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

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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](image1.jpg)

**Figure 1.** The hydrogen bonding network around the flavin that includes the key residues Y21, Q63, W104, H44, N45 and M106. 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 cycle.

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](image2.jpg)

**Figure 2.** A. Evolution associated spectra of WT AppA*met* 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 fluoro tyrosine.

These fluorinated derivatives of tyrosine alter the redox potential and the pKₐ providing a range of pKₐ’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

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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.

In contrast to the primary step and structural reorganization phases which occur 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.  

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 pKa of the fluorotyrosine, suggesting a key role for a proton transfer reaction.

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References


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

<table>
<thead>
<tr>
<th>Sample</th>
<th>pK_a</th>
<th>Recovery time, s</th>
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<tbody>
<tr>
<td>AppA_RBLUF Y56F</td>
<td>10</td>
<td>4440</td>
</tr>
<tr>
<td>2-FY21 AppA_BLUF Y56F</td>
<td>9</td>
<td>486</td>
</tr>
<tr>
<td>3-FY21 AppA_BLUF Y56F</td>
<td>8.4</td>
<td>96</td>
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<tr>
<td>2,3-FY21 AppA_BLUF Y56F</td>
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<td>3,5-FY21 AppA_BLUF Y56F</td>
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<td>2,3,5-FY21 AppA_BLUF Y56F</td>
<td>6.1</td>
<td>0.7</td>
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</table>

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

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.