Research paper

Femtosecond Stimulated Raman Study of the Photoactive Flavoprotein AppA$_{BLUF}$

Christopher R. Hall, Ismael Heisler, Garth Jones, James Frost, Agnieszka A. Gil, Peter J. Tonge, Stephen R. Meech

PII: S0009-2614(17)30250-6
DOI: http://dx.doi.org/10.1016/j.cplett.2017.03.030
Reference: CPLETT 34630

To appear in: *Chemical Physics Letters*

Received Date: 30 January 2017
Revised Date: 9 March 2017
Accepted Date: 10 March 2017

Please cite this article as: C.R. Hall, I. Heisler, G. Jones, J. Frost, A.A. Gil, P.J. Tonge, S.R. Meech, Femtosecond Stimulated Raman Study of the Photoactive Flavoprotein AppA$_{BLUF}$, *Chemical Physics Letters* (2017), doi: http://dx.doi.org/10.1016/j.cplett.2017.03.030

Femtosecond Stimulated Raman Study of the Photoactive Flavoprotein $AppA_{BLUF}$

Christopher R. Hall,[1] Ismael Heisler,[1] Garth Jones,[1] James Frost,[1] Agnieszka A. Gil,[2] Peter J. Tonge[2] and Stephen R. Meech[1]*

[1]School of Chemistry, University of East Anglia, Norwich, NR4 7TJ, UK and [2]Department of Chemistry, Stony Brook University, Stony Brook, New York 11794-3400, USA

Abstract

Femtosecond stimulated Raman Spectroscopy (FSRS) is applied to study the photocycle of a blue light using flavin (BLUF) domain photoreceptor, $AppA_{BLUF}$. It is shown that FSRS spectra are sensitive to the light adapted state of the protein and probe its excited state dynamics. The dominant contribution to the most sensitive excited state Raman active modes is from flavin ring modes. However, TD-DFT calculations for excited state structures indicate that reproduction and assignment of the experimentally observed spectral shift will require high level calculations on the flavin in its specific protein environment.

Introduction

Time resolved vibrational spectroscopy has proven to be a rich source of information on a wide variety of light driven processes in proteins.[1, 2] Two complementary methods have found wide application, time resolved infra-red (TRIR)[3] and transient Raman,[4] especially femtosecond stimulated Raman spectroscopy (FSRS)[5]. TRIR has been applied to a very wide range of photoactive proteins, from blue light sensing flavoproteins[6-9] to the red sensitive phytochromes[10] (including some proteins in which photoactivity has been artificially induced[11]) with sub 100 fs time resolution and an accessible time window spanning femtoseconds to seconds. However, the method is somewhat limited by strong IR absorption of the aqueous medium and the difficulty of distinguishing evolution of vibrational modes associated with the chromophore from those associated with structural changes amongst the surrounding amino acids, necessitating extensive isotope labelling experiments.[12, 13] In contrast to TRIR, FSRS is an all optical method which can exploit electronic resonances which selectively highlight chromophore or (in the case of a UV Raman pulse)-amino acid localised modes. This has led to a surge in interest in applying FSRS to photoactive proteins, but to date the palette of proteins probed by the method is much more limited than for TRIR, including the robust green fluorescent protein family,[14-16] photoactive yellow protein (PYP)[17, 18] and isolated examples from the families of phytochromes,[19] bacteriorhodopsins[20, 21] and light harvesting complexes.[22] In particular, there are as yet no reports on the FSRS of the three distinct families of the blue light sensitive photoactive flavoproteins,[23] which utilise the isoalloxazine chromophore in the co-factors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). These represent an important class of proteins responsible for mediating the effects of light on a wide range of plants and bacteria.[23, 24] Moreover, a number of such proteins have been adopted as photoswitchable elements in the emerging field of optogenetics.[25] Thus, the application of Raman methods to probe the excited state vibrational dynamics providing essential details on the mode of action of these photoswitchable proteins is of a great interest, especially for their future application.

In this communication we apply FSRS to probe the transient spectroscopy of the BLUF domain protein - $AppA_{BLUF}$, and complement those measurements with preliminary time dependent density functional theory (TD-DFT) calculations. $AppA_{BLUF}$ has previously been studied by TRIR and transient electronic spectroscopy.[7, 8, 26] Excitation of the flavin ring in dark adapted $AppA_{BLUF}$ ($dAppA_{BLUF}$) gives rise to a light induced structural change in an adjacent glutamine residue (Q63) which in turn leads to a change in the H-bonding environment of the isoalloxazine ring of the FAD. (Figure 1) This change is also observed through a 10 - 15 nm red shift in the FAD absorption spectrum of the light adapted form, $lAppA_{BLUF}$, which relaxes back to the dark state in minutes.[27] The mechanism of the light driven structure change and the subsequent evolution in the protein structure has been discussed elsewhere.[6, 7, 28] In this letter we demonstrate that the excited state Raman spectrum is sensitive to the light adaptation state of $AppA_{BLUF}$, and thus that FSRS can be applied to probe photodynamics in the three families of blue light sensing flavoproteins. In doing so, we build on the comprehensive transient electronic and FSRS study of FAD in solution reported by Weigel et al.[29]

Experimental

The (FSRS) experiment is driven by an amplified 1 kHz titanium:sapphire laser that provides the input to two optical parametric amplifiers (OPA) and a second harmonic bandwidth compressor (SHBC). One OPA generates pump pulses for sample excitation (80 fs pulse duration) at 440 nm. The second generates pulses centred at 1100 nm which are focused onto a sapphire window to generate a white-light continuum. The pulsed continuum spans a 480 – 1000 nm spectral region and is used as the broadband probe in the FSRS experiment. The narrowband (10 cm^{-1}) SHBC output pumps an additional picosecond OPA to generate a Raman pump pulse at 750 nm, which is resonant with the excited state absorption of the photoexcited isoalloxazine chromophore.[29] The resonant (440 nm) pump and white-light probe pulses were focused at the sample position by reflective optics to spot sizes (FWHM) of 170 µm and 30 µm, respectively. For all measurements the pump pulse energy was

1 µJ (excitation intensity of 4 W cm^{-2}). The Raman pulse (which is not resonant with the ground state absorption) was focused and overlapped with the pump and probe pulses. The Raman pulse spot size is 100 µm with a pulse energy of 4 µJ (intensity 50 W cm^{-2}). All pulses had the same polarization. Detection with spectral resolution <10 cm^{-1}; was achieved with a SPEX 500M spectrometer and a single CCD detector.

In our experiment the spectra were recorded at 1 kHz, with the pump and Raman pulses modulated by synchronized and phase locked optical choppers operating at 500 Hz and 250 Hz, respectively. This resulted in a four pulse measurement sequence: Excited state (ES) FSRS spectrum (Pump + Probe + Raman pulses); ground state (GS) FSRS spectrum (Raman + Probe pulses); transient absorption (TA) spectrum (Pump + Probe pulses); probe spectrum (Probe only). The ES-FSRS spectrum, measured when the actinic pump, Raman pump and probe pulses are all incident on the sample, consists of Raman signal pathways originating from the sample excited by the actinic pump pulse, and typically includes background Raman signals from unexcited sample and solvent.[30] To extract the transient FSRS signal we calculate:

$$\text{Raman Gain} = Log\left(\frac{Pump + Probe + Raman}{Probe + Raman}\right)$$

The FSRS signal typically appears on top of a strong background TA signal, which is fit to a polynomial and subtracted from the unprocessed FSRS signal. Following this, an additional baseline fit is made using an appropriate order polynomial to correct for a remaining baseline offset, which arises from the interaction between the Raman pulse and the excited state population.[29] An additional step is to remove the contribution of the depleted ground state Raman spectrum, which was not implemented as no ground state Raman spectrum was observed under the excitation conditions used. Finally, a five pixel moving filter is applied to remove high frequency noise that accumulates during the initial difference calculation. The effect of the filter on the spectral resolution was shown to be negligible

For all measurements $AppA_{BLUF}$ samples were prepared using expression and purification protocols described previously [7]. In all measurements $AppA_{BLUF}$ in 50 mM sodium phosphate buffer at pH 8.0 had an optical density of 0.45 at 450 nm in a 200 µm pathlength flow cell. The samples were flowed at a rate sufficient to refresh the sample between each laser shot. Data sets are presented as the average of 10 scans. Each data set was acquired over a period of 100 mins. The $dAppA_{BLUF}$ sample was converted to $lAppA_{BLUF}$ by illumination with a 400 mW 455 nm LED weakly focused onto the sample reservoir (0.13 W cm^{-2}) during the experiment.

Geometry optimisations of the ground state have been carried out on lumiflavin, lumiflavin surrounded by four water molecules situated at the C2O, N3H, C4O and N5 positions, and lumiflavin surrounded by three water molecules with the water located at N5 removed, somewhat analogous to the dark and light adaptations respectively (Figure 1). The calculations were carried out at the B3LYP[31-33]/TZVP[34] level of theory using the Gaussian 09 program[35]. These optimised ground state structures were subsequently characterised through a frequency analysis and shown to be at legitimate minimum energy, stationary point geometries (see supporting information). Single-point TD-DFT calculations were performed on the ground state optimised structures in order to determine the vertical excitation energies of each of the systems. The vertical excitation energies recovered were in good agreement with literature values.[29, 36] Significantly, the breaking of the H-bond at N5 did not result in a red shift in the calculated excitation energy, as observed in the experiments. Finally, following identification of the first ten excited states, a TD-DFT/TZVP geometry optimisation was performed on the first singlet excited state.

Results and Discussion

In Figure 2A we compare FSRS spectra for three samples, FAD in water, $dAppA_{BLUF}$, and $lAppA_{BLUF}$. These spectra are time integrated between 0 and 50 ps to improve signal-to-noise ratio. For the aqueous FAD solution there is very good agreement between the present measurement and the

data of Weigel et al..[29] Significantly there are clear differences between the FSRS spectra for lAppA and dAppA$_{BLUF}$, showing that FSRS is indeed a complementary tool to TRIR, which can probe BLUF domain protein (and therefore presumably other photoactive flavoprotein) dynamics, yielding information on excited state structure and the evolution of that structure in response to time dependent changes in the chromophore environment. Confirmation that the FSRS spectra indeed arise from the excited states of the two different forms of AppA$_{BLUF}$ is obtained from the FSRS signals measured as a function of time after the pump pulse. These are measured and compared with the TA of the electronically excited singlet state in Figure 2B. It was previously shown that the lAppA$_{BLUF}$ excited state is more strongly quenched than that of dAppA$_{BLUF}$, due to efficient electron transfer to the excited flavin in lAppA$_{BLUF}$ from nearby tyrosine and tryptophan residues.[26] The FSRS intensity accurately tracks the excited state absorption intensity in both cases (Figure 2B) confirming the assignment of the spectra to the dark and light adapted forms.

Significantly the FSRS spectra measured separately for dAppA$_{BLUF}$ and lAppA$_{BLUF}$ are themselves independent of time, as shown in supporting information (Figure S2). This is consistent with previous transient IR studies for dAppA$_{BLUF}$, where the main changes in the vibrational spectrum were ultrafast and associated with an excitation induced perturbation of amino acids around FAD, rather than a structural transformation of the chromophore itself. We expect that the resonant FSRS signal will be more focused on the chromophore.[26, 28]

The sensitivity to light adaptation shown in Figure 2A must reflect the changes in the H-bond environment illustrated in Figure 1. The effect of this change on vibrational spectra of the ground electronic state has already been reported in IR experiments, where photoinduced reorganisation of the H-bond network gives rise to significant shifts in IR spectra associated with the C2=O and C4=O carbonyls stretches (1663 cm^{-1} and 1706 cm^{-1} respectively), which are coupled with the N3-H wag.[13] The red shift of the C=O modes on irradiation was ascribed to the formation of the additional H-bond interaction at C4=O and the breaking of an H-bond to N5 (Figure 1).

The C=O modes are weak in both ground and excited state Raman experiments (Figure S2 shows FSRS to 1750 cm^{-1}, with no carbonyl stretch signal observed above the noise). Instead the change in H-bond structure is manifest in significant blue shifts in the strong Raman active modes at 1390 cm^{-1} and 1205 cm^{-1}. Similar small blue shifts on formation of $lAppA_{BLUF}$ were reported in ground state Raman measurements.[37] In contrast other well resolved modes in the excited state are unshifted on formation of the light adapted state, *e.g.* at 1505 cm^{-1} (Figure 2A). Two calculations have already been performed on the excited state vibrational modes of the flavin ring containing molecule riboflavin.[29, 36] On the basis of those it is difficult to propose a clear assignment for the differential sensitivity of the FSRS bands to their environment. For example, the normal mode analyses reveal that both the blue shifted 1390 cm^{-1} and the unshifted 1505 cm^{-1} bands both have major contributions from C4a-C10a and C5a-C9a ring II stretches. However, the same analyses shows multiple contributions to each calculated mode and also that a number of different modes are able to contribute to the broad bands observed experimentally (Figure 2).[29, 36] Thus a more detailed assignment is not possible without further experiments, such as isotope exchange.

In an effort to gauge the role of the different H-bond configurations in determining the vibrational frequencies, we compared the optimized geometries of the flavin ring in its singlet excited state in the presence and absence of the H-bond at N5, here represented by water molecules (Figure 3). As can be seen, there are small changes in the optimized structures, but these are not apparent in the central ring (ring II) of the flavin, where the C4a-C10a and C5a-C9 stretches are important. From this we conclude that calculations, and hence assignments of the experimental spectral shifts observed in FSRS, will require more sophisticated calculations, which should at least include interactions with the amino acid residue Q63, responsible for the H-bond switch in the S_1 excited state (Figure 1). This conclusion is in line with observation in TRIR measurements, which suggest that the strong interactions between the flavin ring and its binding pocket have a profound influence on the TRIR spectra.[37]

Conclusion

Excited state vibrational spectra of a photoactive flavoprotein, $AppA_{BLUF}$, have been measured for the first time. It has been shown that the excited state Raman spectra are sensitive to changes in the H-bonding environment of the flavin chromophore, and are thus a useful new tool in characterising the structural dynamics induced by electronic excitation. The largest shifts observed on photoconversion were blue shifts in a number of flavin ring modes. The origin of those shifts could not be easily assigned to specific interactions, and was not reproduced by simplistic TD-DFT calculations. These results point to the requirement for TD-DFT calculations incorporating at least the most strongly interacting amino acid residues to be developed for the assignment of the FSRS spectra. A combination of FSRS and such TD-DFT calculations will prove a valuable new approach to probing the photocycle of photoactive proteins.

Acknowledgements

SRM and PJT are grateful to EPSRC (EP/G002916, EP/ J009481 and EP/N033647/1) and NSF (CHE-1223819) for funding and JF thanks EPSRC for the award of a studentship. The calculations presented in this paper were carried out on the High Performance Computing Cluster supported by the Research and Specialist Computing Support service at the University of East Anglia.

References

[1] J.T.M. Kennis, M.L. Groot, Ultrafast spectroscopy of biological photoreceptors, Current Opinion in Structural Biology 17 (2007) 623-630.
[2] P.J. Tonge, S.R. Meech, Excited state dynamics in the green fluorescent protein, J. Photochem. Photobiol. A: Chem. 205 (2009) 1-11.
[3] E.T.J. Nibbering, H. Fidder, E. Pines, Ultrafast chemistry: Using time-resolved vibrational spectroscopy for interrogation of structural dynamics, Annual Review of Physical Chemistry2005, pp. 337-367.
[4] N. Fujii, M. Mizuno, H. Ishikawa, Y. Mizutani, Observing Vibrational Energy Flow in a Protein with the Spatial Resolution of a Single Amino Acid Residue, Journal of Physical Chemistry Letters 5 (2014) 3269-3273.
[5] P. Kukura, D.W. McCamant, R.A. Mathies, Femtosecond stimulated Raman spectroscopy, Annual Review of Physical Chemistry2007, pp. 461-488.
[6] R. Brust, A. Lukacs, A. Haigney, K. Addison, A. Gil, M. Towrie, I.P. Clark, G.M. Greetham, P.J. Tonge, S.R. Meech, Proteins in Action: Femtosecond to Millisecond Structural Dynamics of a Photoactive Flavoprotein, Journal of the American Chemical Society 135 (2013) 16168-16174.
[7] A.A. Gil, A. Haigney, S.P. Laptenok, R. Brust, A. Lukacs, J.N. Iuliano, J. Jeng, E.H. Melief, R.-K. Zhao, E. Yoon, I.P. Clark, M. Towrie, G.M. Greetham, A. Ng, J.J. Truglio, J.B. French, S.R. Meech, P.J. Tonge, Mechanism of the AppA(BLUF) Photocycle Probed by Site-Specific Incorporation of Fluorotyrosine Residues: Effect of the Y21 pK(a) on the Forward and Reverse Ground-State Reactions, Journal of the American Chemical Society 138 (2016) 926-935.
[8] M. Gauden, I.H.M. van Stokkum, J.M. Key, D.C. Luhrs, R. Van Grondelle, P. Hegemann, J.T.M. Kennis, Hydrogen-bond switching through a radical pair mechanism in a flavin-binding photoreceptor, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 10895-10900.
[9] J.T.M. Kennis, I.H.M. van Stokkum, S. Crosson, M. Gauden, K. Moffat, R. van Grondelle, The LOV2 domain of phototropin: A reversible photochromic switch, Journal of the American Chemical Society 126 (2004) 4512-4513.
[10] K.C. Toh, E.A. Stojkovic, A.B. Rupenyan, I.H.M. van Stokkum, M. Salumbides, M.L. Groot, K. Moffat, J.T.M. Kennis, Primary Reactions of Bacteriophytochrome Observed with Ultrafast Mid-Infrared Spectroscopy, Journal of Physical Chemistry A 115 (2011) 3778-3786.
[11] B. Buchli, S.A. Waldauer, R. Walser, M.L. Donten, R. Pfister, N. Blochliger, S. Steiner, A. Caflisch, O. Zerbe, P. Hamm, Kinetic response of a photoperturbed allosteric protein, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 11725-11730.
[12] A. Haigney, A. Lukacs, R. Brust, R.K. Zhao, M. Towrie, G.M. Greetham, I. Clark, B. Illarionov, A. Bacher, R.R. Kim, M. Fischer, S.R. Meech, P.J. Tonge, Vibrational Assignment of the Ultrafast Infrared Spectrum of the Photoactivatable Flavoprotein AppA, Journal of Physical Chemistry B 116 (2012) 10722-10729.
[13] A. Haigney, A. Lukacs, R.K. Zhao, A.L. Stelling, R. Brust, R.R. Kim, M. Kondo, I. Clark, M. Towrie, G.M. Greetham, B. Illarionov, A. Bacher, W. Romisch-Margl, M. Fischer, S.R. Meech, P.J. Tonge, Ultrafast Infrared Spectroscopy of an Isotope-Labeled Photoactivatable Flavoprotein, Biochemistry 50 (2011) 1321-1328.
[14] C. Fang, R.R. Frontiera, R. Tran, R.A. Mathies, Mapping GFP structure evolution during proton transfer with femtosecond Raman spectroscopy, Nature 462 (2009) 200-U274.
[15] T. Fujisawa, H. Kuramochi, H. Hosoi, S. Takeuchi, T. Tahara, Role of Coherent Low-Frequency Motion in Excited-State Proton Transfer of Green Fluorescent Protein Studied by Time-Resolved Impulsive Stimulated Raman Spectroscopy, Journal of the American Chemical Society 138 (2016) 3942-3945.
[16] B.G. Oscar, W.M. Liu, Y.X. Zhao, L.T. Tang, Y.L. Wang, R.E. Campbell, C. Fang, Excited-state structural dynamics of a dual-emission calmodulin-green fluorescent protein sensor for calcium ion imaging, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 10191-10196.

[17] M. Creelman, M. Kumauchi, W.D. Hoff, R.A. Mathies, Chromophore Dynamics in the PYP Photocycle from Femtosecond Stimulated Raman Spectroscopy, Journal of Physical Chemistry B 118 (2014) 659-667.
[18] R. Nakamura, N. Hamada, K. Abe, M. Yoshizawa, Ultrafast Hydrogen-Bonding Dynamics in the Electronic Excited State of Photoactive Yellow Protein Revealed by Femtosecond Stimulated Raman Spectroscopy, Journal of Physical Chemistry B 116 (2012) 14768-14775.
[19] J. Dasgupta, R.R. Frontiera, K.C. Taylor, J.C. Lagarias, R.A. Mathies, Ultrafast excited-state isomerization in phytochrome revealed by femtosecond stimulated Raman spectroscopy, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 1784-1789.
[20] Y. Hontani, K. Inoue, M. Kloz, Y. Kato, H. Kandori, J.T.M. Kennis, The photochemistry of sodium ion pump rhodopsin observed by watermarked femto- to submillisecond stimulated Raman spectroscopy, Physical Chemistry Chemical Physics 18 (2016) 24729-24736.
[21] A. Wand, N. Friedman, M. Sheves, S. Ruhman, Ultrafast Photochemistry of Light-Adapted and Dark-Adapted Bacteriorhodopsin: Effects of the Initial Retinal Configuration, Journal of Physical Chemistry B 116 (2012) 10444-10452.
[22] P.C. Arpin, D.B. Turner, S.D. McClure, C.C. Jumper, T. Mirkovic, J.R. Challa, J. Lee, C.Y. Teng, B.R. Green, K.E. Wilk, P.M.G. Curmi, K. Hoef-Emden, D.W. McCamant, G.D. Scholes, Spectroscopic Studies of Cryptophyte Light Harvesting Proteins: Vibrations and Coherent Oscillations, Journal of Physical Chemistry B 119 (2015) 10025-10034.
[23] A. Losi, W. Gartner, Old Chromophores, New Photoactivation Paradigms, Trendy Applications: Flavins in Blue Light-Sensing Photoreceptors, Photochemistry and Photobiology 87 (2010) 491-510.
[24] A. Moglich, X.J. Yang, R.A. Ayers, K. Moffat, Structure and Function of Plant Photoreceptors, in: S. Merchant, W.R. Briggs, D. Ort (Eds.), Annual Review of Plant Biology, Vol 61, Annual Reviews, Palo Alto, 2010, pp. 21-47.
[25] A. Moeglich, K. Moffat, Engineered photoreceptors as novel optogenetic tools, Photochemical & Photobiological Sciences 9 (2010) 1286-1300.
[26] A.L. Stelling, K.L. Ronayne, J. Nappa, P.J. Tonge, S.R. Meech, Ultrafast structural dynamics in BLUF domains: Transient infrared spectroscopy of AppA and its mutants, Journal of the American Chemical Society 129 (2007) 15556-15564.
[27] S. Masuda, Light Detection and Signal Transduction in the BLUF Photoreceptors, Plant and Cell Physiology 54 (2013) 171-179.
[28] A. Lukacs, R. Brust, A. Haigney, S.P. Laptenok, K. Addison, A. Gil, M. Towrie, G.M. Greetham, P.J. Tonge, S.R. Meech, BLUF Domain Function Does Not Require a Metastable Radical Intermediate State, Journal of the American Chemical Society 136 (2014) 4605-4615.
[29] A. Weigel, A. Dobryakov, B. Klaumunzer, M. Sajadi, P. Saalfrank, N.P. Ernsting, Femtosecond Stimulated Raman Spectroscopy of Flavin after Optical Excitation, Journal of Physical Chemistry B 115 (2011) 3656-3680.
[30] D.W. McCamant, P. Kukura, R.A. Mathies, Femtosecond Stimulated Raman Study of Excited-State Evolution in Bacteriorhodopsin, The Journal of Physical Chemistry B 109 (2005) 10449-10457.
[31] A.D. Becke, DENSITY-FUNCTIONAL THERMOCHEMISTRY .3. THE ROLE OF EXACT EXCHANGE, Journal of Chemical Physics 98 (1993) 5648-5652.
[32] C.T. Lee, W.T. Yang, R.G. Parr, DEVELOPMENT OF THE COLLE-SALVETTI CORRELATION-ENERGY FORMULA INTO A FUNCTIONAL OF THE ELECTRON-DENSITY, Physical Review B 37 (1988) 785-789.
[33] P.J. Stephens, F.J. Devlin, C.F. Chabalowski, M.J. Frisch, AB-INITIO CALCULATION OF VIBRATIONAL ABSORPTION AND CIRCULAR-DICHROISM SPECTRA USING DENSITY-FUNCTIONAL FORCE-FIELDS, Journal of Physical Chemistry 98 (1994) 11623-11627.
[34] A. Schafer, C. Huber, R. Ahlrichs, FULLY OPTIMIZED CONTRACTED GAUSSIAN-BASIS SETS OF TRIPLE ZETA VALENCE QUALITY FOR ATOMS LI TO KR, Journal of Chemical Physics 100 (1994) 5829-5835.
[35] M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G.A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H.P. Hratchian, A.F. Izmaylov, J.

Bloino, G. Zheng, J.L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J.A. Montgomery, J.E. Peralta, F. Ogliaro, M. Bearpark, J.J. Heyd, E. Brothers, K.N. Kudin, V.N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J.C. Burant, S.S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J.M. Millam, M. Klene, J.E. Knox, J.B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R.E. Stratmann, O. Yazyev, A.J. Austin, R. Cammi, C. Pomelli, J.W. Ochterski, R.L. Martin, K. Morokuma, V.G. Zakrzewski, G.A. Voth, P. Salvador, J.J. Dannenberg, S. Dapprich, A.D. Daniels, Farkas, J.B. Foresman, J.V. Ortiz, J. Cioslowski, D.J. Fox, Gaussian 09, Revision B.01, Wallingford CT, 2009.
[36] B. Klaumunzer, D. Kroner, P. Saalfrank, (TD-)DFT Calculation of Vibrational and Vibronic Spectra of Riboflavin in Solution, Journal of Physical Chemistry B 114 (2010) 10826-10834.
[37] A. Lukacs, A. Haigney, R. Brust, R.K. Zhao, A.L. Stelling, I.P. Clark, M. Towrie, G.M. Greetham, S.R. Meech, P.J. Tonge, Photoexcitation of the Blue Light Using FAD Photoreceptor AppA Results in Ultrafast Changes to the Protein Matrix, Journal of the American Chemical Society 133 (2011) 16893-16900.

Figure Legends

Figure 1. A representation of the structure change in the environment of the flavin chromophore accompanying the $dAppA_{BLUF}$ to $lAppA_{BLUF}$ photoconversion.

Figure 2. FSRS spectra and time dependence. A. The FSRS spectra were obtained using an actinic pulse at 440 nm with the Raman pulse tuned to the excited state absorption at 750 nm for FAD in water (red), $dAppA_{BLUF}$ (blue) and $lAppA_{BLUF}$ (green). Data are integrated over the first 50 ps. B. Transient FSRS intensity compared with transient absorption. The red and aqua coloured lines plot the normalised band integral for the observable excited state absorption region (786 nm – 851 nm). The blue and green lines plot the band integral for the ~1500 cm^{-1} peak in the FSRS spectrum. The amplitudes of the blue and green lines have been scaled to demonstrate their similar relaxation dynamics.

Figure 3. TD-DFT optimised structure for S_1 state in the presence and absence of the specific water H-bonded at N5 (cf Figure 1). The four water system, presented in cyan, has been overlaid onto the three water system, shown in red. The dashed lines have been added in manually to highlight the H-bonds present in these systems.

Figure 1

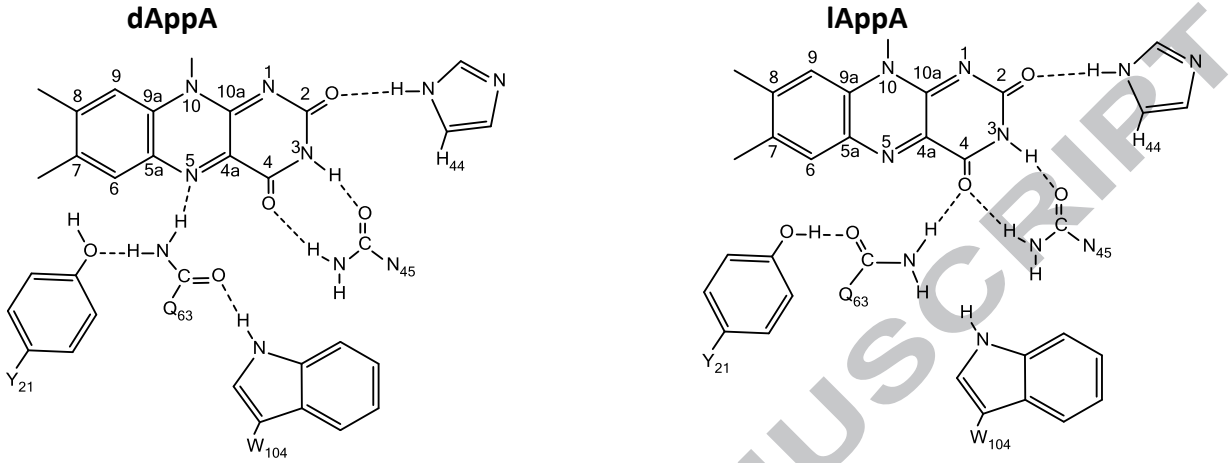


Figure 2

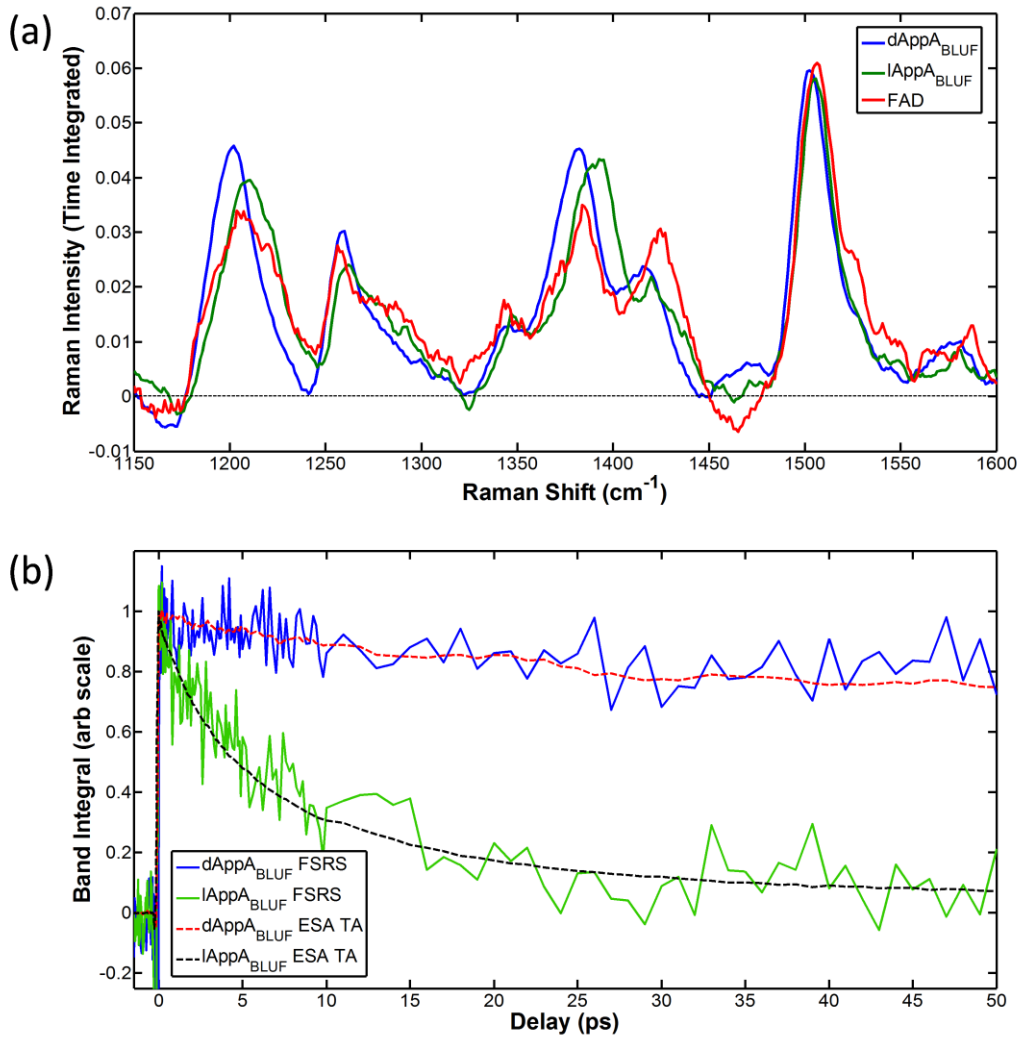
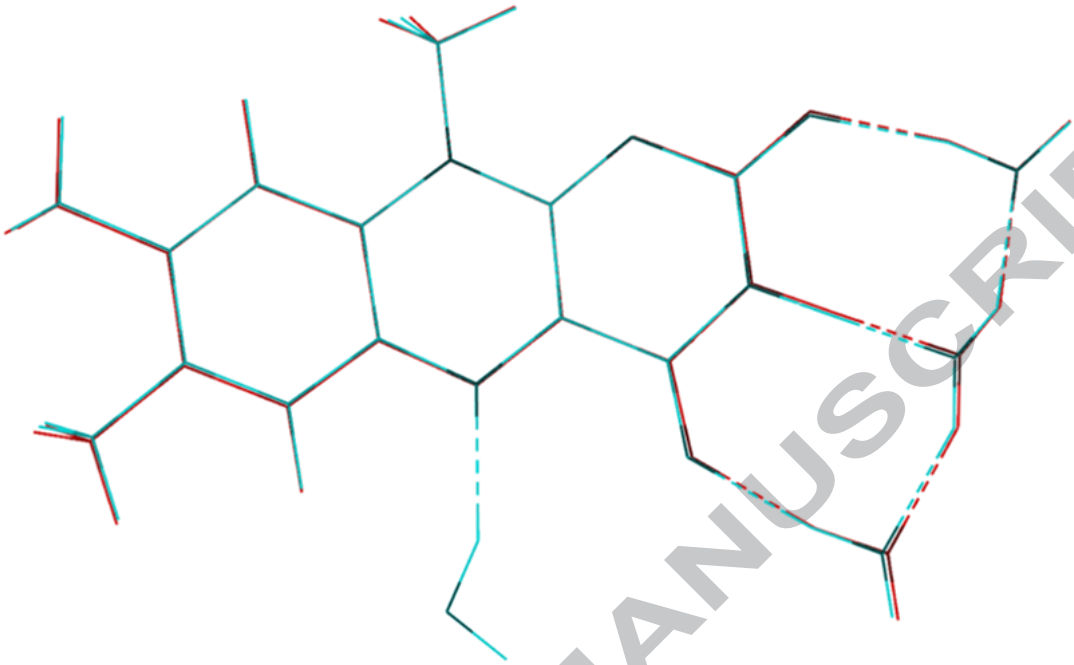
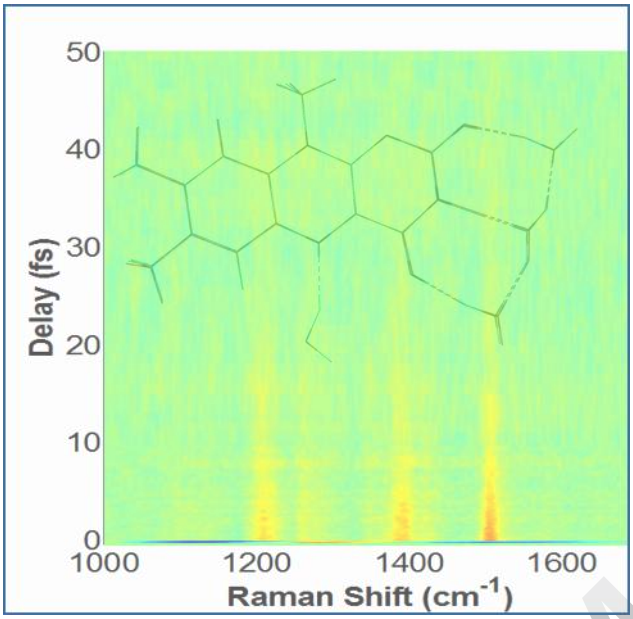


Figure 3



Graphical abstract



Highlights

- Femtosecond Stimulated Raman Spectra are measured for a photoactive flavoprotein, $AppA_{BLUF}$.
- FSRS can probe photodynamics in flavoproteins.
- The FSRS spectra for dark and signalling states are disctinct.
- Simulationo of spectral shifts require calculations incorporating the chromophore environment.