the mutant signal was found to be much slower than that of the corresponding WT for PCC6803 as well as PCC7942. The difference which we observed between the rise kinetics of the MSP-free mutant signal and the WT signal is comparable to the data previously published by Burnap et al. (1992) for cells of a MSP-free PCC6803 mutant without photoactivation. In general it can be concluded that the slower rise time of the O_2 signal from the mutant cells indicates that the S_3 to (S_4) to S_0 transition is slower in both MSP-free mutants than in the corresponding WT.

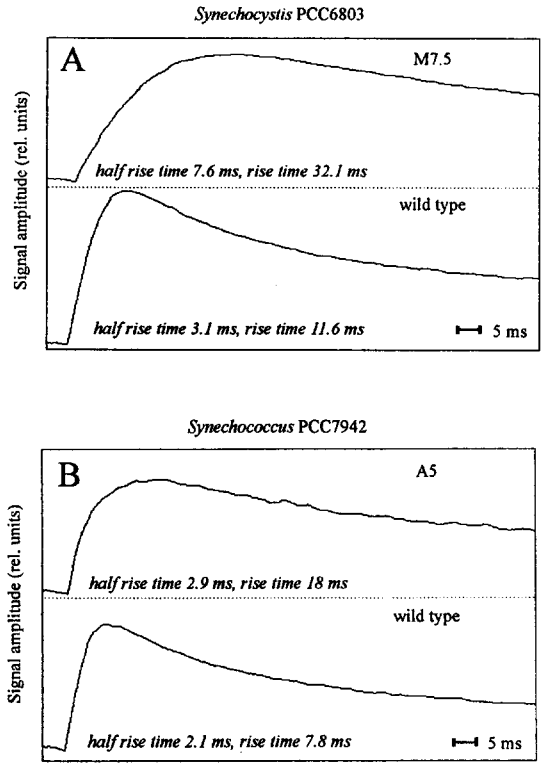

Fig. 8. Oxygen signal rise kinetics of WT and MSP-free mutant cells of *Synechocystis* PCC6803 (A) and *Synechococcus* PCC7942 (B). The tracings are presented in time resolution and correspond to the third signal from a flash sequence given to cells which had been dark adapted for 10 min. A detailed signal analysis as previously published by Schulder et al. (1990) for other preparations was not performed for these experiments.

The effect of hydroxylamine and ammonia on O_2 evolution of WT and mutant cells

50% inhibition of O_2 evolution was obtained with about 75 μM or 40 μM NH_2OH for PCC6803 WT or photoactivated mutant, respectively (not shown). The degree of NH_2OH inhibition of WT and mutant increased with increasing dark incubation periods, since – besides its regular inhibitory effect – NH_2OH greatly accelerates the dark inactivation of the WOE in the mutant cells and also causes a partial reversible inactivation of the WOE in the WT. The influence of NH_2OH on the S-state distribution of the WOE in PCC6803 is given in Table 6. As expected, with increasing hydroxylamine concentrations the S-state distribution shifts to more reduced S-states at the expense of the S_1 and S_0 state, resulting in substantial reduction of the O_2 yield under the third and fourth flash of the respective sequences (not shown). A significant difference between WT and mutant was that at high hydroxylamine concentrations, an approximately equal population of the S_{-2} state and the S_{-1} state was observed in WT. In contrast, in the MSP-free mutant only the S_{-1} state was populated, while no S_{-2} could be detected. In both cell types S_{-1} seemed to be mainly formed on the expense of S_1, and S_{-2} in WT seemed to be formed on the expense of S_0 and, at higher NH_2OH concentrations, on the expense of S_{-1}. Besides the absence of a detectable S_{-2} state, there was another pecularity which we observed in the mutant. When mutant cells were illuminated with at least one flash before NH_2OH was added, then the S-state distribution approached the S-state distribution and the dark inactivation rate observed without NH_2OH addition, although inhibition of the steady-state O_2 signal was obtained. Our results indicate that in the 'Mn free WOE' a one step oxidation could occur and that the formed state was very stable, since it was neither influenced by photoactivation under our conditions nor reversed during dark periods up to 45 min (not shown). The chemical nature of this state is presently unknown. In contrast, in WT cells no difference in S-state distribution was observed whether NH_2OH was added in the dark or after cells were pre-illuminated with one flash. Because of the substantial irregularities observed for the O_2 pattern of PCC7942 WT and mutant, these experiments were not performed with PCC7942.

Moreover, we could show that O_2 evolution in the MSP-free mutant of PCC6803 and PCC7942 was more sensitive towards ammonia inhibition – especially in case of PCC6803 which could rapidly take up ammonia in the dark. For these experiments the cells were preincubated with different NH_4Cl concentrations for 40 min, then pre-illuminated with 800 flashes and 10 s after this treatment the steady state O_2 amplitude was determined in a sequence of 15 flashes. In mutant cells of PCC6803 50% inhibition of O_2 evolution was obtained by addition of 3–6 mM NH_4Cl, while in PCC6803 WT cells 30–50 mM NH_4Cl was required. For PCC7942 50% inhibition of O_2 evolution was obtained with 8–12 mM NH_4Cl in the MSP-free mutant and with 30–40 mM NH_4Cl in WT. Assuming that ammonia uptake in WT and mutant cells is similar, then these results indicate that the WOE in the absence of MSP becomes much more accessible for endogenous metabolites and is therefore more sensitive towards ammonia inhibition. It is likely that the actual ammonia concentration in the cyanobacterial cells was lower than the NH_4Cl concentration in the incubation medium and therefore might come close

to physiological ammonia concentrations – indicating that ammonia might be a physiological inhibitor of the WOE in the absence of MSP.

Discussion

After an extended period of dark adaptation, no oscillating O_2 evolving activity was detectable with cell suspensions from the MSP-free mutants of *Synechococcus* PCC7942 and *Synechocystis* PCC6803, when O_2 evolution was measured on a bare platinum electrode with a sequence of short saturating light flashes. In contrast, O_2 evolving activities in cell susupensions of PCC7942 WT and PCC6803 WT were only slightly reduced after such a dark adaptation. Although the O_2 evolution pattern of PCC7942 WT and PCC6803 WT showed substantial differences (see Tables 5 and 6 and Figs. 2 and 6), the WOE in the MSP-free mutants responded in the same way to dark incubation. This leaves no doubt that the observed dark inactivation of the WOE in the mutants is indeed a consequence of the lacking MSP.

Thus, our results show that O_2 evolution in the MSP-free cyanobacterial mutant cells under flash illumination is greatly dependent on the previous treatment of the cells. This property of the WOE in the MSP-free mutant cells most likely explains the low and somewhat variable O_2 yields under flash illumination reported by Burnap et al. (1992) and Vass et al. (1992) for their MSP-free PCC6803 mutants. The dark inactivation of the WOE which we observed in intact mutant cells is not an irreversible process but can be reversed by light, flash light being more effective than continuous light. $MnCl_2$ addition to whole cells was not required for photoactivation – indicating that the endogenous Mn concentration of such cells is sufficient. Proof that photoactivation of the WOE in the MSP-free mutant cells is indeed due to photoligation of Mn came from experiments with isolated thylakoid membranes of the MSP-free PCC7942. Here it could be shown that the process of photoactivation had a requirement for added Mn^{2+} and Ca^{2+}, while Cl^- addition was not required under our experimental conditions.

Our results showing that dark adaptation of the two cyanobacterial mutants lacking MSP leads to a reversible inactivation of the WOE adds one more condition to the list of conditions under which photoactivation of the WOE can be observed (see e.g. review by Radmer and Cheniae 1977). More recently, extensive *in vitro* studies on conditions leading to photoligation of Mn^{2+} and photoactivation of O_2 evolution have been carried out with Tris extracted and/or NH_2OH treated PS II membranes and show that photoligation of Mn^{2+} is a low quantum yield process which requires Mn^{2+}, Ca^{2+}, Cl^- and light and an artifical electron donor (Tamura and Cheniae 1987; Miller and Brudvig 1990; Miyao and Inoue 1991a, b). The here reported conditions required for photoactivation of the inactive WOE in dark incubated MSP-free mutant cells basically agree with those in the literature, suggesting that dark incubation of the mutants leads to Mn loss resulting in the absence of a functional S-state system.

Since it is suggested that Mn is preferentially lost when the WOE is in reduced states (Cheniae and Martin 1971; Yocum et al. 1981), it should be expected that reduced S-states (such as S_0 as well as S_{-1} and S_{-2} when formed) might prevail in the MSP-free mutant cells to explain the observed inactivation of the WOE. However, analysis of the S-state distribution of the photoactivated WOE in the mutant cells only showed that, although the S_{-1} population in the mutants was higher compared to the one in the corresponding WT, significantly high values for the S_{-1} population were only observed for the PCC7942 mutant. Moreover, in both mutants practically no S_{-2} was detectable. Taking into consideration that in the MSP-free mutant of PCC7942 the reversible dark inactivation of the WOE caused by the loss of Mn is twice as fast as in the PCC6803 mutant, one might speculate that in the mutant WOE the formation of a 'Mn-free state', which requires photoactivation before contributing to the detectable S-state distribution, occurs via S_{-1}. Although the S_{-1} state is also present in the corresponding WTs, it might be shielded from further reduction and loss of Mn might be prevented by MSP providing additional ligands to the Mn cluster. Evidence that this could be a possible explanation came from experiments with hydroxylamine which is very suited for whole cell experiments and which as a redox active substance can undergo one or two electron reducing reactions with S_2, S_1, and S_0 (see for review Rutherford 1992 and more recent papers: Guiles et al. 1990; Messinger et al. 1991; Mei and Yocum 1992, 1993). Our results show that in PCC6803 WT hydroxylamine addition causes S_{-2} formation, while in the mutant cells no significant S_{-2} was detectable. Since it is unlikely that in the mutants no S_{-2} is formed by NH_2OH treatment while formed in WT, the most likely explanation is that in the mutant cells Mn is lost from this state leading to the observed accelerat-

ed dark inactivation of the WOE in the mutant cells, while in WT loss of Mn from the WOE (even in the S_{-2} state) is prevented by the presence of MSP. If in the mutants S_{-2} is short lived as a consequence of Mn loss, then the equilibrium between S_{-2} and S_{-1} is most likely permanently changed in favour of replenishing the S_{-2} population on the expense of the S_{-1} population and consequently the detectable S_{-1} state never accumulates beyond a certain level. This might explain why – besides the absence of detectable S_{-2} – no substantial increase in S_{-1} is observed. Hydroxylamine is a non-physiological reductant of the WOE, but a number of endogenous reductants of the WOE could be envisaged. H_2O_2 is a redox active substance known to interact with PS II and to be produced in cyanobacteria (Patterson and Myers 1973; Roncel et al. 1989). There is also indication that H_2O_2 could be formed in a PS II pathway (Bader and Schmid 1988; Klimov et al. 1993; Hillier and Wydrzynski 1993). Due to the better accessibility of the WOE for endogenous metabolites in absence of MSP, a reduction of the WOE with concomittent loss of Mn might occur in the mutants – although Mn must remain in close proximity to its binding site, since reactivation of the WOE in intact mutant cells can easily be achieved by illumination without addition of $MnCl_2$. In conclusion, it can be said that the most likely explanation for the inactivation phenomenon observed for the WOE in MSP-free mutant cells during dark adaptation is that endogenous reductants cause a shift of the S-state population to more reduced states. Due to a greater lability of these states in the absence of MSP, loss of Mn from the WOE in the mutants occurs. The WOE in WT is protected from this inactivation mainly for two reasons: The WOE is not equally well accessible for reductants and when becoming partially reduced, loss of Mn is greatly prevented by the presence of MSP which properly provides additional ligands for the Mn cluster. Thus, besides having a cofactor stabilizing function MSP also has a role in protecting the WOE from endogenous metabolites.

Another question which we raised was whether in the absence of MSP the WOE is more susceptible to inhibition by such physiological metabolites as NH_3. Our results show that the sensitivity of the WOE for NH_3 inhibition was substantially higher in the MSP-free mutants of *Synechococcus* PCC7942 as well as *Synechocystis* PCC6803 than in the corresponding WTs. This result suggests that NH_3 could indeed be considered to be a physiological inhibitor of the WOE in the absence of MSP. Therefore, it would be worthwhile to investigate whether conditions can be found under which MSP is specifically degraded or detached from the PS II complex – temporarily leading to an inactive WOE.

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