Cold-Sensitive Assembly of a Mutant Manganese-Stabilizing Protein Caused by a Val to Ala Replacement†

Scott D. Betts, Jeannine R. Ross, Eran Pichersky, and Charles F. Yocum*

Departments of Biology and Chemistry, The University of Michigan, Ann Arbor, Michigan 48109-1048

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ABSTRACT: Photosystem II (PSII) is a multisubunit transmembrane protein complex that oxidizes water and evolves O_2. A tetranuclear manganese cluster associated with integral membrane subunits of PSII catalyzes water oxidation. The 33-kDa water-soluble PSII subunit, or manganese-stabilizing protein (MSP), stabilizes the O_2-evolving manganese cluster and accelerates O_2 evolution. Spinach PSII can be depleted of native MSP under conditions which retain a functional manganese cluster. Reconstition of MSP-depleted PSII with recombinant MSP was equally efficient at 4 and 22 °C. Replacement of Val235 (a conserved residue near the C-terminus of MSP) with Ala inhibited assembly of MSP at 4 °C, but not at 22 °C. Once assembled, [V235A]MSP remained bound to PSII even at 4 °C and in the presence of low concentrations of urea. Results from far-UV circular dichroism spectrometry indicated that [V235A]-MSP was destabilized by low temperature to a greater extent than the wild-type protein. However, the effect of temperature on the secondary structure of both the mutant and wild-type proteins was small compared to the temperature-independent destabilization of secondary structure induced by the mutation. These results demonstrate that the V235A mutation introduces an activation energy barrier for assembly of MSP into PSII, and it is suggested that the mutation acts by inhibiting isomerization of one or more prolyl peptide bonds required for assembly.

Photosystem II (PSII)[1] oxidizes H_2O as a source of electrons for the photosynthetic electron transfer chain, evolving O_2 as a byproduct. Water oxidation is catalyzed by a cluster of four manganese atoms that bind to one or more subunits in the reaction center of PSII [see Debus (1992)]. The 33-kDa manganese-stabilizing protein (MSP), one of three extrinsic subunits of PSII, stabilizes the O_2-evolving manganese cluster. Effects of MSP extraction on PSII activity have been characterized using PSII membrane preparations first washed with 1 M NaCl, which releases the 17- and 23-kDa extrinsic subunits; subsequent exposure of salt-washed PSII membranes to either 1 M $CaCl_2$ or 2−3 M urea specifically releases MSP. However, MSP-depleted membranes resuspended in low Cl^- concentrations (<10 mM) rapidly lose half of the manganese atoms required for O_2 evolution (Kuwabara et al., 1985; Miyao & Murata, 1984a; Ono & Inoue, 1984). In contrast, storage of MSP-depleted PSII in the presence of high, nonphysiological concentrations of Cl^- (>100 mM) preserves a functional manganese cluster (Bricker, 1992; Miyao & Murata, 1984b; Ono & Inoue, 1983).

The Cl^--stabilized manganese cluster is kinetically impaired in MSP-depleted PSII. Specific extraction of MSP from salt-washed PSII membranes reduced the maximum rate of O_2 evolution to 40−50% (Betts et al., 1994; Miyao & Murata, 1984b). A similar result was obtained when *psbO*, the gene encoding MSP, was deleted from the genome of *Synechocystis* sp. PCC6803. Photosystem II in thylakoid membrane samples obtained from a *psbO*-deletion mutant of *Synechocystis* evolved O_2 at 36% of the rate measured for wild-type PSII (with DCBQ as the electron acceptor; Burnap et al., 1992).

The absence of MSP from PSII also reduces the long-term stability of PSII activity. The steady-state rate of O_2 evolution decays faster during prolonged illumination in MSP-deficient PSII both *in vivo* and *in vitro* (Betts et al., 1994; Engels et al., 1994; Mayes et al., 1991; Philbrick et al., 1991). This inhibitory effect of intense light on sustained O_2 evolution activity likely results from the accumulation of strongly oxidizing electron transfer cofactors in PSII due to limited rates of electron donation from the kinetically impaired manganese cluster (Vass et al., 1992). Reconstitution of PSII with MSP *in vitro* suppresses the kinetic lesion in manganese-catalyzed water oxidation caused by MSP extraction (Miyao et al., 1987; Ono & Inoue, 1986).

Several laboratories have demonstrated highly efficient assembly of MSP into PSII *in vitro* (Betts et al., 1994; Kuwabara et al., 1985; Miyao & Murata, 1984b; Seidler, 1994; Seidler & Michel, 1990). Efforts to relate the various functions of MSP to its structure have identified two structural features, both near the N-terminus, that are required for binding of the subunit to PSII: an intramolecular disulfide bridge, which forms between Cys28 and Cys51 (Tanaka & Wada, 1988), and the first 16 amino acids of the polypeptide (Eaton-Rye & Murata, 1989). Seidler (1994) observed an

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[1] Abbreviations: Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; BSA, bovine serum albumin; CD, circular dichroism; Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid disodium salt; MES, 2-(*N*-morpholino)ethanesulfonic acid; MSP, Manganese-stabilizing protein; OEC, O_2-evolving complex; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; pET, plasmid for expression with T7 RNA polymerase; preMSP, *psbO* gene product and precursor polypeptide of MSP; *psbO* cDNA clone encoding preMSP; PS, photosystem; SDS, sodium dodecyl sulfate; TMA^+, tetramethylammonium; Tris, tris(hydroxymethyl)aminomethane.

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inhibitory effect on MSP assembly when a six-residue His tail extended the C-terminus of spinach MSP. By comparison, an even longer extension of the N-terminus, which also included six contiguous His residues, had no effect on assembly of MSP into PSII. Here we report the characterization of an MSP mutant with a conditional assembly defect induced by a mutation near the C-terminus. This mutant assembled efficiently into PSII when *in vitro* reconstitution experiments were carried out at 22 °C; however, assembly was inhibited at 4 °C. The mutation was identified as a single amino acid substitution of Ala for a conserved Val near the carboxy terminus of the protein. The effect of this mutation was examined with respect to MSP folding, assembly, and function.

EXPERIMENTAL PROCEDURES

Cloning of Spinach psbO. Spinach *psbO* was amplified by PCR from a spinach cDNA library (gift of W. Gruissem and J. Norita) using synthetic primers whose sequences were based on the nucleotide sequence of *psbO* (Tyagi et al., 1987). An *Nco*I restriction cloning site was introduced at the position of the translation initiation codon, an *Eco*R1 restriction cloning site was introduced immediately upstream of the *Nco*I site, and a second *Eco*R1 site was introduced in the 3′ noncoding sequence. No changes were made to the coding sequence.

The PCR product was cloned into the TA cloning vector, pCRII (Invitrogen), and the resulting construct was used to transform *Escherichia coli* XL1. Transformants were screened by dideoxy DNA sequencing (Sanger et al., 1977). A clone of wild-type *psbO* was identified; its DNA sequence was identical to that reported by Tyagi et al. (1987). A second clone of spinach *psbO* was obtained with a point mutation most likely caused by a PCR error. The mutation converted codon 235 from GTG to GCG, encoding Ala instead of the wild-type residue Val.

The two cDNA clones were subcloned into the *Nco*I and *Eco*RI sites of the expression vector, pET8c (Studier et al., 1990). The resulting pET(psbO) constructs were used to transform the *E. coli* expression strain BL21(DE3)pLysS. Conditions for bacterial growth, induction of *psbO* expression, and cell storage were the same as described previously (Betts et al., 1996). The *psbO* gene encodes preMSP, which when expressed under these conditions is processed efficiently and correctly by *E. coli*, yielding the mature polypeptide (Betts et al., 1994).

Purification and Folding of MSP. Inclusion bodies were purified from cell lysates and dissolved in urea as described previously (Betts et al., 1994). MSP was purified from the supernatant fraction of the solubilized inclusion bodies by FPLC anion-exchange chromatography. The column (6-mL Resource Q, Pharmacia) was equilibrated with 20 mM Bis-tris (pH 6.4), 3 M urea, 5% betaine, and 10 mM NaCl (buffer A). A NaCl gradient was applied to the column, and eluted MSP (in ≈100 mM NaCl) was diluted with buffer A to 1 mg/mL. The concentration of MSP was estimated from the absorbance at 276 nm using an extinction coefficient of 16 mM^{-1} (Xu & Bricker, 1992).

Urea was removed from MSP preparations by dialysis. The first two dialysis steps (2−3 h each, 4 °C) were against 15 volumes of 100 mM Tris (pH 8) containing 100 mM NaCl. The final dialysis step (4 °C) was against 20 volumes of 50 mM MES (pH 6) containing 10 mM NaCl. Insoluble material was removed from the retentate by centrifugation (48000*g*, 4 °C, 10 min), and MSP was purified from the supernatant fraction by FPLC anion-exchange chromatography (1−mL Mono-Q column, Pharmacia). The column was equilibrated with 50 mM MES (pH 6), 10 mM NaCl, and 5% betaine. A NaCl gradient was applied to the column, and the major peak contained pure MSP in about 100 mM NaCl. The concentration of MSP used in reconstitution experiments was determined by the Lowry assay (Lowry et al., 1951). Sucrose (0.4 M) was added and samples were stored at −70 °C.

Purification of PSII Membranes. Photosystem II membranes were isolated from thylakoids by detergent extraction according to the method of Berthold et al. (1981) with three modifications (Ghanotakis & Babcock, 1983; Ghanotakis et al., 1984a,b). The three extrinsic proteins were extracted from PSII membranes as described in Betts et al. (1994). The 17- and 23-kDa extrinsic subunits were released from PSII membranes by incubation in 2 M NaCl, and native MSP was extracted from salt-washed PSII membranes by incubation in 2.6 M urea/200 mM NaCl.

Reconstitution of PSII and Measurement of O_2 Evolution. Urea-washed PSII membranes were incubated with either wild-type or [V235A]MSP for 30 min at 4 °C and then transferred to 22 °C for an additional 1 h. Reconstitution medium included 50 mM MES-NaOH (pH 6), 0.4 M sucrose, 20 mM $CaCl_2$, 60 mM NaCl, 100 μg BSA/mL, 2% betaine (w/v), and MSP. Photosystem II membranes were present at 0.2 mg of Chl/mL (1 μM PSII reaction center complexes based on 250 Chl/PSII). Reconstitution mixtures at 4 or 22 °C were diluted 20-fold (final Chl concentration was 10 μg/mL) into 0.4 M sucrose, 50 mM MES-TMAOH (pH 6), 60 mM TMACl, and 20 mM $CaCl_2$ (SMTC) equilibrated to room temperature. The assay buffer (SMTC) was supplemented with 100 μg of BSA/mL and the electron acceptor, DCBQ.

Oxygen evolution was measured polarographically using a Clark-type O_2 electrode (YSI-4004) at 25 °C. The rate of steady-state O_2 evolution was measured 10−30 s after the start of saturating illumination in the presence of 300 μM DCBQ. The stability of O_2 evolution activity ("O_2 yield") was measured as the total O_2 produced during 4 min of continuous illumination (80% of saturation) in the presence of 600 μM DCBQ.

Analysis of Reconstituted PSII Membranes by SDS−PAGE and Densitometry. PSII-bound MSP was quantified by laser densitometry of Coomassie-stained polyacrylamide gels. PSII membranes were sedimented to remove unbound MSP (12000*g*, 4 °C, 10 min) and washed once in assay buffer. Samples were analyzed by SDS−PAGE (10% acrylamide/4.7 M urea) according to the method of Piccioni et al. (1982) using the Neville buffer system. Coomassie-stained gels were scanned using a laser densitometer (LKB 2222-010 UltroScan XL). Absorbance of the laser beam at 663 nm was recorded, and the amount of MSP in PSII samples was quantified by digital integration of densitogram peaks. Within the range of MSP concentrations analyzed, the relationship between staining intensity and protein concentration was linear (data not shown).

Circular Dichroism Spectrometry. MSP was prepared for CD spectroscopy by gel filtration chromatography (Superose-12, HR 10/30 column, Pharmacia) in 10 mM KH_2PO_4−

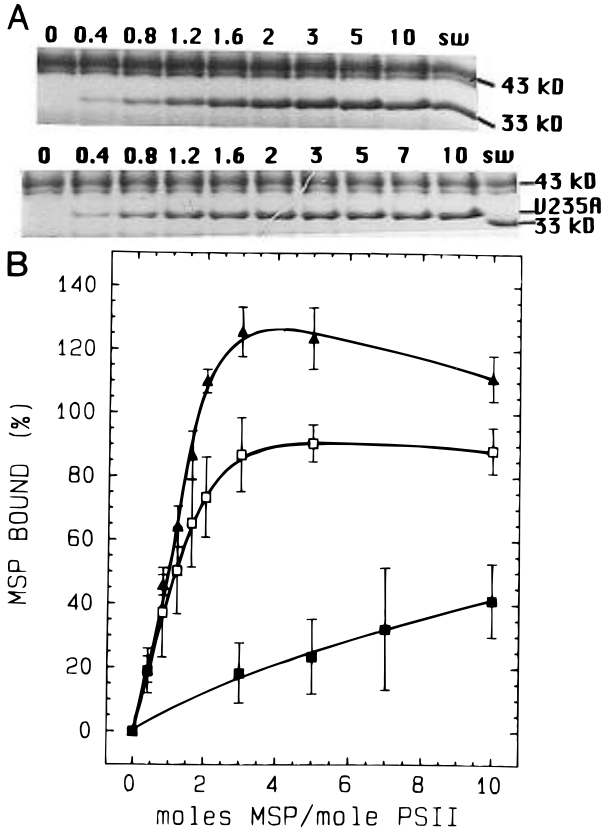

FIGURE 1: Assembly of wild-type MSP and [V235A]MSP at 4 and 22 °C. (A) SDS−PAGE analysis of urea-washed PSII reconstituted at 22 °C with wild-type MSP (top) and [V235A]MSP (bottom). Numbers above lanes indicate moles of MSP added per mole of PSII; sw, salt-washed (control) PSII. (B) Densitometric analysis of MSP in polyacrylamide gels (examples are shown in panel A). The amount of MSP bound to reconstituted PSII is plotted as the percentage of native MSP retained by salt-washed PSII (=100%). Triangles, wild-type MSP assembled at 4 and 22 °C; solid squares, [V235A]MSP assembled at 4 °C; open squares, [V235A]MSP assembled at 22 °C. Each symbol represents the average value from 2−4 replicate experiments. Error bars = ±1 standard deviation.

NaOH (pH 6) at 22 °C. Spectra were obtained using an AVIV 62 DS CD spectrometer. The concentration of MSP following gel filtration was determined using an extinction coefficient of 16 mM^{-1} (Xu & Bricker, 1992). The cell path length for CD measurements was 1 mm with a 300 μL sample volume. The concentration of MSP was 10 μM. Data were collected every 1 nm with a bandwidth of 1.5 nm and a 5 s time constant.

RESULTS

V235A Causes a Cold-Sensitive Assembly Defect. Binding curves for wild-type MSP and [V235A]MSP were obtained following reconstitution of urea-washed PSII at 4 and 22 °C. Both proteins assembled efficiently into PSII at 22 °C; saturation was observed in samples with 2−3 mol of MSP added per mol of PSII (Figure 1A,B). When reconstitutions were carried out on ice (4 °C), assembly of wild-type MSP proceeded efficiently, but assembly of the mutant protein was strongly inhibited (Figure 1B). Saturation of MSP binding sites with the mutant protein at 4 °C required the addition of 20−30 mol of the protein per mol of PSII (data not shown).

The amount of bound wild-type MSP exceeded the amount of native MSP present in samples of control salt-washed PSII

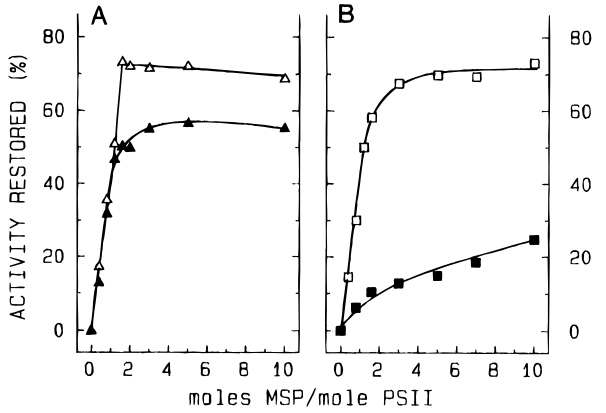

FIGURE 2: Analysis of the rate of steady-state O_2 evolution. (A) Urea-washed PSII + wild-type MSP. (B) Urea-washed PSII + [V235A]MSP. Samples were assayed following consecutive reconstitution incubations at 4 (solid symbols) and 22 °C (open symbols). A *y*-value of zero corresponds to the residual activity of urea-washed PSII samples (see Table 1), and a *y*-value of 100% corresponds to the activity of control salt-washed samples. Each symbol represents the average of 4−5 experiments.

by about 25% (Figure 1B). This apparent net gain of MSP following extraction and reconstitution can be attributed in part to the salt-wash treatment used to prepare control PSII membranes; this treatment extracts some MSP as well as all of the 17- and 23-kDa proteins (data not shown). As a result, a small fraction of MSP binding sites may be vacant in the control (salt-washed) PSII preparations. By comparison to the binding curve for wild-type MSP, urea-washed PSII bound about one-third less [V235A]MSP (Figure 1B; estimate derived from scanning of Coomassie-stained gels). This difference may indicate that the mutant protein does not bind stably to a subpopulation of MSP binding sites. Alternatively, the difference may simply result from non-stoichiometric staining of the wild-type and mutant proteins by Coomassie blue.

The mutant and wild-type proteins were equally effective at restoring the steady-state rate of O_2 evolution following reconstitution of urea-washed PSII at 22 °C (Figure 2). The apparent increase in the maximum activity restored by wild-type MSP after raising the reconstitution temperature to 22 °C (Figure 2A) was primarily due to the lower activity baseline. The activity baseline ($y = 0$) in Figures 2 and 3 represents the residual activity of urea-washed PSII, which was more susceptible to inactivation during 22 °C incubation (in the dark) as compared to salt-washed or reconstituted PSII (Table 1).

After reconstitution at 22 °C, the mutant protein was slightly less effective than the wild-type protein when PSII activity was measured as the total O_2 produced during 4 min of illumination (O_2 yield assay; Figure 3). Following reconstitution at 4 °C, the mutant protein was apparently more effective at stabilizing O_2 evolution activity (Figure 3B) than it was at accelerating O_2 evolution (Figure 2B). This effect may result in part from additional binding of [V235A]MSP to PSII after dilution from 4 °C into assay buffer at 25 °C. The increased duration of the O_2 yield assay may allow sufficient time for [V235A]MSP to assemble and thus stabilize activity before prolonged exposure to the light causes inactivation.

Functional analyses of PSII reconstituted with the wild-type and mutant proteins are thus consistent with the binding

Table 1: O_2 Evolution Activity Following Reconstitution at 4 and 22 °C

PSII	added[a]	rate[b] 4 °C	rate[b] 22 °C	O_2 yield[c] 4 °C	O_2 yield[c] 22 °C
salt-washed	none	100% (397 ± 55)	100% (379 ± 51)	100% (60 ± 9)	100% (53 ± 6)
urea-washed	none	47%	38%	30%	23%
urea-washed	wild-type	77%	83%	77%	81%
salt-washed	none	100% (424 ± 26)	100% (409 ± 42)	100% (58 ± 8)	100% (62 ± 11)
urea-washed	none	42%	38%	26%	21%
urea-washed	V235A		82%		72%

[a] Reconstituted with 5 mol of MSP per mol of PSII. [b] μmol of O_2·(mg of Chl^{-1})·h^{-1}. [c] μmol of O_2 evolved per mg of Chl during a 4 min assay.

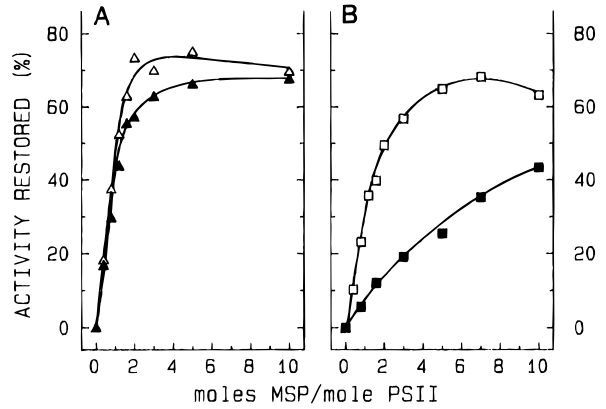

FIGURE 3: Analysis of the stability of O_2 evolution activity. (A) PSII + wild-type MSP. (B) PSII + [V235A]MSP. Treatments and symbols were the same as in Figure 2. Activity was assayed as the total O_2 evolved during 4 min of continuous illumination (O_2 yield). A *y*-value of zero corresponds to the residual activity of urea-washed PSII samples (see Table 1), and a *y*-value of 100% corresponds to the activity of control salt-washed samples. Each symbol represents the average of 2−4 experiments.

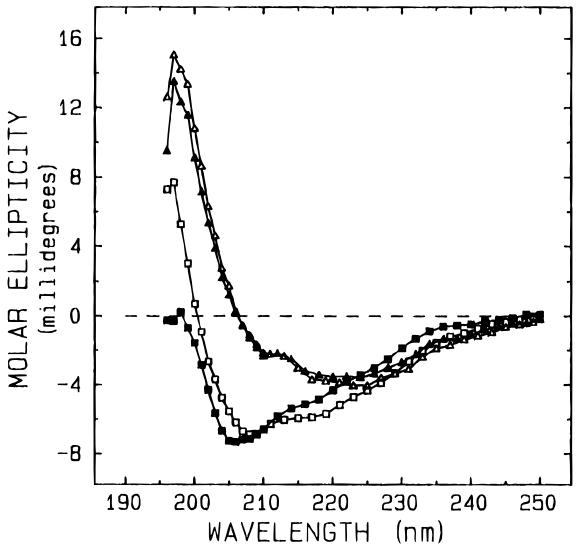

FIGURE 4: Structural comparison of wild-type and [V235A]MSP by far-UV CD. Each spectrum represents the average of 10 spectra scanned consecutively at constant temperature. Triangles, wild-type MSP; squares, [V235A]MSP. Solid symbols, 4 °C; open symbols, 25 °C.

results. Under all conditions tested the activity curves shown in Figures 2 and 3 closely paralleled their corresponding binding curves in Figure 1. We conclude that [V235A]MSP binds to PSII in a functional conformation at both the low and the high temperatures.

V235A Destabilizes Monomeric MSP. The V235A mutation affected the structure of MSP under a wide range of conditions. The mutation caused a reduction in the electrophoretic mobility of MSP in polyacrylamide gels containing 4.7 M urea (Figure 1A). However, the mutant and wild-type proteins comigrated when the polyacrylamide gel included 8 M urea (data not shown). Comigration of the two proteins under strongly denaturing conditions supports the interpretation that the difference in mobility observed in 4.7 M urea gels is due to the presence of residual structure in the wild-type protein. The greater compactness of the wild-type protein under these conditions would explain its faster migration through polyacrylamide gels.

The CD spectra of the wild-type and mutant proteins were compared to determine possible temperature-dependent effects of the V235A substitution on the secondary structure content of MSP. The CD spectra obtained at 25 °C indicate that [V235A]MSP was partially denatured (Figure 4). The ellipticity of the mutant protein was more negative than that of the wild-type protein at wavelengths below about 225 nm. Also, the mutation caused a decrease in the intensity of the positive CD band with a peak at about 197 nm. By comparison to reference CD spectra (Johnson, 1990), these differences most likely reflect an increased content of random coil.

The CD spectra of both wild-type and mutant proteins obtained at 4 °C differed in the same respects from their corresponding CD spectra obtained at 25 °C. At the lower temperature, the ellipticity of both wild-type and mutant proteins was less negative at wavelengths above 210 nm and more negative at lower wavelengths (Figure 4). These changes in the CD spectra at low temperature indicate increased content of random coil, consistent with the well-documented phenomenon of cold denaturation of proteins (Privalov, 1990). The effect of low temperature on the CD spectra was much more pronounced for [V235A]MSP than for wild-type MSP, consistent with the presence of a destabilizing mutation. However, the effect of temperature on the CD spectrum of the mutant protein was small relative to the temperature-independent effect of the amino acid replacement.

[V235A]MSP Remains Tightly Bound to PSII at 4 °C. The effect of the V235A mutation on the binding interaction between MSP and PSII was examined by analyzing the amount of MSP released from reconstituted PSII at various urea concentrations (Figure 5). The mutation caused a displacement of the subunit dissociation curve toward lower urea concentrations; consequently, 50% of the mutant protein was extracted in 1.2 M urea, while 1.6−1.7 M urea was required to extract the same amount of wild-type MSP. This result demonstrates that the mutation destabilized the binding conformation of MSP, at least on ice. Significantly, however, [V235A]MSP remained bound to PSII at 4 °C, a temperature which strongly inhibited its assembly.

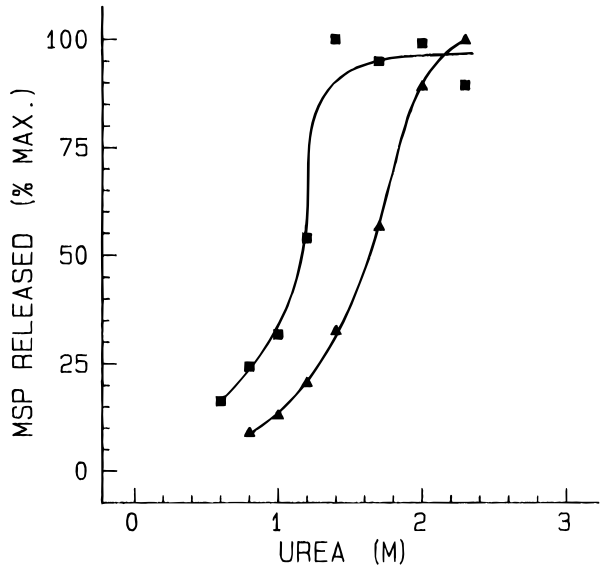

FIGURE 5: Urea dissociation of wild-type MSP and [V235A]MSP from PSII. Reconstituted PSII membranes were incubated at 4 °C for 1 h (0.5 mg of Chl/mL, 200 mM NaCl) in different urea concentrations. The amount of MSP released from PSII was determined by densitometry following SDS−PAGE analysis of supernatant fractions. Triangles, wild-type MSP; squares, [V235A]-MSP. Each symbol represents the average of two experiments.

DISCUSSION

The V235A Mutation Raises the Activation Energy Barrier for MSP Assembly. Substitution of Val235 with Ala induced a cold-sensitive defect in assembly of MSP into PSII. The presence of this mutation inhibited, but did not block, assembly at low temperature. Results from CD spectroscopy indicate that the effect of temperature on the structures of wild-type and [V235A]MSP was relatively small compared to the temperature-independent destabilization caused by the amino acid replacement. However, the effect of temperature on the solution structure of MSP, though small, was still significant. This result is therefore consistent with the possibility that Ala235 causes a cold-sensitive folding or stability defect in MSP.

Once bound, the mutant subunit remained tightly associated to PSII at the same low temperature which inhibited its assembly. The simplest interpretation of this result is that Ala235 destabilizes—at both 4 and 22 °C—a structural feature in MSP which normally facilitates assembly at low temperature. This structural feature is apparently dispensible both for assembly at 22 °C and for stable binding to PSII at 4 and 22 °C. A comparison of the structural context of Val235 in all available MSP sequences is shown in Table 2. The boxed amino acids in the spinach sequence were predicted as a β-sheet in the structural model proposed by Xu et al. (1994). Substitution of Val235 by Ala may destabilize this β-sheet relatively independent of temperature; the resulting loss of secondary structure content would be consistent with the CD data.

Possible Mechanism of the Cold-Sensitive Assembly Defect. The results presented here support the hypothesis that the V235A mutation acts by inhibiting isomerization of one or more essential prolyl peptide bonds in MSP. *In vitro* studies have shown that nonenzymatic isomerization of peptide bonds preceding Pro residues is slowed considerably at low temperature; for example, the half-time of Pro isomerization at 0 °C is about 20 min (Creighton, 1986).

Table 2: Structural Context of Val235[a]

	225	235	245
S. oleracea[b]	SDTDLGAKVPKD	V KIEGVWYAQL	EQ
S. tuberosum[c]	--------T---	- ----I-----	-S
P. sativum[d]	--------A---	- ----------	-S
L. esculentum[e]	--------T---	- ----I-----	-S
A. thaliana[f]	----------	- --Q----G--	-*
T. aestivum[g]	-----E-**---	- ----------	-SN
C. reinhardtii[h]	--------P---	I -VT-L-----	K-
E. gracilis[i]	----------	I -TS------I	SPSK
Synechocystis sp.[j]	--------E-L-	- -VR-IF--RV	DTDV
Anabaena sp.[k]	-------DE--E	- --R-IF-ARV	-*
A. nidulans[l]	----M-G-EAV-	- -LV-QF--RI	-PADA

[a] All sequences are shown through the C-terminus. The boxed sequence indicates predicted β-sheet (Xu & Bricker, 1994). A dash (−) indicates an identical amino acid, and an asterisk (*) indicates a gap in the sequence. [b] Tyagi et al. (1987). [c] van Spanje et al. (1991). [d] Wales et al. (1989). [e] Ko et al. (1990) and Betts et al. (1994). [f] Meadows et al. (1991). [g] Görlach et al. (1993). [h] Mayfield et al. (1989). [i] Shigemori et al. (1994). [j] Philbrick and Zilinskas (1988). [k] Borthakur and Haselkorn (1989). [l] Kuwabara et al. (1987).

Results from gel electrophoresis may also be explained by an effect of the mutation on Pro isomerization. Although [V235A]MSP migrated more slowly through polyacrylamide gels containing 4.7 M urea, it comigrated with wild-type MSP in gels containing 8 M urea. The presence of two or more discrete gel bands of MSP that resolve into a single band in strongly denaturing gels would be predicted, for example, if a sample of wild-type MSP consisted of a mixture of molecules, some containing all correct Pro isomers (MSP has 14 Pro) and some containing one or more incorrect Pro isomers (Creighton, 1986; Creighton & Pain, 1980). Consistent with this prediction, we have observed accumulation of a nonnative form of wild-type MSP which comigrates with [V235A]MSP (data not shown). This slow-migrating form of MSP has not been detected in samples purified from spinach or in inclusion bodies purified from *E. coli*; rather, it only accumulates during renaturation of MSP purified from inclusion bodies. The slow-migrating form of wild-type MSP may represent a stable, misfolded conformation of the protein which accumulates only *in vitro*. The native and nonnative gel bands of wild-type MSP resolved into a single band when gels contained 8 M urea (data not shown), as expected if one or more incorrect Pro isomers are responsible for the low-mobility band.

The V235A mutation could act by inhibiting isomerization of an essential prolyl peptide bond in MSP, either as a direct or as an indirect consequence of the structural destabilization induced by the mutation. If a particular Pro isomer is required for assembly and/or binding, then assembly should be inhibited at low temperature, as demonstrated here for [V235A]MSP. Increased temperature would accelerate isomerization, and the correct Pro isomer would be trapped upon assembly. Such a temperature-dependent increase in the fraction of MSP molecules containing the correct Pro isomer required for assembly and/or binding would not necessarily affect the CD spectrum, consistent with our results.

Comparison to Mutations That Cause Temperature-Sensitive Folding and Assembly Defects in Other Proteins. A defect similar to that caused by [V235A]MSP was observed in studies on an *E. coli* mutant exhibiting cold-sensitive assembly of ribosomal subunit L3 (Lhoest & Colson, 1981). The defect was traced to the failure to

methylate a specific Gln residue in the affected ribosomal protein. The unmethylated protein assembled into ribosomes at the nonrestrictive temperature, and ribosomes containing the unmodified subunit displayed normal stability and function.

Additional evidence for the sensitivity of folding and/or assembly processes to small changes in side-chain bulk comes from the genetic analysis of folding of the P22 tailspike protein. King and co-workers (1987) have identified numerous temperature-sensitive folding (tsf) mutations that block folding and assembly of the trimeric tailspike at the restrictive (high) temperature. Two intragenic mutations were isolated as suppressors of numerous such tsf mutations and were subsequently identified as a substitution of Val for Ala at one site and a substitution of Ala for Val at the other site (Fane et al., 1991). The tsf mutations destabilize the partially folded, unassembled tailspike subunit, and the second-site suppressor mutations apparently act by restoring stability to the monomer (Beissinger et al., 1995; Mitraki & King, 1992). By comparison to [V235A]MSP, the presence of Val at residue 235 in wild-type MSP stabilizes a conformation that is apparently not required for assembly but rather facilitates the process under conditions (in this case, low temperature) that destabilize the structure of MSP in solution.

The structural context of the tsf suppressor mutations in the tailspike protein is similar to that predicted for Val235 in MSP. The secondary structure of MSP and the tailspike protein consist predominantly of β-sheet (Steinbacher et al., 1994; Xu et al., 1994). The sites of the suppressor mutations in the tailspike protein are located, in one case, between two β-sheets, and, in the other case, within a β-sheet. As shown in Table 2, Val235 is predicted as the first amino acid in a β-sheet. The common feature in these diverse systems is the temperature-sensitive effect on assembly caused by the addition or elimination of one or two methyl groups from the side chains of specific amino acids. Such subtle changes in side-chain bulk apparently destabilize structures in unassembled subunits that are required for efficient assembly, but not for stable binding, under extreme conditions.

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