Complex Formation of Vipp1 Depends on Its α-Helical PspA-like Domain*

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Vipp1 (vesicle-inducing protein in plastids 1) is found in Cyanobacteria and chloroplasts of photosynthetic eukaryotes where it is essential for the formation of the thylakoid membrane. Vipp1 is closely related to the phage shock protein A (PspA), a bacterial protein induced under diverse stress conditions. Vipp1 proteins differ from PspA by an additional C-terminal domain that is required for Vipp1 function in thylakoid biogenesis. We show here that in Cyanobacteria, green algae, and vascular plants, Vipp1 is part of a high molecular mass complex. The complex is formed by multiple copies of Vipp1, and complex formation involves interaction of the central α-helical domain that is common to Vipp1 as well as to PspA proteins. In chloroplasts of vascular plants, the Vipp1 complex can be visualized by green fluorescent protein fusion in discrete locations at the inner envelope. Green fluorescent protein fusion analysis furthermore revealed that complex formation is important for proper positioning of Vipp1 at the inner envelope of chloroplasts.

Oxygenic photosynthesis is a specific feature of Cyanobacteria and chloroplasts of plants and algae. Characteristic of oxygenic photosynthesis is a specialized membrane system, the thylakoids, on which the components of the photosynthetic machinery are located (1). Despite its importance in the process of oxygenic photosynthesis, very little is known about the origin of the thylakoid membrane system in the ancestry of present day Cyanobacteria. Furthermore, other factors that are involved in the formation and maintenance of the thylakoid membrane in either Cyanobacteria or chloroplasts are not well defined.

We have shown recently that in Arabidopsis as well as in Synechocystis the Vipp1 protein is essential for thylakoid biogenesis (2, 3). Δ-vipp1 mutants, in which the expression of the vipp1 gene is greatly reduced, have lost their ability to build up a thylakoid membrane system and to perform oxygenic photosynthesis (2, 3). Interestingly, disruption of the vipp1 gene in Arabidopsis also abolished chloroplast vesicle transport, which has an alleged function in thylakoid formation in the chloroplasts of higher plants (2, 4). Vipp1 is closely related to PspA, a bacterial protein that is induced under distinct stress conditions, i.e. invasion by filamentous phages, inhibition of lipid biosynthesis, secretion defects, or the saturation of the twin arginine pathway (5–8). PspA and Vipp1 are distinguished by a C-terminal domain of about 20–30 amino acids which is present in all Vipp1 proteins but lacking in PspA (3). It is presumed that this C-terminal extension is important for the role of Vipp1 in thylakoid biogenesis. Most Cyanobacteria possess genes coding for both PspA and Vipp1. Chloroplasts seem to have retained solely Vipp1, indicating that the function of PspA is no longer required in this organelle.

So far, the exact function of PspA and Vipp1 remains elusive. In bacteria, PspA is part of a larger operon encoding four more proteins, PspB–PspE. PspA is induced under a number of different stress conditions, and it is believed that the protein helps to sustain the proton motive force across the plasma membrane (5, 6). PspA was furthermore shown to repress its own transcription by binding to the AAA-ATPase PspF, which subsequently binds to the pspA promoter (9, 10). PspA can associate with the plasma membrane by interaction with two membrane-bound members of the psp operon, PspB and PspC (11). A recent study by DeLisa and co-workers (8) suggests that one of the functions of PspA is the enhancement of protein translocation via the twin arginine pathway. They showed that blockage of the twin arginine translocon by overexpression of a substrate could be released by coexpression of PspA. In similar experiments, the coexpression of Vipp1 from Synechocystis had the same effect as PspA, indicating that under these conditions Vipp1 might be able to replace PspA. Our experiments in Synechocystis, on the other hand, indicated that the function of Vipp1 could not be replaced by PspA (3).

Here we show that the Vipp1 protein of Cyanobacteria, green algae, and vascular plants is organized in a high molecular mass complex. The complex consists of several Vipp1 molecules and does not seem to comprise any additional proteins. In chloroplasts, the Vipp1 complex is found closely associated with the inner envelope membrane but not within the thylakoids. Complex formation requires the interaction of a central α-helical domain of the Vipp1 molecules which is also common to PspA proteins. It does not require the C-terminal domain that distinguishes Vipp1 from PspA.

EXPERIMENTAL PROCEDURES

Cloning of GFP and RFP Fusion Constructs—Full-length and C-terminally truncated vipp1 from Arabidopsis thaliana were amplified by PCR using Arabidopsis cDNA as template. Primers V-forward (5′-ggatcgtatggtcttcaatggacct-3′) and V-reverse (5′-ggggtcactattaccttttac-3′) were used to obtain the full-length vipp1 PCR product;
primers V-forward and Vm-reverse (5′-ggaagaccaattctttttctag-3′) were used to amplify the 3′-terminal part of vipp1 (1–859 bp). The PCR products were cloned into pOL-LP vector, N-terminal to the coding sequence for GFP, thereby creating the plasmids pVipp1-GFP and pVipp1m-GFP (12). pVipp1α-helix-GFP was created by restriction of pVipp1-GFP with Eco72I at positions 475 bp and 637 bp followed by religation of the plasmid. pVipp1-RFP were created by subcloning vipp1 into pOL-LP where the GFP sequence was replaced with RFP (12).

Western Blotting Analysis—Proteins were separated by 3–13% blue native (BN)-PAGE and 12.5% SDS-PAGE and electroblotted onto nitrocellulose membrane (Protran, Schleicher & Schuell) following the supplier’s instructions. Immunodecorations with α-Vipp1 were performed as described previously (3). α-Vterm antisera was prepared against the C-terminal 42 amino acids of the pea Vipp1 protein. Base pairs 915–1041 of the vipp1 coding region were amplified by PCR with primers 5′-ggagagcttcctcctggaaga-3′ and 5′-taaagctttgatctttct3′. The PCR product was cloned into the pCR T7/NT-TOPO expression vector (Invitrogen) with an N-terminal His8 tag. Heterologously expressed protein was purified on a Ni-NTA Superflow column (Qiagen) under conditions following the manufacturer’s instruction. Antibodies were made by injection of purified protein into rabbit by Fineda antibody service.

Cross-linking—Chloroplast inner envelope membranes from pea were purified according to the method of Keegstra and Youssif (13) modified by Waegemann and Soll (14). Isolated inner envelope membranes were sonicated in 50 mM HEPES, pH 7.1, and cross-linked with 0.5 mM bis(sulfosuccinimidyl)suberate (BS3) for 1 h at room temperature. The reaction was stopped by the addition of 10 mM Tris, pH 7.5. Proteins were separated on 12.5% SDS-PAGE and analyzed by Western blot analysis using α-Vipp1 and α-Vterm antisera.

Trypsin Digestion—Inner envelope membrane of pea and total Escherichia coli membranes were incubated with trypsin as indicated at 25 °C for 90 s in buffer containing 50 mM Hepes and 5 mM CaCl2. To stop the reaction, 10 mM phenylmethylsulfonyl fluoride was added, and the samples were incubated for 10 min on ice. Proteins were subsequently separated on 12.5% SDS-PAGE and analyzed by Western blotting using α-Vipp1, α-Vterm, and α-PspA antisera.

RNase A Separation was carried out on 3–13% gradient gels according to the method of Schägger and von Jagow (15).

Arabidopsis chloroplasts were isolated from 3–4-week-old plants, purified on 40–80% Percoll gradients, and washed with isolation medium (50 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.33 mM sorbitol, 143 mM β-mercaptoethanol). Chlorophyll concentrations were determined by the method of Arnon (16). Synecochysis membranes were isolated as described in Shukla et al. (17). Fractions containing 50 μg of chlorophyll for chloroplasts and 10 μg of chlorophyll for Synecochystis were solubilized in 60 μl of buffer containing 750 mM mnaacropic acid, 50 mM BisTris, pH 7.0, 0.5 mM EDTA-Na2, and incubated for 3–5 min on ice. n-Dodecyl-β-maltoside (DM) was added to a final concentration of 1%, and the samples were sonicated on ice for 5 min. After a 24-h centrifugation at 4 °C and 21,000 × g the supernatant was collected and loaded buffer (5% Serva Blue G, 750 mM mnaacropic acid) was added to 1/10th of the sample volume. Proteins samples were separated by BN-PAGE in the first and SDS-PAGE in the second dimension.

Transient Expression in Tobacco Protoplasts—Seedlings of Nicotiana tabacum cv. petite Havana were germinated on 85-modified medium (18), and leaves of 3–4-week-old plants were used in all experiments. Protoplast isolation and transient transformation were performed as described previously (19). pVipp1-GFP, pVipp1α-helix-GFP, and pVipp1α-helix-GFP were used for single transformations at 50 μg of DNA/5 × 105 cells; for co-transformation, 25 μg of pVipp1-RFP and pVipp1m-GFP vectors were used. For subsequent BN-PAGE analysis, 2 × 106 protoplasts were transformed, collected, and resuspended in isolation medium (330 mM sorbitol, 20 mM MOPS, 13 mM Tris-HCl, pH 7.9, 1 mM MgCl2, 0.02% bovine serum albumin, 1 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride). Cells were lysed on ice for 30 min, and chloroplasts were isolated by filtering the suspension through 25-mm polycarbonate filter disks. For BN-PAGE separation and induction with 0.4 mM isopropyl-1-thio-b-D-galactopyranoside. 24 h after induction, cells were harvested by centrifugation, resuspended in 50 mM Hepes, pH 7.6, and lysed by passage through a French pressure cell.

For BN-PAGE analysis the suspension was centrifuged at 15,000 × g for 10 min, and the pellet was resuspended in 50 mM Hepes, pH 7.6. DM was added to 1% of the total volume, and the suspension was incubated for 15–20 min on ice. The insoluble fraction was discarded after centrifugation (1 h, 15,000 × g), and the supernatant, representing the DM-solubilized fraction, was analyzed on BN-PAGE followed by SDS-PAGE and Western blotting.

Size exclusion chromatography of Vipp1-His6 was performed under different conditions. First, E. coli cells overexpressing Vipp1-His6, were collected, resuspended in lysis buffer (100 mM Tris-HCl, pH 7.6, 50 mM NaCl, 75 mM NaSCN, and lysed using a French pressure cell. The suspension was centrifuged at 15,000 × g for 10 min, the pellet was resuspended in lysis buffer supplemented with 1.1% CHAPS, 800 mM NaCl, pH 7.6, and agitated for 2 h. Second, Vipp1 protein was purified under 15,000 × g for 1 h at 15,000 × g. The supernatant, containing solubilized proteins, was applied onto a Ni-NTA column in binding buffer (50 mM Hepes-HCl, pH 7.6, 300 mM NaCl, 10 mM imidazol, 75 mM NaSCN, 0.05% CHAPS), and Vipp1-His6 was purified under native conditions according to the manufacturer’s instructions with the exception that 75 mM NaSCN was added to washing and elution buffer. Eluted Vipp1 was centrifuged at 10 min at 21,000 × g and analyzed on Superose 6 HR 10/300 column. The column was equilibrated with 20 mM Tris, pH 7.6, 50 mM NaCl, 75 mM NaSCN. The column was calibrated with the following molecular mass markers: blue dextran (2,000 kDa), catalase (223 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), and chymotrypsinogen (25 kDa). Size exclusion was performed at a flow rate of 0.3 ml/min. Fractions (0.25 ml) were collected and analyzed by Western blotting with α-Vipp1 antisera.

Second, E. coli cells overexpressing Vipp1-His6, were harvested by centrifugation, resuspended in 50 mM Hepes, pH 7.6, and lysed by passage through a French pressure cell. The suspension was centrifuged at 15,000 × g for 10 min, and the pellet was resuspended in buffer containing 4 mM urea, 50 mM NaCl, 50 mM NaH2PO4, 10 mM Hepes, 5 mM Tris-HCl, pH 8.0, and incubated for approximately 20 min at room temperature with agitation. Nonsolubilized and aggregated protein was removed by centrifugation (10 min, 15,000 × g). After estimation of the protein concentration the supernatant was diluted 1:20 (v/v) in renaturation buffer (55 mM Tris, pH 8.2, 10.56 mM NaCl, 0.055% polyethylene glycol 3350, 550 mM guanidine HCl, 1.1 mM EDTA, 440 mM sucrose, 1.0 mM dithiothreitol, 0.3 mM DTT) to yield a final concentration of 1 mg/ml protein and incubated for 14 h at 4 °C with rotating. The sample was centrifuged at 15,000 × g for 10 min to remove aggregated proteins, and the supernatant was dialyzed against 20 mM Hepes, pH 7.6, 50 mM NaCl, 300 μM phenylmethylsulfonyl fluoride, centrifuged again (21,000 × g, 10 min, 4 °C) before being applied to the Superose 6 HR 10/300 column. The column was equilibrated with 20 mM Hepes, pH 7.6, 50 mM NaCl and calibrated as described above.

Electron Microscopy—Samples were prepared for EM using the conventional negative staining procedure. Briefly, one drop of purified Vipp1 complex diluted 1:10 was placed onto a carbon-coated grid hydrophilized by charge glow. After 2 min the protein solution was removed, and the grid was dried on air. Grids spread with the Vipp1 complex were treated for 1 min with 1% uranyl acetate and 0.01% glucose in water, briefly rinsed with a drop of water, and then air dried. The samples were examined with a Zeiss EM 912 transmission electron microscope operated with the Omega energy filter in the zero-loss mode.

Imaging—All fluorescence images were obtained by an epifluorescence microscope (polychrome IV System, Till Photonics, Munich, Germany) using GFP, fluorescein isothiocyanate, and rhodamine filter sets. Pictures were taken with a cooled IR CCD camera and visualized by the TILLvision 4.0 software.

RESULTS

Cross-linking of Inner Envelope Vesicles from Pea—Inner envelope vesicles derived from pea chloroplasts were cross-linked with BS3, and proteins were subsequently separated by SDS-PAGE. Vipp1 protein was detected by Western blot analysis using an antibody raised against the full-length protein from pea (Fig. 1A). As shown previously, the monomeric Vipp1 can be detected as a band of 33 kDa, in accordance with its deduced molecular mass (2, 20). A second band of about 35 kDa can be observed frequently after long exposure to reducing conditions and was identified previously as Vipp1 by protein sequencing (2). The chemical alteration that causes the difference in the running behavior is not known. Incubation of inner...
envelope vesicles with BS3 created several immunoreactive bands of higher molecular mass. A characteristic band pattern was observed repeatedly in all our experiments, indicating the formation of specific cross-link products (Fig. 1A). A band of about 66 kDa became visible early on during the cross-linking reaction, and it remained the most prominent immunoreactive band even after prolonged incubation with BS3. The reaction created two more immunoreactive bands of about 100 and well over 200 kDa. A similar pattern was observed with chloroplasts of other vascular plants and with a different cross-linker (data not shown) supporting the specificity of the cross-link reaction. These results were the first indication that Vipp1 is in close proximity to other proteins to which it can be cross-linked. The cross-linking pattern suggests that Vipp1 primarily forms a homodimer or that it interacts with a protein of a very similar molecular mass (Fig. 1A). From their size, the higher molecular mass products could well present tetramers and octamers of Vipp1.

**BN-PAGE Analysis of Cyanobacteria and Chloroplast Vipp1**—To investigate a potential complex formation by Vipp1 we separated native protein complexes from *Arabidopsis* chloroplasts by BN-PAGE. Isolated chloroplasts were solubilized in 1% DM, and nonsolubilized proteins were removed by centrifugation. Under these conditions most of the proteins were found in the supernatant including about 90% of the Vipp1 protein as determined by Western blot analysis (data not shown). The solubilized proteins and protein complexes were subsequently separated by BN-PAGE in the first (Fig. 1B, upper panel) and SDS-PAGE in the second dimension. Western blot analysis of the second dimension revealed two distinct positions corresponding to the monomeric protein and a complex of well over 1,000 kDa (lower panel). Immunodecoration shows Vipp1 as a high molecular mass complex. Similar results were obtained for the Vipp1 protein from pea, tobacco, or *Chlamydo-monas* (data not shown). Thus, the organization of Vipp1 in a high molecular mass complex seems common to vascular plants as well as green algae.

We wanted to elucidate whether a similar organization of Vipp1 can be found in Cyanobacteria. Therefore, detergent-solubilized membrane proteins from *Synechocystis* were separated by BN-PAGE in the first (Fig. 1C, upper panel) and SDS-PAGE in the second dimension. The proteins were subsequently analyzed by Western blot with α-Vipp1 against the pea protein, which also recognizes Vipp1 from *Synechocystis* (Fig. 1C, lower panel). The immunoblot showed very little monomeric Vipp1 protein in *Synechocystis*, whereas most of the Vipp1 was present in complexes of similar sizes compared with higher plants. It was shown recently that PspA from *E. coli* forms a ring-like homomultimer of 1,023 kDa. Our results suggest that a similar structure is also formed by Vipp1.

**Size Exclusion Chromatography of Heterologously Expressed Vipp1**—To elucidate whether the Vipp1 complex is homo-oligomeric, we analyzed the organization of full-length Vipp1 from *Arabidopsis* that was heterologously expressed in *E. coli*. Cells were disrupted, and the membrane fraction was recovered by centrifugation. The majority of the heterologously expressed Vipp1 protein was found in this fraction. The membrane fraction was subsequently treated with 1% DM, and solubilized proteins were separated by BN-PAGE and SDS-PAGE as before. Western blot analysis revealed that more than half of Vipp1 was present as monomeric protein (Fig. 2A). Nevertheless, a significant amount was found in a high molecular mass complex of approximately the same size as native Vipp1 from chloroplasts. These results indicated that heterologously expressed Vipp1 protein alone seems to be sufficient for complex formation, and no further chloroplast proteins are required for this process.

The chromatogram displayed a major protein peak corresponding to a molecular mass of about 2,000 kDa. Western blot analysis identified Vipp1 in this high molecular mass peak (Fig. 2B), confirming the results from the BN-PAGE analysis. The behavior of the heterologously expressed Vipp1 protein during size exclusion is identical to Vipp1 purified from detergent-solubilized chloroplasts, providing confirmation that the heterologously expressed Vipp1 behaves like the native protein (data not shown).

To obtain a larger quantity of purified Vipp1 for negative stain EM analysis, the protein was isolated from inclusion bodies under denaturing conditions and was subsequently renatured in a buffer that included guanidine-HCl, polyethylene glycol, and sucrose. Most of the Vipp1 protein was soluble after renaturation, and this fraction was then likewise analyzed by size exclusion chromatography (Fig. 2C). The chromatogram obtained from this fraction was very similar to the one from Vipp1 purified under nondenaturing conditions. The Vipp1 protein was again found predominantly in the peak corresponding to a molecular mass of approximately 2,000 kDa. Thus we concluded that the denaturing-renaturing process did not interfere with the ability of Vipp1 to form a high molecular mass complex, and the protein eluting in the peak fraction was used for EM studies.
Analysis of Purified Vipp1 Complex by Negative Staining EM—The Vipp1 complex purified under nondenaturing conditions by size exclusion chromatography was analyzed by negative stain EM after treatment with uranyl acetate. Because of the limited amount of protein the sample yielded only very few particles (Fig. 2 Da). To obtain more data on the Vipp1 particles, we analyzed Vipp1 complexes isolated under denaturing conditions, which had been renatured and subsequently purified by size exclusion (Fig. 2 Db and Dc). These samples yielded a much larger set of particles. Several different samples obtained from two separate purifications were analyzed to get an impression of the structure of the Vipp1 complex. The majority of singular particles appeared ring-shaped, with a darker area in the center (Fig. 2Da, a, b, d, and e), and they were classified as top view onto the complex. Other particles appeared tilted by 90° in relation to the plane of the grid, representing a side view on the complex (Fig. 2Db, b and g). These particles were often found in stacks of two or more. We assume that this stacking is most likely the result of a higher complex concentration in this sample. In some rare cases particles could be found which were tilted at a less than 90° angle (Fig. 2Dc). In these cases

trypsin digestion of inner envelope vesicles and overexpressed Vipp1 protein—We had observed previously that treatment of inner envelope vesicles with trypsin resulted in the formation of a 25-kDa proteolytic fragment of Vipp1 that remained fairly resistant to proteolysis (Fig. 3A, left panel). This fragment is about the same size as the Vipp1 protein without its C-terminal extension or as PspA from E. coli (Fig. 3A, right panel). We tested this assumption by raising an antibody against the last 42 amino acids of the pea Vipp1 protein (a-Vterm). The antisera recognized the full-length Vipp1 protein in untreated inner envelope. In contrast, the 25-kDa peptide that remained after trypsin digestion was not recognized by this antibody (Fig. 3A, center panel). Thus, it is indeed the Vipp1-specific C-terminal extension that is cleaved from the Vipp1 protein. These results are consistent with a hypothetical
structure of the Vipp1 where the N-terminal PspA-like domain is being protected from proteolysis by a tight complex formation, whereas the C-terminal extension is accessible to the protease. To test this possibility further, we trypsin treated chloroplast proteins after solubilization with 1% DM. SDS-PAGE analysis shows that the N-terminal PspA-like domain of Vipp1 is protected (Fig. 3B). When proteins were subsequently analyzed by BN-PAGE and SDS-PAGE, no monomeric Vipp1 protein was visible, whereas the 25-kDa proteolytic fragment was still present at the complex size (Fig. 3C). These results support the idea that complex formation depends on the N-terminal PspA-like domain of Vipp1 and that it protects the Vipp1 protein from trypsin digestion. Accessibility to trypsin digestion furthermore indicates that the Vipp1-specific C-terminal extension is not buried within the complex.

**Transient Expression of Vipp1 in Protoplast**—The fact that most of Vipp1 is organized in a high molecular mass complex raised the question of where this large complex is localized within the chloroplast. We transformed protoplasts from both tobacco (Fig. 4B, a–c) and Arabidopsis (data not shown) with pVipp1-GFP, which contains the vipp1 gene of Arabidopsis including its presequence as an N-terminal fusion to GFP expression of Vipp1-GFP. a, chlorophyll fluorescence; b, GFP fluorescence; c, overlay of a and b. Vipp1-GFP builds a ring-like pattern around the chloroplasts, indicating that Vipp1-GFP is dispersed within the chloroplast stroma.

**BN-PAGE Analysis of Protoplasts Transformed with GFP Fusion Proteins**—To support the fluorescence microscopy data further, we isolated chloroplasts from protoplasts transformed with various GFP fusion constructs. Proteins were separated
with the fluorescence microscopy data suggest that disruption of the endogenous Vipp1, whereas Vipp1 is a protein found in Cyanobacteria and chloroplasts, where it plays an essential role in the formation of the thylakoid membrane (2, 3). So far, the exact function of Vipp1 in this process remains elusive, and it is not known whether it requires specific interaction with other proteins. In search of potential interaction partners of Vipp1 we found that the protein is present as a high molecular mass complex. The complex formation seems to be formed exclusively by Vipp1 without involvement of other proteins. Complex formation of Vipp1 was found in all organisms analyzed, which included Cyanobacteria, chloroplasts of the green alga *Chlamydomonas reinhardti*, and several different vascular plants. We therefore assume that it is a common feature of Vipp1 and that it is important for the function of Vipp1 in thylakoid biogenesis.

Vipp1 was first noticed because of its apparent dual location in the envelope and the thylakoids of pea chloroplasts (20). It was later shown that in Cyanobacteria Vipp1 is located exclusively in the plasma membrane (3). Our analysis of Vipp1-GFP fusion protein suggests that in chloroplasts, Vipp1 resides only at the inner envelope membrane and not within the thylakoids. There are two possible explanations for this discrepancy between previous results on the Vipp1 localization (2, 20) and our GFP fusion data. First, we cannot exclude that part of the Vipp1 protein is indeed located in the thylakoid membrane, but that it is not abundant enough for the GFP signal to become visible. Second, the presence of Vipp1 in isolated thylakoid membrane fractions could result from a contamination of the thylakoids during the preparation. Our experiments indicate that the Vipp1 complexes are loosely associated with the inner envelope, and they might partially dissociate during membrane preparation in vitro. Because of its size the complex could then pellet together with the thylakoid membranes. More importantly, our results suggest that the localization of Vipp1 within the chloroplast is determined by its complex formation. When the N-terminal α-helical domain of Vipp1 is disrupted we can no longer observe Vipp1 complexes, and at the same time the GFP fluorescence signal becomes dispersed within the chloroplasts.

Structurally, the Vipp1 protein can be divided in three domains. The major part (PspA-like domain), comprising the first 300 amino acids of the mature protein, is mostly α-helical with two potential coiled coil regions (21). This structure is not only found in Vipp1 but is also conserved in all PspA proteins. The PspA-like domain of Vipp1 is followed by a random coil spacer. This spacer can vary in size among different Vipp1 proteins and has no significant conservation in its amino acid sequence. This spacer connects the PspA-like domain to a short α-helical domain, which contains a leucine zipper-like motif. This C-terminal extension is specific for Vipp1 and not found in any PspA protein. In our opinion the α-helical structure within the PspA-like domain of Vipp1 has the greatest potential to be involved in complex formation, and this speculation was supported by our findings. BN-PAGE analysis as well as GFP fusion data show that Vipp1 without its C-terminal domain is capable of complex formation. Furthermore, BS2 cross-linking of native Vipp1 after proteolytic removal of the C terminus still results in oligomer formation, indicating that this Vipp1-specific domain is not involved in the interaction of the Vipp1 molecules within the complex (data not shown). In contrast, if the α-helical part of the PspA-like domain is disrupted, the protein can only be found in its monomeric form. Interestingly, it seems to be the complex formation that protects the major part of Vipp1 from proteolytic digestion because the free protein can be completely degraded. The sequence of pea Vipp1 contains a recognition site for trypsin at amino acid residue 282, just before the start of the random coil spacer. Proteolytic digestion at this position would create a fragment of about 25 kDa, identical to what we observed in our experiments. The fact that the C terminus can be removed by trypsin treatment suggests that it is not buried within the complex but rather protrudes from it and might therefore be able to facilitate further interactions with other proteins.

It was shown very recently (21) that PspA from *E. coli* forms a homo-oligomeric complex of 1,023 kDa, which is created by 36 PspA molecules. Three-dimensional reconstruction indicated that they form a 9-fold rotationally symmetric ring composed of nine PspA tetramers. The analysis by Hankamer and co-workers (21) as well as previous studies by Elderkin et al. (10) have furthermore shown that a dimer of PspA is the smallest unit within this complex. Our data support a similar complex formation for Vipp1. The most dominant cross-linking product of Vipp1 is about double the size of the mature protein and most likely represents a Vipp1 dimer, the minimal building unit for the oligomer. Other prominent cross-linking products for Vipp1 suggest the formation of tetramers and possibly octamers. The latter might be derived from the cross-linking of two neighboring Vipp1 tetramers. We cannot completely exclude that some of the cross-link products are formed by interaction of Vipp1 with other proteins, but it seems unlikely from our data. Negative stain EM of the purified Vipp1 complex revealed a ring-shaped structure similar to the one observed for PspA. Compared with the dimension found for PspA from *E. coli*, the complex formed by the *Arabidopsis* Vipp1 protein appears to be double in size. This was consistent to the data from BN-PAGE and size exclusion chromatography. Further studies will have to show whether the increase in size of the Vipp1 complex is the
result of a larger number of molecules in the complex. Alternatively, the Vipp1-specific C-terminal extension might cause a somewhat different arrangement of the subunits within the complex. Aside from the size, we propose a structure for Vipp1 very similar to the one suggested by Hankamer and co-workers for PspA, with a ring-shaped complex formed by several Vipp1 dimers or tetramers. It is tempting to speculate that the increase in dimension of the Vipp1 complex might have been required for the new function of Vipp1 in thylakoid biogenesis.

To facilitate this function the Vipp1 complex appears to be attached to the inner envelope of chloroplasts either by interaction of the Vipp1 molecules with other proteins or by a direct association of the complex with the lipid interface. This is a clear distinction from PspA, which alternates between a membrane-attached and a soluble form. The C-terminal extension that so clearly distinguishes Vipp1 from PspA is not involved in either complex formation or membrane association but instead protrudes from the complex into the stroma of chloroplasts or the cytosol in Cyanobacteria, where it would be accessible for other interaction partners.

Together our findings suggest that Vipp1 and PspA adopt a very similar structure thereby raising the question whether the function of PspA and Vipp1 is also conserved more closely than previously anticipated. In Cyanobacteria, the first organisms to contain thylakoid membranes, Vipp1 has evolved, whereas PspA still exists, indicating that the function of the two proteins is not redundant. In chloroplasts, it is specifically the function of Vipp1 that was retained because PspA is not found in photosynthetic eukaryotes. Nevertheless, it also underlines the importance of the C-terminal domain as a distinction between the two proteins, and it is important for the future to elucidate the specific property of this domain.

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