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SHORT COMMUNICATION



# Insights into topology and membrane interaction characteristics of plastoglobule-localized AtFBN1a and AtLOX2

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**ABSTRACT**

Plant chloroplasts harbor ubiquitous lipid droplets called plastoglobules. While physically connected to the thylakoid membrane, they are characterized by a unique set of about 30 proteins specifically associated with the plastoglobule. How these proteins selectively target the plastoglobule remains unknown. Protease shaving assays with isolated *Arabidopsis thaliana* thylakoid and plastoglobule show that a ca. 25 kD portion of the abundant structural protein of plastoglobules, Fibrillin 1a, is protected from protease digestion. Mapping of protease cleavage sites and experimentally identified phosphorylation sites onto a homology model of Fibrillin 1a indicates that this protected sequence corresponds to the C-terminal lipocalin-like domain, implicated in specific lipid binding. In contrast, protease shaving and membrane washing assays with another plastoglobule-associated protein harboring a C-terminal PLAT domain, Lipoxygenase 2, is consistent with an exposed PLAT domain positioned parallel with, and upon, the surface of the plastoglobule. We propose a model where conserved lipid-binding domains associate with either the surface or neutral core of the lipid droplet. Our study provides insight into the topology and membrane interactions of two plastoglobule-localized proteins.

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Plastoglobules are ubiquitous lipid droplets of plant chloroplasts formed by a monolayer glycerolipid leaflet enclosing a neutral lipid core.[1] The plastoglobule surface is contiguous with the outer leaflet of the thylakoid membrane from which they are derived, reminiscent of cytosolic lipid droplets derived from the endoplasmic reticulum.[2,3] A collection of 30 proteins, most of which are characterized or putative lipid metabolic enzymes, exclusively associate with plastoglobules.[4] Due to the hydrophobic nature of the plastoglobule core, these proteins presumably interact peripherally with the surface monolayer. However, the mechanism(s) of protein interaction with the plastoglobule, or the identification of requisite protein domains for plastoglobule interaction, remains unclear. Microscopy studies monitoring the localization of various truncation mutants of the structural plastoglobule protein from *Arabidopsis thaliana*, fibrillin 7a (AtFBN7a), determined that most of the AtFBN7a sequence was necessary for proper association with the plastoglobule but could not identify conserved plastoglobule-targeting domains or suggest molecular mechanisms of plastoglobule association.[5] The determination of plastoglobule protein-targeting mechanisms is important for predicting additional plastoglobule proteins from amino acid sequences and harnessing the plastoglobule for biotechnological approaches. To address how selected proteins interact with the plastoglobule surface from a biochemical perspective, we have combined protease shaving and membrane washing assays with homology modeling of protein structure to provide evidence that two plastoglobule proteins interact through β-barrels likely involved in specific lipid binding.

Protease shaving and membrane washing assays used isolated *Arabidopsis thaliana* thylakoid membranes with their associated plastoglobules (thylakoid/plastoglobule membranes), collected by pelleting unstressed total leaf homogenate and resuspending in buffer. The protease shaving assay probes the extent of protein exposure from the membrane by incubating isolated membranes with trypsin or an alternative protease.[6] Protected regions reveal sequence domains in close association with the membrane, precluding access by the proteases. Membrane washes test the type of interactions involved in protein-membrane binding by interfering with specific interactions, such as electrostatic interactions (by addition of NaCl), or protein structure (by addition of the chaotrope, urea).

We selected a pair of plastoglobule-associated proteins to test with protease shaving and membrane washing assays, i) the structural fibrillin protein, AtFBN1a,[7] and ii) the 13-lipoxygenase, AtLOX2, acting in the early, plastid-localized pathway of jasmonic acid biosynthesis.[8] Both proteins share homology with lipid-binding domains characterized by terminal β-barrels believed to provide lipid-binding capacity. These correspond to the lipocalin-like and PLAT (for polycystin-1, lipoxygenase and alpha toxin) domains found in AtFBN1a and AtLOX2, respectively.[9,10] Importantly, both proteins have close protein homologs (>20% identity) with crystal structures available.[11,12] This permits the development of meaningful homology models of our *A. thaliana* proteins, which we used to interpret our biochemical results as described below. Immunoblots used commercial primary antibodies raised

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against each full-length protein sequence (Agrisera AS07 258 and AS06 116); hence, trypsin truncation products from throughout the protein sequence may be recognized in immunoblots.

While AtLOX2 was completely degraded by trypsin within 5 minutes, revealing no apparent protected intermediates, AtFBN1a showed a slow rate of digestion and a ca. 25 kD intermediate that resisted further digestion within the 20-min incubation period (Figure 1a). This protected fragment likely represents protein sequence in close association with the glycerolipid surface, some of which may be embedded into the neutral lipid core. This experiment was repeated with the orthogonal protease, thermolysin, which provided the same patterns as seen with trypsin digestion. The lumenal oxygen evolving protein 33 (OE33) and the peripheral thylakoid protein ATPase α/β subunit were used as negative and positive controls, respectively (Figure 1a).

We continued by washing our thylakoid/plastoglobule membranes with buffers containing 1 M NaCl or 3 M urea to assess if electrostatic or protein structure-based interactions, respectively, were necessary for their attachment to plastoglobules. Both plastoglobule proteins remained membrane-associated in the presence of 1 M NaCl, indicating protein association was not solely dependent on electrostatic interactions. When washing with urea, AtFBN1a was very efficiently dissociated from the membrane fraction. This result suggests that protein conformation, and possibly protein–protein interaction, is critical for AtFBN1a association. AtFBN1a has been demonstrated to dimerize with itself and with the closely related AtFBN1b.[13] Our results with the urea support a possible role for dimerization in proper plastoglobule interaction. In contrast, neither the salt nor chaotrope treatment was capable of removing AtLOX2 from the membrane surface, suggesting that, although significantly exposed to the soluble fraction, as evidenced by the protease shaving assay, its interaction with membranes is particularly tenacious, comparable to an integral membrane protein such as the Photosystem II subunit, D1, in regard to membrane-binding strength (Figure 1b). While studies with other lipoxygenases in animal model species have indicated that protein–protein interactions may be required for membrane localization,[14,15] our results with the urea treatment suggest that AtLOX2 does not solely rely on protein–protein interaction to mediate its association to the plastoglobule, although protein–protein interactions within the membrane may still be involved in plastoglobule association. As a positive control for the washes, the peripheral thylakoid protein, ATPase alpha/beta subunit was tested (Figure 1b).

To help interpret our biochemical results, we generated homology models of both tested proteins. AtFBN1a shares 21.4% sequence identity and 31.8% sequence similarity with the lipocalin-like protein from *Diploptera punctata* (PDB: 5EPQ).[11] Likewise, AtLOX2 shares 42.6% sequence identity and 57.1% sequence similarity with soybean lipoxygenase-B (PDB: 2IUJ).[12] Homology modeling using the server Robetta[16] produced high confidence models for both proteins, achieving local distance difference test (lDDT) values of 0.67 and 0.86, for AtFBN1a and AtLOX2, respectively.[17] The modeled structure for AtFBN1a displays two distinct domains with a short N-terminal domain (ca. 8 kDa) characterized by a helix-loop-helix structure and a larger C-terminal domain (ca. 21 kDa) encompassing the lipocalin–like β-barrel comprised eight antiparallel β-sheets interspersed with a single, long α-helix (Figure 2a). This is comparable to the homology model of the plastoglobule-localized AtFBN4 produced by Lohscheider et al.,[18] which also identified a distinct C-terminal lipocalin-like domain and a shorter N-terminus with a helix-loop-helix. The structural features represented by the homology model could

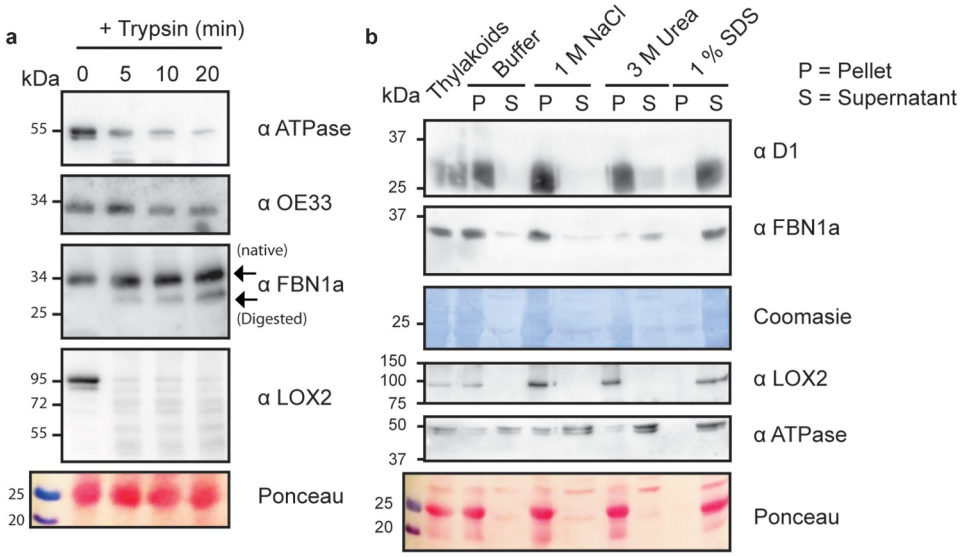


**Figure 1.** Membrane shaving and washing suggests differing membrane interaction strategies for AtLOX2 and AtFBN1a. (a) Isolated thylakoid/plastoglobule membranes were extracted from 4 week-old wild-type *A. thaliana* plants and treated with trypsin for protease digestion (12 mg of chlorophyll content treated with 60 µg of trypsin in 4 ml of buffer at 4°C). The reaction was stopped with SDS loading buffer after the specified incubation time. The thylakoid lumen OE33 (oxygen evolving complex subunit 33) and peripheral thylakoid ATPase alpha-beta subunits were used as negative and positive controls, respectively. (b) The isolated thylakoid/plastoglobule membranes were washed with specified buffer and separated by centrifugation into pellet (p) or supernatant (s) fractions. As a negative control for membrane washing, the integral thylakoid Photosystem II D1 subunit and the peripheral thylakoid ATPase alpha-beta subunits were used as negative and positive controls, respectively. Images are representative of results from at least three independent experimental replicates.

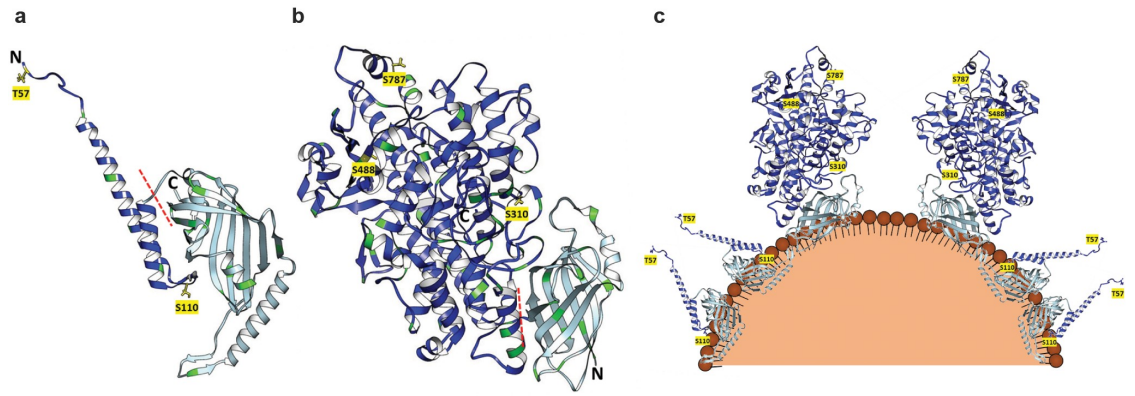


**Figure 2.** Structural predictions of AtFBN1a and AtLOX2. Structural predictions of mature AtFBN1a (amino acids 56–318) (a) and AtLOX2 (amino acids 72–896) (b) employed the Robetta server using homology modeling and the modeling method "TrRefineRosetta". The predicted membrane-associated β-barrel domain is presented in pale blue and the predicted exposed domain in deep blue. The experimentally identified phospho-serine and phospho-threonine residues identified from PhosPHAT 4.0 are highlighted in yellow, and the trypsin digestion sites in green. The structural models were visualized using UCSF Chimera. (c) A model illustrating the differing binding strategies suggested by the membrane shaving and washing assays. The protection of a large portion of the C-terminus of AtFBN1a supports a deep embedding of its lipocalin-like β-barrel within the plastoglobule core, although specific orientations or models of the embedding is premature in the absence of additional experimental or molecular dynamic data. In contrast, we present AtLOX2 with its N-terminal β-barrel laying parallel with the plastoglobule surface and largely exposed to the aqueous stroma. Note that the membrane curvature is not drawn to the scale of the protein models.

likely be extrapolated to the other Fibrillins found in plastoglobules based on their sequence conservation. The AtLOX2 homology model also presents two distinct domains, including a larger catalytic C-terminus (ca. 79 kDa) comprised mostly of α-helices, and a short N-terminus (ca. 14 kDa) harboring a β-barrel PLAT domain (Figure 2b).

Mapping of trypsin cleavage sites (identified in green in Figure 2) confirmed that the lack of digestion in the 25 kD AtFBN1a fragment was due to protection against the protease treatment rather than a lack of digestion sites. Notably, cleavage after the R116 residue (Figure 2a, red dashed line) would be expected to release a ca. 8 kD fragment from the mature protein sequence, which matches the size shift upon trypsin digestion and corresponds to the size of the N-terminal domain of the AtFBN1a. Consistent with the apparent sensitivity of the N-terminus, mapping of experimentally identified phosphorylation sites (compiled from the PhosPhat Database[19]) place all phosphorylation activity on the N-terminal domain. Collectively, this strongly suggests that the N-terminal domain of AtFBN1a is exposed in the aqueous stroma, while the C-terminal lipocalin-like domain is nestled into the glycerolipid monolayer and neutral lipid core of the plastoglobule (Figure 2c). It is interesting to consider what portions of the lipocalin-like domain may embed within the neutral core, but it would be difficult to hypothesize without additional experimental or molecular dynamics data. It is also possible that putative protein–protein interactions, such as the homo- or hetero-dimerization, described above may be involved in lipid or membrane interaction, accounting for some of the protease protection seen with AtFBN1a. The lack of detectable, protected fragments from trypsin (or thermolysin) incubation of AtLOX2 makes the assessment of its topology on the plastoglobule more difficult. However, the complete digestion of AtLOX2 by trypsin indicates that it does not embed into the neutral core as suggested for the AtFBN1a. Instead, we suggest that the PLAT domain of AtLOX2 lies along the surface of the plastoglobule using bulky hydrophobic residues to interact below the membrane–water interface of the plastoglobule (Figure 2c). This is consistent with our observed complete trypsin digestion of AtLOX2 and with the membrane topology suggested for the human 5-lipoxygenase ortholog that is associated with nuclear membranes.[20,21] Furthermore, AtLOX2 phosphorylation site mapping shows that the three experimentally identified phospho-sites are located in the catalytic C-terminus domain, which our model presents in an exposed fashion. The phosphorylation of these and other plastoglobule proteins could influence plastoglobule localization through impacts on protein conformation or protein–protein interactions.[22] Collectively, our data provide insights into the topology of two plastoglobule proteins and suggest possible mechanisms of interaction with the plastoglobule.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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