The extension peptide of plant ferritin from sea lettuce contributes to shell stability and surface hydrophobicity

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Received 20 January 2012; Accepted 9 March 2012

DOI: 10.1002/pro.2061

Published online 14 March 2012 proteinscience.org

Abstract: Plant ferritins have some unique structural and functional features. Most of these features can be related to the plant-specific “extension peptide” (EP), which exists in the N-terminus of the mature region of a plant ferritin. Recent crystallographic analysis of a plant ferritin revealed the structure of the EP, however, two points remain unclear: (i) whether the structures of well-conserved EP of plant ferritins are common in all plants, and (ii) whether the EP truly contributes to the shell stability of the plant ferritin oligomer. To clarify these matters, we have cloned a green-plant-type ferritin cDNA from a green alga, *Ulva pertusa*, and investigated its crystal structure. *Ulva pertusa* ferritin (UpFER) has a plant-ferritin-specific extension peptide composed of 28 amino acid residues. In the crystal structure of UpFER, the EP lay on and interacted with the neighboring threefold symmetry-related subunit. The amino acid residues involved in the interaction were very highly conserved among plant ferritins. The EPs masked the hydrophobic pockets on the ferritin shell surface by lying on them, and this made the ferritin oligomer more hydrophilic. Furthermore, differential scanning calorimetric analysis of the native and its EP-deletion mutant suggested that the EP contributed to the thermal stability of the plant ferritin shell. Thus, the shell stability and surface hydrophobicity of plant ferritin were controlled by the presence or absence of the plant-ferritin-specific EP. This regulation can account for those processes such as shell stability, degradation, and association of plant ferritin, which are significantly related to iron utilization in plants.

Keywords: ferritin; oligomer; processing; assembly; stability; surface hydrophobicity; extension peptide

Abbreviations: ASA, accessible surface area; DSC, differential scanning calorimeter; EP, extension peptide; ICP, inductively coupled plasma spectrometry; PAGE, polyacrylamide gel electrophoresis; PEG, polyethyleneglycol; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; TP, transit peptide; UpFER, plant ferritin from *Ulva pertusa*.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Grants-in-Aid for Young Scientists from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; Grant number: 22780303.

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Introduction

Ferritin is a class of iron storage protein distributed in virtually all living kingdoms. Proteins of this class commonly form spherical protein nanocages, each of which is usually composed of 24 similar or identical subunits.^{1–4} This cage-like 24-mer has a large inner cavity and shows highly symmetrical architecture, i.e., the 24 subunits are related by four-, three-, and twofold symmetries (432 symmetry). A subunit of ferritin forms a four-helix bundle composed of helices A to D and a short fifth helix E, which is also a common structural feature of all ferritins.⁵ Many intersubunit interactions have been observed in the crystal structures of ferritin from various organisms,⁶ giving the ferritin superfamily its super thermal stability.^{7,8} The ferritin superfamily can be divided into a vertebrate type, a plant type, a bacterial type, and so on.^{1,2} Bacterial ferritin can be further divided into heme-containing bacterioferritin (Bfr), nonheme ferritin (Ftn),⁹ and Dps (DNA binding protein from starved cells), the latter of which exceptionally forms a dodecameric protein shell with two- and threefold symmetry axes (mini-ferritin).^{10,11} Most of the ferritins possess a di-iron oxidoreductase site (ferroxidase site) responsible for iron oxidation in the center of the four-helix bundle of the monomeric subunit, except for mammalian L chain ferritin.^{1–3}

Among ferritins, the plant-type ferritin has some physiologically and functionally distinct features.^{1,2} Most of these features can be related to the presence of two plant-specific amino acid sequences: transit peptide (TP) and extension peptide (EP). The primary structure of the plant ferritin core region without TP and EP has a relatively high level of similarity to the vertebrate H-type ferritins (~40%).¹ The TP that is responsible for targeting plant ferritin to plastid exists in the N-terminus of the plant ferritin sequence. The TP is removed from the mature region of plant ferritin after entering to the plastid.¹² On the other hand, EP is present downstream from TP, so it exists in the N-terminus of the mature region. It has been suggested that EP is cleaved from the core region in the germination process in legume seeds,¹³ and that this cleavage induces the destabilization of the plant ferritin shell.¹⁴ Further, recent studies suggest that the EP, a specific domain found only in plant ferritin, has some significant role in regulating the function of plant ferritin.¹⁵ First, it was suggested that the EP functions as a second ferroxidase site, which assists in effective iron oxidation and incorporation especially in high iron/ferritin stoichiometry.^{16,17} However, unlike the well-characterized ferroxidase site in the four-helix-bundle, the structural and enzymatical basis of this EP-involved second ferroxidase site has not been fully elucidated. Second, the EP controls the association and dissociation states of plant ferri-

tin 24-mer.¹⁸ Concerning this function, Fu *et al.* suggested that plant ferritins tend to form large aggregations in the presence of excess ferrous iron, whereas such oligomer association is not observed in plant ferritin lacking the EP. Another EP function was suggested to be related to the mechanism of iron release from the plant ferritin shell: that is, EP removal accelerates the iron release from plant ferritin oligomer.¹⁹ Recently, we presented the first crystal structure of plant ferritin from soybean.²⁰ The crystal structure revealed that the EP forms a helix and lay on the outer surface of the plant ferritin shell. Some apolar contacts are observed between the EP and the neighboring subunit on the shell surface, although these regions are relatively disordered in the crystal structure of soybean ferritin.²⁰

Since ferritin plays an important role in iron storage especially in legume seeds, research into the purification and characterization of plant ferritin from legume (soybean and pea) seeds has begun.^{12,13,21–23} During the last decade, many ferritin genes and their products have been identified and characterized from various dicots and monocots. In general, their primary sequences are highly conserved, i.e., they all possess plant-specific TP and EP.²⁴ Recently, ferritin genes have been identified and characterized not only from land plants, such as monocots or dicots, but also from algae.^{25–29} Among them, green algae have plant-ferritin-like molecules,^{28,29} whereas ferritin from cyanobacteria and diatoms have diverged sequences, which are rather similar to bacterioferritin (Bfr).^{25–27} To elucidate the structure and function of plant ferritin, we here cloned a plant-ferritin-like cDNA from an evolutionally distant species, multicellular green alga, sea lettuce (*Ulva pertusa*), and crystallized it. The crystal belonged to a highly symmetrical cubic crystal (space group *I*432), that enabled a more precise EP structural determination and elucidation of its interaction with the neighboring subunit. Furthermore, it was demonstrated that the EP contributed to the thermal stability of the plant ferritin shell, and that its deletion caused drastic change in the surface hydrophobicity of plant ferritin. These results can explain the molecular mechanisms underlying the previously described functions of the EP, such as stabilization, association, and dissociation of the plant ferritin shell.

Results

Sequence description of plant ferritin from *Ulva pertusa* (UpFER)

cDNA of UpFER was isolated using degenerate PCR and 5'/3'-rapid amplified cDNA ends (RACE). The obtained cDNA sequence contained 2453 bp with a predicted 723 bp open reading frame encoding 240 amino acids (Supporting Information Fig. S1). In the deduced amino acid sequence, a putative transit peptide sequence was present (Supporting Information

Figure 2. (a) Overall structure of UpFER. A single subunit is highlighted in green. (b) and (c) The metal entry channel around a threefold symmetry axis. This image is viewed down a threefold symmetry axis from outside of the shell (b) and viewed perpendicular to the axes (c). Calcium ions are shown as red balls. Water molecules (Oxygen) are shown as blue balls. The electron densities are positive maps of the $|F_o| - |F_c|$ map (contoured at 3.0σ , which was obtained from the model without metal ions, are shown as mesh (c). Asp159 and Glu162 of UpFER are corresponding to Asp131 and Glu134 of human H chain ferritin. (d) and (e) The structure of UpFER around the fourfold symmetry axis. The fourfold channels are represented by two different orientations: (d) aligned on the fourfold axis and (b) perpendicular to the axis. Val190 and Leu194 of UpFER are corresponding to Leu165 and Leu169 of human H chain ferritin, respectively.

monomer per subunit in an asymmetric unit. The structure was solved by molecular replacement with the structure of a monomer of soybean ferritin

(SFER4, PDB code: 3A68)²⁰ as the search model. As shown in Table I, root mean square deviations of band lengths and angles from ideality were

Table II. *Metal Contents in the Crystallized Protein Preparation*

	Fe	Ca	Mg	Zn
Metal concentration ^a (ppb)	183 ± 0.250	230 ± 57.0	16.8 ± 5.44	0.283 ± 0.0244
Atom per assembled Native UpFER	4.84	8.29	1.02	—

Concentrations of metal ions in the recombinant native UpFER were analyzed by ICP. Concentrations were determined from three independent experiments and are shown as the mean ± standard deviation.

^a Concentration of 4 mL of solutions containing 1.5 mg of native UpFER.

calculated to be 0.008° and 0.998 Å. These values are well within accepted limits, indicating that the structure has tight stereochemical constraints. A Ramachandran plot of the final structure showed that 99.5 and 0.5% of all residues were in the most favored and allowed regions, respectively.

The final model contained 195 amino acid residues of a subunit of UpFER (from Val8 to Glu202), in which N-terminal seven and C-terminal two residues were missing. Figure 2(a) shows that the plant-specific EP region forms a helix composed of Gln10 to Gln24 (see below) and a long loop which is followed by the conserved core helical region [Fig. 2(a)]. The conserved core helical region of UpFER is composed of A-helix (from Ile38 to Ala66), B-helix (from Pro73 to Arg100), C-helix (from Glu123 to His152), D-helix (from Ala 155 to Val186), and E-helix (from Val190 to Glu202) [Figs. 1 and 2(a)]. The overall structure was greatly conserved when compared with that of soybean ferritin H-4 [Fig. 2(a)]. The main-chain root mean square deviation of determined 195 C α atoms was calculated to be 0.707 Å.

There are eight hydrophilic channels around the threefold symmetry axes of the shell. This channel has been suggested to contribute to the specific metal ion incorporation.^{32,33} Similar to the case of the vertebrate and the previously solved structure of soybean ferritin, the channel of UpFER was lined with acidic side chains of three symmetrically related subunits [Fig. 2(b)]. The negatively charged channel was composed of two layers of acidic side chains, that is, the outer layer was formed by Glu162 and the inner one by Asp159 [Fig. 2(c)]. These two acidic residues are conserved among vertebrate and plant ferritins. These are corresponding to Glu134 and Asp131 in human H chain ferritin. Especially, Glu162 forms a bottleneck in the channel and contributes to specific metal incorporation.^{32,33} In the crystal structure of UpFER, the electron densities of three metal ions were seen in the channel. However, the metal ions are relatively disordered, especially on the outer side. The omit map of the metal ions shows three blobs of electron densities in this channel [Fig. 2(c)]. Metal content of the recombinant UpFER showed that it contained 8.29 and 4.84 atoms of calcium and iron per assembled 24-mer (Table II). Since the anomalous signals of the putative metal ion sites around the channel were

faint, we have assigned the metals as calcium and refined the structure with an occupancy factor of 0.3. The outer metal ion was coordinated with Glu162 [Fig. 2(c)], which corresponds to Glu167 of soybean ferritin (SFER4).²⁰ The middle one was coordinated by Glu162 and Asp159, whereas the inner one was coordinated by Asp159 [Fig. 2(c)]. This metal ion coordination was very similar to that in soybean ferritin.

The structure of the ferroxidase site was nearly identical to that of soybean ferritin. However, site A in the ferroxidase site of UpFER was empty or occupied with a trace amount of metal, unlike the case of the soybean ferritin, whose ferroxidase site is fully occupied by calcium ion. The difference in the occupation of ferroxidase sites may depend on whether the metal ion was contained in crystallization solutions, because the components of a ferroxidase site are fully conserved between UpFER and soybean ferritin.

Another channel of a ferritin shell is the fourfold symmetry channel, which was more tightly packed than the threefold channel, resulting in a narrow channel diameter [Fig. 2(d,e)]. In general, the fourfold channels of vertebrate ferritin are lined with hydrophobic residues, mainly leucine,⁶ forming three layers, whereas this channel of plant ferritin from soybean is lined with two layers of histidine cluster.²⁰ The channel of UpFER was composed of hydrophobic residues [Fig. 2(d)], valine (Val190) and leucine (Leu194), rather similar to that of the vertebrate type (corresponding to Leu165 and Leu169 of human H chain ferritin). However, this channel was composed of two layers similar to the case of plant ferritin from soybean [Fig. 2(e)].

EP structure and interaction with ferritin shell

One of the most prominent features of plant ferritin is the presence of the EP in its N-terminus. In our previous paper, we suggested that the EP domain interacts with the ferritin shell via apolar contacts.²⁰ Although the crystal structure of soybean ferritin was solved at 1.8 Å resolution, the structure of the EP region was a little disordered, partly because the crystal contained the whole 24-mer in the asymmetric unit. On the other hand, in this study, the structure of the EP region of UpFER can be unambiguously determined despite the lower resolution (up to 2.4 Å). Hence, the crystal structure of UpFER revealed the precise structure of the EP and its

Figure 3. Interaction between the EP and the conserved helical region. (a) Relative position of the EP (short helix shown as a green ribbon diagram) and its interaction with the helical region. Surface area is also shown as the corresponding color to that in the ribbon diagram. (b) Side chains of amino acid residues that are related to the interaction between the EP and the core helical region. The residues, Phe9, Pro11, Phe12, Glu14, Glu18, Leu19, and Val22, are the components of the EP (shown as green), whereas Ile50, Val57 (A-helix), Leu130, Leu132, Leu134, Gln140, and Lys141 (C-helix) are residues in conserved helical region of another subunit (shown as magenta). The amino acid residues interacting with the EP (Ile50, Val57, Leu130, Leu132, and Leu134) are corresponding to Leu26, Val33, Cys102, Leu104, and Leu106, respectively. Electrostatic interactions are shown as broken lines.

interaction with the core helical region [Fig. 3(a,b)]. As shown in Figure 3(a), the EP forms a short helix that lay on the neighboring threefold symmetry-related subunit. The helix of the EP was composed of 15 amino acid residues (from Gln10 to Gln24). The N-terminal half of this helix has a bent structure because of a kink in Glu14, whereas the C-terminal half has a normal pitch of α -helix [Fig. 3(a,b)]. This bent helical structure enables the close interaction with conserved four-helical region. Electrostatic interactions or charged hydrogen bonds were observed between the side chains of Glu14 (EP) and Lys141 (C-helix), and of Glu18 (EP) and Gln140 (C-helix) [Fig. 3(b)]. However, most of the interactions were formed mainly between hydrophobic side chains in the EP and those in helix C or A of the core four-helix bundle via the apolar contact [Fig. 3(b)]. As shown in Table II, the residues contributing to the apolar interactions were Phe9, Pro11, Phe12, Val15, Leu19, and Val22 in the EP; Ile50 and Val57 in the helix A; and Leu130, Leu132, and Leu134 in helix C. Generally, the EP region forms a helix on the outer surface of the ferritin shell. In the structure of UpFER, the outer side of the EP helix (exposed to solvent) was mainly composed of hydrophilic or charged amino acid residues (Ser13, Gln16, Ser20, and Gln24) [Fig. 1(b)], whereas the hydrophobic ones were positioned on the inner side and were interacting with hydrophobic residues on the protein shell surface [Fig. 3(b)]. Therefore, we hypothesized as follows: (1) The EP region contributes to shell stability by interacting with its neighboring subunit. (2) The EP region masks the hydrophobic patch on the ferritin shell, rendering it more hydrophilic.

Shell stabilities and surface hydrophobicities of native UpFER and its EP-deletion mutant

The thermal stabilities of the native UpFER and its EP deletion mutant shells were assayed using differential scanning calorimetry (DSC). Two EP deletion mutants, delEP1 and delEP2, were prepared. The former lacked the whole EP region (residues from Ala1 to Thr23 were deleted), whereas the latter lacked a front part of the EP (residues from Ala1 to Met7 were deleted). These EP-deleted mutants had similar elution property to the native UpFER in gel filtration, suggesting that these were properly assembled to 24-mer. First, we analyzed the thermal stabilities of UpFER and its EP-deletion variants in phosphate buffer (20 mM Sodium-phosphate buffer, pH 7.6). However, denaturation temperatures (T_m) could not be determined because plant ferritin possesses high thermal stability, as is the case with vertebrate ferritin.⁸ So, we analyzed the denaturation temperature of UpFER and its variants in phosphate buffer containing 1.5M of guanidine hydrochloride. In this condition, the T_m for each sample—native, delEP1, and delEP2—was $81.5 \pm 0.161^\circ\text{C}$, $77.8 \pm 0.0945^\circ\text{C}$, and $82.1 \pm 0.381^\circ\text{C}$ (Fig. 4), respectively; the T_m of the delEP1 was significantly lower than those of native and delEP2. This indicated that the UpFer_delEP1 mutant lacking the whole EP region was significantly less stable against high temperature. In contrast, the thermal stability of the UpFER_delEP2, lacking the N-terminal seven residues of the EP, was not affected. These results suggested that the EP, especially from Val8 to Thr23, contributed to UpFER shell stability.

Figure 4. Thermostabilities of UpFER and its EP-deleted variants. The thermostabilities of the native UpFER and its EP-deleted variants (delEP1 and delEP2) were assessed by differential scanning calorimetry. delEP1 lacked the whole EP region (residues from Ala1 to Thr23 were deleted), whereas delEP2 lacked a front part of the EP (residues from Ala1 to Met7 were deleted). The melting temperature of each sample is shown as T_m in the figure. For clarify, the data are arbitrarily shifted on the ordinate scale. Melting temperatures were determined from three independent experiments and are shown as the mean $\pm$ standard deviation. Means with different letters are significantly different at the $P < 0.01$ level. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Surface hydrophobicity of native UpFER and its EP-deletion mutant

The EP of UpFER interacted with the hydrophobic side chains on the ferritin shell surface, whereas the side chains of charged or hydrophilic residues of the EP were exposed to solvent. Therefore, we have postulated that the presence of the EP affects the surface hydrophobicity of the ferritin shell. To test our hypothesis, we investigated the surface hydrophobicities of UpFER and its EP-deleted mutant (delEP1) by determining the concentration of salt precipitation of each preparation. Figure 5 shows that purified native UpFER needed the addition of 45% saturation of ammonium sulfate to be precipitated, whereas delEP1 began to be precipitated by the addition of only 25% saturation of ammonium sulfate (Fig. 5). These results indicated that the presence of the EP made the UpFER shell surface more hydrophilic. The removal of the EP region made it more hydrophobic and enhanced the association of ferritin shells.

Discussion

To date, ferritin from higher plants, green algae, diatoms, and blue-green algae have been identified and/or characterized as phototrophic ferritins. Sequence analyses have revealed that ferritins from diatom and blue-green algae are rather similar to bacterioferritins (Bfr).^{26,27} In this study, we have

demonstrated that ferritin from multicellular green alga, sea lettuce (*Ulva pertusa*), possesses the structural features of ferritin from higher plants, in contrast to that from diatoms or blue-green algae. In general, plant ferritin possesses the EP in the N-terminal region, which renders it unique in the ferritin superfamily. Recently, we determined the crystal structure of plant ferritin from soybean for the first time.²⁰ In this study, the crystal structure of plant ferritin from green alga, *Ulva pertusa*, allowed a more precise observation of the three-dimensional structure of the EP and its interaction with the ferritin shell. The EP interacted with the neighboring subunit on the outer surface of the ferritin shell via apolar contacts (Fig. 3); these contacts contributed to whole-shell stability (Fig. 4). Furthermore, the presence or absence of the EP greatly affected the surface hydrophobicity of UpFER (Fig. 5), because the removal of the EP would expose the surface hydrophobic residues of conserved helical regions to solvent (Fig. 6). The accessible surface area (ASA) of hydrophobic residues (Leu, Ile, Val, and Phe) of native UpFER [Fig. 6(a)] and of the EP-deletion mutant of UpFER (delEP1) [Fig. 6(b)] were calculated to be 424.2 and 639.5 Å², respectively. These results suggested that the EP masked the hydrophobic amino acid residues and that removal of the EP induced drastic changes in the surface hydrophobicity of plant ferritin. In fact, the variant delEP1, which lacked the whole EP region, tended to form aggregate when expressed in *E. coli* at high temperature (30–37°C) or prepared in high concentration (above 5 mg mL⁻¹) (Data not shown), whereas the native UpFER can be concentrated to at least 15 mg·mL⁻¹ (see Materials and methods). These observations support the hypothesis that the EP keeps the surface of sea lettuce ferritin hydrophilic by masking the hydrophobic patch.

The amino acid residues in the EP (such as Phe9, Pro11, Phe12, Glu14, Val15, and Val22 of UpFER), which were related to the interaction with

Figure 5. Comparison of the surface hydrophobicities of native UpFER and its EP-deleted mutant (delEP1) by ammonium sulfate precipitation. The precipitants of 20, 25, 30, 40, and 45% saturations of ammonium sulfate were solved in 20 mM Tris HCl buffer pH 7.6 containing 0.15M NaCl. Then, each preparation (5 µg) was separated by SDS-PAGE. Lane M represents the size marker, which contained 25 kDa (upper) and 20 kDa (lower) bands. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Viewed from

Two-fold symmetry axis

Three-fold symmetry axis

Figure 6. Comparison of the positions of hydrophobic residues on molecular surfaces of native UpFER and its EP-deleted mutant (delEP1). Hydrophobic residues (Leu, Ile, Val, and Phe) of the native UpFER and its EP-deleted mutant (delEP1) are shown in red. The hydrophobic pockets, which are generated by the deletion of the EP, are indicated by circles (right panel, delEP1). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the four-helix bundle region (ferritin shell) of neighboring threefold symmetry subunit, were highly conserved among UpFER and other ferritins from

Table III. Interactions Between the Extension Peptide (EP) and Conserved Helical Regions

Extension peptide	Conserved helical region	Distance (Å)	Corresponding helical region of human H chain
Phe9 CZ	Ile50 CG2	4.1	Leu26
Phe9 CE2	Ile54 CG1	4.9	Ala30
Pro11 CG	Val57 CG2	4.3	Val33
Phe12 CZ	Val57 CG1	3.8	
Phe12 CE	Leu130 CD1	4.0	Cys102
Val15 CG2	Leu134 CD2	3.7	Leu106
Val15 CG2	Leu137 CG	4.4	Asn109
Leu19 CD1	Tyr126 CE2	4.5	Asn98
Val22 CG2	Leu132 CG	4.2	Leu104
Glu14 OE2	Lys141 NZ	3.4	Ser113
Glu18 OE2	Gln140 NE	3.0	Gln112
Thr23 O	Lys121 NZ	3.2	Absent

Amino acid residues of UpFER, which contribute to the interactions between the EP and conserved helical region, are listed with its distances. The corresponding amino acid residues in conserved helical region of human H chain ferritin are also listed in the right column. For example, Ile50 of UpFER is corresponding to Leu26 of human H chain ferritin. The amino acid residue corresponding to Lys121 of UpFER is absent in the sequence of human H chain ferritin, because this part forms a plant-specific loop.

higher plants [Fig.1(b)]. Furthermore, the residues in the four-helix bundle region that interacted with the EP—Leu130, Leu134, Leu137, and Lys136—were also well conserved and corresponded to Leu135, Leu139, Leu142, and Lys141 of SFER4,²⁰ respectively. However, some of the amino acid residues that corresponded to those that interact with the EP of plant ferritins are not present in vertebrate ferritin sequences (Table III), suggesting that these residues have a plant-ferritin-specific significance. Judging from the three-dimensional and primary structure, there is no such hydrophobic cluster on the surface of human H chain ferritin. On the other hand, the amino acid residues exposed to the outer side of the EP were relatively variable, although they are commonly charged or hydrophilic residues [Fig. 1(b)]. The ability of metal ion-induced association¹⁸ of the full-length plant ferritin may depend on the residues on the outer surface of the EP that are exposed to solvent.

Our results indicated that the interaction between the EP and ferritin shell surface affect not only their solubility or hydrophobicity, but also thermal stability of plant ferritin shell. Although the thermal stability of UpFER was significantly decreased by the deletion of the EP, we cannot conclude that vertebrate or bacterial ferritins, which do not possess the EP, have decreased thermal stability. The reported T_m of recombinant human H chain

ferritin was 59.8°C.⁷ Since the experimental condition (containing 2.0M of guanidine hydrochloride) was a little different from this study, the T_m of sea lettuce ferritin (UpFER) and vertebrate ferritin cannot be compared directly.

Until now, the maturation and enzymatic properties of plant ferritin have been discussed in relation to the function of the EP. First, Sczekan and Joshi observed two subunits, 28 kDa and 22 kDa, in the cotyledons of soybean seeds soaked for several days in water.²¹ Those authors also indicated that the 22 kDa subunit is generated by the degradation of the 28 kDa subunit and functions as phytosiderin, which is an insoluble form of plant ferritin and abundant in iron content. In accordance with this observation, other authors have suggested that the EP region is easy to be removed from the N-terminus of the conserved helical region.^{16,18,19} Our data demonstrated that the deletion of the EP rendered UpFER more hydrophobic and less stable. In accordance with our observations, the instability and insolubility of EP-deleted plant ferritin have been indicated previously.¹⁴ Therefore, it is possible that the EP-processed 22 kDa form would tend to aggregate *in vivo*.

Thus, we can hypothesize on the maturation and fate of plant ferritin as follows. First, plant ferritin is synthesized as a full-length precursor polypeptide (larger than 30 kDa), then processed to mature form (26.5–28 kDa) upon its entrance to plastid.¹² Second, the mature form is subject to further degradation of the N-terminal EP¹³ or C-terminal E-helix part.³⁴ The former EP processing generates the 22 kDa form, which is easy to associate to form insoluble phytosiderin. This form may facilitate iron release from the ferritin shell because of its instability. Since the contribution of ferritin to whole iron content in cells or tissues varies among species or organs, the contribution of UpFER to whole iron storage in green alga, *Ulva pertusa*, needs to be elucidated. We are now investigating the gene expression and iron content of UpFER in sea lettuce.

Materials and Methods

Materials

Ulva pertusa Kjellman was collected at the Marine Station of Kyoto University in Maizuru, Kyoto Prefecture in September 2010 and transferred to the laboratory at Kyoto University. The rhizoid was removed and other parts were used for the experiments after washing with distilled water. Nucleic acid extract was prepared from the samples frozen at –80°C.

cDNA cloning of *Ulva pertusa* ferritin gene (UpFER)

Total RNA was extracted using the Sepasol reagent (Nacalai, Kyoto, Japan) according to the manufac-

turer's instructions. First-strand cDNA was synthesized from the total RNA using PrimeScript reverse transcriptase (Takara, Ohtsu, Japan) and random 6-mer as a primer. Degenerate PCR was performed using a primer set of 2Fw1 (5'-AAYGARCARTCAAYGTNGARTAC-3') and 2Rv3 (5'-GATTCCTTRAARAAAYTTKGCAAGNCC-3'), followed by nested PCR using a primer set of 2Fw2 (5'-TCNTATGTKTAYCAYGCNHTG-3') and 2Rv3 (see above). The resultant specific fragment was cloned into a T-vector, followed by sequence determination by an ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA).

3'- and 5'-RACE (rapid amplification cDNA ends) were performed using the Smarter RACE cDNA synthesis kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. The gene-specific primers (GSP) and nested GSP primers for RACE experiments were 3'-GSP1 : 5'-GCGCTGCACTCTACTTCGCCCCGTG-3' and 3'-NGSP1 : 5'-ACAATGTTGGACTTCCTGGCCTTGC-3' for 3'-RACE, and 5'-GSP1 : 5'-CGTGCTCCTTCACAGCGTCTACCTGCTC-3' and 5'-NGSP1 5'-GCTTGGTCTGGTAGTCCATC-3' for 5'-RACE. The resulting PCR fragment generated from the 5'/3'-RACE experiments were cloned into a T-vector followed by sequence determination by the ABI Prism 3100 sequencer.

The similarity analysis of the nucleotide sequence was carried out using BLAST2.0 at DDBJ. The theoretical isoelectric point (pI) and molecular weight (M_w) of a protein was calculated from the deduced amino acid sequence of UpFER using the pI/ M_w tool (<http://au.expasy.org/tools/>). Multiple alignment of the ferritin sequences was performed using the ClustalW program (<http://clustalw.ddbj.nig.ac.jp/top-j.html>) program. The phylogenetic tree was created by ClustalW and viewed by the TreeView program.

Preparation of recombinant UpFER (rUpFER)

A DNA sequence encoding a deduced mature region of UpFER, which starts from Ala41, was amplified by PCR using a primer set of (5'-GCTGACCATGGCACAAGAAGTCACCGGCAT-3') and (5'-TATACTCAGCTACGCCGCTCCTCTCC-3'), containing *Nco*I and *Xho*I restriction sites, respectively. To construct the expression plasmid of native UpFER (pET_UpFER_Nat), the resulting fragment, which was lacking the TP sequence, was ligated to the *Nco*I/*Xho*I restriction site on the expression vector pET21d (Novagen, San Diego, CA). The expression plasmids for EP-deletion mutants, UpFER_delEP1 and UpFER_delEP2, were constructed using a primer set of delEP1 (5'-CAGGCACCTGTGACAGATTTC-3') and ATGrev (5'-CATGGTAATATCTCTTC-3'), and a primer set of delEP2 (5'-GTCTTCCAGCCATTCTCGAG-3') and ATGrev by inverse PCR using pET_UpFER_Nat as a template. The expression plasmids were transformed into the *Escherichia coli* strain

BL21(DE3) (Novagen, San Diego, CA). Protein expressions were performed at 20°C after isopropyl- β -D-thiogalactopyranoside induction. The cells were harvested and disrupted by sonication, followed by protein extraction with phosphate-buffered saline containing a protease inhibitor cocktail (Nacalai). UpFER and its EP-deleted variants were expressed as soluble proteins and were further purified by ammonium sulfate precipitation, anion exchange chromatography by Q-sepharose (GE Healthcare, Piscataway, NJ) and size exclusion chromatography using a Superdex 200 pg 16/60 column (GE Healthcare). Concentrations of purified proteins were adjusted by the absorbance at 280 nm.³⁵

Crystallization, data collection, and processing of native UpFER

Initial crystals were obtained in a week using a crystallization screening kit (PEG Rx; Hampton Research, Aliso Viejo, CA). The crystallization condition was optimized using the hanging-drop vapor-diffusion method. The optimized crystallization drops were prepared by mixing equal volumes of mother liquid (21% PEG400, 0.1M Bicine pH 9.0) and protein solution containing 15 mg mL⁻¹ of recombinant native UpFER. Cubic crystals (space group *I*432; $a = b = c = 151.9$ Å; $V_M = 3.19$ Å³/Da for one subunit per asymmetric unit) appeared in a week at 20°C. Diffraction data on a native crystal were collected to 2.4 Å resolution at 100 K at the SPring-8 beamline 38B1 after flush cooling. No cryoprotectant was needed for the crystals obtained in this condition. Diffraction data were processed, merged, and scaled with HKL-2000 (HKL Research, Charlottesville, CA).³⁶ Data processing statistics are shown in Table I.

Structure determination and refinement

The structure of UpFER was determined by molecular replacement using the Molrep program³⁷ in CCP4i suite 1.4.4³⁸ using the structure of a subunit of soybean ferritin (PDB code: 3A68)²⁰ as a search model. Refinement was performed using the REFMAC5³⁹ and PHENIX⁴⁰ programs. The structure was visualized and rebuilt using COOT 0.6.2⁴¹ and further modified on σ -weighted ($2|F_o| - |F_c|$) and ($|F_o| - |F_c|$) electron density maps. Figures 2, 3, and 6 were produced by PyMOL (DeLano Scientific, San Carlos, CA). The accessible surface area of UpFER and its EP-deleted mutant were calculated with the "Accessible Surface Area" application in the CCP4i suite. The input PDB files for this calculation were 3VNX (native UpFER) and modified a PDB file by lacking the EP region (From Val8 to Thr28).

Metal content measurement

A total of 100 μ L of aforementioned protein solution (15 mg mL⁻¹) for crystallization was diluted to 4 mL by adding 1N nitric acid. Then, the precipitated protein was removed by centrifugation. Fe, Ca, Mg, Zn,

Cu, Ni, and Co concentrations were measured by using inductively coupled plasma spectrometry CIROS-160 EOP (Rigaku, Tokyo, Japan). Background metal content in the protein-free buffer was subtracted. The metal concentrations were measured from three independent preparations.

DSC analysis

Purified UpFER and its EP-deleted variants were dialyzed against 20 mM sodium phosphate buffer (pH 7.6). The concentrations of these samples were adjusted to 1.2 mg/mL, followed by the addition of 1/4 volume of 6M guanidine hydrochloride solution, followed by filtration using 0.22 μ m filter (Millipore). These samples were analyzed with a differential scanning calorimeter (MCS-DSC, MicroCal, Piscataway, NJ) at a heating rate of 1°C/min. Data were obtained from three independent experiments, and the results were used for statistical analysis.

Surface hydrophobicity analysis

The concentrations of purified UpFER and its EP-deleted mutant were adjusted to 1.0 mg mL⁻¹ in 20 mM Tris-HCl buffer containing 0.15M NaCl. Subsequently, fractured ammonium sulfate was added to 20, 25, 30, 35, 40, and 45% saturation to the 1 mL of the protein solution described above. The resultant ammonium sulfate-containing solutions were stored at 4°C overnight, followed by centrifugation. The precipitants were resolved in 1 mL of the same buffer. The samples were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue R-250 stain.

Accession numbers

The cDNA sequence of UpFER has been submitted to GenBank (Accession No. AB691549). Coordinates and structural factors of UpFER have been deposited in Protein Data Bank with accession number 3VNX.

Acknowledgments

The synchrotron radiation experiments were performed at BL38B1 at SPring-8 with the approval of the Japan Synchrotron Radiation Research Institute (JASRI) (proposal number 2011B1327). The authors thank Dr. Kenji Fukui, Dr. Takehiko Tosha, and Prof. Seiki Kuramitsu (RIKEN SPring-8 Center) for assistance and suggestions for the metal content measurement.

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