Signal sensing and transduction in the blue-light photoreceptor AppA and the cyanobacterial phytochrome Cph1
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Chapter 5
On the mechanism of photo-activation of the BLUF domain of AppA

Wouter Laan, Magdalena Gauden, Sergey Yeremenko, John Kennis and Klaas J. Hellingwerf
Chapter 5

5.1 Abstract

AppA, a transcriptional anti-repressor, regulates the steady expression of photosynthesis genes in *Rhodobacter sphaeroides*, in response to high-intensity blue light and to redox signals. Its blue-light sensing is mediated by an N-terminal BLUF domain, a member of a novel flavin fold. The photocycle of this domain (AppA5,125) shows formation of a slightly red-shifted long-lived signaling state, which is formed directly from the singlet excited state of the flavin with a half-life of 590 picoseconds (Gauden et al., 2005). The red-shift of the absorption spectrum of this signaling state has been attributed to an altered stacking of the flavin with (an) aromatic amino acid(s) and/or rearrangement of its hydrogen bonding interactions with the surrounding apo-protein. In agreement with this it was shown that its Y21F mutant is photochemically non-responsive.

In this study we have characterized a second AppA mutant with an altered aromatic amino acid: W104F. This mutant exhibits an increased life-time of the singlet excited state of the flavin chromophore. Most strikingly, however, it shows a 1.5-fold increase in the quantum yield of signaling state formation. In addition, it shows a slightly increased rate of ground-state recovery. On top of this, the presence of imidazole, both in this mutant protein as well as in the wild type BLUF domain, significantly accelerates the rate of ground-state recovery, suggesting that this rate is limited by rearrangement of (a) hydrogen bond(s). In total, a ~ 700 fold increase in recovery rate has been obtained, which makes the W104F BLUF domain e.g. suitable for future analyses with step-scan FTIR.

The rate of ground-state recovery of the BLUF domain of AppA follows Arrhenius kinetics. This suggests that this domain itself does not undergo large structural changes upon illumination, and that the structural transitions in AppA are dominated by inter-domain rearrangements.

5.2 Introduction

Photoreceptor proteins contain at least one domain that monitors the spectral and intensity changes of the environmental light conditions and elicit an appropriate response in living organisms. Several photoreceptor families have evolved, absorbing light in different regions of the solar spectrum ranging from UV-A to the far-red. Four different families of blue-light absorbing photoreceptors are known: the Xanthopsins, the LOV domains, the
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Cryptochromes and the recently discovered family of the BLUF domains.

The Xanthopsins are mainly found in purple bacteria and Photoactive Yellow Protein (PYP) from *Halorhodospira halophila* is the best characterized of its members. PYP is the prototypical PAS domain and functions as the photoreceptor for negative phototaxis. PAS domains are a ubiquitous signaling module found in proteins from all kingdoms of life. Detailed knowledge is available for the primary photochemistry and the structural dynamics relevant for PYP function. Its photochemistry is based on *cis/trans* isomerization of an ethylene bond in its 4-hydroxy-cinnamic acid chromophore (Kort et al., 1996), which ultimately results in partial unfolding of the protein in its signaling state (Chen et al., 2003; Craven et al., 2000; Rubinstein et al., 1998; Van Brederode et al., 1996).

Interestingly, the other three families all use flavin as their chromophore, but each with very different photochemistry. LOV domains occur in many signal transduction proteins like the phototropins from plants (Huila et al., 1997), VIVID and WC-1 from the fungus *Neurospora crassa* (Ballario et al., 1998; Heintzen et al., 2001) and YtvA from *Bacillus subtilis* (Losi et al., 2002). They mediate different light responses like phototropism, chloroplast movement, control of the circadian rhythm, etc. Signaling state formation in the LOV domains is based on light-induced formation of a meta-stable covalent adduct between the C4 atom of the flavin and the sulfur atom of a conserved cysteine (Crosson and Moffat, 2002). Adduct formation results in conformational changes in the LOV domain (Crosson and Moffat, 2002; Fedorov et al., 2003; Salomon et al., 2001) which are relayed to the surface where they may lead to disruption of a conserved salt-bridge (Crosson et al., 2003). Phototropins are serine/threonine kinases and exhibit blue-light dependent autophosphorylation (Crosson et al., 2003). All phototropins contain 2 LOV domains of which the second (*i.e.* LOV2) is the most important one for the light-regulated kinase activity (Christie et al., 2002). It has been shown for a phototropin LOV2 domain that the light-induced structural changes in the canonical LOV domain lead to unfolding of an α-helix outside the PAS fold, which is involved in regulation of the kinase activity (Harper et al., 2004; Harper et al., 2003).

The cryptochromes are found in lower and higher eukaryotes (including mammals like *Homo sapiens*), insects (*Drosophila*), plants, algae (*Chlamydomonas*) and in one prokaryote (*Synechocystis*), where they are involved in processes like synchronization of the circadian clock, seed germination and regulation of pigment synthesis (Lin and Shalitin, 2003). They share sequence and structural homology with (bacterial) photolyases.
The photochemistry of cryptochromes is thought to be based on (reversible) electron transfer to the flavin (Galland and Tolle, 2003; Giovani et al., 2003), but little is known about the changes in tertiary structure after light activation.

Recently, a novel family of blue-light photoreceptors emerged, the BLUF domains (for sensors of blue light using flavin (Gomelsky and Klug, 2002)). BLUF domains are mainly found in prokaryotes and in one eukaryote, (Euglena gracilis) and are involved in photophobic responses in E. gracilis, (Iseki et al., 2002), in transcriptional regulation in Rhodobacter sphaeroides (Masuda and Bauer, 2002), and in phototaxis in Synechocystis. Secondary structure predictions suggest that the fold of the BLUF domain is different from all the known flavin-binding folds. The photochemistry and structural dynamics underlying signaling state formation in the BLUF domains is at present poorly understood. Four members of this family have been characterized by various spectroscopic techniques: AppA from Rb. sphaeroides (e.g. (Kraft et al., 2003; Laan et al., 2003)), Slr1694 from Synechocystis PCC6803 (Masuda et al., 2004), BlrP from Escherichia coli (previously designated YcgF (Ragapogal et al, 2004)) and the photoactivated adenyl cyclase (PAC) from E. gracilis (Iseki et al., 2002). The most extensively studied BLUF-domain containing protein, with respect to both function and photochemistry, is AppA from Rb. sphaeroides (Braatsch et al., 2002; Gauden et al., 2005; Kraft et al., 2003; Laan et al., 2003; Masuda and Bauer, 2002; Masuda et al., 2005). AppA is a regulatory protein that integrates light- and redox signals and functions as a transcriptional antirepressor, controlling the expression of the photosynthesis gene clusters via redox and light-modulated interaction with the repressor PpsR (Masuda and Bauer, 2002). When oxygen levels decrease, AppA binds to PpsR, thereby preventing it from binding to its DNA targets and allowing transcription of the photosynthesis genes. Blue-light illumination disrupts this interaction, restoring the repressor activity of PpsR. The ground-state of AppA shows the two main absorption bands typical for a flavin, located at around 370 and 447 nm. Upon illumination, an intermediate is formed which exhibits a ~10 nm red shifted absorption spectrum, which decays back to the ground state with a rate of ~ 1 x 10^{-3} s^{-1} (Laan et al., 2003; Masuda and Bauer, 2002). The recovery rate of YcgF is about two fold faster, whereas that of Slr1694 ~ 180 fold (Masuda et al., 2004; Rajagopal et al., 2004). The signaling state is formed directly from the singlet excited state of the flavin on a sub-ns timescale with a quantum yield of 0.24 (Gauden et al., 2005). It has been suggested that the photochemistry of AppA is based on a change in π-π stacking between the flavin and an aromatic residue.
probably a conserved tyrosine and a change in hydrogen bonding between the flavin N5 and the conserved tyrosine (Kraft et al., 2003). In agreement with this, mutation of this tyrosine has been shown to abolish the primary photochemical reaction (Kraft et al., 2003; Laan et al., 2003). In addition, FTIR measurements on Slr1694 and the BLUF domain of AppA indicate that signaling state formation in BLUF domains is accompanied by increased hydrogen bonding between the carbonyl groups of the flavin and residues lining the chromophore binding pocket (Masuda et al., 2004). The fact that the recovery reaction of Slr1694 is almost four times slower in D$_2$O compared to H$_2$O indicates that (a) rate limiting proton-transfer step(s) and/or a change in hydrogen bonding is involved in the recovery process (Masuda et al., 2004).

In full-length AppA, and in the C-terminally truncated variant AppA$_{1-156}$ (containing residues 1-156), the formation of the signaling state is accompanied by a conformational change that increases the Stokes radius and/or the aggregation state of the protein, as detected by size-exclusion chromatography (Kraft et al., 2003; Masuda and Bauer, 2002). Also FTIR measurements on the BLUF domain of AppA and Slr1694 revealed light-induced structural changes in the protein, contrasted by little structural changes of the flavin (Masuda et al., 2004; Masuda et al., 2005). Similar as in PYP and in LOV domains, the light-induced structural changes in BLUF proteins might reflect functional unfolding.

Here we report additional studies on the mechanism of photo-activation of AppA. We characterize a second site-directed mutant of the BLUF domain with an altered aromatic amino acid (W104F), which – very surprisingly – turns out to display a 1.5-fold increase in quantum yield of signaling state formation as compared to the corresponding wild type protein. In addition, we show that the unfolding of AppA in its signaling state most probably is very limited in the isolated BLUF domain (residues 5-125). This conclusion is based on the linear Arrhenius kinetics of its ground state recovery rate. By combining different effects, this recovery rate can be accelerated up to 700-fold, which makes this BLUF domain suitable for studies that require high repetition rates of photoactivation, like step-scan FTIR.

### 5.3 Materials and methods

#### 5.3.1 Strains and growth conditions

Cloning was performed using *Escherichia coli* XL1-Blue grown in LB medium according to established protocols. Protein overproduction was performed in *Escherichia coli* M15
(pREP4) grown in Production Broth (PB: 20 g L\(^{-1}\) tryptone, 10 g L\(^{-1}\) yeast extract, 5 g L\(^{-1}\) dextrose, 5 g L\(^{-1}\) NaCl and 8.7 g L\(^{-1}\) K\(_2\)HPO\(_4\), pH 7.0). Ampicillin and kanamycin were used at 100 and 50 \(\mu\)g ml\(^{-1}\), respectively.

### 5.3.2 Site-directed mutagenesis

The BLUF domain with the W104F mutation was constructed with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). pQEAppA\(_{5-125}\) (Laan et al., 2003) was used as the template for the PCR reaction using the primers: AppA W104F F: 5′-GCTTTCGCGGATTTCACATGCAGCTCTCCTGC-3′ and AppA W104F R: 5′-GCAGGAGAGCTGCATGTGAAATCCCGCAAAGC-3′. Mutated bases are indicated in bold. The constructs were verified by sequencing (BaseClear, Leiden, The Netherlands).

### 5.3.3 Protein production and purification

Wild type AppA\(_{5-125}\) and the AppA\(_{5-125}\) W104F mutant were both expressed and purified essentially as described previously (Laan et al., 2004). Before proceeding with the nickel-purification, the cell-free extracts were incubated for 1 h on ice with a large molar excess of FAD. Purified proteins were dialyzed to 10 mM Tris-HCl pH 8.0 and stored at -20°C. Purity of the samples was checked by SDS-PAGE using the PHAST-System (Amersham Biosciences) and UV-Vis spectroscopy. The flavin composition of each variant was determined by thin-layer chromatography (TLC) as described (Laan et al., 2004).

### 5.3.4 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 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 (WT or W104F in 10 mM Tris-HCl, pH 8.0 with or without different concentrations of salt or imidazol as indicated in the Results section) 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 were fitted with a mono-exponential decay function using Origin software. For isotopic replacement, the protein was concentrated, and subsequently diluted with buffered D₂O, at least three times.

5.3.5 Time-resolved fluorescence spectroscopy

Time-resolved fluorescence experiments were performed with a synchroscan streak camera setup described earlier (Gobets et al., 2001). The time resolved fluorescence kinetics were recorded upon excitation at 400 nm at a power of 500 µW/pulse. Pulses of 100 fs duration were generated with 50 kHz repetition rate using an amplified titanium:sapphire laser system (Mira-Rega, Coherent Inc., Mountain View CA). Fluorescence was collected with a right-angle detection geometry using achromatic lenses and detected through a sheet polarizer set at the magic angle (54.7°) with a Hamamatsu C5680 synchroscan camera and a Chromex 250IS spectrograph. The streak images were recorded with a cooled (-55°C) Hamamatsu C4880 CCD camera.

5.3.6 Calculation thermodynamic parameters

Thermodynamic parameters were calculated using Eq. 1, in which \( k_{gr} \) is the rate of ground state recovery, \( h \), \( k_b \) and \( R \) are the Planck, Boltzmann and universal gas constant, respectively.

\[
\ln \frac{k_c \cdot h}{k_s \cdot T} = \frac{\Delta S^* (T_0)}{R} - \frac{\Delta H^* (T_0)}{RT} - \frac{\Delta C^*_p}{R} \left( 1 - \frac{T_0}{T} + \ln \frac{T_0}{T} \right)
\]  

(1)

5.3.7 Quantum yield signaling state formation AppA5-125 W104F

The quantum yield of signaling state formation of the W104F mutant was determined using a relative actinometric method with Ru(II)trisbipyridine (Ru(bpy)_3²⁺) as a reference, as described for the wild-type (Gauden et al., 2005), assuming the same extinction coefficient at 447 nm as for the wild-type protein (8500 M⁻¹ cm⁻¹ (Kraft et al., 2003)). The quantum yield of signaling state formation in AppA was determined using the following expression:

\[
\Phi_{\text{type}, t} = \Phi_{\text{type}, r} \frac{\Delta OD_{\text{type}, t} \cdot \Delta \epsilon_{\text{type}, t} \cdot \Delta \epsilon_{\text{type}, r}}{\Delta OD_{\text{type}, r} \cdot \Delta \epsilon_{\text{type}, r} \cdot \Delta \epsilon_{\text{type}, t}}
\]  

(2)
where $\Phi_{ref}$ is the quantum yield of the photoreaction of Ru\((bpy)_3^{2+}\), $\Delta\varepsilon_{\text{qy}}^{(450)}$ (M$^{-1}$ cm$^{-1}$) is the change in the absorption coefficient of Ru\((bpy)_3^{2+}\) at 450 nm, $\Delta\varepsilon_{\text{qy}}^{(495)}$ (M$^{-1}$ cm$^{-1}$) is the change in the absorption coefficient of AppA at 495 nm and $\Delta$OD is the optical density change.

5.4 Results

5.4.1 Characterization of AppA W104F

Ultrafast fluorescence and femto-nanosecond transient absorption spectroscopy measurements on AppA have revealed that the signaling state of AppA is formed directly from the singlet excited state of the flavin on a sub-ns timescale with a quantum yield of 0.24 (Gauden et al., 2005). During similar measurements on photoinactive Y21 mutants, spectral features indicating the presence of a neutral flavin semiquinone were observed, a species which is not observed in the wild-type (Gauden et al., unpublished data). Since the samples did not contain any reducing agent, the hydrogen atom taken up by the flavin must originate from the protein environment. Tryptophan residues are well known as electron donors, and a tryptophan, at position 104 in the BLUF domain of AppA, is highly conserved in all known BLUF sequences. To explore the potential role of this residue in the photochemistry of AppA, we changed this tryptophan to a phenylalanine, and produced and purified this W104F protein. The protein was produced with yields comparable to the

![Figure 1](https://via.placeholder.com/150)

**Figure 1. UV-Vis absorption characteristics of the W104F mutant.** (A) The absorption spectra of the W104F variant (in 10 mM Tris-HCl, pH 8.0 at 25°C) in the dark (black line) and after illumination (grey line). (B) Light-dark difference spectra of the W104F mutant (black line) and wild-type AppA$_{5125}$ (grey line).
wild-type and could be fully reconstituted with FAD exclusively (data not shown). The variant was photoactive and the UV-Vis absorption spectra of the ground- and signaling state and the light-dark difference spectrum are similar to those of the wild-type, although the spectrum of the signaling state is a little less-far red-shifted, exhibiting absorption maxima at 457 and 380 nm (460 and 381 in wild-type (Fig. 1)). As in the wild-type, the signaling state of the mutant protein returns back to the groundstate mono-exponentially; however, with a 2.7 fold increased recovery rate (Fig. 2). The ratio of the absorption at 270 over the absorption of the flavin peak at 447 is ~ 90% of that of the wild-type, which can be accounted for by a decrease in the intensity of the 270 nm peak by replacement of a tryptophan ($\varepsilon_{270} = 5200 \text{ M}^{-1} \text{ cm}^{-1}$) by a phenylalanine ($\varepsilon_{259} = 200 \text{ M}^{-1} \text{ cm}^{-1}$). This indicates that the absorption coefficient of the flavin is unaffected by the mutation.

![Figure 2. Recovery kinetics of the W104F mutant. AppA_{5-125} W104F (squares) and wild-type AppA_{5-125} (triangles, both in 10 mM Tris-HCl, pH 8.0 at 25 °C) were converted to the signaling state by illumination with white light and allowed to revert back to the ground-state in the dark. Plotted are the absorption changes at 495 nm. The solid lines represent fits through the data using a mono-exponential function. The corresponding recovery rates are given in Table 2.](image)

We also determined the quantum yield of signaling state formation using the relative actinometric method with Ru(II)trisbipyridine (Ru(bpy)$_3^{2+}$) as a reference, that we have previously used to determine the quantum yield of wild-type AppA. The photoreactions were monitored by the detection of transient absorption changes at 450 nm and 495 nm for the reference and AppA, respectively, after excitation of either at 450 nm. To determine the quantum yield, absorbance changes of the samples at the time of maximal
concentration of the photo-product state were assessed (Fig. 3). Very surprisingly, the W104F mutant protein displayed larger absorption changes at 495 nm than the wild-type protein (see inset Fig. 3), suggesting a higher quantum yield for the former. Indeed, using actual experimental parameters, the quantum yield of the photo-transformation is determined as follows: \( \Phi_{\text{AppA W104F}} = \frac{(0.013 \times 7900)}{(0.08 \times 3500)} = 0.37 \pm 0.07 \), which is 1.5 fold higher than the quantum yield determined for the wild-type.

![Figure 3. Quantum yield determination of signaling state formation of AppA W104F. Transient absorption kinetics of solutions of Ru(bpy)_3^2+ (circles) and AppA W104F (squares) measured at 450 and 495 nm respectively, after excitation by a laser pulse at 450 nm. The inset shows the absorption changes after excitation at 450 nm for both the wild-type and the mutant protein. The quantum yield was calculated using equation 2. Note that the signal at 495 nm shows some variation as a result from interference with the Q-switch from the Nd:YAG laser.](image)

5.4.2 Time resolved fluorescence spectroscopy
To try to determine the basis for the observed increased quantum yield of the W104F mutant, we have performed synchroscan streak camera fluorescence experiments to examine its excited-state dynamics, and compared these with results obtained on the wild-type protein in a previous study (Gauden et al., 2005). The selected excitation wavelength was 400 nm, which implies that a mixture of S_1 and S_2 singlet states of FAD was generated. The fluorescence was monitored in a spectral window from 460 nm to 650 nm. A kinetic trace (circles) measured at the maximum of the fluorescence emission band (505 nm) is depicted in Fig. 4B, together with a trace measured on the WT protein for comparison. The application of a global analysis procedure yielded three decay
components, with lifetimes of 47 ps, 555 ps and 2.5 ns. The solid lines in Fig. 4B show the fitted curves. The decay-associated spectrum (DAS) of each component (Fig. 4A) has a maximum around 505 nm and a shoulder around 530 nm. The DAS of the first component is blue-shifted compared to the other two, but this is ascribed to a significant contribution of scatter. The 555 ps component is dominant with a decay amplitude of 0.63, with smaller contributions by the 47 ps component (amplitude 0.14) and the 2.5 ns component (0.23). These data are significantly different from those obtained on the wild-type protein. In the wild-type, four components were observed: 25 ps (amplitude 0.10), 150 ps (amplitude 0.32), 670 ps (amplitude 0.56) and 3.8 ns (amplitude 0.02). The kinetics of the fastest component in AppA W104F is similar to that of the fastest component in the wild-type protein, also with respect to amplitude. The dominant 555 ps may be compared with the 670 ps component in the wild-type. The slowest component of the mutant has a shorter life-time than the slowest component in the wild-type, but a 10 fold increased contribution, whereas the 150 ps component in the wild-type has no counterpart in the mutant. This results in an overall increase in the life-time of the singlet excited state of the chromophore in the mutant, as compared to the wild-type protein, which presumably increases the quantum yield of signaling state formation.
5.4.3 D$_2$O and imidazol affect the ground state recovery rate

Fig. 5 shows the decay kinetics of the signaling state of AppA$_{5,125}$ in H$_2$O and D$_2$O. The deuterated sample exhibits a 4.7 fold slower recovery rate, comparable to what has been observed for Sln1694, although in absolute terms the rates in the latter are ~ 200 fold higher. During purification of the BLUF domain of AppA it was observed that the recovery rate of the protein directly after elution from the nickel-affinity column, thus in the presence of 0.5 M NaCl plus 0.5 M imidazol, was significantly higher than after dialysis against buffer without salt and imidazol. Analysis of the recovery rate in the presence of either NaCl or imidazol revealed that the observed effect is mainly due to imidazol (Table 2). The recovery rate increased 1.5 fold in the presence of 1 M NaCl, whereas an equal concentration of imidazol increased the recovery rate 27 fold (Fig. 6). Concentrations of imidazol up to 2 M, at which the recovery is ~ 80 times faster, did not significantly affect the UV-vis spectra of the ground- and signaling state, but higher concentrations resulted in release of the flavin from the apo-protein, as indicated by a simultaneous loss of fine structure and red-shift of the ground-state UV-Vis spectrum. The recovery rate shows a linear dependence on the imidazol concentration, indicating a first order effect. The recovery was mono-exponential, indicating the imidazol does not induce formation of additional intermediates (Fig. 6). Imidazol has a pK of 6.95, and the fact that the increase in recovery rate in the presence of 1 M imidazol is about 2 fold smaller at pH 7 (53 %

![Normalized Absorbance vs Time](image.png)
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neutral imidazol) compared to pH 8 (92 % neutral imidazol) suggests that it is the neutral form of imidazol that affects the recovery kinetics. The recovery kinetics of the protein before the addition of imidazol and after removal of the imidazol by dialysis were identical, showing that the effect exerted by the imidazol is reversible. As observed for the wild-type protein, imidazol also significantly increased the ground state recovery rate of AppA W104F, ultimately resulting in a ~700 fold acceleration at 2 M imidazol compared to wild-type protein without imidazol (Table 1).

5.4.4 Thermodynamics of the recovery

It has been suggested, based on size exclusion chromatography of full-length AppA and the C-terminally truncated variant AppA156, that light induces a conformational change in the protein that increases the Stokes radius. Similar experiments also show that the full-length protein is a monomer, whereas AppA156 forms dimers (Kraft et al., 2003; Masuda and Bauer, 2002). Analysis of AppA5-125 by size-exclusion chromatography revealed no significant difference in elution profile between the ground- and signaling state of AppA. Both states elute at a volume corresponding to a 40 kDa protein (data not shown). Since the mass of the protein is 15.5 kDa, this indicates that also this variant forms dimers. The
Table 1. NaCl and imidazole increase the recovery rate of AppA. The recovery of wild-type AppA_{5-125} and the W104F mutant protein was measured at 25 °C in 10 mM Tris-HCl, pH 7.0 or 8.0 in the absence or presence of NaCl and imidazole. The absorption changes at 495 nm were fitted with a mono-exponential function. The values shown are the mean of at least two experiments and the standard deviation.

<table>
<thead>
<tr>
<th>Protein</th>
<th>pH</th>
<th>NaCl (M)</th>
<th>Imidazole (M)</th>
<th>Rate (x 10^{-5} \text{s}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>8.0</td>
<td>0</td>
<td>0</td>
<td>1.30 ± 0.10</td>
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<tr>
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<td>0.5</td>
<td>0</td>
<td>1.62 ± 0.03</td>
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<tr>
<td>Wild-type</td>
<td>8.0</td>
<td>1.0</td>
<td>0</td>
<td>2.04 ± 0.20</td>
</tr>
<tr>
<td>Wild-type</td>
<td>8.0</td>
<td>0</td>
<td>0.5</td>
<td>19.50 ± 1.50</td>
</tr>
<tr>
<td>Wild-type</td>
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<td>0</td>
<td>1.0</td>
<td>34.70 ± 0.20</td>
</tr>
<tr>
<td>Wild-type</td>
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<td>0</td>
<td>1.5</td>
<td>68.30 ± 1.68</td>
</tr>
<tr>
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</tr>
<tr>
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<td>0</td>
<td>1.50 ± 0.08</td>
</tr>
<tr>
<td>Wild-type</td>
<td>7.0</td>
<td>0</td>
<td>1</td>
<td>18.68 ± 0.30</td>
</tr>
<tr>
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</tr>
<tr>
<td>W104F</td>
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<td>0</td>
<td>0.5</td>
<td>82.60 ± 1.00</td>
</tr>
<tr>
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</tr>
<tr>
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<td>479.40 ± 17.0</td>
</tr>
<tr>
<td>W104F</td>
<td>8.0</td>
<td>0</td>
<td>2.0</td>
<td>730.56 ± 33.7</td>
</tr>
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</table>

Table 2. Thermodynamic parameters of the ground state recovery of AppA. The values of the thermodynamic activation parameters associated with the recovery of AppA_{5-125} were calculated from the fit of the data from Fig. 7. Values at 298 K are shown, followed by the standard deviation in the thermodynamic parameter, according to the least square fit of the data to equation 1.

<table>
<thead>
<tr>
<th>ΔS^a (J/mol/K)</th>
<th>ΔH^a (kJ/mol)</th>
<th>ΔG^a (kJ/mol)</th>
<th>ΔC_p^a (J/mol/K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>71 ± 11</td>
<td>82 ± 3</td>
<td>81 ± 9</td>
<td>570 ± 490</td>
</tr>
</tbody>
</table>

apparent molecular weight (40 kDa) is higher than the weight of a dimer calculated from the amino-acid sequence (31 kDa), suggesting AppA_{5-125} forms dimers with a molecular shape which deviates from a sphere (Nakasako et al., 2005). A change in the tertiary structure of a protein, like partial unfolding, may result in a change in heat-capacity, as has been shown for PYP (Hoff et al., 1999; Van Brederode et al., 1996; van der Horst et al., 2001). The change in heat capacity associated with the transition from the signaling- to the ground-state of a photoreceptor can be calculated from the degree of curvature of a plot of the natural logarithm of the recovery rate against reciprocal temperature, i.e. an Arrhenius plot. For the BLUF domain of AppA, an essentially linear plot is obtained (Fig. 7). From this plot, a change in heat capacity associated with the transition from the signaling- to the ground state of 570 J/mol/K can be calculated, albeit with a large uncertainty (Table 2). This value is about 5-fold smaller than the value reported for PYP (- 2.5 KJ/mol/K (Van Brederode et al., 1996; van der Horst et al., 2001)).
Figure 7. Thermodynamic analysis of the rate of ground-state recovery of AppA<sub>S-125</sub>. The natural logarithm of the rate constant k is plotted as a function of reciprocal temperature. The points shown are the mean of at least two experiments, the error in each point is less than 5%. The solid line represents the fit to the data using equation 1.

5.5 Discussion
The W104F mutant represents the first photoactive mutant of a BLUF domain. The fact that the recovery rate is slightly increased compared to the wild-type might indicate that the signaling state of this variant is less stable than the light-induced state of the wild-type. The reduced temperature stability of the mutant is in agreement with this (data not shown). The increased quantum yield can be explained by a longer living singlet excited state of the flavin, as shown by time-resolved fluorescence measurements. However, this remains to be confirmed by a complementary analysis of the transient absorption changes as has been performed for the wild-type protein. The fact that neutral semiquinones are observed in Y21 mutants suggests that a residue capable of transferring a hydrogen atom (or: an electron and a proton) to the flavin is in close proximity to the flavin. It has been shown in flavo-enzymes already, that efficient electron and proton transfer may occur from aromatic residues to the flavin, after which the radical pair rapidly recombines on the ps timescale to the ground-state (Mataga et al., 2002; Zhong and Zewail, 2001). The possibility exists that in wild type AppA, the FAD singlet excited state is (partially) deactivated via a hydrogen transfer from Trp104 to FAD, and subsequent rapid recombination to the ground state. If this recombination is faster than the initial electron/hydrogen transfer, the semiquinone will not transiently accumulate in an appreciable concentration, and thus will not be detected. In the W104F mutant, the contribution of this deactivation pathway would be significantly
reduced due to the lower electron/proton transfer capacities of the phenylalanine, resulting in a longer excited state life-time and increased quantum yield of signaling state formation. The rate of electron/hydrogen transfer is dependent on the donor/acceptor distance. In flavoproteins where ultrafast electron/hydrogen transfer from a Trp residue to the flavin is observed (e.g. in flavodoxin, photolyase), the edge to edge distance between the Trp and the flavin is about 3 to 4 Å (Byrdi et al., 2003; Mataga et al., 2002; Zhong and Zewail, 2001). This suggests a similar distance between the Trp104 and the flavin in the BLUF domain of AppA. Trp104 is almost completely conserved in all the BLUF sequences known to date. The exceptions are the second BLUF domain of the PACα subunit from E. gracilis, (which contains a leucine at this position), the two BLUF domains from Klebsiella pneumoniae (threonine and valine) and YcgF from E. coli (alanine). It would be of interest to determine the quantum yield of signaling state formation of these BLUF domains and assess whether the introduction of a Trp at this position will also decrease the quantum yield in these BLUF domains.

The reduction of the ground-state recovery rate of AppA in D₂O is comparable to the reduction observed in Slr1694, but is significantly larger than the 2 fold reduction reported by (Masuda et al., 2005) for the BLUF domain of AppA. The reason for this difference is at present unclear. The effect of deuteriation indicates that ground-state recovery involves a rate-limiting rearrangement of hydrogen-bonds and/or a proton-transfer step. Formation of the signaling state has been proposed to involve a change in hydrogen bonding between the conserved tyrosine and the flavin (Kraft et al., 2003). In addition, light-induced strengthening of hydrogen bonds to the carbonyl-groups of the flavin combined with the weakening of hydrogen bonding to a residue in a β-sheet structure of the protein has been proposed by (Masuda et al., 2005). The rearrangement of these light-induced changes in hydrogen-bonding during the ground-state recovery may well be affected by deuteration. In contrast, the presence of imidazol significantly accelerates the ground-state recovery. The structure of imidazol is analogous to the side-chain of histidine, which is capable of accepting and donating hydrogen bonds. Imidazol might increase the rate of ground-state recovery by catalyzing the relaxation of the light-induced changes in the hydrogen-bonding network of AppA. However, the fact that the UV-Vis spectra of both the ground- and the signaling state are virtually unaffected by the presence of imidazol suggests that imidazol affects the protein outside the chromophore binding pocket. By combining the effects of the W104F mutation and those of addition of
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imidazol. ground-state recovery rates are obtained which are compatible with step-scan FTIR. This will be very useful to gain more insight in the detailed structural dynamics underlying the BLUF photocycle.

We conclude that the BLUF domain of AppA does not undergo large structural changes upon illumination, although a recent FTIR study did reveal light-induced structural changes in the BLUF domain of AppA (Masuda et al., 2005). The linear Arrhenius behavior of the ground-state recovery of the BLUF domain of AppA indicates there is no significant change in heat capacity between the ground- and signaling state. Although this does not exclude the possibility that illumination elicits a large structural change without exposure or shielding of a hydrophobic surface, both our results of size-exclusion chromatography and the fact that we observe very little changes in the structure of AppA upon illumination in HSQC-NMR spectra (Grinstead et al., unpublished data) argue against such a large conformational transition, and suggest that these changes are minor. The positive value for the change in heat capacity suggests that, in contrast to what is observed in PYP, signaling state formation in AppA_{5-125} results in contraction of the protein. The fact that an apparent increase in size upon illumination is observed with the BLUF domain in the context of longer fragments of AppA suggests that the minor structural changes in the BLUF domain upon formation of the signaling state propagate to residues outside the domain, and lead to larger structural changes in elements outside the BLUF domain. A fragment of AppA lacking the entire BLUF domain is still functional with respect to redox-signaling, implying that the C-terminal part of AppA interacts with PpsR. Upon illumination of the full-length protein this interaction is disrupted, suggesting the light-induced structural changes in the BLUF domain are propagated to the C-terminal domain of AppA. A similar mechanism is observed in PYP and LOV domains, where light induced structural changes in the PAS core of the protein lead to partial unfolding of helical segments outside the PAS domain. It would therefore be of interest to determine whether longer fragments of AppA show non-linear Arrhenius behaviour of their ground-state recovery kinetics.

The fact that AppA_{5-125} and AppA_{1-156} both form dimers, whereas the full-length protein is a monomer in solution, suggests that truncation of AppA exposes a hydrophobic region in the protein which is stabilized by protein-protein interactions. In the full-length protein, this putative interaction surface might relay the structural changes in the BLUF domain to the C-terminus.