Structural Studies of the Manganese Stabilizing Subunit in Photosystem II

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ABSTRACT Photosystem II (PSII) is the plant photosynthetic reaction center that carries out the light driven oxidation of water. The water splitting reactions are catalyzed at a tetranuclear manganese cluster. The manganese stabilizing protein (MSP) of PSII stabilizes the manganese cluster and accelerates the rate of oxygen evolution. MSP can be removed from PSII, with an accompanying decrease in activity. Either an *Escherichia coli* expressed version of MSP or native, plant MSP can be rebound to the PSII reaction center; MSP reconstitution reverses the deleterious effects associated with MSP removal. We have employed Fourier transform infrared (FTIR) spectroscopy and solution small angle x-ray scattering (SAXS) techniques to investigate the structure of MSP in solution and to define the structural changes that occur before and after reconstitution to PSII. FTIR and SAXS are complementary, because FTIR spectroscopy detects changes in MSP secondary structure and SAXS detects changes in MSP size/shape. From the SAXS data, we conclude that the size/shape and domain structure of MSP do not change when MSP binds to PSII. From FTIR data acquired before and after reconstitution, we conclude that the reconstitution-induced increase in β-sheet content, which was previously reported, persists after MSP is removed from the PSII reaction center. However, the secondary structural change in MSP is metastable after removal from PSII, which indicates that this form of MSP is not the lowest energy conformation in solution.

INTRODUCTION

In higher plants, algae, and cyanobacteria, photosystem II (PSII) performs the light-driven reduction of plastoquinone and the oxidation of water. The PSII complex is composed of multiple polypeptide subunits, but the integral membrane proteins, D1 and D2, form the heterdimer core of the PSII reaction center and bind most of the redox components necessary for the primary electron transfer reactions. Oxidation of water to molecular oxygen is catalyzed by the oxygen evolving complex (OEC), which contains four manganese atoms. The OEC accumulates the four oxidizing equivalents that are required for water oxidation. These intermediate oxidation states are called the S-states (reviewed in Britt (1996)).

In plants, the integrity and optimal function of the OEC depend on the presence of extrinsic proteins with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) predicted masses of 18, 24, and 33 kDa (Seidler, 1996). The 33-kDa protein or manganese stabilizing protein (MSP) plays a central role in maintaining the integrity and activity of the manganese cluster. Treatment of PSII with calcium chloride or urea extracts MSP; extraction lowers activity and decreases the stability of OEC manganese (Miyao and Murata, 1983; Ono and Inoue, 1983). Reconstitution of MSP reverses these effects (for a review see Bricker and Frankel (1998)). Hydrodynamic (Zubrzycki et al., 1998) and small angle x-ray scattering (SAXS) (Svensson et al., 2002) measurements show that plant MSP has an extended, prolate ellipsoid shape in solution. Several lines of experimentation have suggested that the structure of MSP in solution is unusual for a globular protein (Lydakis-Simantiris et al., 1999; Shutova et al., 2000). For example, to explain the high thermal stability and the unusual hydrodynamic properties of MSP, it was proposed that MSP is a natively unfolded or intrinsically disordered protein (Lydakis-Simantiris et al., 1999).

In solution, Fourier transform infrared (FTIR) spectroscopy (Ahmed et al., 1995; Hutchison et al., 1998; Lydakis-Simantiris et al., 1999) and circular dichroism (CD) spectroscopy (Shutova et al., 1997; Xu et al., 1994) indicate that MSP has a high β-sheet content, although variability in β-sheet content has also been reported (Hutchison et al., 1998). Isotope editing was used to show that MSP undergoes a change in secondary structure when MSP is reconstituted. This change in secondary structure was consistent with an increase in β-sheet content and a decrease in random structure (Hutchison et al., 1998). A reconstitution-induced change in structure has also been described in cross-linking studies (Enami et al., 1998). In addition, manganese stabilizing proteins isolated from different organisms had different solution structures, as assessed in limited proteolysis experiments (Tohri et al., 2002). Recently, it was reported that millimolar concentrations of calcium induced a 7–10% change in MSP secondary structural content (Heredia and De Las Rivas, 2003).

Using secondary structure prediction methods, folding models for MSP have been presented (Bricker and Frankel, 1998; De Las Rivas and Heredia, 1999). Threading methods have been used to suggest a possible two domain, β-sand-

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wich model for MSP (Pazos et al., 2001). Low resolution electron microscopy data also provided insight in the shape and position of MSP in the PSII complex (Boekema et al., 2000; Hasler et al., 1997; Holzenburg et al., 1996; Nield et al., 2002).

Recently, x-ray diffraction studies have given structural information concerning *Synechococcus elongatus* and *Thermosynechococcus vulcanus* PSII (Kamiya and Shen, 2003; Zouni et al., 2001). The structures have been determined at 3.7- and 3.8-Å resolution, respectively (Kamiya and Shen, 2003; Zouni et al., 2001). In the structure of Zouni et al. (2001), electron density has been assigned to MSP, and MSP is a 35-Å β-barrel. In the structure of Kamiya and Shen, 2003, MSP has been assigned as a cylindrical β-sheet structure with a diameter of 20 Å and a length of 45 Å. In Kamiya and Shen (2003), additional electron density, corresponding to a 25-Å loop extending from one end of the cylinder, has also been assigned to MSP. These crystallographic structures are still below atomic resolution, so the position of amino acid side chains in MSP is not known.

In this report, we use x-ray scattering and FTIR spectroscopy to acquire more information concerning the structural alterations that occur when MSP binds to PSII. A direct comparison of experimental scattering data and computed scattering curves (Svergun et al., 1995; Zhang et al., 2000) is used to evaluate specific atomic models for MSP. One novel finding of this work is that the reconstitution-induced change in MSP secondary structure, which was reported previously (Hutchison et al., 1998), is metastable after MSP is removed from PSII. Also, we find that, despite this secondary structural change, MSP domain structure and size/shape are similar when MSP is in solution and when MSP is bound to PSII.

MATERIALS AND METHODS

Purification of MSP from spinach PSII membranes (Anderson et al., 2000; Berthold et al., 1981) was performed using calcium chloride extraction, dialysis, and ion exchange chromatography (Kuwabara et al., 1986). Spinach MSP was expressed in and purified from *Escherichia coli* by methods previously described (Betts et al., 1994; Hutchison et al., 1998). *E. coli* expressed MSP differs from native spinach MSP in the replacement of the transit sequence with an additional methionine at the amino terminus. MSP reconstitution to PSII centers, depleted of native MSP but retaining manganese, was carried out by standard methods (Betts et al., 1994; Hutchison et al., 1998). The MSP concentration was determined from the absorbance at 276 nm using an extinction coefficient of 16 mM^{-1} (Kuwabara et al., 1986; Xu et al., 1994). Purified MSP was concentrated to the range of 0.2–0.3 mM and exchanged into a buffer, containing 5 mM MES (2-(*N*-morpholino)ethanesulfonic acid)-NaOH, pH 6.0, through the use of Centricon-10 centrifugal filter devices (Millipore, Bedford, MA).

The purity and homogeneity of the MSP was assessed through the use of SDS-PAGE and matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. The polyacrylamide gel and protein samples were prepared for SDS-PAGE using the Neville method; to visualize protein bands, the gels were stained using Coomassie (Ouellette et al., 1998; Piccioni et al., 1982). One stained band was observed for the MSP preparations employed here. MALDI-TOF studies were performed at the University of Minnesota Mass Spectroscopy Consortium on a Bruker BiFlexIII (Billerica, MA) instrument as previously described (Svensson et al., 2002). Samples were diluted with 0.1% trifluoroacetic acid and were mixed with a matrix of sinapinic acid. Mass standards of cytochrome c and trypsinogen were used to calibrate the instrument (Svensson et al., 2002). As determined by MALDI-TOF, the measured mass of *E. coli* expressed MSP was 26.63 kDa. This measured mass is consistent with the addition of the N-terminal methionine to the expression product (Oh-oka et al., 1986). For comparison, the mass of native, calcium chloride extracted MSP was 26.54 kDa (Svensson et al., 2002). However, *E. coli* expressed MSP, which had been reconstituted and extracted from PSII, had a measured mass of 26.52 kDa. The difference in mass between reconstituted and nonreconstituted preparations of *E. coli* MSP is attributed to the loss of one amino acid in the primary sequence by an unknown mechanism. Loss of this single amino acid is interesting and is still under investigation, but will not alter the results of our FTIR or SAXS experiments.

FTIR data were acquired by methods previously described (Hutchison et al., 1998) on a Thermo-Nicolet Magna II spectrometer (Madison, WI) using a Harrick (Ossining, NY) temperature control cell, fitted with CaF_2 windows. The spectral resolution was 2 cm^{-1}, and a Happ-Genzel apodization function and one additional level of zero filling were employed. The temperature was maintained at 20.0 $\pm$ 0.2°C. The mirror velocity was 2.53 cm s^{-1}, and 2000 mirror scans were accumulated. Spectral analysis and curve fitting were performed using OMNIC (Thermo-Nicolet), IGOR-PRO (WaveMetrics, Lake Oswego, OR), or GRAMS (Galactic Industries, Salem, NH) software, as described (Hutchison et al., 1998).

X-ray scattering experiments were performed at the BESSRC beamline 12-ID of the Advanced Photon Source (APS) at Argonne National Laboratory. As described (Svensson et al., 2002), the x-ray wavelength was 1.028 Å, and the sample-to-detector distance was set so that the detecting range was $0.004 < q < 0.8$ $Å^{-1}$. Transmission coefficients for the sample and buffer background were measured using a PIN photodiode mounted on the beamstop. The scattering vector, q, was calibrated by reference to the (001) powder diffraction peak at $q = 1.076$ $Å^{-1}$ of a silver behenate standard (Huang et al., 1993). Precautions to prevent radiation damage to the sample include the use of a flow cell and short exposure times of 2 s per image. Data from 10 images were averaged. Calculations and curve fitting were performed with Origin 6.1 (Microcal Software, Northampton, MA). The $P(r)$ distance-distance distribution function was calculated from the scattering data using the program GNOM (Svergun, 1991). Simulation of x-ray scattering curves from protein structures was performed using the computer programs CRYSOL (Svergun et al., 1995) and SCAT2D (Zhang et al., 2000). The distance pair-distribution function, $P(r)$, was also calculated from the coordinates of protein structures, as obtained from the PDB database.

RESULTS AND DISCUSSION

In Fig. 1, we present FTIR data acquired from MSP before (Fig. 1 *A*) or after (Fig. 1 *B*) reconstitution to PSII. In Fig. 1 *A*, MSP was produced in *E. coli*, purified, and then exchanged into a ^2H_2O buffer. In Fig. 1 *B*, MSP was produced in *E. coli* and then reconstituted to PSII. After this reconstitution, MSP was extracted from PSII with $CaCl_2$, and then exchanged into a ^2H_2O buffer (Fig. 1 *B*). The FTIR data in Fig. 1 show the amide I′ band, which is the C=O stretch of the peptide bond in ^2H_2O buffer (Krimm and Bandekar, 1986). Previous work has derived correlations between the frequency of the amide I′ band and the secondary structural content of proteins (reviewed in Surewicz et al., 1993). Therefore, comparison of Fig. 1, *A* and *B*, reveals any changes in MSP secondary structure, which are

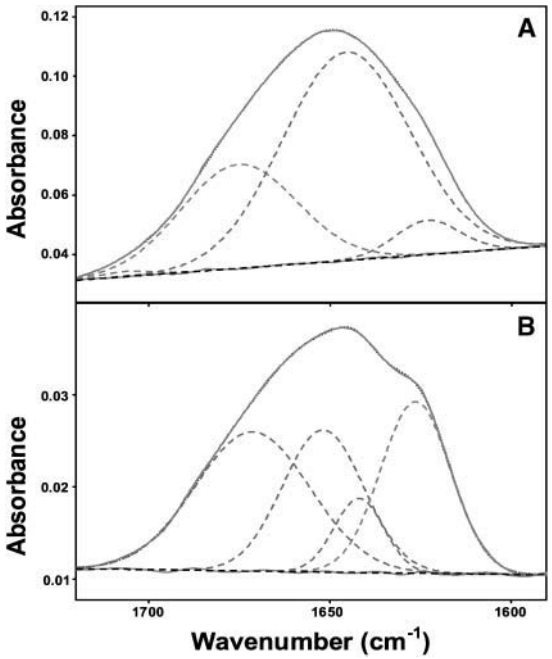

FIGURE 1 The amide I′ band of *E. coli* expressed MSP in (*A*) ^2H_2O buffer or (*B*) ^2H_2O buffer after reconstitution and subsequent calcium chloride extraction from PSII. In *A* and *B*, the solid lines represent experimental data; the dashed lines show one possible fit to the spectra, the baseline, and the residual. See Materials and Methods for spectral conditions.

induced by reconstitution to PSII and which persist after extraction.

Fig. 1 shows that the amide I′ lineshape of MSP differs when reconstituted and extracted samples (Fig. 1 *B*) are compared to samples that were not reconstituted (Fig. 1 *A*). Analysis of the spectral difference observed in Fig. 1 indicates that spectral components at 1675 and 1625 cm^{-1} increase in amplitude when MSP is reconstituted. There is an accompanying decrease in a spectral component at 1640 cm^{-1}. Bands with a frequency of 1640 cm^{-1} in ^2H_2O are assigned to random structure in proteins. Bands with frequencies of 1675 and 1625 cm^{-1} are assigned to β-sheet structural elements (Krimm and Bandekar, 1986; Surewicz et al., 1993). It should be noted that some reconstituted/extracted MSP samples showed less dramatic changes at 1675, 1640, and 1625 cm^{-1} (data not shown). Also, prolonged storage of reconstituted/extracted MSP samples caused loss of the observed, additional β-sheet structure (data not shown). These results suggest that the induced conformational changes are metastable in solution. For this reason, we have not attempted a quantitative analysis of the spectral change, but we qualitatively conclude that increases in MSP β-strand content are associated with reconstitution and extraction. Previous work, using isotope editing, has shown that substantial MSP hydrogen bonding changes occur when MSP is bound to PSII (Hutchison et al., 1998). Therefore, we interpret these new data to show that this hydrogen bonding change persists when MSP is removed from the reaction center, but that the structural change is not completely stable after extraction.

Recently, it has been reported that millimolar concentrations of calcium chloride induce a small (7–10%) decrease in MSP β-sheet content (Heredia and De Las Rivas, 2003). In our work, MSP, as expressed and purified from *E. coli*, was not exposed to high concentrations of calcium chloride, whereas removal of MSP from the PSII reaction center employed high calcium chloride concentrations. However, this difference in calcium exposure cannot explain the FTIR spectral differences reported here. The secondary structural change reported in this work is more dramatic than the calcium-induced change (Heredia and De Las Rivas, 2003), and the ^2H_2O spectrum shown in Fig. 1 *B* is clearly distinguishable from the ^2H_2O spectrum reported in Heredia and De Las Rivas (2003). Note that in our previous work (Hutchison et al., 1998), we reported the FTIR lineshapes acquired from urea-extracted and calcium chloride-extracted MSP, and a substantial spectral difference was indeed observed. However, the spectrum shown in Fig. 1 *B* actually resembles data previously acquired from plant MSP, which had been extracted with urea (Hutchison et al., 1998; Lydakis-Simantiris et al., 1999). Variability in the plant MSP lineshape has been reported previously (Hutchison et al., 1998; Lydakis-Simantiris et al., 1999); this variability may arise from the metastable nature of the reconstitution-induced spectral changes.

Fig. 2 shows the low angle region of the x-ray scattering data acquired from MSP in solution. Two curves (Fig. 2, *A*

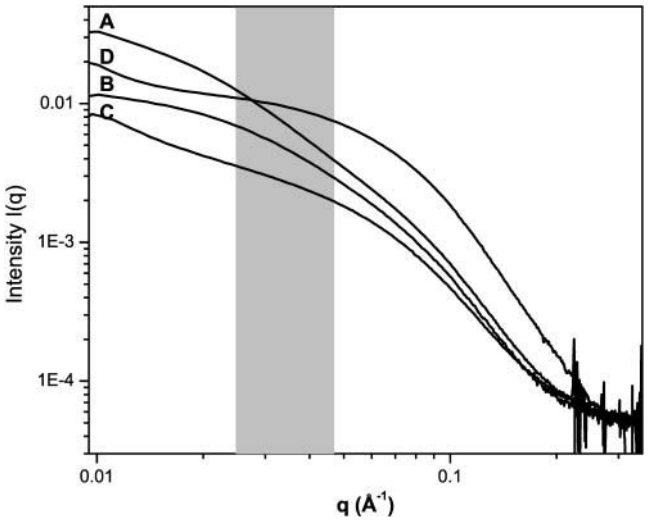

FIGURE 2 X-ray scattering data derived from MSP. In *A*, *E. coli* expressed MSP was purified using two rounds of ion exchange chromatography. In *B*, *E. coli* expressed MSP was purified using two rounds of ion exchange chromatography and one additional gel filtration step. In *C*, *E. coli* expressed MSP was purified, reconstituted, and then calcium chloride extracted from PSII. In *D*, native MSP was calcium chloride extracted from spinach PSII. The shaded area represents the Guinier region. See Materials and Methods for data acquisition parameters.

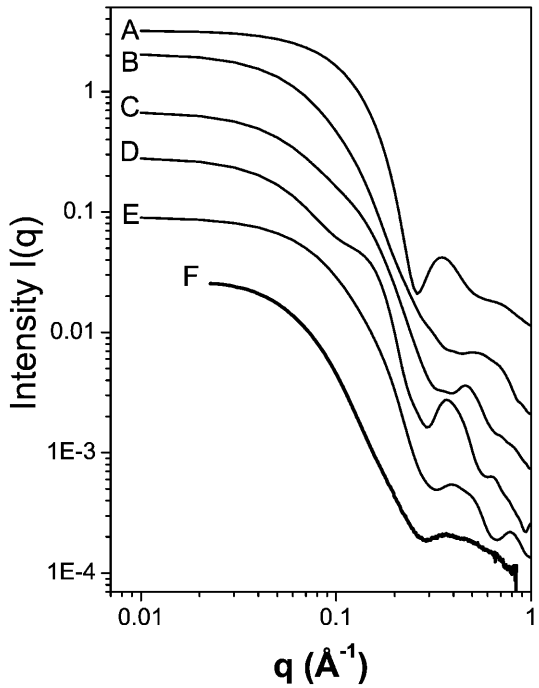

FIGURE 3 X-ray scattering data calculated from known protein structures (*A–E*) and acquired from native MSP, calcium chloride extracted from spinach PSII (*F*). The program CRYSOL was used to generate the x-ray scattering curve from structures in the PDB database (Svergun et al., 1995). The curves represent (*A*) cytochrome c (1HRC), (*B*) cytochrome f lumenal domain (1EWH), (*C*) CD2 T-lymphocyte adhesion glycoprotein (1HNF), (*D*) β-B2-crystallin (2BB2), and (*E*) outer membrane protein A, OmpA (1BXW).

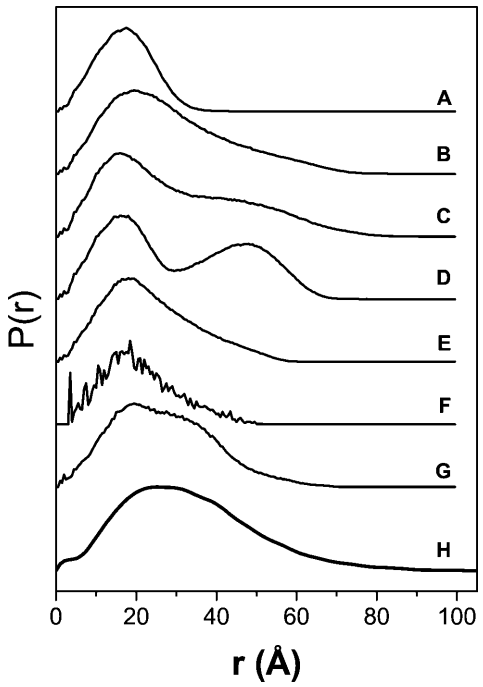

FIGURE 4 The distance pair-distribution function, $P(r)$, for known protein structures (*A–G*) and for MSP (*H*). In *H*, the function was generated from the x-ray scattering data using the program GNOM (Svergun, 1991). In *A–E*, the functions were generated from the PDB structures of (*A*) cytochrome c (1HRC), (*B*) cytochrome f lumenal domain (1EWH), (*C*) CD2 T-lymphocyte adhesion glycoprotein (1HNF), (*D*) β-B2-crystallin (2BB2), and (*E*) outer membrane protein A, OmpA (1BXW). In *F*, the function was generated from assigned MSP density in the *Synechococcus* PSII structure at 3.8-Å resolution (1FE1). The assigned density consists only of C-α carbons and constitutes ~50% of the MSP peptide backbone. In *G*, the function was generated from assigned MSP density in the *Thermosynechococcus* PSII structure at 3.7-Å resolution (1IZL). The assigned density consists of a polyalanine chain and represents ~80% of the MSP peptide backbone.

and *B*) correspond to *E. coli* expressed MSP before reconstitution. In Fig. 2 *A*, MSP was purified from the *E. coli* expression system by the published protocol, which involves two rounds of ion exchange chromatography (Betts et al., 1994; Hutchison et al., 1998). In Fig. 2 *B*, MSP was purified by the published protocol and then subjected to an additional round of gel filtration chromatography. This was performed to remove any small percentage of oligomeric MSP in solution. When Fig. 2, *A* or *B*, is compared to MSP that was reconstituted and then extracted from PSII (Fig. 2 *C*), changes in the x-ray scattering curve are observed.

The shape of the curve in the Guinier region can be used to determine the radius of gyration, which in turn gives information about the shape and size of MSP (Svensson et al., 2002). In *E. coli* expressed MSP, purified with two rounds of ion exchange chromatography (Fig. 2 *A*), the Guinier region is not linear, indicating that the sample is not sufficiently homogeneous for quantitative analysis. When gel filtration is performed (Fig. 2 *B*), a radius of gyration can be determined from the Guinier region, and the value is 38 Å. When MSP has been bound and then extracted from PSII, the radius of gyration is determined to be 33 Å (Fig. 2 *C*). This value is slightly larger than the radius of gyration determined for native, spinach, calcium chloride extracted MSP (Fig. 2 *D*), where a value of 26 Å was reported (Svensson et al., 2002). Note that our previous SAXS measurements (Svensson et al., 2002) have shown that there is no significant difference in the radius of gyration when MSP is extracted with calcium chloride or when MSP is extracted with urea. The results in Fig. 2 are consistent either with a change of MSP size/shape with reconstitution or with some remaining oligomeric heterogeneity in the *E. coli* expressed MSP preparation.

To give more information about MSP tertiary structure, wide angle x-ray scattering data were acquired from native, plant MSP (Fig. 3 *F*). Analysis of wide angle x-ray scattering data gives more detailed information about protein structure (Zhang et al., 2000; Tiede et al., 2002). For comparison, x-ray scattering profiles were calculated from PDB database structures of representative proteins. Proteins with different domain structures were chosen for comparison (Fig. 3, *A–E*). The proteins employed were cytochrome c, which has one α-helical domain (Fig. 3 *A*), cytochrome f, which has one

large and one small β-domain (Fig. 3 *B*), CD2 T-lymphocyte adhesion glycoprotein, which has two equally sized β-domains (Fig. 3 *C*), β-B2-crystallin, which also has two equally sized β-domains (Fig. 3 *D*), and OmpA, which has one β-barrel domain (Fig. 3 *E*). Based on a comparison of the curves in the wide angle region, the domain structure of MSP (Fig. 3 *F*) appears to be closest to that of cytochrome f (Fig. 3 *B*) or OmpA (Fig. 3 *E*). Proteins with two equally sized β-domains, such as CD2 T-lymphocyte adhesion glycoprotein and β-B2-crystallin, are distinguishable from MSP with this approach.

To investigate this conclusion in more detail, the distance pair distribution function was generated, either from protein structures in the PDB database (Fig. 4, *A–G*) or from the wide angle x-ray scattering data on native MSP (Fig. 4 *H*). In addition to the proteins listed above (Fig. 4, *A–E*), the distance pair distribution function was also generated for the MSP subunit, as assigned in the crystal structures of *Synechococcus* (Fig. 4 *F*) and *Thermosynechococcus* PSII (Fig. 4 *G*), respectively. The distribution function for *Synechococcus* PSII contains fewer points, because only a portion of the C-α backbone was assigned in that work (Fig. 4 *F*). Comparison of the data (Fig. 4 *H*) with the distribution functions generated from the PDB structures (Fig. 4, *A–G*) shows that the experimental data, derived from solution MSP, matches well with the function generated from MSP density in *Thermosynechococcus* PSII (Fig. 4 *G*). This comparison demonstrates that the overall domain structure of MSP is the same in solution and as assigned in the crystal structure of *Thermosynechococccus* PSII.

SUMMARY

These data agree with previous results, derived from isotope editing (Hutchison et al., 1998), which showed that MSP hydrogen bonding is altered when MSP is reconstituted to PSII. The hydrogen bonding change in this and in previous work is consistent with an increase in β-structure. This conformational change is observed to persist in a metastable manner after extraction. This result implies that binding to PSII provides the free energy to maintain MSP in a secondary structural form, which is not the lowest energy structure of MSP in solution. These observations can explain previous variability observed in the structure of MSP in solution (Hutchison et al., 1998; Lydakis-Simantiris et al., 1999). This work has also shown that both the size/shape and the domain structure of MSP are similar in solution and as assigned in the crystal structure of cyanobacterial PSII.

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