Molecular Cloning and Functional Expression of a Water-soluble Chlorophyll Protein, a Putative Carrier of Chlorophyll Molecules in Cauliflower*

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A cDNA for a water-soluble chlorophyll (Chl) protein (WSCP) from cauliflower (Brassica oleracea L. var botrys) was cloned and sequenced. The cDNA contained an open reading frame encoding 19 residues for a signal peptide and 199 residues for the mature form of WSCP. The sequence showed extensive homology to drought-stress-related, 22-kDa proteins in some Brassicaceae plants. Functional WSCP was expressed in Escherichia coli as a fusion protein with a maltose-binding protein (MBP). When the recombinant MBP-WSCP was incubated with thylakoid membranes, the MBP-WSCP removed Chls from these membranes. During this process, the monomer of the apo-MBP-WSCP successfully bound Chls and was converted into tetrameric holo-MBP-WSCP. The reconstituted MBP-WSCP exhibited absorption and fluorescent spectra identical to those of the native WSCP purified from cauliflower leaves. The Chl a/b ratio in native WSCP indicates a high content of Chl a, which was mainly due to the higher affinity of MBP-WSCP for Chl a. WSCP is the first example of a hydrophilic protein that can transfer Chls from thylakoid hydrophobic proteins. Possible functions of WSCP are discussed.

A water-soluble chlorophyll protein (WSCP) in Brassicaceae plants has been purified from Lepidium virginicum (1), Brassica nigra (wild mustard) (1), Brassica oleracea var. botrys (cauliflower) (2, 3), Brassica oleracea var. gennifera (Brussels sprouts) (4), and Brassica napus var. oleifera (rapeseed) (3). Although all these WSCPs exhibit similar properties in terms of hydrophilicity, molecular size, and subunit conformation, they can be categorized into two groups on the basis of their hydrophilicity, molecular size, and subunit conformation.

Although some physicochemical and biochemical characterizations of WSCPs have been performed, almost nothing has been determined in regard to the physiological function of these proteins in plant tissues. In recent studies, it was suggested that the WSCPs from cauliflower (3) and Brussels sprouts (4) may be identical to a drought-induced 22-kDa protein in rapeseed, BnD22, which possesses a motif of the Kunitz-type protease inhibitor family (7, 8). Although WSCPs from cauliflower (3), rapeseed (3), Brussels sprouts (4), and L. virginicum (Y. Oka, K. Fujiyama, H. Satoh, K. Nakayama, and M. Okada (1996) DDBJ accession no. AB002589) possess this motif, those from cauliflower and Brussels sprouts have been reported not to inhibit trypsin (3, 4). On the other hand, it has been speculated that BnD22 controls proteolytic pathways in plants, based on its ability to slightly inhibit chymotrypsin activity (10).

In addition to their predicted function as a protease inhibitor, WSCPs have been postulated to function as a Chl carrier (4, 5). However, there are no experimental data in support of this hypothesis. In this study, we have cloned the cauliflower WSCP cDNA and expressed it in Escherichia coli as a fusion protein linked to maltose-binding protein (MBP). The recombinant MBP-WSCP successfully bound Chl molecules in the same fashion as did the native WSCP when the protein was mixed in aqueous solution with thylakoid membranes from spinach leaves. This result makes WSCP the first documented hydrophilic protein capable of removing Chls from thylakoid pigment proteins, and suggests that the WSCP may function as a Chl carrier in plants.

EXPERIMENTAL PROCEDURES

Plant Material and Purification of Native WSCP—Seeds of cauliflower (B. oleracea L. var. botrys) were germinated on cubes of glass fiber irrigated with half-strength Hoagland’s solution. Seedlings were grown hydroponically with the same medium in a greenhouse under a 16-h photoperiod at 23 °C. The volume of medium was maintained at the original level by addition of water every other day, and the culture medium was changed every 2 weeks. Cauliflower plants for purification of native WSCP were obtained from a local farmer in Chiba, Japan. About 2 kg of cauliflower leaves were used for the WSCP purification (3). The molecular weight of native WSCP was determined by mass spectrometry (Voyager, PerSeptive Biosystems).

The protein concentration was determined by the bicinchoninic acid method using a kit from Sigma. Bovine serum albumin was used as a standard.

Amplification and Cloning of the WSCP cDNA from Cauliflower Leaves—cDNA of cauliflower-WSCP was amplified by rapid amplification of cDNA ends (RACE) (11). Two-month-old cauliflowers grown hydroponically were transferred, along with their bases, from the growth medium and left to dry for 2 days in the greenhouse. Total leaf RNA was extracted by the guanidinium thiocyanate method (12). First strand cDNA was synthesized by reverse transcription of total RNA by priming the reaction with an oligo(dT)12-18 anchor (5'-GGTTCCTCCAGTCACGACTTTTTTTTTTTT-3'). The reverse-transcription reaction was performed at 42 °C for 1 h with 3 μg of RNA and

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† The abbreviations used are: WSCP, water-soluble chlorophyll protein; Chl, chlorophyll; PAGE, polyacrylamide gel electrophoresis; MBP, maltose binding protein; RACE, rapid amplification of cDNA ends; IPTG, isopropyl-1-thio-β-D-galactopyranoside; ER, endoplasmic reticulum; PCR, polymerase chain reaction; CBB, Coomassie Brilliant Blue.
300 units of reverse transcriptase XL (Takara Biomedicals, Japan) in a 10-μl mixture.

Previously, 46 amino acid residues from the NH$_2$ terminus of native WSCP from cauliflower were determined (3). The amplification was performed using Ex Taq DNA polymerase (Takara Biomedicals) in the presence of sense primer (5'-AGAGAACAGGTGAAGGA-3') corresponding to NH$_2$-terminal residues REQVKD, and anchor (5'-GTTTTC-CCAGTCACGAC-3') primer. PCR (GeneAmp 2400; Perkin-Elmer) was conducted for 30 cycles using the profile: 94 °C for 2 min, 50 °C for 2 min, and 72 °C for 2 min. An additional 10 min at 72 °C was used for the last cycle.

The PCR product was cloned into pBluescript II KS(1) vector (Stratagene) at the EcoRV site. Six independent recombinant clones were sequenced using an ALFred DNA sequencer with a Thermo Sequenase or an Autoread DNA Sequencing Kit (all from Amersham Pharmacia Biotech).

The nucleotide sequence of the cDNA confirmed that it encoded a WSCP with 19 residues for the signal peptides and 199 residues for the mature form of WSCP.

Expression of Recombinant MBP-WSCP in E. coli—To express the mature form of WSCP, a cDNA without the signal peptide was excised and cloned into a procaryotic expression vector, pMAL-cRI (New England Biolabs). Cauliflower WSCP was expressed in E. coli as a fusion protein linked to the MBP. Expression of MBP-WSCP and affinity purification by amylose column chromatography were performed according to the manufacturer’s instructions. Profiles of the expressed protein and its purification were monitored by SDS-polyacrylamide gel electrophoresis (PAGE) (18).

Reconstitution of the MBP-WSCP with Chls by Mixing with Thylakoid Membrane Preparations—Thylakoid membranes were prepared from spinach chloroplasts according to the methods of Nakayama et al. (14). Five nmol of WSCP was mixed with membrane aliquots containing 0, 1.25, 5 or 20 nmol of Chls in an aqueous solution of 10 mM sodium phosphate buffer (pH 7.2), 0.5 M NaCl, 1 mM EDTA, and 10 mM maltose. After 30 min at 22 °C, the water-soluble fraction was recovered by centrifugation at 10,000 g for 30 min and subjected to PAGE (detergent-free) using an 8% polyacrylamide gel. Fluorescence of Chls bound to MBP-WSCP was detected on a long wave UV transilluminator. Protein bands were visualized by Coomassie Brilliant Blue (CBB) staining.

To analyze the spectrophotometric properties of the reconstituted MBP-WSCP, thylakoid membranes with an 8-fold molar excess of Chls relative to MBP-WSCP were mixed with MBP-WSCP. After centrifugation, the water-soluble fraction, as well as native WSCP purified from cauliflower, were analyzed by absorption and fluorescence spectrometries using a U-3300 spectrophotometer (Hitachi Co., Ltd.) and a FluoroMax spectrofluorometer (SPEX Industries). The absorption spectral data at 0.2-nm intervals were analyzed as fourth derivative spectra (15). Spectra were resolved into their components (16) using an Igor Multi-peak Fit package (Wave Metrics, Inc.).

To determine the ratio of Chl molecules to MBP-WSCP, a thylakoid membrane preparation with 200 nmol of Chls was incubated with 40 nmol of MBP-WSCP at 23 °C for 30 min and centrifuged (10,000 × g, 15 min). An aliquot of the supernatant was then subjected to protein quantitation. SDS solution was added to the remaining supernatant to a final concentration of 1%. Chls were extracted from holo-MBP-WSCP by diethyl ether, and the concentrations of Chls were determined spectrophotometrically in 80% acetone (17). The stoichiometry was calculated from the amounts of protein and Chls in reconstituted MBP-WSCP.
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RESULTS AND DISCUSSION

**Molecular Cloning of a cDNA for Cauliflower WSCP and Analysis of Its Deduced Amino Acid Sequence**—A cDNA for cauliflower WSCP was amplified by 3'-RACE PCR with a sense primer deduced from residues 1–6 (REQVKD) of the native WSCP (3), and cloned. Six independently isolated recombinants contained identical sequences. These 729-base pair cDNAs contained an open reading frame encoding 199 amino acids, and a 3'-untranslated region of 129 bases. All 48 amino acids, and a 3'-untranslated region of 129 bases. All 48 amino acids in the native WSCP (3) corresponded to the deduced amino acid sequence of the cDNA starting at residue 1 of the mature form of WSCP.

Our previous work suggested that cauliflower WSCP and BnD22, a drought-induced protein in rapeseed, were identical to that of the fragment amplified with a sense primer corresponding to the NH2 terminus of a precursor of BnD22 for the mature form of WSCP.

To verify this identity, we used a sense primer corresponding to residues 1–6 (REQVKD) of the native WSCP (3), and cloned. Six independently isolated recombinants contained identical sequences. These 729-base pair cDNAs contained an open reading frame encoding 199 amino acids, and a 3'-untranslated region of 129 bases. All 48 amino acids in the native WSCP (3) corresponded to the deduced amino acid sequence of the cDNA starting at residue 1 of the mature form of WSCP.

BnD22 (B. napus) and P22 (R. sativus), drought-induced 22-kDa protein in rape (1993) GenBank accession no. Z25770) and Arabidopsis thaliana (24) showed a much lower homology (44%) to that from cauliflower. In Raphanus sativus, a cDNA encoding a protein that is 80% homologous to WSCP, designated P22, has been cloned as a salt- and drought-stress-inducible protein (22). Similar drought-related proteins have been cloned from Brassica rapa (C. Shin, S. Song, and Y. Choi (1993) GenBank accession no. Z25770) and Arabidopsis thaliana (24). The sequence homologies of cauliflower WSCP to these two proteins are 51% and 26%, respectively. Although it is unclear whether or not these proteins can bind Chls, it can be concluded that proteins homologous to WSCP are widespread in Brassicaceae.

**Sequence Comparisons of WSCP to Its Homologs in Brassicaceae Plants**—The deduced amino acid sequence of the cDNA was compared with a data base by the BLASTp program (20). Sequences of proteins in Brassicaceae with high homology to WSCP are listed in Fig. 2. All proteins listed in the figure contain a motif for the Kunitz-type protease inhibitor family (PROSITE (21) accession no. PS00283, indicated by the thick bar in Fig. 2), although the functional significance remains obscure.

Reconstitution of the MBP-WSCP with Purified Chls—Photosynthetic pigments were extracted from spinach leaves with chloroform (18) after the leaves were dried by grinding with a silica gel (19). Chls a and b were separated by reversed-phase high performance liquid chromatography (Unilc Pack C18; Gasakuro Kogyo, Japan) using 100% methanol as an eluent.

Five hundred pmol of MBP-WSCP was mixed with 0, 12.5, 125, 250, or 500 pmol of purified Chl a in 20% ethanol and 0.1 M sodium phosphate buffer (pH 7.2). The mixtures were incubated at 22 °C for 30 min and subjected to 8% PAGE (detergent-free). The Chls and proteins were visualized. 

**Conclusions**—A cDNA for a Water-soluble Chlorophyll Protein in Cauliflower has been cloned as a salt- and drought-induced protein. The deduced amino acid sequence of the cDNA was compared with a data base by the BLASTp program (20). Sequences of proteins in Brassicaceae with high homology to WSCP are listed in Fig. 2. All proteins listed in the figure contain a motif for the Kunitz-type protease inhibitor family (PROSITE (21) accession no. PS00283, indicated by the thick bar in Fig. 2), although the functional significance remains obscure.

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The WSCP sequence of cauliflower (B. oleracea) shows extensive homology (94%) to BnD22 of rapeseed (B. napus), supporting the identity of both proteins. In contrast, the WSCP in L. virgatum (DDBJ accession no. AB002589) and AtDr4, drought-suppressed protein in Arabidopsis thaliana (24). The sequence homologies of cauliflower WSCP to these two proteins are 51% and 26%, respectively. Although it is unclear whether or not these proteins can bind Chls, it can be concluded that proteins homologous to WSCP are widespread in Brassicaceae.
lutein (25). This suggests the possibility that this motif in WSCP might play a role in Chl binding.

Although the sequence homology in the COOH-terminal half is relatively low, the COOH-terminal 11 amino acids, EKLGL/K/R/MFPFY are highly conserved. Interestingly, we found that 10 COOH-terminal residues in this hydrophobic region were missing in the mature form of WSCP. The molecular weight of native WSCP, as determined by mass spectrometry, was 20,226.4, which corresponds to the calculated molecular weight of residues 1–189 (with an error of 0.048%) of the predicted 199-amino acid sequence of mature WSCP. This conserved hydrophobic region may function in targeting to a particular compartment; however, this region may not affect the Chl-binding properties of WSCP, since the recombinant MBP-WSCP that possesses this region still binds Chls correctly (Figs. 4–9, discussed below).

Expression and Purification of Recombinant MBP-WSCP Fusion Protein in E. coli Cells—Recombinant WSCP was expressed in E. coli as a fusion protein with MBP. Fig. 3 shows the SDS-PAGE profiles of the MBP-WSCP fusion protein during the expression and purification steps. Addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to the bacterial culture medium induced a prominent polypeptide of 66 kDa (indicated by an arrow; compare lanes 1 and 2); this polypeptide represents a fusion of MBP (44 kDa) and WSCP (22 kDa). Following sonication, much of the MBP-WSCP was found in the soluble fraction of the cell extracts (compare lanes 3 and 4). The 66-kDa MBP-WSCP bound to an amylose column was specifically eluted with 10 mM maltose (lane 5). Proteins expressed in pMAL-cRI can be cleaved with a proteinase, factor Xa. The expressed MBP-WSCP was cleaved by the proteinase and analyzed by SDS-PAGE. The profile displayed a prominent 44-kDa band corresponding to MBP and several faint bands between 21 and 30 kDa (data not shown). Because we could not obtain a distinct band for WSCP following factor-Xa digestion, we used the fusion protein to analyze Chl binding.

Binding Activity of MBP-WSCP to Chls in Thylakoid Membranes—To examine the function of WSCP as a carrier of Chl, MBP-WSCP was mixed in various ratios with thylakoid membranes (Fig. 4). When thylakoid membranes were not added, a ladder of oligomeric MBP-WSCP formed (panel A, lane 1), with the major form being the monomer. When the MBP-WSCP was mixed with thylakoid membranes, the major form became the tetramer, and the hexamer form was also apparent (lanes 2–4 in panel A). Interestingly, only the tetramers and hexamers emitted fluorescence (panel B). These results indicate that the MBP-WSCP monomer removed Chls from thylakoid membranes to form tetrameric and hexameric structures. The dimeric form of apo-MBP-WSCP was also prominent (lane 1 in panel A), but its level was not substantially altered upon addition of thylakoid membranes. Further, the dimer and trimer, unlike the tetramer and hexamer, did not emit fluorescence. This raises the possibility that dimers and trimers may be formed artificially (discussed below). Densitometric analysis of the gel presented in panel A revealed that, prior to the addition of Chl (lane 1), the ratio of monomer:dimer was about 3:1. Forms other than monomers and dimers made up less than about 3% of the total. Hence, at least 75% of the MBP-WSCP in the preparation could bind Chl. This activity coefficient of 0.75 was used in subsequent analyses of the data.

The stoichiometry of Chl in MBP-WSCP, which may be fully saturated with Chl, was examined. A thylakoid membrane and MBP-WSCP were mixed with a molar ratio of Chl:MBP-WSCP of 200:30. From the amount of protein and Chl, the stoichiometry was calculated to be 2 Chls per tetramer, as in the case of the native WSCP.

To compare the Chl-binding properties of the MBP-WSCP with those of the native WSCP, absorption (Figs. 5 and 6) and
fluorescence (Fig. 7) spectra were measured. The absorption spectra of MBP-WSCP and native WSCP exhibited a similar shape in both the red and Soret bands (Fig. 5A). MBP-WSCP showed a shoulder at 485 nm, which was not as intense in the spectrum of the native WSCP. This shoulder may represent one of the protein-bound forms of Chl \(b\), although this speculation remains to be verified. The Chl \(a/b\) ratio in native WSCP displays variations among the different WSCP preparations. Our previously determined absorption spectrum for cauliflower leaves (Fig. 3) indicates that of MBP-WSCP. After incubation at 22 °C for 30 min, proteins (panel B) were separated on PAGE. Proteins (panel A) and Chls (panel B) were deconvoluted it into four major components assigned to be Chl \(a\) peaked at 663, 671, 677, and 685 nm, and one component for Chl \(b\) peaked at 653 nm. Although the intensities of the component with a peaked at 677 nm were slightly different between the two spectra, other components exhibited good agreement in both intensity and wavelength between the two spectra.

Peak wavelengths of absorption spectra of each MBP-WSCP and native WSCP in the red band were 672 and 673 nm, respectively (Fig. 5B). The fourth derivative spectra (Fig. 5B) and a curve-fitting analysis of the red band of native WSCP (Fig. 6) deconvoluted it into four major Chl \(a\)-components (Ca663, Ca671, Ca677, and Ca685) and a single component for Chl \(b\) (Cb653) (Fig. 6). This deconvolution profile was very similar to that for the native WSCP purified from wild mustard (27). The intensity of each component in both MBP-WSCP and native WSCP exhibited good agreement between the two proteins with the exception of one component, Ca677 (Fig. 6). The intensity of Ca677 was greater in native WSCP than in MBP-WSCP. This difference caused a slight shift of the absorption peak between the two spectra (Fig. 5B). However, in both proteins, peak wavelengths of all Chl components were identical (Fig. 6).

Fluorescence emission spectra of both proteins excited at 440 nm (Fig. 7, panels A and B) and at 460 nm (Fig. 7, panels C and D) were measured. These fluorescence spectra were resolved into four major components assigned for Chl \(a\) with peaks at 667, 672, 679, and 686 nm. Identical emission peaks were obtained for native WSCP and MBP-WSCP at both excitation wavelengths (Fig. 7, panels A and B, C and D). When Chl \(a\) was excited, the major fluorescence was emitted from Ca671 (F672) (panel A), whereas excitation of Chl \(b\) gave a more intense signal from Ca677 (F679) (panel C). Because very low level fluorescence was emitted from Chl \(b\), excited Chl \(b\) molecules might efficiently transfer their energy to Chl \(a\) molecules in the complex, as reported in WSCP from L. virgatum (28). It should also be noted that considerable fluorescence was emitted from all Chl \(a\) forms when Chl \(b\) was primarily excited, suggesting that energy transfer also occurred between Chl \(a\) molecules. These spectrofluorometric properties of the native WSCP were exactly reproduced in the reconstituted MBP-WSCP (panels B and D). However, it is possible that interaction of MBP-WSCP with thylakoid pigment proteins may not be perfectly comparable to that of the native WSCP, because MBP-WSCP is much larger than the native WSCP.

**Binding Activity of MBP-WSCP to Purified Chls**—The original ratio of Chls \(a/b\) in thylakoid membranes from spinach was 2.89. After Chls were extracted from the membranes by mixing them with MBP-WSCP in a 0.75:4 molar ratio, the Chl \(a/b\) ratio in the remaining thylakoid membranes decreased to 2.41. This indicates that MBP-WSCP preferentially removes Chl \(a\). In addition, native WSCP contains more Chl \(a\) than Chl \(b\) (Fig. 4A). To address whether this preference of Chl \(a\) is due mainly to the nature of WSCP or to the properties of thylakoid membranes, the Chl-binding activity of the MBP-WSCP was assayed using purified Chls \(a\) and \(b\) (Fig. 8). Increasing amounts of Chl \(a\) (lanes 1–5) or Chl \(b\) (lanes 6–10) were mixed with MBP-WSCP. After incubation at 22 °C for 30 min, proteins were separated on PAGE. Proteins (panel A) and Chls (panel B)
were visualized as described above. In this experiment, the batch of MBP-WSCP was different from that used in Fig. 4 and the dimeric form of apo-MBP-WSCP (lane 1 in panel A) was not as prominent; this supports the conjecture that the presence of the dimer observed in Fig. 4A may be artifactual. Because the amounts of forms other than the monomer of apo-MBP-WSCP were negligible, the activity coefficient of this MBP-WSCP preparation was taken as 1.0.

Again, as shown in Fig. 8, the binding of purified Chls to MBP-WSCP caused tetramer formation (panel A), and only the tetramer exhibited an intense Chl fluorescence (panel B). The binding of Chl a to MBP-WSCP in a 0.5:1 molar ratio (lane 4 in panel A) resulted in almost no residual monomeric form of the protein. The stoichiometry of Chl to the MBP-WSCP tetramer was determined to be 2:1 (described above), suggesting that two molecules of Chl a are sufficient to produce one MBP-WSCP tetramer. In contrast, a 1:1 molar ratio of Chl b to MBP-WSCP (lane 10 in panel A) did not eliminate all of the monomeric MBP-WSCP, indicating that the MBP-WSCP possesses a higher affinity for Chl a than for Chl b.

When MBP-WSCP was reconstituted with thylakoid membranes, the resulting hexamer emitted more intense Chl fluorescence (Fig. 4B, lane 4) than the hexamer formed during reconstitution with purified Chl (Fig. 8B, lane 5). Since different batches of MBP-WSCP were used in these experiments, the same batch of MBP-WSCP was used for the binding analysis to address the cause of this difference (Fig. 9). Panel A indicates CBB staining and panel B, visualization by fluorescence. For an unknown reason, some of the oligomeric structures showed split bands with CBB staining (panel A). However, reconstitution with thylakoid membranes led to the appearance of a prominent hexameric band (lane 3 in panels A and B), while less of the hexamer was formed when MBP-WSCP was reconstituted with purified Chl a (lane 2 in panels A and B). Of note was that reconstitution with purified Chl a also yielded pentamers, heptamers, etc., which emitted fluorescence (lane 2 in panel B). In contrast, no oligomeric forms other than the tetramer and hexamer were detected during the electrophoretic purification of native WSCP from cauliflower (data not shown). These results indicate that the degree of hexamer formation is dependent on the substrate environment. In thylakoid membranes, almost all Chls are bound with proteins (29), whereas in ethanol solution, Chls are freely solubilized. Furthermore, other proteins in thylakoid membranes may affect the transfer. These results suggest that reconstitution of MBP-WSCP with thylakoid membranes produces a WSCP quaternary structure identical to that of native WSCP from cauliflower.
Putative Chl Carrier—The precursor of WSCP possesses 19 residues in the NH$_2$-terminal extension. Unexpectedly, this extension possesses the signature of a signal peptide entering into a secretory pathway (for review, see Ref. 30). In addition, the cleavage site of the WSCP extension follows the “−1, −3 rule” (31, 32) for secretory proteins. These characteristics of the extension polypeptide suggest that the WSCP precursor may be first transported into the endoplasmic reticulum (ER). Band22 was also predicted to be imported into the ER (7). The fact that WSCP is a Chl-binding protein seems to conflict with the idea that WSCP could be transported into ER. A hypothetical contact site of WSCP with Chl could be outside the chloroplast. The various enzymes that increase during senescence and seem likely to participate in chloroplast breakdown are not targeted to the chloroplast (for review, see Ref. 33). Senescing chloroplasts exude lipidic blebs containing Chl or a similar fluorescent material. These blebs could represent chloroplast components in transit to degradation in the cytoplasm or vacuoles (33). As a different pathway of chloroplast degradation, the removal of whole chloroplasts all at once, as in phagocytosis, has been observed (33). In both degradative pathways, Chl should be catabolized in the cytosol or vacuoles. If WSCP is distributed in the ER or vacuoles, WSCP may participate in Chl breakdown that occurs outside the chloroplasts.

On the other hand, it has been reported that native WSCP was detectable in the water-soluble fraction of Percoll-purified class 1 chloroplasts of _L. virginicum_ (34). As described, this seems to conflict with the notion that the NH$_2$-terminal extension of cauliflower WSCP may be a signal peptide. However, in some organisms, several proteins are routed through the ER to plastids (35–37). Therefore, it is possible that WSCP is transported to chloroplasts via a secretory pathway. Subcellular fractionation of cauliflower cells indicated the equivocal distribution of WSCP (data not shown). The intracellular targeting function of the signal peptide of WSCP must be tested experimentally and is currently under examination in a series of transgenic studies in our laboratory.

Although the intracellular distribution of WSCP remains ambiguous, on the other hand, the necessity of a water-soluble Chl carrier in the chloroplast can be postulated as follows. The photosynthetic apparatus is reorganized during acclimation to various light environments (38, 39). Formation of photosynthetic Chl-protein complexes by redistribution of Chl has been reported (40–42); however, the detailed process of Chl redistribution has not been clarified. It is conceivable that WSCP may play a role during this process, because WSCP is able to extract Chl from thylakoid. However, to support this hypothesis, it must be demonstrated that WSCP transports Chl molecules into thylakoid pigment proteins.

When pea plants were subjected to drought stress (leaf water potential, −1.3 MPa), Chl α declined by approximately 18% (43). Senescence (for review, see Ref. 44), as well as drought stress, may cause Chl degradation. Although chlorophyllase, the enzyme catalyzing the first step of the Chl degradation, is present in chloroplasts, its activity is latent and observed only after detergent (45) or organic solvent treatments (9). Recently, it has been shown that chlorophyllase activity occurs on the inner chloroplastic envelope (23). Matile et al. (23) hypothesized the existence of a water-soluble carrier protein that transports Chls from thylakoid pigment-protein complex to the inner chloroplastic envelope in senescent leaves (23, 44). WSCP is highly hydrophilic, may be located in chloroplasts (34), is able to extract Chls from thylakoid pigment proteins (Fig. 4), and is induced under stress conditions (3, 7, 8). All these features of WSCP are in accord with the hypothesis that WSCP serves as a Chl carrier.
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