Functional dynamics of a single tryptophan residue in a BLUF protein revealed by fluorescence spectroscopy

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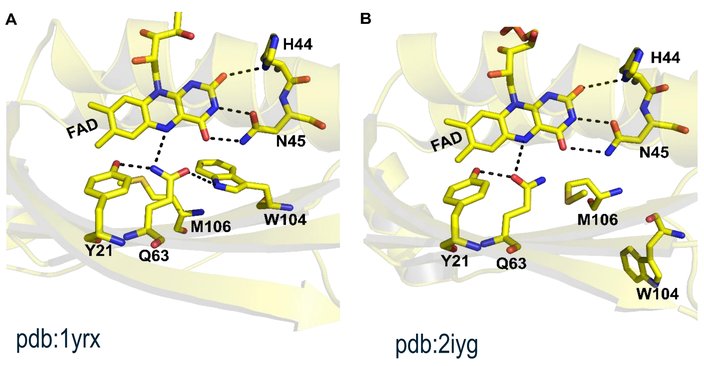
Introduction

AppA is a member of the 'Blue Light Using FAD' (BLUF) class of photoreceptors that are found in both prokaryotes and eukaryotes and is involved in the transcriptional control of genes required for photosynthesis in the bacterium *Rhodobacter sphaeroides* [1,2]. In BLUF domains, blue light excitation results in a signaling state (light-adapted state) that is characterized by a reorganization of the hydrogen bond network around FAD and the Tyr-Gln-Trp(Met) tetrad. This is revealed by a characteristic 10-15nm red shift of the first π→π* transition and a 20cm^{-1} downshift of the flavin C_4=O stretching vibration compared to the dark-adapted state [3,4].



Ribbon diagrams of the BLUF domain crystal structures from WT (1yrx) and C20S AppA (2iyg)

W104 is a key player in the photocycle in communicating the electronic excitation of the flavin ring to the protein backbone[5]. The exact conformation of W104 during the photoactivation process in AppA and other BLUF domains has been a controversial topic in the field. The first crystal structure of AppA (pdb:1yrx) showed that W104 in the dark-adapted state is located close to flavin in the so called Trp_{in} conformation. In a subsequent structure (pdb:2iyg), W104 is pointing away from the flavin (Trp_{out} conformation).

Computational and spectroscopic studies have tried to address the question of the Trp flip in AppA, leading to opposite conclusions. In this work, we revisit the question using a different approach.

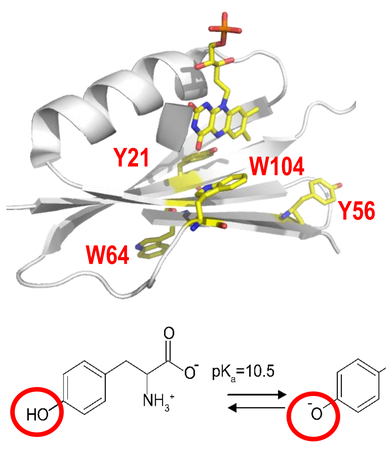
Results

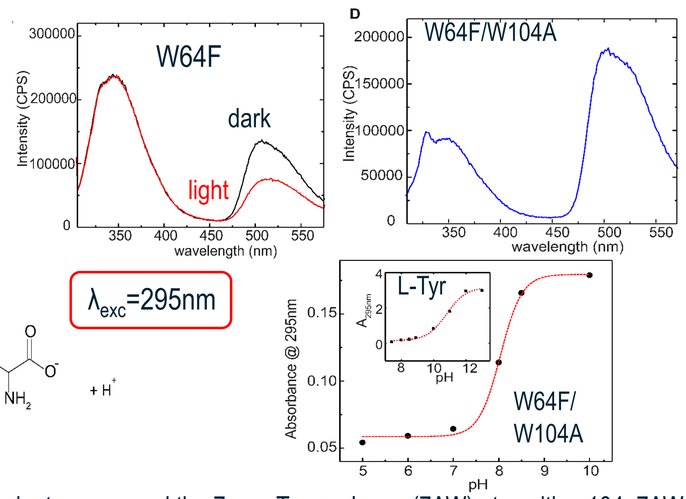
We have used fluorescence resonance energy transfer measurements (FRET) to provide quantitative information on the position of W104 during the photoactivation process in AppA.

$$E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}$$

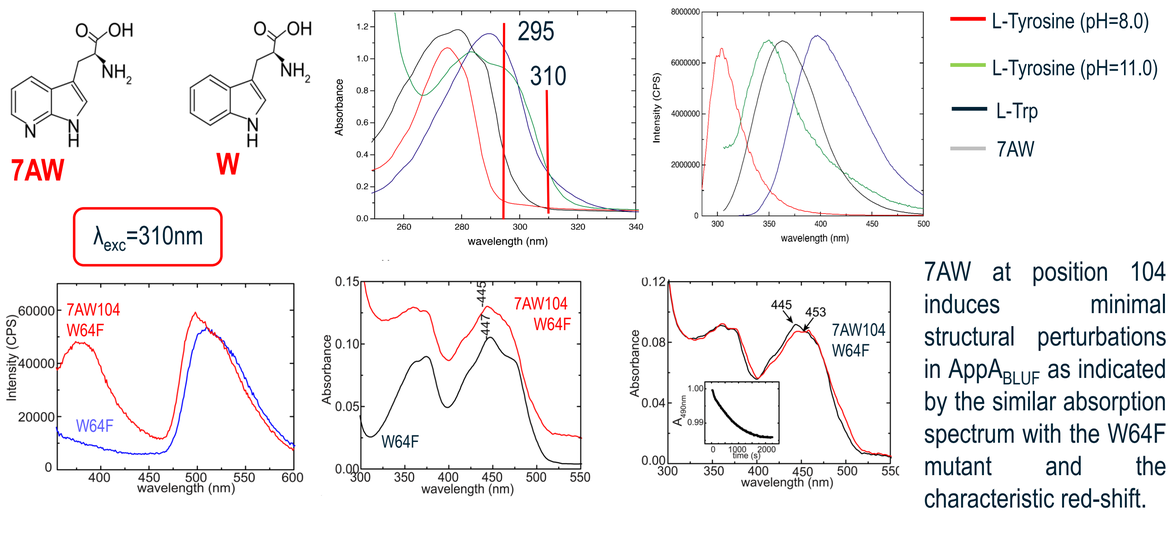
where E is the FRET efficiency, R is the distance between the donor (W104) and the acceptor (FAD) fluorophores and R_0 is the distance at 50% transfer efficiency.

To eliminate FRET contributions from W64 and tyrosines, we worked with the W64F mutant using λexc=295nm. However, tyrosinates in W64F $AppA_{BLUF}$ formed at pH 8.0 give strong fluorescence emission at 345 nm.



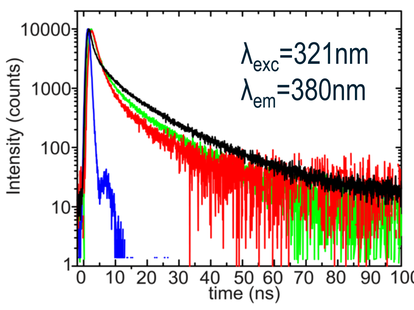


In order to eliminate any contribution from tyrosinates, we used the 7-aza-Trp analogue (7AW) at position 104. 7AW has absorption and fluorescence maxima red-shifted by 10 nm and 50 nm, respectively, which allows selective excitation of the tryptophan W104 (analogue) using λ_{exc}=310nm, avoiding the tyrosinate's absorbance at 295nm.



7AW at position 104 induces minimal structural perturbations in $AppA_{BLUF}$ as indicated by the similar absorption spectrum with the W64F mutant and the characteristic red-shift.

FRET: fluorescence lifetime measurements of 7AW104/W64F $AppA_{BLUF}$



Using time correlated single photon counting (TCSPC) measurements, we calculated the FRET efficiency in the dark- and light-adapted states using the equation $E = 1 - \frac{\tau_{DA}}{\tau_D}$

where τ_{DA} is the fluorescence lifetime of the donor (7AW) in the presence of the acceptor (FAD) and τ_D is the fluorescence lifetime of the donor (7AW) in the absence of the acceptor (FAD).

We obtained the following values: E_{dark}=0.23 → **R=20.5 Å**

E_{light}=0.40 → **R=9.5 Å**

References

1. Gomelsky, M. and Kaplan, S. (1998) AppA a redox regulator of photosystem formation in *Rhodobacter Sphaeroides* 2.4.1, is a flavoprotein-identification of a novel FAD binding domain J.Biol.Chem. 273, 35319-35325.
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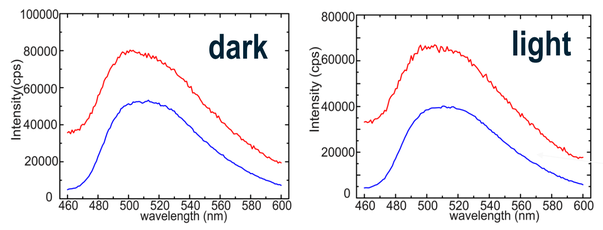
4. Lukacs, A. et al. (2014) BLUF domain function does not require metastable radical intermediate state J. Am. Chem. Soc. 136, 4605-4615.
5. Masuda, S. et al. (2005) Tryptophan at position 104 is involved in transforming light signal into changes of beta-sheet structure for the signaling state in the BLUF domain of AppA Plant. Cell.Physiol. 46, 1894-1901.
6. Karadi, K. et al. (2020) Functional dynamics of a single tryptophan residue in a BLUF protein revealed by fluorescence spectroscopy. Scientific Reports 10:2061

FRET: acceptor enhancement method

FRET efficiency between 7AW104 and the flavin was calculated by measuring the intensity enhancement of the emission of the flavin due to the resonance energy transfer using the equation:

$$E = \frac{\varepsilon_A(\lambda_D^{ex})}{\varepsilon_D(\lambda_D^{ex})}\left[\frac{I_{AD}(\lambda_A^{em})}{I_A(\lambda_A^{em})} - 1\right]$$

λ_{exc}=310nm



7AW104/W64F

calculated acceptor (FAD) only spectrum

E_{dark}=0.42 → **R=17.7 Å**

E_{light}=0.59 → **R=8.3 Å**

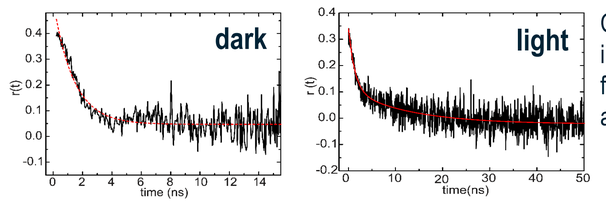
Estimated distances between W104 and FAD obtained from the two fluorescent measurements are in relatively good agreement for the dark-adapted state (20.5 Å and 17.7 Å).

Protein	pdb/FRET	Distance (Å)
$AppA_{BLUF}$(17-133)	1yrx	5.9
$AppA_{BLUF}$(5-125)	2bun	6.2
C20S $AppA_{BLUF}$(1-124)	2iyg (dark)	16.0
C20S $AppA_{BLUF}$(1-124)	2iyi (light)	16.2
C20S AppA Δ399	4hh0	15.7
wt AppA Δ399	4hh1	15.7
$TePixD_{BLUF}$(2-143)	1x0p	16.1
$Slr1694_{BLUF}$(2-140)	2hfn	15.7, 5.7
OaPAC (1-366)	4yus	16.6
$BrlB_{BLUF}$(1-140)	2byc	16.0
7AW104W64F$AppA_{BLUF}$ (dark)	FRET:FL	20.5
7AW104W64F$AppA_{BLUF}$ (light)	FRET:FL	9.5
7AW104W64F$AppA_{BLUF}$ (dark)	FRET:AEM	17.7
7AW104W64F$AppA_{BLUF}$ (light)	FRET:AEM	8.3

Estimated distances from the fluorescent measurements are:

- in good agreement with those from the crystal structures $AppA_{BLUF}$:2iyg, 2iyi and AppAΔCys:44hh0, 4hh1 (Trp_{out} conformation)
- significantly deviate from those in the first solved crystal structure of $AppA_{BLUF}$ (1yrx) and the NMR solution structure (2bun) (Trp_{in} conformation)
- The significantly shorter distance between W104 and FAD for the light-adapted state obtained from the fluorescence measurements, suggest that W104 adopts a Trp_{in} conformation in the light-adapted state

7AW104 has restricted movement in the light-adapted state



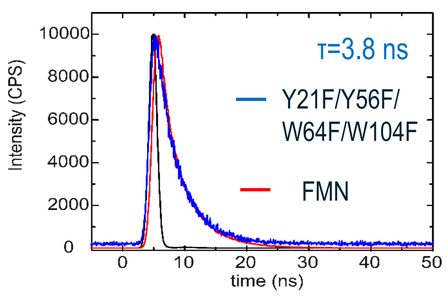
Conformational dynamics of W104 and steric restrictions in 7aza-W104/W64F $AppA_{BLUF}$ were probed by time-resolved fluorescence anisotropy decay measurements in the dark- and light-adapted states.

λ_{exc}=321nm, λ_{em}=380nm

τ_{dark}=1.5± 0.06 ns reflects a less restricted rotation of the tryptophan (θ =~0.6 ns of L-tryptophan in buffer)

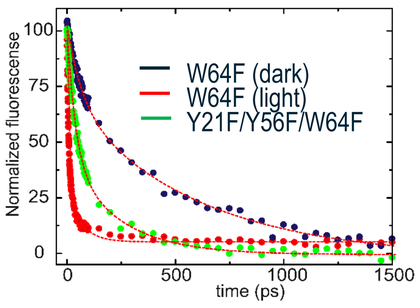
τ_{light}=11.1± 0.5 ns corresponds to the rotation of the whole protein and suggests that W104 moves closer to the flavin

Fluorescence lifetimes of the flavin in the dark- and light-adapted state



λ_{exc}=455nm
λ_{em}=520nm

λ_{exc}=390nm
λ_{em}=520nm



In the absence of electron donors the fluorescence lifetime of the flavin is similar to that in solution.

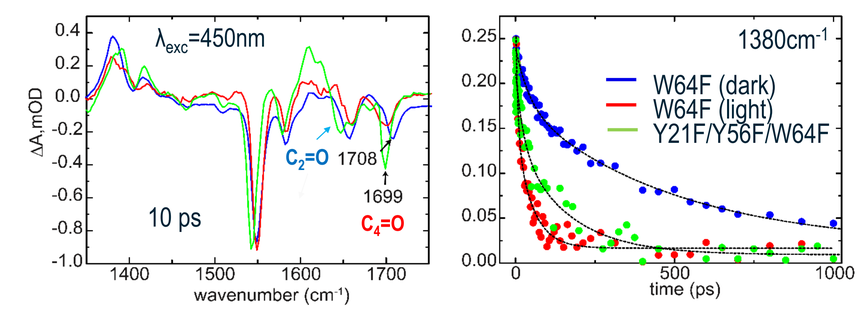
Fluorescence decay constants of the flavin

Protein	A1	T1(ps)	A2	T2(ps)	R1 (Å)	R2 (Å)
W64F dark	0.7	500	0.3	70	9.5	8.03
W64F light	0.13	230	0.87	11	8.9	6.7
Y21F/Y56F/W64F	0.43	200	0.57	36	8.8	7.6

The faster decay in the light-adapted state (11 ps) suggests that the excited state of the flavin is quenched by electron transfer from close-by aromatic residues. Distances are calculated using the Dutton ruler:

$$\log k_{ET} = 15 - 0.6R - 3.1\frac{(\Delta G + \lambda)^2}{\lambda}$$

Protein dynamics revealed by transient infrared measurements



Decay time constants at 1380 cm^{-1} (excited state of the flavin)

Protein	τ_1 (ps)	α_1	τ_2 (ps)	α_2
W64F dark	41	0.27	482	0.73
W64F light	23	0.86	221	0.14
Y21F/Y56F/W64F	23	0.36	160	0.64

A ~9cm^{-1} downshift of the C_4=O band in the light-adapted state is attributed to the formation of a new H-bond to C_4=O.

The kinetics are in line with the findings from the ps fluorescence lifetime measurements.

Summary

- in the light-adapted state, W104 is in a restricted environment with an enhanced H-bond network[6]
- in the dark-adapted state, W104 is present in a less restricted environment pointing away from the flavin[6]

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