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Functional reconstitution of photosystem II with recombinant manganese-stabilizing proteins containing mutations that remove the disulfide bridge

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Abstract

The 33-kDa extrinsic subunit of PSII stabilizes the O_2-evolving tetranuclear Mn cluster and accelerates O_2 evolution. We have used site-directed mutagenesis to replace one or both Cys residues in spinach MSP with Ala. Previous experiments using native and reduced MSP led to the conclusion that a disulfide bridge between these two cysteines is essential both for its binding and its functional properties. We report here that the disulfide bridge, though essential for MSP stability, is otherwise dispensible. The mutation C51A by itself had a delayed effect on MSP function: [C51A]MSP restored normal rates of O_2 evolution to PSII but was defective in stabilizing this activity during extended illumination. In contrast, the Cys-free double mutant, [C28A,C51A]MSP, was functionally identical to the wild-type protein. Based on results presented here, we propose a light-dependent interaction between MSP and PSII that occurs only during the redox cycling of the Mn cluster and which is destabilized by the single mutation, C51A.

Keywords: Assembly; Cysteine; psbO; Site-directed mutagenesis; Size-exclusion chromatography; Protein, 33 kDa

1. Introduction

Photosystem II (PSII) oxidizes water in a reaction activated by Ca^{2+} and Cl^- and catalyzed by a cluster of 4 Mn atoms. Three extrinsic subunits shield these cofactors from bulk solvent and regulate their binding to intrinsic PSII subunits. The active site of water oxidation and the three extrinsic subunits together constitute the O_2-evolving complex (OEC) of PSII. The 33-kDa manganese-stabilizing protein (MSP) is the largest and most tightly bound of the OEC polypeptides. Following extraction of MSP, two of four Mn atoms are released from the OEC, and as a result O_2 evolution activity is lost [1]. Denaturation of the Mn cluster is prevented in MSP-depleted PSII when elevated Cl^- levels (> 100 mM) are included in the suspending medium [1,2]. If Ca^{2+} is also added, MSP-depleted PSII evolves O_2 at 24% of the rate measured for intact PSII membranes that retain all three extrinsic proteins [3]. Genetic deletion of *psbO*, the gene that encodes MSP, from the cyanobacterium *Synechocystis* sp. PCC6803 yields a similar result in vivo: cells lacking MSP evolve O_2 at a rate 36% that measured for wild-type cells [4].

Biochemical and genetic studies indicate that a disulfide bridge, proposed to exist in vivo between the only two cysteines in spinach MSP, is required for folding, assembly, and function of the protein. These two cysteines are conserved in MSP from all 8 eukaryotic species and all 4

Abbreviations: Bis-tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; BSA, bovine serum albumin; Chl, chlorophyll; cyt, cytochrome; DCBQ, 2,6-dichloro-*p*-benzoquinone; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid disodium salt; IPTG, isopropyl-β-D-thiogalactopyranoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; MSP, manganese-stabilizing protein; OD, optical density; OEC, O_2-evolving complex; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; pET, plasmid for expression with T7 RNA polymerase; preMSP, MSP precursor protein encoded by *psbO*; *psbO*, cDNA clone encoding preMSP; PMSF, phenylmethylsulfonyl fluoride; PS, photosystem; SDS, sodium dodecyl sulfate; TMA^+, tetramethylammonium; Tris, Tris(hydroxymethyl)aminomethane.

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prokaryotic species for which sequence information is available. Chemical reduction of the disulfide bridge in purified spinach MSP, with either DTT or mercaptoethanol, caused denaturation of the protein to occur [5,6]. Starting with fully reduced, denatured spinach MSP, Tanaka and Wada [5] regenerated the disulfide bridge in vitro and observed a correlation between binding of the protein to PSII and the disappearance of free sulfhydryl groups. The disulfide in MSP was also reported to be essential for O_2 evolution when similar disulfide reduction/reoxidation experiments were performed in situ, using a PSII membrane preparation [7]. Finally, substitution of one of the two homologous Cys residues in *Synechocystis* MSP with Ser (C20S) blocked cellular accumulation of MSP [8], a result consistent with the in vitro disulfide bond reduction experiments using spinach MSP.

We have employed site-directed mutagenesis to eliminate the -S-S- bridge in recombinant spinach MSP as a way to assess the role, if any, of this structural feature in the folding, assembly, and function of the subunit using an in vitro PSII reconstitution assay. We reasoned that the structural defect observed for reduced native MSP may result from the inability of the native conformation to accommodate two sulfhydryl groups in the position normally occupied by the two sulfur atoms of the disulfide bridge. Substitution of Ser for Cys, to replace sulfhydryl groups with hydroxyl groups, may be structurally incompatible as well. Therefore, we replaced Cys with Ala so as to either minimize or eliminate the possibility of steric interference in the folded protein. Here we demonstrate that the double mutant, [C28A,C51A]MSP, assembles into PSII and functions normally. The single mutant, [C51A]MSP, also assembles into PSII, but with impaired function.

2. Materials and methods

2.1. PSII preparation and extractions

Photosystem II membranes were purified from market spinach according to Berthold et al. [9] with modifications [10–12]. Extrinsic proteins were extracted sequentially as described previously [13]. The 23- and 17-kDa proteins were extracted by incubation of PSII membranes in 2 M NaCl/1 mM EDTA. To specifically release MSP, 'salt-washed' PSII membranes were incubated in 2.6 M urea/200 mM NaCl to produce 'urea-washed' PSII membranes that lack all three extrinsic proteins.

2.2. Mutagenesis and expression of psbO

Spinach *psbO* was amplified by PCR from a spinach cDNA library (gift of W. Gruissem and J. Norita) using primers designed based on the nucleotide sequence [14]. The PCR product was cloned into the TA cloning vector, pCRII (Invitrogen), and then subcloned into the phagemid, pBluescript SK + . This construct was called pBS(*psbO*) and was used to transform *E. coli* strain CJ236, which incorporates uracil instead of thymine into DNA. Site-directed mutations were introduced into *psbO* using the method of Kunkel [15]. Second-strand synthesis, using circular single-stranded pBS(*psbO*) DNA, was primed with mutagenic oligonucleotides. The codons encoding Cys28 and Cys51 were changed from TGC to GCC, which encodes Ala. The products of synthesis were used to transform *E. coli* strain XL1. Transformants were screened by dideoxy DNA sequencing [16]. Clones encoding the single and double MSP mutants, [C51A]MSP and [C28A,C51A]MSP were cloned into the expression vector pET8c [17].

Expression of *psbO* was induced by addition of 20–30 μM IPTG to exponential phase cultures of *E. coli* strain BL21(DE3)pLysS transformed with pET(*psbO*). Cells were grown in Fernbach flasks in 1-liter of LB medium (50 μg ampicillin/ml, 25 μg chloramphenicol/ml), and harvested 3–5 h after induction. Pelleted cells were resuspended in 50 mM Tris (pH 8), 100 mM NaCl, 1 mM EDTA, 1 mM PMSF and stored at −70°C [13].

2.3. Purification of MSP and size-exclusion chromatography

As observed previously for *Arabidopsis* preMSP [13], the transit peptide of spinach preMSP was correctly processed in *E. coli*, and the mature protein accumulated as inclusion bodies. Cells were lysed by sonication and inclusion bodies were purified as described in Ref. [13]. Total inclusion bodies isolated from each 1-liter cell culture were suspended to a final volume of 50 ml in solubilization/chromatography medium (3 M urea, 5% betaine (w/v), 10 mM NaCl, 20 mM Bis-Tris (pH 6.4)) for 2 h at room temperature. Following centrifugation (48 000 × *g*, 4°C, 30 min), MSP was purified from the supernatant fraction by anion-exchange chromatography with 3 M urea present in chromatography buffers [13]. The single mutant, [C51A]MSP, was not treated further. The double mutant, [C28A,C51A]MSP, was renatured by dialysis against 50 mM MES (pH 6), 100 mM NaCl. Recombinant wild-type MSP used for control reconstitutions was purified as above by chromatography in 3 M urea, and was then renatured by dialysis first against 100 mM Tris (pH 8), 100 mM NaCl and then against 50 mM MES (pH 6), 100 mM NaCl. Following dialysis, wild-type MSP was purified to homogeneity by an additional anion-exchange step under non-denaturing conditions [18]. The N-terminal amino acid sequence of recombinant spinach wild-type MSP was determined by automated Edman degradation [13]. The first four amino acids were Glu-Gly-Gly-Lys-, identical to the N-terminus of native spinach MSP [19].

Some inclusion body preparations of wild-type or mutant MSP also contained the corresponding precursor pro-

tein. The precursor and mature forms of wild-type MSP can be separated by the non-denaturing anion-exchange step described above. However, the two mutant proteins could not be eluted from the anion-exchange column in the absence of urea (see Section 3.2). It was therefore necessary to use relatively crude preparations of the mutant proteins purified by a single anion-exchange step under partially denaturing conditions (3 M urea). Consequently, a small but detectable amount of the precursor protein of the MSP mutants was present as a minor contaminant in some preparations used for reconstitution experiments (see Fig. 2A).

Conditions for size-exclusion chromatography were the same as those used in anion-exchange chromatography, except the NaCl concentration was constant (100 mM). Samples of MSP (0.2 ml, 0.2 mg/ml) were injected into a Superose 12 FPLC column (HR 10/30, Pharmacia) at a flow rate of 0.5 ml/min. Absorbance of the eluent was monitored continuously at 280 nm.

2.4. Reconstitution of PSII and functional analysis

Urea-washed PSII and MSP were mixed in a solution containing 50 mM MES (pH 6), 20 mM $CaCl_2$, 60 mM NaCl, 100 μg BSA/ml, 0.4 M sucrose, 2% betaine (w/v) at a Chl concentration of 200 μg/ml. Chlorophyll concentration was measured in 80% acetone [20], and the concentration of PSII was calculated based on a stoichiometry of 250 Chl per 4 Mn [9]. The concentration of MSP was estimated spectrophotometrically using the extinction coefficient, 16 mM^{-1}, determined for the native protein at a wavelength of 276 nm [21]. Urea-washed PSII membranes were incubated with zero to 10 mol of MSP per mol of PSII for 1 h at room temperature in the dark, and unbound MSP was removed by centrifugation (10 min, 12000 $\times$ g, 4°C). Pellets, containing PSII membranes and bound MSP, were washed once in two volumes of 0.4 M sucrose, 50 mM MES (pH 6), 20 mM $CaCl_2$, 60 mM TMACl (SMTC), recentrifuged, and resuspended to 1 mg Chl/ml.

Samples were analyzed by SDS-PAGE (10% acrylamide, 4.7 M urea) using Neville buffers [22]. Protein samples were diluted with an equal volume of 2 $\times$ electrophoresis sample buffer (5.2 M urea, 4% SDS, 100 mM Na_2CO_3, Bromphenol blue). Where indicated, DTT (120 mM) was also present in the 2 $\times$ buffer. Proteins were visualized by staining with Coomassie brilliant blue R-250. Gels were analyzed by laser densitometry (LKB 2222-010 Ultroscan XL). Absorbance of the laser beam at 663 nm was recorded, and the concentration of MSP in gel bands was determined by integration using GelScan XL software. Plots of the integration results for MSP gel bands in a dilution series of PSII membranes (4–30 μg Chl/lane, within the range of MSP concentrations analyzed here) demonstrated linearity of staining intensity.

Oxygen evolution was assayed using a Clark-type electrode (YSI4004). The assay buffer contained 0.4 M sucrose, 50 mM MES (pH 6.0), 60 mM TMACl, and 20 mM $CaCl_2$ (SMTC) and was supplemented with BSA (100 μg/ml). The concentration of PSII Chl was 10 μg/ml for all assays. The rate of steady-state O_2 evolution was measured at 25°C in the presence of 300 μM DCBQ and saturating light. The stability of O_2 evolution activity was measured as the total O_2 produced (O_2 yield) at 25°C with 600 μM DCBQ; light intensity was 80% of saturation.

3. Results

3.1. [C28A,C51A]MSP functions normally, but [C51A]MSP is defective in the stabilization of O_2 evolution activity

The presence of the Cys-to-Ala mutations in MSP, first identified by sequencing of *psbO*, was confirmed at the protein level by analysis of the electrophoretic behavior of the MSP mutants. A simple method for distinguishing the wild-type, single mutant, and double mutant proteins (which have two, one, and zero Cys residues, respectively) is to analyze duplicate samples, one with DTT and one without DTT, in adjacent lanes on a polyacrylamide gel. DTT present in the reduced sample will diffuse laterally through the stacking gel (6% acrylamide) a few millimeters into the adjacent DTT-free lane, breaking disulfide bonds before proteins enter the resolving gel. Using this technique, mobility shifts resulting from intramolecular or intermolecular disulfide bond cleavage can be observed within a single gel band. Fig. 1 shows the differential effects of DTT on the electrophretic mobility of the three proteins. Reduction of the intramolecular -S-S- bridge in wild-type MSP with mercaptoethanol has been shown to decrease the electrophoretic mobility of the protein [23]. We also observed this effect with recombinant wild-type MSP (Fig. 1, lanes 4 and 6). (Unless otherwise indicated,

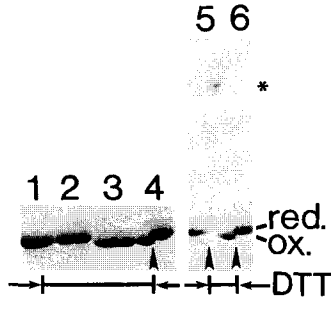

Fig. 1. SDS-PAGE analysis of MSP mutants. Samples of MSP mutants used in reconstitution experiments were analyzed by gel electrophoresis. DTT was omitted from the electrophoresis sample buffer for all lanes pictured here, but it was present in samples loaded in adjacent gel lanes (not pictured). Lanes 1 and 2, [C28A,C51A]MSP; 3, 4, and 6, wild-type MSP; 5, [C51A]MSP. Red., reduced MSP. Ox., oxidized MSP. Asterisk indicates dimer of [C51A]MSP. At bottom, vertical arrowheads indicate MSP mobility shift; horizontal arrows indicate direction and extent of DTT diffusion from adjacent gel lanes (not pictured); and bars indicate DTT-free areas of the gel.

Table 1
O_2 evolution activity and MSP binding for control PSII preparations.

PSII	MSP added	O_2 evolution		MSP bound [c]
		rate [a]	O_2 yield [b]	
Salt-washed	none	460 (100%)	16.0 (100%)	– (100%)
Urea-washed	none	125 (27%)	2.6 (16%)	–
Urea-washed	wild-type [d]	374 (81%)	12.8 (80%)	– (115%)

[a] μmol O_2 /mg Chl/h.
[b] μmol O_2 evolved per mg Chl during 4 min assay.
[c] relative concentrations of MSP in Coomassie-stained gels.
[d] 5 mol MSP per mol PSII.

'wild-type' refers to recombinant wild-type MSP from *E. coli*, and 'native' MSP refers to the protein isolated from spinach). Due to elimination of the disulfide bridge by mutagenesis, the double mutant should co-migrate with reduced wild-type MSP both in the presence and absence of DTT. Fig. 1 demonstrates this predicted behavior for [C28A,C51A]MSP (lanes 1 and 2). In contrast, [C51A]MSP migrated as a monomer in the presence of DTT and as a dimer in its absence (Fig. 1, lane 5). The single mutant has one Cys residue (C28), and dimerization therefore occurred by inter-molecular -S-S- formation in the highly oxidizing electrophoresis sample buffer. Analysis by size-exclusion chromatography demonstrated that [C51A]MSP used in reconstitution studies was monomeric (data not shown). The absence of dimers in samples of the double mutant confirms the replacement of both Cys.

Absolute values for assays of O_2 evolution using control PSII preparations are shown, for reference, in Table 1. Extraction of MSP reduced the rate of O_2 evolution and the long-term O_2 production ('O_2 yield') to 27% and 16%, respectively, of the values obtained for salt-washed PSII. Reconstitution of urea-washed PSII with wild-type MSP restored both measures of O_2 evolution activity to 80% of the control level. One explanation of incomplete reconstitution is that some PSII centers lose Mn in the absence of MSP, as noted previously [13]. At the same time, the amount of recombinant wild-type MSP bound to urea-washed PSII slightly exceeded the amount of native MSP in salt-washed PSII. This result is consistent with the observation that a trace of MSP is lost during extraction of the 17- and 23-kDa polypeptides (data not shown).

In PSII reconstitution experiments using both MSP mutants (Fig. 2B and Fig. 3B), activity curves obtained from plots of the rate of O_2 evolution (triangles) closely paralleled the corresponding MSP binding curves (circles). The activity values plotted in Fig. 2B and Fig. 3B do not represent absolute values for O_2 evolution, but rather show only the activity *restored* to urea-washed PSII as a percentage of that restored with wild-type MSP in control samples. All control samples were reconstituted with 5 mol of wild-type MSP per mol of PSII. When $\geq$ 5 mol of the

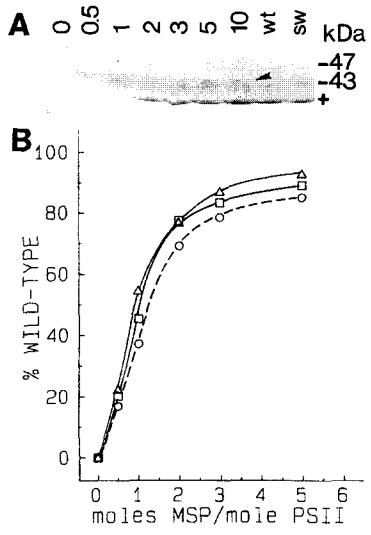

Fig. 2. Reconstitution of PSII with [C28A,C51A]MSP. A: SDS-PAGE analysis of reconstituted and washed PSII. Numbers above lanes indicate moles MSP/mol PSII; wt, reconstitution control using wild-type MSP (5 mol per mol PSII); sw, salt-washed PSII retaining native MSP; +, MSP. The 47- and 43-kDa PSII Chl-binding core subunits are labelled for reference. The arrowhead indicates MSP precursor protein which was present as a minor contaminant in the [C28A,C51A]MSP preparation used for this reconstitution experiment (see Section 2.3) and which appeared to bind to PSII. B: Functional analysis of reconstituted and washed PSII. Circles, PSII-bound MSP. Maximum (100%) MSP bound equals the average integrated area for the wild-type MSP gel band in two control lanes on individual gels. Triangles, rate of steady-state O_2 evolution. Maximum rate of O_2 evolution (100%) equals the rate measured for PSII reconstituted with wild-type MSP minus that for urea-washed PSII. Squares, total O_2 produced during 4 min of continuous illumination. Maximum O_2 yield (100%) equals O_2 produced by PSII reconsituted with wild-type MSP minus that for urea-washed PSII. Each point on the two activity curves is the average value from two independent experiments in which each value was the average of two assays. See Table 1 for absolute values and description of units.

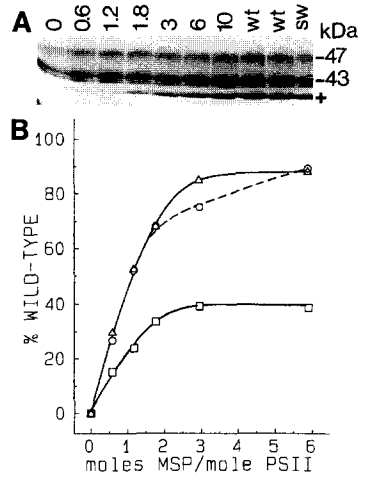

Fig. 3. Reconstitution of PSII with [C51A]MSP. A: SDS-PAGE analysis of reconstituted and washed PSII. Labels same as in Fig. 2A. B: Functional analysis of [C51A]MSP. Circles, PSII-bound [C51A]MSP; triangles, rate of O_2 evolution; squares, O_2 yield. See Fig. 2 legend for details.

Table 2
Effects of amino acid substitutions on O_2 evolution activity and MSP binding

PSII	MSP added [a]	O_2 evolution		MSP bound [b]
		rate [b]	O_2 yield [b]	
Urea-washed	wild-type	100%	100%	100%
Urea-washed	A28,A51	96%	92%	85%
Urea-washed	A51	94%	52%	93%

[a] 5–6 mol MSP per mol PSII.
[b] See Table 1 for units.

mutant proteins were added to PSII, the double and single mutants were, respectively, 88% and 85% as effective as wild-type MSP in accelerating O_2 evolution (see Fig. 2B and Fig. 3B, triangles).

The only effect of either mutation on O_2 evolution was observed with MSP containing the single mutation, C51A. The O_2 evolution activity recovered following reconstitution with [C51A]MSP decayed rapidly during extended illumination. Consequently, [C51A]MSP was only 40% as effective as the wild-type protein in stabilizing O_2 evolution activity (Fig. 3B, squares). In contrast, replacement of the second Cys with Ala suppressed this effect (Fig. 2B, squares). The assay for O_2 yield was designed to detect possible effects of mutations on the stability of O_2 evolution during long-term illumination. The differential effect of the C51A mutation on the rate and stability of O_2 evolution activity provides the first evidence that different structural features in MSP regulate the rate of O_2 evolution and the stability of O_2 evolution activity. Table 2 summarizes the absolute values of O_2 evolution activity for urea-washed PSII following reconstitution with wild-type MSP and the two MSP mutants.

It should be noted that folding and assembly of [C51A]MSP were combined into a single step because folding by dialysis against pH 6 buffer resulted in extensive dimerization (gel filtration data not shown). Preparations of [C51A]MSP were fully monomeric when eluted from the anion-exchange column in 3 M urea (immediately following solubilization from inclusion bodies). The truncated procedure involved mixing MSP in 3 M urea with urea-washed PSII in the reconstitution medium. Urea was thus removed by dilution and subsequently by pelleting and washing the reconstituted PSII membranes. When the double mutant was reconstituted using this one-step procedure, the same results were obtained as in Fig. 2B, where [C28A,C51A]MSP was renatured by dialysis before being used in reconstitution experiments (data not shown). The reduced O_2 yield observed for PSII-[C51A]MSP is therefore not an artifact of the combined folding and assembly protocol.

3.2. Elimination of the disulfide bridge destabilizes both the unbound and PSII-bound conformations of MSP

Neither MSP mutant could be purified by anion-exchange chromatography under non-denaturing conditions. 'Refolded' mutant protein did not elute from the Mono-Q column even in 1 M NaCl. Consequently, the second (non-denaturing) anion-exchange step used to purify wild-type MSP to homogeneity was by necessity omitted. The tendency of the MSP mutants to bind strongly to the anion-exchange resin under non-denaturing conditions suggested that the mutations were destabilizing the structure of the unbound subunit.

We therefore examined possible structural effects of the amino acid replacements by comparing the relative sizes of MSP mutants and the wild-type protein at a range of denaturant concentrations. The apparent sizes of wild-type MSP and the two mutants determined by size-exclusion chromatography in the absence of urea were 37 kDa and 43 kDa, respectively. The anomalously large apparent size of MSP has been noted previously [5]. The increased size of the mutant proteins likely resulted at least in part from decreased compactness due to the absence of the -S-S- bridge.

The apparent sizes of the MSP mutants increased considerably in low concentrations of urea (up to 1.75 M), across which range only small increases were observed in the apparent sizes of native and recombinant wild-type MSP (Fig. 4). In contrast, a sharp increase in the apparent sizes of both the native and wild-type proteins was observed between 1.75 and 3 M urea (Fig. 4). Only small additional increases in apparent size occurred between 3 M and 6 M urea for the mutant and wild-type proteins (data not shown), demonstrating that the unfolding (or other type of protein conformational change) responsible for the size increase was extensive in 3 M urea. To summarize, the mutant proteins are structurally destabilized and their apparent sizes increase considerably in 1 M urea, while > 1.75 M urea is required to produce a similar effect with the wild-type protein.

While the yield of wild-type MSP from the size-exclusion column was constant across all urea concentrations (0–6 M), the yield of both mutant proteins decreased with decreasing urea concentrations (data not shown). As with anion-exchange chromatography carried out under non-denaturing conditions, the MSP mutants apparently adsorbed to the gel-filtration matrix. Adsorption likely resulted from increased exposure of hydrophobic surfaces in the structurally destabilized mutant proteins. Increased intermolecular interactions as well as increased protein-column interactions would contribute to the increase in apparent size (Fig. 4) and to the loss of protein observed in the absence of urea.

Possible effects of the mutations on the binding interaction between MSP and PSII were tested by measuring MSP released from reconstituted PSII following exposure to a range of denaturant concentrations. Incubation in 1.2 M urea was sufficient to extract 50% of each MSP mutant, while 1.6–1.7 M urea was required to extract 50% of the wild-type protein (Fig. 5). The dissociation of native MSP from salt-washed membranes was not analyzed because we

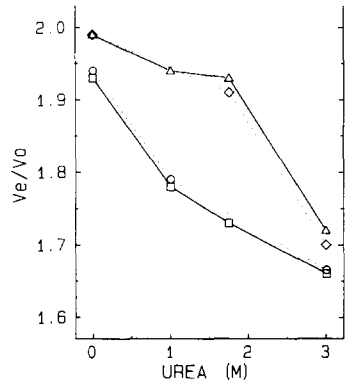

Fig. 4. Size-exclusion chromatography at different urea concentrations. Samples of MSP were not exposed to the indicated urea concentration until injection. Diamonds, native wild-type MSP; triangles, recombinant wild-type MSP; circles, [C51A]MSP; squares, [C28A,C51A]MSP. The V_e / V_o values for the wild-type and mutant proteins in the absence of urea corresponded to sizes of 37 kDa and 43 kDa, respectively, determined from a standard curve using BSA (66 kDa), carbonic anhydrase (29 kDa), and cyt c (12.4 kDa). The void volume (6.7 ml) was determined with Blue dextran.

were only interested in comparing the relative binding strengths of recombinant MSP in reconstituted PSII. However, Miyao and Murata [24] previously observed that incubation in 2 M urea was sufficient to release 50% of native MSP from spinach PSII membranes, identical to the result reported here (Fig. 5, triangles) using recombinant wild-type MSP. For comparison of monomer gel filtration curves and subunit dissociation curves, the data in Fig. 4 were replotted so that V_e/V_o values decrease instead of increase on the ordinate (Fig. 5, inset). As plotted in Fig. 5, the gel filtration and dissociation curves have almost identical shapes.

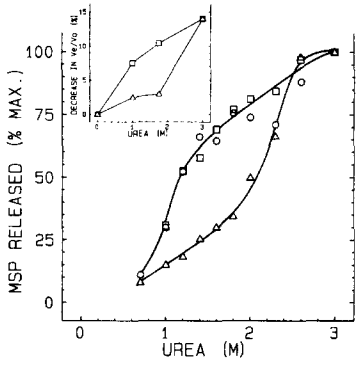

Fig. 5. Urea-dissocation of MSP from PSII. Urea-washed PSII were reconstituted with 5 mol MSP/mol PSII. Washed samples were incubated in the indicated concentrations of urea for 30 min at room temperature followed by centrifugation. The relative level of MSP in gel bands was measured by densitometry. Triangles, recombinant wild-type MSP; circles, [C51A]MSP; squares, [C28A,C51A]MSP. Maximum MSP released (100%) equals the integrated area of the MSP gel band from the 3 M urea-wash supernatant. Inset: Fig. 4 was replotted for direct comparison. Only the data for wild-type and [C28A,C51A]MSP were plotted at the reduced size for the sake of clarity. Ordinate values represent the decrease in V_e / V_o values relative to that obtained in the absence of urea.

4. Discussion

4.1. The disulfide bridge is not essential for MSP function

The -S-S- bridge in native spinach MSP has been shown to be required for stability and binding in vitro [5,6]. It has also been reported that chemical reduction of the disulfide bridge in PSII-bound MSP (i.e., in situ) completely (but reversibly) inactivates O_2 evolution [7]. A similar treatment of MSP in situ also inactivated O_2 evolution and even initiated O_2 consumption, although reversibility was not reported [25]. The complete inactivation of O_2 evolution brought about by reduction of the disulfide bridge in PSII-bound MSP is inconsistent with the well-established observation that PSII evolves O_2 in the absence of MSP [3,26–28]. Treatment of PSII with a disulfide reducing agent would therefore not be expected to completely inhibit O_2 evolution, unless the reductant is attacking additional targets, such as the Mn cluster.

We have presented evidence here that a cysteine-free double mutant of spinach MSP, though structurally destabilized, functions normally in PSII. This result demonstrates that the two conserved Cys residues in MSP, whether present in the reduced or oxidized forms, do not participate in the acceleration of O_2 evolution observed upon reconstitution of PSII with MSP. Because we used mutagenized MSP instead of reduced wild-type MSP in our studies, we cannot rule out the hypothesis that reduction of the disulfide bridge in situ inactivates O_2 evolution. It is possible, for example, that breaking the disulfide bridge in the bound subunit alters the structure and/or function of the Mn cluster, either directly through interaction with free sulfhydryls or through gross structural rearrangements. Another possibility is that reduced MSP blocks access of substrate H_2O to its binding site in the OEC. Our results do exclude, however, any functional role for MSP that would depend on a specific interaction between the Mn cluster and the two sulfur atoms in residues C28 and C51, as proposed by Raval et al. [29].

4.2. Different structural bases for effects of MSP on the rate and stability of O_2 evolution

Analysis of [C51A]MSP has provided the first evidence that different structural features in MSP are responsible for two of its functions, namely the acceleration of O_2 evolution [30] and stabilization of O_2 evolution activity [13,28,31]. Photosystem II reconstituted with [C51A]MSP evolved O_2 at a normal rate. In contrast, when activity was measured as O_2 yield, PSII evolved 50% less O_2 with [C51A]MSP than with the wild-type or double-mutant proteins. Decreased O_2 yield may result from failure of the mutant protein to protect PSII from photo-oxidative damage (i.e., photoinhibition). Another possible cause of lower O_2 yields by PSII-[C51A]MSP is a reduced quantum yield for O_2 evolution. Saturating light intensity was used for

rate assays, but a lower intensity was used for O_2 yield assays (80% light saturation). However, the rates of O_2 evolution at the beginning (first 15–30 s) of O_2 yield assays were the same with either wild-type or [C51A]MSP, ruling out an effect on PSII quantum yield. After 30 s of illumination, the rate of O_2 evolution by PSII-[C51A]MSP decreased dramatically and continued to decay at a greater rate than observed for control samples (data not shown). We conclude that the single mutant, [C51A]MSP, fails to stabilize O_2 evolution activity.

4.3. Structural considerations

The functional defect associated with C51A disappears when the second Cys is also changed to Ala. One possible explanation for this reversion to wild-type function is that the conformation of bound MSP cannot accommodate the sulfhydryl group of Cys28 in the position normally occupied by the disulfide bridge. Such structural incompatibility would also explain the complete loss of binding observed by Tanaka and Wada [5] following reduction of the disulfide bridge in native MSP.

Elimination of the disulfide bridge destabilized MSP to the same extent whether in solution or while bound to PSII. The gel filtration curves for the mutant and wild-type proteins, measured in urea, were almost superimposable with their respective urea-dissocation curves (Fig. 5). The simplest interpretation of this result is that the conformation of MSP required for tight binding to PSII forms in solution, and that the disulfide bridge stabilizes this conformation. This interpretation is consistent with the prediction of extensive secondary structure in monomeric MSP based on its far-UV CD spectrum [32] and the presence of tertiary structure demonstrated by analysis of the near-UV CD and fluorescence spectra [6].

The two MSP mutants were destabilized to the same extent both in solution and when bound to PSII. This result suggests that the functional defect caused by C51A does not stem from a general conformational defect which would be expected to weaken MSP binding. Furthermore, because the C51A mutation by itself did not affect O_2 evolution until after at least 30 s in intense light, it would therefore not necessarily be expected to further weaken binding in the dark, relative to the double mutant. Therefore, to account for the delayed effect of C51A on O_2 evolution, we suggest that this mutation perturbs a structural interaction between MSP and PSII which is present only in the light, during the redox cycling of the Mn cluster. Light has been shown to facilitate release of MSP by urea [24]. The consequence of this proposed light-dependent binding defect is the acceleration of photoinhibition, demonstrated here as decreased O_2 yield by PSII-[C51A]MSP. Further experiments will test the effect of light on the binding interaction between MSP and PSII, and efforts are under way to obtain the complementary single mutant, [C28A]MSP, for analysis.

5. Conclusions

The disulfide bridge in MSP stabilizes both the unbound and PSII-bound conformations of the protein, but is not required for its function. The unique phenotype of the single mutant, [C51A]MSP, has allowed dissection of the structural bases of two different MSP functions. This MSP mutant retains the structural elements required to accelerate O_2 evolution, while an apparently separate structural feature that normally stabilizes O_2 evolution activity has been perturbed. We propose that this structural feature is a light-dependent interaction that occurs between MSP and PSII during the redox cycling of the Mn cluster.

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