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The PYP chromophore acts as a 'photoacid' in an isolated hydrogen bonded complex

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The light-induced response of the neutral Photoactive Yellow Protein chromophore in the hydrogen-bonded complex with a proton acceptor has been studied by dual-detection action absorption spectroscopy and density functional theory. We show that the chromophore is a 'photoacid' and that ultrafast excited-state proton transfer might be operative in the isolated complex.

1. Introduction

The photoactive yellow protein (PYP) is a relatively small (14 kDa) water-soluble protein found in the purple bacterium Halorhodospira halophila[1–3]. With its absorption in the blue (λ_{max} = 446 nm) it presumably serves as a signalling photoreceptor for avoiding blue-light photodamage. The chromophore in PYP is the deprotonated *para*-coumaric acid (pCA^-), covalently bound to Cys69 via a thioester linkage. The PYP chromophore is one of the simplest model systems for studying spectral tuning in protein environments[4–7]. The protein has absorption maximum at 446 nm (in the initial pG_{446} *trans* state)[1], while different chromophore models *in vacuo* have maxima from 430 nm to 460 nm[8–10]. Variations are for the main part ascribed to the different chemical structures of the model chromophores with maxima clustering around the absorption maximum of the protein. A similar result has earlier been found for the chromophore of the green fluorescent protein (GFP)[11]. Interactions with the protein, however lower the electronic energy levels of the chromophore and prevent electron detachment from the anionic chromophore upon excitation to the first excited state S_1[6,12].

The photocycle of PYP has been studied extensively experimentally and theoretically[13–16]. Upon photoexcitation, the deprotonated chromophore undergoes a photocycle which first involves *trans-cis* isomerization with internal conversion to the ground state within a few ps, forming the intermediate pR_{465} state, and then a protonation of the ground state chromophore on the μs time scale, forming the pB_{355} state[13]. The initial, dark-adapted state is recovered thermally upon a reverse deprotonation and reisomerization. The recovery can also be triggered by light, and the neutral chromophore undergoes excited-state proton transfer (ESPT) on a sub-ps time scale followed by reisomerization[17]. Important for the present work is the significant spectroscopic shift between the anionic pG_{446} and neutral pB_{355} states which enables easy identification of the state of protonation, as well as the ultrafast ESPT time scale observed inside the protein.

Photo-induced proton transfer and photo-isomerization-induced internal conversion are the fundamental light-triggered chemical reactions in photocycles of many photoactive proteins. To address the role played by the protein environment in these primary events, knowledge of the intrinsic photoresponse of the chromophores is required as a reference for the protein functioning. The photo-induced dynamics of the isolated PYP chromophore anion has previously been studied by using time-resolved photoelectron spectroscopy, and internal conversion through a conical intersection has been found to occur on a timescale of tens of picoseconds, an order of magnitude slower than that occurring inside the PYP protein[12]. At the same time, studies of photo-induced proton transfer, in particular ESPT, requires a model system, where the neutral chromophore is bound to a proton acceptor through a hydrogen bond. The photophysics and photochemistry of such interacting molecular systems, where the interplay between proton transfer and internal conversion in the excited-state decay can be assessed, have not been addressed so far.

Here, we study the light-induced response of the PYP-model chromophore, *trans-p*-coumaric acid (pCA), in the presence of a Cl^- proton acceptor – see Fig. 1. We are working with the complex in vacuum and hence in an environment devoid of external perturbations. The goal is to understand the intrinsic

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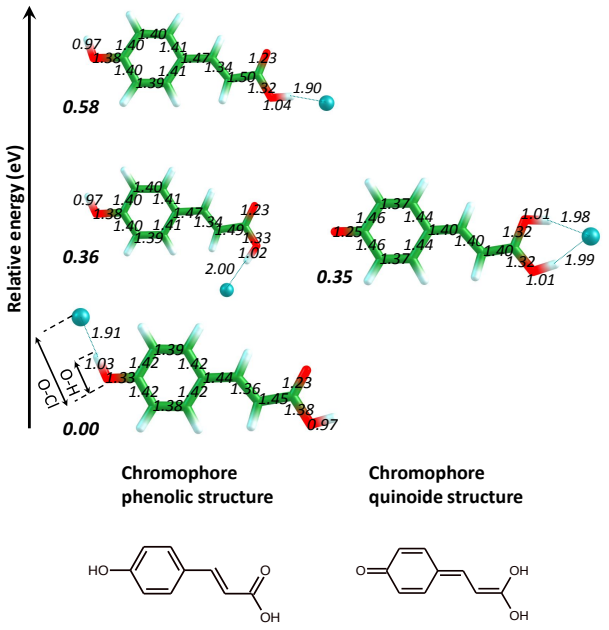

Fig. 1 Isomers of the Cl^-·pCA complex. Minimum-energy structures are calculated by DFT (B3LYP/6-31+G(d,p)) and relative energies are listed in eV. Bond lengths are shown in Å. The lowest-energy structure corresponds to the biologically relevant one with proton exchange taking place at the phenolic oxygen.

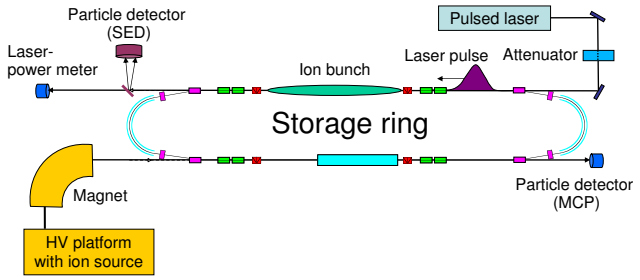

Fig. 2 The ELISA storage ring equipped with a secondary electron emission detector (SED) for counting neutral photoproducts, which are formed after dissociation of the hydrogen-bonded complex induced by photoexcitation, and a multichannel-plate detector (MCP) for mass selected ionic fragments. In the storage ring, electrostatic fields are used to store ions of specific mass and energy. The same fields were promptly changed after firing the laser to identify charged photoproducts by first storing them in the ring and then dumping them on the MCP detector several revolutions later. Details of this dual-detection action absorption spectroscopy technique can be found in Ref. 18.

photoacid properties of the model PYP chromophore by studying the mechanism of photoinduced proton transfer in the isolated gas-phase complex of pCA bound to a proton acceptor through a hydrogen bond. We use the significant spectral shift upon deprotonation of the pCA chromophore to establish the initial position of the proton prior to photoexcitation. As a result of excitation to the first electronically excited state, the hydrogen-bonded complex breaks apart and we use photofragment spectroscopy to identify the final position of the proton after photoexcitation. The experimental findings are discussed on the basis of density functional theory (DFT) calculations and we specifically discuss the possibility of having excited-state proton transfer taking place.

2. Experiment

The action spectroscopy studies of the hydrogen bonded Cl^-·pCA complex, which provide the absorption cross section as a function of wavelength, were carried out at the electrostatic ion-storage ring ELISA[19] (Fig. 2). The chromophore complex was generated by an electrospray ion source, with a solution of pCA in methanol with a small trace of NaCl added. The assignment of the chemical structure of the complex was supported by the presence of the two Cl isotopes (mass 35 and 37) as seen in Fig. 3. In the ion source, the ions were first collected in a 22-pole ion trap with helium as a buffer gas at room temperature. At a rate of 20 Hz, the trap was emptied for about 30 μs and extracted as a bunch. Ions of mass 199 amu, corresponding to $Cl_{35}{}^-$·pCA, were mass-analyzed by a magnet and injected into the ion-storage ring. After ~5 ms of storage in ELISA the ions were exposed to a single 0.01 mJ laser pulse of 4 ns duration. The photoexcitation causes the H-bonded complex to break apart and both a neutral and a charged fragment are produced. Neutrals fly on straight trajectories directly into the SED detector immediately after the interaction region (see Fig. 2), whereas charged fragments were identified by electrostatic mass/energy selection in the deflectors in the ring and subsequently dumped on the MCP detector in the other side of the ring. The photoabsorption (action) spectra were recorded by measuring, at given wavelength λ, the counts of neutrals as well as charged photofragments generated after laser excitation. The yield was normalized to the number of photons and ions (see Ref. 20).

To obtain the most reliable values for the branching ratio of daughter fragments (see later discussion) we performed independent photoexcitation measurements at the Sep1 accelerator facility[21,22], which is equipped with a precision mass spectrometer as well as a similar ion source and laser system as at ELISA. Here, Cl^-·pCA ions were produced by electrospray ionization and stored in an octopole ion trap. The ions were gently extracted from the trap in 20-μs bunches at a rate of 40 Hz and accelerated to 50 keV. A bending magnet was used to select the ions of interest by their mass-to-charge ratio. Every second ion bunch was overlapped with a laser pulse in a collinear geometry. A hemispherical electrostatic energy analyzer was used to separate the daughter ions, which were counted with a channeltron detector. The photodissociation action signal was obtained as the difference between the interleaved "laser on" and "laser off" signals.

3. Experimental results

The vertical detachment energy of Cl^- is 3.6 eV (340 nm), which is increased by the presence of pCA. It is therefore unlikely that electron detachment occurs in the S_1 spectral region, and the action resulting from photoexcitation has two primary channels:

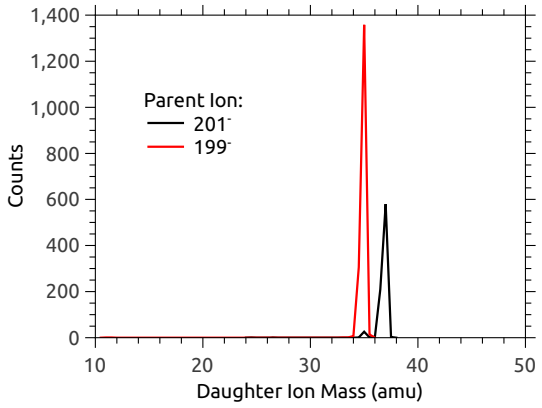

Fig. 3 Daughter photo-fragment ions produced by photoexcitation of Cl^-·pCA at 340 nm. Seen are the two Cl^- isotopes of mass 35 and 37, which are created exclusively from the corresponding $Cl_{35}{}^-$·pCA and $Cl_{37}{}^-$·pCA complexes of mass 199 and 201, respectively.

$$Cl^- \cdot pCA + h\nu \rightarrow \begin{cases} Cl^- + pCA & (A) \\ HCl + pCA^- & (B) \end{cases} \quad (1)$$

By storing the excited negatively charged parent complexes in the ring on an extended time scale (several ms), we found no sign of delayed action. This ensures that all photofragments are formed in a time shorter than the ion-flight time from the point of interaction to the SED detector (about 15 μs). This is consistent with a relatively low binding energy of the H-bonded complex. The total photoabsorption spectrum is thus obtained by analyzing the 'neutral' SED signal since both channels give exactly one neutral fragment. By measuring the yield of given charged photofragments, on the other hand, a channel-specific, action (absorption) spectrum is registered. Such a dual-detection technique allows us to identify the photo-fragmentation pathways.

Figure 4 shows absorption profiles of the isolated pCA^- chromophore and the Cl^-·pCA complex *in vacuo* compared to PYP (protein) spectra. Clearly, the state of protonation is of crucial importance for the location of the absorption band. Both in vacuum and in the partially unfolded protein do we see absorption from the neutral chromophore in the 350 nm region. The anionic chromophore on the other hand absorbs close to 450 nm in the protein, and the pCA^- model chromophore absorbs at 430 nm *in vacuo*[8]. When situated in the partially unfolded protein with exposure to water, the deprotonated PYP chromophore experiences a significant blue shift of 50 nm.

The very close similarity between the total absorption profile of the Cl^-·pCA complex and the action spectrum of channel B (Fig. 4, top) confirms that electron detachment does not occur or at least is only a minor channel at wavelengths longer than 340 nm. This also indicates that the branching ratio between channels A and B is not particularly sensitive to excitation wavelengths. This is due to the fact that only one channel (channel B) is a predominant photodissociation pathway, as we show below.

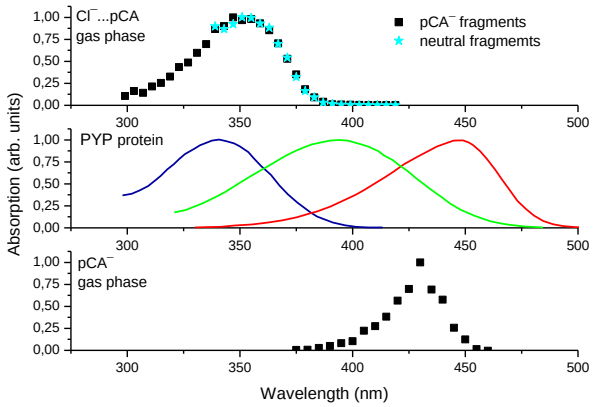

Fig. 4 Absorption spectra of the PYP chromophore in various protonation states and media. Top: The absorption by the Cl^-·pCA complex *in vacuo*. Shown are data obtained from the SED detector (neutrals) as well as pCA^- (mass 163 amu), measured by the MCP detector after mass analysis in ELISA. Since the laser light destroys the SED detector coating at wavelengths below 340 nm, prompt neutral products were only detected at wavelengths above 340 nm. Below this wavelength the SED detector was removed and only the MCP detector was used. Middle: The absorption by neutral pCA (blue) and anionic pCA^- (green) in a partially unfolded protein in water and the absorption by the native PYP protein with the deprotonated pCA^- chromophore (red), adapted from Ref. 23. Bottom: The absorption by bare pCA^- *in vacuo* from Ref. 8.

4. Discussion

The lowest-energy structure of the Cl^-·pCA complex has been calculated at the B3LYP/6-31+G(d,p) level of theory using Gaussian[24] (see Fig. 1). At the minimum, the proton is located 1.03 Å from the phenolate oxygen and 1.91 Å from the chloride anion. With fixed O–Cl distance of 2.94 Å and all other structural parameters optimized, the density distribution of the proton in the ground-state potential (and hence also the nature of the hydrogen bond) was calculated by solving the one-dimensional Schrödinger equation within the basis set of eigenstates of the infinite square well potential[25]. The result shows that the proton is indeed localized on the chromophore with very little density on the cloride anion in the vibrational ground state (Fig. 5). In other words, the pCA chromophore is protonated (neutral) in the ground-state complex in accordance with the spectroscopic data (Fig. 4)[26]. This is in contrast to the situation with the recently studied H-bonded dimer between anionic and neutral GFP chromophores, where the proton is less localized[18].

Figure 6 shows a B3LYP/6-31+G(d,p) geometry-relaxed ground state 3D-potential-energy surface as a function of the proton transfer O–H and dissociation O–Cl coordinates. Zero-point energy corrections does not significantly change the dissociation energy of 1.3 eV for channel A (Cl^- release), whereas it reduces the dissociation energy by almost 0.2 eV for channel B (HCl release), which after correction also becomes 1.3 eV, i.e., it is equally expensive to loose Cl^- and HCl. Accordingly, daughter-

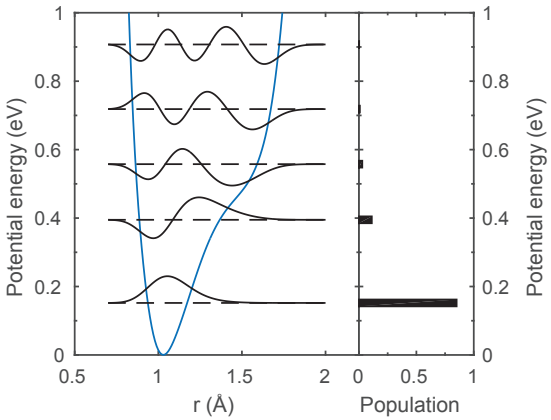

Fig. 5 The ground-state adiabatic potential that hinders the proton transfer dynamics between pCA and Cl^- for the lowest-energy isomer of the complex (r = O−H, see Fig. 1). The O−Cl distance is kept fixed at the equilibrium value of 2.94 Å. Shown are also the wave functions in the lowest vibrational states. The statistical state population *after* absorption of a 350 nm photon, corresponding to internal energy of 3.81 eV and microcanonical temperature of 1388 K, is shown to the right.

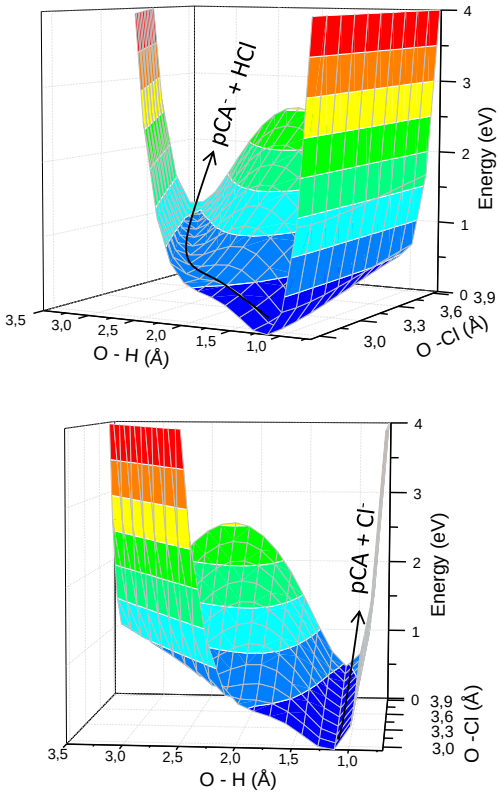

Fig. 6 Calculated B3LYP/6-31+G(d,p) ground state potential-energy surface along the proton transfer (O−H) and dissociation (O−Cl) coordinates (Fig. 1). Shown are the two possible dissociation pathways.

mass measurements should give an almost equal branching between channels A and B, if following internal conversion, the photo-induced dissociation dynamics takes place exclusively in the hot electronic ground state in a fully statistical manner. We note, however, that channels A and B are non-equivalent on the ground-state potential energy surface, and the release of HCl is a two step process, where proton transfer has to precede the dissociation (in this case with a highly localized density function for the proton location even after absorption of a photon, see the average population of vibrational states in Fig. 5).

If there were no proton transfer in the excited state upon photoexcitation, the system returns to the hot ground state with additional 3.4 eV energy (360 nm) and we expect about equal dissociation into the A and B channels based on the almost identical dissociation energy in the ground state. Moreover, if we assume a non-statistical dissociation in the ground state and no ESPT, the maximum branching ratio of channel B (pCA^-) over channel A (Cl^-) would not exceed 1 either, since highly excited vibrational levels for the proton transfer coordinate would result in proton delocalization within the complex, whereas the low-energy levels would only disfavor channel B (see Fig. 5). In other words, the proton can be transferred with maximum probability of about 50% in the electronic ground state. We find, however, that at 360 nm 4.2±0.6 times *more* mass 163 (pCA^-) compared to mass 35 (Cl^-) is produced, indicating that proton transfer happens in the excited state prior to internal conversion to the hot ground state, and the pCA^-·HCl complex with the transferred proton, at least partially, dissociates directly in S_1, or in S_0 in a non-statistical manner. Naturally, the excited-state dynamics needs further theoretical and experimental verification gained for example through fs pump-probe experiments in combination with electron spectroscopy[27] or at our new ion-storage device SAPHIRA[28].

In conclusion, the anionic Cl^-·pCA complex has a stable structure with the proton attached to pCA^-, thus providing a neutral chromophore with a large blue-shifted absorption relative to that of the deprotonated pCA^- chromophore. The spectral shift of the absorption associated with protonation/deprotonation of the chromophore in the gas phase very closely resembles that of the *photochromic* PYP protein with different protonation states of the chromophore in its photocycle. Our work shows that even in very small model systems *in vacuo* can one study fundamental photo-induced reactions occurring in nature. The PYP chromophore here acts as an efficient light-induced proton donor, and to our knowledge, this is the first study of the photoresponse of an isolated interacting molecular system, where ultrafast ESPT may happen.

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References

1 T. E. Meyer, *Biochim. Biophys. Acta*, 1985, **806**, 175–183.
2 J. J. van Beeumen, B. V. Devreese, S. M. van Bun, W. D. Hoff, K. J. Hellingwerf, T. E. Meyer, M. A. Cusanovich and D. E. Mcree, *Prot. Sci.*, 1993, **2**, 1114–1125.

3 M. Baca, G. E. O. Borgstahl, M. Boissinot, P. M. Burke, D. R. Williams, K. A. Slater and E. D. Getzoff, *Biochemistry*, 1994, **33**, 14369–14377.
4 A. Sergi, M. Grüning, M. Ferrario and F. Buda, *J. Phys. Chem. B*, 2001, **105**, 4386.
5 E. V. Gromov, I. Burghardt, J. T. Hynes, H. Köppel and L. S. Cederbaum, *J. Photochem. Photobiol. A*, 2007, **190**, 241–257.
6 E. V. Gromov, I. Burghardt, H. Koppel and L. S. Cederbaum, *J. Am. Chem. Soc.*, 2007, **129**, 6798–6806.
7 D. Zuev, K. B. Bravaya, T. Crawford, R. Lindh and A. Krylov, *J. Chem. Phys.*, 2011, **134**, 034310.
8 I. Nielsen, S. Boye-Peronne, M. E. Ghazaly, M. Kristensen, S. B. Nielsen and L. H. Andersen, *Biophys. J.*, 2005, **89**, 2597–2604.
9 T. Rocha-Rinza, O. Christiansen, J. Rajput, G. Aravind, D. B. Rahbek, L. H. Andersen, A. V. Bochenkova, A. A. Granovsky, K. B. Bravaya, A. V. Nemukhin, K. L. Christiansen and M. B. Nielsen, *J. Phys. Chem. A*, 2009, **113**, 9442–9449.
10 T. Rocha-Rinza, O. Christiansen, D. B. Rahbek, B. Klærke, L. H. Andersen, K. Lincke and M. B. Nielsen, *Chem. Eur. J.*, 2010, **16**, 11977–11984.
11 S. B. Nielsen, A. Lapierre, J. U. Andersen, U. V. Pedersen, S. Tomita and L. H. Andersen, *Phys. Rev. Lett.*, 2001, **87**, 228102.
12 I. Lee, W. Lee and A. H. Zewail, *Proc. Natl. Acad. Sci. USA*, 2006, **103**, 258–262.
13 A. Xie, W. D. Hoff, A. R. Kroon and K. J. Hellingwerf, *Biochemistry*, 1996, **35**, 14671–14678.
14 M. L. Groot, L. J. G. W. van Wilderen, D. S. Larsen, M. A. van der Horst, I. H. M. van Stokkum, K. J. Hellingwerf and R. van Grondelle, *Biochemistry*, 2003, **42**, 10054–10059.
15 J. Tenboer, S. Basu, N. Zatsepin, K. Pande, D. Milathianaki, M. Frank, M. Hunter, S. Boutet, G. J. Williams, J. E. Koglin, D. Oberthuer, M. Heymann, C. Kupitz, C. Conrad, J. Coe, S. Roy-Chowdhury, U. Weierstall, D. James, D. Wang, T. Grant, A. Barty, O. Yefanov, J. Scales, C. Gati, C. Seuring, V. Srajer, R. Henning, P. Schwander, R. Fromme, A. Ourmazd, K. Moffat, J. J. Van Thor, J. C. H. Spence, P. Fromme, H. N. Chapman and M. Schmidt, *Science*, 2014, **346**, 1242–1246.
16 V. Molina and M. Merchán, *Proc. Natl. Acad. Sci. USA*, 2001, **98**, 4299–4304.
17 E. C. Carroll, S.-H. Song, M. Kumauchi, I. H. M. van Stokkum, A. Jailaubekov, W. D. Hoff and D. S. Larsen, *J. Phys. Chem. Lett.*, 2010, **1**, 2793–2799.
18 H. V. Kiefer, E. Lattouf, N. W. Persen, A. V. Bochenkova and L. H. Andersen, *Phys. Chem. Chem. Phys.*, 2015, **17**, 20056–20060.
19 S. P. Møller, *Nucl. Instr. Meth. Phys. Res. A*, 1997, **394**, 281–286.
20 L. H. Andersen, H. Bluhme, S. Boyé, T. J. D. Jørgensen, H. Krogh, I. B. Nielsen, S. B. Nielsen and A. Svendsen, *Phys. Chem. Chem. Phys.*, 2004, **6**, 2617–2627.
21 K. Støchkel, B. F. Milne and S. B. Nielsen, *J. Phys. Chem. A*, 2011, **115**, 2155–2159.
22 J. A. Wyer and S. B. Nielsen, *Angew. Chem. Int. Ed*, 2012, **51**, 10256–10260.
23 A. F. Philip, R. A. Nome, G. A. Papadantonakis, N. F. Scherer and W. D. Hoff, *Proc. Natl. Acad. Sci. USA*, 2010, **107**, 5821–5826.
24 M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, Montgomery, Jr., J. A., T. Vreven, K. N. Kudin, J. C. Burant, J. M. Millam, S. S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G. A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J. E. Knox, H. P. Hratchian, 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, P. Y. Ayala, K. Morokuma, G. A. Voth, P. Salvador, J. J. Dannenberg, V. G. Zakrzewski, S. Dapprich, A. D. Daniels, M. C. Strain, O. Farkas, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. V. Ortiz, Q. Cui, A. G. Baboul, S. Clifford, J. Cioslowski, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, M. Challacombe, P. M. W. Gill, B. Johnson, W. Chen, M. W. Wong, C. Gonzalez and J. A. Pople, *Gaussian 03*, Gaussian, Inc., Wallingford, CT, 2004.
25 F. Marsiglio, *Am. J. Phys.*, 2008, **77**, 253–258.
26 This may also explain the blueshift reported by Rajput *et al.*, Biophys. J., 2010, **98**, 488-492.
27 C. R. S. Mooney, D. A. Horke, A. S. Chatterley, A. Simperler, H. H. Fielding and J. R. R. Verlet, *Chem. Sci.*, 2013, **4**, 921–927.
28 H. B. Pedersen, A. Svendsen, L. S. Harbo, H. V. Kiefer, H. Kjeldsen, L. Lammich, Y. Toker and L. H. Andersen, *Rev. Sci. Instr.*, 2015, **86**, 063107.