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Electronic structure and dynamics of torsion-locked photoactive yellow protein chromophores†

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The photocycle of photoactive yellow protein (PYP) begins with small-scale torsional motions of the chromophore leading to large-scale movements of the protein scaffold triggering a biological response. The role of single-bond torsional molecular motions of the chromophore in the initial steps of the PYP photocycle are not fully understood. Here, we employ anion photoelectron spectroscopy measurements and quantum chemistry calculations to investigate the electronic relaxation dynamics following photoexcitation of four model chromophores, *para*-coumaric acid, its methyl ester, and two analogues with aliphatic bridges hindering torsional motions around the single bonds adjacent to the alkene group. Following direct photoexcitation of S_1 at 400 nm, we find that both single bond rotations play a role in steering the PYP chromophore through the S_1/S_0 conical intersection but that rotation around the single bond between the alkene moiety and the phenoxide group is particularly important. Following photoexcitation of higher lying electronic states in the range 346–310 nm, we find that rotation around the single bond between the alkene and phenoxide groups also plays a key role in the electronic relaxation from higher lying states to the S_1 state. These results have potential applications in tuning the photoresponse of photoactive proteins and materials with chromophores based on PYP.

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1 Introduction

Photoreceptor proteins play a vital role in nature facilitating various biological processes, such as ion transport, vision and signal transduction. Following photoexcitation, the small chromophores that are embedded in these proteins undergo fundamental photochemical reactions, such as isomerisation, excited-state proton transfer or excited-state electron transfer.¹ The small-scale structural changes of the chromophore then propagate to large-scale structural changes in the surrounding protein framework that generate longer-lived signalling states to which the host organism may respond. It is important to understand the role the protein plays in orchestrating the photoresponse of the host organism by optimising the efficiency of the process and minimising competing pathways.

Photoactive yellow protein (PYP) is the primary photoreceptor for the negative phototactic response of the *Halorhodospira halophila* bacterium to blue light that limits its exposure to

potentially harmful UV light.³ The chromophore that lies at the centre of PYP is a deprotonated *trans-para*-coumaric acid anion (pCA^-) covalently bound to a cysteine residue *via* a thio-ester linkage and stabilised by nearby residues through a hydrogen bond network (Fig. 1). Upon photoexcitation, PYP undergoes a photocycle that begins with a *trans-cis* isomerisation of the chromophore and is followed by restructuring of the surrounding hydrogen bond network to accommodate the *cis* conformation, partial unfolding of the protein and protonation of the chromophore to form the signalling state. Subsequent *cis-trans* isomerisation of the chromophore, refolding of the protein and



Fig. 1 Left: PYP protein structure² with the chromophore enclosed. Right: PYP chromophore as it exists in the protein showing the torsional rotations that are the subject of this paper.

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2 Experimental and computational methods

2.1 Chromophores

pCA was purchased from Sigma-Aldrich and *pCE* was purchased from Tokyo Chemical Industry, both were used without further purification. *PpCE* and *NpCE* were synthesised by standard chemistry. See ESI† for further details.

2.2 Photoelectron spectroscopy

Anion photoelectron spectra were recorded using our apparatus that has also been described elsewhere.^{19–22} In brief, we obtain gas-phase deprotonated anions of *pCA*, *pCE*, *PpCE* and *NpCE* by electrospray ionisation of ~1 mM methanol solutions of each chromophore with a few drops of aqueous ammonia added. The anions are mass-selected by a quadrupole and guided into a hexapole ion trap. The anions are released from the ion trap at 20 Hz and focused into the source region of a collinear velocity map imaging spectrometer. Nanosecond laser pulses of wavelength 310–346 nm were generated by frequency-doubling the output of a Nd:YAG pumped dye laser. Femtosecond laser pulses (> 50 fs, 250 Hz) of wavelength centred at 400 nm were produced by frequency-doubling the output of a commercial amplified Ti:Sapphire femtosecond laser system. Photoelectrons generated in the interaction region were accelerated towards a position sensitive detector and imaged using a CCD camera. Laser-only images were recorded without the pulsed ion-beam and subtracted from the overall signal to remove background counts from ionisation of residual gas or scattered laser light. Photoelectron velocity distributions were obtained using the pBasex inversion method.²³ Electron kinetic energy (eKE) spectra were obtained by calibrating the radial photoelectron velocity distribution against the photoelectron spectrum of iodide²⁴ (310–346 nm) and *p*-HBDI^{–25} (400 nm). The energy resolution of the 310–346 nm spectra is <5% and the error in eKE is around 0.05 eV.

2.3 Calculations

The structures of the anionic chromophores and their corresponding neutral radicals were optimised using density functional theory with the B3LYP hybrid functional^{26–29} and 6-311++G(3df,3pd) basis set.^{30–32} Vibrational analysis was performed to confirm the optimisations resulted in minima.

Vertical excitation energies (VEEs) of the singlet electronic excited states of the chromophores were calculated using time-dependent density functional theory (TD-DFT) and the algebraic diagrammatic construction method to second order (ADC(2)).^{33,34} TD-DFT calculations were performed with CAM-B3LYP³⁵/6-311++G(3df,3pd), a method that has been shown to provide qualitative agreement with measured electronic excitation energies of PYP chromophores.¹⁸ ADC(2)/6-31+G** calculations have also been performed and these have shown to calculate the VEEs accurately and reliably with a reasonable computational expense (see Table S1 in ESI† for benchmarking VEE calculations).

Vertical detachment energies (VDEs) were calculated using various methods to compare to the measured values we have

obtained. We have used electron propagator theory (EPT)³⁶ with the 6-311++G(3df,3pd) basis set, a method that has proved to agree well with experimental VDEs in earlier work.^{18,25} Also, we have used the equation-of-motion coupled cluster method with single and double excitations for the calculation of ionisation potentials (EOM-IP-CCSD),³⁷ performed using the aug-cc-pVDZ basis set³⁸ which has shown previously to calculate detachment energies to higher-lying excited states of similar neutral radicals effectively.^{25,39} The VDEs have also been calculated as the anion-neutral radical energy difference using DFT (B3LYP/6-311++G(3df,3pd)) at the optimised geometry of the anion. Calculating the VDEs with this method gives a more direct comparison with the calculated adiabatic detachment energies (ADEs) which are found using energies from DFT (B3LYP/6-311++G(3df,3pd)) calculations. ADEs are determined by the difference in energy between the S_0 minima and D_0 minima (0-0 transition), accounting for zero-point energies.

Geometry optimisations, vibrational frequencies, TD-DFT and EPT calculations were performed using the Gaussian09 software suite,⁴⁰ while EOM-CCSD and ADC(2) calculations were carried out using the Q-Chem software package.⁴¹

3 Results and discussion

3.1 Photoelectron spectra and computational results

Photoelectron spectra of the deprotonated chromophores (Fig. 2) were recorded as a function of electron kinetic energy (eKE) and are also presented as a function of electron binding energy, $eBE = h\nu - eKE$ (Fig. 3). Lines on the spectra mark calculated VDEs and VEEs.

Possible direct and indirect photodetachment (PD) processes are illustrated on the Jablonski diagram presented in Fig. 4. The 400 nm spectra are dominated by a strong feature at $eKE \approx 0$ ($eBE \approx h\nu$). The eKE profiles are those of structured exponential decays, characteristic of thermionic emission (TE).^{42,43} In contrast, the 310–346 nm photoelectron spectra are dominated by broad, unresolved features at low eBEs. The sharp, low eBE edges of these broad features remain at constant eBE for all photon energies, signifying a direct PD process. There is also evidence for direct PD in the 400 nm spectra, although it is particularly weak for *pCA*[–] and *pCE*[–]. For *pCA*[–], *pCE*[–] and *PpCE*[–], the low eBE features broaden on their high eBE edges with increasing photon energy, indicative of an indirect PD process following resonant excitation of an excited electronic state. This broadening is less pronounced for *NpCE*[–]. The 310–346 nm spectra also have features at $eBE \approx h\nu$ ($eKE \approx 0$) that increase in intensity with respect to the direct detachment features as the photon energy is increased. These $eBE \approx h\nu$ features are less intense in the *NpCE*[–] photoelectron spectra compared to those of *pCA*[–], *pCE*[–] and *PpCE*[–].

Approximate VDEs are determined from the maxima of the direct PD features at low eBE in the 346 nm spectra since this wavelength corresponds to a minimum in the action absorption spectra of *pCE*[–].⁴⁴ These maxima are presented in Table 1 along with calculated VDEs and ADEs. For D_0 , VDEs and ADEs





Fig. 3 Photoelectron spectra of pCA^- , pCE^- , $PpCE^-$ and $NpCE^-$ recorded at 310 nm (4.00 eV), 315 nm (3.94 eV), 318 nm (3.90 eV), 330 nm (3.76 eV), 346 nm (3.58 eV) and 400 nm (3.10 eV). Intensities of the spectra plotted as a function of eKE (left) have been scaled so that the low eKE features lie on top of one another and are normalised to the maximum of the low eKE feature. Intensities of the spectra plotted as a function of eBE (right) have been scaled to align the rising edges and are normalised to the maximum of the rising edges of the 346 nm spectra. Note the scale changes used to plot the 400 nm data with the shorter wavelength data shown by the grey dashed lines. Combs mark the EPT calculated VDEs (grey) and the first three singlet excited states of the anion calculated using ADC(2)/6-31+G** (black).

increase in the order $NpCE^- < pCE^- < PpCE^- < pCA^-$. The trend in the calculated and experimentally measured detachment thresholds can be explained in terms of the electronic effects of the acyl substituents and structural strain on the resonant stabilisation of the anion. The slight difference

between the pCA^- and pCE^- detachment thresholds can be attributed to the hydroxyl group of pCA^- being a slightly weaker electron donor than the OMe group. This is intuitive as the methyl group is electron releasing and in this instance can be shown by the sum of the ChelpG⁴⁵ partial charges on



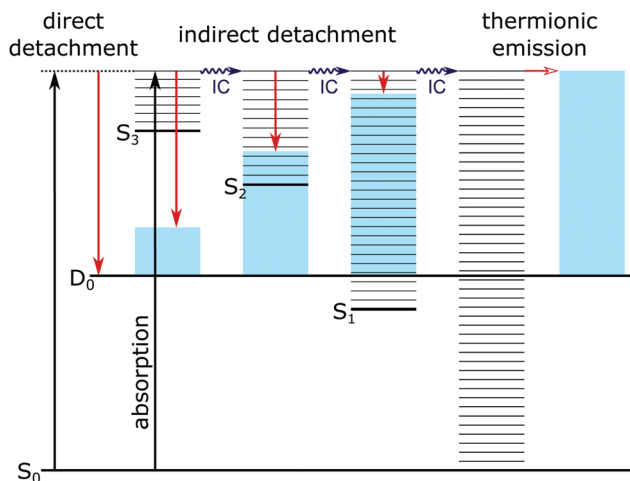


Fig. 4 Jablonski diagram illustrating the major photodetachment pathways (red arrows) available for the model PYP chromophores employed in this work (Fig. 2) following UV photoexcitation (346–310 nm) including internal conversion (IC) and thermionic emission (horizontal red arrow). Horizontal black lines represent the vibrational levels of the excited electronic states of the anion and the blue shaded areas represent the vibrational energy left in the neutral radical upon electron detachment as a result of the propensity for conserving vibrational energy.

Table 1 Vertical detachment energies (VDEs), B3LYP/6-311++G(3df,3pd) D_0 – S_0 adiabatic detachment energies (ADEs) (0–0 transition) and maxima of the experimental 346 nm photoelectron spectra in eV for each chromophore. VDEs were calculated using EPT/6-311++G(3df,3pd) (pole strengths in parentheses), EOM-IP-CCSD/aug-cc-pVDZ and B3LYP/6-311++G(3df,3pd) methods. EPT and EOM-IP-CCSD have two values listed corresponding to detachment to the D_0 and D_1 states

	Vertical detachment energy			ADE(0–0)	Expt.
	EPT	EOM-IP-CCSD	DFT		
pCA^-	2.99 (0.879)	2.97	3.10	2.99	2.92 ± 0.05
	5.25 (0.872)	5.45			
pCE^-	2.91 (0.879)	2.78	3.01	2.91	2.85 ± 0.05
	5.18 (0.873)	4.42			
$PpCE^-$	2.96 (0.878)	2.83	3.03	2.93	2.89 ± 0.05
	5.23 (0.871)	4.48			
$NpCE^-$	2.67 (0.879)	2.71	2.93	2.82	2.78 ± 0.05
	5.02 (0.868)	4.33			

the OH group ($-0.296 e$) compared to that of the OMe group ($-0.275 e$). In pCE^- the effect of $n_{O(sp^2)} \rightarrow \sigma^*_{C=O}$ donation will destabilise the anion. This stereoelectronic effect is not possible with $PpCE^-$ so it is surprising that the VDEs of $PpCE^-$ and pCE^- are similar. The expected greater stabilising effect of the lactone ring in $PpCE^-$ may be offset by the conformation induced by the tether compromising the planarity of the system with respect to the simple ester in pCE^- . The detachment threshold of $NpCE^-$ is most likely lowered by the ring strain introduced by the rotation lock (half-chair conformation) inducing a small reduction in the planarity (see Fig. S2 in ESI†) and thus hindering resonant stabilisation of the anion.

It is worth noting that pCA can deprotonate at two sites to form either the carboxylate or the phenolate tautomer. The VDE of carboxylate pCA^- ($4.68 eV$)¹⁷ is significantly higher than that

of phenolate pCA^- and the photon energies used in this work ($<4 eV$). Additionally, the spectra of pCA^- and pCE^- are very similar. Hence, although both forms of pCA^- could be present in our instrument, we are only sensitive to the phenolate form.

The DFT VDEs are close to the ADEs, suggesting that the geometries of the anions and their corresponding neutral radicals are similar, in agreement with our earlier work on model PYP chromophores.^{17,18}

In terms of the calculated VDEs for D_0 , the EPT method has determined the VDEs of pCA^- , pCE^- and $PpCE^-$ to be just slightly higher than the experimental maxima ($\sim 0.05 eV$) and slightly lower for $NpCE^-$ ($0.115 eV$). The EPT/6-311++G(3df,3pd) method was employed to calculate the first VDE of the phenoxide anion previously⁴⁶ and it was found to be within 1% of the experimental value of Gunion *et al.*⁴⁷ Here, we find a 2% difference between the EPT calculated VDE and the experimental maxima for pCA^- , pCE^- and $PpCE^-$, and a 4% difference for $NpCE^-$. The VDEs calculated using DFT are systematically higher ($\sim 0.15 eV$) than the experimental maxima. The EOM-IP-CCSD calculations have predicted the VDEs of the chromophores to within $\sim 0.06 eV$ of the experimental maxima, although the deviations are less systematic than with DFT; for example, the EOM-IP-CCSD VDE of pCA^- is higher than the experimental maximum whereas the calculated VDE of pCE^- is lower than the experimental maximum. It should be noted that the accuracy of our measurement is $\pm 0.05 eV$ and that the high density of vibrational transitions contributing to these vibrationally unresolved photoelectron spectra can easily shift the maxima away from the true VDE. Thus, we conclude that all the calculated VDEs are in good agreement with experiment and note that the simple, computationally inexpensive DFT method performs just as well as the other methods. Calculated VDEs for D_1 are also listed in Table 1 and are all significantly higher than the photon energies used in this study suggesting that direct PD to D_1 is unlikely.

The first three singlet excited states of the chromophores are $1^1\pi\pi^*$, $1^1n\pi^*$ and $2^1\pi\pi^*$, as illustrated in Fig. 5. The relevant orbitals of all the chromophores are similar to those of pCA^- (shown), as one may expect from a set of structural analogues without any significant changes to functionality (see Tables S2–S5 and Fig. S3–S6, ESI†). The π and π_1^* orbitals are delocalised across the whole chromophore whereas the n and π_2^* orbitals are more localised on the phenoxide end of the anion. Hence, the $S_3(2^1\pi\pi^*)$ and the $S_2(1^1n\pi^*)$ transitions have charge transfer character. The delocalised π orbital is the highest occupied molecular orbital and the configuration of the D_0 state of the neutral radical corresponds to the removal of an electron from this orbital.

Vertical excitation energies (VEEs) for these first three singlet excited states calculated using CAM-B3LYP/6-311++G(3df,3pd) and ADC(2)/6-31+G** methods are presented in Table 2. They are compared with SA-CASSCF(14,12)-PT2/cc-pVDZ results by Fernández García-Prieto *et al.*⁴⁸ and experimental values recorded by Rocha-Rinza *et al.*⁴⁹ as these have been shown to be in excellent agreement with one another and provide a benchmark for our own calculations.

The calculated oscillator strength for the $S_1 \leftarrow S_0$ transition is near unity for each of the chromophores whereas that for the





Fig. 5 Left: The main molecular orbitals involved in the transitions to the first three singlet excited states of the anion. Although the MOs plotted are calculated for pCA^- , they are similar for all the chromophores shown in Fig. 2 (see Tables S2–S5 and Fig. S3–S6, ESI†). Right: The electron configurations associated with the ground state of the anion (S_0) and neutral radical (D_0) and the first three singlet excited states of the anion for all the chromophores.

$S_2 \leftarrow S_0$ transition is zero. The $S_3 \leftarrow S_0$ transition has a non-zero oscillator strength for each chromophore of approximately 0.1. From Tables 1 and 2, and the VDEs and VEEs plotted in Fig. 3, it is clear that the D_0 and S_1 states lie close in energy, particularly for pCA^- , pCE^- and $PpCE^-$. Due to the good agreement between the literature and ADC(2) methods, the ADC(2) values are plotted on the spectra in Fig. 3. The CAM-B3LYP VEEs are similar to those calculated for PYP chromophores in earlier work using the same method;¹⁸ however, the VEEs for pCA^- and pCE^- are approximately 0.5 eV higher than those calculated using higher level theory⁴⁸ and obtained from action absorption spectra.⁴⁹ The VEEs calculated using the ADC(2)/6-31+G** method for pCA^- and pCE^- are in good agreement with those calculated using the SA-CASSCF(14,12)-PT2/cc-pVDZ method⁴⁸ and with the available experimental measurements,⁴⁹ so we use the VEEs calculated with this method to assist with the interpretation of our experimental measurements.

The electron configurations of the accessible electronically excited states of the anions and the corresponding neutral radicals (Fig. 5) are useful in the consideration of coupling strengths of the excited states of the anion to the detachment

continua. Indirect PD from the S_1 state to the D_0 continuum involves removing a single electron from the π_1^* orbital to yield the D_0 electron configuration. Vibrational states of S_1 that lie in the D_0 continuum have shape resonance character and are coupled strongly to the continuum. Indirect PD from S_3 involves removing an electron from the π_2^* orbital. The S_3 state has excited shape resonance character with respect to the D_0 continuum and electron detachment through this channel is expected to be very fast. In contrast, indirect PD from the S_2 state to the D_0 continuum involves removing a π_2^* electron and making the $\pi \leftarrow n$ electronic transition. Thus S_2 has Feshbach resonance character with respect to the D_0 continuum and indirect detachment from this state is expected to be relatively slow. Although the D_1 VDEs are higher than the photon energies employed in this work, adiabatic detachment to D_1 from the S_2 and S_3 states cannot be ruled out. The D_1 state has electronic configuration $n^1\pi^2\pi_1^*\pi_2^*/n^1\pi^1\pi_1^*\pi_2^*$ so the S_3 state has Feshbach resonance character with respect to the D_1 continuum suggesting that indirect PD to D_1 will not be able to compete with indirect PD to D_0 or ultrafast IC to lower-lying states. The S_2 state is an excited shape resonance with respect to D_1 , suggesting that indirect PD from S_2 to D_1 is possible; however, there would still be competition from internal conversion to S_1 or S_0 . Overall, it seems most likely that the photoelectron spectra are dominated by direct and indirect electron emission to the D_0 continuum.

3.2 The role of torsional motion

Photoexcitation at 400 nm populates vibrationally excited states of S_1 lying in the D_0 detachment continuum. All the photoelectron spectra are dominated by $eKE \approx 0$ ($eBE \approx h\nu$) photoelectrons with an overall exponential decay profile, suggesting that the dominant electronic relaxation mechanism is IC to S_0 followed by TE. However, the ratio of photoelectrons attributed to TE compared with those attributed to direct PD decreases in the order $pCA^- \approx pCE^- \gg PpCE^- > NpCE^-$. This suggests that torsional motions around both single bonds adjacent to the alkene group are involved in steering the PYP chromophore towards the S_1/S_0 CI but that rotation around the single bond

Table 2 Vertical excitation energies (VEEs) calculated using CAM-B3LYP/6-311++G(3df,3pd) and ADC(2)/6-31+G** compared with SA-CASSCF(14,12)-PT2/cc-pVDZ⁴⁸ calculated values and experimental values.⁴⁹ Oscillator strengths are shown in brackets

			pCA^-	pCE^-	$PpCE^-$	$NpCE^-$
CAM-B3LYP 6-311++G(3df,3pd)	$1^1\pi\pi^*$	S_1	3.45 (0.910)	3.43 (0.949)	3.49 (0.952)	3.36 (0.812)
	$1^1n\pi^*$	S_2	4.10 (0.000)	4.12 (0.000)	4.15 (0.000)	4.10 (0.000)
	$2^1\pi\pi^*$	S_3	4.33 (0.071)	4.31 (0.110)	4.34 (0.056)	4.40 (0.026)
ADC(2) 6-31+G**	$1^1\pi\pi^*$	S_1	2.96 (1.000)	2.96 (1.021)	3.02 (1.032)	2.82 (0.885)
	$1^1n\pi^*$	S_2	3.35 (0.000)	3.37 (0.000)	3.39 (0.000)	3.26 (0.000)
	$2^1\pi\pi^*$	S_3	4.13 (0.091)	4.10 (0.097)	4.10 (0.061)	3.94 (0.065)
SA-CASSCF (14,12)-PT2 cc-pVDZ	$1^1\pi\pi^*$	S_1	2.96	2.94	—	—
	$1^1n\pi^*$	S_2	3.65	3.65	—	—
	$2^1\pi\pi^*$	S_3	3.82	3.82	—	—
Experiment	$1^1\pi\pi^*$	S_1	2.89	2.88	—	—



between the alkene moiety and the phenoxide group plays a more important role.

Photoexcitation at 346 nm results predominantly in direct detachment from S_0 ; however, the observation of $eKE \approx 0$ ($eBE \approx h\nu$) photoelectrons with an overall exponential decay profile suggests that there is some contribution from resonant excitation of excited states of the anion followed by IC to S_0 and subsequent TE. We cannot assign the resonant excitation process to any particular excited state of the anion, but the high vibrational levels of S_1 , low vibrational levels of S_3 or the S_2 state, through vibronic mixing with the bright S_1 state, are all possibilities. In terms of the role of torsional motions, the ratio of photoelectrons attributed to TE compared with those from direct PD is lower for $NpCE^-$ than the other three chromophores, supporting the idea that rotation around the single bond between the alkene moiety and the phenoxide group plays an important role in IC from S_1 to S_0 .

Increasing the photon energy to 330–310 nm results in a significant contribution from indirect PD from S_1 and TE from S_0 for pCA^- , pCE^- and $PpCE^-$. In contrast, indirect PD from S_1 is far less pronounced for $NpCE^-$ and the contribution from TE only increases slightly. The most likely explanation for these observations is that as the photon energy increases, increasing population of S_3 followed by internal conversion to S_1 occurs and subsequent autodetachment to D_0 or IC to S_0 followed by TE. Locking the rotation around the single bond between the alkene moiety and the phenoxide group in $NpCE^-$ inhibits these pathways, which suggests that rotation around the single bond between the alkene moiety and the phenoxide group also plays a role in IC from S_3 to S_1 .

4 Conclusions

From the combination of photoelectron spectroscopy measurements and computational chemistry calculations, we have improved our understanding of the role of torsional motions around the single bonds either side of the alkene moiety in the electronic relaxation of isolated PYP chromophores in the gas-phase. We have found that single bond rotations are involved in steering the PYP chromophore towards the S_1/S_0 CI and that rotation around the single bond between the alkene moiety and the phenoxide group plays a particularly important role. From measurements at higher photon energies, we also find that rotation around this single bond between the alkene moiety and the phenoxide group plays a key role in IC from S_3 to S_1 . This suggests that torsional motions around the single bonds, and particularly around the one between the alkene and phenoxide groups are not restricted in the protein. This supports the conclusion of the earlier time-resolved study by Lee *et al.* on the ketone analogue of the PYP chromophore in which isomerisation occurred on a similar time scale to the protein, suggesting entry into the photocycle must be unhindered by torsional motion and almost frictionless.¹¹ These results have potential applications in biology and nanotechnology in terms of improving our understanding of how to tune the photoresponse of

proteins or materials with a chromophore based on the PYP chromophore.

Conflicts of interest

There are no conflicts to declare.

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