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Flavin-based sensorial photoreceptors: from bacteria to plants

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ABSTRACTS OF THE PRESENTED PAPERS (presenters are underlined)

I1. **Flavin based photoreceptors: ubiquitous "eyes for the blue"?** Aba Losi *University of Parma - Dept. of Physics*, *Parma, **Italy*** losia@fis.unipr.it
Biological photoreceptors are integrated systems, with a dedicated chromophore embedded into a protein moiety. The are able to exploit light as a source of energy (e.g. during photosynthesis) or as a sensory input (e.g. in the process of vision). Blue-light photoreceptors binding flavins as chromophores mediate a variety of sensorial responses in plants and animals, ranging from phototropism to regulation of circadian rhythms. Genome digging, bioinformatics tools and molecular biology are now revealing that assessed and/or putative flavin-based photoreceptors are spread also in lower organisms, e.g bacteria and fungi. Accordingly, flavins are ubiquitous, non-toxic and chemically versatile. Light excitation further extends the chemical versatility of flavins by altering their redox potential, by enhancing the rate of addition reactions or/and by inducing the formation of transient species that absorb light in a large spectral range. Up to now three photosensing mechanisms have been discovered: (i) the LOV (Light Oxygen and Sensing) protein domains present in plant phototropins, in fungi photoreceptors and in bacterial proteins. In the LOV domains the flavin chromophore forms a covalent bond with a conserved cysteine after light excitation. (ii) the cryptochromes, whose flavin chromophore has been recently shown to be redox active in a light dependent way (iii) the emerging BLUF (Blue-Light sensing Using FAD) domain paradigm, present in bacteria and plant photosensors, whose photocycle is poorly understood. Despite the considerable progress in the last decade, key questions in the field of flavin-based blue-light sensing are still open, concerning the molecular mechanisms of light-driven reactions, the light-to-signal transduction pathways, the physiological role of flavin-based photoreceptors in lower organisms and the yield/quality of protein preparations for molecular studies.

F1A. **Photoreactivity of LOV1 and LOV2 of the Blue-light receptor Phot from *Chlamydomonas reinhardtii*** Peter Hegemann, *Institut für Biochemie, Universität Regensburg, Germany* peter.hegemann@biologie.uni-regensburg.de
The green alga *Chlamydomonas reinhardtii* possesses a blue light-sensitive Phot photoreceptor, that is related to phototropins of higher plants. The Phot-receptor is responsible for the conversion of vegetative cells into gametes. After illumination the LOV1-domain undergoes a photocycle comprising two triplet states that both convert into the thioadduct with time constant of $t_{1/2}$ = 0.9 and 4 μs respectively (LOV-390) [1]. The thioadduct reconverts to the dark form with $t_{1/2}$ = 200 s at neutral pH and t1/2 = 1000 s at pH 4. The 1.9 Å crystal structure [2] revealed that already in darkness the reactive cysteine is present in two conformations. In conformation-2 (30%) the cysteine is closer to the C4a-atom of the FMN originally suggesting that this is the fast responding conformer. However, this conclusion is in conflict to the finding that 80% of the triplet is reacting with faster kinetics which forced us to the conclusion that conformer-1 is the fast reacting isoform. Since the reaction mechanism is still under debate, we have employed the mutants C57S, C57M. C57S is completely unreactive which is explained by the less acidic O-H group. However, C57M is reactive and forming an N5-C-radical-adduct, which is stable indefinitely [3]. This can only be explained by a H• transfer. This hypothesis was extended by splitting the transfer into an e-transfer followed by a fast H^+-transfer. This conclusion is in full agreement with the reactivity of all mutants. C57S is unreactive because the radical cannot be stabilized. We also can explain the faster reactivity of conformer-1 if we anticipate that the e- transfer onto C5 is the rate limiting step.
We also began to investigate LOV2. This photorective domain is forming a thioadduct but, the reactivity is faster ($t_{1/2}$ = 0.5 μs), the quantum efficiency is higher (> 90%) and we have indications for an involvement of an e- transfer within the singlet state [4]. This need to be confirmed by ultrafast spectroscopy.

[1] Kottke, T., Heberle, J., Dick, B. and Hegemann, P. (2003) Phot-LOV1: Photocycle of Blue-Licht Receptor Domain from the Green Alga *Chlamydomonas reinhardtii*. *Biophys. J.* 84, 1192-1201.
[2] Fedorov, R., Schlichting, I., Hofmann, E., Domratcheva, T., Fuhrmann, M. and Hegemann, P. (2003) Crystal structures and molecular mechanism of a light induced signaling switch: The Phot-Lov1 domain from *Chlamydomonas reinhardtii. Biophys. J.* 84, 2474 – 2482.
[3] Kottke, T., Dick, B., Fedorov. R., Schlichting, I., Deutzmann, R. and Hegemann, P. (2003) Irreversible Photoreduction of Flavin in a Mutated Phot-LOV1 Domain. *Biochemistry* 42, 9854-9862.
[4] W. Holzer, A. Penzkofer, T. Susdorf, M. Álvarez[1], Sh. D. M. P. Hegemann Islam (2004) Absorption and Emission Spectroscopic Characterisation of the LOV2-Domain of Phot from *Chlamydomonas Reinhardtii* fused to a Maltose Binding Protein. *Chem Phys.* (in press)

F1B. **Mechanism of action of cryptochrome photoreceptors** Bouly, Jean-Pierre (1); Giovani, Baldissera(1,2) Bakrim, Nadia (1), Zeugner, Anke (1); Byrdin, Martin (2); Brettel, Klaus (2); Batschauer, Alfred (3); Galland, Paul (3); Black, Robert (4) and Ahmad, Margaret (1,4) ahmad@ccr.jussieu.fr
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Cryptochrome photoreceptors are blue-light absorbing flavoproteins with marked amino acid sequence similarity to DNA photolyases, a class of blue-light sensing DNA repair enzyme. Despite their similar sequence, cryptochromes differ from photolyases in that they do not repair DNA, and instead have evolved a novel role in signaling. In our prior studies, we have demonstrated that cryptochromes, like photolyases, catalyse a blue-light dependent electron transfer reaction known as photoactivation (1). Purified preparations of Arabidopsis cryptochrome-1 expressed in baculovirus expression system have been obtained and shown to undergo a light-dependent photoreduction reaction in vitro. These studies have been extended by laser flash absorption spectroscopy techniques that demonstrated that intramolecular electron transfer is involved in the photoreduction of flavin, and that this reaction implicates several of the trypophan and tyrosine residues of the apoprotein. Therefore, a redox reaction involving excitation of the oxidized flavin, followed by a chain of tryptophan radicals resulting from electon transfer from the flavin to tyrosine residues at the surface of the protein, probably represents one of the primary light-dependent reactions catalyzed by cryptochrome These *in vitro* data have been extended by producing mutant forms of the cryptochrome photoreceptors with single amino acid substitutions at the trypophan residues implicated in intramolecular electron transfer. Such mutant proteins have been expressed in recombinant Arabidopsis plants and shown to lack photoreceptor activity, indicating that this pathway of redox activatin is likely to be important for the function of the photoreceptor *in vitro*.. In addition to their similarity to photolyases, we have in addition determined that several cryptochrome photoreceptors undergo a light-regulated autophosphorylation reaction, presumably to cause conformational changes implicated in signaling (2). Additional differences in the mechanism of action of cryptochromes and photolyases, and the means whereby these mechanisms are transduced into the signaling pathways, have been discussed.
1. Giovani,B., Byrdin,M., **Ahmad, M.** & **Brettel, K**.(2003). *Nature Struc. Biol*, 10, 489-90.
2. .Bouly, J-P, Giovani,, B: Djamei, A., Mueller,M, Zeugner,A, Dudkin,E. Batschauer, A. & **Ahmad, M.** (2003)..*European J. Biochem*, 270, 2921 – 2928.

F1C. **Cofactors of blue-light photoreceptors studied by EPR spectroscopy**

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Paramagnetic states of cofactors are involved in a variety of photo-induced biological processes. These states can favorably be investigated by EPR spectroscopic methods. Here

several EPR techniques will be discussed with the focus on two blue-light receptors. A specific LOV1 mutant with the replacement of the reactive cysteine by methione (see talk by P. Hegemann) shows a radical species as final product after light excitation. Results of our EPR studies on this species and their implications on the likely mechanism of photo-adduct generation in the wild-type will be presented. Secondly, initial EPR experiments on the radical state of the flavin cofactor in cryptochrome will be shown.

F1D. **Vibrational spectroscopy explores the mechanism of blue-light photoreceptors**
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The two LOV domains, LOV1 and LOV2, from *Chlamydomonas reinhardtii* and the LOV domain of *Bacillus subtilis (*YtvA-LOV) have been studied by vibrational spectroscopy. The FMN modes of the dark state of LOV1 were identified by pre-resonance Raman spectroscopy and assigned to molecular vibrations. By comparing the bluelight-induced FT-IR difference spectrum with the pre-resonance Raman spectrum, most of the differences are due to FMN modes. The band at 2567 cm^{-1} is assigned to the S-H stretching vibration of C57, the residue that forms the transient thio-adduct with the chromophore FMN. The occurrence of this band is evidence that C57 is protonated in the dark state of LOV1. Moreover, it is shown that the two S—H conformations of the reactive LOV1 cysteine C57 (1) are exposed to environments of different hydrogen bonding strength. Exchange of the two other cysteines of LOV1 (C32S and C83S) does not alter the S—H stretching band providing evidence that this band feature arises solely from C57. The reactive cysteine of LOV2 from *Chlamydomonas reinhardtii* (C250) and of YtvA-LOV (C62) exhibit both a homogenous S—H stretching vibrational band which suggests a single conformer of the amino acid side chain. Finally, the FT-IR difference spectrum of YtvA from *Bacillus subtilis* comprising the light absorbing LOV domain and the putative signaling STAS (sulfate transporter/antisigma-factor antagonist) domain, reveals conformational changes in the latter after blue-light excitation.

F1E. Primary reactions of flavin-based photoreceptors John Kennis, *Department of Biophysics Vrije Universiteit, Amsterdam, The Netherlands* john@nat.vu.nl
Light, Oxygen or Voltage (LOV) domains constitute a new class of photoreceptor proteins that are sensitive to blue light through a noncovalently bound flavin mononucleotide (FMN) chromophore. Blue-light absorption by the LOV2 domain initiates a photochemical reaction that leads to formation of a long-lived covalent adduct between a conserved cysteine residue and the FMN. Here, we report the primary photophysics and photochemistry of LOV2 domains of phototropin 1 of *Avena sativa* (oat) and of the phy3 photoreceptor of *Adiantum capillus-veneris* (maidenhair fern). We find that the FMN triplet state is the reactive species from which the photoreaction occurs and demonstrate that it is the primary photoproduct in the LOV2 photocycle, generated at 60% efficiency. No spectroscopically distinguishable intermediates precede the FMN triplet on the femtosecond to nanosecond timescale, indicating that it is formed directly via intersystem crossing (ISC) from the singlet state. Our results indicate that the majority of the FMN triplets in LOV2 exist in the protonated form. We propose a reaction mechanism that involves excited-state proton transfer, on the nanosecond timescale or faster, from the sulfhydryl group of the conserved cysteine to the N5 atom of FMN. This event promotes adduct formation by increasing the electrophilicity of C(4a) and subsequent nucleophilic attack by the cysteine's thiolate anion. Comparison to free FMN in solution shows that the protein environment of LOV2 increases the ISC rate of FMN by a factor of 2.4, thus improving the yield of the cysteinyl-flavin adduct and the efficiency of phototropin-mediated signaling processes. We have applied ultrafast spectroscopy on the photoaccumulated covalent adduct state of LOV2, and find that upon absorption of a near-UV photon by the adduct state, the covalent bond between the flavin and the conserved cysteine is broken and the blue-light sensitive ground state is regained on an ultrafast timescale of 100 ps. We thus demonstrate that the LOV2 domain is a reversible photochromic switch, which can be activated by blue light, and deactivated by near-UV light. AppA is a novel blue-light sensor from *Rhodobacter sphaeroides* which is involved in light-dependent regulation of photosynthesis gene expression. It binds FAD as a cofactor and undergoes a blue-light dependent photocycle which leads to a long-lived red-shifted

intermediate, which is believed to be the signaling state. Time-resolved fluorescence experiments utilizing a synchroscan streak camera have indicated a strongly multiphasic decay of the FAD singlet-excited state with time constants of 40 ps (30%), 600 ps (65%) and 4.5 ns (5%). Ultrafast transient absorption experiments with multichannel detection (340-700 nm spectral range) have confirmed the multiphasic decay of the FAD singlet-excited state with similar time constants and amplitudes. No long-lived FAD photoproduct with appreciable yield has been found to exist on timescales longer than 5 ns, which implies that either the quantum yield of signaling-state formation is very low in AppA, or that a photoproduct is formed that involves only a chemical change in the protein, and not on the FAD cofactor.

F1F. **NMR Investigations on LOV Domains from the Photoreceptor Phototropin**
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Perception of light in various wavelength ranges is essential for plants. They have photoreceptors for UV, blue, red and far red light spanning the spectral range of about 280-800 nm. Phototropism is a phenomenon that is known for a long time to be regulated by blue light. Nevertheless, the corresponding receptor phototropin has been identified only recently. FMN serves as a chromophore. Phototropin *PHOT1* of oat (*Avena sativa*) is a protein with 923 amino acids and comprises two FMN-binding LOV domains and a serine/threonine kinase domain. We reconstituted recombinant wild type and mutant LOV2 domains with ^{13}C labeled FMN. ^{1}H, ^{13}C, and ^{31}P NMR spectra measured in the darkness and with blue light illumination identify the light-mediated reversible formation of a covalent cysteinyl (C4a) adduct as the trigger for conformational modulation of the protein.
The recombinant LOV2 domain has absorbance maxima at 380, 425, 450 and 475 nm. The protein can be photobleached by blue light irradiation and has then an absorbance maximum at 390 nm suggesting the formation of a 4a thiol adduct of the FMN chromophore. Replacement of the conserved cysteine 450 by alanine affords an FMN-binding mutant protein that is no longer susceptible to photobleaching. Comparison with the spectral properties of model systems suggested that photobleaching involves the formation of a covalent adduct of the sulfhydryl group of cysteine 450 to C-4a of the flavin chromophore. The recombinant fusion protein was reconstituted with various isotope-labeled FMN samples. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded without illumination and subsequently with continuous blue light illumination. Chemical shift modulations caused by blue light irradiation in the ^{1}H, ^{13}C, and ^{31}P spectra were fully reversible in the dark. Signal assignments in the ^{13}C spectra were based on (i) comparison of proteins reconstituted with different isotope-labeled FMN samples, (ii) $^{13}C^{13}C$ coupling patterns, (iii) comparison with other flavoproteins and (iv) comparison with flavin model compounds.

The signal of C-4a experiences an upfield shift of 69.5 ppm upon illumination. The observed upfield shift, as well as the decreased coupling constant between C-4 and C-4a provide strong evidence for sp^3 hybridisation of C-4a. The detected chemical shift of 65.0 ppm for C-4a in LOV2 is well in line with a C-4a-cysteinyl adduct.
The ribityl side chain carbon atoms undergo substantial chemical shift changes which are proposed to result from conformational modification. Similarly, the reversible upfield shift of 0.6 ppm experienced by the phosphate residue of FMN upon illumination indicates conformational modification of the phosphate environment. Last but not least, significant differences between the dark and the light state are observed in ^{1}H-NMR spectra. Major changes were detected in the region of 6 – 10 ppm where backbone NH-protons resonate.
NMR spectroscopy on a C450A mutant LOV2 domain
Illumination of a C450A mutant domain of LOV2 was conducive to the formation of a blue form with absorbance maxima at 580 nm and 620 nm suggesting the formation of a flavin neutral radical. NMR spectra measured with [U-$^{13}C_{17}$]FMN incorporated in the mutant protein were very similar to the corresponding spectra of the wild type protein in the dark while major differences were observed upon illumination. Most notably, some signals of the isoalloxazine moiety were inverted, probably due to the formation of a radical. The signals of the ribityl side chain were not shifted upon illumination as observed with the wildtype protein. This gives

further evidence that no adduct can be formed in the mutant protein and that the residue Cys450 is the partner for the formation of a (C-4a)-adduct.

F1G. **Structure-Function studies of LOV- and BLUF-domain containing proteins by (time-resolved) crystallography** Astrid Jung *Max Planck Institute for Medical Research, Department of Biomolecular Mechanisms, Heidelberg, Germany* Jung@mpimf-heidelberg.mpg.de

LOV-domains are Flavin-based photoreceptors that are combined with enzymatic and other domains into multi-domain proteins with diverse light-driven functions. Therefore, a common LOV photochemistry has to be coupled to the regulation of many different cellular activities.Single isolated LOV-domains are well characterized: We and others have determined their molecular structure in the ground as well as in the signalling state, and we obtained a detailed structure-based understanding of their photocycles using quantum mechanical approaches. But how does the light signal propagate from the Flavin-cofactor, where photon absorption takes place, out to the photoreceptor's surface and then to effector domains? In order to identify the structural basis of this process three proposals were made last year: Crosson et al. highlighted a pathway of structural connectivities that leads from the FMN cofactor of a LOV2 construct to a conserved salt-bridge at the domain surface, whereas our group implicated changes around the cofactor's phosphate moiety. Gardner and coworkers identified a C-terminal α-helix outside the canonical LOV domain to be important for the communication between photoreceptor and partner domains.
However, no structure of a full-length LOV protein is available up to now. Thus, we still do not know how the light signal is translated into a biological effect. To answer this question we plan to study proteins that contain – beside the photoreceptor domain – at least one additional functional effector domain. We are accomplishing time-resolved crystallographic experiments on these model systems as we successfully did for an isolated LOV domain from *Chlamydomonas rheinhardii*. The time-resolved approach especially enables structure determination of unstable species like a LOV protein in its signalling state that only temporary occurs during the photocyle. Thereby we hope to identify the general structural prerequisites that allow LOV proteins to act as light-driven molecular switches. If possible we would like to extend our work to BLUF domain containing proteins as very similar fundamental questions (will) occur on the way to better understand these protein's functionality.

F1H. **On the Reaction Mechanism of Adduct Formation in LOV Domains of the Plant Blue-Light Receptor Phototropin – A Time-Resolved EPR Study** Stefan Weber, *Fachbereich Physik, Freie Universität Berlin, Germany* Stefan.Weber@physik.fu-berlin.de
The blue-light sensitive photoreceptor, phototropin, is a flavoprotein which regulates the phototropism response of higher plants. The photo-induced triplet state and the photoreactivity of the flavin-mononucleotide (FMN) cofactor in two LOV domains of *Avena sativa*, *Adiantum capillus-veneris* and *Chlamydomonas reinhardtii* phototropin have been studied by time-resolved electron paramagnetic resonance (EPR) and UV–vis spectroscopy at low temperatures (T = 80 K). Differences in the electronic structure of the FMN as reflected by altered zero-field splitting parameters of the triplet state could be correlated with changes in the amino-acid composition of the binding pocket in wild-type LOV1 and LOV2 as well as in mutant LOV domains. Even at cryogenic temperatures, time-resolved EPR experiments indicate photoreactivity of the wild-type LOV domains, which was further characterized by UV–vis spectroscopy. Wild-type LOV1 and LOV2 were found to form an adduct between the FMN cofactor and the functional cysteine with a yield of 22% and 68%, respectively. The absorption maximum of the low-temperature photoproduct of wild-type LOV2 is red-shifted by about 15 nm as compared with the FMN C(4a)-cysteinyl adduct formed at room temperature. In light of these observations we discuss a radical-pair reaction mechanism for the primary photoreaction in LOV domains.

F2A. **Light induction of transcription is mediated by histone tails modifications** B. Grimaldi, P.Coiro, P. Filetici°, J. Dolby* ,E. Selker * and P. Ballario § *Dipartimento di*

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Control of blue light-induced transcription of specific genes in the paradigmatic model of *Neurospora crassa* is exerted by White Collar-1 (WC-1) and WhiteCollar-2 (WC-2). The two proteins form a functional heterodimer: the White Collar Complex (WCC) that controls signal transduction and transcriptional activation of light-dependent genes the *al* genes involved in carotenoids biosynthesis and *frq*, the main regulator of the circadian rhythm. Here we report evidences that chromatin modifications are also necessary for light control of gene expression. We observed in the LRR (light responding region) of the *al-3* promoter a transient acetylation of residue K14 of histone H3 N-terminal tail, upon light pulse. Increase of acetylation of H3 K14 and of *al-3* mRNA amount both follow the same transient kinetic, with a maximum at 20 minutes after a light pulse. Histone H3 acetylation response is dependent on the presence of WC-1 protein. Using single point mutants with a replacement of Q instead of K at residue 14 of H3 N-terminal tail we obtained Neurospora transformants that appear as phenocopies of a *wc* phenotype. Northern and ChIP analysis confirme at molecular level the influence of this substitution on *al3* gene expression. Finally we have found that an homologue of Gcn5, the yeast histone acetyl transferase, is responsible of light dependent H3 K14Ac. We observed that the phenotype of a ripped *Ncgcn5* is very similar to that of a *wc* mutant, and the expression of *al-3* in the mutant appears to be constitutive, thus suggesting an essential role of NcGcn5 as coactivator in the transcriptional response to light induction.
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F2B. Role of Neurospora PKC in the regulation of light signal transduction and circadian rhythm. **Lisa Franchi** *Dipartimento di Biotecnologie Cellulari ed Ematologia Sezione di Genetica Molecolare, Policlinico Umberto I° Roma,* ***Italy.*** Franchi@bce.uniroma1.it (abstract not available); reference: Merrow M, **Franchi L**, Dragovic Z, Gorl M, Johnson J, Brunner M, Macino G, Roenneberg T *Circadian regulation of the light input pathway in Neurospora crassa* **EMBO J. 2001,** 20: 307-315.

F2C. **Phototropin receptor signaling** John Christie **,** *Plant Science Group,University of Glasgow,University Avenue, Glasgow, UK* J.Christie@bio.gla.ac.uk
Plants depend heavily on light to direct their growth and development. In particular, blue light acts to regulate a variety of important plant responses. These include phototropism, stomatal opening and chloroplast movement. The phototropins (phot1 and phot2) are blue light-activated receptor kinases that induce these afore mentioned responses. Both photoreceptors contain a serine/threonine kinase domain located at their C-terminus that function to catalyze autophosphorylation of the receptor protein in response to blue light irradiation. The N-terminal regions of phot1 and phot2 contain a repeated motif designated LOV1 and LOV2 that function as the light sensors for the receptor protein. At present, little information is known regarding the mechanism(s) that couple blue light excitation of the LOV domains to activation of the C-terminal kinase domain. Moreover, little is known about the molecular events that immediately follow photoreceptor activation. Therefore, we are carrying out site-directed mutagenesis analyses to further investigate the reaction mechanism associated with phototropin receptor activation. In addition, we are using yeast two-hybrid analyses to identify proteins that potentially interact with phot1 and phot2. Progress on both these research areas will be discussed.

F3A. **Progress in expression and purification of plant cryptochromes for structural and spectroscopic studies** Alfred Batschauer, *Philipps-University, Marburg, Germany* batschau@staff.uni-marburg.de

Cryptochromes were the first flavin-based photoreceptors discovered in plants about one decade ago. They are related to DNA-repair enzymes, the DNA-photolyase, and carry like DNA-photolyase besides the flavin FAD the pterin cofactor methenyltetrahydrofolate.
In the model plant *Arabidopsis thaliana*, three cryptochromes (CRY1-3) have been identified. cry1 and cry2 are nuclear proteins whereas cry3 is targeted to organelles. The biological function of the plant cryptochromes with the exception of cry3 was elucidated by mutant analysis showing that cryptochromes play important roles in the blue light-regulated expression of numerous genes, and thus regulate growth and differentiation of plants throughout their complete life cycle. In addition, significant progress has been made in recent years to reveal the signal transduction pathway of plant cryptochromes by characterizing proteins that interact with cryptochromes, identifying amino acid positions and domains within cryptochromes essential for their biological function, and demonstrating that cryptochromes are phosphorylated in blue light at least in part through autophosphorylation. In addition, the groups of Ahmad and Brettl have shown, that light excitation of the oxidized flavin in cry1 causes formation of a semi-reduced FAD mechanistically similar to the photoreduction of DNA-photolyase. In order to gain deeper insight into the mechanism of cryptochrome photoreceptor action at the molecular and atomic level it is required to produce recombinant cryptochromes for in vitro studies such as EPR, ultra-fast optical spectroscopy, vibrational spectroscopy, and X-ray crystallography. This goal was hampered for some time by the fact that expression of cryptochromes in *E. coli* and yeast cells resulted in aggregated and mostly insoluble protein preparations. More recently, we succeeded in producing *Arabidopsis* His-tagged cry2 in Sf-21 insect cells with yields of up to 50 mg soluble protein per liter cell culture. The protein can be purified close to homogeneity by Ni-NTA affinity and few additional chromatography steps. Although the MTHF cofactor cannot be detected in the purified samples, at least 60% of the cry2 molecules carry FAD. Similar results were obtained for cry1 in the group of Margaret Ahmad. Besides cry2, cry3 can be expressed as soluble and cofactor-containing protein. Although cry3 yields are about 1 mg per liter culture this does not cause severe problems because cry3 can be expressed in *E. coli*, and up-scaling the expression is feasible. Taken together, the recent progress made in the expression of soluble, cofactor-containing full-length plant cryptochromes makes these photoreceptors now accessible for spectroscopic studies to enlighten the primary mechanism of cryptochrome signaling.

F3B. **Progress and problems in the expression of bacterial phototropin-related proteins** Aba Losi[1] and Wolfgang Gärtner[2] . [1] *Department of Physics, University of Parma, Italy;* [2] *Max-Planck-Institut for Bio-Inorganic Chemistry, Muelheim an der Ruhr, Germany* gaertner@mpi-muelheim.mpg.de
The bacterial counterparts to the plant phototropins,(blue-light photoreceptors) are presented with respect to their phylogenetic distribution, their modular-built tertiary structure and their photochemistry. The similarities and differences to the eukaryotic equivalents will be outlined. Only two prokaryotic representatives up-to-now have been obtained in recombinant form (YtvA from B. subtilis and Q9ABE3-LOV-His kinase from C. crescentus). Despite this still insufficient provision of samples, their advantages over the plant photosensors is obvious due to the fact that they can be generated as full length proteins, making studies on the blue-light driven signal transduction possible. Following a complete characterization of the photochemical reactions, a comparison of fluorescence data from solely the LOV domain of YtvA and the full length protein reveals domain-domain interaction sites within the protein as putative mechanisms for the transmittance of the signal, being generated by the photoreaction of the FMN chromophore. Fluorescence measurements reveal distances between the chromophore and various aromatic amino acids.

F3C. **Structural characterization of flavin-based photoreceptors** Wouter Laan and Klaas J. Hellingwerf* *Laboratory for Microbiology, Swammerdam Institute for Lifesciences, BioCentrum Amsterdam, University of Amsterdam, The Netherlands*
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Six well-characterized photoreceptor families function in Nature to mediate light-induced signal transduction: the rhodopsins, phytochromes, xanthopsins, cryptochromes,

phototropins, and BLUF (for: *B*lue-*L*ight sensing *U*sing *F*lavin) proteins. The first three catalyze *E*/*Z* isomerization (of retinal, phytochromobilin, and *p*-coumaric acid, respectively), while the last three all have a different flavin-based photochemistry. For many of these photoreceptor proteins, the details of the conversion of the light-induced change in configuration of their chromophore into a signaling state and eventually a biological response have been well resolved. Several members, particularly of the rhodopsins, the xanthopsins, and the phototropins, are so well characterized that they function as model systems to study (receptor) protein dynamics and (un)-folding.

The photochemistry in the flavin-based photoreceptors families is still partially unresolved. The LOV domains of phototropins are activated through transient covalent bond formation between the C(4) atom of the flavin and the sulfur atom of a nearby flavin. For the BLUF-domain containing photoreceptors different working hypotheses have been formulated: (i) transient stacking of aromatic amino acids onto the flavin and (ii) intramolecular proton transfer between the flavin and a nearby tyrosine. The photochemistry of cryptochrome activation is still largely a mystery.

For these reasons it is of key importance to further characterize in particular these flavin-containing photoreceptor proteins. Heterologous overexpression is an important step in many of such experiments. Upon heterologous (over) expression of the BLUF domain from AppA, a transcriptional anti-repressor from *Rhodobacter sphaeroides* representing an entirely new 'flavin fold', 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 photoreception 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. Addition of excess flavin during purification allows one to produce homogeneous photoreceptor protein with any of these three naturally occurring flavins selected. The nature of the particular flavin bound significantly affects the (photochemical) properties of the N-terminal domain of AppA. For the LOV domain of phototropin much slower flavin exchange kinetics is observed than for the BLUF domain.

Structure and function of plant phototropins

F4A. Structure and function of plant phototropins John Christie , *Plant Science Group,University of Glasgow,University Avenue, Glasgow, UK* J.Christie@bio.gla.ac.uk

Phototropin 1 (phot1) is an autophosphorylating serine/threonine kinase that functions as a photoreceptor for phototropism, stomatal opening and chloroplast movement. The N-terminal region of phot1 contains two specialized PAS domains, designated LOV1 and LOV2, which bind flavin mononucleotide (FMN). Both LOV1 and LOV2 undergo a self-contained photocycle involving the formation of a covalent adduct between the FMN chromophore and a conserved active site cysteine (C39). Replacement of C39 with alanine abolishes the light-induced photochemical reaction of LOV1 and LOV2. The LOV domains of phot1 exhibit different photosensitivities and reaction kinetics, suggesting that LOV1 and LOV2 might have different functional roles. We have used the C39A mutation to investigate the role of the LOV domains in regulating phot1 function. Our studies indicate that LOV2 plays a major role in phot1 photochemistry and phot1 kinase activity whereas LOV1 has a minor role. Similarly, photochemically active LOV2 alone in full-length phot1 is sufficient to elicit hypocotyl phototropism in transgenic *Arabidopsis*, whereas photochemically active LOV1 alone is not. The significance for the different roles of the phototropin LOV domains along with further structure-function analyses will be discussed.

F4B. **The photoreceptor for phototaxis in the flagellate *Euglena gracilis***

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The unicellular photosynthetic flagellate *Euglena gracilis* orients itself in the water column using light and gravity as external clues. It responds to light showing photokinesis (swimming velocity dependent on light intensity), phobic responses (transient changes in direction upon

a sudden increase or decrease in light intensity) and phototaxis (swimming toward or away from the light source).
Iseki et al. (2002) have identified the photoreceptor for step-up photophobic responses in the wild-type green *E. gracilis*. The photoreceptor, a photoactivated adenylyl cyclase (PAC), a 400 kDa protein consisting of 2 α and 2 β subunits, is located in the paraxonemal body (PAB) attached to the flagellum of *Euglena gracilis*. FAD serves as the chromophoric group absorbing blue light (max. 370, 450 nm) that leads to activation of an adenylyl cyclase domain located on the same protein. The synthesis of cAMP modulates the flagellar beat and thus causes light-dependent responses. We found that step-up photophobic responses (but not step-down responses) are controlled by PAC in the non-phototactic, colorless relative *Astasia* as well as in a number of *Euglena* mutant strains. PAC also mediates positive and negative phototaxis as has been shown by RNA interference (RNAi). Some mutant strains do not possess a PAB as indicated by confocal immunofluorescence microscopy and autofluorescence. In these strains, which do not show phototaxis, the photoreceptor seems to be located on the flagella. RNAi of a key enzyme in the pterin synthesis indicates that pterins are necessary for positive and negative phototaxis, but not for photophobic responses. The protein component has not yet been identified. Currently, work is in progress to construct a homologous and a heterologous expression system for *Euglena* to overexpress PAC for crystal structural analysis.

F4C. **Blue light photoreceptors in *Rhodobacter***
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While it is known for many years that a variety of photosensor proteins exist in eukaryotes it was only recently that light receptors were identified in bacteria. The flavin-binding BLUF domain functions as blue light receptor in eukaryotes and bacteria. The heterotetrameric PAC protein from the flagellate *Euglena gracilis* acts as photoactivated adenylyl cyclase, which mediates a photophobic response. AppA functions as transcriptional antirepressor, controlling photosynthesis gene expression in the purple bacterium *Rhodobacter sphaeroides.* We fused the PACα1-BLUF domain from *Euglena* to the C-terminal part of AppA. Our results show that the hybrid protein is fully functional in light dependent gene repression in *R. sphaeroides*.

F4D. **The hypogeous fungus *Tuber borchii* responds to blue light and contains a gene (tbwc-1) homologous to the blue-light photoreceptor of *Neurospora crassa*.**
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Truffles form a group of plant-symbiotic Ascomycetes whose hypogeous life cycle is poorly understood. Here we present initial evidence for the influence of light on *Tuber borchii* mycelial growth and the identification and cloning of a gene, *Tbwc-1,* homologous to a blue-light photoreceptor of *Neurospora crassa*. Blue-light irradiation of *T. borchii* colonies inhibits their apical growth. It also alters apical growth in *N. crassa.* In *Neurospora*, the response is controlled by a nuclear photoreceptor, NcWC-1 (White Collar-1), which consists of a sensor domain (LOV) and a transcriptional factor moiety. We isolated a gene (*Tbwc-1)* whose deduced amino acid sequence shows a high similarity and colinearity of domains with NcWC-1, except for the polyglutamine regions. As previously found in *Neurospora, Tbwc-1* mRNA is under light control and its steady state level increases upon irradiation. *In silico* analysis of the TbWC-1 sensor domain (LOV) supports the hypothesis that TbWC-1 is a photoreceptor, while the absence of the two polyglutamine regions involved in transcriptional activation in *Neurospora* suggests that this function in *Tuber* could be absent.

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F4E. **Blue light perception in cyanobacteria** Nicole Tandeau de Marsac
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Cyanobacteria constitute the most diverse group of microorganisms performing oxygenic photosynthesis like plants and algae. BLAST searches have revealed a number of putative light sensors in the cyanobacterial genomes sequenced during the last decade but thus far relatively little is known on blue light perception in these micro-organisms.

In the unicellular freshwater cyanobacterium *Synechococcus* PCC 7942, cells respond to blue light treatment by rapidly synthesising new polypeptides [1], in particular the expression of the three *psbA* genes encoding D1, the reaction centre protein of photosystem II, is reversibly controlled by blue and red lights [2]. In contrast, in the unicellular euryhaline strain *Synechocystis* PCC 6803, a redox- rather than a light-sensory mechanism controls the expression of *psbA2-3* and *psaE* (encoding a component of photosystem I) [3]. In the same cyanobacterium, a blue light sensor PlpA that might act as one of the receptors involved in light-dependent regulation of cell growth [4] and two putative sensors for phototaxis, Cph2 [5, 6] and TaxD1 (also called PisJ1) [7] have been characterised. Cph2 has been shown to inhibit positive phototaxis towards blue light but whether Cph2 is a blue light receptor or a protein that interacts with a red (or far-red) receptor involved in phototaxis remains to be established. TaxD1 controls positive phototaxis under red light and influences the fluence dependency for negative phototaxis under blue light. A putative blue light sensor (Cry DASH) sharing similarities with eukaryotic cryptochromes has also been characterised in *Synechocystis* PCC 6803 [8, 9]. This protein behaves as a transcriptional repressor. The 3-D structure of Cry DASH, that binds FAD as a cofactor, has been determined at 1.9 Angströms. BLAST searches did not reveal the existence of orthologs of Cry DASH in the different cyanobacterial genomes, except *Gloeobacter violaceus.* The similarity between Cry DASH and Glr0835 is, however, restricted to the C-terminal part of the proteins.

BLAST searches of the cyanobacterial genome sequences available to date revealed that several hypothetical proteins carry LOV motifs commonly found in blue light sensors. Interestingly, none of them were detected in the genomes of the marine cyanobacteria. More generally, no putative proteins sharing similarities with phytochromes, phototropins or rhodopsins were found in marine strains. Photolyases and putative cryptochromes were identified in only two of the four marine cyanobacteria whose genomes have been sequenced, *Synechococcus* WH8102 and *Prochlorococcus* MED4 [10, 11, 12]. Interestingly, however, *Prochlorococcus* strains have conserved some of the genes encoding phycoerythrin but synthesise only minute amounts of this pigment. Whether phycoerythrin could play the role of blue light receptor in these cyanobacteria remains an open question [13].

[1] Pandey, R. et al. Curr. Microbiol. 40: 297-301 (2000).
[2] Tsinoremas, N. F. et al. J. Biol. Chem. 269: 16143-16147 (1994).
[3] El Bissati, K. and Kirilovsky, D. Plant Physiol. 125: 1988-2000 (2001)
[4] Wilde, A. et al. FEBS Lett. 406: 89-92 (1997).
[5] Wu, S.-H. and Lagarias, J. C. Biochemistry 39: 13487-13495 (2000).
[6] Wilde, A. et al. Mol. Microbiol. 44: 981-988 (2002).
[7] Ng, W.-O. et al. J. Bacteriol. 185: 1599-1607 (2003).
[8] Hitomi, K. et al. Nucleic Acid Res. 28:2353-2362 (2000).
[9] Brudler, R. et al. Mol. Cell 11: 59-67 (2003).
[10] Rocap, G. et al. Nature 424: 1042-1047 (2003).
[11] Dufresne, A. et al. Proc. Natl. Acad. Sci. USA. 100: 10020-10025 (2003).
[12] Palenik, B. et al. Nature 424: 1037-1042 (2003).
[13] Hess, W. R. et al. Photosynth. Res. 70: 53-71 (2001).

P1. **Photoactivated adenylyl cyclase in *Euglena gracilis, Astasia longa,* and mutant strains** M. Ntefidou, M. Lebert, D.-P. Häder University of Erlangen, Department of Plant Ecophysiology, Erlangen, Germany ntefidou@biologie.uni-erlangen.de

Euglena gracilis is a unicellular flagellate found in freshwater. *Euglena* exhibits different light responses: step-up and step-down photophobic responses, positive and negative phototaxis and photokinesis. A novel blue light receptor, photoactivated adenylyl cyclase (PAC) has been identified to be the photoreceptor for step-up photophobic responses in *E. gracilis,* but it is not the photoreceptor of step-down photophobic responses. In addition, PAC is involved in positive and negative phototaxis. PAC is a heterotetramer; each subunit consists of two domains with adenylyl cyclase activity and two FAD binding BLUF domains (sensing blue-light using FAD). Upon absorption of blue light by the BLUF domain the adenylyl cyclase domain synthesizes cAMP which initiates the sensory transduction chain that alters the flagellar beat. We examined the functions of PAC in mutant strains of *Euglena gracilis* 1F, FB, St- and in *Astasia longa*, a phylogenetically close relative of *Euglena gracilis*, by RNA interference and sequenced the cDNA of both subunits in all strains to gain further insight to PAC.

P2. **Irreversible Photoreduction of Flavin in the Mutant C57M of a Phot-LOV1 Domain**
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Sensory photoreceptors regulate the response of organisms to the light conditions in the environment. In several blue-light photoreceptors 'Light-, Oxygen- and Voltage-sensitive' (LOV) domains are responsible for light sensing. They noncovalently bind flavin mononucleotide (FMN) as a chromophore.In Phototropin and its homologs, the Phot proteins, two LOV domains are present. Phot proteins mediate responses such as phototropic plant movement, chloroplast relocation, and stomatal opening in guard cells as well as gametogenesis in green algae[1].The photocycle of the LOV1 domain of the Phot protein from the green alga *Chlamydomonas reinhardtii* was investigated by time-resolved absorption spectroscopy[2]. Upon absorption of blue light, the triplet excited state of the chromophore FMN is formed (LOV1-715) and decays into a covalent adduct with the adjacent cysteine 57 (LOV1-390). The adduct reverts to the dark form within minutes, the time constant depending strongly on the conditions in the medium. In order to clarify the mechanism of adduct formation, the reactive cysteine in the LOV1 domain was replaced by a methionine[3]. In the mutant C57M, flavin reacts with the terminal methyl group of methionine. In a secondary, thermal reaction the adduct is oxidized to a stable neutral flavin radical. In the mutant, an ionic mechanism of adduct formation via deprotonation of the methionine and protonation of the flavin triplet state can be excluded. More likely, a radical mechanism takes place, that may also be present in the wild-type system.
[1] Briggs, W. R., and Christie, J. M. (2002) Phototropins 1 and 2: versatile plant blue-light receptors, *Trends in Plant Sci.* 7, 204-210. [2] Kottke, T., Heberle, J., Hehn, D., Dick, B., and Hegemann, P. (2003) Phot-LOV1: Photocycle of a blue light receptor domain from the green alga *Chlamydomonas reinhardtii*, *Biophys. J. 84*, 1192-1201. [3] Kottke, T., Dick, B., Fedorov, R., Schlichting, I., Deutzmann, I., and Hegemann, P. (2003) Irreversible photoreduction of flavin in a mutated Phot-LOV1 domain, *Biochemistry 42*, 9854-9862.

P3. **Tryptophan fluorescence in the *Bacillus subtilis* phototropin-related protein YtvA as a marker of interdomain interaction**
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The *Bacillus subtilis* protein YtvA, related to plant phototropins (phot), binds flavin-mononucleotide (FMN) within the N-terminal LOV (Light Oxygen and Voltage) domain. The photocycle of YtvA and phot involves the reversible formation of a covalent photoadduct between FMN and a cysteine residue. As all phot-LOV domains, YtvA contains a single tryptophan residue, W103. Here we show that the fluorescence parameters of W103 in YtvA-LOV are markedly different from those observed in the full-length Ytva. The fluorescence quantum yields are ca. 0.03 and 0.08 respectively. In YtvA-LOV the maximum is red shifted (ca 345 vs. 335 nm) and the average fluorescence lifetime shorter (2.7 vs 4.7 ns). These

data indicate that W103 is located in a site of tight contact between the two domains of YtvA. In the FMN-cys adduct, selective excitation of W103 at 295 nm, results in minimal changes of the fluorescence parameters with respect to the dark state. Upon 280 nm excitation however, there is a detectable decrease of the fluorescence emitted from tyrosines, with concomitant increase of W103 fluorescence. This effect is reversible in the dark and might arise from a light regulated energy transfer process from a yet unidentified tyrosine to W103.