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From primary photochemistry to biological function in the blue-light photoreceptors PYP and AppA†

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To properly respond to changes in fluency conditions, Nature has developed a variety of photosensors that modulate gene expression, enzyme activity and/or motility. Dedicated types have evolved, which can be classified in six families: rhodopsins, phytochromes, xanthopsins, cryptochromes, phototropins and BLUF-proteins. The photochemistry of the first three families is based on *cis*/*trans* isomerization of an ethylene bond. Surprisingly, the latter three all use flavin as their chromophore, but each with very different photochemistry. In this contribution we will discuss the molecular basis of signal generation in a xanthopsin (Photoactive Yellow Protein (PYP) from *Halorhodospira halophila*), a photoreceptor for negative phototaxis, and in a BLUF protein (AppA from *Rhodobacter sphaeroides*), a transcriptional anti-repressor. PYP is activated through *trans*/*cis* isomerization of the 7,8-vinyl bond of its 4-hydroxycinnamic acid chromophore. This initiates a photocycle with multiple intermediates, like pB, which is formed after intramolecular proton transfer. The negative charge thus formed in the interior of the protein triggers formation of a partially unfolded signaling state. For AppA much less is known about the underlying photochemistry. Available evidence suggests that it is based on a light-induced change in the hydrogen-bonding of its flavin chromophore and/or a change in hydrophobic stacking between the flavin and/or nearby aromatic amino acids like Y21. A signaling state is formed within microseconds, which recovers with a rate of $\sim 10^{-3}$ s^{-1}. The change in conformation between receptor- and signaling-state in AppA, however, appear to be minute as compared to those in PYP. Here we review the underlying chemistry in the various steps of the photocycle of these two photoreceptor proteins and provide new data on their mechanism and function.

Introduction

Photoreceptor proteins with a signal transduction function are optimally suited to relate the role of dynamical alterations in protein structure to biological function. First, photo-triggering of the reaction allows resolving their dynamics with high precision by optical methods. Second, one may anticipate large conformational transitions to be involved in their signaling state formation, which is indeed borne out by the experiments (*e.g.* ref. 1). Third, the structural transitions of these proteins are reflected in specific changes of their optical properties that provide an excellent indicator to reveal the timescale (and mechanism) of these transitions. Unsurpassed insight along these lines is being obtained for a number of different photoreceptor proteins. Hence they have been referred to as "star actors"[2] in the pursuit to understand atomic detail of structure/function relations in proteins, like in enzyme catalysis and in the large structural changes required for some signal transduction processes, structural changes that are best described as a partial unfolding.

The many different photoreceptor proteins that have been described in the literature can be classified into a limited number of families. The most rational approach is to base this classification on the chemical structure of the light-absorbing chromophores involved, but in addition, arguments derived from protein sequence alignments have to be used to discriminate the many photoreceptor proteins that bind a flavin derivative (*i.e.* in the last three families). Accordingly, the most important families are the rhodopsins, the phytochromes, the xanthopsins, the cryptochromes, the phototropins and the BLUF proteins (see *e.g.* ref. 2 for a review). The primary photochemistry of activation of these photoreceptor proteins is based upon a change in the configuration of the chromophore involved. For the first three families this change in configuration equals a chemical isomerization (*e.g.* between *Z* and *E*, or: *cis* and *trans*). In the flavin-containing photoreceptor proteins other types of photochemistry have been uncovered (like transient cysteinyl-adduct formation in the LOV domains of phototropins[3]). This change in configuration subsequently must lead to formation of a signaling state of sufficient stability to communicate the process of photon absorption to a downstream signal transduction partner.

Below, we will provide an update of current understanding, *via* review and presentation of unpublished experiments, of the process of signal generation in a xanthopsin (Photoactive Yellow Protein), containing an isomerising cinnamyl chromophore, and in a BLUF protein, which is activated by flavin photochemistry, from the process of electronic excitation to the formation of a transient signaling state.

Materials and methods

DNA sequencing and cloning of pyp(B)

DNA sequencing was performed using standard procedures.

A polymerase chain reaction (PCR) was performed to amplify DNA encoding the *pyp*(*b*) gene using the HotStarTaq-Kit (Qiagen, Hilden, Germany). PCRs were performed according to the manufacturer's instructions and using *Halorhodospira halophila* SL-1 chromosomal DNA as template. The primers used were: 5′-CGATGGATCCGATGACGATGACAAAATG-GGCACACTCATCTTCGGCCGCC-3′ and 5′-CGATAAGC-TTTCAGGCTGCCGGGGCGCTGATC-3′. The expression-vector pMH014 was constructed by ligating BamHI/HindIII digested PCR fragment in pQE30 (Qiagen) digested with the

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same restriction enzymes. pMH014 was then retransformed to the expression strain *Escherichia coli* M15/pREP4 (Qiagen).

Overexpression and purification of PYP(B)

E. coli M15/pMH014 was cultured at 37 °C, protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 0.2 mM final concentration) at OD_{600} ~0.8. After 3 hours cells were harvested by centrifugation. Cell pellets were resuspended in 50 mM Tris-HCl, 8M Urea, pH 8, a purification procedure adapted from.[4] Solublized cells were diluted 1 : 1 with 50 mM Tris/HCl, pH 8.0. Apo-PYP(B) was then reconstituted with the chromophore by adding small aliquots of imidazole-activated chromophore,[5] Reconstituted PYP(B) was purified from the cell extracts with Ni^{2+} affinity chromatography, using Ni-NTA-agarose (Qiagen). PYP from *H. halophila* BN9626 was expressed and purified as described previously (see *e.g.* ref. 5).

Overexpression and purification of the BLUF domain of AppA

The BLUF domain containing the N-terminal residues 5–125 of AppA was cloned, heterologously expressed in *E. coli* and purified with metal-chelate affinity- and ion-exchange chromatography as described in refs. 6 and 7. A large excess FAD was added during reconstitution.

Steady-state and transient (millisecond/second) UV/Vis measurements

Steady-state protein spectra and photocycle kinetics on a millisecond to second time-scale were measured with a model 8453 Hewlett Packard diode array spectrophotometer (Portland, OR; minimal time resolution 100 ms). Protein solutions of ~10 μM in 50 mM Tris/HCl, pH 8 were used, without removal of the N-terminal hexahistidine tag from the holoproteins.

Results and discussion

Photoactive Yellow Protein

Blue-light activation of PYP initiates a fully reversible self-contained photocycle, with multiple intermediates that can be detected with time-resolved analyses of techniques like UV/Vis-, IR- and Raman spectroscopy, X-ray diffraction, *etc.* The schematic representation of the PYP photocycle is shown in Fig. 1, in which of all intermediates the corresponding UV-Vis absorption spectrum and the chromophore configuration are displayed. The absorption spectra are derived from femtosecond-picosecond[8,9] and nanosecond-millisecond[10] transient absorption studies. The temporal evolution of the concentration of each intermediate is shown in the inset. The electronic excitation of the system of conjugated n,π electrons of the 4-hydroxycinnamyl chromophore of PYP brings an electron into an anti-bonding orbital, which creates a rotary force on the atoms of its aliphatic tail.[11] In the first ground-state transient intermediate (I_0), formed within ~3 ps, this has led to the formation of a *cis* configuration of the cinnamyl moiety of PYP, in such a way that the hydrogen bonding network, that stabilizes the negative charge on the cinnamate inside the main hydrophobic core of the PYP protein, remains intact. This interpretation is based on many UV/Vis and some IR spectroscopic studies (*e.g.* ref. 12). In parallel with this (biologically) functional pathway, which has a quantum yield of 0.35, several other decay channels are available to the electronically excited state of PYP, such as fluorescence and non-radiative ground state recovery. Apart from this, in ultra-fast studies, when the sample is excited with femtosecond pulses at 400 nm, such processes as resonant release of a hydrated electron can also contribute.[9] The use of chromophore model systems in UV-Vis spectroscopic experiments has guided the interpretation(s) given above. Recent advances in the method of Laue diffraction allowed structural resolution on timescales

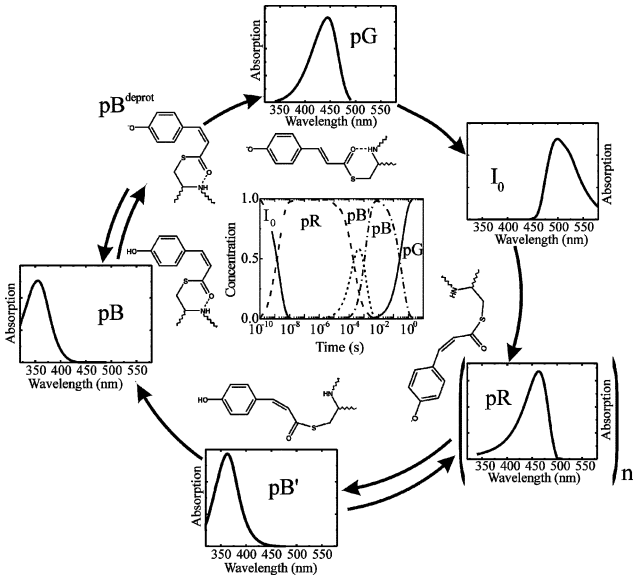

Fig. 1 Schematic overview of the photocycle of Photoactive Yellow Protein. The intermediate states are illustrated by their absorption spectra in the UV-Vis region and a schematic representation of the chromophore configuration. The absorption spectra are derived from femtosecond–picosecond and nanosecond–millisecond transient absorption studies. The brackets with subscript *n* in the pR state denote the possible existence of multiple intermediates on this timescale with similar spectral characteristics. The temporal evolution of the concentration of each intermediate is depicted in the inset.

as short as a nanosecond; however, this technique does not yet provide the structural information on the initial events, which take place on the (sub)picosecond timescale, involving the cinnamyl *cis–trans* transition. The structure that presumably comes closest to the configuration of the chromophore, immediately after isomerization, is the low-temperature trapped structure that was resolved with maximal correction for radiation (by visible radiation and X-rays) damage.[13,14] I_0 relaxes into pR, still with a deprotonated chromophore, in ~2 ns.[15] Actually, multiple pR-like intermediates may be involved, that all have a similar red-shifted absorption spectrum, as indicated by UV/Vis, Raman and photoacoustics (*e.g.* ref. 16). The time-resolved Laue diffraction experiments reveal that in pR the hydrogen bond between the phenolate oxygen and E46 is disrupted.[17] The interpretation of this recent resonance-Raman study suggests that subsequently E46 releases a proton (*e.g.* to a water molecule), leading to formation of PYP_L[1] (*i.e.* a late pR-like intermediate). The next step then would be that this proton protonates the chromophore, which leads to a dramatic blue shift in the visible absorption of the latter, hence the first of a series of blue-shifted (*i.e.* pB) intermediates is formed. The first of these was named pB′; this intermediate is in thermal equilibrium with the slowest of the red-shifted intermediates.[10]

The deprotonation of E46 generates a negative charge in a hydrophobic core with much less possibility for delocalisation of the negative charge than on the phenolate. This significantly destabilizes the structure of the PYP protein.[18] This leads to the long-lived intermediate pB, which presumably is the signaling state involved in the photophobic response generated by light-absorption of PYP in *Halorhodospira halophila*. The precise nature of this unfolding, however, is strongly dependent on the mesoscopic context of the protein. This is relevant because the techniques with highest resolution to resolve spatial biomolecular structure (*i.e.* X-ray diffraction and multidimensional NMR solution spectroscopy) require a mutually exclusive molecular context of the protein under study.

Caught in a crystalline lattice the conformational transitions after photon absorption expand through the protein like a 'quake'. They arrive at the surface of the protein in the N-terminal domain in about a millisecond. The conformational

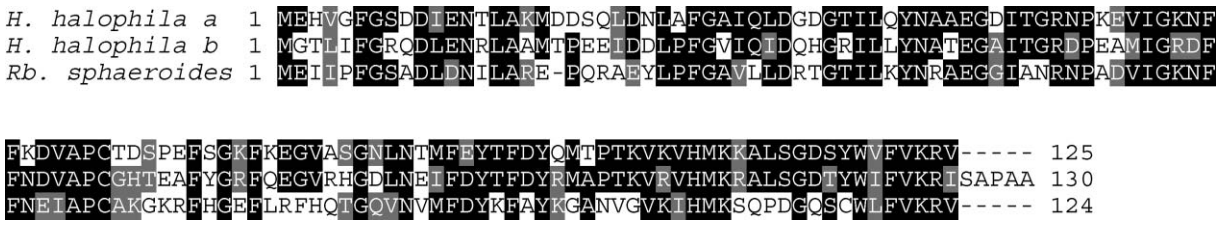

Fig. 2 Photoactive Yellow Protein multiple sequence alignment. Alignment of the PYP from *Rb. sphaeroides* and the two PYP's found in *H. halophila* SL1.

transitions, observed with X-ray diffraction, nevertheless are restricted to very small backbone displacements plus chromophore- and side-chain rearrangements; all elements of secondary structure remain intact. The most important rearrangement is the release of the protonated chromophore towards solvent, in such a position that it now hydrogen bonds to R-52.[17]

When PYP is dissolved in aqueous solution, in contrast to the crystalline state, elements of its secondary structure partially unfold upon formation of the signaling state, in particular the N-terminal helices[19–21] and the helices that cover the chromophore in the main hydrophobic core. Because of its unfolded nature the pB state in solution has been very refractory to spatial characterization. Recently, however, both the use of an N-terminally truncated variant of PYP (Δ25-PYP, which increases the lifetime of the pB state 1000-fold[22]) in NMR spectroscopy, and a computational prediction, have independently led to a detailed model confirming the above description.[23,24] Both studies reveal that key groups from the chromophore binding pocket are exposed to aqueous solvent in the signaling state pB.

Recovery of the receptor (=ground) state of PYP requires: (i) re-isomerization of the coumaryl chromophore to the *trans* configuration and (ii) deprotonation of the chromophore plus protonation of E46. In the wild type protein both steps have rate control in the recovery reaction. The blue-light induced acceleration of the recovery[25] illustrates the contribution of the isomerization reaction to this rate control (highly accentuated *e.g.* in the M100 mutants), whereas the occurrence of the pB^{deprot} intermediate can be taken as an indication of the rate control of the proton transfer processes. The latter is strongly accentuated in the E46A mutant.[26] For catalysis of the re-isomerization a p-orbital of the sulfur atom of M100 and/or deprotonation of the chromophore has to increase the electron density on the C7 atom. This decreases the bond order of the C7=C8 double bond, through which the isomerization barrier is significantly lowered.[27]

PYP(B), the second Photoactive Yellow Protein in *H. halophila*

So far it has not been revealed which signaling partner would pick up the signal from PYP in *H. halophila* cells. That makes it difficult to choose which of the two contexts referred to above (*i.e.* free in solution or in a crystalline-lattice like complex) is most relevant for understanding the physiological function of PYP and impossible to follow this signal on its route to true biological function. A *pyp* gene, encoding a Photoactive Yellow Protein, has been discovered in seven organisms, all of which are purple bacteria. In *H. halophila*, PYP was proposed to be involved in negative phototaxis towards blue light.[28] This function was implied because the wavelength dependence of this response coincides with the PYP absorption spectrum. However, genetic techniques are poorly developed in this organism, hampering attempts to obtain further genetic proof. Only in one of the PYPs found – the PYP/phytochrome hybrid Ppr from *Rhodocista centenaria* – a genetic proof for its function has been provided: it was shown to regulate expression of the enzyme chalcone synthase (a key enzyme in (protective) pigment synthesis[29]). Since mutant selection is technically very challenging in *H. halophila* it is highly relevant that a second *pyp* gene was recently discovered in the genome sequencing project for this organism (Oesterhelt *et al.*, unpublished results). Fig. 2 shows the alignment of the encoded protein with the other PYP from this *H. halophila* strain and the PYP found in *Rb. sphaeroides*. The gene encodes an authentic PYP protein (Fig. 3): its product can be reconstituted with 4-hydroxycinnamic acid into yellow holoprotein, with maximal absorbance at 443 nm, closely resembling the spectrum of PYP from *H. halophila* BN9626. Furthermore, upon blue-light excitation, this holoprotein shows an authentic, reversible photocycle (see Fig. 3(B)). We refer to this newly discovered photoreceptor protein as PYP(B). After a photoflash, a blue-shifted intermediate is formed, similar to that in the BN9626-PYP photocycle, and the protein recovers to its ground state very slowly, with a rate constant of 1.3×10^{-2} s^{-1}, as determined by fitting the trace shown in Fig. 3(B). Note that, as a result of the slow recovery rate, the measuring light also has a slight photoactivation effect on the protein.

Significantly, the genomic context of the *pyp*(*B*) gene contains several open reading frames encoding proteins that show high similarity to well-characterized signal-transduction components (*i.e.* sensors and regulators from two-component systems). Ongoing experiments aim at expression of these latter reading frames to test whether the encoded proteins interact with PYP(B). If so, then this will be an excellent system to characterize inter-molecular signal transfer in a xanthopsin-based system.

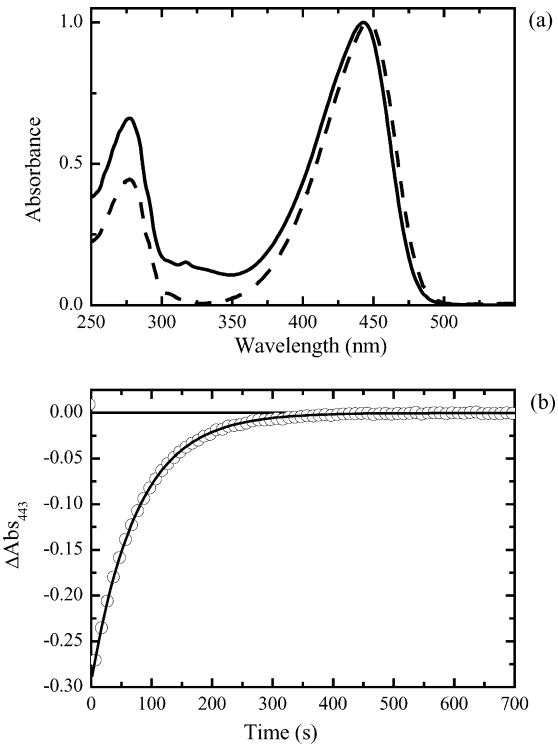

Fig. 3 Spectroscopic characterization of the second Xanthopsin (PYP(B)) in *Halorhodospira halophila*. (A) Absorption spectra of PYP(B) from *H. halophila* SL-1 (—) and PYP from *H. halophila* BN9626 (---). (B) Recovery trace after blue light excitation, showing time-dependent absorption changes at 443 nm. The solid line represents a mono-exponential fit to these data. Samples were measured in 10 mM Tris-HCl, pH 8.

The transcriptional anti-repressor AppA

The information available for AppA structure and function mirrors the situation of PYP. For AppA the biological role has clearly been established: It is a transcriptional anti-repressor that is released from its interaction partner PpsR upon blue-light illumination. PpsR can then repress the operons encoding the photosynthetic machinery in *Rhodobacter sphaeroides*[30,31] This light sensing is catalysed by the N-terminal domain of AppA, whereas its C-terminus is involved in relaying redox signals. The spatial structure of AppA, however, is still unresolved.

The N-terminal domain of AppA belongs to the BLUF domain family of proteins, a newly discovered family of photoreceptor proteins which bind a flavin non-covalently. Secondary structure prediction programs predict that it represents a new flavin-binding fold.[31] Ongoing NMR studies have recently confirmed this (Hsu *et al.*, unpublished experiments). Upon light absorption AppA goes through a functional photocycle with a relatively low quantum yield and only the minimum number of states: Besides the receptor- or ground state and an electronically excited state so far only a single transient intermediate has been found to have a function in the photocycle of AppA. This transient ground state intermediate, formed on the timescale of ~400 ps, by definition would then be expected to be the signaling state of AppA. It has a ~10 nm red-shifted absorption spectrum and a very long lifetime: it recovers in ~900 s.

Additional intermediates do exist: Also an electronically excited triplet state is formed from the singlet excited state; formation of this intermediate, however, presents a side reaction, which does not lead to signaling state formation (Yeremenko *et al.*, unpublished work). In this respect the AppA protein is very different from the best-characterized family of flavin-containing photoreceptors: The LOV domains. In the latter, the electronically excited singlet state relaxes to a longer living triplet state, from which a covalent adduct is formed between the C(4a) atom of the isoalloxazine ring and a nearby cysteine.[3,32] Similar photocycle characteristics as measured for AppA have been reported for other members of the BLUF family, like Slr1694 from *Synechocystis* PCC6803,[33] for PAC from *Euglena gracilis*[34] and for YcgF from *E. coli*.[35]

The red-shifted absorption spectrum of the signaling state of AppA was originally proposed to result from altered π–π stacking interaction between a tyrosine (Y21), conserved in all BLUF sequences known to date, and the flavin isoalloxazine ring, along with a hydrogen bond rearrangement between the N5 of the flavin ring and the tyrosine. However, the FAD cofactor might also deprotonate in the signaling state.[6] FTIR spectroscopy on Slr1694[36] showed that light absorption causes a hydrogen-bond rearrangement, so that the carbonyls of the isoalloxazine ring become more strongly hydrogen-bonded with residues lining the flavin-binding pocket. In addition, *via* the Amide I region of the spectra, a conformational rearrangement of the protein was detected. It is relevant to keep in mind, however, that this cyanobacterial BLUF domain shows a 180-fold increased recovery rate as compared to AppA.[33]

It was reported, based on size exclusion chromatography of full-length AppA and of the C-terminally truncated variant $AppA_{1–156}$, that light induces a conformational change in the protein that increases its Stokes radius and/or its dynamics. Similar experiments also show that the full-length protein is monomeric, whereas $AppA_{1–156}$ forms dimers.[30,37] Analysis of $AppA_{5–125}$[6] by size-exclusion chromatography revealed no significant difference in elution profile between the ground- and signaling-state of AppA (data not shown). Both states elute at an elution volume that corresponds to a 40 kDa protein. Since the mass of the protein is 15.5 kDa, this indicates also this $AppA_{5–125}$ variant forms dimers.

In PYP, formation of the signaling state results in partial unfolding of the secondary structure of the protein. 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.[19] 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, suggesting there is no significant change in heat capacity between the ground- and signaling-state. Thus it appears that the BLUF domain does not show a significant increase in hydrophobic contact surface upon illumination, which is supported by the results of our own unpublished size-exclusion experiments and the fact that we observe little structural changes in the structure of AppA, upon illumination, using NMR (Hsu *et al.*, unpublished data). 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 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[38,39] (and made likely, using small angle X-ray scattering techniques, by Nakasako *et al.*[39]).

During purification of the BLUF domain of AppA, it was observed that the recovery rate of the protein directly after elution from the nickel-column, thus in the presence of 0.5 M NaCl and 0.5 M imidazole, was significantly higher than after dialysis against buffer without salt and imidazole. Analysis of the recovery rate in the presence of either NaCl or imidazole revealed that the observed effect is mainly due to imidazole. The recovery rate increased 1.5-fold in the presence of 1 M NaCl, whereas an equal concentration of imidazole increased the recovery rate 27-fold. Concentrations of imidazole 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, indicated by a simultaneous loss of fine structure and red-shift of the ground-state spectrum. The recovery was mono-exponential, indicating the imidazole does not lead to formation of additional intermediates (Fig. 4). Imidazole has a pK_a of 6.95, and the fact that the increase in recovery rate in the presence of 1 M imidazole

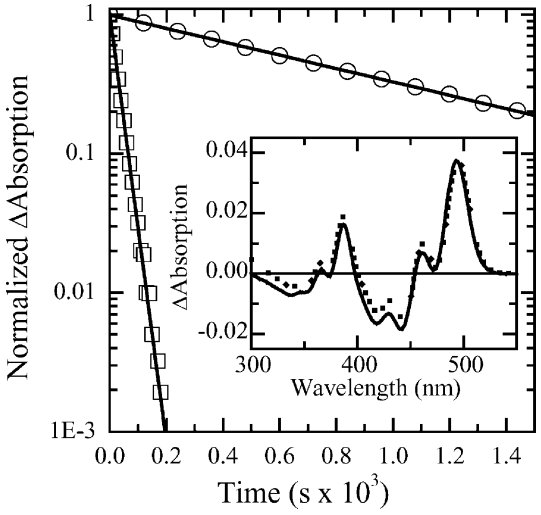

Fig. 4 Imidazole increases the recovery rate of the BLUF domain of AppA. $AppA_{5–125}$ (in 10 mM Tris-HCl, pH 8.0) with (□) or without (○) 1 M imidazole was converted to the signaling state by illumination with white light and allowed to revert back to the ground state in the dark. The solid lines represent fits through the data points using a mono-exponential (decay) function. The inset shows the UV/Vis light–dark difference spectra of AppA with (—) and without 1 M imidazole (---).

is about two-fold smaller at pH 7 (11% neutral imidazole) compared to pH 8 (91% neutral imidazole) suggests that it is the neutral form of imidazole that affects the recovery kinetics. The recovery kinetics of the protein before the addition of imidazole and after removal of the imidazole by dialysis were identical, showing that the effect exerted by the imidazole is reversible.

As imidazole has been demonstrated in other systems also to catalyze rearrangements of hydrogen bonding,[40] we do think that its role may be similar in its effect on the rate of ground-state recovery in AppA. Together with unpublished information from structural studies, this leads to models in which Y21 is indirectly linked (*e.g. via* an aspartyl side chain) to one of the carbonyls of the flavin isoalloxazine ring. Light absorption might then alter hydrogen-bonding in this chain to relay the information of photon absorption to the surface of the BLUF domain so that its interaction with the carboxy-terminal domain of AppA can result in proper alteration of PpsR binding.

Conclusion

In this perspective paper we discuss recent findings in the two photoreceptor proteins PYP and AppA. Both are bacterial blue-light photoreceptor proteins, but they differ substantially, both in the molecular mechanisms that underlie their functioning, and in the extent to which they have been characterized. The molecular details of the light-induced binding to the signaling partner are poorly understood in both cases. In the case of AppA the partner protein is known, but there is no structural information available for either protein; in the case of PYP high resolution structures are available, also of the signaling state, but the signaling partner remains unknown. Presumably, the finding of new xanthopsins, such as the PYP described here, will help with the identification of a signaling partner protein.

As soon as the spatial structure of the BLUF domain of AppA is available (which is very near), the next step will have to be the analysis of full-length, multi-domain proteins, as opposed to the single domains that have mostly been studied until now. This will allow us to make models of the entire pathway of signal transfer, from light absorption all the way to the regulation of expression of photosynthesis genes, in unprecedented detail. Such models may eventually allow us to computationally predict the outcome of alterations in the electronically excited state of the photoreceptor on its biological performance.

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