Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 8230–8234, December 1987
Biochemistry

Nucleotide sequence of the gene from the cyanobacterium *Anacystis nidulans* R2 encoding the Mn-stabilizing protein involved in photosystem II water oxidation

(O_2 evolution/Mn-binding protein/*woxA*/protein processing/λgt11)

T. KUWABARA*†, K. J. REDDY‡, AND L. A. SHERMAN‡

‡Division of Biological Sciences, Tucker Hall, University of Missouri, Columbia, MO 65211; and *Department of Chemistry, Faculty of Science, Toho University, Miyama, Funabashi 274, Japan

Communicated by E. R. Sears, July 27, 1987

ABSTRACT **The gene for the Mn-stabilizing protein (MSP; the so-called extrinsic 33-kDa protein) that is involved in photosystem II water oxidation was cloned and sequenced from the genome of the cyanobacterium *Anacystis nidulans* R2. The gene (here designated *woxA*) was shown to be present in a single copy. The deduced amino acid sequence indicated that the translation product consisted of 277 amino acid residues with a M_r of 29,306. The comparison of the sequence with that of mature MSP from spinach chloroplasts suggested that the translation product is a precursor whose amino-terminal 28 amino acid residues represent the signal peptide for the protein to cross the thylakoid membrane into the lumen. The length of the putative signal peptide was less than half that of the transit peptide for thylakoid-lumenal proteins of higher plants, whereas the structural profile of the putative signal peptide was similar to that of the carboxyl-terminal portion of the higher plant transit peptides. The amino acid sequence of the mature *A. nidulans* R2 MSP showed rather low homology (48–49%) to higher plant MSPs, but the conserved amino acid residues appeared to be clustered. Five clusters were tentatively assigned, in which the homology values were in a range of 66–70%. Domains essential for the functioning of MSP are expected to be situated in these clusters. It is of note that the two cysteine residues in MSP were conserved, and the disulfide linkage between them may play an important role in maintaining the tertiary structure of MSP.**

Photosynthetic water oxidation is closely associated with photosystem II (PSII) activity, and many of the structural components responsible for this mechanism are located at the lumenal surface of the thylakoid membrane. The minimum unit capable of photooxidizing water seems to contain five intrinsic proteins and one extrinsic protein (1–3)—namely, the 47-kDa and 43-kDa chlorophyll-a-binding proteins, the D1 and D2 proteins, cytochrome b_{559}, and the Mn-stabilizing protein (MSP; the so-called extrinsic 33-kDa protein). The MSP is involved in water oxidation but not in the photochemical charge separation or in electron transport on the reducing side of PSII (4). Biochemical studies on O_2-evolving PSII preparations suggest that MSP binds to the putative Mn-binding protein and keeps two of the four Mn atoms associated with PSII (5). The intrinsic Mn-binding protein has not yet been identified but may be the D1 or D2 protein (6). The MSP seems to stabilize the Mn cluster and allows the water-oxidizing center to operate with the best efficiency (5). The spinach MSP is physicochemically and chemically well characterized (7), and its amino acid sequence is reported to have partial homology to the putative Mn-binding site of bacterial Mn superoxide dismutases (8). In higher plants, it is suggested that the MSP is encoded by nuclear DNA and that the precursor synthesized in the cytoplasm is posttranslationally transported into the chloroplast (9, 10). The precursor must undergo at least two processing steps to reach its final destination, the thylakoid lumen (11).

Cyanobacterial MSP has been characterized mostly in reference to the spinach MSP. The MSPs of *Synechococcus vulcanus* and spinach are functionally interchangeable to some extent in the reconstitution of water oxidation (12). The cyanobacteria *Phormidium laminosum, Synechococcus leopoliensis*, and *Anabaena variabilis* possess a polypeptide of about 33 kDa that cross-reacts to antiserum raised against spinach MSP (13). However, very little is known as to the molecular characteristics, biogenesis, or processing of cyanobacterial MSP. In this study, we have cloned and sequenced the MSP gene (termed *woxA*, for water oxidation) of the cyanobacterium *Anacystis nidulans* R2.§ From the deduced amino acid sequence, we conclude that this protein is synthesized as a precursor with a 28-amino acid signal peptide that is responsible for transit across the thylakoid membrane into the lumen. The structures of this transit peptide and the mature protein are compared with related proteins from higher plant chloroplasts. Such analyses provide important information as to the functional domains of MSP, potential Mn-binding regions, and signal sequences involved in transit across chloroplast envelope and thylakoid membranes.

MATERIALS AND METHODS

DNA Preparation. *A. nidulans* R2 was grown axenically in BG-11 medium (14). Cells in the late-logarithmic phase were harvested, washed with 120 mM NaCl/50 mM Na_2EDTA, pH 8.0, and stored overnight at −20°C. The pellet was suspended in 25% sucrose/50 mM Tris·HCl, pH 8.0/100 mM Na_2EDTA and incubated with 5 mg of egg-white lysozyme per ml at 37°C for 1.5 hr with gentle stirring. After the lysozyme treatment, cells were treated with 1% $NaDodSO_4$ for 1 hr at 37°C. Cell debris were removed by $NaDodSO_4$/NaCl precipitation and the DNA in the supernatant was precipitated by the addition of polyethylene glycol to a final concentration of 10%. The pellet was resuspended in 7 ml of buffer containing 50 mM Tris·HCl (pH 8.0), 5 mM EDTA, and 50 mM NaCl. CsCl and propidium iodide were added to the suspension to final concentrations of 0.86 g/ml and 0.2 mg/ml, respectively. The suspension was subjected to equilibrium centrifugation at 140,000 × *g* for 72 hr at 20°C. The band containing chromosomal DNA was collected and the dye was removed by ex-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PS, photosystem; MSP, Mn-stabilizing protein.

†To whom reprint requests should be addressed.

§This sequence is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03002).

traction with isopropyl alcohol saturated with 3.0 M NaCl/0.3 M Na_3 citrate, pH 7.0. The aqueous phase containing the DNA was dialyzed against 10 mM Tris·HCl, pH 7.6/1 mM Na_2EDTA.

Screening of a λgt11 Genomic Library. A genomic library of *A. nidulans* R2 DNA was constructed (15) in the bacteriophage expression vector λgt11 (Promega Biotec, Madison, WI). The clones containing a part of the MSP gene were isolated on a lawn of host cell *Escherichia coli* Y1090 according to Young *et al.* (16) using rabbit antiserum raised against spinach MSP (17).

DNA–DNA Hybridization. Southern blot hybridization (18) and plaque hybridization (19) were performed as specified. ^{32}P-labeled probe DNA was prepared according to Feinberg and Vogelstein (20). Unless otherwise stated, a 1.9-kilobase pair (kbp) *A. nidulans* DNA fragment carrying a part of the MSP gene in λmsp13 was used as a DNA probe.

Cloning. *A. nidulans* R2 chromosomal DNA was digested by *Kpn* I and *Xba* I and fractionated by agarose gel electrophoresis. DNA fragments of 1.9–2.5 kbp were electroeluted from a gel segment using Tris acetate buffer (40 mM Tris acetate, pH 8.3/1 mM Na_2EDTA) and subsequently cloned into bacteriophage M13mp18. The recombinant clone with the 2.2-kbp *Xba* I–*Kpn* I fragment containing the MSP gene was isolated by plaque hybridization and named M13mTK1. Clones with sequential deletions from the *Xba* I site in the fragment were constructed from M13mTK1 with BAL-31 exonuclease according to Poncz *et al.* (21) as follows. Aliquots of M13mTK1 replicative form DNA were linearized by *Xba* I and treated with BAL-31 exonuclease (New England Biolabs) for different times. The samples were digested by *Sst* I and subcloned into the *Sma* I–*Sst* I site of M13mp18. The clones with deletions in the MSP gene were isolated by plaque hybridization and used to determine the nucleotide sequence of the noncoding strand. Since the sequence indicated that a unique *Hin*dIII site in the *Xba* I–*Kpn* I fragment is located 126 base pairs (bp) downstream from the termination codon of the gene, the *Xba* I–*Hin*dIII fragment was cloned from the replicative form of M13mTK1 DNA into pUC19 to produce pTK1 for the sequencing of the coding strand. The pTK1 was treated with *Hin*dIII and BAL-31 to generate various degrees of deletions from the *Hin*dIII site. The samples were digested by *Xba* I and subcloned into the *Sma* I–*Xba* I site of M13mp19. Recombinants were isolated with a DNA probe prepared from the 2.2-kbp *Xba* I–*Kpn* I fragment from M13mTK1.

Sequencing. The M13 recombinants were propagated in *E. coli* JM101. DNA sequencing was performed by the dideoxy method (22) using deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate (New England Nuclear, 500 Ci/mmol; 1 Ci = 37 GBq) and the ^{35}S nucleotide reagents kit (Pharmacia) according to Pharmacia's sequencing manual.

RESULTS AND DISCUSSION

Identification of the MSP of *A. nidulans* R2. When polypeptides of thylakoid membranes of *A. nidulans* R2 were separated by $NaDodSO_4$/PAGE and subjected to immunodecoration with antiserum raised against spinach MSP, only one band at an approximate molecular mass of 30 kDa was detected (Fig. 1). This band was relatively faint upon Coomassie blue staining, but the cross-reactivity, as well as the size of the polypeptide, suggested that the 30-kDa polypeptide was the MSP of *A. nidulans*. The finding that the *A. nidulans* MSP was smaller than the spinach protein, whose apparent molecular mass was 33 kDa (7), was confirmed by comparing their electrophoretic mobilities on slab gels (data not shown).

Cloning and Sequencing of the MSP Gene. A genomic library of *A. nidulans* R2 DNA was constructed in the bacteriophage expression vector λgt11 (15). Recombinant phages carrying a part of the MSP gene were obtained using the spinach MSP antiserum. Eleven positive recombinants surveyed for the size of the expression products showed immunoreactive polypeptides of >110 kDa. This finding suggested the MSP was expressed in those clones as a fusion protein to β-galactosidase.

One of the recombinants, λmsp13, had a 1.9-kbp *A. nidulans* R2 DNA fragment (Fig. 2*A*). This insert possessed a unique *Kpn* I site about 200 bp from the 3′ end of the noncoding strand. Several restriction sites were mapped on the insert in reference to the unique *Kpn* I site, and the insert was used as a DNA probe for Southern hybridization experiments (18) against chromosomal DNA. The hybridization experiments suggested that *woxA* is present in a single copy (data not shown) and allowed us to determine the restriction map of the *woxA* gene region (Fig. 2*B*).

Since λmsp13 and the other positive recombinants were lacking the N-terminal portion of MSP, it was necessary to

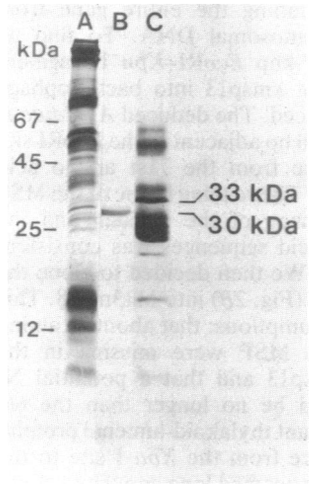

FIG. 1. Polypeptide profiles of *A. nidulans* R2 thylakoid membranes and spinach PSII particles on $NaDodSO_4$/PAGE. Lane A, *A. nidulans* R2 thylakoid membranes stained with Coomassie blue; lane B, *A. nidulans* R2 thylakoid membranes immunodecorated with antiserum raised against spinach MSP; lane C, spinach PSII particles stained with Coomassie blue. The *A. nidulans* R2 thylakoid membranes (23) and the spinach PSII particles (24) were prepared as specified. The immunodecoration was performed according to Towbin *et al.* (25).

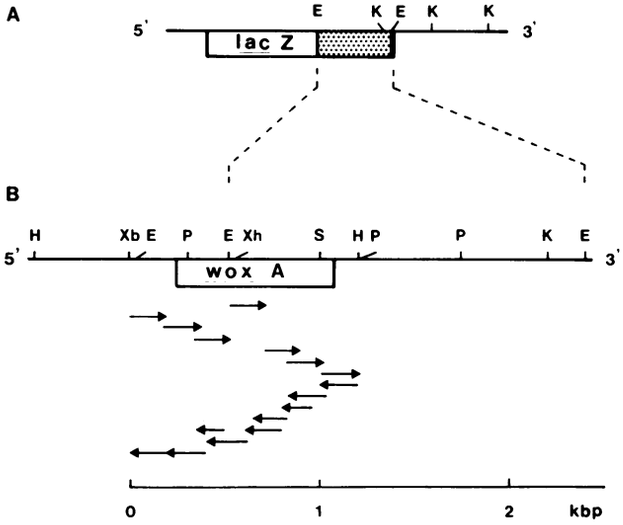

FIG. 2. (*A*) Schematic illustration of a 1.9-kbp *A. nidulans* DNA fragment, carrying a part of the MSP gene (*woxA*), inserted into the *Eco*RI cloning site of λgt11 DNA. The noncoding strand for *lacZ* in a recombinant λgt11, λmsp13, is presented with the *A. nidulans* DNA fragment stippled. (*B*) Restriction map and sequencing strategy diagram of the chromosomal DNA region carrying the *woxA* gene. The restriction enzymes are abbreviated as follows: E, *Eco*RI; H, *Hin*dIII; K, *Kpn* I; P, *Pst* I; S, *Sal* I; Xb, *Xba* I; Xh, *Xho* I. The horizontal arrows indicate the direction and extent of the DNA regions sequenced.

clone a DNA fragment containing the entire gene from restriction fragments of chromosomal DNA. To find an appropriate fragment, the 1.7-kbp *Eco*RI–*Kpn* I fragment (Fig. 2*B*) was subcloned from λmsp13 into bacteriophage M13mp19 and partially sequenced. The deduced *A. nidulans* amino acid sequence for the 200 bp adjacent to the *Eco*RI site corresponded to the sequence from the 71st amino acid residue of the spinach MSP (8). The reading frame of the MSP gene derived by the comparison of the spinach and the deduced *A. nidulans* amino acid sequences was consistent with that for *lacZ* in λmsp13. We then decided to clone the 2.2-kbp *Xba* I–*Kpn* I fragment (Fig. 2*B*) into M13mp18. This strategy was based on two assumptions: that about 70 amino acid residues of the mature MSP were missing in the polypeptide produced in λmsp13 and that a potential N-terminal signal peptide would be no longer than the 66-residue peptide of the higher plant thylakoid-lumenal protein, plastocyanin (26). The distance from the *Xba* I site to the *Eco*RI site was >500 bp, which seemed long enough to cover the translation initiation site.

The nucleotide sequence of the 1215-bp *Xba* I–*Hin*dIII region was determined, applying a strategy of ordered deletions with BAL-31 exonuclease (21). Fig. 3 shows the nucleotide sequence and the deduced amino acid sequence of *A. nidulans* MSP. The sequenced DNA contained an 834-bp coding region and 250-bp 5′ and 131-bp 3′ flanking regions. There was only one ATG codon upstream from the *Eco*RI site in the correct reading frame. A putative ribosome-binding site (27), GAGGA, and an in-frame stop codon were located 6–10 bp and eight triplets upstream from this ATG codon, respectively. These findings suggest that this ATG is the translation-initiation site. The deduced amino acid sequence indicated that the translation product consists of 277 amino acid residues, yielding a M_r of 29,306. In comparison, the M_r of mature MSP from spinach is 26,663 (8). This suggests that the 277-amino-acid translation product should be a precursor, since the mature *A. nidulans* MSP migrated ahead of the mature spinach MSP upon gel electrophoresis (Fig. 1). Comparison of the two MSP sequences suggested the presence of an extra N-terminal peptide, methionine-1 to alanine-28, in the *A. nidulans* precursor MSP. This polypeptide segment is a good candidate for a signal peptide, as discussed in the next section. If the processing site is actually located between alanine-28 and aspartic acid-29, the total amino acid number and the M_r of mature *A. nidulans* MSP are 249 and 26,462, respectively. It seems to be characteristic of MSP species that the apparent molecular mass estimated on acrylamide gels tends to be larger than the M_r value.

Structural Profile of the Extra N-Terminal Peptide of *A. nidulans* MSP. A structural profile of the N-terminal region of the *A. nidulans* precursor MSP (Fig. 4*a*) supports the identification of the polypeptide segment from methionine-1 to alanine-28 as the signal peptide required for the protein to cross the thylakoid membrane. This analysis shows (*i*) a hydrophobic stretch that is predicted to form an α-helix and a β-sheet, (*ii*) a couple of positively charged amino acid residues on the amino side of the hydrophobic stretch, (*iii*) a small and neutral residue (alanine) at the −1 position of the putative processing site, and (*iv*) two amino acid residues known to break ordered secondary structure (proline and glycine) (32) at the −4 and −5 positions. It is expected that the positively charged residues serve to attach the precursor to the negatively charged thylakoid membrane surface and that the hydrophobic stretch spans the thylakoid membrane to induce the translocation of the protein. The structure near the putative processing site represented by *iii* and *iv* above is consistent with that of many known processing sites (33). The distribution of charged residues near the putative processing site seems noteworthy; there is a negatively charged residue on the mature protein side but no charged residue on the signal peptide side. This distribution should be contrasted to the processing sites in precursors of nuclear-encoded stromal or thylakoidal proteins of higher plants (34), which are supposed to pass through the chloroplast envelope but not the thylakoid membrane.

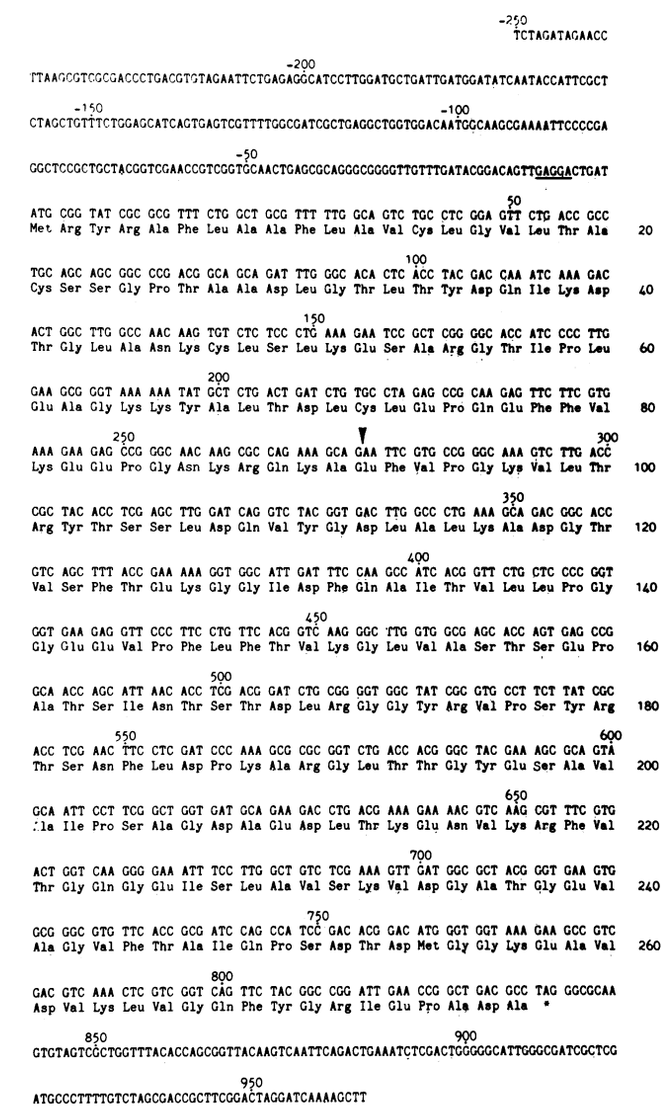

FIG. 3. Nucleotide sequence of the 1215-bp *Xba* I–*Hin*dIII region carrying *woxA* and the deduced amino acid sequence of MSP of *A. nidulans* R2. The noncoding strand is presented. The putative ribosome-binding site is underlined, and the *Eco*RI site in the gene is shown by an arrowhead.

The N-terminal amino acid sequence of mature MSP from *Synechocystis* sp. PCC6803 has recently been determined (L. McIntosh, personal communication), and this protein contains valine as the N-terminal residue with aspartic acid at the +2 position. This information would be consistent with an alternative prediction for the *A. nidulans* MSP processing site, which would be located between alanine-27 and alanine-28. However, this prediction would seem unlikely at this stage since it would place a proline residue at the −3 position. To date, there are no examples of a proline residue at the −3 position in signal sequences (33).

The length of the *A. nidulans* MSP signal sequence is less than half that of the transit peptide found on plastocyanin, a nuclear-encoded, chloroplast protein (26) that is also located in the thylakoid lumen. However, the structure of the *A. nidulans* MSP signal sequence resembles the C-terminal

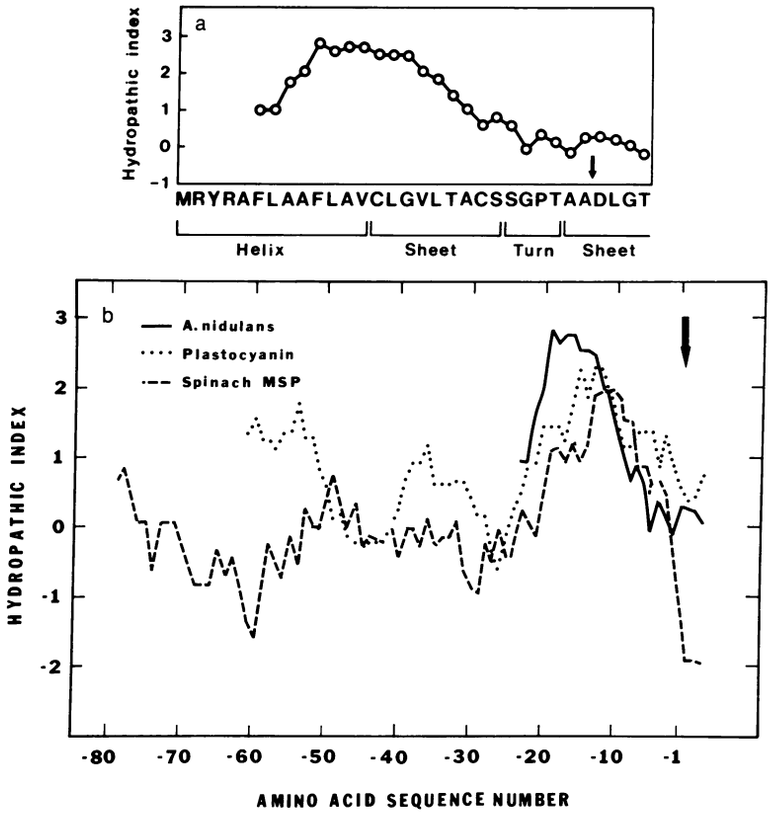

FIG. 4. (*a*) Structural profile of the N-terminal region of *A. nidulans* R2 precursor MSP. The amino acid sequence is depicted with single-letter abbreviations, and the putative processing site is shown by an arrow. Hydropathic indices were calculated according to Kyte and Doolittle (28) with a window of 11 amino acid residues. Secondary structures were predicted according to Garnier *et al.* (29). (*b*) Comparison of the hydropathy profiles of the N-terminal regions of *A. nidulans* MSP, spinach MSP (30), and plastocyanin (31). Hydropathic indices were calculated as above. The arrow indicates the cleavage site for spinach MSP and plastocyanin and the proposed cleavage site for *A. nidulans* MSP.

portion of the plastocyanin transit peptide, even though very little homology is found in their amino acid sequences. This is demonstrated by comparison of the hydropathic index of the *A. nidulans* putative signal sequence with that of plastocyanin and spinach MSP (Fig. 4*b*). It was interesting to note that the profile of the *A. nidulans* putative signal sequence was similar to the "thylakoid transfer domain" reported for plastocyanin (31). The transit sequence of spinach MSP, which was recently revealed by Tyagi *et al.* (30), also shows a similar C-terminal structure. It has been suggested that the C-terminal portion of the transit peptide is involved in translocation of the protein across the thylakoid membrane (11, 31). It is thus not surprising that the protein from the prokaryotic cyanobacterium lacks the N-terminal portion of

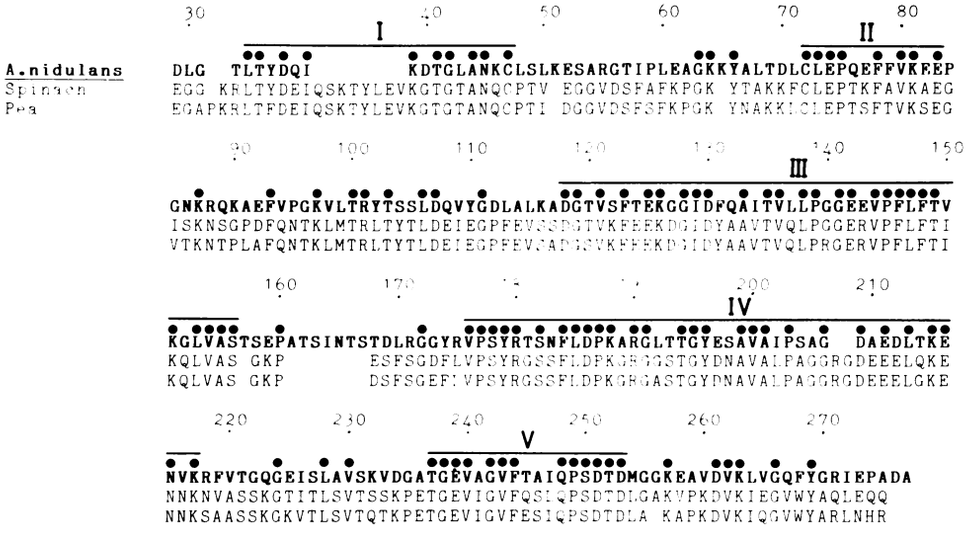

FIG. 5. Alignment of mature MSP sequences of *A. nidulans* R2, spinach, and pea. Amino acid residues conserved in the three MSP species are indicated by black circles. Tentatively assigned clusters of conserved amino acid residues are shown by horizontal bars and roman numerals. The numbering of residues is according to the *A. nidulans* precursor MSP; the presumptive signal sequence (residues 1–28) is not shown.

the transit peptide that is typical of nuclear-encoded, chloroplast thylakoid-lumenal proteins. It is likely that N-terminal portion of the transit peptide is involved with the mechanism by which the higher plant precursor protein crosses the chloroplast envelope (11, 31), a step that does not occur in cyanobacteria.

Comparison of Mature MSP Sequences of *A. nidulans* R2 and Higher Plants. As seen above, the comparison of cyanobacterial and higher plant proteins may allow us to discuss structure/function relationships in proteins. In this context, the mature MSP sequence of *A. nidulans* was compared with higher plant MSPs (Fig. 5). The sequences of MSPs from spinach (8) and pea (11) are quite similar, with homology of 85%. In comparison, the *A. nidulans* MSP sequence is not as well conserved. It has a rather low homology of 48–49% relative to the higher plant proteins; furthermore, it possesses several deletions and insertions. However, the *A. nidulans* MSP reveals that the conserved amino acid residues are clustered rather than evenly distributed. Five clusters, leucine-33 to cysteine-47, cysteine-72 to glutamic acid-83, aspartic acid-118 to serine-156, valine-176 to lysine-217, and threonine-237 to lysine-257 (numbered according to the precursor) were tentatively assigned and designated clusters I–V, respectively. The homology values in these clusters are in the range of 66–70%, and the structures essential for the functioning of the protein are expected to be situated in these clusters.

Importantly, the two cysteine residues in the protein are conserved (residue 47 in cluster I and residue 72 in cluster II). These cysteine residues are likely to form a disulfide bond, and the spinach MSP has been shown to change its electrophoretic mobility after treatment with 2-mercaptoethanol (7). The conservation of these cysteine residues suggests that they may play an important role in maintaining the tertiary structure of MSP in cyanobacteria as well as in chloroplasts. The cysteine residue in cluster I and the neighboring regions in spinach MSP have been proposed as a candidate for the site interacting with Mn atom(s), based on partial sequence homology to the putative Mn-binding site of bacterial Mn superoxide dismutases (8). However, the local sequence of the spinach MSP near cluster I is not strictly conserved in the *A. nidulans* MSP, and the homology of the *A. nidulans* MSP to the superoxide dismutases is lower than that of the spinach MSP. This finding may reduce the significance of the sequence homology, although the possibility of the interaction cannot be excluded. If the interaction does occur, the responsible group is likely to be the bridged disulfur with the involvement of the cysteine residue in cluster II.

The cloning and sequencing of the cyanobacterial *woxA* gene is significant from a number of perspectives. As indicated above, the comparison of the deduced amino acid sequence with the sequences from chloroplast proteins may generate important clues as to functional domains and for the interaction with Mn. The *A. nidulans woxA* gene also provides an excellent system for analyzing membrane assembly and posttranslational processing in cyanobacteria, since, to our knowledge, a cyanobacterial signal sequence has not been reported previously. The availability of the *woxA* gene and the cyanobacterial transformation systems will also enable us to perform site-directed mutagenesis to help elucidate the importance of specific amino acids in photosynthetic water oxidation.

We are grateful to Dr. Carolyn Vann for continued help and advice during the course of this work and to Ms. Lauren Playl and Ms. Jill Cunningham for technical assistance. We also thank Dr. Akira Watanabe for sharing anti-spinach MSP antiserum and providing the pea MSP sequence before publication. This work was performed under the Japan–U.S. Cooperative Photoconversion and Photosynthesis Research program and supported by Grants GM21827 from the National Institutes of Health and FG02-86ER13516 from the U.S. Department of Energy to L.A.S.

1. Tang, X.-S. & Satoh, K. (1985) *FEBS Lett.* **179,** 60–64.
2. Satoh, K., Ohno, T. & Katoh, S. (1985) *FEBS Lett.* **180,** 326–330.
3. Ikeuchi, M., Yuasa, M. & Inoue, Y. (1985) *FEBS Lett.* **185,** 316–322.
4. Kuwabara, T. & Murata, N. (1982) *Plant Cell Physiol.* **23,** 533–539.
5. Kuwabara, T., Miyao, M., Murata, T. & Murata, N. (1985) *Biochim. Biophys. Acta* **806,** 283–289.
6. Metz, J. G., Wong, J. & Bishop, N. I. (1980) *FEBS Lett.* **114,** 61–66.
7. Kuwabara, T. & Murata, N. (1979) *Biochim. Biophys. Acta* **581,** 228–236.
8. Oh-oka, H., Tanaka, S., Wada, K., Kuwabara, T. & Murata, N. (1986) *FEBS Lett.* **197,** 63–66.
9. Westhoff, P., Jansson, C., Klein-Hitpa, B. L., Berzborn, R., Larsson, C. & Bartlett, S. G. (1985) *Plant Mol. Biol.* **4,** 137–146.
10. Minami, E., Shinohara, K., Kuwabara, T. & Watanabe, A. (1986) *Arch. Biochem. Biophys.* **244,** 517–527.
11. Watanabe, A., Minami, E., Murase, M., Shinohara, K., Kuwabara, T. & Murata, N. (1987) in *Progress in Photosynthesis Research*, ed. Biggins, J. (Nijhoff, The Hague), Vol. 4, pp. 629–636.
12. Koike, H. & Inoue, Y. (1985) *Biochim. Biophys. Acta* **807,** 64–73.
13. Stewart, A. C., Ljungberg, U., Akerlund, H.-E. & Andersson, B. (1985) *Biochim. Biophys. Acta* **808,** 353–362.
14. Sherman, D. M. & Sherman, L. A. (1983) *J. Bacteriol.* **156,** 393–401.
15. Reddy, K. J., Vann, C. & Sherman, L. A. (1987) in *Progress in Photosynthesis Research*, ed. Biggins, J. (Nijhoff, The Hague), Vol. 4, pp. 777–780.
16. Young, R. A., Bloom, B. R., Grosskinsky, C. M., Ivanyi, J., Thomas, D. & Davis, R. W. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2583–2587.
17. Murata, N., Miyao, M., Omata, T., Matsunami, H. & Kuwabara, T. (1984) *Biochim. Biophys. Acta* **765,** 363–369.
18. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
19. Silhavy, T. J., Berman, M. L. & Enquist, L. W. (1984) *Experiments with Gene Fusions* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), p. 196.
20. Feinberg, A. P. & Vogelstein, B. (1984) *Anal. Biochem.* **137,** 266–267.
21. Poncz, M., Solowiejczyk, D., Ballantine, M., Schwartz, E. & Surrey, S. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4298–4302.
22. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
23. Pakrasi, H. B., Riethman, H. C. & Sherman, L. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 6903–6907.
24. Kuwabara, T., Murata, T., Miyao, M. & Murata, N. (1986) *Biochim. Biophys. Acta* **850,** 146–155.
25. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
26. Smeekens, S., de Groot, M., van Binsbergen, J. & Weisbeek, P. (1985) *Nature (London)* **317,** 456–458.
27. Shine, J. & Dalgarno, L. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 1342–1346.
28. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132.
29. Garnier, J., Osguthorpe, D. J. & Robson, B. (1978) *J. Mol. Biol.* **120,** 97–120.
30. Tyagi, A., Hermans, J., Steppuhn, J., Jansson, C., Vater, F. & Herrmann, R. G. (1987) *Mol. Gen. Genet.* **207,** 288–293.
31. Smeekens, S., Bauerle, C., Hageman, J., Keegstra, K. & Weisbeek, P. (1986) *Cell* **46,** 365–375.
32. Chou, P. Y. & Fasman, G. D. (1978) *Annu. Rev. Biochem.* **47,** 251–276.
33. Von Heijne, G. (1986) *Nucleic Acids Res.* **14,** 4683–4690.
34. Karlin-Neumann, G. A. & Tobin, E. M. (1986) *EMBO J.* **5,** 9–13.