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Chapter 3

Chromophore composition of a heterologously expressed BLUF-domain

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Chapter 3

3.1 Abstract

Upon heterologous expression of the BLUF (for: Blue-Light sensing Using Flavin) domain from AppA, a transcriptional anti-repressor from *Rhodobacter sphaeroides*, in *Escherichia coli*, photoactive holo-protein is formed through non-covalent binding of a flavin. Whereas it is generally assumed that FAD is the physiological chromophore of this photo-perception domain *in vivo*, *E. coli* can (and does) insert, depending on the growth conditions, all naturally occurring flavins, *i.e.* riboflavin, FMN and FAD into this protein domain. The nature of the particular flavin bound affects the photochemical- and particularly the fluorescence properties of the N-terminal domain of this photosensory protein.

3.2 Introduction

The general interest in photoreceptor proteins has significantly increased during the last decade because of the very significant increase in the opportunities to over-express such proteins to amounts that are compatible with detailed biophysical characterization. This development has made these photoreceptor proteins as excellent model systems for studies of the dynamical and functional transitions in protein structure (van der Horst and Hellingwerf, 2004). The possibility to activate these proteins with (laser) light provides very high time resolution for such functional characterisations.

The most common heterologous overexpression hosts would be *Escherichia coli*, *Saccharomyces cerevisiae* and insect cell cultures. For many heterologously expressed photoreceptors the physiological chromophore is not available in these hosts. This is specifically true for members of the photoreceptor families that base their photoactivation on the E/Z isomerization of their chromophore, *i.e.* the phytochromes, the rhodopsins and the xanthopsins. For these proteins therefore specific arrangements have to be made to allow for holoprotein formation. One way to achieve this is via complementation of the heterologous host with a biosynthetic route for the desired chromophore (Gambetta and Lagarias, 2001; Kyndt et al., 2003; Landgraf et al., 2001). The alternative is to reconstitute apo-protein with (a) chromophore (derivative), either during growth or during or after purification of the photoreceptor protein (*e.g.* (Imamoto et al., 1995; Shimono et al., 1997)).

This situation is different for members of the photoreceptor families that make use of a flavin-based chromophore. Flavins, or more specifically: riboflavin, flavin
mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) are an intrinsic part of the metabolite repertoire of every living cell. During the past five years several such flavin-containing photoreceptor families have been characterized, of which the photochemistry is based on flavin photoexcitation, i.e. (i) the cryptochromes, (ii) the phototropins and (iii) the family of the BLUF domain proteins.

For members of these latter three families it cannot be taken for granted that the proper chromophore is inserted upon heterologous overexpression. Here we report that for the BLUF domain of the transcriptional anti-repressor AppA, from the purple non-sulfur anoxygenotrophic bacterium *Rhodobacter sphaeroides*, significant variation in chromophore composition occurs upon heterologous overexpression in *Escherichia coli*.

However, incubation of cell-free extract with a large molar excess of a desired flavin does allow one to produce homogeneous holoprotein with respect to chromophore composition. Significantly, variation of the nature of the flavin affects the spectral and fluorescence properties of the reconstituted holoprotein.

### 3.3 Materials and methods

#### 3.3.1 Strains and growth conditions

Cloning experiments were performed with *Escherichia coli* XL1-Blue grown in Luria-Bertani (LB) medium using established protocols. Heterologous overexpression of AppA<sub>5-125</sub> (i.e. the N-terminal domain of AppA truncated between amino-acids 5 and 125) was performed in *Escherichia coli* M15 (pREP4) grown in production broth (PB) medium (20 g L<sup>-1</sup> tryptone, 10 g L<sup>-1</sup> yeast extract, 5 g L<sup>-1</sup> dextrose, 5 g L<sup>-1</sup> NaCl, 8.7 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, pH 7.0). Ampicillin and kanamycin were used at 100 and 50 µg ml<sup>-1</sup>, respectively.

#### 3.3.2 Protein expression and purification

AppA<sub>5-125</sub> was cloned, expressed and nickel-affinity purified as described previously (Laan et al., 2003). Where indicated, cell-free extracts were incubated with a large molar excess of FAD, FMN or riboflavin (assuming a yield of ~ 100 mg L<sup>-1</sup> of *E. coli* culture (final OD<sub>600</sub> ~5.5 (Novaspec II spectrophotometer, Pharmacia Biotech), which is equivalent to a dry weight of ~ 2.3 mg ml<sup>-1</sup>) for 1 h on ice before proceeding with the nickel-affinity chromatography. Nickel-affinity purified protein was dialyzed overnight against 10 mM Tris-HCl, pH 8.0 and further purified by anion-exchange chromatography on a ResourceQ (6
or a MonoQ (1 ml) column (Amersham Biosciences; see also Results section). The proteins were eluted using a gradient of 0 to 1 M NaCl in 10 mM Tris-HCl, pH 8.0 and detected at 280 nm. Eluted proteins with similar UV-Vis absorption spectra were pooled and dialyzed to 10 mM Tris-HCl, pH 8.0 and stored at 253 K. Purity of the samples was checked with SDS-PAGE using the PHAST-System (Amersham Biosciences).

3.3.3 TLC analysis of the flavin cofactors associated with AppA<sub>5-125</sub>
Chromophore was released from the protein by boiling samples in 70% ethanol for 1 min. After centrifugation at 23,000 x g, the flavin-containing supernatants were lyophilized to dryness with a speed-vac concentrator and resuspended in 35% (v/v) ethanol. TLC analysis was performed on silica gel 60 W plates (Merck, Darmstadt) with n-butanol/acetic acid/water, 3:1:1 (v/v) as developing solvent. Also standards of FAD, FMN and riboflavin (2 mg ml<sup>-1</sup> in 35% (v/v) ethanol) were spotted. Flavin-containing spots were detected via their UV-induced fluorescence emission. A calibration with a mixture of flavins showed that a contaminating flavin could not be detected if it was present at less than 5 % of the total amount of flavin in the sample, in the concentration range tested.

3.3.4 Transient UV-Vis absorption spectroscopy
Steady state UV-Vis absorption spectra and receptor state recovery kinetics were recorded using a Hewlett Packard 8453 spectrophotometer (Portland, Oregon). Measurements of the rate of receptor state recovery were performed at 298 K using a "Kraayenhof vessel" (Kraayenhof et al., 1982) placed in the sample compartment of the spectrophotometer. One port of the vessel was used to convert AppA (in 10 mM Tris-HCl, pH 8.0) to the signaling state by illumination with actinic light from a Schott KL1500 light source (containing a 150 W halogen lamp). Two ports were used for the measuring beam, and the fourth port was used to monitor the pH by using a Mettler Toledo (micro) combination pH electrode (InLab423) connected to a Dulas Engineering amplifier. The data was fitted with a mono-exponential decay function using Origin software.

3.3.5 Relative quantum yield determination
Solutions of AppA<sub>5-125</sub> containing either riboflavin, FMN or FAD as the only chromophore and with an identical final absorption at 446 nm of ~0.3, were converted to the signaling
state by illumination with light from a Schott KL1500 light source (containing a 150 W halogen lamp) passed through a 450 nm band-pass filter (fwhm 10 nm). UV/Vis absorbance spectra (from 300 to 600 nm) were measured every second. To reduce signaling state formation by the measuring beam, a neutral density filter (19.6 % transmission) was placed between the beam and the sample.

3.3.6 Fluorescence spectroscopy

Fluorescence emission spectra were recorded at room temperature with an Aminco Bowman Series 2 Luminescence Spectrometer using 20 μM samples of AppA (in 10 mM Tris-HCl, pH 8.0), with excitation at 450 nm. After recording the fluorescence spectra of the receptor state of these proteins the samples were converted to their signaling state by illumination with light from a Schott KL1500 light source. To reduce signaling state formation by the excitation beam, a neutral density filter (19.6 % transmission) was placed in the beam before it reached the sample compartment. The quantum yield of fluorescence of the AppA variants at 527 nm was determined by comparison to the fluorescence at 527 nm of a sample of FAD excited at 450 nm (in 10 mM Tris-HCl, pH 8.0, with an identical absorption at 450 nm as the protein samples), assuming a quantum yield of 0.03 for FAD (Weber, 1950).

3.3.7 FT-IR spectroscopy

FT-IR difference spectroscopy was performed using a Bruker IFS 66v spectrometer (Bruker Analytische Metechnik). The preparation of the sample for the conventional transmission technique was as follows: a droplet of a concentrated AppA solution (830 μM, in 10 mM Tris-HCl, pH 8) was put on a BaF$_2$ window and dried by a stream of nitrogen. After rehydration in a chamber at 100 % relative humidity, the sample was sealed by a second BaF$_2$ window and inserted into the spectrometer (for more details, see (Ataka et al., 2003)). For sample illumination, an intense blue-emitting LED (emission maximum at 445, fwhm 22 nm with a power of 130 mW) was used. The temperature of the sample was set to 293 K by a circulating water bath.
3.4 Results

3.4.1 Separation of BLUF domains containing different chromophores

For spectroscopic studies of the primary photochemistry of AppA we routinely have produced its N-terminal BLUF domain as a poly-histidine tagged protein in *E. coli*. To optimize production, the producer cells are grown in rich media, based on peptone and yeast extract. Using the production procedure described in Materials and Methods, we routinely obtain a yield of ~100 mg L\(^{-1}\) of culture broth, under conditions at which the cultures reach a final OD\(_{600}\) of ~5.5 (2.3 mg ml\(^{-1}\) cellular dry weight).

For a sample with a protein : flavin molar ratio of 1:1, the ratio of the absorption at 270 nm (\(\varepsilon_{270} = 35,800 \text{ M}^{-1} \text{ cm}^{-1}\); from the combined spectral contributions from the two tryptophan and two tyrosine residues and the flavin (Kraft et al., 2003)) over the absorption at the flavin peak (\(\varepsilon_{446} = 8,500 \text{ M}^{-1} \text{ cm}^{-1}\) (Kraft et al., 2003)) should be ~4.2. However, when the BLUF domain is produced in *E. coli*, grown on PB medium, and subsequently purified with Ni-affinity chromatography, the UV-Vis absorption spectrum of the purified protein shows a 280/446 nm ratio that varies between ~6 and 8. Because SDS-PAGE analysis shows that the samples are routinely >90% pure, the additional absorption at 280 nm is not caused by contaminating proteins, but rather by the presence of apo-AppA, *i.e.* AppA protein lacking a chromophore. When we tried to separate the holo- and apo-protein with ion-exchange chromatography, using FPLC and a MonoQ column, the elution pattern as shown in Fig.
Chromophore composition of the BLUF domain of AppA

1A was obtained. Indeed colorless apo-protein (peak 3) could be separated from holo-protein (peaks 1 and 2). Initial trials using a ResourceQ column, which has a higher binding capacity and allows for higher flow rates, resulted in a similar elution profile for the holo-protein, but the apo-protein could not be eluted from this latter column with the selected range of elution buffers. The purified apo-protein can be functionally reconstituted with FAD (data not shown).

It was also unexpected that the holo-protein would elute in two separate peaks (peaks 1 and 2) upon ion-exchange chromatography. Because analysis with gel electrophoresis (SDS-PAGE) and mass-spectrometry revealed that the apo-protein in these two fractions was indistinguishable (data not shown), we decided to analyze their chromophore content. The result - obtained with thin-layer chromatography (TLC) and displayed in Fig. 2A - reveals that the different elution behavior of the two fractions on the ion-exchange column can be explained by the difference in chromophore structure in these holoproteins: In fraction 1 the chromophore is riboflavin (lane 5), whereas protein in fraction 2 (predominantly) contains FMN (lane 6). As a control, FAD was subjected to the same procedure as used for the extraction of the flavins from the protein. This control revealed that the extraction procedure does not result in degradation of the flavin (lane 4).

Figure 2. TLC-analysis of flavins in ResourceQ peaks. Flavins associated with the protein present in the peaks eluted from the ResourceQ anion-exchange column were analyzed by TLC (see Materials and methods). (A) flavins from the 2 peaks of holoprotein of nickel-purified AppA<sub>5,125</sub>. Lane 1: FAD, lane 2: FMN, lane 3: riboflavin, lane 4: FAD treated as protein-associated flavin, lane 5: flavin extracted from peak 1, lane 6: flavin extracted from peak 2 (see also Fig. 1A). (B) flavins in nickel-purified AppA<sub>5,125</sub> with and without prior incubation of the cell-extract with different flavins. Lane 1: no pre-incubation, lane 2: incubation with FAD, lane 3: incubation with FMN, lane 4: incubation with riboflavin.
These results are rather surprising because it is generally assumed that FAD is the physiological chromophore of the BLUF domain in *Rb. sphaeroides*. As a next step we repeated the production- and purification procedure, but now with an excess FAD, FMN or riboflavin added to the cell-extract before initiation of the purification steps. As can be concluded from the elution patterns from the Resource Q column (see Fig. 1B) this adaptation of the procedure resulted in the production of a (nearly) homogeneous fraction of purified protein. Furthermore, analysis of the chromophore complement of these purified proteins (see Fig. 2B) revealed that the respective apo-proteins had combined with FAD, FMN or riboflavin, exclusively.

![Figure 3. UV-Vis absorption spectra and light-dark difference spectra of the N-terminal domain of AppA reconstituted with different flavins.](image)

(A) AppA containing riboflavin. (B) AppA containing FMN. (C) AppA containing FAD. In A – C the solid line represents the receptor state (i.e. dark- or ground state), and the dotted line the signalling state (obtained after illumination). (D) light-dark difference spectra of AppA containing FAD (black line), FMN (dotted line), or riboflavin (dashed line).
3.4.2 Comparison of BLUF domains containing different chromophores

The procedures described above allowed us to prepare separate and pure fractions of the BLUF domain, each containing one of the three flavins exclusively. When tested for spectral properties, these fractions revealed significant differences. Fig. 3 shows that the spectra of the receptor- and signaling state of AppA with FAD and FMN are indistinguishable, with absorption maxima at 375 and 447 nm for the ground state and 381 and 460 nm for the signaling state, respectively. The spectra of AppA with riboflavin bound are similar to the spectra of AppA with FAD bound, albeit with a small blue-shift, particularly in the longer wavelength absorption band, now exhibiting absorption maxima at 375 and 444 nm in the ground state and 380 and 457 nm in the signaling state. From this it is clear that all 3 AppA variants exhibit similar spectral changes upon illumination, as shown by the similarity of the light-dark difference spectra (Fig. 3D).

Photocycle kinetics are also slightly affected by the chemical structure of the chromophore (see Table 1), but are very similar.

<table>
<thead>
<tr>
<th>Flavin</th>
<th>k (x 10^{-3} s^{-1})</th>
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<tr>
<td>riboflavin</td>
<td>1.49 ± 0.03</td>
</tr>
<tr>
<td>FMN</td>
<td>1.61 ± 0.03</td>
</tr>
<tr>
<td>FAD</td>
<td>1.33 ± 0.08</td>
</tr>
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Table 1. Recovery rate of receptor state formation in AppA containing the three different flavins.

Figure 4. The relative quantum yield of signalling state formation of AppA with FAD, FMN or riboflavin. Samples of AppA containing either FAD (filled squares), FMN (circles) or riboflavin (triangles) with identical absorption at 446, were converted to the signalling state by illumination. The graph shows the initial part of the traces shown in the insert. The lines through the data points were obtained through linear regression of the data obtained between 1 and 6 seconds after illumination.
To determine whether the nature of the flavin affects the quantum yield of photochemistry, we compared the initial rates of photoconversion of samples, containing one of the three different flavins, with identical initial absorption at 450 nm. Fig. 4 shows that these rates are very similar, irrespective of the flavin bound, indicating that this quantum yield is similar for all 3 AppA variants. Comparable results were obtained with different light intensities and/or different filters (see table 2).

<table>
<thead>
<tr>
<th>Flavin</th>
<th>Relative quantum yield (%)</th>
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<tbody>
<tr>
<td>FAD</td>
<td>100</td>
</tr>
<tr>
<td>FMN</td>
<td>112 ± 5</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>111 ± 24</td>
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Table 2. The relative quantum yield of signaling state formation of AppA containing the three different flavins. From plots of the decrease in absorption at 446 upon illumination of AppA containing either FAD, FMN or riboflavin, the initial parts were fitted with linear regression using the Origin software (see also legend to Fig. 4). Given is the mean of the slopes of three different experiments using different filters and light intensities, relative to the slope determined for AppA with FAD, which is set at 100 %.

The fluorescence emission spectra of the ground states of the 3 AppA variants, upon excitation at 450 nm, are very similar in shape, showing an emission peak at 502 nm and a shoulder at ~ 525 nm (see Fig. 5). FMN-containing AppA shows the highest emission, followed by FAD- and riboflavin-containing AppA (see table 3). Illumination of all 3 variants (to convert them into their signaling state) results in significant quenching of the fluorescence (see table 3) and on average a 13 nm red-shift of the emission spectrum, comparable to the red-shift observed in the UV-Vis absorbance spectra. The fluorescence quantum-yield of the different variants at 527 nm (with excitation at 450 nm) is given in table 3.

<table>
<thead>
<tr>
<th>Flavin</th>
<th>Φfluorescence</th>
<th>Decrease in fluorescence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>riboflavin</td>
<td>0.016</td>
<td>89</td>
</tr>
<tr>
<td>FMN</td>
<td>0.029</td>
<td>93</td>
</tr>
<tr>
<td>FAD</td>
<td>0.023</td>
<td>94</td>
</tr>
</tbody>
</table>

Table 3. Fluorescence emission of receptor- and signaling state of AppA. The quantum yield of fluorescence is given for the receptor state; the decrease in fluorescence emission refers to signalling state formation.
We have also studied possible functional differences among the three reconstituted AppA proteins by FT-IR spectroscopy. Fig. 6 shows the light-induced FT-IR difference spectra of AppA supplied with FAD, FMN, and riboflavin, respectively. The very close similarity of the difference spectra shows that the protein undergoes very similar molecular changes after photoexcitation, irrespective of which flavin was incorporated as its chromophore.

Figure 5. Fluorescence emission spectra of AppA with different flavins bound. Emission spectra of dark-adapted and light-excited AppA with FAD (solid lines), FMN (dotted lines), riboflavin (dashed lines). The top 3 spectra are from the groundstates, whereas the bottom 3 spectra are from signalling states. Excitation under all conditions was performed at 450 nm (see also (Kraft et al., 2003)).

Figure 6. Light-induced FT-IR difference spectra of AppA with FAD, FMN or riboflavin. Spectra have been scaled to yield identical difference absorbance at 1710 cm⁻¹ where the differential absorbance is -9 x 10⁻³ for AppA with FAD (top spectrum).
3.5 Discussion

We have shown that the BLUF-domain of AppA can incorporate all three naturally occurring flavins. However, by proper adjustment of the purification procedure, AppA protein can be produced that contains any of the three flavins exclusively. The nature of the flavin chromophore hardly affects the capacity of AppA to photocycle, in contrast to the intensity of its fluorescence emission, which decreases by approximately 50% upon incorporation of riboflavin, as compared to FAD-containing AppA.

Binding of both FAD and FMN is also reported for another heterologously expressed BLUF-domain protein, the photoactivated adenylyl cyclase (PAC) from *Euglena gracilis* (Watanabe M., 2004), suggesting that the heterogeneous flavin binding may be a general property of BLUF domains. Although AppA was previously shown to bind FAD (Gomelsky and Kaplan, 1998) and the binding-affinity of the BLUF-domain may be highest for FAD, we assume that the high expression level reached in our system leads to depletion of free FAD in the cells and consequent binding of riboflavin and FMN to the heterologously expressed BLUF domain. The level of incorporation of each flavin in the BLUF domain may in fact reflect the flavin-composition within the cell and the affinity of this domain for the three flavins. Although unlikely, because the construct used in this study extends at both the N- and C-terminus beyond the protein region predicted to form the flavin-binding BLUF domain (residues 16-107 (Gomelsky and Klug, 2002)), the truncation of the protein may have altered the specificity for flavin incorporation. To test this, the full-length protein will be expressed (both in *E. coli* and in *Rhodobacter sphaeroides*; see further below), purified and the flavin-complement analyzed.

The fact that AppA with riboflavin as the chromophore behaves very similar to AppA with FAD indicates that neither the AMP- nor the phosphate group of FAD are critically required for signaling state formation. In this respect AppA may differ from LOV-domain containing proteins (Losi *et al.*, 2004a). AppA with riboflavin bound has a slightly blue-shifted absorption maximum compared to FMN- and FAD-AppA, suggesting less hydrogen bonding to the flavin and/or reduced π-π stacking, possibly with the conserved Y21 (Kraft *et al.*, 2003). The heterogeneity in chromophore composition, however, may strongly affect crystallization of AppA, as is suggested by the results of the ion exchange chromatography. In agreement with this, the initial reports on AppA crystals (see abstract to the 14th ICP, Jeju, Korea, 2004) mentioned poor resolution in diffraction studies. The difference spectra between signaling- and receptor state show a slight increase in
vibrational fine structure for FAD- and FMN-containing AppA, which is absent in the riboflavin derivative.

Our ground state fluorescence spectra show a peak at 502 nm, which is not seen in the spectrum published by (Kraft et al., 2003). The reason for this is unclear at present. The fluorescence quantum yield of riboflavin and FMN in solution ($\Phi_f = 0.26$ (Weber, 1950)) is significantly higher than that of FAD ($\Phi_f = 0.03$ (Weber, 1950)), because in the latter an intramolecular complex is formed between the flavin and the adenine moiety (see (Islam et al., 2003) and references therein). Bound to AppA, the three flavins have fluorescence quantum yields similar to FAD in solution. Assuming that FAD is bound to its apo-protein in an extended conformation, i.e. that an intra-chromophore quenching complex is not present in FAD when it is bound to AppA, this indicates that the protein significantly quenches the fluorescence of all three flavins. Again, stacking of the flavins with aromatic residues, like the conserved tyrosine-21, may be responsible for this quenching. In the signaling state of AppA this quenching is even far more pronounced because the fluorescence emission decreases with 80 to 94 %. In Slr1694, which is also a member of the family of BLUF-domain containing proteins, quenching of fluorescence in the signaling state – as compared to the receptor state- has been reported too (Masuda et al., 2004). In the latter protein however, due to the faster photocycle recovery rates, the exact ratio of the quantum yields in these two states could not be determined. We are not aware of analysis of the flavin composition of heterologously produced Slr1694. Nevertheless, FTIR difference spectra of the signaling- and receptor state of these proteins are very similar too (see further below).

Whereas UV/Vis- and fluorescence spectroscopy provide information about the electronic states of the chromophore, FT-IR spectroscopy goes beyond this and provides direct evidence for the vibrational modes of the chromophore and apo-protein that are involved in the conversion of photon energy into the structure of the signaling state. It is premature to try and assign all the bands observed in the difference spectra. Therefore, we refrain from a discussion on the molecular origin of the bands. In this work, we rather used functional FT-IR difference spectroscopy as a very sensitive tool to resolve even the slightest dissimilarities of AppA with respect to the incorporated chromophore. Both the UV/Vis- and the FT-IR studies on AppA shown here, show that AppA undergoes similar spectral and molecular changes upon photoexcitation, regardless of the chemical nature of the inserted flavin. The FT-IR difference spectra show close similarity to those published
for another BLUF domain containing protein, Str1694. In previous FT-IR experiments on AppA from *R. sphaeroides* (Laan et al., 2003), some regions of the mid-IR difference spectrum have been obscured due to the strong absorption of the rather concentrated sample.

The fact that the photocycle seems largely independent of the nature of the flavin suggests that it is possible that AppA may be functional in *Rhodobacter sphaeroides* (see also above) with either FAD, FMN or riboflavin bound. Analysis of the flavins incorporated in AppA produced in and purified from *Rhodobacter sphaeroides* will shed light on this. The importance of identifying the *in vivo* chromophore of photoreceptors is exemplified by the recent isolation of a bacterial phytochrome from *Synechocystis* PCC 6803 (Yoshihara and Ikeuchi, 2004). Surprisingly, this phytochrome absorbs blue and green light, in contrast to absorption in the red/far-red region of the spectrum commonly found for (bacterio)phytochromes. This suggests that this protein (i.e. PixJ1) binds a different tetrapyrrole than the ones known to bind to (bacterio)phytochromes and usually used for *in vitro* reconstitution, like phycoerythrin and biliverdin (Yoshihara and Ikeuchi, 2004).

It remains to be established to what extent the versatility in flavin binding reported here for the BLUF domain of AppA is relevant for other members of the three flavin-containing photoreceptor families. Heterologously expressed cryptochromes have not been reported to contain any other flavin than FAD as their 'catalytic chromophore'. LOV domains mostly contain FMN. However, the PAS domain of White Collar-1 isolated from *Neurospora crassa* contains FAD, whereas heterologously expressed VIVID, from the same organism, binds both FAD and FMN (He et al., 2002; Schwerdtfeger and Linden, 2003). Incubation of the LOV2 domain of Phototropin 1 from *Avena sativa* (oat), with a 1,000-fold excess of FAD in the dark did not result in measurable exchange of these two flavins (J. Arents, unpublished experiments); this does not exclude possible flavin exchangeability in the light, however.

Based on the observations reported here it is advisable to test the nature of the flavin complement of heterologously produced flavin containing photoreceptors against their physiological chromophore composition. The very sensitive test based on TLC in combination with fluorescence detection provides a convenient assay for this.
3.6 Acknowledgements

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