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Ultrafast Structural Dynamics of BlsA, a Photoreceptor from the Pathogenic Bacterium *Acinetobacter baumannii*

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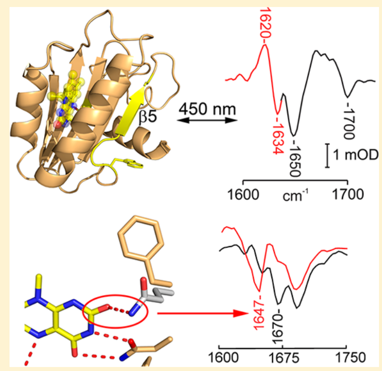
Ⓢ *Supporting Information*

ABSTRACT: *Acinetobacter baumannii* is an important human pathogen that can form biofilms and persist under harsh environmental conditions. Biofilm formation and virulence are modulated by blue light, which is thought to be regulated by a BLUF protein, BlsA. To understand the molecular mechanism of light sensing, we have used steady-state and ultrafast vibrational spectroscopy to compare the photoactivation mechanism of BlsA to the BLUF photosensor AppA from *Rhodobacter sphaeroides*. Although similar photocycles are observed, vibrational data together with homology modeling identify significant differences in the $\beta 5$ strand in BlsA caused by photoactivation, which are proposed to be directly linked to downstream signaling.



SECTION: Biophysical Chemistry and Biomolecules

Acinetobacter baumannii is a gram-negative opportunistic pathogen that poses a significant threat to human health due to its resistance to many frontline antibiotics and ability to survive in harsh environments.[2] In particular, the ability of *A. baumannii* to form biofilms has been attributed to its ability to survive nutrient depletion and sterilization in hospitals.[3] Although the relationship between *A. baumannii* biofilm formation and drug resistance has been extensively studied,[3,4] little is understood concerning how *A. baumannii* responds to its environment and if biofilm formation is sensitive to external stimuli. It has been discovered that *A. baumannii* can sense and respond to light and that biofilm formation is abolished in the presence of blue light, while virulence toward eukaryotic cells is enhanced.[5] The ability of *A. baumannii* to sense blue light is a result of a blue-light-sensing using flavin (BLUF) protein, BlsA. BLUF proteins have been found in numerous organisms,[6] where they are either directly fused within a multidomain protein to an additional domain that regulates the photoresponse or standalone domains that interact noncovalently with an output protein. BlsA falls into the latter category, although currently, the identity of the protein binding partner(s) for BlsA is unknown. Recently, it was established that photocontrol of motility and biofilm formation is in fact widespread within the genus *Acinetobacter*, adding further urgency to delineating the mechanistic role that BlsA plays in light regulation.[7,8]

Similar to other BLUF proteins, photoactivation of dark-adapted BlsA (dBlsA) to form the light-activated state (lBlsA) results in a 14 nm red shift in the flavin absorption band at 456 nm.[5] lBlsA relaxes back to dBlsA with a half-life of 8 min, which is similar to that of the well-characterized BLUF protein, AppA ($t_{1/2}$ = 14 min) (Figure S1 and Table S1, Supporting Information).[9]

To investigate similarities and differences between BlsA and AppA, we used ultrafast time-resolved infrared (TRIR) spectroscopy to probe the primary structural changes associated with photoexcitation of dBlsA. The TRIR method reports on the evolution of the pump-on minus pump-off transient difference spectra following excitation of the flavin chromo-

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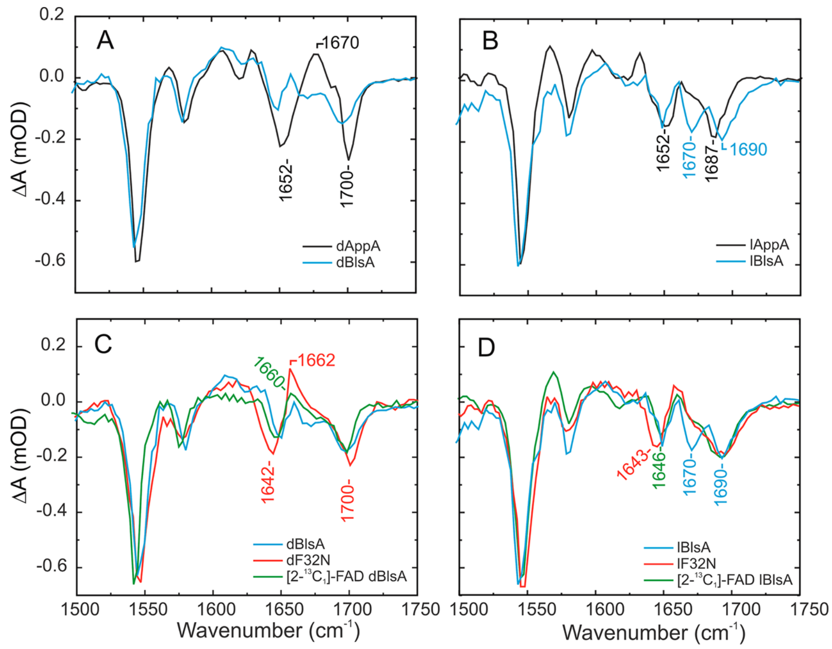

Figure 1. TRIR spectra of $AppA_{BLUF}$ (black), BlsA (blue), F32N (red), and BlsA reconstituted with [2-$^{13}C_1$]FAD (green) taken 3 ps post excitation. (A,C) Spectra of dark-adapted states. (B,D) Spectra of light-adapted states.

phore with a 100 fs visible (450 nm) pulse. The resulting difference spectrum contains information on excited- as well as ground-state events as a function of time after excitation. Transients (positive ΔA) formed instantaneously are excited-state modes resulting from photoexcitation, whereas bleaches (negative ΔA) are the result of the depletion of ground-state modes. The temporal evolution of the TRIR spectra reveals changes in the protein structure and its interaction with the flavin ring.

We measured the TRIR spectra of dBlsA and lBlsA, comparing them to AppA (Figure 1A and B and Figure S2, Supporting Information). The early time (3 ps) spectra (Figure 1) are dominated by modes arising from the chromophore and report on the interaction of the isoalloxazine ring with the protein. While the spectra are quite similar, an important instantaneously formed transient at 1670 cm^{-1} observed in dAppA is absent in dBlsA (Figure 1A). This mode is also absent in free flavin, lAppA, and photoinactive mutants of AppA and has been proposed to be a marker for photoactivity in AppA, possibly arising from the conserved glutamine, Q63.[9,10] Because BlsA is a photoactive BLUF protein and also has the conserved glutamine (Q51, Figure S3, Supporting Information), this transient mode must either be absent or obscured in dBlsA. Similar to $AppA_{BLUF}$,[9,11,12] a bleach at 1700 cm^{-1} in dBlsA is shifted by 10 cm^{-1} to 1690 cm^{-1} in lBlsA and is assigned as the C4═O carbonyl of the flavin. A decrease in frequency is indicative of an increase of H-bonding to the flavin in the light-adapted state.[13] However, a new bleach is observed in lBlsA at 1670 cm^{-1} that does not appear to have a counterpart in the reported spectrum of lAppA (Figure 1)[11,12] and appears to be absent in dBlsA. Finally, as observed for AppA, lBlsA recovers its ground state much faster than dBlsA.

To aid in assignment of the TRIR spectra of BlsA, uniform ^{13}C labeling of the protein was performed, and the purified BlsA was back exchanged with unlabeled FAD to ensure that only protein modes would be affected by labeling. Little effect on the carbonyl region of the TRIR spectra (Figure S4, Supporting Information) was observed, indicating that the 1670 cm^{-1} bleach in the lBlsA mode arises from the flavin. To make a more definite assignment, we incorporated [2-$^{13}C_1$]FAD into the protein using a protocol previously described for AppA.[11,12] In dBlsA reconstituted with [2-$^{13}C_1$]FAD, an enhanced transient is observed at 1660 cm^{-1} when compared to BlsA reconstituted with unlabeled FAD (Figure 1C). This 1660 cm^{-1} transient is not observed in FAD in solution, which indicates a mode associated with the protein environment. This recalls the behavior associated with dAppA where a 1670 cm^{-1} mode was observed and associated with photoactivity. In the [2-$^{13}C_1$]FAD reconstituted lBlsA, the 1670 cm^{-1} bleach is shifted to 1646 cm^{-1}; this spectral shift reveals this mode to be the C2═O of the flavin and that the position of this mode masks the 1660 cm^{-1} transient observed in $AppA_{BLUF}$ and its photoactive mutants. Therefore, we propose that the flavin C2═O carbonyl bleach is at 1670 cm^{-1} in both dBlsA and lBlsA and that this mode masks the presence of the transient in dBlsA previously proposed to be a marker for photoactivity in dAppA, making it a unique feature that has not been observed in other BLUF systems to date.

Because no crystal structure is available for BlsA, to investigate the 20 cm^{-1} blue shift in the flavin C2═O mode relative to AppA, we employed homology modeling. The BLUF protein PixD (Slr1694) from *Synechocystis* PCC6803 (2HFN.pdb)[14] was chosen as a template to generate a structure of BlsA (Figure S3, Supporting Information) on the basis of their sequence similarity The model for BlsA was then compared with the structure of AppA[15] (Figure 2). This analysis demonstrated that residues that H-bond to the C2═O of the flavin in AppA (H44) or PixD (N31) are replaced by F32 in BlsA. This is a rather surprising result because in all sequenced BLUF proteins, there is a residue at this position that is capable of H-bonding to the flavin C2═O carbonyl.[16,17] Thus, the homology model of BlsA suggests that the C2═O vibration is blue-shifted relative to the position of this mode in

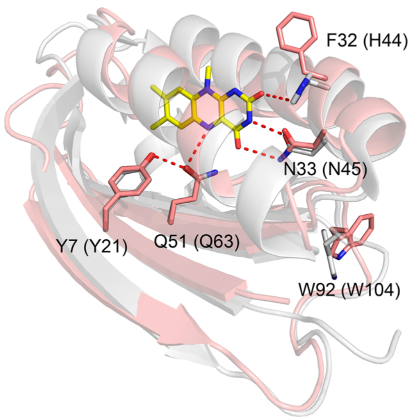

Figure 2. Structure of $AppA_{BLUF}$ overlaid with the homology model of BlsA. $AppA_{BLUF}$ (PDB: 2IYG) is in gray, and BlsA is in pink. Residue numbering is for BlsA, with the corresponding residues for $AppA_{BLUF}$ in parentheses. H-bonds between the flavin and the protein are depicted by red dots and are shown for $AppA_{BLUF}$. The figure was made using Pymol.[1]

AppA because the side chain of F32 is incapable of forming a H-bond.

To determine the role of F32 in the photocycle, we replaced this residue with the homologous residues in AppA (His) and PixD (Asn) that are capable of H-bonding and characterized these mutants by steady-state and TRIR spectroscopy. Mutations to this position resulted in faster dark-state recoveries, as observed by UV−vis spectroscopy (Figure S5 and Table S1, Supporting Information), suggesting that the F32 in BlsA may have been selected during evolution to reduce the rate of dark-state recovery of the *A. baumannii* light-sensing protein.

TRIR spectroscopy was performed on the F32N (Figure 1C and D) and F32H (Figures S6 and S7, Supporting Information) BlsA mutants. While the F32H mutation did not significantly alter the TRIR spectra in either the dark- or light-adapted states, the spectrum of dark-adapted F32N BlsA revealed a new transient at 1662 cm^{-1} that is absent in lF32N BlsA and is in the region where one would expect a transient for a photoactive BLUF protein.[9,11,18] In addition, the bleach observed at 1670 cm^{-1} in lBlsA is absent in both dF32N and lF32N BlsA, while a strong bleach is observed at 1642(3) cm^{-1} in both dF32N BlsA and lF32N BlsA. The 1642(3) cm^{-1} band is similar to the frequency of the C2═O observed in $AppA_{BLUF}$[11,12] and is unaffected by ^{13}C labeling of the protein, indicating that it is a flavin mode. Because isotopic editing of the flavin chromophore revealed that the 1670 cm^{-1} bleach in wild-type lBlsA is the C2═O carbonyl, the simplest explanation is that the Asn at position 32 in F32N BlsA H-bonds to the C2═O, resulting in a red shift in the frequency of this vibration. Indeed, the position of the C2═O in F32N BlsA is ∼7 cm^{-1} red-shifted even compared to that observed in the TRIR spectra of $AppA_{BLUF}$ (Figure 1A and B), indicating stronger H-bonding interactions in the F32N mutant. The change in frequency of the C2═O band allows for the protein transient observed in the dark state of photoactive BLUF proteins to be seen, here at 1662 cm^{-1}.

In addition, ground-state (GS) recovery data are reported at 1547 cm^{-1} for AppA, BlsA, and the F32H/N mutants (Table S2 and Figure S8, Supporting Information). Average lifetimes for AppA and BlsA are in good agreement with each other. The F32 mutants exhibited roughly a 2-fold increase in the rate of GS recovery for the dark states. A slight increase is observed in GS recovery for the light-adapted states compared to lAppA, 1.4-fold for lF32N and 1.1-fold for F32H. These results suggest a faster radiationless decay of the flavin excited state in the dark states of F32 mutants compared to wild-type BlsA with a moderate increase in light-adapted states.

The TRIR data reveal the ground- and excited-state vibrational modes associated with electronic excitation of the flavin chromophore. To provide additional information on the structural change accompanying light state formation, we measured the light minus dark steady-state FTIR difference spectrum of BlsA and compared it to the analogous spectrum obtained for $AppA_{BLUF}$ (Figure 3). These difference spectra

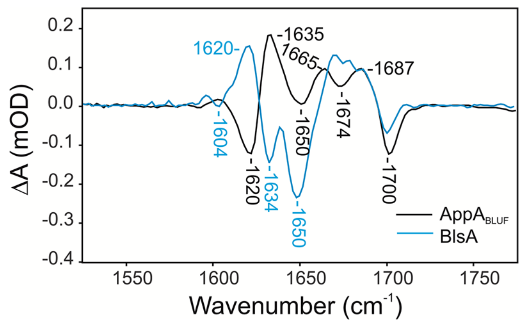

Figure 3. FTIR light minus dark difference spectra of $AppA_{BLUF}$ (black) and BlsA (blue). The light spectra were obtained after 3 min of irradiation with a 460 nm LED diode.

reveal the overall change in both the protein and chromophore structure on transitioning from the dark- to the light-adapted state, in contrast to the 3 ps TRIR in Figure 1, which primarily contains chromophore-specific modes. Both spectra exhibit the 1700(−)/1687(+) cm^{-1} difference band assigned to changes in H-bonding to the flavin C4═O associated with rotation of Q51 (Q63) between dark and light states of $AppA_{BLUF}$.[11] In addition, photoexcitation also leads to formation of a 1634(5)/1620 cm^{-1} difference band in both $AppA_{BLUF}$ and BlsA in a region where β-sheet secondary structure can be observed.[19−21] In BlsA, this difference mode shifts to 1590/1578 upon U−^{13}C labeling of the protein, confirming the assignment of these bands to protein (Figure S9, Supporting Information). In addition, although this difference band is not observed on the picosecond time scale,[9,11,12] recent time-resolved multiple probe spectroscopy experiments reveal its appearance on the microsecond time scale.[22] Significantly, this difference mode has opposite signs in $AppA_{BLUF}$ (1635(+)/1620(−) cm^{-1}) and BlsA (1634(−)/1620(+) cm^{-1}). The sign of this protein mode is a unique feature for BlsA because the FTIR difference spectra of other BLUF proteins such as PixD resemble $AppA_{BLUF}$.[13,23−26] Consequently, the present results for BlsA point to greater diversity in the structural changes induced by light than hitherto realized.

In $AppA_{BLUF}$, the 1635(+)/1620(−) cm^{-1} difference mode has been attributed to structural rearrangement of the BLUF β-sheet, consistent with the notion that the β-sheet, and β5 strand in particular (Figure 2), is involved in signal transduction.[23,27,28] Due to the opposite sign of the difference mode, we propose that the secondary structure content of dBlsA resembles that of $lAppA_{BLUF}$, while the secondary structure content of lBlsA resembles $dAppA_{BLUF}$. In AppA, the N-terminal residue of the β5 strand is a tryptophan (W104; W92 in BlsA), which is hypothesized to move upon light-state formation,[27,28] and significantly, the 1635(+)/1620(−) cm^{-1} difference mode is much weaker in the W104A photoactive $AppA_{BLUF}$ mutant.[23] Although it is not clear how changes in the β5 strand in $AppA_{BLUF}$ modulate the structure of the C-terminal domain of this protein, it is known that in the related BLUF protein PixD, photoactivation leads to dissociation of PixD from the output protein PixE.[29] PixD and $AppA_{BLUF}$ have similar FTIR difference spectra in the 1620−1635 cm^{-1} region,[30] with PixD showing the characteristic 1635(+)/1620(−) cm^{-1} difference mode. We propose that the weakening of H-bonding in the β-sheet that occurs upon photoactivation in PixD is directly related to dissociation of PixE from PixD. Conversely then, the strengthening of H-bonding in the BlsA β-sheet upon photoactivation, revealed by the change in sign of the β-strand marker mode, supports a model for BlsA photoreceptor function in which photoactivation leads to *formation* of a complex with the downstream target protein rather than dissociation.

In conclusion, we report the initial characterization of the photosensor BlsA from *A. baumannii* using infrared spectroscopy. TRIR spectra reveal a blue-shifted flavin C2═O frequency that is proposed to result from loss of H-bonding interactions with the protein that are normally present in other BLUF proteins such as AppA and PixD. In addition, steady-state IR data reveal that photoactivation of BlsA causes changes to the β-sheet structure of the protein, particularly, the β5 strand, that are fundamentally different from those observed in other BLUF proteins and that are proposed to be directly relevant to the light-activated function of *A. baumannii*.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Methods for protein purification, steady-state and TRIR spectroscopy, and homology modeling. Photorecovery data for the F32 mutants and the TRIR spectra for the F32H mutant together with kinetic data and sequence alignment of BlsA and other BLUF proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

FAD, flavin adenosine dinucleotide; BLUF, blue-light-sensing using FAD; BlsA, blue-light-sensing A protein; FTIR, Fourier transform infrared; TRIR, time-resolved infrared

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