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Insertional inactivation of the *psb*O gene encoding the manganese stabilizing protein of photosystem II in the cyanobacterium *Synechococcus* PCC7942

Effect on photosynthetic water oxidation and L-amino acid oxidase activity

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A *Synechococcus* PCC7942 mutant in which the *psb*O gene was inactivated by insertion of a chloramphenicol interposon and which did not contain any detectable manganese stabilizing protein in immunoblot experiments, was constructed. Such a *Synechococcus* mutant was able to grow under photoautotrophic conditions. Isolated thylakoid membranes from the mutant required addition of $CaCl_2$ and $MnCl_2$ for photosynthetic O_2 evolution, and the detectable L-amino acid oxidase activity in the isolated thylakoid membranes from the mutant was approximately four times higher than in wild-type thylakoids. The results are discussed with respect to our model suggesting that the water-oxidizing enzyme may have evolved from a flavoprotein with L-amino acid dehydrogenase/oxidase activity.

Photosystem II; O_2 evolution; *psb*O gene; Manganese stabilizing protein; L-Amino acid oxidase

1. INTRODUCTION

Photosynthetic water oxidation requires three inorganic cofactors: Mn, Ca^{2+} and Cl^-. However, it is still unknown to which peptide (or peptides) in PS II these cofactors are bound (see review in [1]). A number of observations suggest that the reaction center peptides D1 and D2 are involved in Mn binding [1–4]. This would imply that the peptides catalyzing photochemical charge separation can also catalyze water oxidation. If the number of polypeptides present in a minimal O_2-evolving PS II complex actually is 8 (gene products of *psb*A, -B, -C, -D, -E, -F, -I and -O), then D1 and D2 seem to be the best candidates for Mn binding, since Burnap and Sherman [5] have recently shown by deletion mutagenesis in the cyanobacterium *Synechocystis* PCC6803 that the *psb*O gene product (MSP) is not obligatory for water oxidation but only helps to stabilize Mn at the water-oxidizing enzyme.

However, our model of PS II predicts that the water-oxidizing enzyme is a separate protein (distinct from D1 and D2), being an additional peptide to the 8 peptides listed above [6]. Based on our results with the cyanobacterium *Synechococcus* PCC6301 we suggested that the water-oxidizing enzyme evolved from a substrate dehydrogenase/oxidase type enzyme which originally mediated electron flow from basic L-amino acids, e.g. L-arginine, to the plastoquinone pool of the electron transport chain [7]. Later in evolution this flavoprotein could also interact with O_2, and this activity is mainly measured in *Synechococcus* PCC6301 [7] and in *Synechococcus* PCC7942 (unpublished results). The L-AOX activity of this protein is totally suppressed in the presence of cations, such as Mn or Ca^{2+}. Our model suggests that during evolution from anoxygenic to oxygenic photosynthesis this flavoprotein became modified with additional cofactors (Mn, Ca^{2+}, Cl^-) and additional peptides (such as the MSP) and that this modified flavoprotein in combination with the D1/D2/cytochrome b_{559} complex became the present-day water plastoquinone oxidoreductase. In this paper a *Synechococcus* mutant lacking the MSP was constructed. Thylakoid membranes of such a mutant were investigated to see what effect the lack of the MSP might have on the O_2 evolving activity and the detectable L-AOX activity.

Abbreviations: L-AOX, L-amino acid oxidase; chl, chlorophyll; MSP, manganese stabilizing protein; PS, photosystem; WT, wild-type.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

The *Escherichia coli* and *Synechococcus* strains and plasmids used in this study have previously been described [8–11].

2.2. *Media and growth conditions*

Synechococcus PCC6301 (*Anacystis nidulans* – SAUG B1402-1) was grown as previously described [7], and *Synechococcus* PCC7942 (*Ana-*

cystis nidulans R2) was grown in BG 11 medium [12] under the same conditions. *E. coli* JM103 [8] was cultivated at 37°C in LB medium [13]. Antibiotics were added at the following concentrations: 150 mg/l ampicillin (Ap) for *E. coli*, 0.5 mg/l for *Synechococcus*; 50 mg/l chloramphenicol (Cm) for *E. coli*, 7.5 mg/l for *Synechococcus*; 25 mg/l kanamycin (Km) for *E. coli*.

2.3. *Cloning procedures*

Total DNA from *Synechococcus* PCC6301 was isolated by the Sarkosyl method and purified through isopycnic centrifugation in CsCl–ethidium bromide gradients as described for *Rhodobacter capsulatus* [14]. *Synechococcus* DNA was digested with *Hin*dIII, and fragments were size-fractionated on NaCl gradients and ligated with *Hin*dIII-linearized dephosphorylated DNA of vector plasmid pUC19. All other recombinant DNA techniques were performed using established techniques [15]. Restriction endonucleases, terminale transferase, T4-DNA-polymerase, T4-DNA-ligase and phosphatase were purchased from Bethesda Research Laboratories or Boehringer. All enzymatic reactions were performed as recommended by the manufacturers.

2.4. *DNA filter hybridizations*

Agarose gel electrophoresis and Southern blotting were carried out as described in [14]. A synthetic oligonucleotide (5'-CGAGCTTG-GATCAGGTCTACGGTGA-3') from the structural *Synechococcus* PCC7942 *psb*O gene was used as a probe in hybridization experiments. The oligonucleotide was synthesized with an Applied Biosystems DNA Synthesizer Model 380B using the phosphoramitide method [16,17]. OPC-purification of the oligonucleotide was carried out as recommended by Applied Biosystems. The oligonucleotide was labelled with DIG-dUTP by the terminal transferase and detected by using the DNA-labelling and detection kit from Boehringer.

2.5. *Isolation of thylakoid membranes and measurement of photosynthetic O_2 evolution and L-AOX activity*

Thylakoid membranes from WT and mutant A5 of *Synechococcus* PCC7942 were isolated as previously described for *Synechococcus* PCC6301 [18]. However, thylakoid membranes obtained after centrifugation were washed once with 50 mM HEPES–NaOH, pH 7, containing 400 mM sucrose, and after recentrifugation resuspended in the same buffer to give a chlorophyll concentration of approximately 1 mg chl/ml.

Photosynthetic O_2 evolution and L-AOX activity in thylakoid membranes were measured as described previously [18]. For photosynthetic O_2 evolution the reaction mixture contained in a total volume of 1.9 ml: 50 mM HEPES–NaOH, pH 7, 1.6 mM potassium ferricyanide, washed thylakoid membranes containing 4–15 μg chl, and $CaCl_2$ and $MnCl_2$ as indicated. O_2 evolution rates of whole cells were measured under the same conditions but in a reaction mixture (1.9 ml) containing: 50 mM HEPES-NaOH, pH 7, 15 mM $NaHCO_3$, and 5–20 μl cells. The reaction mixture for the L-AOX assay (1.9 ml) contained 50 mM HEPES-NaOH, pH 7, 10 mM EDTA, pH 7, 10 mM L-arginine–HEPES, pH 7, and thylakoid membranes containing 40–150 μg chl.

SDS-PAGE and immunoblot experiments were performed as described previously [6]. The antiserum was raised against the MSP isolated from oat and used with a dilution of 1:50.

3. RESULTS

3.1. *Insertional inactivation of the* psb*O gene from* Synechococcus *PCC7942*

The DNA sequence of a 1215 bp *Xba*I-*Hin*dIII fragment encoding the *Synechococcus* PCC7942 *psb*O gene has been published previously [19]. In order to inactivate the *psb*O gene, a 1.7 kb *Hin*dIII fragment (shown in Fig. 1) was chosen as a basis for insertional mutagenesis. Since *Synechococcus* strains PCC7942 and PCC6301 are very closely related [9], a clone carrying the corresponding 1.7 kb *Hin*dIII fragment from *Synechococcus* PCC6301, was used in this study. This clone was isolated from a partial *Synechococcus* PCC6301 gene bank consisting of size-fractionated *Hin*dIII fragments cloned into the vector plasmid pUC19 [10]. A clone carrying the *psb*O gene was identified by colony hybridization using a synthetic 25-mer oligonucleotide as a probe. The resulting recombinant plasmid was called pRB29. In order to abolish the vector-encoded *Pst*I site, plasmid pRB29 was digested with *Sph*I and *Bam*HI. Protruding ends were blunt-ended by T4-DNA-polymerase treatment prior to religation. The resulting hybrid plasmid pRB3 contained a single *Pst*I site approximately 60 bp downstream the starting codon of the structural *psb*O gene (Fig. 1). This *Pst*I site was used to clone a 1871 bp *Pst*I interposon carrying a chloramphenicol resistance gene from plasmid pSUP401 [11]. The chloramphenicol resistance gene in the resulting plasmid pRB5 is orientated in the same direction as the *psb*O gene. Plasmid pRB5 was transformed into *Synechococcus* PCC7942. Selection for chloramphenicol resistance resulted in single and double recombination events because plasmid pRB5 cannot replicate in *Synechococcus* PCC7942. About 80% of chloramphenicol-resistant *Synechococcus* colonies had lost the vector-encoded ampicillin resistance. Correct insertion of the chloramphenicol interposon into the chromosomal *psb*O gene by marker rescue was proofed by Southern analysis (data not shown). The corresponding *Synechococcus* PCC7942 strain carrying a mutated *psb*O gene was called *Synechococcus* PCC7942 – A5.

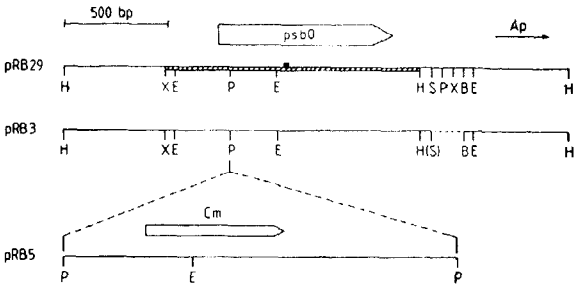

Fig. 1. Insertional inactivation of the *Synechococcus* PCC7942 *psb*O gene. The physical map of a 1.7 kb DNA fragment carrying the *Synechococcus psb*O gene is given for the enzymes *Hin*dIII (H), *Xba*I (X), *Eco*RI (E), *Pst*I (P), *Sph*I (S) and *Bam*HI (B). Plasmid pRB29 contains the 1.7 kb *Hin*dIII fragment cloned into pUC19. The pUC19 part including the multiple cloning site is not drawn to scale. The hatched bar marks an 1215 bp *Xba*I-*Hin*dIII fragment which has been sequenced [19] and the black box indicates the location of the synthetic oligonucleotide used as a hybridization probe. Plasmid pRB3 was derived from plasmid pRB29 by deletion of the *Sph*I-*Bam*HI fragment from the multiple cloning site. The orientation of the chloramphenicol resistance gene (Cm) within the interposon inserted in the single *Pst*I site of plasmid pRB3 is indicated for the resulting plasmid pRB5.

3.2. *Comparative analysis of* Synechococcus *PCC7942 and mutant A5*

The *Synechococcus* PCC7942 mutant A5 carrying a *psb*O gene inactivated by insertion of a chloramphenicol interposon was able to grow photoautotrophically. The growth rate was approximately 75% the rate of WT (not shown). Immunoblot analysis of WT and mutant (Fig. 2) with an antiserum raised against the MSP clearly showed that the mutant did not contain any detectable MSP. Under the applied conditions, the antiserum would be able to detect 1% of MSP present in WT. Immunoblots with the antisera raised against D1 and the L-AOX protein indicated that both proteins were present in comparable amounts in WT and mutant A5 (not shown). Photosynthetic O_2 evolution of WT and mutant cells using CO_2 (as $NaHCO_3$) as terminal electron acceptor was in the range of 50–60 μmol O_2 evolved/mg chlorophyll·h for both cell types. In the assays with whole cells neither Ca^{2+} nor Mn^{2+} had to be added indicating that in whole cells both cations were associated with the water oxidizing enzyme as could be expected from the growth rates.

However, the difference in the requirement for added cations became quite obvious when thylakoid membranes were isolated from WT and mutant. As shown in Table I, thylakoid membranes of WT *Synechococcus* PCC7942 required the addition of Ca^{2+} for photosynthetic O_2 evolution (as previously shown for *Synechococcus* PCC6301) [20,21], but they did not need the addition of Mn^{2+}, since loss of Mn was prevented by the MSP. However, Ca^{2+} and in addition Mn^{2+} were required for optimal O_2 evolution measured with thylakoid membranes from the mutant. The rate with Ca^{2+} alone was approximately 10% of the maximal rate. Measurements of the L-AOX activity in thylakoid membranes of WT and mutant showed that L-AOX activity in the mutant thylakoids was approximately four times higher than in WT (Table I). The O_2 uptake was inhibited by $CaCl_2$, clearly demonstrating that the O_2 uptake measured with thylakoid membranes in the presence of L-arginine was due to the L-AOX protein. One of our main reasons to believe that photosynthetic O_2 evolution and L-AOX activity in the thylakoid membranes are interrelated is due to the observation that $CaCl_2$ has an antagonistic effect on the two activities: $CaCl_2$ stimulates O_2 evolution, but inhibits the L-AOX activity [7,21]. In the thylakoid membranes of the mutant this antagonistic effect of metal ions on the two reactions examined could now be demonstrated for $MnCl_2$ as well as for $CaCl_2$ (Fig. 3).

Table I

Photosynthetic O_2 evolution and L-amino acid oxidase activity in isolated thylakoid membranes from *Synechococcus* PCC7942 wild-type and mutant A5

Additions	Photosynthetic O_2 evolution (μmol O_2 evolved/mg chl·h)	
	Wild-type	Mutant A5
None	3.6	0
$MnCl_2$	28.4	0
$CaCl_2$	79.9	6.6
$MnCl_2$ and $CaCl_2$	72.8	53.6
	L-Amino acid oxidase activity (μmol O_2 taken up/mg chl·h)	
	Wild-type	Mutant A5
EDTA	3.2	12.9
$CaCl_2$	0	0

The activity was measured as described in Materials and Methods. The concentrations of cations and EDTA in the reaction mixtures were: 50 mM $CaCl_2$, 1 mM $MnCl_2$, and 10 mM EDTA (pH 7).

4. DISCUSSION

Burnap and Sherman [5] have recently shown that a *Synechocystis* PCC6803 mutant in which the entire *psb*O gene encoding the MSP had been deleted, was capable of photoautotrophic growth. Our results with the *Synechococcus* PCC7942 mutant carrying an insertional inactivated *psb*O gene and containing no detectable MSP, confirm these results and clearly show that at least in the two cyanobacteria so far investigated the MSP is not obligatory for photoautotrophic growth.

Our model of the water-oxidizing enzyme (Fig. 4) predicts that the two cations (Mn and Ca^{2+}) required for

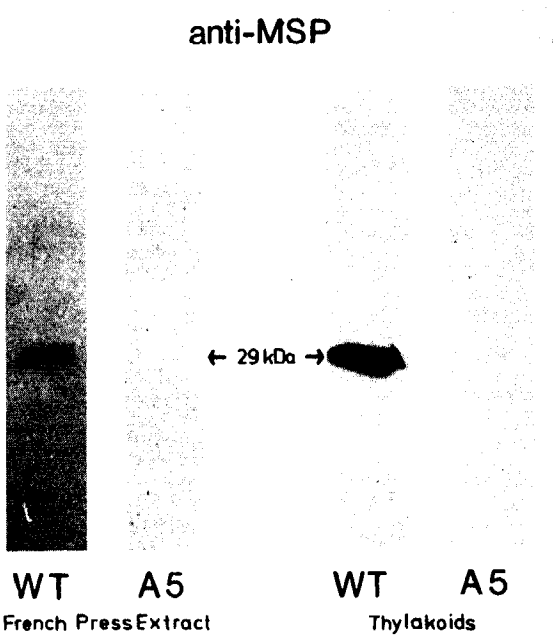

Fig. 2. Immunoblots with the antiserum raised against the MSP. (Left-hand side) Immunoblots of total French press extracts of *Synechococcus* PCC7942 WT and mutant A5. (Right-hand side) Immunoblots of isolated thylakoid membranes of *Synechococcus* PCC7942 WT and mutant A5.

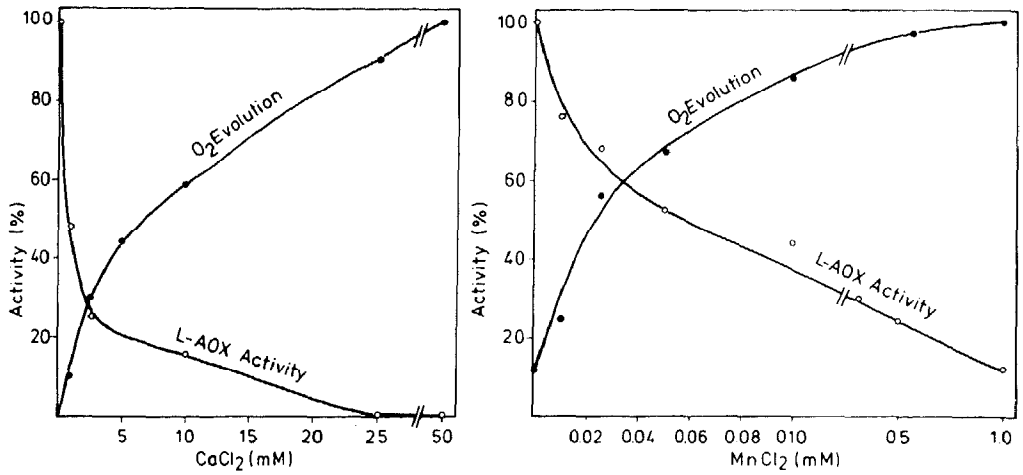

Fig. 3. Antagonistic effect of $CaCl_2$ and $MnCl_2$ on photosynthetic O_2 evolution and L-AOX activity in washed thylakoid membranes of the *Synechococcus* PCC7942 mutant A5. The activity measurements were performed as described in Materials and Methods with the following alterations: in the reaction mixture for the L-AOX assay EDTA was omitted when cations were added to the reaction mixture. The reaction mixture for photosynthetic O_2 evolution contained either 1 mM $MnCl_2$ and $CaCl_2$ as indicated in the figure (left-hand side) or 50 mM $CaCl_2$ and $MnCl_2$ as indicated in the figure (right-hand side).

water oxidation are bound to a flavoprotein which has been shown to be associated with PS II complexes and which has an L-AOX activity (L-arginine being the best substrate) in the absence of cations in *Synechococcus* PCC6301 [6,7] as well as in *Synechococcus* PCC7942 (unpublished results). This protein by itself has a relatively poor affinity for both cations as previously shown [21]. The results presented here indicate that, in absence of the MSP, Mn^{2+} as well as Ca^{2+} rapidly dissociates from the water-oxidizing enzyme, and that under such conditions (when the MSP is removed and the L-AOX protein is more easily accessible for the hydrophilic substrate L-arginine), the L-AOX activity detectable in thylakoid membranes is higher in the mutant than in WT thylakoids. Moreover, it could be shown that the concentrations of Ca^{2+} as well as Mn^{2+} required for activating photosynthetic O_2 evolution correlate quite well with the concentrations required to suppress the L-AOX activity, indicating that the water-oxidizing enzyme had approximately the same affinities for Ca^{2+} and Mn^{2+} as the L-AOX protein. These results strongly support our hypothesis suggesting that Mn and Ca^{2+} in PS II are bound to this flavoprotein. By performing a comparable inactivation experiment of the gene encoding the L-AOX protein, we hope to find a final answer to the question whether our hypothesis proves to be correct.

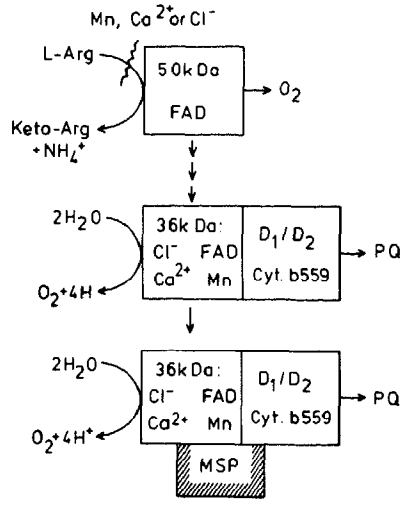

Fig. 4. Hypothetical model of the conversion of an L-arginine dehydrogenase/oxidase to the present-day water plastoquinone oxidoreductase. Our model predicts that the water plastoquinone oxidoreductase consists of a flavoprotein which originally had an L-arginine metabolizing activity, and of the D1/D2/cytochrome b_{559} complex which in our model can only catalyze the photochemical charge separation. In our model Mn is stabilized at this flavoprotein by the MSP.

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