Signal sensing and transduction in the blue-light photoreceptor AppA and the cyanobacterial phytochrome Cph1
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Summary

Light plays a crucial role for life on earth. The energy of the sun is converted to chemical energy by plants and several micro-organisms via photosynthesis. In addition, light also functions as an information carrier, for example in vision and regulation of the flowering time in plants. To be able to monitor their light environment and respond to changes in the light-climate, organisms have developed elaborate systems for perception and transduction of light signals, allowing them to adjust growth and development optimally to the prevailing light conditions. The proteins functioning as the sensors of light signals are known as photoreceptors. To date, 6 different photoreceptor families are known. This thesis describes the study of members of two of these families: the novel blue-light photoreceptor AppA, a member of the BLUF-domain containing protein family, and the red/far-red light photoreceptor Cph1, a member of the phytochromes.

AppA is the prototype of the recently discovered family of BLUF-domain containing proteins. BLUF is an abbreviation for: sensors of blue light using flavin. These proteins have a yellow color, due to the non-covalently bound flavin chromophore. Interestingly, the BLUF domains adopt a novel flavin-binding fold and exhibit a new type of primary photochemistry. AppA is found in the bacterium Rhodobacter sphaeroides, where it functions in the regulation of expression of the photosynthetic machinery in response to light and redox signals. Under aerobic conditions the photosynthetic machinery is not expressed. When the oxygen levels decrease, AppA binds to the repressor protein PpsR, allowing expression of the photosynthetic apparatus. However, absorption of blue light by the BLUF domain of AppA results in a conformational change in the protein and release of PpsR, thus restoring repression. Blue-light absorption by AppA has been shown to result in the formation of a metastable intermediate (signaling state) with a ~ 10 nm red-shifted absorption spectrum and a lifetime of 1800 s. When the work on AppA in this thesis was initiated, the structure of the BLUF domain and the mechanism of photochemistry were not resolved. The major part of this thesis describes the study of the mechanism underlying the formation of the signaling state in the BLUF domain of AppA.

Chapter 2 describes the cloning, expression and purification of this BLUF domain of AppA. Characterization of this protein by UV-Vis absorption- and vibrational spectroscopy led to the hypothesis that absorption of blue light results in deprotonation of the chromophore. Based on the fact that a mutant protein, in which a tyrosine, (conserved in
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all BLUF sequences), has been replaced by an isoleucine, is not responsive to light, it was proposed that the tyrosine might be the acceptor for the proton.

The finding that the BLUF domain is capable of binding the flavins riboflavin, FMN and FAD, and a method to produce homogeneous samples containing each one of these exclusively, is described in Chapter 3. Surprisingly, characterization of these three variants by UV-Vis absorption-, fluorescence- and vibrational spectroscopy revealed that the photochemical properties of the BLUF domain are largely independent of the nature of the flavin. This finding casts doubt on the nature of the chromophore incorporated by the BLUF domain in *Rhodobacter sphaeroides*, which is thought to be FAD.

Chapter 4 describes the kinetics of, and intermediate states through which the photochemistry of the BLUF domain of AppA proceeds. Using ultra-fast fluorescence-, femtosecond transient absorption- and nano-second time-resolved UV-Vis spectroscopy, it is shown that the signaling state, with the red-shifted absorption spectrum, is formed within 1 ns, without any preceding intermediates, with a quantum yield of 24%. Moreover, the results clearly demonstrate that the signaling state is formed directly from the singlet excited state of the flavin. The triplet state, which is also formed, is part of a non-productive pathway. This contrasts the situation in another flavin-binding photoreceptor family, the LOV domains, where the triplet state of the flavin is an on-pathway reactive intermediate.

Chapter 5 describes the increase of the quantum yield of signaling state formation and ground-state recovery rates as revealed by site-directed mutagenesis and the effect of addition of imidazol. It is shown that a W104F mutant exhibits an increased recovery rate and a 1.5 fold increased quantum yield. This suggests that in the wild-type protein W104 is involved in deactivation of the excited state of the flavin by transient electron transfer. The presence of imidazol significantly accelerates the ground-state recovery. By combining the effects of the W104 mutation and imidazol we have achieved a remarkable ~700 fold increase in recovery rate, which will be useful for the analysis of the protein with spectroscopic techniques that require a high repetition rate. In addition, analysis of the protein in D₂O suggests that signaling state formation is accompanied by a proton-transfer step, a common theme in the activation of photoreceptor proteins. Signaling state formation in photoreceptor proteins is generally accompanied by structural changes of the protein, which relay the light-signal to their interaction partner(s). Characterization of the temperature-dependence of the recovery rate of AppA indicates that the BLUF domain
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does not undergo large conformational changes upon illumination, suggesting that small light-induced changes of the BLUF domain are transduced to elements outside the BLUF domain, resulting in its inability to interact with its interaction partner, PpsR.

Whereas the studies on AppA in this thesis are mainly aimed at elucidating the initial events after photon-absorption, i.e. signal sensing, the study on Cph1 described in Chapter 6 deals more with the molecular properties of signal transduction. The cyanobacterial phytochrome Cph1 is a multi-domain protein. It is a member of the phytochrome family, which are found in bacteria, but were originally discover in plants, where they regulate numerous light-dependent processes. It contains a N-terminal chromophore binding domain, a PHY domain, and a C-terminal histidine kinase domain. The protein can be interconverted between two stable states: the red-light absorbing Pr state and the far-red light absorbing Pfr state. The Pr state exhibits the highest kinase activity, which activates its interaction partner Rcp1. Whereas this mechanism of photochemistry is firmly established, both the structure of the protein (domains) and how absorption of a photon ultimately results in regulation of the kinase domain are unknown.

To gain more insight in the function of the PHY domain of Cph1, we analysed its interaction with the N-terminal chromophore-binding domain. It is shown that the PHY domain is able to form dimers and is involved in stabilization of the Pfr state. Addition of non-covalently bound PHY domain restores the properties of the chromophore binding domain. This indicates that a covalent interaction is not required for functional interaction of these domains. In addition, the preliminary results of homology modeling of the domains of Cph1 are presented, yielding valuable insight in their structure, which will be very useful to understand the mechanisms of signal transduction in phytochromes.

In Chapter 7 the wealth of information on the structure and photochemistry of BLUF domains that has become available through the above described studies and the work of others during the preparation of this thesis is discussed. Most importantly, the very recently determined spatial structure of two BLUF-domains, the BLUF domain of AppA and the protein TII0078, allowed for a critical evaluation of proposed models for photoactivation, resulting in the formulation of a plausible mechanism of photoactivation of BLUF domains.