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Dimerization of the plant photoreceptor phototropin is probably mediated by the LOV1 domain

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Abstract Phototropin is a membrane-bound UV-A/blue light photoreceptor of plants responsible for phototropism, chloroplast migration and stomatal opening. Characteristic are two LOV domains, each binding one flavin mononucleotide, in the N-terminal half and having a serine/threonine kinase domain in the C-terminal half of the molecule. We purified the N-terminal half of oat phototropin 1, containing LOV1 and LOV2 domains, as a soluble fusion protein with the calmodulin binding peptide (CBP) by expression in *Escherichia coli*. Gel chromatography showed that it was dimeric in solution. While the fusion protein CBP-LOV2 was exclusively monomeric in solution, the fusion protein CBP-LOV1 occurred as monomer and dimer. The proportion of dimer increased on prolonged incubation. We conclude that native phototropin is a dimer and that the LOV1 domain is probably responsible for dimerization.

Keywords: Blue light photoreceptor; Gel chromatography; Protein domain; *Avena sativa* L.

1. Introduction

Phototropins are a family of plant blue/UV-A photoreceptors with at least two members, phot1 and phot2, involved in phototropism, chloroplast migration, stomatal opening, and rapid inhibition of stem growth [1]. Characteristic for phototropins are a serine/threonine kinase domain in the C-terminal half and two FMN-binding domains (LOV1 and LOV2) in the N-terminal half of the molecule; the FMN-binding domains have been named LOV domains because they show sequence homology to domains of receptor proteins that are regulated by light, oxygen, or voltage [2,3]. Illumination with UV-A or blue light results in a photocycle characterized by the reversible formation of a cysteinyl-flavin adduct connected with reversible bleaching of the major bands in the UV-A and blue region [4–6]; in addition to a thermal back reaction, elimination of the cysteine from the adduct can be achieved photochemically [7].

Much of the present knowledge on phototropin structure and function stems from the use of mutants, including phot1/phot2 double mutants which show none of the physiological responses mentioned above [1]. *Arabidopsis* transformants in which the LOV2 domain of phot1 was inactivated failed to show hypocotyl phototropism, while inactivation of LOV1 yielded transformants with high phototropic activity, showing that an active LOV2 domain alone was sufficient to establish phototropic curvature [8]. While this result showed the importance of LOV2 in regulating phototropin activity, the exact role of LOV1 remained unclear.

LOV domains are a subgroup of the larger group of PAS (Per-Arnt-Sim) domains of various sensor proteins; PAS domains are structurally characterized by three helical segments and a five-stranded antiparallel β-sheet, also found in LOV2 from the fern *Adiantum* [9]. Besides binding of cofactors, PAS domains are involved in protein–protein interactions [10]. Crosson and Moffat [9] speculated about interdomain dimerization between LOV1 and LOV2 in a single phototropin molecule or between distinct molecules within a signaling complex. Here, we show that a soluble phototropin fragment lacking the kinase domain is a dimer and further that LOV1 forms dimers in solution while LOV2 does not show this tendency.

2. Materials and methods

Subdomains of phototropin were generated by PCR amplification of the corresponding regions in the PHOT1a cDNA from *Avena sativa* and used for preparation of fusion proteins with the CBP as previously described [3,4]. The fusion protein CBP-LOV1 contained amino acids 135–276, CBP-LOV2, the amino acids 409–559 and CBP-NL1/2, the amino acids 1–525 of the PHOT1a sequence; together with the amino acids from the CBP vector, the calculated molecular sizes were 20.8, 26.2, and 62.6 kDa, respectively. Site-directed mutagenesis of LOV1 and LOV2 was carried out as described [4].

For gel chromatography, a column (1×49 cm) was packed with Superdex 200 (Amersham Pharmacia Biotech, Freiburg, Germany) and equilibrated with high salt buffer (50 mM Tris–HCl, pH 8.0, 1 M NaCl, and 2 mM EGTA). At a flow rate of 0.5 ml min^{-1}, the column was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), katalase (230 kDa), aldolase (160 kDa), albumin (67 kDa), and LOV2 (26.2 kDa) and used for analysis of CBP-NL1/2 (see Fig. 1). For analysis of CBP-LOV1 and CBP-LOV2 under otherwise identical conditions, the column was packed with Superdex 75 and calibrated with albumin (67 kDa), ovalbumin (43 kDa), chymotrypsin A (25 kDa), and ribonuclease A (13.7 kDa), the results are shown in Figs. 2 and 3. All analyses were carried out in darkness or under dim light unless stated otherwise.

3. Results and discussion

For determination of molecular size by gel chromatography, the proteins to be investigated must be soluble. Expression of

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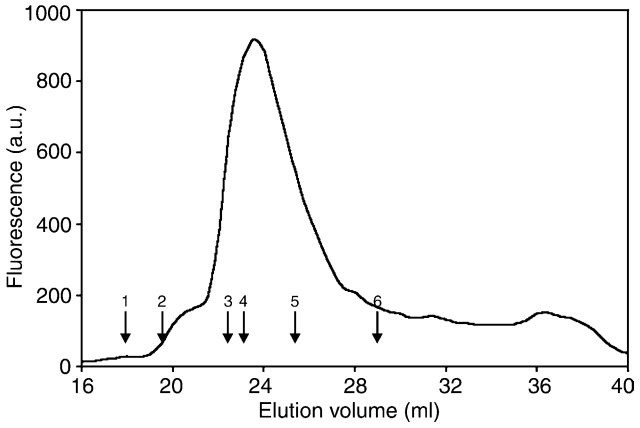

Fig. 1. Elution profile of the fusion protein CBP-NL1/2 on Superdex 200. The peak position of standard proteins used for calibration of the column are indicated by arrows as follows: 1, thyroglobulin; 2, ferritin; 3, catalase; 4, aldolase; 5, albumin; 6, LOV2 of phototropin. The fusion protein that contains the N-terminal half of phototropin including LOV1 and LOV2 domains is a dimer.

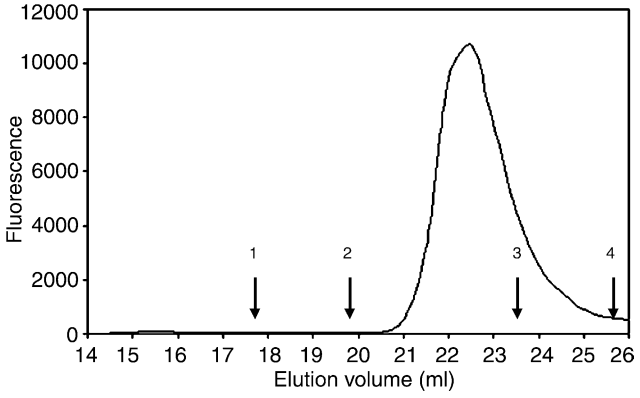

Fig. 2. Elution profile of the fusion protein CBP-LOV2 on Superdex 75. The peak position of standard proteins used for calibration of the column are indicated by arrows as follows: 1, albumin; 2, ovalbumin; 3, chymotrypsin A; 4, ribonuclease. Only the monomeric species of CBP-LOV2 is detected.

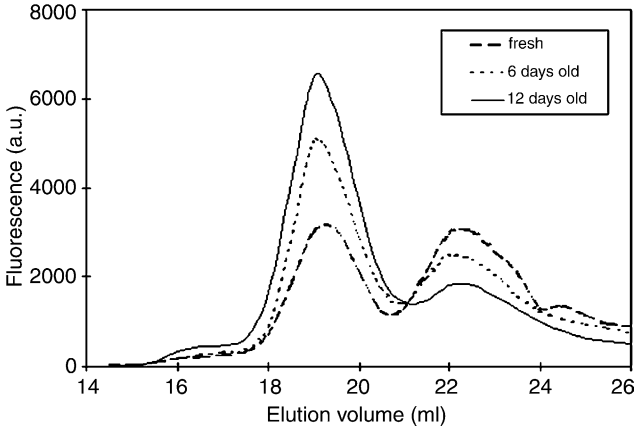

Fig. 3. Elution profile of the fusion protein CBP-LOV1 on Superdex 75. CBP-LOV1 shows increasing dimerization on prolonged incubation. For calibration see Fig. 2.

full-length phot1 in *Escherichia coli* cells resulted only in denatured phot1 in the pelletable fraction (data not shown), and with the insect cell system transfected with recombinant baculovirus, Christie et al. [11] obtained active phot1 that, although showing light-dependent autophosphorylation, was mostly insoluble. On the other hand, phot1 fragments expressed in *E. coli* as fusion proteins with the calmodulin binding peptide (CBP) proved to be soluble [3,4]. The largest soluble fragment CBP-NL1/2 contained, besides 47 amino acids of the CBP vector, amino acids 1 to 525 of the phot1 sequence [12]. Gel chromatography of this fragment on a calibrated column filled with Superdex 200 showed one peak with a maximum at 23.5 ml (Fig. 1); this corresponds to an apparent size of about 150 kDa. Since the calculated size based on the amino acid sequence is 62.6 kDa, the fragment CBP-NL1/2 clearly exists as a dimer in solution. The peak is asymmetric showing some tailing, either some monomers or proteolytic degradation products may contribute to the elution profile; SDS–PAGE before and after gel chromatography showed that a considerable part of CBP-NL1/2 was proteolytically degraded during the run (data not shown).

The fusion protein CBP-NL1/2 lacks the kinase domain of phototropin but otherwise contains the complete phot1 sequence including both LOV domains. Given that this fusion protein is dimeric in solution, one can conclude that also native phototropin forms dimers because it is unlikely that the kinase domain counteracts the dimerization. The best candidates causing dimerization are the two LOV domains: they belong to the type of PAS domains that have been reported to mediate dimerization and protein–protein interaction [13,14]. However, we must still exclude the possibility that the CBP sequence causes dimerization of the fusion protein CBP-NL1/2.

Gel chromatography of the fragment CBP-LOV2 on a calibrated column filled with Superdex 75 resulted in an elution profile showing a single peak with a maximum at 22.5 ml (Fig. 2). The result shown in Fig. 2 was obtained under safelight, and an otherwise identical run where the column was permanently illuminated with strong blue light gave an identical profile (data not shown). Comparison of the size determined by gel chromatography (30.5 kDa) and the size calculated from the sequence (26.2 kDa) revealed that the fragment is entirely monomeric in solution; thus, LOV2 does not show any tendency to form dimers. Further, this result also demonstrates that the CBP sequence is not responsible for the dimerization of CBP-NL1/2.

The analogous analysis with the fragment CBP-LOV1 gave a different result. With all preparations, we found two peaks in the elution profile (Fig. 3). The first maximum at 18.5 ml corresponded to a size of 53 kDa, the second maximum at 22.5 ml to a size of 25 kDa. Compared with the size of 20.8 kDa calculated from the amino acid sequence, the two species must be dimeric and monomeric CBL-LOV1. The apparent size of both species derived from the retention time was about 20% larger than that calculated from the amino acid sequence, and the same was true for CBP-LOV2 and CBP-NL1/2 (see above). Such behavior is typical for non-globular proteins. The X-ray analysis of the LOV domain of *Adiantum* phytochrome 3 revealed a relatively compact structure around the flavin mononucleotide chromophore with an almost globular overall shape [6,9], and identical UV/vis absorption spectra and photocycle indicate that folding around the chromophore must be very similar in the fusion proteins investigated here. The

ability of the CBP domain to bind calmodulin indicates that it has its own "native" fold, and it is suggestive to assume that the combination of two domains that fold independently in a fusion protein leads to an extended overall shape.

The relative amplitude of dimer and monomer varied from preparation to preparation. This was independent from light conditions: illumination of the sample and the column during the run with blue light that induced the photocycle of LOV1 did not result in any change in the proportions of the two peaks, and the mutated fragment CBP-LOV1-C39A that showed no photocycle [4] gave the same two peaks (data not shown). Finally, we found that prolonged incubation resulted in an increase of the dimer to monomer ratio (Fig. 3). By contrast, CBP-LOV2 and CBP-LOV2-C39A showed only the peak of the monomer even after prolonged incubation (data not shown).

It is unlikely that the kinetics of dimerization are as slow in vivo as we see in vitro. We consider it more likely that the dimerization process requires a defined conformation of LOV1, and that heterologous expression yields a relatively unstable and conformationally flexible protein. The conformational change necessary to obtain dimerization may be the rate limiting step for the observed reaction. While we cannot exclude the remote possibility that LOV2 can exist in a conformation that also leads to dimerization, the spectral properties of this domain indicate a stable and natively folded protein [4]. As such, the striking difference in the elution profiles between LOV1 and LOV2 suggests that LOV1 is most likely the domain responsible for dimerization of phototropin.

The assumption that phototropin consists of dimers in the cell like many other photoreceptors and sensor proteins is so far supported merely by indirect evidence. Native electrophoresis of extracts from etiolated pea seedlings yielded a 350-kDa complex that contained phototropin and other non-identified proteins [15]. The authors did not determine whether phototropin was monomeric or dimeric in the complex. Full-length phototropin expressed in insect cells transfected with recombinant baculovirus was mainly insoluble [11], the aggregation state of the small soluble part was not determined. Here, we show that a soluble construct that contains the N-terminal half of phototropin 1 and lacks the C-terminal kinase domain indeed forms dimers. As mentioned above, we argue that this result can be taken as indirect evidence for full-length phototropin forming dimers, because it is unlikely that the kinase domain prevents dimerization. While our results indicate dimerization as a function of the LOV1 domain, we wish to emphasize that this is not necessarily the only function of this domain. The lack of phototropic response and kinase activation by LOV1, when the photocycle of LOV2 was abolished by mutation, was found at low light intensity or fluence (W m^{-2}) [8]. The photocycle for activation of LOV1 requires higher fluences than that of LOV2: photoproduct formation is slower and dark regeneration is faster in LOV1 than in LOV2 [4]. The experimental approach of Christie et al. [8] did not allow one to decide whether LOV1 may function in kinase activation and phototropism at high fluence; further, its possible involvement in responses different from phototropism was not tested. It remains to be shown as to which functions other than dimerization can be attributed to the LOV1 domain.

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