PhD thesis

Unraveling the functional dynamics of OaPAC using ultrafast spectroscopy

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INTRODUCTION

All living organisms need to be able to sense their environment in order to respond appropriately to the prevailing conditions around them, and the ability to adapt to different levels of sunlight is particularly important, even for many non-photosynthetic organisms. Higher plants have more types of sensory photoreceptors to optimise the energy yield from photosynthesis while minimising UV-mediated damage. Various photoreceptor types using different chromophores have evolved for different purposes and are receptive to different wavelengths of light [1].

In this work we focus on BLUF (Blue Light Using Flavin) proteins belonging to photoactive flavoprotein family containing flavin molecule – one of the most important cofactors in enzymatic functions – responsible for sensing the light [2]. BLUF domains were independently discovered by different research groups in and around 2002, in the unicellular flagellate *Euglena gracilis* [3] and in the purple bacterium *Rhodobacter sphaeroides* [1], [4]. Many BLUF proteins including AppA (**A**ctivation of **P**hotopigment and ***P****uc* expression **A)** and PAC (Photoactivated Adenilate Cyclase), carry an extra domain downstream from the BLUF domain, with enzymatic or other properties [1]. Illuminating these proteins by – an appropriate wavelength of – light causes activation of the BLUF domain and initiate the signal transduction towards the extra domain. The light induced signal reaching the extra domain can trigger different (e.g., enzymatic) functions of the protein [1]. The photoresponse depends on interactions of the isoalloxazine ring with several nearby conserved residues [5]. In all BLUF proteins tyrosine, glutamine and metionin are conserved amino acids and almost all of them (excepting only one protein) also contains tryptophan. These amino acids settle close to the flavin cofactor in all BLUF proteins and are critical to the signalling mechanism containing photoinduced charge- and H-bond rearrangement and conformation changing [1], [5]. Excitation of the flavin to the S1 singlet state is quenched in as short a time as ~1-10 ps by ET (electrontransfer) from neighbouring aromatic residues [5], because the excited state flavin is ready to oxidise its environment. The primary electron donor is a close tyrosine and in some cases a tryptophan. This transient charge-separated state rearranges the hydrogen bonding of the active center to produce a much longer-lived metastable conformation for propagating signals. The flavin absorption spectra of BLUF domains undergo a rapid ~10 nm redshift upon conversion from the dark to light-adapted states that can persist from seconds to minutes [5].

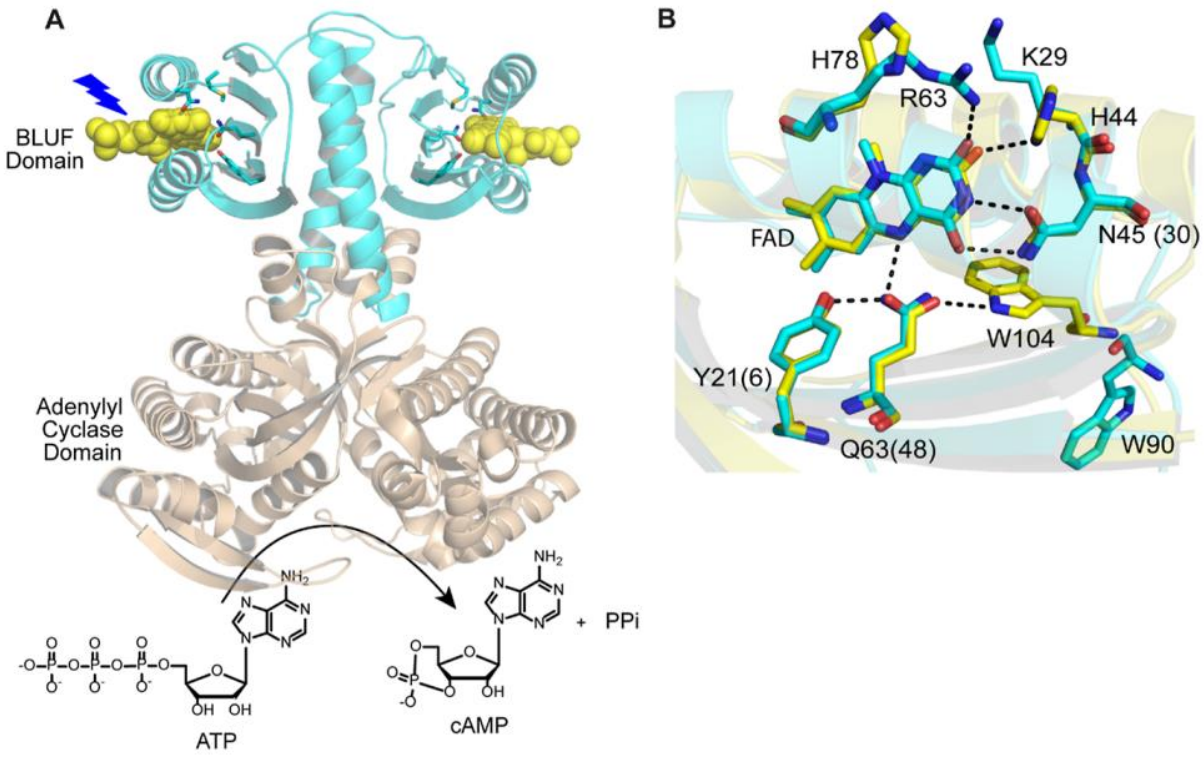

Figure 1. Structure of the OaPAC (A, B)

One of the BLUF proteins in our focus is the OaPAC that belongs to photoactivated AC (Adenylate Cyclase) family. OaPAC is an adenylate cyclase from the photosynthetic cyanobacterium *Oscillatoria acuminata* that can respond to light. The primary function of PAC proteins is to produce cAMP (cyclic Adenosine Monophosphate) a second messenger molecule caused by illumination of blue light. PAC proteins can be found both procaryotes and eukaryotes and can be used as optogenetic tools in controlling the intracellular cAMP level. In our work we investigate the photoactivation of the OaPAC using femtosecond transient visible- and infrared spectroscopy.

The other protein that important in our work is AppA from the purple bacterium *Rhodobacter sphaeroides* that takes part in the transcription regulation. Mark Gomelsky and Samuel Kaplan identified the protein in 1995 and named AppA (**A**ctivation of **P**hotopigment and ***Puc*** expression **A)** [6], [7]. In dark and low oxygen level conditions AppA binds the PpsR2 transcriptional repressor, whereas under high intensity blue light or high oxygen level the complex dissociates enabling PpsR2 to bind the DNS whereby halt the biosynthesis of the photosynthetic genes [8]. The first step of the photocycle of AppA is a PCET (proton coupled electron transfer) triggered by light absorption. The source of both the electron- and proton

transfer is the tyrosine (Tyr21) and PCET cause changes in the redox state of the flavin and some rearrangement of the hydrogen bonding system around the flavin [8].

AIMS OF THE THESIS

The aims of our work can be summarised in the following points

- determining the vibrational marker of the tyrosine cation radical using transient infrared spectroscopy
- investigation of the photoactivity of OaPAC using unnatural amino acid mutagenesis
- investigation of the photoactivity of OaPAC using ultrafast spectroscopy

MATERIALS AND METHODS

Particularly in the case of flavoprotein photochemistry transient radicals exceedingly investigable by the complementary techniques: ultrafast time resolved visible- and infrared spectroscopy.

Transient absorption (TA) spectroscopy

TA measurements performed at University of Pécs at the Medical School, on the TA spectrometer found in the laser lab of Biophysics Department. Transient visible spectra were recorded with 100 fs temporal resolution by a transient absorption (TA) spectrometer applying ~800 μJ laser pulses centered at 800 nm at a repetition rate of 200 Hz. The high energy, ultrashort (100 fs duration) laser pulses were obtained from a *Spitfire Ace* (Ti: sapphire) regenerative amplifier seeded by a femtosecond *Mai Tai* mode-locked (Ti: sapphire) laser oscillator and pumped by an *Empower 45* multi-kilohertz, intracavity-doubled, green (Nd: YLF) pump laser [9].

The output of the amplifier was split in the ratio 1:9 to set the so-called pump-probe arrangement. The pump arm was generated from the higher energy pulses by second harmonic generation (SHG) in a BBO crystal to get 400 nm exciting beam then attenuated to ~200-400 nJ/pulse before reaching the investigated molecular system. The probe beam is provided by the lesser energy laser pulses via white continuum generation (WCG) in a rastered CaF_2 crystal. The train of both the pump and probe pulses were overlapped in the sample and the polarization of the probe was set to magic angle compared to excitation. To avoid photodegradation the samples were moved with the help of a homemade Lissajous scanner, simultaneously flowed by a peristaltic pump and kept at 12 °C temperature during the whole measurement. A *Newport (IMS Series High-Performance Long Travel Linear Stages 600 PP*) delay stage was fixed in the beam path of the pump arm to set the different delays comparing to the probe. Exciting pulses were chopped by a *Thorlabs MC2000* optical chopper (to the half of the output repetition rate) to generate "pump ON" and "pump OFF" states of the sample. Absorption spectra were recorded by an *Andor Newton* CCD operating well at -80 °C. Absorption data matrices were collected, and the absorption changes calculated and stored by the home written *NI LabView* (visual programming language) data acquisition and control software. The absorption changes were reported as pump on minus pump off normalized difference spectra. The obtained data matrices were also analysed by the Glotaran software.

Transient infrared (TRIR = Time Resolved InfraRed) spectroscopy

Time resolved infrared experiments were performed on the ULTRA in the Appleton Laboratories Central Laser Facility in United Kingdom. ULTRA system can be used with a range of advanced time resolved linear and nonlinear vibrational spectroscopy techniques that utilize a narrow-band picosecond pump and broadband femtosecond probe [10].

At the heart of the spectrometer is a custom dual Ti: sapphire chirped pulse amplifier (Thales Laser) seeded from a single <20 fs, >50 nm bandwidth oscillator (Femtolaser). Both amplifier arms (we need only the femtosecond one) consist of regenerative and subsequent two-pass amplification stages. The femtosecond amplifier arm generates 10 kHz, 40-80 fs, 0,8 mJ pulses at 800 nm for measurements of TRIR, 2DIR etc. The amplifiers are pumped by three ~55 W Touaryx Ny: YAG lasers. The system comprises two femtosecond OPAs (Optical Parametric Amplifier) to generate WCG (White Light Continuum) and SHG (Second Harmonic Generation) for TRIR experiments.

The system utilizes high-rate-readout linear detectors sensitive from the ultraviolet to the mid-infrared wavelengths. Three different detector types are available and can be mounted on the spectrograph to probe from the UV to mid-IR wavelengths: 512-element silicon (Quantum Detector, QD) 256-element indium gallium arsenide (InGaAs, QD), and 128- and 64-element mercury cadmium telluride (MCT, IR Associates) arrays. These detectors all connect to a common data acquisition (DAQ) system (QD) and two of these, each up to 512 channels, may be synchronized and read to a computer at any repetition rate up to 20 kHz. The DAQ hardware, computer connections and software processing are the same for all detectors, allowing use of common software control and processing systems across the different experimental configurations [10]. Data acquisition was controlled by LabView software.

We used TRIR part of ULTRA to record transient infrared spectra of TrmFO (folate or FAD dependent tRNS metiltransferase), GOX (glucose oxidase), AppA and OaPAC. A femtosecond OPA generates the 100 fs, 5 kHz, 450 nm pump pulses and the probe pulses covering $1400 - 1800\ \mathrm{cm}^{-1}$ wavenumber range. The resolution of the system is limited by the pulselegth of the laser pulses. Pump-probe arrangement of TRIR is like that of TA arrangement. TRIR

method is applicable to detect vibrational modes of the chromophore or the protein in transient infrared spectra.

RESULTS

Identification of the vibrational marker of tyrosine cation radical

We employed ultrafast infrared absorption measurements to identify the vibrational markers of the cation and neutral tyrosine radical in C51A and C51A/Y343F variants of TrmFO. We further investigated the formation of the same radical in wild type GOX and W104Y AppABLUF mutant in the light state. Comparing our previous visible transient absorption data measured on TrmFO mutants and GOX with the infrared measurements, we identify the 1483 cm^{-1} vibrational mode as a vibrational marker of $TyrOH^{\bullet+}$. Tryptophan cation radical ($TrpH^{\bullet+}$) has a vibrational signature around 1488–1490 cm^{-1}. We were able to identify a vibrational marker for the neutral tyrosine radical at ~1502 cm^{-1}. The exact position of both the tyrosine cation and neutral radical strongly depends on the hydrogen bonding environment, on the solvent, so their frequencies can differ in a water-based buffer as in this work the samples were dissolved in deuterated buffer. The observed shift in a water-based buffer is expected to be smaller in the case of the neutral tyrosine radical and more significant in the case of the tyrosine cation radical.

This finding is in good agreement with earlier assignments of this vibrational mode with the pioneering work on phenoxyl and tyrosine radicals. Time-resolved Resonance Raman spectroscopy measurements – using 400 nm excitation – were done on phenoxyl radicals produced in aqueous solutions by pulse radiolysis. These measurements found a strong Raman peak at 1505 cm^{-1} which the authors assigned to the C=O stretch of the phenol group [11]. A strong vibration for the tyrosine radical was found using UV Resonance Raman spectroscopy at 1510 cm^{-1} [12].

The role of tyrosine as electron donor in Photosystem II was first proved by Gerken, Brettel et al. using a nanosecond UV flash photolysis system; the authors proved that due to the electron transfer neutral tyrosine radical is formed [13]. Formation of neutral tyrosine radical in Photosystem II from Synechocystis sp. PCC 6803 was investigated by difference FTIR and the vibrational marker of $Tyr_D^{\bullet}$ and $Tyr_Z^{\bullet}$ radicals were found at 1503 and 1512 cm^{-1}, respectively [14].

Using these new findings on the vibrational feature of neutral and cation tyrosine radical, we characterized the photochemistry of AppA W104Y mutant which was tailored to enhance and identify the formation of tyrosine radicals. This mutation significantly altered the photochemistry of the protein but as expected from earlier work [15], [16], it did not stop the

formation of the light-adapted state, except it altered the dark-adapted state recovery to ~100-fold faster compared to WT AppA. Blue-light irradiation of BLUF domain proteins results in a ~ 10 nm shift of the 447 nm absorption peak of flavin due the reorganization of the hydrogen bonding network around the isoalloxazine ring. Stopping the irradiation, the absorption peak turns back to the original value in dark-adapted state. This recovery from light-adapted to dark-adapted state happens in ~ 30 min in AppA and much faster (couple of seconds) in PixD or OaPAC. Replacement of W104 supresses the red shift in the full-length protein [16] and results in a faster recovery of the dark-adapted state in the BLUF domain; in W104A, the recovery was 80-fold faster than in wild type [17].

We performed transient infrared measurements on the light state of W104Y, and we observed one step formation of neutral tyrosine radical accompanying with a neutral flavin radical at ~ 2 ps after excitation. This suggests that in this mutant proton-coupled electron transfer occurs, with both the electron and proton donor being a tyrosine. PCET is not unexpected during the photoactivation of photoactive flavoproteins, especially if the primary electron donor is a tyrosine. Proton coupled electron transfer was observed for example in the case of two BLUF domain proteins, PixD and PapB [18]. In the case of PixD, we observed a sequential radical formation: first, Y8 donated an electron to the flavin and $FAD^{\bullet-}$ radical formed, neutral flavin radical was formed concomitantly in ~ 100 ps [19]. Based on theoretical calculations, in the case of PixD protonation of the flavin happens sequentially: after excitation the tyrosine gives a proton to an adjacent glutamine which finally protonates the flavin, stabilizing the neutral radical state[20], [21]. In the case of PaPB, a BLUF protein found in the purple bacterium *Rhodopseudomonas palustris*, $FADH^{\bullet}$ was formed directly – as in the case of the AppA W104Y mutant – via a proton-coupled electron transfer process [22].

Our experimental data indicate that excitation of flavin in C51A TrmFO mutant also leads to a sequential proton coupled electron transfer to a very small extent (<10 %), like in some of the BLUF domain proteins: tyrosine 343 gives an electron to the flavin forming the anionic flavin radical, subsequently fast deprotonation of the tyrosine leads to the forming the neutral flavin radical. We also have seen evidence for proton-coupled electron transfer in GOX, where an electron is transferred upon excitation from one of the close tyrosines, followed by a proton transfer – from the tyrosine which gave the electron – stabilizing the $FADH^{\bullet}$ state of flavin. Photochemistry of GOX was observed to be heterogenous and complex since besides the tyrosines, one of the close tryptophans can also donate an electron to the excited flavin.

Altogether, this work exemplifies how extremely sensitive and selective TRIR method is in elucidating even minor reaction pathways involving aromatic residue radicals.

Investigation of OaPAC photoactivity

Although the photochemistry of BLUF photoreceptors has been extensively studied using a variety of time-resolved approaches, these studies have been largely confined to BLUF domain proteins lacking the biologically relevant output partner [19], [21], [23]–[25]. In the present work, we extend our analysis of the BLUF photocycle to OaPAC in which both the BLUF and adenylate cyclase output domains are contained in a single protein. Using TRIR and TRMPS, we investigated the OaPAC photoactivation mechanism, and compared it with other BLUF proteins. We further explored the impact of modulating the acidity of the conserved tyrosine Y6 on the light-controlled adenylate cyclase reaction. The photoactivation of the BLUF photoreceptors can be broadly distinguished based on the presence or absence of radical intermediates during light-state formation. Whereas no radical intermediates can be observed during light-state formation in AppA, BlrB and BlsA, the photoactivation of PixD (Slr1694) and PapB involves PCET on the reaction pathway leading to the light state. In the case of PixD, photoactivation of the flavin leads to a sequential formation of anionic ($FAD^{\bullet-}$) and neutral ($FADH^{\bullet}$) flavin radicals, while in PapB, a neutral flavin semiquinone FADH radical ($FADH^{\bullet}$) was observed as the intermediate before the formation of the signalling state [24], [26], [27].

The TRIR and visible TA data indicate that two different processes can occur in the OaPAC photocycle after excitation. TRIR measurements on the wild-type protein and the Y6F mutant illustrate that if W90 is present, there is an electron transfer process from W90 to the flavin, forming the $FAD^{\bullet-}$ $TrpOH^{\bullet+}$ radical pair. TRIR and visible transient measurements on the wild-type and W90F mutant also indicate that after excitation, a concerted proton-coupled electron transfer process occurs from the Y6 to the flavin, generating the $FADH^{\bullet}$ $-Tyr^{\bullet}$ radical pair.

The role of proton transfer in the function of OaPAC BLUF was examined in a recent paper by the Zhong group where the authors replaced the tyrosine by a tryptophan (Y6W). Kang et al. observed a sequential electron transfer process: formation of the flavin anionic radical, followed by the formation of the neutral semiquinone. The authors proposed that they observed proton rocking in which the tryptophan transiently donated a proton to the flavin to form the neutral semiquinone followed by a reverse PT; the rates of the forward and reverse PT were very close, being 51 ps and 52 ps, respectively. We have observed

enhancement of the electron transfer process in other BLUF domain proteins (AppA, PixD) when the conserved tyrosine (Y21 and Y8, respectively) was replaced with a tryptophan, although this mutation resulted in the loss of protein activity. We expect that a similar effect occurs in the case of the Y6W OaPAC mutant and plan to observe the effect of this mutation on the cAMP production.

The conserved tyrosine in BLUF domain proteins is essential for photoactivity, and in every case, including OaPAC, replacement of this residue with phenylalanine results in a photoinactive protein. However, the precise role of the conserved tyrosine in the photoactivation mechanism depends on whether radical intermediates are present in the photocycle. In PixD, the 3000-fold increase in acidity of Y8 resulting from replacing Y8 with 2,3,5-F3Y6 halts the photocycle at $FAD^{\bullet-}$ presumably because the tyrosine is ionized and can no longer function as the proton donor required for the formation of $FADH^{\bullet}$. In contrast, replacement of Y21 in AppA has only a slight impact on the kinetics of light-state formation and every n-FY6 variant is photoactive. The biggest alteration in AppA BLUF was on the dark-state recovery where the change in Y21 pKa led to a 3000-fold increase in the rate of dark-state recovery in H_2O, while in PixD the change was only 15-fold [19]. The studies here show again that OaPAC photochemistry resembles PixD. For 3,5- F2Y6 and 2,3,5-F3Y6, the two variants with the most acidic phenol groups (pKa 7.2 and 6.4), no light state can be observed in either the absorption spectrum or the TRIR spectrum. In contrast for 3-FY6, where the phenol pKa is only 1.5 pH units more acidic than tyrosine, a 10 nm red shift is observed in the flavin absorption spectrum upon excitation and the TRIR/ TRMPS spectra are very similar to wild-type OaPAC. Finally, although a ~5 nm red shift in the flavin absorbance at 450 nm can be observed upon irradiation of 2,3-F2Y6 OaPAC, no light state transient at 1694 cm^{-1} can be observed in the TRIR and the photocycle apparently stalls at $FAD^{\bullet-}$. We speculate that the lack of observable light state in the TRIR data is because the yield of light state is low in this mutant given that this (TRMPS in this case) is a single shot experiment.

The presence of a covalently attached adenylate cyclase domain in OaPAC provides a unique opportunity to directly link the photochemistry of the BLUF domain with activation of the output domain. Using a coupled assay, light-dependent conversion of ATP into cAMP occurs with kcat, KM, and kcat/ KM values of 205 ± 11 min^{-1}, 0.12 ± 0.01 mM, and 1888 mM^{-1} min^{-1}, respectively. These values have not been previously reported for OaPAC; however, Ohki et al. reported an ~20-fold change in enzymatic activity between dark and light states at a single ATP concentration[28]. In the pyrophosphate spectrophotometric assay, we observe a ~100-fold increase in activity for wild-type OaPAC upon photoexcitation. In agreement with time-

resolved spectroscopy, 3-FY6 OaPAC has adenylate cyclase activity that is comparable to that of the wild-type protein, whereas 3,5-FY6 and 2,3,5-FY6 show no light-dependent catalytic activity. Interestingly, 2,3-F_2Y6 has a similar kcat/KM value to wild-type OaPAC even though no light state can be observed in the TRIR spectrum, and the steady-state FTIR difference spectrum showed smaller light-induced changes in the protein modes in contrast to the wild-type. As noted above, a small red shift can be observed in the flavin absorption band at 450 nm, suggesting that light state can be formed when the protein is continuously illuminated. Also, a population of 2,3-F_2Y6 variant is in a pseudo-lit state because in the assay, we observed conversion of ATP to cAMP in the absence of light. Therefore, in the case of the steady-state IR difference measurement, the appearance of protein modes should appear suppressed compared to the wild-type.

CONCLUSIONS

Identification of the vibration marker of tyrosine cation radical

In this work using transient infrared spectroscopy we identified the vibration markers of tyrosine cation radical and tyrosine neutral radical in a model system TrmFO suitable for this purpose. We observed the same radicals in glucose oxidase, and we perform measurements on a light state of BLUF domain protein AppA BLUF W104Y mutant also. Based on our results the tyrosine cation radical is represented in the TRIR spectrum by the 1483 cm^{-1} peak and the vibrational marker of the tyrosine neutral radical is around ~1502 cm^{-1}. We also found the fingerprint of the tryptophan cation radical around 1488-1490 cm^{-1} region. Based on our experiments we can state that the PCET takes place sequentially in TrmFO C51A thus we were able to identify the tyrosine cation radical in this mutant, whereas the TRIR spectrum of C51A Y343F double mutant resemble the FAD* spectra and do not evolve in time as it is lacking the potential electron donor. In another model system, in glucose oxidase we observed sequential proton coupled electron transfer, confirmed by the global analysis results (EAS spectra) of the raw data. We also performed measurements on the light state of a BLUF domain protein AppA BLUF W104Y, which shows new tones of knowledge of the photochemistry of AppA protein. The global analysis of the recorded spectra let us to conclude that PCET in AppA takes place in a concomitant way ~2 ps after the excitation and the source of both the electron and the proton is a close tyrosine. Our results also suitable for identifying the tyrosine cation and neutral radicals and also shine a light on how exquisitely sensitive (regarding low rate of radical intermediates) method is the TRIR spectroscopy.

Investigation of photoactivity of OaPAC

Our time-resolved experiments on BLUF domain photoreceptors have been extended to OaPAC in which the BLUF domain is covalently attached to an AC output domain. OaPAC is thus a good model system not only for studying photochemistry but also for elucidating signal transduction between a BLUF domain and an output domain by monitoring the light-stimulated conversion of ATP into cAMP. Our work illustrates the direct impact of the photochemical processes in the BLUF domain on the output domain, which has not previously been shown in AppA, PixD, or any other BLUF domain. Using ultrafast infrared and transient absorption spectroscopy, we show that the photoactivation mechanism of OaPAC involves concerted proton coupled electron transfer from the conserved Y6 to the excited state of FAD (FAD*). This mechanism is slightly different from the one observed in PixD, where the

photoactivation mechanism involves a sequential proton-coupled electron transfer from Y8 to FAD*. Instead, in the case of OaPAC protonation of the flavin occurs together with the electron transfer step from Y6 to the flavin.

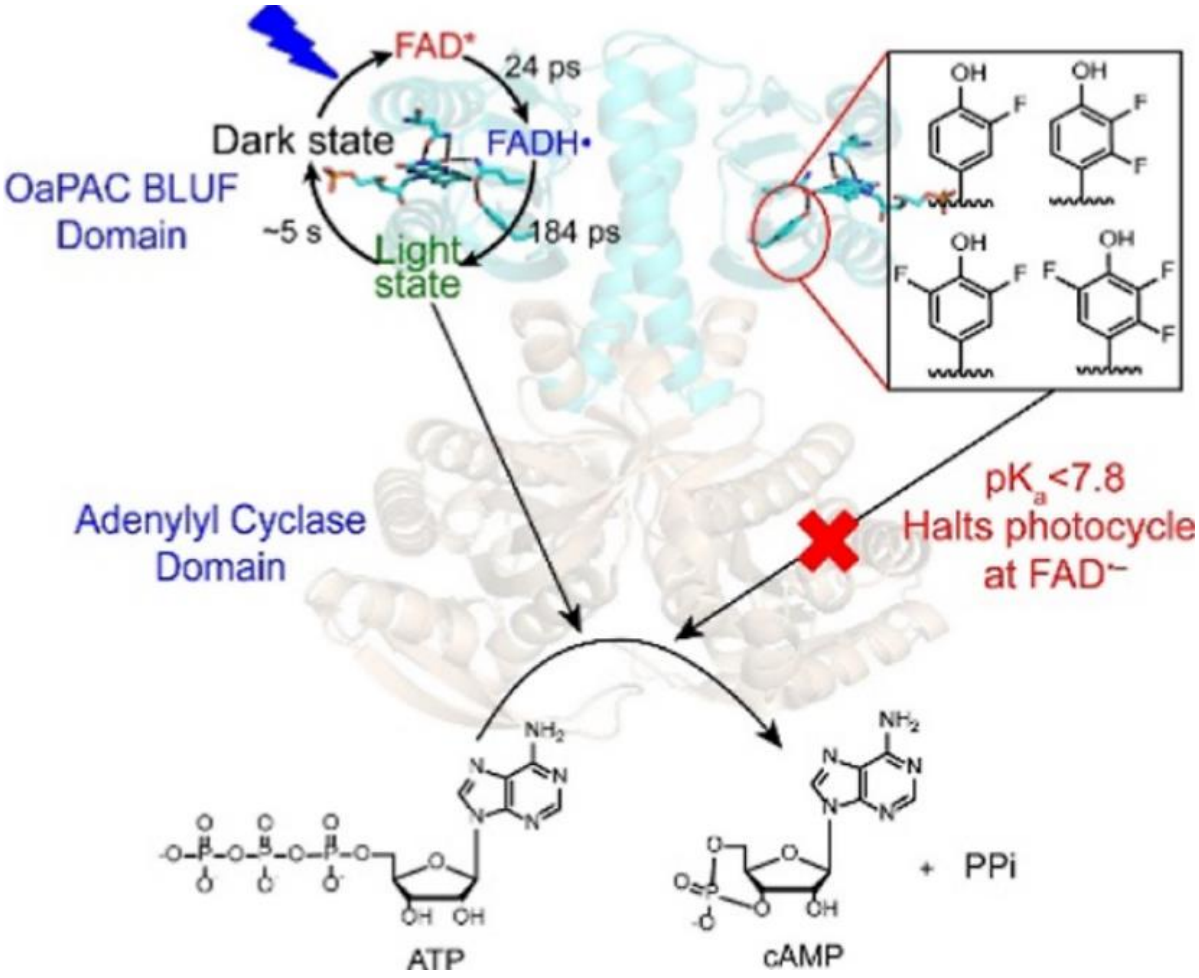

Figure 2. Photocycle of the OaPAC

The role of Y6 in the photocycle of OaPAC was probed via UAA mutagenesis. Replacement of Y6 with n-FY analogues increases the acidity of the phenol hydroxyl group and reduces the rate of electron transfer. Specifically, altering the pKa and/or reduction potential of the flavin had a profound impact on light-state formation for 2,3-F2Y6, 3,5-F2Y6, and 2,3,5-F3Y6 where the photocycle is halted at FAD•-.

Using an enzyme assay that couples PPi production to the consumption of NADH, we quantified the adenylate cyclase activity of wildtype OaPAC and also of the n-FY6 variants to interrogate the impact of the Y6 pKa on the light-activated conversion of ATP into cAMP and PPi. Only the n-FY6 variants with pKa values of 7.8 or higher were able to catalyse cAMP formation, while variants with lower pKa values were inactive because the photocycle was halted at FAD•-. While the 2,3-F2Y6 OaPAC variant (pKa 7.8) had light-dependent adenylate

cyclase activity, no light state was observed in the TRIR which we propose is due to the low yield of product formation given the single-shot format of the experiment. Collectively, the results shed new light on the photoactivation mechanism of BLUF domain photoreceptors.

PUBLICATIONS

1.

„Unraveling the Photoactivation Mechanism of a Light-Activated Adenylyl Cyclase Using Ultrafast Spectroscopy Coupled with Unnatural Amino Acid Mutagenesis"

Jinnette Tolentino Collado, James N. Iuliano, Katalin Pirisi, Samruddhi Jewlikar, Katrin Adamczyk, Gregory M. Greetham, Michael Towrie, Jeremy R. H. Tame, Stephen R. Meech, Peter J. Tonge, and Andras Lukacs

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2.

"Identification of the vibrational marker of tyrosine cation radical using ultrafast transient infrared spectroscopy of flavoprotein systems,"

Katalin Pirisi, Lipsa Nag, Zsuzsanna Fekete, James N. Iuliano, Jinnette Tolentino Collado, Ian P. Clark, Ildikó Pécsi, Pierre Sourina, Ursula Liebl, Gregory M. Greetham, Peter J. Tonge, Stephen R. Meech, Marten H. Vos, András Lukács

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