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Structure and activity of the Photosystem II manganese-stabilizing protein: role of the conserved disulfide bond

Aaron J. Wyman[1,3] & Charles F. Yocum[1,2,*]
[1]*Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA;* [2]*Department of Chemistry, University of Michigan, Ann Arbor, MI 48109-1048, USA;* [3]*Department of Biochemistry, Purdue University, West Lafayette, IN 47906, USA; *Author for correspondence (e-mail: cyocum@umich.edu; fax: +1734-647-0884)*

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Abstract

The 33-kDa manganese-stabilizing protein (MSP) of Photosystem II (PS II) maintains the functional stability of the Mn cluster in the enzyme's active site. This protein has been shown to possess characteristics similar to those of the intrinsically disordered, or natively unfolded proteins [Lydakis-Simantiris et al. (1999b) Biochemistry 38: 404–414]. Alternately it was proposed that MSP should be classified as a molten globule, based in part on the hypothesis that its lone disulfide bridge is necessary for structural stability and function in solution [Shutova et al. (2000) FEBS Lett. 467: 137–140]. A site-directed mutant MSP (C28A,C51A) that eliminates the disulfide bond reconstitutes O_2 evolution activity and binds to MSP-free PS II preparations at wild-type levels [Betts et al. (1996) Biochim. Biophys. Acta 1274: 135–142]. This mutant was further characterized by incubation at 90 °C to determine the effect of loss of the disulfide bridge on MSP thermostability and solution structure. After heating at 90 °C for 20 min, C28A,C51A MSP was still able to bind to PS II preparations at molar stoichiometries similar to those of WT MSP and reconstitute O_2 evolution activity. A fraction of the protein aggregates upon heating, but after resolubilization, it regains the ability to bind to PS II and reconstitute O_2 evolution activity. Characterization of the solution structure of C28A,C51A MSP, using CD spectroscopy, UV absorption spectroscopy, and gel filtration chromatography, revealed that the mutant has a more disordered solution structure than WT MSP. The disulfide bond is therefore unnecessary for MSP function and the intrinsically disordered characteristics of MSP are not dependent on its presence. However, the disulfide bond does play a role in the solution structure of MSP *in vivo*, as evidenced by the lability of a C20S MSP mutation in *Synechocystis* 6803 [Burnap et al. (1994) Biochemistry 33: 13712–13718].

Abbreviations: CD – circular dichroism; Chl – chlorophyll; CP – chloroplast protein; DCBQ – 2,6-dichloro-*p*-benzoquinone; EDC – 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EDTA – ethylenediamine tetraacetic acid; FT-IR – Fourier transform infrared; IPTG – isopropyl-β-D-thiogalactopyranoside; MES – 2-(N-morpholino) ethanesulfonic acid; MSP – manganese-stabilizing protein; OEC – O_2-evolving complex; PAGE – polyacrylamide gel electrophoresis; PS – photosystem; *psbO* – gene encoding precursor MSP; SDS – sodium dodecyl sulfate; SMN – buffer composed of sucrose (0.4 M), MES (50 mM, pH 6.0), NaCl (10 mM)); SW-PS II – Photosystem II preparation treated with 2 M NaCl to extract Ca^{2+} and the 23 and 17 kDa extrinsic polypeptides; USW-PS II – Photosystem II preparation treated with 2 M NaCl to extract Ca^{2+} and the 23 and 17 kDa extrinsic polypeptides, followed by incubation with 3.1 M urea and 240 mM NaCl to remove the manganese-stabilizing protein

Introduction

Photosystem II (PS II) is the multisubunit enzyme that catalyzes water oxidation. The site of this reaction, called the O_2-evolving complex (OEC), is composed of several intrinsic and extrinsic proteins, a tetranuclear Mn cluster, and Ca^{+2} and Cl^- (Debus 1992). Substrate H_2O is oxidized as the Mn cluster cycles through five distinct redox states, termed S-states (S_n, where $n = 0$–4) (Kok et al. 1970). The intrinsic proteins of PS II include the reaction center subunits D1 and D2, proteins that bind accessory antenna Chl *a* molecules (CP47 and CP43), the α and β subunits of cytochrome *b*-559, and a number of small polypeptides of uncertain function (Ikeuchi and Inoue 1988; Debus 1992). The intrinsic proteins stabilize the OEC by forming a membrane-associated core, which provides binding sites for the Mn cluster and Ca^{+2}, as well as for the extrinsic proteins that reside on the lumenal side of PS II. In eukaryotes, these proteins are the 17, 23, and 33 kDa polypeptides (Seidler 1996). The 17 and 23 kDa proteins form part of a structure that retains Cl^- and Ca^{+2} ions within the OEC (Ghanotakis et al. 1984; Miyao and Murata 1984). These extrinsic subunits are replaced by cytochrome *c*-550 (PsbV) and a 12 kDa subunit (PsbU) in cyanobacteria (Shen and Inoue 1993; Shen et al. 1998), where crystal structures of three PS II preparations from *Thermosynechococcus elongatus* (Zouni et al. 2001; Ferreira et al. 2004) and *Thermosynechococcus vulcanus* (Kamiya and Shen 2003) at resolutions from 3.8 to 3.5 Å are available.

The 33-kDa extrinsic polypeptide, also referred to as manganese-stabilizing protein (MSP), plays a critical role in the structure and function of the OEC. It is required for binding of the 17 and 23 kDa proteins and maintains the stability of the Mn cluster (Ono and Inoue 1983, 1986; Miyao and Murata 1984; Miyao et al. 1987; Bricker 1992; Bricker and Frankel 1998). Two copies of MSP bind to the OEC of eukaryotic photosynthetic organisms (Xu and Bricker 1992; Seidler 1996; Popelkova et al. 2002a, b) but the crystal structures of PS II from cyanobacteria contain only one copy of MSP (Zouni et al. 2001; Kamiya and Shen 2003; Ferreira et al. 2004). This difference may be due to the absence in cyanobacterial MSP of a N-terminal amino acid sequence that is present in eukaryotic MSP (Popelkova et al. 2002b). When MSP is removed from the OEC during dark incubations, loss of Cl^- and Ca^{+2} occurs along with destabilization of the Mn cluster; a slow release of two Mn atoms and loss of O_2 evolution activity is observed (Miyao and Murata 1984). Disintegration of the Mn cluster and loss of low levels of O_2 evolution activity can be prevented if high, non-physiological concentrations of Ca^{2+} and Cl^- are present (Ono and Inoue 1983, 1986; Miyao and Murata 1984; Miyao et al. 1987; Bricker 1992; Seidler 1996; Bricker and Frankel 1998). A *psbO* gene encodes the mature lumenal form of MSP as a 247-amino acid polypeptide with a predicted molecular mass of 26.5 kDa (Tyagi et al. 1987). Its primary structure includes a disulfide bridge, conserved among all MSP sequences examined so far, that encloses a loop of 22–24 amino acids (De Las Rivas and Heredia 1999; Motoki et al. 2002).

Manganese-stabilizing protein behaves in solution as if it was an intrinsically disordered or natively unfolded protein (Lydakis-Simantiris et al. 1999b). Proteins of this type share a similar set of unique physical and chemical characteristics. In many cases, they are components of multisubunit complexes that carry out diverse functions, such as protein phosphorylation, ribosomal translation, synapse formation, and maintenance of cytoskeletal structure (Weinreb et al. 1996; Uversky et al. 2000; Dunker et al. 2001; Uversky 2002a, b). Characteristics of intrinsically disordered proteins include thermostability, secondary structures containing large amounts of random coil and turn, and primary amino acid sequences with high ratios of charged to hydrophobic residues. These proteins also possess extended solution structures, as determined by gel filtration chromatography and anomalous mobility on SDS-PAGE, and by unusually large Stokes radii (R_s) and sedimentation coefficient (S_e) values, and also by very acidic or basic pI values (Weinreb et al. 1996; Uversky et al. 2000; Dunker et al. 2001; Uversky 2002a, b). The secondary structure of WT MSP in solution has been shown to consist of about 50–55% random coil and turns (Xu et al. 1994; Ahmed et al. 1995; Sonoyama et al. 1996; Zhang et al. 1996; Shutova et al. 1997; Hutchison et al. 1998; Lydakis-Simantiris et al. 1999a, b; Motoki et al. 2002; Popelkova et al. 2002a, b) and it is an acidic protein (pI = 5.2) whose estimated molecular mass depends on the technique used for analysis. The calculated molecular mass from the *psbO* sequence is 26.5 kDa, and a similar value is

obtained with MALDI-TOF mass spectroscopy (Tyagi et al. 1987; Zubrzycki et al. 1998; Svensson et al. 2002). However, on SDS-PAGE MSP exhibits an apparent molecular mass of 33 kDa, and in gel filtration experiments, it produces an estimated molecular mass of 38–41 kDa (Kuwabara and Murata 1979; Tyagi et al. 1987; Betts et al. 1998; Lydakis-Simantiris et al. 1999b; Popelkova et al. 2002a, b, 2003a). The protein has a R_s value of 31 Å, and its sedimentation coefficient, determined by analytical ultracentrifugation, is 2.26 S; both values being larger than expected for a protein of its predicted mass (Betts et al. 1998; Zubrzycki et al. 1998). In addition, MSP has calculated axial ratios of 4.2 (from sedimentation experiments (Zubrzycki et al. 1998)) and 4.8 (small-angle X-ray scattering (Svensson et al. 2002)), results that suggest that WT MSP has a prolate ellipsoid shape in solution (Zubrzycki et al. 1998). This may account for part of its unusual behavior in gel filtration experiments, but not for its low content of ordered secondary structure. Hutchison et al. (1998) employed FTIR spectroscopy to show that MSP in solution appears to sample a number of conformational states; more recently, Nowaczyk et al. (2004) used NMR to probe the structure of *Thermosynechococcus elongatus* MSP and found that the soluble protein consists of both a well-ordered core as well as regions of flexibility, features previously associated with intrinsically disordered proteins (Dunker et al. 2001; Uversky 2002a, b). Loll et al. (2005) characterized native and recombinant forms of the same protein using FTIR, and concluded that it was more disordered in solution than was spinach MSP. Finally, although MSP is found in mesophilic and thermophilic organisms, it is unique on account of thermostability, as defined by its resistance to heat-induced denaturation, observed in the spinach protein (Lydakis-Simantiris et al. 1999b).

Recently, it has been proposed that MSP has properties consistent with those of a molten globule, rather than of an intrinsically disordered protein (Shutova et al. 2000). This suggestion was based on several criteria: the presence of secondary structure (α-helices, β-sheet, turns), a more compact structure than the unfolded state, significant flexibility, absence of a defined tertiary structure, and the predicted existence of a hydrophobic core. Other properties presented to compare intrinsically disordered and molten globule proteins included the number of amino acid residues present in the protein, the ratio of the protein's molecular compactness ($R_{s(MG)}$) to that predicted for the unfolded state ($R_{s(N)}$), the percentage of charged amino acid residues, the presence of near-UV CD features, and the R_s value of the protein. Several of the criteria used to designate a protein as a molten globule (Shutova et al. 2000) are also characteristics of intrinsically disordered proteins (Weinreb et al. 1996; Lydakis-Simantiris et al. 1999b; Uversky et al. 2000; Uversky 2002a, b). A critical factor used to classify MSP as a molten globule protein was the presence of the disulfide bridge; Shutova et al. (2000) proposed that this covalent bond stabilizes a hydrophobic core made up of β-sheet structure in MSP, and that this is responsible for the protein's thermostability.

A characterization of the effects of deletion of one or both Cys residues on MSP's function and solution structure (Betts et al. 1996) showed that C28A,C51A MSP could reconstitute O_2 evolution activity and bind to USW-PS II at levels similar to that of WT MSP. Although removal of a single Cys (C51A) from MSP did not interfere with MSP binding to USW-PS II or with reconstitution of O_2 evolution activity, C51A MSP was unable to stabilize O_2 evolution activity under long-term illumination conditions. These results suggest that while the disulfide bridge may have a role in maintaining the solution structure of MSP, it does not have a pronounced effect on the protein's ability to bind to PS II and reconstitute O_2 evolution activity. To determine the structural role of the disulfide bond in MSP, and how it might affect the protein's classification as intrinsically disordered or as a molten globule, the C28A,C51A MSP mutant was subjected to further characterization. In the work reported here, we have further characterized the function of MSP's disulfide bridge with respect to the protein's thermostability and ability to reconstitute O_2 evolution activity. The secondary and tertiary structure of C28A,C51A MSP was characterized using far-UV CD and near-UV CD spectroscopy, UV absorbance spectroscopy, and gel-filtration chromatography to determine how the solution structure of the mutant protein differs from that of WT MSP. Elimination of the disulfide bridge creates additional disorder in MSP's solution structure and causes the protein to bind non-specifically to PS II. However, C28A,C51A MSP retains other properties, including thermostability, attributed to intrinsically disordered proteins.

Materials and methods

Overexpression and purification of recombinant WT MSP and C28A,C51A MSP

Recombinant WT MSP and C28A,C51A MSP were overexpressed in *E. coli* and inclusion bodies were isolated as previously described (Betts et al. 1994, 1996), with the following modifications: only ampicillin (50 μg ml^{-1}) was added to the LB media during overexpression, and IPTG at concentrations of 25–32.5 μM was used to induce protein overexpression. Both proteins were solubilized from inclusion bodies as described previously (Betts et al. 1994, 1996), with the following modification: inclusion bodies were incubated overnight at 4 °C with stirring in 3 M urea, 20 mM Bis-Tris (pH 6.4), and 5 mM NaCl (solubilization buffer) (Popelkova et al. 2002b, 2003a), The resulting suspension was centrifuged at 48,000×*g* for 30 min at 4 °C, and the supernatant was loaded onto a Pharmacia Resource Q column equilibrated with solubilization buffer containing 5% betaine. Proteins were eluted from the column using a linear salt gradient (5–250 mM NaCl). The MSP's were eluted between 100–200 mM NaCl. The partially purified protein fractions were combined, dialyzed overnight in solubilization buffer minus betaine at 4 °C, and loaded onto the same Resource Q column using solubilization buffer containing 5% betaine. The second round of ion exchange chromatography utilized a stepwise NaCl gradient (30, 70, 170, and 1000 mM NaCl); MSP eluted at 170 mM NaCl. In order to prevent MSP from aggregating when frozen (−70 °C), the purified proteins were either stored in solubilization buffer, or were dialyzed overnight at 4 °C in 0.4 M sucrose, 50 mM MES (pH 6.0), and 10 mM NaCl (SMN); both solutions were found to prevent protein aggregation upon freezing. The protein concentration was determined spectrophotometrically at 276 nm using an extinction coefficient of 16 mM^{-1} (Xu and Bricker 1992).

PS II membranes: preparation and extraction of extrinsic polypeptides

PS II membranes were isolated as described in Berthold et al. (1981) with modifications (Ghanotakis et al. 1984). Extrinsic proteins were removed sequentially; the 17 and 23 kDa proteins were extracted first by incubation in 2 M NaCl, 1 mM EDTA, and 50 mM MES (pH 6.0) to yield a SW–SII preparation. Natively bound MSP was then removed from SW–PS II preparations by a 30 min incubation in the dark, on ice, in 50 mM MES (pH 6.0), 3.1 M urea, and 240 mM NaCl to produce USW–PS II samples (Betts et al. 1994). These samples were washed in SMN-200 buffer (0.4 M sucrose, 50 mM MES (pH 6.0), and 200 mM NaCl) prior to suspension in SMN-200 and storage at −70 °C. Activities for the intact PS II preparations were 600–700 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}, for SW-PS II preparations, 300–400 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}, and for USW–PS II samples, 80–150 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}.

Reconstitution of PS II with WT or C28A,C51A MSP

Reconstitutions and O_2 evolution assays were carried out as previously described (Popelkova et al. 2002a, b, 2003a) with the following modifications: assays of control O_2 evolution rates in SW–PS II samples used 300 μM DCBQ, while for determination of the recombinant MSP-reconstituted PS II activities, 600 μM DCBQ was added. WT MSP and C28A,C51A MSP samples stored in solubilization buffer were dialyzed overnight at 4 °C against SMN buffer prior to use in reconstitution reactions. Solutions of WT MSP and C28A,C51A MSP both aggregated upon heating at 90 °C and cooling to 25 °C (Wyman and Yocum 2005). Heat-treated proteins were centrifuged for 10 min at 12,000 × *g* to remove all aggregated, insoluble proteins, which amounted to about 50% of the total protein present in the sample before heating. The concentration of soluble MSP in the supernatant fraction was determined spectrophotometrically, and this protein was then used in PS II reconstitution experiments. Heat-aggregated MSP was resolubilized from centrifuged pellets by dialyzing the insoluble protein overnight at 4 °C against 50 mM MES (pH 6.0), 10 mM NaCl, and 3 M urea. The protein was then dialyzed overnight a second time against SMN buffer to remove urea. The concentration of the resolubilized protein was determined as described above.

Reconstitution of USW-PS II with WT MSP and C28A,C51A MSP, and analysis of the extent of rebinding to PS II, were carried out as described

in Betts et al. (1996) and Popelkova et al. (2003a). The relative concentrations of MSP bound to PS II were determined by integration of Coomassie-stained MSP bands after SDS-PAGE using Sigmagel software (Jandel Scientific). The intensity of the MSP band present in a SW–PS II sample was used as the 100% MSP control. All staining intensities were normalized to the Coomassie-stained 47 kDa band (CP47) to correct for variations that arise from loading protein samples onto the gel. The O_2 evolution activity and MSP rebinding data represent the average of 3–6 assays for each point on the graphs.

Circular dichroism spectroscopy, UV absorbance spectroscopy, and size-exclusion chromatography

Solutions of C28A,C51A MSP were dialyzed overnight against 10 mM KH_2PO_4 (pH 6.0) buffer for far-UV CD and near-UV CD spectroscopy carried out using an AVIV 62 DS CD spectrometer calibrated with (+)10-camphorsulfonic acid (1 mg ml^{-1} in H_2O) (Johnson 1990). Experimental conditions for the far-UV and near-UV CD spectral measurements are given in the legends for Figure 3 and Figure 4. The secondary structural contents of C28A,C51A MSP from its far-UV CD spectra were estimated using the CONTIN/LL and CDSSTR programs from the CDPro package (Sreerama and Woody 2000) available for downloading from the Internet (http://lamar.colostate.edu/~sreeram/CDPro). UV spectra were obtained using an OLIS-modified Cary-17 instrument. Experimental conditions for the UV spectra are given in the legend of Figure 5. A superose-12 column connected to a Pharmacia FPLC system was used for size-exclusion chromatography; a calibration curve was produced with protein standards used previously (Lydakis-Simantiris et al. 1999b). The column was equilibrated with 20 mM Tris (pH 8.0), and 50 mM NaCl was used, instead of 150 mM NaCl, which was necessary to prevent C28A,C51A MSP from aggregating on the gel filtration column.

Results

C28A,C51A MSP reconstitutes O_2 evolution activity after exposure to high temperatures

Previously, it was shown that C28A,C51A MSP is more difficult to manipulate during purification; urea must be included in buffers to prevent the mutant protein from clogging ion exchange chromatography columns (Betts et al. 1996). This protein is nevertheless capable of rebinding to USW–PS II preparations and of reconstituting O_2 evolution activities (Betts et al. 1996). To determine whether the presence of the disulfide bridge in WT MSP was critical for MSP thermostability, C28A,C51A MSP was incubated at 25 °C, or at 90 °C followed by cooling to 25 °C, and both samples were then assayed for their ability to reconstitute O_2 evolution. As can be seen in Figure 1a, unheated C28A,C51A MSP restored O_2 rates and yields up to 35–45% of SW–PS II control levels upon rebinding of about 2 moles C28A,C51A MSP/mol PS II. These activities were unchanged when additional MSP was added to the reconstitution mixtures. Binding of C28A,C51A MSP to USW–PS II preparations, however, failed to saturate at any protein concentration tested, whereas WT MSP binding to PS II saturates at about 2 moles MSP/mol PS II (Betts et al. 1994). The adventitious binding exhibited by C28A,C51A MSP is probably due to non-specific binding by the mutant protein to USW–PS II samples. This binding behavior has been previously detected with other MSP mutants from both eukaryotic and prokaryotic sources (Popelkova et al. 2002a, b; Motoki et al. 2002; Popelkova et al. 2003a). Non-specific MSP binding was confirmed by incubating C28A,C51A MSP with intact PS II samples (which have native WT MSP bound with high affinity to the specific MSP binding sites), as previously described (data not shown) (Popelkova et al. 2002a, b, 2003a) Following incubation, the PS II samples were washed to remove any weakly bound C28A,C51A MSP. With no specific binding sites available, C28A,C51A MSP should only be able to bind non-specifically to these PS II samples. It was found that incubation of 0–2 moles of C28A,C51A MSP per mol intact PS II produced no detectable non-specific binding, but incubation of 4 moles of C28A,C51A MSP per mol intact PS II resulted in non-specific binding of about 1 mol of C28A,C51A MSP per mol intact PS II. Incubation of 8 moles of C28A,C51A MSP per mol intact PS II produced a non-specific binding of about 2 moles of C28A,C51A MSP per mol intact PS II (data not shown).

Figure 1b presents data on the reconstitution of O_2 evolution activity by C28A,C51A MSP that was

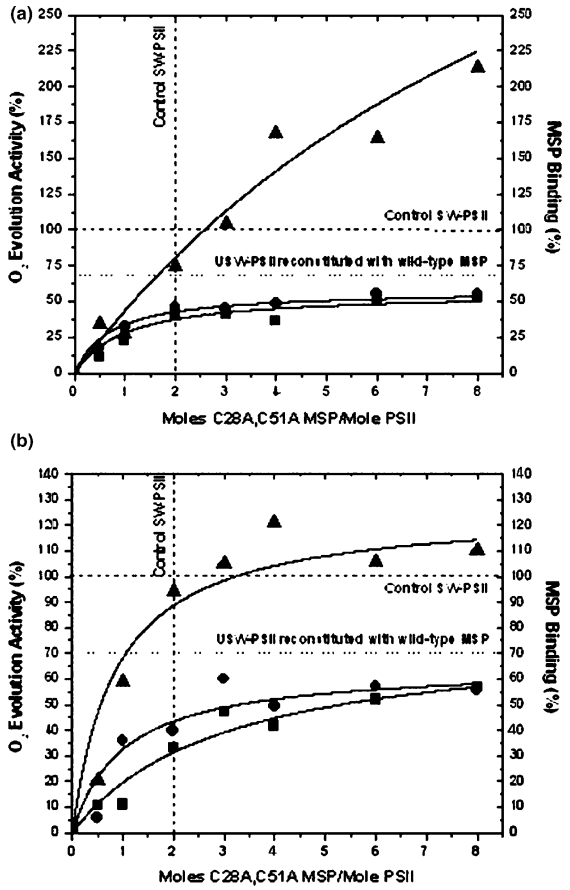

Figure 1. Reconstitution of USW–PS II preparations with recombinant C28A,C51A MSP. C28A,C51A MSP was incubated for 20 min at room temperature (a) or 90 °C (b) prior to addition to the USW–PS II samples. MSP binding curves are represented by (▲). The O_2 rates (●) and O_2 yields (■) were obtained from assays carried out under continuous illumination for 1 or 4 min., respectively. Reconstitution mixtures were incubated on ice for 60 min and then assayed for O_2 evolution activity. Residual activity from USW–PS II preparations was subtracted from the reconstituted samples, and control (100% activity) was that of SW–PS II samples. The dotted lines indicate WT MSP activity levels, while the dashed lines represent WT MSP binding levels (Betts et al. 1996a; Popelkova et al. 2003a).

incubated at 90 °C, and then cooled prior to use. Approximately 50% of the C28A,C51A MSP initially in solution forms insoluble aggregates upon heating under the conditions used here (a detailed characterization of MSP aggregation is given in Wyman and Yocum 2005). After cooling to 25 °C, the sample was centrifuged to remove aggregated protein and the supernatant was used for the rebinding experiments, as described in Materials and methods section. Reconstitution of O_2 rates and O_2 yields by the heated, soluble MSP fraction attained 30–35% the SW–PS II control levels at about 2 moles of C28A,C51A MSP per mol PS II, and ultimately reached a plateau at 6 moles of C28A,C51A MSP per mol PS II, with O_2 rates and O_2 yields of 50% of SW–PS II values. Comparison of these results with those in Figure 1a reveals that binding of the heated C28A,C51A MSP to USW–PS II parallels recovery of O_2 evolution activity, but non-specific binding of heated C28A,C51A MSP was not observed.

The activity of the heat-aggregated MSP was also characterized. In its aggregated form, C28A,C51A MSP was unable to reconstitute O_2 evolution activity upon incubation with USW–PS II (data not shown). However, when the aggregated protein was solubilized as described in Materials and methods section and used in reconstitution assays, O_2 rates and yields were obtained that saturated at about 2 moles C28A,C51A MSP/mol PS II, yielding 25% and 55% of the SW–PS II control O_2 rates and O_2 yields, respectively (Figure 2). The solubilized protein rebinds to USW–PS II to about 80% of SW–PS II control levels in incubation mixtures containing 2 moles of C28A,C51A MSP per mol PS II. At 3–6 moles of C28A,C51A MSP per mol PS II, the solubilized protein bound at levels comparable to the WT MSP content of SW–PS II. However, upon incubation of USW–PS II with 8 moles of solubilized C28A,C51A MSP per mol PS II, both specific and non-specific binding were observed; about 2 moles of solubilized

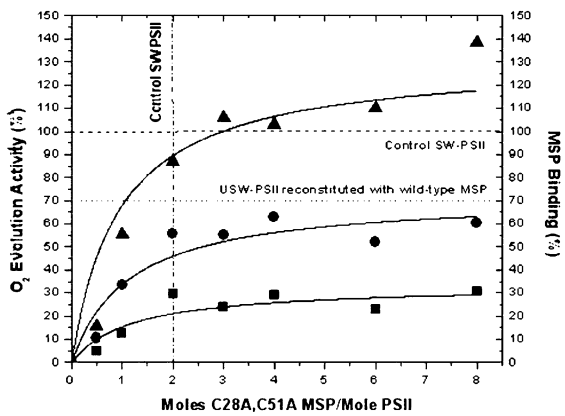

Figure 2. Reconstitution of USW-PS II preparations with resolubilized recombinant C28A,C51A MSP. Heat aggregated C28A,C51A MSP renatured and incubated for 20 min at room temperature prior to use in O_2 evolution assays. Other conditions are given in the legend of Figure 1.

C28A,C51A MSP per mol PS II bound specifically, and about 1 mole of C28A,C51A MSP per mol PS II was non-specifically bound (Figure 2). Thus, the ability of C28A,C51A MSP to bind non-specifically to PS II preparations appears to be decreased upon heating. The origin of this behavior is unknown, although it might, in theory, be due to an altered MSP solution conformation induced by the heating and cooling steps. It is clear that C28A,C51A MSP is thermostable, as shown by the results in Figures 1 and 2, retaining its activity even after exposure to high temperatures for an extended period of time, or after aggregation during heating. This behavior is comparable to the thermostability reported for WT MSP (Lydakis-Simantiris et al. 1999b). WT MSP at sufficiently high concentrations also aggregated upon heating (Wyman and Yocum 2005). The O_2 evolution activity and binding levels of WT MSP from the soluble supernatant and the resolubilized aggregated fractions were found to be similar to those produced from the same fractions from C28A,C51A MSP (data not shown).

Solution structure of C28A,C51A MSP

Further evidence for the thermostability of C28A,C51A MSP was obtained from analyses of the protein's secondary structure using far-UV CD spectroscopy. The far-UV CD spectra shown in Figure 3 were obtained with a C28A,C51A MSP sample that was heated and cooled. Although the 25 °C far-UV CD spectrum of C28A,C51A MSP differs from that obtained with WT MSP under similar experimental conditions (Lydakis-Simantiris et al. 1999b), heating and subsequent cooling do not produce drastic changes in the overall appearance of the C28A,C51A MSP spectrum when compared to that of the WT protein. At 25 °C, C28A,C51A MSP exhibits a deep trough at 202 nm (indicative of random coil), and a slight trough between 205–225 nm (associated with the presence of α-helix). Upon heating C28A,C51A MSP to 90 °C, the troughs at 202 nm and 225 nm deepened, indicating increased levels of both random coil and α-helix; cooling to 25 °C restored a large fraction of the original spectrum, consistent with earlier results obtained with WT MSP (Lydakis-Simantiris et al. 1999b). It should also be noted that the far-UV CD spectrum of unheated C28A,C51A MSP at 25 °C shown in Figure 3 is very

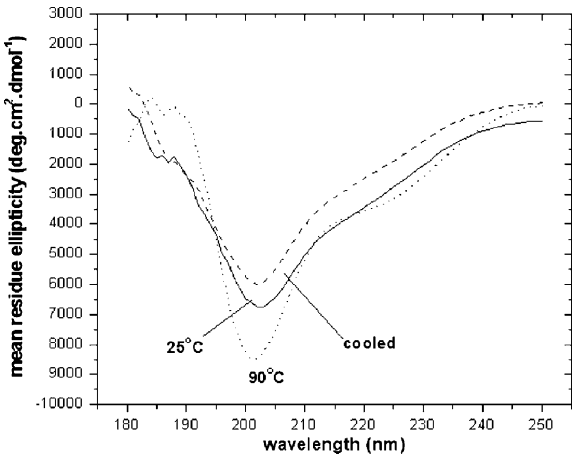

Figure 3. Effect of heating on the far-UV CD spectrum of C28A,C51A MSP. Samples of C28A,C51A MSP (13.7 μM) in 10 mM KH_2PO_4 buffer, pH 6.0 and 5.7 (calculated using the buffer's temperature coefficients at 25 °C and 90 °C) were equilibrated at the indicated temperatures (25 °C (———), 90 °C (·····), cooled to 25 °C (- - - -)) and 20 scans were averaged at each temperature. Predicted secondary structure compositions for the protein at each temperature are presented in Table 1. Experimental conditions: scan width, 250–180 nm; time constant, 1 s; bandwidth, 1 nm; path length, 1 mm; sample volume, 300 μl.

similar to the spectrum of WT MSP at 90 °C (Lydakis-Simantiris et al. 1999b), and agrees well with the far-UV CD spectrum of reduced native WT MSP (Tanaka et al. 1989).

Table 1 presents the calculated α-helix, β-sheet, and random coil contents of C28A,C51A MSP at 25 °C and 90 °C. These data were obtained from analyses of the C28A,C51A MSP far-UV CD spectra by several computational methods (CONTIL/LL, CDSSTR), which compare the far-UV CD spectra of C28A,C51A MSP to spectra of proteins with known secondary structures, allowing one to estimate the percentage of secondary structures (α-helix, β-sheet/turn, and random coil) in C28A,C51A MSP (Sreerama and Woody 2000). Of the two groups of protein basis sets available for comparisons, basis sets 6 and 7 are considered to be more suitable for estimations of MSP secondary structure because they contain structural data for five denatured proteins, which are hypothesized to have secondary structural contents more similar to that of intrinsically disordered proteins (Sreerama and Woody 2000). For comparison, CD spectra of WT MSP were also taken at 25 °C, 90 °C, and after cooling to 25 °C. The spectra, which were similar to those described

Table 1. Secondary structure predictions for WT MSP and C28A,C51A MSP based on analyses of far-UV CD spectra

Protein	Basis sets 1,3,4,6, and 7 calculated% of:				Basis sets 6 and 7 only calculated% of:			
	α-helix	β-sheet	Turn + unordered	Total	α-helix	β-sheet	Turn + unordered	Total
WT (25 °C)	7	36	56	99	4	36	60	99
WT (90 °C)	7	30	62	99	6	25	68	99
WT (Cooled)	5	37	56	99	3	37	59	99
C28A,C51A (25 °C)	5	37	58	100	4	34	63	101
C28A,C51A (90 °C)	7	29	63	99	6	28	65	99
C28A,C51A (Cooled)	5	34	60	99	3	31	65	99

Proteins were assayed in 10 mM KH_2PO_4 buffer at pH 6.0 and 5.7, as calculated from the buffer's temperature coefficients at 25 °C and 90 °C. For a detailed basis set description, see Sreerama and Woody (2000). The numbers shown are averages of results obtained from the basis sets shown and from both CONTIN/LL and CDSSTR methods.

in Lydakis-Simantiris et al. (1999) (data not shown), were used for the WT secondary structure predictions shown in Table 1. Use of basis sets 6 and 7 indicates that at 25 °C, C28A,C51A MSP shows increased levels of random coil and α-helix, along with decreased amounts of β-sheet, when compared to WT MSP. Upon heating to 90 °C, C28A,C51A MSP is predicted to gain random coil and α-helix at the expense of β-sheet; WT MSP at 90 °C is predicted to contain similar fractions of secondary structure, indicating that the values reported here may represent the maximum extent to which MSP, plus or minus the disulfide bond, can lose secondary structure at 90 °C. After cooling to 25 °C, C28A,C51A MSP regains its original secondary structure content. These findings, along with the results of the reconstitution experiments using heated C28A,C51A MSP, indicate that MSP retains its thermostability in the absence of the disulfide bridge.

Analyses of the tertiary structure of C28A,C51A MSP were carried out using near-UV CD spectroscopy and gel filtration chromatography. The intensity of CD signals from aromatic amino acids (Phe, Tyr, Trp) that absorb between 250 and 300 nm is dependent on the local protein environment; hydrophobicity produces stronger signals, while increased hydrophilicity leads to weaker signals (Kelly and Price 1997). Because there are 13 Phe and 8 Tyr residues distributed throughout the MSP sequence, monitoring their near-UV CD signals provides a useful indicator of structural changes in the protein (Tanaka et al. 1989; Shutova et al. 1997; Lydakis-Simantiris et al. 1999a, b; Motoki et al. 2002; Popelkova et al. 2002a, b, 2003a; Wyman and Yocum 2005). The lone Trp residue, located at position 241 near the C-terminus of the MSP primary amino acid sequence, produces an intense near-UV CD signal that has been used to follow changes in this domain's solution structure (Lydakis-Simantiris et al. 1999a, b; Yu et al. 2001; Motoki et al. 2002; Popelkova et al. 2002a, b, 2003a). For WT MSP, the large, well-defined peaks at 292 nm and 285 nm shown in Figure 4 have been attributed to Trp and Tyr residues, respectively, while two smaller, broad peaks at 258 and 264 nm are assigned to Phe (Kelly and Price 1997; Shutova et al. 1997; Lydakis-Simantiris et al. 1999a, b; Motoki et al. 2002; Popelkova et al. 2002a, b, 2003a). As shown in Figure 4, no signals from the Trp or Tyr residues are observed in the near-UV CD spectrum obtained from C28A,C51A MSP; the only signals present are weak Phe peaks centered at 258 nm and 264 nm. In good agreement with these findings, reduced native WT MSP was found to produce a very similar near-UV CD spectrum (Tanaka et al. 1989). An earlier near-UV CD spectrum for C28A,C51A MSP showed two small, broad peaks at 264 and 272 nm (Popelkova et al. 2003b). It appears that those signals were due to noise; a higher concentration of C28A,C51A MSP was used to obtain the near-UV CD spectrum presented in Figure 4, and this resulted in improved signal to noise ratio and more clearly defined peaks for Phe. These data suggest that the structure of C28A,C51A MSP has been modified so that the protein environments containing the Trp and Tyr residue(s) responsible for the near-UV CD signals seen in the WT MSP spectrum at 25 °C have been shifted

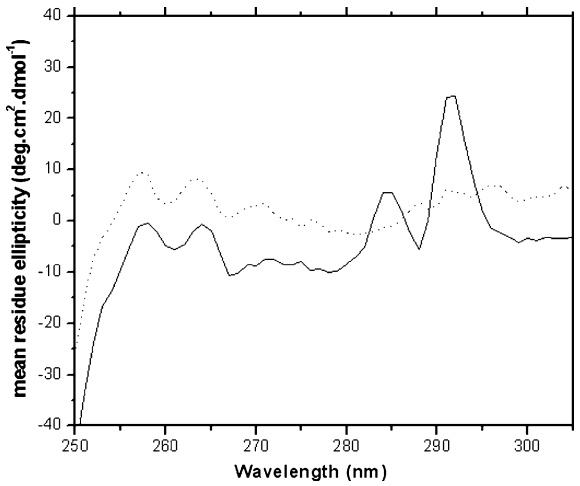

Figure 4. Near-UV CD spectra of WT MSP and C28A,C51A MSP. Near-UV CD spectra were recorded after allowing WT MSP (——) and C28A,C51A MSP (……) to equilibrate to 25 °C. Each spectrum is the average of 20 scans. The protein concentrations were 50.5 μM (WT MSP) and 28.1 μM (C28A,C51A MSP). The buffer in both experiments was 10 mM KH_2PO_4, pH 6.0. Experimental conditions: scan width, 320–250 nm; time constant, 1s; bandwidth, 1 nm; path length, 1 cm; sample volume, 1 ml.

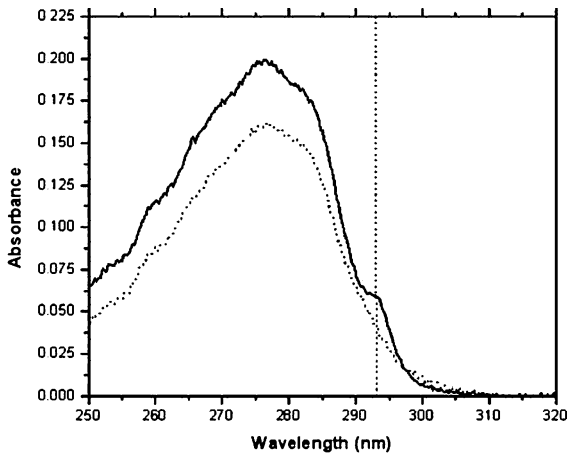

Figure 5. UV absorption spectra of WT MSP and C28A,C51A MSP. The protein concentrations were 12.9 μM (WT MSP, (——)) and 10.6 μM (C28A,C51A MSP, (……)). Both proteins were dissolved in 10 mM KH_2PO_4, pH 6.0 buffer. Experimental conditions: scan width, 320–250 nm; temperature, 25 °C; path length, 1 cm; sample volume, 1 ml. The vertical dotted line denotes 293 nm, the position of the shoulder assigned to Trp.

from hydrophobic to more solvent-exposed domains, resulting in a loss of signal from these aromatic residues. Wild-type MSP heated to 90 °C produces a similar near-UV CD spectrum to that of C28A,C51A MSP at 25 °C (Lydakis-Simantiris et al. 1999b; Popelkova et al. 2003b; data not shown). In this case, the loss of signals from the Trp and Tyr are proposed to arise from increased disorder caused by the elevated temperature.

Further evidence that C28A,C51A MSP is more disordered in solution than WT MSP is evident in a comparison of the UV absorption spectra of these proteins. The intensity of UV absorption by aromatic amino acids in proteins depends on the environment surrounding them (Schmid 1997). Aromatic amino acids in regions of greater hydrophobicity exhibit increased absorption; conversely, when exposed to the solvent, signals from these residues are blue-shifted (Schmid 1997). As shown in Figure 5, WT MSP exhibits a characteristic, well-defined shoulder at 293 nm from Trp (Schmid 1997). This shoulder is absent from the C28A,C51A MSP UV spectrum (Figure 5), indicating that loss of the disulfide bridge shifts this residue to a more solvent exposed environment.

One of the key characteristics of intrinsically disordered proteins is their anomalously large apparent molecular masses (Weinreb et al. 1996; Lydakis-Simantiris et al. 1999; Uversky, 2002a, b). While the *psbO* gene sequence predicts a molecular mass of 26,540 Da for WT MSP (Tyagi et al. 1987), SDS-PAGE gives apparent molecular mass of 33 kDa (Kuwabara and Murata, 1979), and an even larger value (38–41 kDa (Lydakis-Simantiris et al. 1999; Popelkova et al. 2002a, b, 2003a)) is obtained with gel filtration chromatography. The mutant has a predicted molecular mass of 26,476 Da due to replacements of Cys by Ala residues. This was confirmed by MALDI-TOF mass spectroscopy (data not shown). The electrophoretic mobility of the double mutant protein was consistent with a molecular mass of 33 kDa on SDS-PAGE gel electrophoresis (Betts et al. 1996). However, characterization of C28A,C51A MSP by size-exclusion chromatography gave the results presented in Table 2. The double mutant protein exhibits an apparent molecular mass of about 51 kDa, which is larger than WT MSP and some N-terminal MSP mutants (Popelkova et al. 2002a, b, 2003a), and which approaches the molecular masses of some C-terminal truncation mutants of MSP (Lydakis-Simantiris et al. 1999a).

Table 2. Apparent molecular masses of WT and mutants of MSP based on size exclusion chromatography

Protein	Apparent molecular mass (kDa)	Reference
WT	38	This work
ΔT7M	34	Popelkova et al. 2003a
ΔT15M	33	Popelkova et al. 2003a
E246K	41	Lydakis-Simantiris et al. 1999a
C28A,C51A	51	This work
L245@	52	Lydakis-Simantiris et al. 1999a
L245E	62	Lydakis-Simantiris et al. 1999a

Discussion

Intrinsically disordered, or natively unfolded proteins as they are also called, are characterized by smaller than usual amounts of estimated secondary structure, abnormally high estimated molecular masses determined by several techniques (gel filtration, SDS-PAGE, analytical ultracentrifugation), and by a thermostability that is unusual for proteins derived from mesophiles (Weinreb et al. 1996; Dunker et al. 2001; Uversky 2002b). From the available high resolution structural information on these proteins, it appears that in some cases such proteins possess regions of ordered secondary and tertiary structure as well as domains that are disordered (Dunker et al. 2001). The basis for classification of MSP as either an intrinsically disordered protein or as a molten globule is described in Lydakis-Simantiris et al. (1999b) and Shutova et al. (2000), respectively. A significant factor in designating MSP as a molten globule in solution was that its heat resistance or thermostability, could, in theory, be explained by the presence of the disulfide bridge, which was hypothesized to stabilize a hydrophobic core of β-sheet structure (Shutova et al. 2000). Thermostability can be conferred on proteins by the introduction of non-native disulfide bridges, as, for example, in the cases of T4 lysozyme (containing no native disulfide bridges) (Perry and Wetzel 1984; Matsumura et al. 1989), ribonuclease H, whose C-terminal region (residues 88–155) is intrinsically disordered (Kanaya et al. 1991), and xylanase (Wakarchuk et al. 1994). This increased thermostability is hypothesized to be due to decreased conformational entropy in these proteins' peptide backbones that is caused by the new disulfide bridges (Matsumura et al. 1989). However, introduction of disulfide bridges does not always lead to increased protein thermostability, as in the case of addition of disulfide bridges to dihydrofolate reductase (Villafranca et al. 1987) or subtilisin BPN' (Mitchinson and Wells 1989).

Although thermostability is a property of intrinsically disordered proteins, the possibility that the disulfide bridge stabilizes MSP against thermal denaturation, as proposed by Shutova et al. (2000), could not be ruled out. Experiments with C28A,C51A MSP show that the unheated protein rebinds to USW–PS II preparations and catalyzes O_2 evolution activity as reported previously (Betts et al. 1996); however, in our assays, it exhibited both specific and non-specific binding, a behavior that was not seen previously and is probably a consequence of using higher concentrations of the protein in the new reconstitution experiments. When the thermostability of C28A,C51A MSP was tested at 90 °C, the mutant was found to aggregate upon heating, a behavior also detected in native WT MSP when the disulfide bridge was chemically reduced (Tanaka and Wada 1988; Tanaka et al. 1989), and during the purification of C28A,C51A MSP (Betts et al. 1996). However, heated C28A,C51A MSP from both the supernatant fraction and the urea-solubilized aggregated material were reconstitutively active (Figures 1 and 2).

Far-UV CD spectroscopic analysis was used to probe the effects of heating on the secondary structure of C28A,C51A MSP. At high temperature, C28A,C51A MSP showed a loss of β-sheet with modest gains in α-helix and disorder in its secondary structure; however, upon cooling, it regained a substantial portion of its original secondary structure, a behavior also seen in the far-UV CD spectrum of WT MSP upon heating and cooling (Figure 3; Table 1; Lydakis-Simantiris et al.1999b; data not shown). Another characteristic of intrinsically disordered proteins is their extended conformations in solution, which is evidenced by their anomalous mobilities in gel filtration analyses (Weinreb et al. 1996; Lydakis-Simantiris et al. 1999b; Uversky et al. 2000; Dunker et al. 2001; Uversky 2002a, b). While Betts et al. (1996) found that the molecular mass of C28A,C51A MSP was 33 kDa on SDS-PAGE, the gel filtration analyses reported here indicate that it

has an apparent molecular mass of 51 kDa, greater than the value found for WT MSP, and closer in size to some MSP C-terminal mutants (Table 2; Lydakis-Simantiris et al. 1999a, b; Popelkova et al. 2003b). Additional data on the disordered state of C28A,C51A MSP is provided by its near-UV CD and UV absorbance spectra. The well-defined near-UV CD signals from the Trp and Tyr residues, seen in WT MSP, are absent in C28A,C51A MSP, suggesting that these residues (and the domains in which they are located) have been shifted from hydrophobic environments to more hydrophilic surroundings (Figure 4). The UV absorbance spectrum of C28A,C51A MSP, showing a loss of the distinctive Trp shoulder seen at 293 nm in WT MSP, is consistent with exposure of this residue to a more hydrophilic environment (Figure 5).

It is apparent that the presence of an intact disulfide bridge in MSP is necessary for the protein's proper solution structure, most importantly for a hydrophobic C-terminal domain in the vicinity of Trp 241. However, MSP retains its thermostable behavior in the absence of these structural features. It has been shown by isotope editing FT-IR spectroscopy that MSP gains β-sheet at the expense of random coil upon binding to PS II (Hutchison et al. 1998). This finding is supported by a crystal structure of PS II, which indicates that PS II-bound MSP is elongated and contains large amounts of β-sheet (Ferreira et al. 2004). Because C28A,C51A MSP retains its ability to bind specifically to PS II and reconstitute O_2 evolution activity, the presence of a stabilizing disulfide bridge within a hydrophobic core of β-sheet structure in WT MSP does not appear to be necessary for MSP function. These observations also demonstrate that the thermostability of MSP is not dependent on the presence of the disulfide bond to maintain a specific hydrophobic core of β-sheet structure. This would suggest that the solution structure of MSP is probably not related to that of molten globules.

It is interesting to note that C28A,C51A MSP, whose mutations reside near the N-terminus of the protein, has characteristics similar to those of C-terminal MSP mutants; non-specific binding to USW–PS II samples, increased apparent molecular size by gel filtration, and some loss of β-sheet structure in favor of random coil (Betts et al. 1998; Lydakis-Simantiris et al. 1999a, b; Popelkova et al. 2003b). As outlined in Popelkova et al. (2003b), these similarities support the hypothesis of Enami et al. (1998) that there is an interaction between the MSP N- and C-termini in solution, based on the finding that the zero-length, water-soluble cross-linker carbodiimide EDC catalyzed bond formation between a MSP C-terminal residue (E246) to a residue (K48) present in the disulfide loop. In addition, several N-terminal MSP truncation mutants produced by Popelkova et al. (2002a, 2002b, 2003a) also exhibited properties in solution similar to those of C28A,C51A MSP and the MSP C-terminal mutants, suggesting that the disulfide bridge and loop and MSP C-terminus are also in proximity to the MSP N-terminus. These findings were used to generate models for the solution structures of WT MSP and C28A,C51A MSP (Popelkova et al. 2003b).

It has been hypothesized that of the eight Tyr residues present in MSP, only one (Y242), which is highly conserved in all known MSP sequences (De Las Rivas and Heredia 1999; Motoki et al. 2002), is responsible for the near-UV CD Tyr signal detected in WT MSP (Shutova et al. 1997; Motoki et al. 2002; Popelkova et al. 2003a, b). This hypothesis is supported by our finding that although there are a number of Tyr and Phe residues distributed throughout the primary sequence of MSP, only Phe signals are detected in the C28A,C51A MSP near-UV CD spectrum (Figure 4). The absence of Trp and Tyr signals suggests that the environment around W241 and Y242 in C28A,C51A MSP has become more hydrophilic. This is consistent with the report of Zhang et al. (2005) who have shown that one Tyr in MSP is resistant to modification by N-acetylimidazole unless the protein is first unfolded in 8 M urea.

Uversky et al. (2000) have shown that a defining property of intrinsically disordered proteins is the ratio of charged to hydrophobic residues in their primary amino acid sequences. They postulated that these proteins' high ratios of charged to hydrophobic residues resulted in a lack of regular structure (due to internal charge repulsions) that lead to their thermostability, because these proteins are able to undergo changes in solution structure in a more energetically favorable manner upon exposure to extreme changes in temperature. With an appropriate balance of charged to

hydrophobic residues in their sequences, intrinsically disordered proteins can maintain an equilibrium between the forces of charge repulsion (which favor protein unfolding/disorder), and forces that drive protein folding (hydrophobic interactions). Dunker et al. (2001) have proposed that certain amino acids facilitate protein folding, while others create disorder, based on the relative frequencies of amino acids found in ordered and disordered domains of proteins having determined structures. Using these criteria, amino acids were characterized as 'order-promoting' (W, C, F, I, Y, V, L, N), 'neutral' (having no affect on structure formation) (H, T, M, D), or 'disorder-promoting' (A, R, G, Q, S, P, E, K). The percentages of these types of amino acids were determined for the primary sequences of two intrinsically disordered proteins (WT MSP, NACP) and a molten globule protein (human carbonic anhydrase B, according to Shutova et al. (2000)) (Popelkova et al. 2003b). The intrinsically disordered proteins contained higher amounts of disorder-promoting amino acids (55.5 and 60.8%, respectively) and lower levels of order-promoting residues (30.3 and 24.3%), than did carbonic anhydrase B, a 'normally folded' protein which possessed increased amounts of order-promoting amino acids (34.7%) and decreased levels of disorder-promoting residues (48.3%). These calculations suggest that MSP contains greater levels of intrinsic disorder than does a protein selected as a model for molten globule behavior in solution.

While this research, and that of Betts et al. (1996), show that the presence of the disulfide bridge is unnecessary for MSP to effectively restore O_2 evolution activity *in vitro*, it is important to note that it has also been shown that the disulfide bridge must remain intact *in vivo* in order for MSP to function (Burnap et al. 1994). The highly conserved residue C20 (identical to C28 in spinach MSP) of the cyanobacterium *Synechocystis* sp. PCC6803 was mutated into a Ser, which eliminates the disulfide bond that normally formed with C45 (C51 in spinach). *Synechocystis* carrying the C20S mutation accumulated normal levels of MSP mRNA, but failed to accumulate the protein, and as a result exhibited O_2 evolution rates similar to those of the *Synechocystis* Δ*psbO* mutation. From these findings, the authors concluded that while the lack of a disulfide bridge did not affect the stability of MSP mRNA, it did render the gene product subject to increased rates of protein degradation within the cell because MSP could not fold and bind properly to PS II.

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