

Femtoseconds to Seconds Transient IR Study of BLUF Domain Function Utilizing Unnatural Amino Acid Substitution

Contact S.Meech@uea.ac.uk

A. Gil

Department of Chemistry, Stony Brook University
New York 11794-3400, USA

A. Haigney

Department of Chemistry, Stony Brook University
New York 11794-3400, USA

S. P. Liptonok

School of Chemistry, University of East Anglia
Norwich NR4 7TJ, UK

A. Lukacs

Department of Biophysics, University of Pécs
Szigeti str. 12, H-7624 Pécs, Hungary

G. Greetham

Research Complex at Harwell
Harwell Science and Innovation Campus, Didcot, Oxon OX11

I. V. Sazanovich

Research Complex at Harwell
Harwell Science and Innovation Campus, Didcot, Oxon OX11

P. J. Tonge

Department of Chemistry, Stony Brook University
New York 11794-3400, USA

S. R. Meech

School of Chemistry, University of East Anglia
Norwich NR4 7TJ, UK

Introduction

Light is a fundamental source of energy required for the survival of many organisms. Relatively recently a range of blue-light-sensing flavoproteins have been discovered, and shown to be capable of mediating a large variety of processes in animals, plants, fungi and bacteria. BLUF (Blue Light Using Flavin) domain proteins are an important class of light sensing flavoproteins. They are involved in the control of a variety of light activated functions, including gene expression, phototaxis and biofilm formation. In this work we investigate the well characterised BLUF domain protein AppA, which is an antirepressor regulating expression of genes involved in biosynthesis in the photosynthetic apparatus in *Rhodobacter Sphaeroides*.

Results and discussion

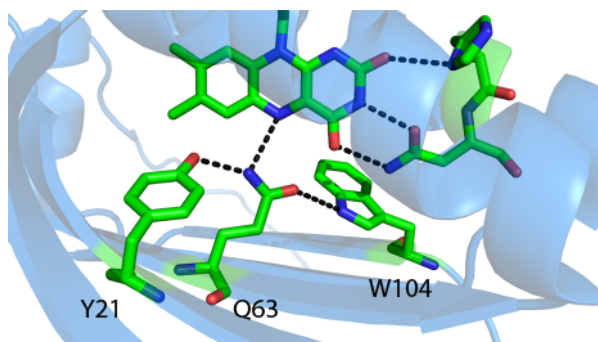


Figure 1. The hydrogen bonding network around the flavin that includes the key residues Y21, Q63, W104, H44, N45 and M106¹.

In the photocycle of AppA light activation to form the signaling state involves changes to the hydrogen bonding network that surrounds the flavin chromophore, while the dark state recovery occurs in a light independent reaction. Conversion of the dark-adapted state of BLUF proteins to the signalling state under blue (~450 nm) light leads to a red-shift in the ground state absorption of the flavin ring by 10–15 nm, with the flavin remaining in its fully oxidized form. The light-adapted state thus formed relaxes back to the dark state in the absence of irradiation. It has been suggested by structural and mutagenesis studies that the residues Y21 and Q63 are vital for the light activated step, while W104 is involved in the light to dark recovery cycle².

It was suggested that the primary step involved photoinduced electron transfer between Y21 and the flavin chromophore,

which results in leads to a structural change in the Q63 residue, which in turn leads to a larger scale longer range allosteric structural relaxation.

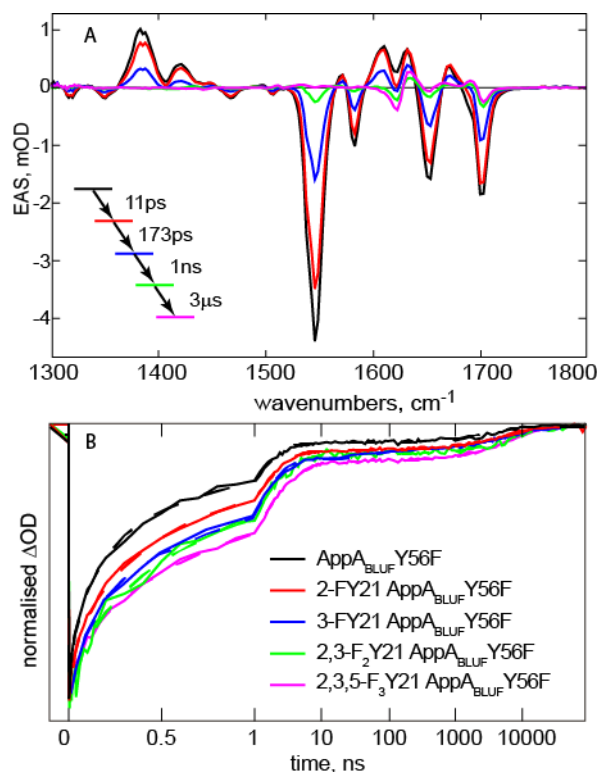


Figure 2. A. Evolution associated spectra of WT AppA_{BLUF} from the global analysis. Estimated lifetimes are shown on the figure; B. Ground state recovery (1548 cm⁻¹) of different FTyr samples, showing differences of the primary steps and structural reorganization phases in dark to light photoconversion.

In this work we investigate the involvement in the photocycle of the critical residue Y21. Since all natural replacements of this residue result in a photoinactive protein, more subtle changes were introduced by incorporation of unnatural amino acid – specifically fluorotyrosine.

These fluorinated derivatives of tyrosine alter the redox potential and the pK_a providing a range of pK_a's from 10 to 6.1 and decrease of the redox potential by 200 mV. An orthogonal aminoacyl-tRNA synthetase (aaRS) – tRNA pair was used to

specifically incorporate mono-, di-, and tri- substituted fluorotyrosines into AppA by suppression of the amber stop codon (TAG). Ultimately, this method provides a very efficient way to obtain a fully labeled protein with 100% of incorporation.

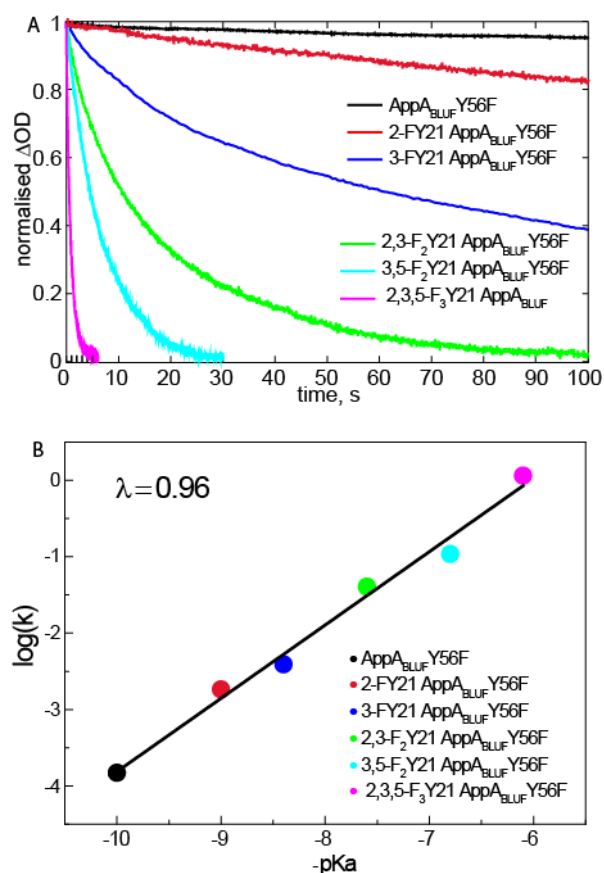


Figure 2. A. Recovery (light to dark) traces of different FTyr samples. B. Bronsted Plot of recovery rates.

Utilising pump-multiple probe spectroscopy (TRMPS) developed in the RCaH we investigated the propagation of the excited state structure change from the flavin binding site in to the protein, which occurs on the time scale of picoseconds to milliseconds. Finally we use rapid scan IR and electronic spectroscopy to probe the recovery of the dark state, which takes from a sub-seconds to 30 minutes. The fluorotyrosine substitution resulted in only a minor effect of about 3 fold on the primary steps and structural reorganization phases which occur on the ps to ms timescale (Figure 2, Table 1). That difference does not correlate with the measured redox potential which is consistent with our earlier proposal that electron transfer was not a rate determining step.³

	τ_1 , ns	τ_2 , ns	τ_3 , ns	τ_4 , ns
AppA _{BLUF} Y56F	0.012	0.17	1.0	3000
2-FY21 AppA _{BLUF} Y56F	0.03	0.33	1.5	4850
3-FY21 AppA _{BLUF} Y56F	0.016	0.42	2.6	4150
2,3-F ₂ Y21 AppA _{BLUF} Y56F	0.08	0.54	2.4	7200
2,3,5-F ₂ Y21 AppA _{BLUF} Y56F	0.065	0.6	2.7	8200

Table 1. Kinetic parameters estimated from the global analysis of the TRMPS data.

In contrast to the primary steps, a remarkable effect was observed in the light to dark recovery with changes of a factor of 3700 when the pK_a of Y21 was decreased from 10 to 6.1. Moreover the log of the rate has a linear correlation with the pK_a with a slope of 0.96, suggesting almost complete proton transfer in the transition state (Figure 3, Table 2).

	pK_a	Recovery time, s
AppA _{BLUF} Y56F	10	4440
2-FY21 AppA _{BLUF} Y56F	9	486
3-FY21 AppA _{BLUF} Y56F	8.4	96
2,3-F ₂ Y21 AppA _{BLUF} Y56F	7.6	27
3,5-F ₂ Y21 AppA _{BLUF} Y56F	6.8	10
2,3,5-F ₂ Y21 AppA _{BLUF} Y56F	6.1	0.7

Table 2. Recovery, light to dark, times of different samples and pK_a of corresponding fTyr

The implications of these results for BLUF domain function led us to propose a detailed light to dark state recovery mechanism (Figure 4) in which we suggest that in the rate determining step (RDS) for relaxation back to the dark state the enolate tautomer form of Q63 abstracts a hydrogen from residue Y21.

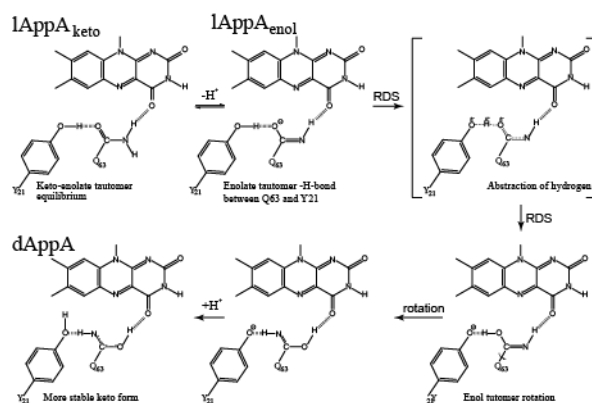


Figure 4. Mechanism of light to dark state recovery in AppA_{BLUF}.

Conclusions

We find that the forward light induced reaction is modestly effected by modulation of the properties of Y21, causing reproducible changes of up to a factor of three in both the picosecond excited state dynamics and the microsecond time scale structural reorganisation leading to signaling state formation. In contrast a dramatic effect of ca 3700 fold is observed for the reverse signaling state to dark adapted relaxation. This effect is shown to be related to the pK_a of the fluorotyrosine, suggesting a key role for a proton transfer reaction.

Acknowledgements

The authors are grateful to STFC for access to the Central Laser Facility. We are also grateful to the Oxford Protein Production Facility (OPPF) for their assistance in sample preparation. We thank EPSRC and NSF for funding (EP/K000764/1, CHE – 1223819)

References

- Anderson, S. *et al.* Structure of a novel photoreceptor, the BLUF domain of AppA from *Rhodobacter sphaeroides*. *Biochemistry* 44, 7998–8005 (2005).
- Laan, W., *et al.* Characterization of the Primary Photochemistry of AppA, a Blue-light-using Flavin Adenine Dinucleotide-domain Containing Transcriptional Antirepressor Protein from *Rhodobacter sphaeroides*: A Key Role for Reversible Intramolecular Proton Transfer. *Photochem. Photobiol.* 78, 290–297 (2007).
- Lukacs, A. *et al.* BLUF domain function does not require a metastable radical intermediate state. *J. Am. Chem. Soc.* 136, 4605–15 (2014).