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Reconstitution of the spinach oxygen-evolving complex with recombinant *Arabidopsis* manganese-stabilizing protein

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Abstract

The *psbO* gene of cyanobacteria, green algae and higher plants encodes the precursor of the 33 kDa manganese-stabilizing protein (MSP), a water-soluble subunit of photosystem II (PSII). Using a pET-T7 cloning/expression system, we have expressed in *Escherichia coli* a full-length cDNA clone of *psbO* from *Arabidopsis thaliana*. Upon induction, high levels of the precursor protein accumulated in cells grown with vigorous aeration. In cells grown under weak aeration, the mature protein accumulated upon induction. In cells grown with moderate aeration, the ratio of precursor to mature MSP decreased as the optical density at induction increased. Both forms of the protein accumulated as inclusion bodies from which the mature protein could be released under mildly denaturing conditions that did not release the precursor. Renatured *Arabidopsis* MSP was 87% as effective as isolated spinach MSP in restoring O_2 evolution activity to MSP-depleted PSII membranes from spinach; however, the heterologous protein binds to spinach PSIIs with about half the affinity of the native protein. We also report a correction to the previously published DNA sequence of *Arabidopsis psbO* (Ko *et al.*, Plant Mol Biol 14 (1990) 217–227).

Introduction

In chloroplasts, the O_2-evolving complex (OEC) of PSII protrudes from the thylakoid membrane into the interior, or lumenal, space. The OEC includes three water-soluble subunits, and some domains of as many as 6 membrane-bound subunits [for review see 11]. These polypeptides provide the structural framework for the essential cofactors Ca^{2+} and Cl^- and for the cluster of four Mn atoms that catalyzes water oxidation [for review see 7].

Upon extraction of the 23 and 17 kDa water-soluble subunits from the OEC, steady-state O_2 evolution activity is reduced to 25% [9]. However, 80% of the original activity can be restored by adding $CaCl_2$ [9]. Subsequent depletion of MSP, the most tightly bound extrinsic subunit, lowers the $CaCl_2$-dependent O_2 evolution activity to 16–31% that of untreated preparations [4]. During and after MSP extraction, 200 mM Cl^- is required in the suspending medium to stabilize the Mn cluster [24]; partial loss of Mn is unavoidable, however, and accounts in part for the

reduced activity of the MSP-depleted preparation [26]. Another factor contributing to the reduction in activity is slower turnover of the enzyme [26]. Other effects observed in the absence of MSP include weakened Ca^{2+} binding [4, 31], increased vulnerability to light (photoinhibition) [31], and loss of specific rebinding of the 23 and 17 kDa subunits [25].

Rebinding of MSP to the OEC reverses all kinetic and stability defects associated with its absence [14, 18, 23, 26, 29, 30, 36]. Parallel results have been obtained in genetic deletion/complementation studies of the *psbO* gene using the O_2-evolving cyanobacterium *Synechocystis* sp. PCC 6803 [5, 21, 31]. The consequences of biochemical and genetic manipulations of MSP on OEC function have been thoroughly studied and await more precise structural analysis for clarification.

Preliminary to directed mutagenesis of MSP, we have performed reconstitution studies of the spinach OEC using isolated spinach MSP and recombinant *Arabidopsis* MSP. Exchangeability of the heterologous protein with the native protein was expected based on the high degree of sequence conservation (84%). We report here that *Arabidopsis* MSP purified from bacterial inclusion bodies is nearly as effective as native spinach MSP in restoring O_2 evolution activity and also in protecting the spinach OEC against photoinhibition.

In addition to the expression and purification of *Arabidopsis* MSP, we report the selective accumulation of *Arabidopsis* MSP precursor (preMSP) in *E. coli*. Previous to the work reported here, a wheat preMSP cDNA was expressed in *E. coli* and, following induction, soluble mature MSP was found in the external periplasmic space [22]. However, because only the mature protein was detected in cell lysates, the authors suggested that accumulation of lumenal protein precursors may be problematic. We demonstrate here that accumulation of either preMSP or MSP, expressed from the same full-length *psbO* cDNA in *E. coli*, can be brought about by controlling growth conditions.

Materials and methods

Construction of expression vector and bacterial host used

The full-length cDNA clone of the *psbO* gene from *Arabidopsis thaliana* described by Ko *et al.* [17] and the pET expression system described by Studier *et al.* [34] were used for these studies. The *psbO* cDNA (a gift from Prof. Anthony Cashmore) was released from the cloning vector pGEM4 by cutting with the restriction endonucleases *Nco* I and *Bgl* II, and then spliced into a pET8c translation vector at the *Nco* I site (which occurs on the translation initiation codon of T7 phage gene 10) and the *Bam* HI site, resulting in the new construct, pET(*psbO*) (Fig. 1). The control region for expression from pET8c includes the promoter and 5′-untranslated sequence for gene 10, thus placing the target gene under the transcriptional control of inducible T7 RNA polymerase. The naturally occurring *Nco* I site of *Arabidopsis psbO* includes the translation initiation codon of this gene; thus the exact coding region of *psbO*, without addition or deletion, should be translated. The *Bgl* II/*Bam* HI fusion site of pET(*psbO*) begins 98 bp downstream of the *psbO* translation stop codon, and is followed by the T7 transcription terminator.

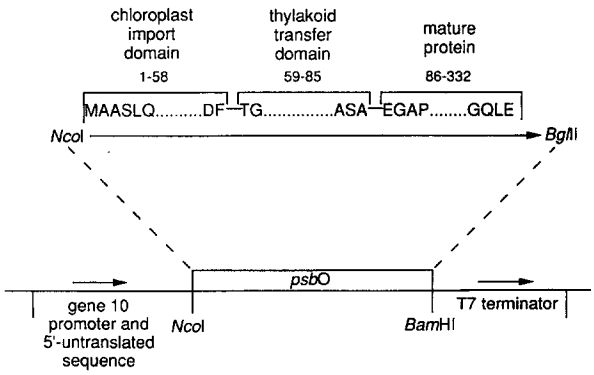

Fig. 1. Schematic representation of the expression vector, pET (*psbO*). The *Nco* I and *Bam* I sites of pET8c and flanking regulatory sequences are shown [34]. The three domains of the *psbO* gene from *Arabidopsis thaliana* were characterized by Ko and Cashmore [16]. Arrows indicate direction of transcription.

The bacterial host for expression was *E. coli* strain BL21(DE3)pLysS (F^- *ompT* $r_B^- mB^-$). Induction was achieved by addition of isopropyl-β-D-thiogalactopyranoside (IPTG; Boehringer Mannheim Biochemicals).

Growth and induction

Bacteria were grown in LB medium supplemented with 50 μg/ml ampicillin and 25 μg/ml chloramphenicol. Analytical cultures were grown in 125 ml Erlenmeyer flasks in a 37 °C water bath with rotary shaking. Growth was monitored optically at 600 nm (OD_{600}) in a Zeiss MQIII single beam spectrophotometer. The 1-cm optical cell was held 6 cm from the phototube. Samples (1 ml) were withdrawn from cultures for each OD measurement.

Three growth conditions were used for analytical induction experiments and are designated by the relative levels of aeration. *Weak aeration:* cultures were inoculated by a 20-fold dilution of an overnight culture to 40% flask volume and shaken at 100 rpm. *Moderate aeration*: cultures were inoculated by a 50-fold dilution of an overnight culture to 40% flask volume and shaken at 180 rpm. *Vigorous aeration*: cultures were inoculated by a 50-fold dilution of an overnight culture to 20% flask volume and shaken at 180 rpm.

For each growth condition described above, at least eight analytical cultures were inoculated at 5–10 min intervals with the same overnight culture. Growth of the first culture was monitored at 15–30 min intervals. When this culture approached OD_{600} 0.7–0.9, expression of preMSP in the other seven cultures was induced by addition of 0.4 mM IPTG. The OD_{600} of each culture was recorded at induction (OD_i) and then every 30–60 min.

Culture samples for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were collected by centrifugation (10500 × *g*, 4 °C) for 5 min, resuspended in a small volume of lysis buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA) and stored at −20 °C. Thawed cells were lysed by four rounds of freezing (1 min in dry ice/ethanol) and thawing (2 min at 2 °C). Lysates were incubated with 50 μg/ml DNase I type II (Sigma Chemical Co.) and 10 mM $MgCl_2$ for 20 min at room temperature.

Purification of inclusion bodies

Preparative cultures were grown and induced as described above for analytical cultures with the following modifications: cultures were grown in Fernbach flasks in an incubator with rotary shaking at rates indicated in the figure legends. Cells were collected 8–10 hours after induction by centrifugation (4000 × *g*, 4 °C) for 5 min, followed by resuspension in lysis buffer to 0.2 g wet weight cells per ml. All insoluble fractions containing precursor and/or mature MSP were homogenized before further treatment. Phenylmethylsulfonyl fluoride was added to 1 mM, and cells were stored at −60 °C.

The procedure for isolating and purifying inclusion bodies was designed as outlined by Marston [20] and by Claassen *et al.* [6]. Thawed cells were lysed by six cycles of sonication (30 s on/1–2 min off) on ice at ≈ 50 Watts (Branson Sonifier Cell Disruptor 185). Sample temperature did not exceed 18 °C during sonication. The lysate was treated with DNase as above for 30 min at room temperature. The volume after DNase addition is designated at the lysate volume. After centrifugation for 15 min (48000 × *g*, 4 °C), the lysate pellet was resuspended in 10 mM NaCl, 50 mM Tris (pH 8), 0.2% dodecyl-β-*D*-maltoside (DM) to one lysate volume and incubated on ice for 30 min. Inclusion bodies were then collected by centrifugation for 10 min (48000 × *g*, 4 °C) and resuspended in 20 mM bis-Tris-HCl (pH 6.4) to one-fifth of the lysate volume.

Solubilization of inclusion bodies and purification of Arabidopsis *MSP*

Inclusion bodies were diluted 10-fold and solubilized by incubation in a medium containing 2.6 M urea, 5 mM dithiothreitol (DTT), and

20 mM bis-Tris (pH 6.4) for 2 h at room temperature. Insoluble material was removed by centrifugation for 30 min (48 000 × *g*, 4 °C). *Arabidopsis* MSP was purified from the supernatant by anion-exchange chromatography. The flow rate was 1 ml/min using a Pharmacia Mono-Q HR 5/5 column (1 ml bed volume) equilibrated with 20 mM bis-Tris (pH 6.4) and 2.6 M urea. Adsorbed material was eluted with a 20 ml gradient from 0–200 mM NaCl; the peak eluting at ca. 95 mM NaCl was highly enriched in MSP. Chromatographic fractions of this peak were pooled and diluted in elution buffer to $A_{276} = 0.5$ (this absorbance value corresponds to a concentration of ca. 800 μg/ml for spinach MSP using the extinction coefficient of 16 $mmol^{-1}$ cm^{-1} [28, 38]). Urea and bis-Tris were removed from pooled Mono-Q fractions by overnight dialysis against 100 volumes of 20 mM MES-NaOH (pH 6) and 50 mM NaCl, followed by two four-hour dialysis steps against 100 volumes of the same buffer, and a final overnight dialysis step against 100 volumes of 0.4 M sucrose, 50 mM MES (pH 6), and 50 mM NaCl. Insoluble material was removed from the dialysis retentate by centrifugation (48 000 × *g*, 4 °C) for 30 min. The retentate supernatant contained pure MSP and was stored at −60 °C.

Preparation of spinach PSII membranes

Photosystem II subchloroplast membranes (PSII membranes) were isolated from market spinach according to the method of Berthold *et al.* [2] with three modifications [8, 9, 10]. Photosystem II membranes were stored in 0.4 M sucrose, 50 mM MES (pH 6), and 10 mM NaCl (SMN) at 3 mg chlorophyll (Chl) per ml and −60 °C. The 23 and 17 kDa polypeptides were removed from PSII membranes by exposure to 2 M NaCl as in Ghanotakis *et al.* [9]; salt-washed membranes were sedimented (48 000 × *g*, 4 °C) for 30 min and resuspended to the original volume in SMN. All membrane fractions were resuspended using a rubber policeman followed by homogenization.

MSP was removed from salt-washed membranes by exposure to 2.6 M urea at ca. 0.5 mg Chl/ml and in the presence of 200 mM NaCl [24]. Membranes were collected by centrifugation as above and washed once in 0.4 M sucrose, 50 mM MES (pH 6), and 200 mM NaCl (SMN-200) by resuspension to ca. 0.25 mg Chl/ml and recentrifugation. Urea-washed membranes were resuspended in SMN-200 to 2–3 mg Chl/ml. Control salt-washed membranes were treated and washed exactly the same as urea-washed membranes, except that urea was omitted. Suspensions (1 ml or less) were frozen by immersion in liquid N_2 and stored at −60 °C.

Purification of spinach MSP

Spinach MSP was isolated from the urea-wash supernatant without further treatment ('native') or following incubation in the presence of DTT. The urea-wash supernatant was prepared for chromatography by dialysis for at least five hours against 10 volumes of 20 mM bis-Tris (pH 6.4) and 5% (w/v) betaine, followed by a second identical dialysis step overnight. Half the dialysis retentate was centrifuged for 15 min (48 000 × *g*, 4 °C), and native spinach MSP was purified from the supernatant by FPLC anion-exchange chromatography as described above for recombinant MSP, except that urea was omitted from chromatography buffers.

To purify DTT-treated spinach MSP, the remaining dialysis retentate from the urea-wash supernatant was incubated for 2 h at room temperature in the presence of 2.6 M urea and 5 mM DTT. Following centrifugation (48 000 × *g*, 4 °C) for 30 min, DTT-treated spinach MSP was purified from the supernatant as described above for recombinant MSP.

Electrophoresis and immunoblotting

SDS-PAGE was performed according to the method of Piccioni *et al.* [32] using the Neville system; the resolving gel included 13.5% acryla-

mide and 4 M urea. Samples were incubated in 2.6 M urea, 2% SDS, 50 mM Na_2CO_3, and 60 mM DTT. Immunoblotting was performed using a Trans-Blot system (BioRad) following the manufacturer's instructions and standard procedures [13]. Proteins were blotted to nitrocellulose and MSP was labeled using rabbit antibody raised against spinach MSP. The polyclonal antisera against spinach MSP was a gift from Prof. Bridgette Barry and was prepared as described in Noren *et al.* [27]. The labeled blot was incubated with protein A-alkaline phosphatase conjugate (Sigma), and MSP was detected after addition of 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and nitroblue tetrazolium (sigma).

Determination of DNA and protein sequences

DNA was sequenced by the chain-termination method [33] using a Sequenase Version 2.0 kit (U.S. Biochemical) and [^{32}P]dATP (ICN Biomedicals). N-terminal protein sequence data was obtained by automated Edman degradation at the University of Michigan Protein and Carbohydrate Structure Core using an Applied Biosystems model 470 or 473 protein sequenator.

Reconstitution of the oxygen-evolving Complex

Urea-washed PSII membranes were incubated with increasing amounts of MSP for 30 min on ice under dim room light with occasional vortexing. The 400 μl rebinding mixture contained 0.4 M sucrose, 50 mM MES (pH 6), bovine serum albumin (BSA; fraction V, Sigma) at 100 μg/ml, 60 mM NaCl, 20 mM $CaCl_2$, 200 μg Chl/ml, and MSP. The concentrations of MSP stocks were determined by the Lowry assay [19] using BSA as a standard.

Measurement of O_2 evolution activity

Oxygen evolution activity was measured polarographically using a Clark-type O_2 electrode (YSI 4004, Yellow Springs Instrument) at 25 °C under saturating light. Tetramethylammonium cation (TMA^+) was substituted for Na^+ in the assay medium according to Waggoner *et al.* [37]. Oxygen evolution activity was assayed after a 20-fold dilution of the reconstitution mixture to a final volume of 1.5 ml; assay medium included 0.4 M sucrose, 50 mM MES-TMAOH, 100 μg BSA/ml, 60 mM TMACl, 20 mM $CaCl_2$, 600 μM 2,6-dichloro-*p*-benzoquinone, and 10 μg Chl/ml.

Results

Arabidopsis psbO *sequence correction*

We have partially sequenced the *Arabidopsis psbO* clone and identified two errors in the published sequence [17]: the two codons between residues 372–375 were reported as CAT-AGC, which specify His-Ser; the corrected sequence is CAG-TGC, which encodes Gln-Cys (the differences are underlined). This Gln residue is conserved among all five published plant MSP sequences, and the Cys residue is conserved among all ten published eukaryotic and prokaryotic MSP sequences.

Expression and identification of MSP

Precursor and mature MSP were initially identified in *E. coli* cell lysates based on their inducibility and apparent molecular masses as determined by SDS-PAGE analysis. Bacterial cultures treated with IPTG accumulated one or both of two proteins with apparent masses of 41 and 33 kDa (Fig. 2B), the expected sizes of precursor and mature *Arabidopsis thaliana* MSP [16]. The identity of preMSP and MSP in bacterial lysates was established with antibody raised against spinach MSP. The antibodies bound the two inducible proteins, and also revealed the presence of minor fragments of MSP (Fig. 2C). Under all conditions tested so far, precursor and mature MSP accumulate as insoluble aggregates or inclusion bodies (see below).

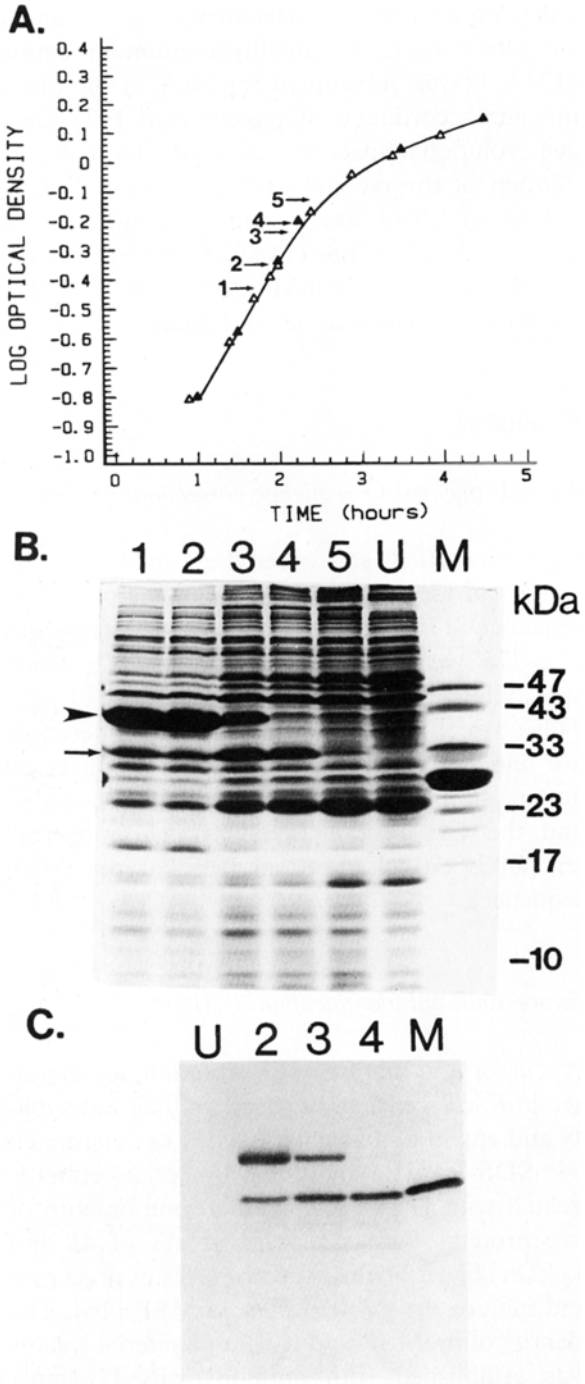

Fig. 2. Effect of cell density at induction on accumulation of precursor and mature MSP in *E. coli*. Analytical cultures were grown with moderate aeration (see 'Materials and methods'). Numbered cultures were induced at the following optical densities: 1, 0.37; 2, 0.45; 3, 0.58; 4, 0.61; 5, 0.74. U, uninduced culture; M, spinach PSII preparation containing MSP (33 kDa) included as molecular mass markers. A. Growth curves of duplicate uninduced cultures. Arrows indicate optical densities at induction for corresponding cultures analyzed in B and C. B. Polyacrylamide gel. Samples were collected 2 h after induction and normalized based on OD_{600} values. Total lysates of concentrated cells were loaded in each lane. Proteins were detected by staining with Coomassie Brilliant Blue R250. Arrowhead and arrow identify preMSP and MSP, respectively. C. Western blot. Samples were prepared as in B. Recombinant MSP was detected with polyclonal antibody against spinach MSP.

Amino-terminal sequencing of renatured MSP produced the sequence EGAP (Fig. 1). Thus the *E. coli* leader peptidase processed the plant preMSP in exactly the same site as predicted by Ko *et al.* [17] for the plant thylakoidal processing peptidase. This result was previously demonstrated *in vitro* using wheat preMSP as substrate for the thylakoidal and *E. coli* signal peptidases [12]. However, identical processing of preMSP in the *E. coli* system has not been previously demonstrated by N-terminal sequence determination.

Effect of optical density at induction on protein accumulation

Initial attempts to express MSP from the expression vector pET (*psbO*) resulted in accumulation of preMSP to high levels and only a trace of the mature protein. Growth conditions and OD_i values were then varied systematically in an attempt to increase the efficiency of MSP maturation. When cultures were grown with moderate aeration, the relative amounts of precursor and mature MSP that accumulated varied dramatically over a small range of OD_i values. Maximum accumulation of preMSP was observed in cultures induced during log phase (Fig. 2, cultures 1–2). In contrast, maximum accumulation of MSP was observed in cultures induced at the beginning of the transition to stationary phase (Fig. 2, cultures 3–4). Neither precursor nor mature MSP accumulated to high levels in cultures induced later (Fig. 2, culture 5). No differences were

apparent between samples taken two and five hours after induction.

These data reveal a narrow range of OD_i values (defined here as an 'induction window'), limited to very late log phase and the beginning of the transition tc stationary phase, where MSP accumulates to maximum levels without the concomitant accumulation of preMSP, as in culture 4 of Fig. 2. The OD_i values for cultures 3 and 5, therefore, are the lower and upper limits, respectively, for the induction window. Based on results from four repetitions of this experiment, the induction window for exclusive, high-level accumulation of MSP spans OD_i values of 0.55 to 0.7.

Effect of aeration level on protein accumulation

In order to identify growth conditions that favor maturation and high-level accumulation of MSP over a wider range of OD_i values than demonstrated above for cultures grown with moderate aeration, we varied three factors that influence growth: the inoculum dilution factor; the ratio of culture volume to flask volume; and the rate of shaking. The growth conditions designated weak aeration and vigorous aeration were optimized for the exclusive accumulation of mature or precursor MSP, respectively. Cultures grown with vigorous aeration accumulated high levels of preMSP, but very little MSP, across all OD_i values (Fig. 3, cultures 1–3). In contrast, cultures grown with weak aeration accumulated moderate to high levels of MSP, but only trace amounts of preMSP, across all OD_i values (Fig. 3, cultures 4–6). Again, no difference was observed between samples taken two and five hours after induction. Under all conditions tested in these experiments, maximum accumulation of preMSP is always much greater than that of MSP (Figs. 2 and 3).

When grown under weak aeration, cells accumulate mature protein to high levels over a wider range of OD_i values than observed for cells grown under moderate aeration. Thus, growth under weak aeration has the desired effect of widening the induction window for exclusive, high-level accumulation of MSP. Growth under vigorous aeration eliminates the induction window, at least within the range of OD_i values tested.

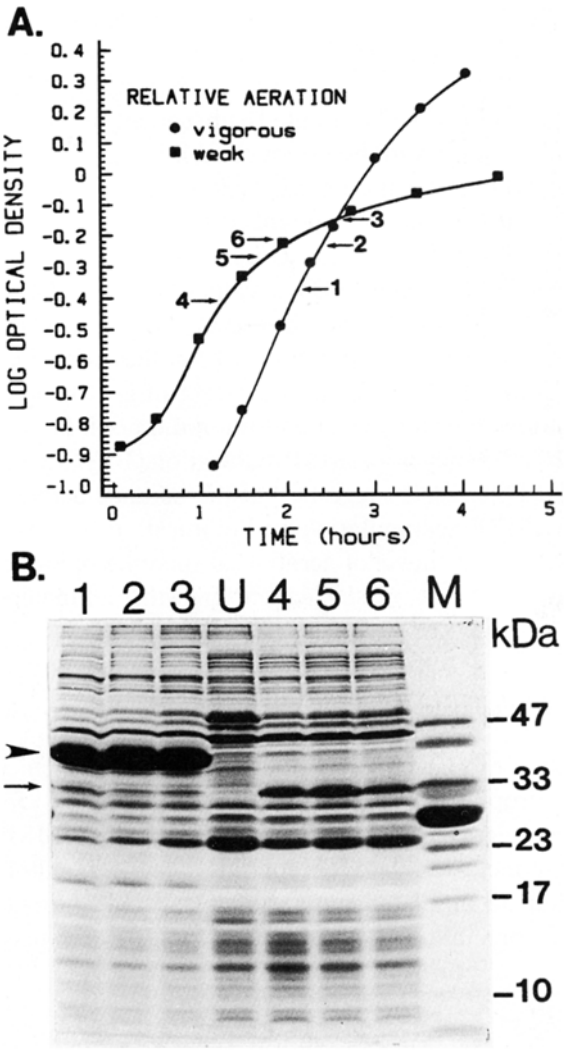

Fig. 3. Accumulation of either precursor or mature MSP in *E. coli* grown with vigorous or weak aeration, respectively. Cultures 1–3 were grown with vigorous aeration, and cultures 4–6 were grown with weak aeration (see 'Materials and methods' for description of growth conditions). Numbered cultures were induced at the following optical densities: 1, 0.42; 2, 0.57; 3, 0.71; 4, 0.39; 5, 0.54; 6, 0.61. A. Growth curves of uninduced cultures. Arrows indicate optical densities at which corresponding cultures analyzed in B were induced. B. Coomassie-stained polyacrylamide gel. Samples were prepared as in Fig. 2B. Arrowhead and arrow identify preMSP and MSP, respectively. U, uninduced culture; M, spinach PSII preparation containing MSP (33 kDa).

Growth inhibition resulting from IPTG addition does not influence preMSP maturation

Addition of IPTG limited subsequent growth of all cultures, and the extent of inhibition decreased as OD_i values increased. This inhibitory effect of IPTG was most pronounced for cultures grown with vigorous aeration (Fig. 4). Induction at the lowest and highest OD_{600} values limited cell density of the stationary phase to 40% and 80%, respectively, of that observed for the uninduced culture. Differences in the extent of growth inhibition caused by IPTG addition did not influence the efficiency of preMSP maturation (Figs. 3B and 4). This result suggests that the balance between preMSP accumulation and maturation is dependent on the level of aeration at the time of induction, but not on subsequent growth parameters.

Characterization of inclusion bodies

Urea was used to characterize the extent to which hydrophobic interactions are responsible for the aggregation of precursor and mature MSP. An inclusion body preparation from a culture that accumulated approximately equal amounts of precursor and mature MSP was used

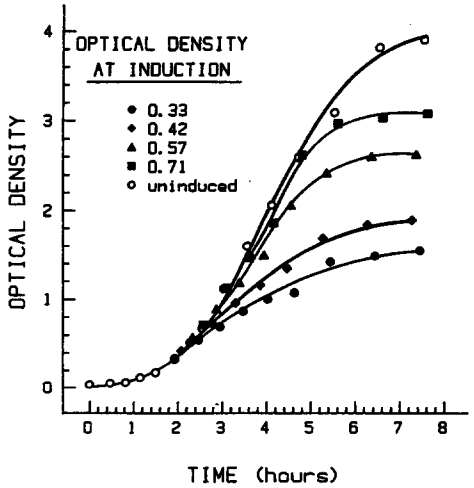

Fig. 4. Effect of IPTG addition on growth of vigorously aerated cultures. See text for description of growth conditions and Fig. 3 for electrophoretic profiles.

(Fig. 5A, IBs). Incubation of inclusion bodies in 2 M urea partially solubilized MSP, and this process was facilitated by DTT (Fig. 5A, lanes 3–4). Incubation in 4 M urea was required for partial solubilization of preMSP; DTT also facilitated this solubilization process (Fig. 5A, lanes 5–6). However, complete solubilization of mature and precursor MSP did not require DTT if inclusion bodies were incubated in 4 M and 6 M urea, respectively (Fig. 5A, lanes 5–8). These data demonstrate that the hydrophobic interactions that aggregate preMSP are stronger than those present in inclusion bodies comprised of MSP.

In order to determine the extent of intermolecular disulfide bond formation in inclusion bodies, we analyzed these preparations by SDS-PAGE under reducing and non-reducing conditions. When inclusion bodies containing mature protein were solubilized in the absence of a reducing agent, at least four new bands were detected on a western blot (Fig. 5B, lane 3). These bands represent dimeric (60 kDa) and oligomeric (155 kDa and larger) forms of MSP. Aggregates of preMSP were also detected by western blot analysis of non-reduced samples, represented by bands at 118 kDa and larger (Fig. 5B, lane 4). These bands persist in a reduced sample (Fig. 5B, lane 2). This result suggests that our conditions for SDS-PAGE sample preparation were not sufficient to achieve complete dissociation of preMSP aggregates. Oligomerization of preMSP therefore is not necessarily due to disulfide bond formation.

A significant fraction of both inclusion body preparations consists of monomeric precursor or mature MSP (Fig. 5B). Monomeric preMSP appears to migrate slightly faster under non-reducing conditions than under reducing conditions (Fig. 5B, lane 2 vs. lane 4). This mobility shift might result from increased compactness of preMSP due to the presence of intramolecular disulfide bonds.

Recovery of MSP from inclusion bodies

The insoluble fraction of the cell lysate is highly enriched in MSP (Fig. 6, lane 2). A significant

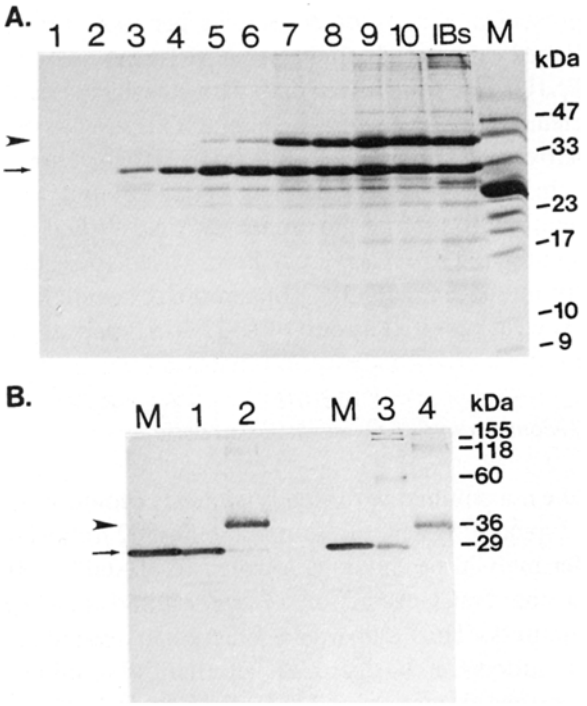

Fig. 5. Characterization of inclusion bodies. A. Differential solubilization of precursor and mature MSP from inclusion bodies. A preparative culture was grown as described in 'Materials and methods' with shaking at 140 rpm and induced at $OD_{600} = 0.67$. Inclusion bodies were incubated for 2 h at room temperature in 50 mM MES (pH 6) containing 0–8 M urea plus or minus 5 mM DTT, followed by centrifugation (10000 × *g*, 4 °C) for 30 min. Supernatants were fractionated by SDS-PAGE and stained with Coomassie Blue. Lanes 1–2, no urea; lanes 3–4, 2 M urea; lanes 5–6, 4 M urea; lanes 7–8, 6 M urea; lanes 9–10, 8 M urea. Odd-numbered lanes, no DTT present during solubilization; even-numbered lanes, DTT present during solubilization. IBs, inclusion bodies before centrifugation; M, spinach PSII preparation containing MSP (33 kDa). Arrowhead and arrow identify preMSP and MSP, respectively. B. Western blot analysis of inclusion body preparations solubilized under reducing or non-reducing conditions. A preparative culture expressing MSP was grown as described in 'Materials and methods', with shaking at 120 rpm, and induced at $OD_{600} = 0.6$. A preparative culture expressing preMSP was grown with shaking at 160 rpm and induced at $OD_{600} = 0.7$. Samples for electrophoresis were prepared by incubation of inclusion bodies for 30 min at 37 °C in 2.6 M urea, 2% SDS, 50 mM Na_2CO_3 and 0.02% bromophenol blue plus or minus 60 mM DTT. Precursor and mature MSP were detected using antibody against spinach MSP as in Fig. 2C. M, spinach PSII preparation. Molecular masses were estimated from Coomassie-stained gels (high-MW standards, Bethesda Research Laboratories, Gaithersburg, MD) (data not shown). Lane 1: inclusion bodies containing mature MSP incubated with DTT; lane 2: inclusion bodies containing preMSP incubated with DTT; lane 3: same as lane 1, but minus DTT; lane 4: same as lane 2, but minus DTT.

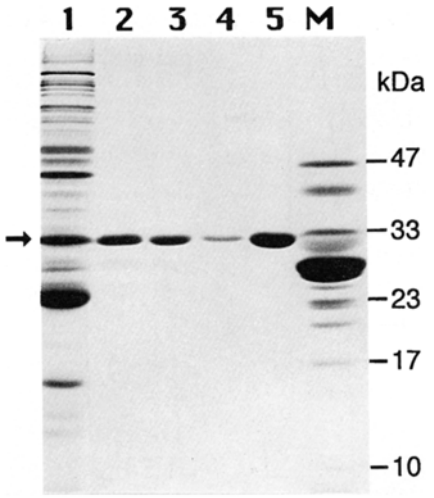

Fig. 6. Purification and renaturation of recombinant *Arabidopsis* MSP. Preparative cultures were grown with shaking at 120 rpm and induced between $OD_{600} = 0.45–0.55$. Concentrated cells from three cultures were pooled and lysed by sonication (see 'Materials and methods'). Arrow identifies MSP. Lanes 1–4 were normalized based on the original lysate volume. Lane 1, cell lysate; lane 2, lysate pellet; lane 3, detergent-washed lysate pellet (inclusion bodies); lane 4, chromatographically purified MSP; lane 5, overloaded sample of renatured MSP. M, spinach PSII membranes.

fraction of MSP in inclusion bodies is lost during chromatography (Fig. 6, compare lanes 3 and 4). This loss is caused at least in part by the formation of aggregates of MSP, as demonstrated by the improved yields that are obtained when 5% betaine (w/v) is included in the solubilization mixture (data not shown). A single chromatographic step purifies the protein to homogeneity (Fig. 6, lane 5). Renatured MSP is routinely recovered in yields of 5–15 mg per liter of culture.

Relative binding affinities of spinach and Arabidopsis *MSP*

The three MSP preparations differed significantly in binding affinity (Fig. 7B). Native spinach MSP restored maximum O_2 yield (see below) when

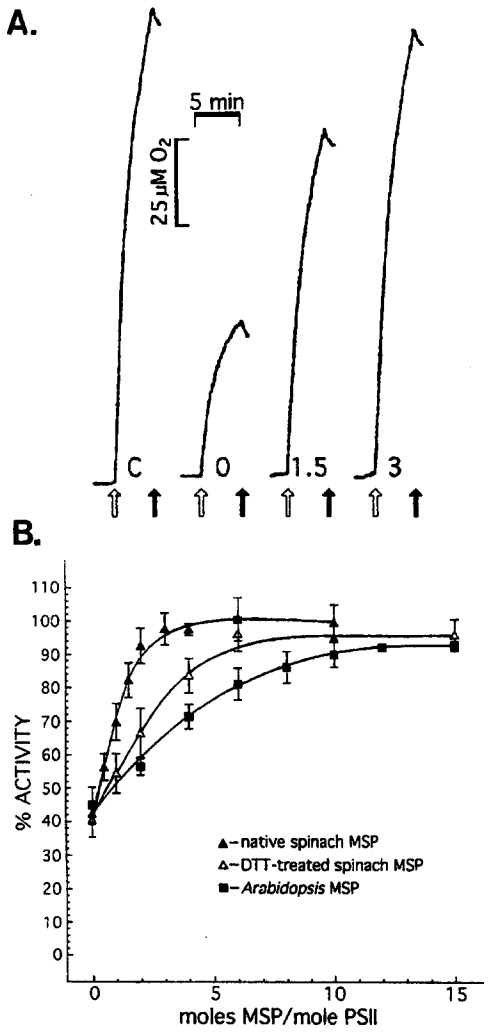

Fig. 7. Reconstitution of the spinach OEC. The procedure for rebinding MSP to urea-washed PSII membranes is described in 'Materials and methods'. The PSII concentration was predicted based on stoichiometries of 250 Chl/PSII [2]. A. Representative assays of long-term stability of O_2 evolution activity. C, control salt-washed PSII membranes; 0, 1.5, and 3 indicate the amount of native spinach MSP (mol MSP/mol RC) added to urea-washed PSII membranes in reconstitution mixtures. B. Determination of relative binding affinities of spinach and *Arabidopsis* MSP. Reconstitution data were obtained for duplicate preparations of native spinach MSP, DTT-treated spinach MSP, and *Arabidopsis* MSP using the same two PSII preparations, for a total of 12 individual reconstitution or binding curves. Each curve represents the average of 4 replicate curves which were generated by averaging 2–3 assays for each point. 100% activity corresponds to the average maximum O_2 yield of a specific urea-washed PSII preparation reconstituted with native spinach MSP. Error bars indicate ± 1 standard error.

present at a ratio of 2–3 moles per mole OEC. Based on a stoichiometry of 2 MSP per spinach PSII [38], this result demonstrates highly efficient reconstitution of the spinach OEC with the native subunit. Twice as much DTT-treated spinach MSP (4–6 per OEC) as native subunit was required to restore maximum O_2 yield. When recombinant *Arabidopsis* MSP was used to reconstitute the spinach OEC, maximum restoration of O_2 yield required a ratio of 8–12 MSP per OEC.

Reconstitution of O_2 evolution activity

The maximum rate of steady-state O_2 evolution of a typical PSII preparation (ca. 600–800 μmol O_2 per mg Chl per hour) is lowered to 50–60% following NaCl extraction of the 23 and 17 kDa subunits. This 'salt-washed' activity, dependent on added $CaCl_2$, decreases another 50% following urea extraction of MSP. Because we are interested specifically in the effects of MSP removal and rebinding on O_2 evolution activity, salt-washed PSII activities are designated as the control. Native and DTT-treated spinach MSP preparations raised the rate of steady-state O_2 evolution of urea-washed PSII membranes from 48% to 82–83% of control rates (Table 1). Recombinant *Arabidopsis* MSP was only slightly less effective.

The O_2 evolution activity of urea-washed PSII membranes is extensively damaged after 4–5 min under saturating light, while salt-washed samples and urea-washed samples reconstituted with MSP continue to evolve O_2 (Fig. 7A). The total O_2 yield of a PSII sample exposed to prolonged illumination thus depends not only on the rate of steady-state O_2 evolution, but also on the stability of the steady-state activity; instability of this activity increases with the susceptibility of the sample to photoinhibition. The O_2 evolution activity of urea-washed samples decreased from 48% of control levels in the rate assay to 34% in the O_2 yield assay (Table 1). Urea-washed samples reconstituted with spinach or *Arabidopsis* MSP are relatively stable under prolonged illumination, as demonstrated by corresponding

Table 1. Steady-state O_2 evolution activity of reconstitution mixtures.

Preparation	Addition	Steady-state activity[1] (%)	
		rate[2]	O_2 yield[3]
Salt-washed	none	100	100
Urea-washed	none	48	34
	native spinach MSP	82	81
	DTT-treated spinach MSP	83	78
	Arabidopsis MSP	78	75

[1] Assay conditions and medium were the same as in Fig. 7. The activity values of reconstituted samples were determined from the plateaus of binding curves (% salt-washed activity vs. mol MSP/mol PSII).

[2] Rate of steady state O_2 evolution. 100% = 388 ± 29 μmol O_2 per mg Chl per hour and 403 ± 69 μmol O_2 per mg Chl per hour for the 2 salt-washed preparations used as controls in this study.

[3] Total O_2 evolved during 4 min under saturating light. 100% = 12.9 ± 0.6 μmol O_2 per mg Chl and 13.6 ± 0.8 μmol O_2 per mg Chl for the 2 control salt-washed preparations. These values correspond to constant steady-state rates of 194 and 204 μmol O_2 per mg Chl per hour, respectively.

decreases of 5% and 3%, respectively, for samples reconstituted with DTT-treated spinach MSP and Arabidopsis MSP.

Discussion

Effects of optical density at induction and aeration level on yield and maturation of recombinant protein

Our experiments reveal that cell density and growth conditions regulate yield and maturation of inducible recombinant protein; excellent yields of precursor or mature MSP can be obtained from cells carrying pET(*psbO*) simply by adjusting culture conditions. Here, we have demonstrated a positive correlation between aeration and preMSP synthesis, but a negative correlation between aeration and preMSP maturation. Cells grown with vigorous aeration and induced with 0.4 mM IPTG accumulate preMSP to very high levels, but are deficient in their ability to process the protein. In contrast, cells grown with weak aeration and induced with 0.4 mM IPTG accumulate lower levels of recombinant protein, but process preMSP efficiently.

In cells grown with moderate aeration, induction below or above a critical OD_{600} value leads to maximum accumulation of preMSP or MSP, respectively. Limits for this threshold OD_i value are defined by the induction window (see 'Results'). We suggest that the threshold OD_i value represents the cell density at which moderately aerated cultures become O_2 limited to the same extent as weakly aerated cultures.

The simplest explanation for the effect of aeration level on the yield of recombinant protein is that IPTG uptake by weakly and moderately aerated cells is proportional to the concentration of dissolved O_2 and therefore to the respiration rate. *E. coli* lactose permease imports galactosides and exports protons in a process driven by the transmembrane proton gradient [15]. Permease activity is thus dependent on respiration to maintain the proton motive force; as a result, weakly aerated cells do not accumulate IPTG to the same extent as vigorously aerated cells.

We have tested the effect of varying the IPTG concentration used to induce cells grown with vigorous aeration. The effect of decreasing the IPTG concentration is identical to that of increasing the OD_i value for cultures grown with moderate aeration. Concentrations of IPTG that induce maximum, exclusive accumulation of MSP fall between 10 and 30 μM (data not shown). The only difference between the effect of varying the OD_i value at constant IPTG concentration and that of IPTG concentration itself is that recombinant protein accumulated much more slowly in the latter and continued to accumulate after two hours. In similar experiments, Bowden and Georgiou [3] showed that the yield of recombinant β-lactamase in *E. coli* was proportional to the concentration of IPTG added to induce expression of pre-β-lactamase. In that study, aggregation of pre-β-lactamase was first detected between IPTG concentrations of 5 and 50 μM, within the range where preMSP aggregation is first observed. Our results indicate that cells grown with

moderate aeration, when treated with 0.4 mM IPTG within the induction window, accumulate the inducer to concentrations between 10 and 30 μM. Finally, we have yet to achieve the same high yields of MSP (on an OD_{600} basis) at low IPTG concentrations as we have at 0.4 mM IPTG in cells grown with weak or moderate aeration. Further experiments are necessary to determine if limited aeration facilitates processing of preMSP. It should be noted that the specific conditions for the maximal expression of processed proteins may vary somewhat from the conditions found here when the copy number of the plasmid used or the strength of the promoter, among other things, vary from that in the pET-T7 system.

Functional analysis of spinach and Arabidopsis *MSP*

Reconstitution of the spinach OEC with native or DTT-treated spinach MSP restored 67% (100 × 35/52; see Table 1) of the steady-state rate of O_2 evolution lost following MSP extraction. Incomplete recovery of control activity results in part from irreversible loss of Mn in a fraction of PSII complexes [26]; another factor contributing to limited recovery may be an irreversible alteration in electron transfer kinetics on the acceptor side of PSII [30]. However, the equal effectiveness of the two spinach MSP preparations demonstrates complete recovery of MSP function following DTT treatment. *Arabidopsis* MSP restored 58% (100 × 30/52; see Table 1) of the steady-state O_2 evolution lost following MSP extraction. We conclude that the heterologous subunit is 87% (100 × 58/67) as effective as the native subunit in restoring O_2 evolution activity to the spinach OEC.

Extraction of MSP causes not only a reduction in the rate of O_2 evolution, but also increases the susceptibility of PSII to photoinhibition. Photoinhibition of PSII is believed to result in part from irreversible photooxidative damage to the quinone-binding D1 subunit, as a result of delayed reduction of the oxidized reaction center Chl $P680^+$ [for review see 1]. The slower turnover of the O_2-evolving enzyme observed following MSP extraction [26] may result in delayed reduction of $P680^+$, thus promoting photoinhibition. Comparisons of O_2 yields allows for a more inclusive analysis of MSP function than comparisons of rate values alone. Our results indicate that recombinant *Arabidopsis* MSP, though slightly less effective at restoring control rates of O_2 evolution activity, is as effective as spinach MSP in stabilizing steady-state O_2 evolution.

The most significant effect of either the urea/DTT treatment or of reconstitution with heterologous MSP was weakened binding of the subunit to the OEC. Two strictly conserved cysteine residues occur near the N-terminus of MSP; on the basis of experiments with spinach MSP, these two cysteines are proposed to form a disulfide bridge that is required for binding of the protein to PSII [35]. Exposure of native spinach MSP to DTT in the presence of urea, followed by sequential removal of the reductant and denaturant caused an estimated 50% reduction in binding affinity. This result, together with the observation that maximum activity is unchanged (steady-state rate) or only slightly reduced (O_2 yield), suggests that our renaturation procedure recovers only ca. 50% of the protein in a conformation capable of functional rebinding to the OEC. Spinach OECs reconstituted with recombinant *Arabidopsis* MSP reach maximum levels of O_2 yield at a ratio of MSP to PSII about twice that observed for DTT-treated spinach MSP. We conclude that *Arabidopsis* MSP binds to the spinach OEC with an affinity about half that of spinach MSP. Our data are consistent with the interpretation that structural features of the MSP protein that are essential for O_2 evolution activity have been conserved to a greater degree than those involved in binding to PSII.

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References

1. Aro E-M, Virgin I, Andersson B: Photoinhibition of photosystem II. Inactivation, protein damage and turnover. Biochim Biophys Acta 1143: 113–134 (1993).
2. Berthold DA, Babcock GT, Yocum CF: A highly resolved, oxygen-evolving photosystem II preparation from spinach thylakoid membranes: EPR and electron-transport properties. FEBS Lett 134: 231–234 (1981).
3. Bowden GA, Georgiou G: Folding and aggregation of β-lactamase in the periplasmic space of *Escherichia coli*. J Biol Chem 265: 16760–16766 (1990).
4. Bricker TM: Oxygen evolution in the absence of the 33-kilodalton manganese-stabilizing protein. Biochemistry 31: 4623–4628 (1992).
5. Burnap R, Shen J-R, Jursinic PA, Inoue Y, Sherman LA: Oxygen yield and thermoluminescence characteristics of a cyanobacterium lacking the manganese-stabilizing protein of photosystem II. Biochemistry 31: 7404–7410 (1992).
6. Claassen LA, Ahn B, Koo H-S, Grossman L: Construction of deletion mutants of the *Escherichia coli* UvrA protein and their purification from inclusion bodies. J Biol Chem 266: 11380–11387 (1991).
7. Debus RJ: The manganese and calcium ions of photosynthetic oxygen evolution. Biochim Biophys Acta 1102: 269–352 (1992).
8. Ghanotakis DF, Babcock GT: Hydroxylamine as an inhibitor between Z and P680 in photosystem II. FEBS Lett 153: 231–234 (1983).
9. Ghanotakis DF, Babcock GT, Yocum CF: Calcium reconstitutes high rates of oxygen evolution in polypeptide depleted photosystem II preparations. FEBS Lett 167: 127–130 (1984).
10. Ghanotakis DF, Babcock GT, Yocum CF: Structural and catalytic properties of the oxygen-evolving complex: correlation of polypeptide and manganese release with the behavior of Z in chloroplasts and a highly resolved preparation of the PSII complex. Biochim Biophys Acta 765: 388–398 (1984).
11. Ghanotakis DF, Yocum CF: Photosystem II and the oxygen-evolving complex. Annu Rev Plant Physiol Plant Mol Biol 41: 255–276 (1990).
12. Halpin C, Elderfield PD, James HE, Zimmermann R, Dunbar B, Robinson C: The reaction specificities of the thylakoidal processing peptidase and *Escherichia coli* leader peptidase are identical. EMBO J 8: 3917–3921 (1989).
13. Harlow E, Lane D: Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988).
14. Imaoka A, Yanagi M, Akabori K, Toyoshima Y: Reconstitution of photosynthetic charge accumulation and oxygen evolution in $CaCl_2$-treated PSII particles. FEBS Lett 176: 341–345 (1984).
15. Kaback HR: Active transport in *Escherichia coli*: passage to permease. Annu Rev Biophys Chem 15: 279–319 (1986).
16. Ko K, Cashmore AR: Targeting of proteins to the thylakoid lumen by the bipartite transit peptide of the 33 kd oxygen-evolving protein. EMBO J 9: 3187–3194 (1989).
17. Ko K, Granell A, Bennett J, Cashmore AR: Isolation and characterization of cDNAs from *Lycopersicon esculentum* and *Arabidopsis thaliana* encoding the 33 kDa protein of the photosystem II-associated oxygen-evolving complex. Plant Mol Biol 14: 217–227 (1990).
18. Kuwabara T, Miyao M, Murata T, Murata N: The function of 33-kDa protein in the photosynthetic oxygen-evolution system studied by reconstitution experiments. Biochim Biophys Acta 806: 283–289 (1985).
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RL: Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275 (1951).
20. Marston FAO: The purification of eukaryotic polypeptides expressed in *Escherichia coli*. In: Glover DM (ed) DNA Cloning: A Practical Approach, Vol. 1, pp 59–87. IRL Press, Oxford (1984).
21. Mayes SR, Cook KM, Self SJ, Zhang Z, Barber J: Deletion of the gene encoding the photosystem II 33 kDa protein from *Synechocystis* PCC 6803 does not inactivate water-splitting but increases vulnerability to photoinhibition. Biochim Biophys Acta 1060: 1–12 (1991).
22. Meadows JW, Robinson C: The full precursor of the 33 kDa oxygenevolving complex protein of wheat is exported by *Escherichia coli* and processed to the mature size. Plant Mol Biol 17: 1241–1243 (1991).
23. Miyao M, Murata N: Partial reconstitution of the photosynthetic oxygen evolution system by rebinding of the 33-kDa polypeptide. FEBS Lett 164: 375–378 (1983).
24. Miyao M, Murata N: Role of the 33-kDa polypeptide in preserving Mn in the photosynthetic oxygen-evolution system and its replacement by chloride ions. FEBS Lett 170: 350–354 (1984).
25. Miyao M, Murata N: The mode of binding of three extrinsic proteins of 33 kDa, 23 kDa and 18 kDa in the photosystem II complex of spinach. Biochim Biophys Acta 977: 315–321 (1989).
26. Miyao M, Murata N, Lavorel J, Maison-Peteri B, Boussac A, Etienne A-L: Effect of the 33-kDa protein on the S-state transitions in photosynthetic oxygen evolution. Biochim Biophys Acta 890: 151–159 (1987).
27. Noren GH, Boerner RJ, Barry BA: EPR characterization of an oxygen-evolving photosystem II preparation from transformable cyanobacterium *Synechocystis* 6803. Biochemistry 30: 3943–3950 (1991).
28. Oh-oka H, Tanaka S, Wada K, Kuwabara T, Murata N:

Complete amino acid sequence of 33 kDa protein isolated from spinach photosystem II particles. FEBS Lett 197: 63–66 (1986).
29. Ono T-A, Inoue Y: Reconstitution of photosynthetic oxygen evolving activity by rebinding of 33 kDa protein to $CaCl_2$-extracted PS II particles. FEBS Lett 166: 381–384 (1984).
30. Ono T-A, Inoue Y: Effects of removal and reconstitution of the extrinsic 33, 24 and 16 kDa proteins on flash oxygen yield in photosystem II particles. Biochim Biophys Acta 850: 380–389 (1986).
31. Philbrick JB, Diner BA, Zilinskas BA: Construction and characterization of cyanobacterial mutants lacking the manganesestabilizing polypeptide of photosystem II. J Biol Chem 266: 13370–13376 (1991).
32. Piccioni R, Bellemare G, Chua N-H: Methods of polyacrylamide gel electrophoresis in the analysis and preparation of plant polypeptides. In: Edelman M, Hallick RB, Chua N-H (eds) Methods in Chloroplast Molecular Biology, pp. 985–1014. Elsevier Biomedical Press, Amsterdam (1982).
33. Sanger R, Nicklen S, Coulsen AR: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74, 5463–5467 (1977).
34. Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW: Use of T7 RNA polymerase to direct expression of cloned genes. Meth Enzymol 185: 60–89 (1990).
35. Tanaka S, Wada K: The status of cysteine residues in the extrinsic 33 kDa protein of spinach photosystem II complexes. Photosyn Res 17: 255–266 (1988).
36. Tang X-S, Satoh K: Reconstitution of photosynthetic water-splitting activity by the addition of 33 kDa polypeptide to urea-treated PS II reaction center complex. FEBS Lett 201: 221–224 (1986).
37. Waggoner CM, Pecoraro V, Yocum CF: Monovalent cations (Na^+, K^+, Cs^+) inhibit calcium activation of photosynthetic oxygen evolution. FEBS Lett 244: 237–240 (1989).
38. Xu Q, Bricker TM: Structural organization of proteins on the oxidizing side of photosystem II: two molecules of the 33-kDa manganese-stabilizing proteins per reaction center. J Biol Chem 267: 25816–25821 (1992).