Chromophore Exchange in the LOV2 Domain of the Plant Photoreceptor Phototropin1 from Oat[†]

Harald Dürr, Michael Salomon, and Wolfhart Rüdiger*

Department of Biology I, Botany, Menzingerstrasse 67, D-80638 München, Germany

Received September 29, 2004; Revised Manuscript Received December 16, 2004

ABSTRACT: Phototropins are a family of plant photoreceptors mediating blue light responses such as phototropism, leaf expansion, chloroplast relocation, and stomatal opening. Characteristic for phototropins are two LOV domains which, when expressed in heterologous systems, each carry a single flavin mononucleotide (FMN) chromophore. Here we describe removal of FMN from the LOV2 domain of *Avena sativa* using a hydrophobic matrix and successful incorporation of flavin adenine dinucleotide (FAD), riboflavin, and 5'-malonyl-riboflavin into the resulting apoprotein; 5-deaza-FMN was not incorporated under the applied conditions. The chromoproteins reconstituted with the various flavins showed absorption spectra and photocycle almost identical to those of the native LOV2 domain and that reconstituted with FMN except for the kinetics: LOV2−riboflavin and LOV2−5'-malonyl-riboflavin showed more rapid regeneration in the dark. LOV2−FAD can be hydrolyzed to LOV2−FMN with phosphodiesterase, indicating that the adenosine part extrudes from the protein. Together with the data from the X-ray structure (Crosson, S., and Moffat, K. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 2995−3000), the results allow us to decide which of the chromophore−protein interactions are essential for the reconstitution process.

Plants use light not only as an energy source for photosynthesis but also as a signal to obtain information about the environment. Light signals are major factors influencing plant growth and development, and several photoreceptors are known to sense specific regions of the visible and UV spectrum: phytochromes for red and far-red light (*1*), cryptochromes and phototropins for blue and UV-A light (*2*). Phototropins are a photoreceptor family with at least the two members phot1 and phot2 (*3*); in addition to phototropism, they are involved in chloroplast migration, stomatal opening, leaf expansion, and the rapid phase of hypocotyl growth inhibition (*4*−*7*). Characteristic of phototropins are two chromophore-binding domains, LOV1 and LOV2,[1] each of which binds one molecule of FMN, and a serine/threonine kinase domain that mediates light-activated autophosphorylation (*3*, *4*). In accordance with a postulate for phototropism, the autophosphorylation of phototropin has been demonstrated to be asymmetric in *Avena* coleoptiles after unilateral illumination (*8*, *9*). The light activation leads to a flavin C(4a)−cysteinyl adduct formed via the flavin triplet state (*10*−*14*); it reverts back to the original educt in the dark within a few minutes (*10*, *15*). LOV domains are not restricted to phototropins: LOV domains were detected in a hybrid phytochrome (phy3) of the fern *Adiantum* (*16*), in FKF1 of *Arabidopsis*, a protein involved in photoperiodism (*17*), and in several prokaryotic photoresponsive proteins (*18*).

LOV domains have a high affinity to the flavin chromophore: both the isolated LOV domains expressed in bacterial cells and full-length phototropin expressed in baculovirus-infected insect cells accumulate with FMN already bound to the protein (*10*, *16*, *19*). Therefore, it is generally assumed that FMN is the natural chromophore of phototropins, although the final proof for that assumption is still missing. In the structure predicted for the LOV2 domain of oat phototropin1a (*10*), strongly supported by the X-ray structure of the LOV2 domain of phy3 from *Adiantum* (*12*, *13*), the isoalloxazine ring and the ribityl side chain are located in a hydrophobic pocket surrounded by the peptide chain, while the end of the ribityl side chain carrying the phosphate group points toward the protein surface, where the phosphate group forms salt bridges with the side chains of two arginines.

We became interested in the question of whether flavins with different substituents at the ribityl side chain are able to bind to LOV domains. We chose the LOV2 domain of *Avena sativa* and the following flavins (see Figure 1): riboflavin (**1**) that lacks the phosphate group of FMN (**2**), FAD (**3**) which is, like FMN, a cofactor of many flavoproteins, and 5'-malonyl-riboflavin (flavinX, **4**) that carries a malonyl group instead of the phosphate group of FMN. **4** has been found in relatively high concentrations in oat coleoptiles (*19*). We also included 5-deaza-FMN (**5**), a chemically modified flavin derivative that contains a carbon instead of the N5 atom but is otherwise identical with FMN (*20*); 5-deaza-flavin derivatives bind to many flavo-enzymes and have often been used as tools to investigate enzyme

[†] This work was supported by the Deutsche Forschungsgemeinschaft (Ru 108/3-4) and the Fonds der Chemischen Industrie.

* To whom correspondence should be addressed. E-mail: ruediger@lrz.uni-muenchen.de. Fax: +49 89 17861 185. Phone: +49 89 17861 244.

[1] Abbreviations: CBP, calmodulin binding peptide; FAD, riboflavin−adenine dinucleotide; FMN, riboflavin-5'-monophosphate; FlavinX, 5'-malonyl-riboflavin; LOV, light, oxygen, voltage; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography.

10.1021/bi0478897 CCC: $30.25
Published on Web 02/04/2005

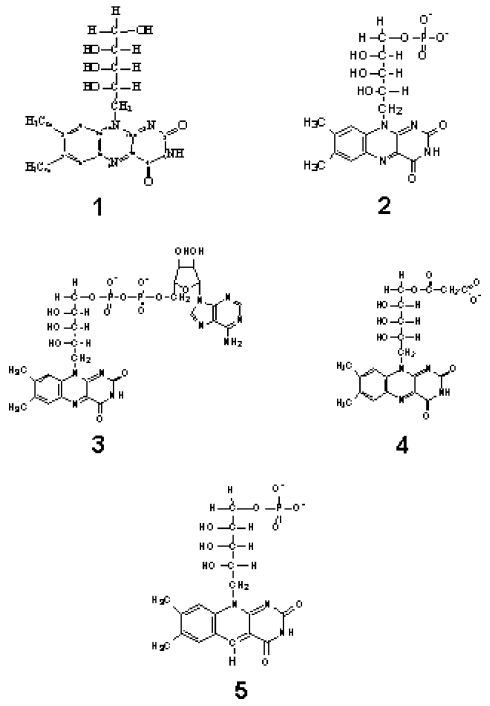

FIGURE 1: Structures of flavins used in reconstitution experiments: **1** = riboflavin, **2** = flavin mononucleotide (FMN), **3** = flavin-adenine dinucleotide (FAD), **4** = 5'-malonyl-riboflavin (flavinX), **5** = 5-deaza-FMN.

mechanisms (*21*−*26*). As mentioned above, LOV domains expressed in heterologous systems are always obtained with FMN bound to them; thus, binding studies with other flavins must imply chromophore exchange. Details of this procedure and the results with compounds **1**−**5** are reported here.

MATERIALS AND METHODS

Preparation of CBP-LOV2 Fusion Proteins. Cloning of the DNA fragment encoding the wild-type LOV2 domain of *A. sativa* phot1a and the mutated domain LOV2R40D into the bacterial expression vector pCAL-n-EK, expression of the fusion proteins with the calmodulin binding peptide (CBP) in *Escherichia coli* strain BL21(DE3), and purification of the fusion proteins CBP-LOV2 and CBP-LOV2R40D on calmodulin resin were performed as previously described (*10*). The purified proteins were stored at 4 °C in high-salt buffer (1 M NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, and 2 mM EGTA).

Chromophore Exchange. The proteins were precipitated from high-salt buffer with 60% ammonium sulfate, collected by centrifugation at 12000*g* for 10 min, dissolved in buffer A (1 M ammonium sulfate, 50 mM K_2HPO_4, pH 7.5), and applied to a column filled with phenyl-Sepharose CL-4B (Sigma) which had been equilibrated with buffer A; the binding capacity was about 4 mg of protein per mL of phenyl-Sepharose. The column was then washed with 3 volumes of buffer A. Removal of bound FMN required several washings with buffer B (1 M ammonium sulfate, 50 mM KH_2PO_4, pH 4.0) until the yellow color and the flavin fluorescence, detectable under UV light, completely disappeared from the column. Subsequently, the pH value was adjusted to 7.5 by repeated washings with buffer A. While the apoprotein remained bound to the column material, a solution of a 10-fold molar excess of the desired flavin in buffer A circulated through the column for 4 h at a flow rate of 1 mL/min at 25 °C. The reconstituted protein was then eluted with elution buffer (25 mM NaH_2PO_4/K_2HPO_4, pH 7.0). Non-reconstituted apoprotein remained on the column under these conditions.

Table 1: Photocycle of the CBP-Lov2 Fusion Protein Reconstituted with Various Chromophores[a]

	formation of photoproduct $t_{1/2}$ (s)		regeneration $t_{1/2}$ (s)		maximal bleaching at 447 nm
chromophore	20 °C	0 °C	20 °C	0 °C	(%)
FMN	4.24	4.93	23.90	192.2	100
FAD	4.43	4.70	14.33	150.5	97
Riboflavin	2.62	4.16	8.78	103.8	75
FlavinX	2.81	4.38	7.84	89.9	67

[a] Presented are half-life values of photoproduct formation during illumination with blue light (300 μmol m^{-2} s^{-1}), the percentage of maximal decrease of absorption at 447 nm after saturating illumination, and half-life values of subsequent regeneration of the absorption at 447 nm by dark incubation.

Chromophore Analysis. The buffer of the protein samples was exchanged by distilled water using an ultrafiltration unit (Nanosep 10 kDa, Pall Gelman Laboratory). Ethanol was added to a final concentration of 70%, and the sample was heated to 80 °C for 2 min and then immediately cooled with ice. The protein precipitate was removed by centrifugation (16000*g* for 10 min), and the supernatant was evaporated to dryness in a SpeedVac at 35 °C. The dry pigment was dissolved in 10 μL of water, of which 5 μL was applied to a thin-layer plate containing silica gel 60 (Merck). The chromatogram was developed with *n*-butanol/acetic acid/water (3:1:1, v:v:v).

Enzymatic Hydrolysis with Phosphodiesterase. A sample of CBP-LOV2−FAD (40 μM in 400 μL of 25 mM phosphate buffer, pH 7.0) was incubated with 0.15 unit of phosphodiesterase I (Sigma) for 30 min at 25 °C. The protein was then precipitated with 60% ammonium sulfate on ice and collected by centrifugation (16000*g* for 10 min); any free flavin chromophore remained in solution. The protein pellet was dissolved in 300 μL of phosphate buffer and used for chromophore analysis (see above). In a control experiment with free FAD, 0.07 unit of phosphodiesterase was sufficient to hydrolyze half of the FAD to FMN within 2 min; after 30 min, FAD had completely disappeared and the sample contained about equal amounts of FMN and riboflavin.

Spectroscopy. Ultraviolet and visible spectra were recorded with a diode array spectrophotometer (HP 8451A, Hewlett-Packard, Palo Alto, CA), and rapid reaction studies of light-induced absorbance changes were done with a Transputer integrated diode array spectrophotometer (TIDAS, J&M Analytische Mess-und Regeltechnik GmbH). Half-times for photoproduct formation and regeneration of the educt (see Table 1) were obtained from semilogarithmic plots of A_0/A_x versus time (not shown); A_0 denotes the amount of educt and photoproduct, respectively, at time zero and A_x the corresponding amount at time *x*, calculated from the respective absorbance changes at 447 nm.

Irradiation was performed with UV-A/blue light using a light source with glass fiber optics (KL 1500 electronic, Schott, Mainz) equipped with a broad-band (380−450 nm) blue glass filter. The fluence was 300 μmol m^{-2} s^{-1} if not stated otherwise.

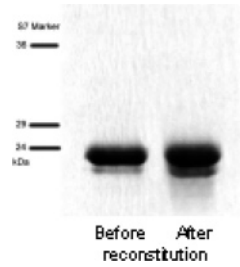

FIGURE 2: Analysis by SDS−PAGE of LOV2 before and after chromophore exchange with FMN.

RESULTS

Method of Chromophore Exchange. Two steps are required for the chromophore exchange of the LOV2 domain: (1) removal of FMN, inserted into the recombinant protein during its biosynthesis in the bacterial cells, and (2) insertion of a new chromophore into the resulting apoprotein. Both processes were separately optimized. A standard method to dissociate flavins from flavoproteins is acidification (*27*, *28*). We found that the LOV2 protein was irreversibly denatured at pH 2−3, while FMN was not completely liberated at pH 5−6, even after prolonged incubation. We used the "hydrophobic-interaction chromatography" described by van Berkel et al. (*28*): the LOV2 holoprotein was bound to phenyl-Sepharose at pH 7.5 on a column, and then the FMN chromophore was set free and quantitatively eluted from the column at pH 4.0. It was essential to check the completeness of removing FMN, e.g., by the sensitive flavin fluorescence under UV light. Elution of the apoprotein with ethylene glycol as used by van Berkel et al. proved to be impossible: the apoprotein remained bound to the phenyl-Sepharose unless the column was treated with hot SDS-containing buffer or 99% ethanol. Attempts to renature the eluted apoprotein failed, and removal of the detergent or solvent always yielded precipitated protein. Apparently, the apoprotein is barely soluble in aqueous buffer and the interaction with the hydrophobic matrix is extremely high, in contrast to the properties of the holoprotein. Therefore, we used the method of "reconstitution on the column". For this purpose, the apoprotein came into contact with a solution of the desired flavin chromophore at pH 7.5 while it was still bound to the hydrophobic matrix. A 10-fold excess of the flavin proved to be optimal: higher flavin concentrations did not increase the yield, while lower flavin concentrations gave lower yields of the holoprotein. To optimize the yield, the flavin solution was recycled several times over the column until the total time of incubation was 3−4 h at 25 °C. The lower yields observed upon incubation for more than 4 h were probably caused by denaturation and irreversible binding of the protein to the matrix. After complete removal of free flavin, the holoprotein was eluted from the column with phosphate buffer. Since the remaining apoprotein was not eluted under these conditions (see above), the reconstituted LOV2 domain was obtained free from apoprotein.

In a control experiment, the reconstitution was performed with CBP-LOV2 and the natural chromophore FMN. Analysis by SDS−PAGE (Figure 2) revealed a marginal increase of a degradation product during the chromophore exchange procedure; the bulk of CBP-LOV2 was intact, however.

Reconstitution with Various Flavins. The reconstitution procedure was then repeated under exactly the same conditions with a variety of flavins (for structures see Figure 1). The first hint for a successful reconstitution was the remaining yellow color on the column after incubation of the apoprotein with the desired flavin and elution of the excess of free pigment. No color remained when we used 5-deaza-FMN (**5**), and no protein was eluted when we subsequently applied phosphate buffer, indicating that no reconstitution occurred with this flavin derivative under the applied conditions. However, we found reconstituted LOV2 domains with compounds **1**−**4**. Interestingly, FAD that shows only a weak fluorescence in its free form became fluorescent like FMN after the reconstitution procedure. The absorption spectra of the eluted proteins were almost identical with the absorption spectrum of "native" LOV2, including the fine structure of the absorption band in the near-UV spectral region (see Figure 3). The yield of reconstituted CBP-LOV2, based on the amount of CBP-LOV2 applied to the phenyl-Sepharose column, was 20−25% with FMN and FAD and about 10% with riboflavin and flavinX, respectively. All four reconstituted LOV2 domains showed the typical bleaching after illumination with blue light (Figure 3). While the formation of the photoproduct was complete under standard conditions with CBP-LOV2−FMN and CBP-LOV2−FAD (Figure 3A,B), we observed only partial bleaching with CBP-LOV2−riboflavin and CBP-LOV2−flavinX (Figure 3C,D). In all cases, the bleaching was reversible: incubation of the bleached forms in the dark restored the original absorption spectra.

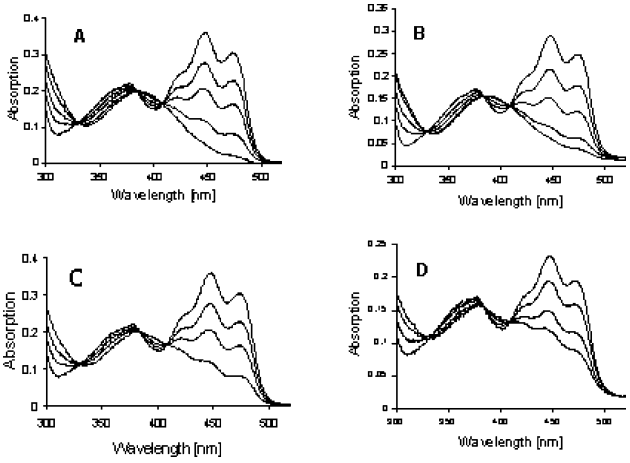

FIGURE 3: Photochemistry of LOV2 reconstituted with FMN (A), FAD (B), riboflavin (C), and flavinX (D). The absorption spectra were determined before irradiation (top) and after irradiation with blue light (300 μmol m^{-2} s^{-1}) for 2, 4, 8, and 12 s (A, B) and 1, 2, and 4 s (C, D).

For a more detailed characterization of the photocycle, the kinetics of bleaching and regeneration of the four reconstituted LOV2 domains was determined by monitoring the absorption at 447 nm (Table 1). The data show a very similar behavior of CBP-LOV2−FMN and CBP-LOV2−FAD and different kinetics for CBP-LOV2−riboflavin and CBP-LOV2−flavinX, especially more rapid regeneration reactions for the latter two compared with the first two products of reconstitution. Rapid regeneration could be a reason for incomplete photoproduct formation.

The Adenosine Part of FAD Is Not inside the Binding Pocket of LOV2. The FAD molecule is nearly twice as large as the FMN molecule, and thus it is unlikely that LOV2 can form a binding pocket which accommodates the complete

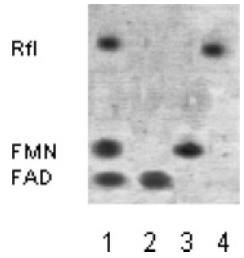

FIGURE 4: Chromophore analysis of reconstituted LOV2 domains. The fusion protein CBP-LOV2 was isolated after reconstitution, and the bound chromophore was liberated by denaturation with 70% ethanol and analyzed by TLC on silica gel with *n*-butanol/acetic acid/water (3:1:1, v:v:v). The reconstitution was carried out with FAD (lane 2), FMN (lane 3), and riboflavin (lane 4). Lane 1 shows the standards (from bottom to top): FAD, FMN, Rfl = riboflavin.

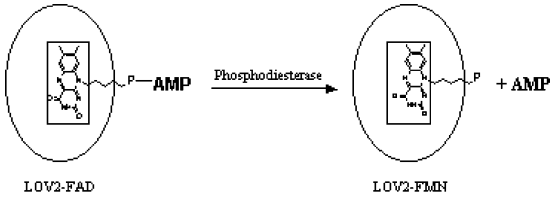

FIGURE 5: Schematic drawing of the supposed position of FAD bound to LOV2 and expected reaction with phosphodiesterase.

FAD molecule. The high similarity in the photochemical properties of LOV2 domains reconstituted with FMN and FAD prompted us to ask whether we had indeed obtained a LOV2 domain reconstituted with FAD or whether our FAD sample had in part been hydrolyzed to FMN before insertion into the protein; in that case, we should find only FMN in the isolated reconstituted protein. Therefore, we analyzed the chromophores in the reconstituted CBP-LOV2 proteins. The TLC analysis clearly showed that the incorporated chromophore was FAD after incubation with this flavin (Figure 4, lane 2). Further, the bound chromophore was riboflavin when this compound was used for incubation (Figure 4, lane 4).

We hypothesized that the binding pocket of LOV2 is, as in all investigated cases, more or less the same as known from the X-ray structure of "native" LOV2: the flavin moiety including the ribityl side chain is surrounded by the protein, and the end of the ribityl side chain points toward the surface of the LOV2 protein. Consequently, the AMP moiety of FAD should extrude from the protein surface, as schematically shown in Figure 5. A first indication for this situation became apparent from the observed change in fluorescence (see above): the AMP part quenches the fluorescence of the flavin part in free FAD, and the observed increase in fluorescence indicates that quenching by AMP was abolished after binding of FAD to the LOV2 domain. Further, the AMP part of FAD should be accessible to enzymes, while the FMN part remains bound to the LOV2 domain. We tested this hypothesis with CBP-LOV2−FAD and phosphodiesterase. The chromophore analysis by TLC revealed that the enzyme hydrolyzed the bound FAD to FMN as expected (Figure 6). Further hydrolysis to riboflavin was not observed, while the same phosphodiesterase sample, which apparently contained phosphatase activity, partially hydrolyzed free FAD to riboflavin under identical experimental conditions (data not shown). The UV/vis absorption spectrum of CBP-LOV2−FAD did not change during the incubation with phosphodiesterase;

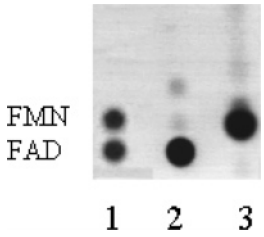

FIGURE 6: Phosphodiesterase digestion of CBP-LOV2−FAD. Chromophore analysis before (lane 2) and after (lane 3) incubation with phosphodiesterase showed quantitative digestion of bound FAD to FMN. Lane 1 shows the standards FAD and FMN.

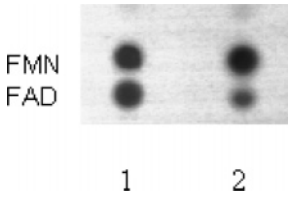

FIGURE 7: Relative binding affinity of the LOV2 domain for FMN and FAD. The reconstitution buffer contained FAD and FMN in the ratio 1:1 (lane 1); the FAD/FMN ratio in the reconstituted CBP-LOV2 fusion protein is about 1:5 (lane 2).

thus, the interaction between chromophore and protein was not significantly altered during the transition from CBP-LOV2−FAD to CBP-LOV2−FMN.

The striking similarity in the properties of CBP-LOV2−FMN and CBP-LOV2−FAD gave us reason to question whether FAD binds to LOV2 apoprotein with the same high affinity as FMN. The physiological background of this question is the fact that no direct analysis of the natural chromophore of plant phototropin has so far been performed. FMN was detected in recombinant phototropin expressed in insect cells (*19*) and in recombinant LOV domains expressed in bacterial cells (*10*, *16*). To approach this question, we performed a reconstitution with a 1:1 mixture of FAD and FMN. To avoid artifacts resulting from changing flavin concentration during prolonged incubation, the reconstitution on the column was terminated after 10 min. Analysis of the bound chromophores showed that the affinity of FAD was significantly lower than that of FMN (Figure 7); this result renders it unlikely that FAD is a natural chromophore of phototropin if chromophore insertion is a spontaneous process also in plants.

Flavin Binding Does Not Require the Interaction of the Phosphate Group of FMN with R40 of LOV2. The X-ray structure of the LOV2 domain of *Adiantum* phy3 revealed that the ribityl chain of FMN terminates at the protein surface on which the phosphate group interacts with the side chains of R40 and R56 (R967 and 983 of the phy3 sequence), forming salt bridges (*12*). The success of the reconstitution with riboflavin (see Figure 3C and Figure 4, lane 4) that does not contain this phosphate group indicates that the salt bridge is not essential for flavin binding of oat phot1a LOV2. However, the mutant protein CBP-LOV2R40D did not contain any flavin when it was expressed in bacterial cells (*10*). In support of this result, we found that reconstitution of CBP-LOV2R40D with FMN under standard conditions failed (data not shown). A possible explanation could be repulsion of the phosphate group by the negative charge of the aspartate side chain; however, we could not from the start exclude the possibility that the mutation changed the

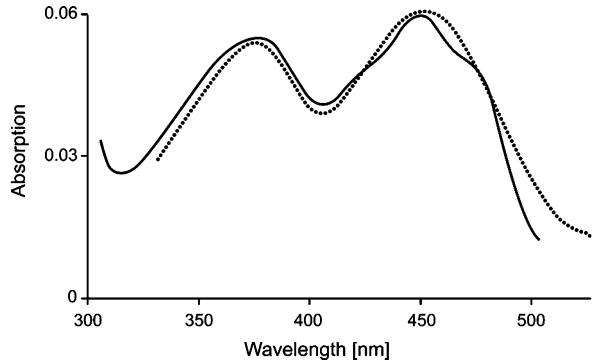

FIGURE 8: Absorption spectrum of the mutant protein CBP-LOV2R40D after reconstitution with riboflavin. The dashed curve is the absorption spectrum of free riboflavin.

spatial structure of the binding pocket, rendering flavin binding impossible. To test this possibility, we tried to reconstitute the mutant protein CBP-LOV2R40D with riboflavin. We obtained a yellow product in about 2% yield, and the absorption spectrum, the quality of which suffered from the low yield, nevertheless showed the fine structure typical for a flavin in the LOV2 binding pocket (Figure 8).

DISCUSSION

Chromophore exchange of CBP-LOV2 had so far been restricted to substitution of the native FMN by isotope-labeled FMN for investigation of the photoreaction of phototropin via NMR spectroscopy (*11*). Here we concentrated our efforts on variation of the flavin structure to get some insight into the properties of the binding pocket of the LOV2 domain. Further, we were able to study the single steps of the exchange reaction in more detail than with isotope-labeled FMN due to its poor availability.

The apoprotein that remained after removal of endogenous FMN from CBP-LOV2 proved to be almost insoluble in water and firmly bound to a hydrophobic matrix, in contrast to "native" CBP-LOV2 that carries the FMN chromophore. Probably, hydrophobic residues that are buried in the holoprotein are exposed to the exterior of the apoprotein. Since hydrophobic interaction is involved in flavin binding (*12*), it is reasonable to assume that residues forming the hydrophobic chromophore-binding pocket in CBP-LOV2 are exposed in the apoprotein and contribute to the hydrophobic interaction with the matrix of the column; this interaction may even partly stabilize the apoprotein, which seems to be more stable on the column than in solution. FMN derivatives will compete with the phenyl resin for the binding site during the procedure of reconstitution, lowering the binding affinity and facilitating elution of the holoprotein. The difference in the solubility of holoprotein and apoprotein also helps us to understand the observation that only the FMN-carrying holoprotein was detected after expression of LOV domains in bacterial cells: any apoprotein produced in excess over available FMN will precipitate; it may have been neglected so far, but we cannot exclude the possibility that such precipitate stops further translation.

Incorporation of flavin compounds **1**−**4** into the apoprotein yielded holoproteins with properties that were almost identical with those of native LOV2, in particular the UV/vis absorption spectra, including the fine structure of the main absorption band, and the same type of photocycle characterized by blue-light-dependent bleaching and complete regeneration of the original state in the dark; differences were detected only for the kinetics of single steps of the photocycle. Since free flavins show neither a corresponding fine structure of the absorption band nor any photocycle, we conclude that flavins **1**−**4** are bound to the protein and that the interaction between chromophore and the protein is at least similar to, if not identical with, that of native LOV2. In the LOV domain of phy3, FMN is bound noncovalently by a network of hydrogen bonds and van der Waals and electrostatic interactions (*12*), and since the participating amino acid residues are conserved, this can be assumed also for LOV2 of *A. sativa*. The salt bridges at the surface of LOV2 between guanidinium groups of two arginine residues and the phosphate group of FMN (*12*) are not essential for reconstitution of the apoprotein with flavins. The reconstitution was successful with riboflavin that lacks the phosphate group and with flavinX that carries a malonyl group instead of the phosphate group; however, the salt bridges seem to contribute to the rigidity of LOV2: the half times of both the photoreaction and the dark regeneration are faster without these salt bridges, and the difference is more apparent at room temperature than at 0 °C (see Table 1). On the other hand, partial neutralization of the positive surface charge by exchange of one arginine by an aspartate in the mutant CBP-LOV2R40D prevented binding of FMN, probably at least in part because of electrostatic repulsion: we showed binding of riboflavin in the mutant (see Figure 8). The low yield of reconstitution in this case may indicate that the overall structure of the protein was also affected by the mutation.

Surprising is the failure to reconstitute a LOV2 holoprotein with 5-deaza-FMN. Crosson and Moffat (*12*) found that there is no hydrogen bond to the N5 atom of the isoalloxazine ring in the LOV domain of phy3, and therefore, substitution of the nitrogen in the isoalloxazine ring by a carbon atom that has about the same spatial requirement as the nitrogen was expected to be tolerated by the LOV2 protein like it is by a number of flavoenzymes, where deaza-flavin derivatives have been valuable probes to study reaction mechanisms (*21*−*26*). Even if there are no hydrogen bonds to N5 in the completely folded state, we cannot exclude the possibility that such bonds may play a role during the folding process. This question needs to be addressed in future investigations.

ACKNOWLEDGMENT

We thank Dr. S. Ghisla for a gift of 5-deaza-FMN and 5'-malonyl-riboflavin.

REFERENCES

1. Fankhauser, C. (2001) The phytochromes, a family of red/far-red absorbing photoreceptors, *J. Biol. Chem. 276*, 11453−11456.
2. Christie, J. M., and Briggs, W. R. (2001) Blue light sensing in higher plants, *J. Biol. Chem. 276*, 11457−11460.
3. Briggs, W. R., Beck, C. F., Cashmore, A. R., Christie, J. M., Hughes, J., Jarillo, J. A., Kagawa, H., Liscum, E., Nagatani, A., Okada, K., Salomon, M., Rüdiger, W., Sakai, T., Takano, M., Wada, M., and Watson, J. C. (2001) The phototropin family of photoreceptors, *Plant Cell 13*, 993−997.
4. Briggs, W. R., and Christie, J. M. (2002). Phototropins 1 and 2: versatile plant blue-light receptors, *Trends Plant Sci. 7*, 204−210.

5. Folta, K. M., and Spalding, E. P. (2001) Unexpected roles for cryptochrome 2 and phototropin revealed by high-resolution analysis of blue light-stimulated hypocotyls growth inhibition, *Plant J. 26*, 471−478.
6. Kagawa, T., and Wada, M. (2002) Blue light-induced chloroplast relocation, *Plant Cell Physiol. 43*, 367−371.
7. Ohgishi, M., Saji, K., Okada, K., and Sakai, T. (2004) Functional analysis of each blue light receptor, cry1, cry2, phot1, and phot2, by using combinatorial multiple mutants in Arabidopsis, *Proc. Natl. Acad. Sci. U.S.A. 101*, 2223−2228.
8. Salomon, M., Zacherl, M., and Rüdiger, W. (1997) Phototropism and protein phosphorylation in higher plants: unilateral blue light irradiation generates a directional gradient of protein phosphorylation across the oat coleoptile, *Bot. Acta 110*, 214−216.
9. Salomon, M., Zacherl, M., and Rüdiger, W. (1997) Asymmetric, blue light-dependent phosphorylation of a 116 kDa plasmamembrane protein can be correlated with the first- and second-positive phototropic curvature of oat (Avena sativa L.) coleoptiles, *Plant Physiol. 115*, 485−491.
10. Salomon, M., Christie, J. M., Knieb, E., Lempert, U., and Briggs, W. R. (2000) Photochemical and mutational analysis of the FMN-binding domains of the plant blue light receptor, phototropin, *Biochemistry 39*, 9401−9410.
11. Salomon, M., Eisenreich, W., Dürr, H., Schleicher, E., Knieb, E., Massey, V., Rüdiger, W., Müller, F., Bacher, A., and Richter, G. (2001) An optomechanical transducer in the blue light receptor phototropin from Avena sativa, *Proc. Natl. Acad. Sci. U.S.A. 98*, 12357−12361.
12. Crosson, S., and Moffat, K. (2001) Structure of a flavin-binding plant photoreceptor domain: insights into light-mediated signal transduction. *Proc. Natl. Acad. Sci. U.S.A. 98*, 2995−3000.
13. Crosson, S., and Moffat, K. (2002) Photoexcited structure of a plant photoreceptor domain reveals a light-driven molecular switch, *Plant Cell 14*, 1067−1075.
14. Schüttrigkeit, T. A., Kompa, C. K., Salomon, M., Rüdiger, W., and Michel-Beyerle, M. E. (2003) Primary photophysics of the FMN binding LOV2 domain of the plant blue light receptor phototropin of Avena sativa, *Chem. Phys. 294*, 501−508.
15. Swartz, T. E., Corchnoy, S. B., Christie, J. M., Lewis, J. W., Szundi, I., Briggs, W. R., and Bogomolni, R. A. (2001) The photocycle of a flavin-binding domain of the blue light photoreceptor phototropin, *J. Biol. Chem. 276*, 36493−36500.
16. Christie, J. M., Salomon, M., Nozue, K., Wada, M., and Briggs, W. R. (1999) LOV (light, oxygen, or voltage) domains of the blue-light photoreceptor phototropin (nph1): binding sites for the chromophore flavin mononucleotide, *Proc. Natl. Acad. Sci. U.S.A. 96*, 8779−8783.
17. Imaizumi, T., Tran, H. G., Swartz, T. E., Briggs, W. R., and Kay, S. A. (2003) FKF1 is essential for photoperiodic-specific light signaling in Arabidopsis, *Nature 426*, 302−306.
18. Crosson, S., Rajagopal, S., and Moffat, K. (2003) The LOV domain family: photoresponsive signaling modules coupled to diverse output domains, *Biochemistry 42*, 2−10.
19. Christie, J. M., Reymond, P., Powell, G. K., Bernasconi, P., Raibekas, A., Liscum, E., and Briggs, W. R. (1998) Arabidopsis NPH1: a flavoprotein with the properties of a photoreceptor for phototropism, *Science 282*, 1698−1701.
20. Walsh, C., Fisher, J., Spencer, R., Donald, W. G., Wallace, T. A., Brown, J. E., Brown, R., and Rogers, E. F. (1978) Chemical and enzymatic properties of riboflavin analogues, *Biochemistry 17*, 1942−1951.
21. Massey, V., and Ghisla, S. (1986) New flavins for old: artificial flavins as active site probes of flavoproteins, *Biochem. J. 239*, 1−12.
22. Payne, G., Wills, M., Walsh, C., and Sancar, A. (1990) Reconstitution of Escherichia coli photolyase with flavins and flavin analogs, *Biochemistry 29*, 5706−5711.
23. Macheroux, P., Bornemann, S., Ghisla, S., and Thorneley, R. N. F. (1996) Studies with flavin analogs provide evidence that a protonated reduced FMN is the substrate-induced transient intermediate in the reaction of Escherichia coli chorismate synthase, *J. Biol. Chem. 271*, 25850−25858.
24. Hazzard, J. T., Govindaraj, S., Poulos, T. L., and Tollin, G. (1997) Electron transfer between the FMN and heme domains of cytochrome P450BM-3. Effects of substrate and CO, *J. Biol. Chem. 272*, 7922−7926.
25. Zhang, H., Gruenke, L., Saribas, A. S., Im, S.-C., Shen, A. L., Kasper, C. B., and Waskell, L. (2003) Preparation and characterization of a 5′-deazaFAD T491V NADPH-cytochrome P450 reductase, *Biochemistry 42*, 6804−6813.
26. Zhang, H., Gruenke, L., Arscott, D., Shen, A., Kasper, C., Harris, D. L., Glavanovich, M., Johnson, R., and Waskell, L. (2003) Determination of the rate of reduction of oxyferrous cytochrome P450 2B4 by 5-deazariboflavin adenine dinucleotide T491V cytochrome P450 reductase, *Biochemistry 42*, 11594−11603.
27. Massey, V., and Husain, M. (1978) Reversible resolution and reconstitution of flavoproteins and free flavin, *Methods Enzymol. 53*, 429−437.
28. van Berkel, W. J. H., van den Berg, W. A. M., and Müller, F. (1988) Large-scale preparation and reconstitution of apo-flavoproteins with spezial reference to butyryl-CoA dehydrogenase from Megasphaera elsdenii, *Eur. J. Biochem. 178*, 197−207.

BI0478897