Chem Soc Rev

This article was published as part of the

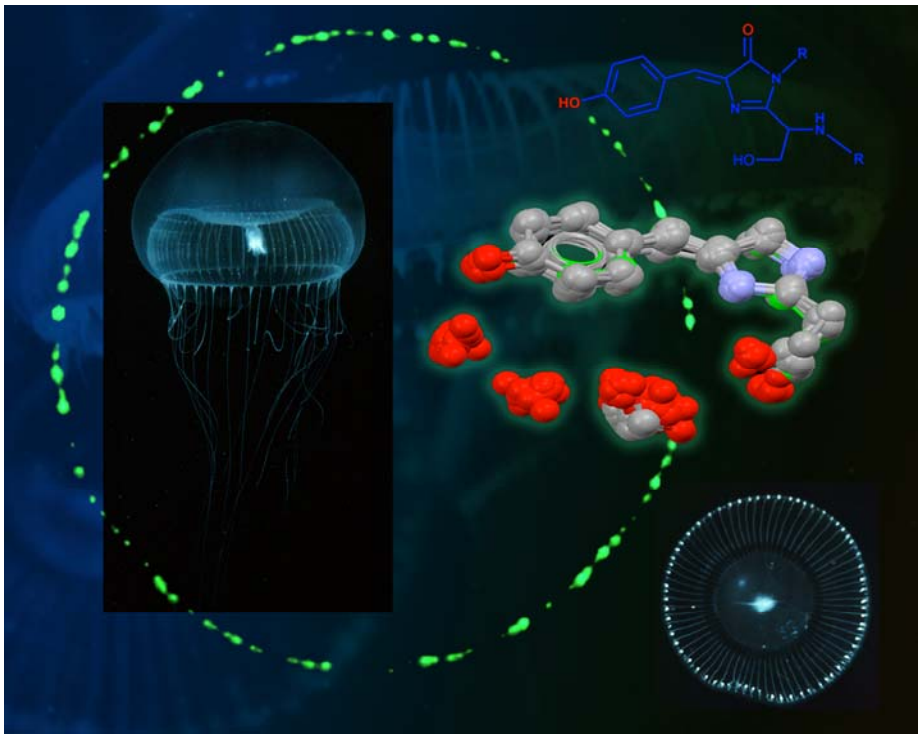
2009 Green Fluorescent Protein issue

Reviewing the latest developments in the science of green fluorescent protein

Guest Editors Dr Sophie Jackson and Professor Jeremy Sanders

All authors contributed to this issue in honour of the 2008 Nobel Prize winners in Chemistry, Professors Osamu Shimomura, Martin Chalfie and Roger Y. Tsien

Please take a look at the issue 10 table of contents to access the other reviews



Excited state reactions in fluorescent proteins†

Stephen R. Meech*

Received 23rd June 2009
First published as an Advance Article on the web 25th August 2009
DOI: 10.1039/b820168b

The green fluorescent protein is a key technology in bioimaging. In this *critical review*, we consider how its various applications can be tailored from knowledge of the excited state chemistry. The photophysics of the basic chromophore in solution are described in detail, and the dominant radiationless decay mechanism is characterised. The quite different photophysics of wild type GFP are described next. The unique excited state proton transfer reaction observed can be used to model proton transfer processes in proteins. Examples where the proton transfer is blocked, or redirected to occur over a low short barrier H-bond are discussed. Finally the photophysics underlying the new generation of photochemically active fluorescent proteins are discussed (155 references).

1. Introduction

In the past 15 years the green fluorescent protein (GFP) has become established as a major tool in many areas of the life sciences.[1–3] In 1994 Chalfie and co-workers showed that the intrinsically fluorescent GFP could be cloned and expressed in living cells, with subsequent detection of the protein through fluorescence microscopy.[4,5] The beautiful images obtained opened the way for GFP to be used as a fluorescent marker protein in living organisms—a revolutionary advance in bioimaging. It is interesting to note that such an illustrious future for GFP was not always anticipated. GFP was first isolated from the jellyfish *Aequorea victoria* by Shimomura and co-workers in the early nineteen sixties,[6,7] and was at that time viewed as something of a novel curiosity, being associated with the bioluminescent aequorin protein and having its intrinsic visible fluorescence excited by energy transfer from aequorin's usually chemiluminescent excited state.[8] The trajectory of GFP from this unpromising origin to being the basis of a major tool in biotechnology and, in 2008, the subject of the Nobel Prize in chemistry, which this themed issue celebrates, is surely a most eloquent justification for curiosity driven research.

The key feature of GFP (and its relatives) underpinning all of its applications is that the fluorescent chromophore is an intrinsic part of the protein backbone. This distinguishes GFP from the very many other coloured proteins, all of which bind their chromophore through non-covalent interactions and may therefore exchange it with the environment (clearly an undesirable event in imaging for example). The mechanism of chromophore formation in GFP has been studied in great detail.[9] In the wild type it involves post translational reactions among three amino acid residues, Ser65, Tyr66 and Gly67.[10–19] The initial folding of the protein into a β-barrel structure places the residues in the correct orientation for a cyclisation reaction to take place. This first reaction is followed by an oxidation reaction (in the presence of dissolved O_2) to yield an extended π-electron system with a core structure of 4′-hydroxybenzylidene-2,3-dimethylimidazolinone (HBDI, Fig. 1a).[20] The entire process results in the chromophore being localised in the centre of the β-barrel structure of GFP (Fig. 1b) and thus well protected from the environment.[21]

Wild type (wt) GFP has a number of features which make it less than optimal for fluorescence imaging.[1] The folding of the protein is quite slow, which is undesirable for time dependent studies. Also the chromophore exists in two forms, protonated (neutral) and deprotonated (anionic) with the dominant form being the former. The neutral form absorbs light at around 400 nm, which is unsuitable for most laser excitation sources, and has lower oscillator strength than the anion. It was thus realized, most notably by Tsien and his co-workers, that there

School of Chemistry, University of East Anglia, Norwich, UK NR1 2QN. E-mail: s.meech@uea.ac.uk

† Part of a themed issue on the topic of green fluorescent protein (GFP) in honour of the 2008 Nobel Prize winners in Chemistry, Professors Osamu Shimomura, Martin Chalfie and Roger Y. Tsien.

Stephen R. Meech

Stephen Meech obtained his PhD degree in photophysics from Southampton University. After periods as a postdoctoral researcher in Detroit and London, he was an EPSRC Fellow at Groningen University, Netherlands. He joined the faculty of Heriot-Watt University in 1986 and was a visiting researcher (Inoue Fellow) at IMS, Japan. In 1994 he moved to the University of East Anglia, where he is Professor of Physical Chemistry. His main research interests are ultrafast molecular dynamics in the condensed phase and at interfaces. Current projects include dynamics in photoactive proteins, reaction dynamics in confined media, and molecular dynamics in complex fluids.

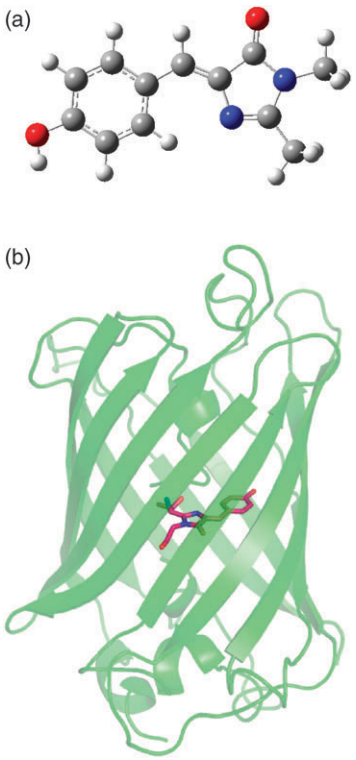


Fig. 1 (a) The structure of the HBDI model chromophore of GFP; (b) the β-barrel structure of GFP, with the chromophore shown in red (structure from ref. 21).

may be much to be gained from studying the mutants of GFP. Developments through mutagenesis enhanced the folding rate, the stability and the maturation rate of GFP.[2] Mutations introduced into the three chromophore forming amino acid residues or into residues surrounding the chromophore broadened the available spectral range, allowing the full potential of GFP and related fluorescent proteins (FPs) to be realised.[22,23]

The colours available as a result of GFP mutagenesis range from blue through cyan and green to yellow. However, shortly after the utility of GFP was demonstrated, the origin of colouring in many other marine organisms was reinvestigated.[24–28] This proved a highly productive exercise—a host of coloured (though sometimes non-fluorescent) GFP-like proteins were isolated, and over recent years the FP family has grown dramatically, and continues to grow.[29,30] The lack of fluorescence in some of the 'FPs' derived from coral argues against a signaling mechanism (assumed to be the function of wtGFP in *A. victoria*) and a number of other functions have been postulated. For example the co-location of a strongly absorbing protein with the photosynthetic apparatus in some organisms, notably coral, is strongly suggestive of a photoprotection role,[28,31] while others have proposed that the colouration is involved in a kind of camouflage.[32] Recently it has even been suggested that FPs may be involved in biological photoinduced electron transfer reactions.[33]

Many of the FPs newly isolated from natural sources have an identical chromophore to GFP itself, while in others a more red-shifted chromophore has been formed through additional reactions with the polypeptide backbone, further extending the conjugation. An early example was the red shifted spectrum of DsRed isolated from coral.[24,34] This modification has the effect of greatly broadening the palette of colours available for imaging research. Even the new FPs possessing a chromophore with the same structure as wtGFP may sometimes have quite different spectra. Changes in the charge state and degree of protonation of the chromophore can have dramatic effects on both the intensity and the frequency of the fluorescence.[15,35,36] Thus with the available range of naturally occurring FPs supplemented and expanded through mutagenesis, the chromophore excitation and emission wavelengths available now extend over most of the visible region of the spectrum—and beyond.[37,38]

The range of application of FPs is certainly too vast to be described here, but some illustrative examples can be mentioned. Among the earliest was the mapping of gene expression in living organisms. The gene which expresses GFP is spliced into the gene expressing the target protein, so that when the target protein is expressed it is attached to its FP partner.[5] Provided that folding and chromophore maturation are sufficiently fast (as they are for the optimised mutants) a fluorescence microscope can map out the location and timing of gene expression in a given cell or an entire organism. Clearly it is potentially advantageous to map out the expression of more than one gene at a time, for example to demonstrate a relationship between them. This is possible if FPs with the same excitation wavelength but different emission spectra are used, in conjunction with some kind of wavelength resolved imaging. Indeed distinct filter sets for specific FP combinations are available, permitting multicolour imaging.[39] In other circumstances the detection of post translational protein–protein interactions may be of significance. Once again these may be visualised by the use of distinct pairs of FPs, specifically a pair whose spectra allow Förster resonance energy transfer (FRET). Thus, when the proteins of interest approach within the Förster radius (typically about 4 nm for FPs) the higher energy fluorescence is quenched and emission from the lower energy partner is observed.[1] This clearly requires distinct absorption and emission spectra.

Finally a number of applications utilising the FP emission as a localised sensor in living systems have been demonstrated. Miyawaki, Tsien and co-workers used a dual labelling approach, placing a FRET donor on calmodulin and an acceptor on calmodulin binding peptide, such that in the presence of Ca^{2+} FRET was greatly enhanced.[40,41] Kneen and co-workers used single labelling with pH sensitive GFP mutants (notably S65T GFP) to show that they could be used as rapid indicators of pH in a range of intracellular compartments.[42]

Clearly many of these applications rely on some degree of control over the photophysics of the FP (absorption/emission maxima, environment sensitivity, *etc.*). Thus the rational design of FPs requires a detailed knowledge of their excited state chemistry. In addition, recent years have seen the growth of a new generation of FPs in which the properties of the protein can be optically modulated through the chromophore's excited state chemistry. These second generation FPs are already finding numerous application in imaging, and have

great potential as components of image or data storage devices. Again the development and utility of these FPs requires a detailed knowledge of the excited state chemistry, which is the main subject of this review. In the next section the photophysics of the chromophore of GFP (HBDI, Fig. 1a) will be considered in detail. We will follow this with a discussion of the quite distinct photophysics of the chromophore in wt GFP. The manipulation of excited state chemistry (particularly excited state proton transfer reactions) through mutagenesis will be described next, and we will finish with an outline of the excited state chemistry and potential applications of some second generation FPs, followed by some concluding remarks.

2. Photophysics of HBDI

Probably the most remarkable feature of the chromophore of the green fluorescent protein is that it does not fluoresce;[20,43] in room temperature aqueous solution the quantum yield is on the order of 2×10^{-4}. Critically this is also true of the denatured protein and of short lengths of peptide containing the chromophore. This result is of course in sharp contrast to wtGFP where the quantum yield for emission is about 0.8;[1] evidently the folded protein structure has a profound effect on the radiationless decay of the chromophore. Significantly a number of the more recently discovered FPs which contain the same basic chromophore as wtGFP, and share essentially the same β-barrel structure, are in fact non-fluorescent[30,44] (so FP is something of a misnomer, and chromoprotein is preferred). In a number intriguing and very important cases (see below) the protein may be optically switched between fluorescent and non-fluorescent states.[45] Clearly then it is critical to develop a good understanding of the factors which influence the quantum yield of the chromophore, and the ideal starting point is the isolated chromophore itself, HBDI, which can be prepared synthetically.[43]

The electronic absorption spectrum of HBDI is shown in Fig. 2 in neutral, acidic and basic conditions. The most red shifted absorption is for the anion, which is deprotonated at the phenolic oxygen. The next longest wavelength transition is associated with the cation, protonated at the unsubstituted

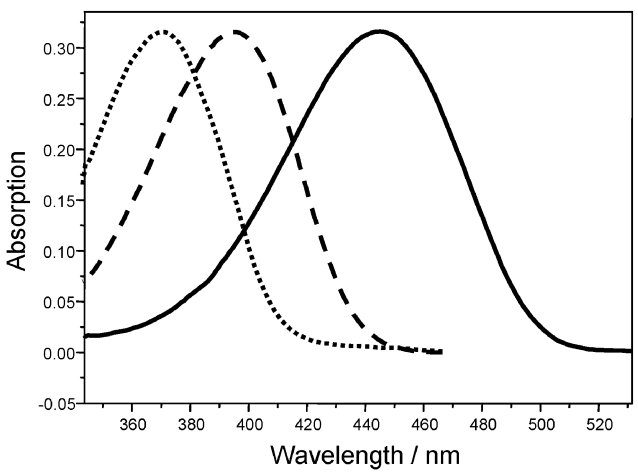


Fig. 2 The absorption spectra of HBDI in neutral (dots) acidic (dash) and basic (solid) solutions.

heterocyclic N atom. The highest energy transition is associated with the neutral protonated form.[46,47] While the pK_a for deprotonation of HBDI to the anion is 8.5 that for protonation is 2.5, which is too low for the cationic state to play a significant role in the protein.[47–49] Thus only neutral, anion and possibly a zwitterion state need be considered.[50] A further significant feature is that all of these transitions in aqueous solution are substantially blue shifted compared with the analogous charge state of the chromophore in wtGFP, which already points to a strong protein–chromophore interaction.[51]

The excited state decay of HBDI in solution has been studied in detail. Both ultrafast polarisation spectroscopy and transient absorption spectroscopy show that after excitation of neutral or anionic HBDI in aqueous or alcohol solutions the ground state is repopulated on a sub-picosecond timescale, so the radiationless decay can be assigned to ultrafast internal conversion (IC).[52–56] The excited state decay of HBDI in alcohol solutions was measured by fluorescence up-conversion.[57,58] The decay is non-single exponential, but the dominant component was measured as only a few hundred femtoseconds, consistent with the very low fluorescence quantum yield. The fact that the excited state decay is slightly faster than the ground state recovery suggests that there may be a very short lived intermediate state in the ground state repopulation pathway, but that intermediate may simply be a vibrationally hot form of the ground state. These measurements were extended to all different charge states of HBDI.[53,57] Essentially the same behaviour was observed—ultrafast decay by IC. The S_1 state of the anionic form is however longer lived than the neutral form by about a factor of two.

Solvent effects on the absorption spectra of HBDI have been investigated.[48,59] In general a larger effect of solvent polarity is observed for the charged than for the neutral form, with the red shifted anion spectrum being the most solvent sensitive. In all solvents the quantum yield is low. In particular pH ranges the zwitterion was observed for a methylated HBDI derivative, with a larger red shift compared even to the anion; however, it is not particularly stable.[59] There is no simple explanation for the solvent effects. In general, H-bonding solvents cause larger spectral shifts than non-H-bonding ones (with aqueous solutions usually having the most blue shifted absorption). However, the exception is the HBDI anion, which has an anomalously red shifted absorption in DMSO and DMF.[48,59] The latter result is interesting in that this represents the only solvent–charge combination which comes close to reproducing the large red shift seen for the chromophore in the protein environment. The significance of this result is unclear, as the shift is not accompanied by a significant increase in quantum yield, and the chromophore in the protein certainly forms H-bonds with surrounding residues. There is no single solvent function (polarity, π*-scale, $E_T(30)$) which adequately fits all the data for HBDI. However, in a detailed study Dong *et al.* were able to obtain a reasonable fit to the data using a combination of solvent acidity, basicity and polarity functions.[59]

The solvent dependence of the IC has also been investigated.[57,58] In all fluid solvents at room temperature the excited state decay of HBDI remains ultrafast, consistent with the absence

of fluorescence, and is non-single exponential. The lifetime of the neutral form appears slightly lengthened in very non-polar solvents, and in aqueous solution both neutral and anion have shortened decay times; however these effects are slight and in all fluid solvents IC dominates the excited state chemistry.

The effect of solvent viscosity has also been studied.[52,53,60,61] One plausible mechanism for radiationless decay in HBDI is an excited state isomerization reaction. For related molecules such behaviour is well characterised in solution, *e.g.* for stilbenes,[62,63] azobenzenes[64] and cyanine dyes.[65] Essentially the decreased bond order for the bridging double bond in the excited ($\pi\pi^*$) state allows nearly free rotation, which increases the ground state energy. At some point on this rotational coordinate the ground and excited state potential surfaces approach or cross at a conical intersection, where rapid deactivation to the ground state occurs. In this simple case the rotational motion on the excited state potential energy surface involves large scale structural reorganisation (*e.g.* a *cis–trans* isomerization) which is opposed by solvent friction. Thus by making measurements of the excited state lifetime as a function of solvent viscosity (*i.e.* by making the hydrodynamic approximation that the microscopic friction experienced by the molecular motion correlates with macroscopic viscosity, which works quite well for the orientational motion of small molecules in fluids[66]) it is possible to extract information about the nature of the coordinate promoting IC. The mean excited state lifetime for anionic HBDI in methanol is 0.5 ps while in ethylene glycol it is 1.6 ps; a forty-fold increase in viscosity causes only a three-fold increase in excited state lifetime.[53] This result suggests that the coordinate promoting IC in HBDI is not very sensitive to solvent friction, so is unlikely to involve a large scale structural change displacing significant volumes of solvent, such as a complete rotation about the exocyclic double bond.

On changing the solvent to modify the viscosity other parameters such as H-bond donation ability and polarity may also change. In principle these may also influence the rate of reaction, for example by modifying the height of any barrier along the reaction coordinate. An alternative route to varying viscosity is through its temperature dependence, in which case only a single solvent is involved. The difficulty with this approach is that the temperature dependent viscosity (which is roughly exponentially activated at temperatures sufficiently far above the glass transition) may be mixed with the exponentially activated reaction rate constant arising from a barrier in the reaction coordinate. The solution to this conundrum is to pick a series of similar solvents and to make measurements at a set of temperatures chosen such that the similar solvents have the same viscosity—an isoviscosity analysis.[67] In that case the temperature dependence observed reflects the activation energy. Assuming the activation energy to be temperature and solvent independent in a given series of solvents then permits an assessment of the viscosity dependence. For HBDI this procedure leads to the conclusion that the reaction is effectively barrierless and is only weakly dependent on viscosity (specifically an $\eta^{0.25}$ dependence).[53] Such a weak dependence suggests that the coordinate promoting IC either does not displace a large solvent volume, so that it is not sensitive to macroscopic friction, or that the potential is not only barrierless, but sufficiently strongly downhill as to be able to substantially overcome solvent friction. In this sense HBDI is more similar to the excited state reaction of *cis* stilbene,[68,69] which is ultrafast and has a weak viscosity dependence, but rather unlike diphenyl- and triphenylmethane dyes,[70] which are barrierless but viscosity dependent, or *trans*-stilbene, which has both a barrier and a viscosity dependence.[71]

A number of measurements on HBDI have been conducted as a function of temperature. It was shown by Niwa and co-workers that strong fluorescence was recovered in HBDI by cooling it in a glass to 77 K.[43] Subsequently Webber *et al.* found that a significant fluorescence yield was recovered in alcohol solvents only at temperatures approaching and below the glass transition temperature—*i.e.* where the viscosity is extremely high.[54] Further, the fluorescence intensity is not exponentially activated over the entire temperature range, but shows a stronger temperature dependence at lower temperature (or in more viscous media), Fig. 3.[53] It was suggested that this change in temperature dependence represents a change in the structure and dynamics of the viscous glass forming solvent.[53]

The requirement for a very high viscosity to recover strong fluorescence is consistent with a barrierless volume conserving

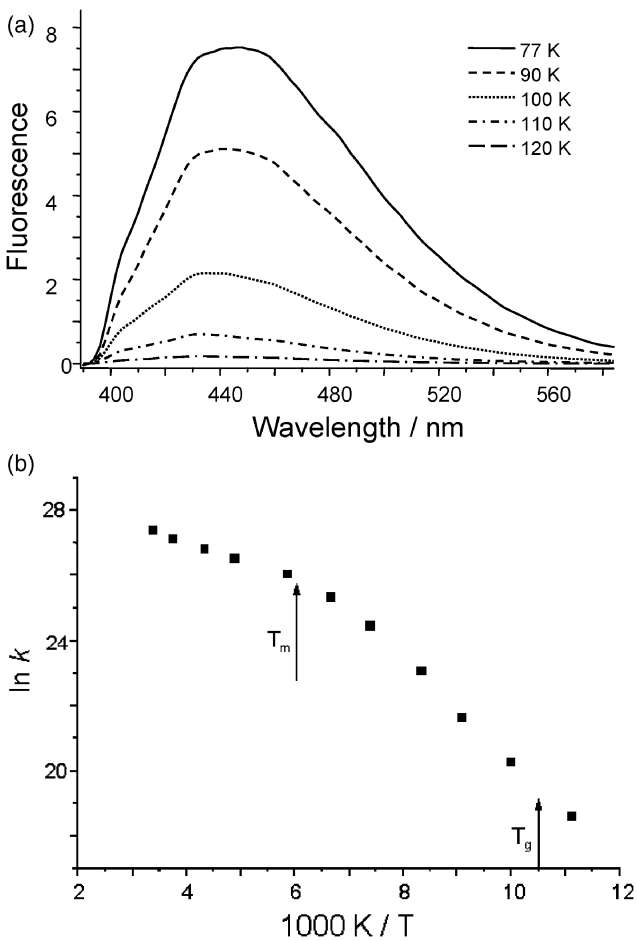


Fig. 3 The temperature dependent fluorescence of HBDI in ethanol. Note the enhancement of fluorescence appears only on approaching the glass transition (a) and does not show a simple Arrhenius dependence (b).[53,54]

coordinate promoting IC. In most measurements conducted to date both low temperature and high viscosity are required to promote HBDI fluorescence, it being non-fluorescent in a PMMA matrix at room temperature for example.[53] However, the synthesis of bridged analogues of HBDI which suppresses rotation about the exocyclic double bond was shown to lead to a large enhancement in the fluorescence quantum yield.[72] Interestingly emission from the crystal phase has recently been reported for some HBDI analogues.[73] Huppert and co-workers analysed in detail the temperature dependent fluorescence lifetime and lineshape for HBDI in glycerol–water solvent.[60] They proposed a distribution of conformers about the phenyl single bond, and were able to model the data assuming thermal activation of a phenyl twisting coordinate, suggesting that this mode specifically is involved in promoting radiationless decay. An inhomogeneous distribution of conformers in the ground state at low temperature is consistent with the observed effect of red edge excitation on the fluorescence spectrum.[48]

There have been a number of reports of modifications to the chemical structure of the isolated HBDI chromophore in an effort to modify its photophysics and to model the effect of mutations in the chromophore-forming residues of GFP. Litvinenko *et al.*[53] studied the phenyl derivative, in which the OH group is replaced by an H-atom (analogous to the blue fluorescent protein $Y66F^1$) and found that it showed essentially the same fast excited state IC as HBDI itself (albeit with the expected differences in the pH dependence of the electronic spectra[48]). Recently Chen *et al.* showed that the *o*-hydroxy derivative of HBDI had a strongly red shifted emission spectrum compared to the *p*-hydroxy form.[74] They assigned this new emission to intramolecular proton transfer between the *o*-OH and the N atom on the imidazolinone ring. This is particularly interesting as it is the first report of proton transfer for the HBDI chromophore in solution (whereas proton transfer dominates the photophysics of wtGFP, see below). Recently Solntsev *et al.* reported that the *m*-OH derivative of HBDI exhibits a longer fluorescent lifetime than *p*-OH HBDI, and that this form is able to support intermolecular proton transfer.[75,76] Yang *et al.* have replaced the phenyl *p*-hydroxy group with an amino group at either the *ortho* or *meta* position.[77] This led to two interesting observations concerning the previously reported[78] photo-isomerization of HBDI. First that substitution greatly changed the solvent dependence of the fluorescence yield for the *m*-amino derivative, and also that the yield of *cis–trans* (or *Z–E*) isomerization was strongly dependent on solvent for both HBDI and the amino derivatives. In HBDI the yield of formation of the *E* isomer depended particularly on the H-bonding ability of the solvent. This was interpreted as indicating an excited state reaction leading to IC which involves rotation about the imidazolinone double bond (generating the *E* isomer) in non-H-bonding solvents. In contrast, in aqueous and alcohol solvents H-bonding interactions mediated the ultrafast IC, leading to a lower yield of the isomer in the ground state. The unexpectedly facile ground state $E \rightarrow Z$ thermal isomerization reaction observed in some solvents was investigated by Dong *et al.*, who proposed that a chemical mechanism (nucleophilic substitution and subsequent elimination) at the bridging carbon atom may be an important process.[79] This mechanism has potentially important implications for the mechanism of operation of photoactive FPs discussed below.

Clearly some more detailed knowledge of the reaction coordinate leading to IC is essential to understand (and control) both the fluorescence enhancement mechanism in wtGFP and the strong variation in quantum yield among GFP mutants and the broader family of FPs. Experiment has been able to characterize the mechanism of radiationless decay, the barrier height, the volume of rotation and identify the formation of new ground state isomers. However, a more detailed insight requires measurement to be complemented by theoretical calculations.

Weber *et al.* considered the energetics of an excited state isomerization reaction involving the bridging bonds of HBDI *via* a 90° rotation about three possible coordinates for three different charge states of the model chromophore.[80] They found that only twisting about the imidazolinone double bond was barrierless for both neutral and ionic states (consistent with experiment) but it did not lead to a crossing of ground and excited states for the anion. They also considered the so called 'hula twist' coordinate originally introduced by Liu and Hammond.[81] This is a volume conserving route to isomerization, and was thus proposed to be consistent with the observed weak dependence of fluorescence lifetime on solvent friction.[53,57] However, it was found that this coordinate only leads to an S_0–S_1 crossing in the HBDI anion *via* a significant energy barrier, in disagreement with the observed barrierless IC mechanism.[80] Voityuk and co-workers considered both of the single bond twist coordinates for the neutral, anionic, cationic and zwitterionic states of HBDI, and found that S_0–S_1 crossing only occurred in the cation, in contrast to observation of a fast internal conversion independent of charge state.[82]

Subsequent theoretical calculations which have built on these early works have considered a variety of plausible reaction coordinates as well as conducting searches for minimum energy pathways and conical intersections between ground and excited states (which may involve motion on more than one coordinate).[83–89] There is now quite broad agreement that major contributions to the coordinate promoting IC arise from zero or low barrier single bond rotation, possibly coupled with a degree of pyramidalization at the bridging carbon atom. In many cases ground and excited states approach or intersect at a 90° twist angle. Pathways involving the 'hula twist' motion are also found to lead to fast IC but only *via* a significant energy barrier. There are conflicting conclusions as to which single bond rotation is most significant in the excited state isomerization. Olivucci and co-workers using solvent free conditions found that two coordinates are important in achieving close approach of S_0 and S_1—a fast stretching coordinate, corresponding to reduced bond order, and rotation about the phenolic single bond.[85] This interpretation is consistent with the model of Gepshtein *et al.* accounting for the temperature dependent HBDI fluorescence,[60] and is in line with calculations of the narrowing of the angular distribution about the phenyl single bond when the chromophore is incorporated into the protein.[90] In contrast

when Altoe *et al*. included a polarizable continuum model of the solvent in their calculations they found that rotation about the imidazolinone double bond was most effective in promoting IC.[83] Martinez and co-workers also identified an important role for the medium in their study of the model chromophore.[86–88] In vacuum, the excited state dynamics primarily involved twisting about the bridging double bond but with a large excursion in the phenyl torsion. These calculations predicted a significant lifetime on the excited state surface. In contrast, the calculation for the water solvated chromophore predicted fast barrierless rotation about the imidazolinone double bond leading to an S_1–S_0 conical intersection, and a sub-picosecond excited state lifetime, as found experimentally. These results point clearly to a significant role for the medium in determining the coordinate leading to IC, which is itself an important conclusion, as it suggests one means by which the protein can modulate the chromophore photophysics.

Thus, calculations which include some representation of the solvent medium suggest that an isomerization about the exocyclic double bond plays a critical role in the excited state decay of HBDI. These calculations reproduce the ultrafast IC observed in experiments. They are also consistent with the picture of photoinduced $Z \rightarrow E$ isomerization in HBDI. However, a simple out-of-plane twist along this coordinate does not appear volume conserving, and thus might be expected to exhibit a stronger dependence on medium viscosity than is in fact observed. This could be explained by a number of factors, for example if the coordinate is strongly downhill, if the S_1–S_0 intersection at which IC becomes efficient occurs earlier on the twisting coordinate than calculated, or if the location of the crossing point itself is solvent dependent. Recent results on HBDI derivatives suggest that the solvent may be more intimately involved in the excited state isomerization than the present calculations allow for.[77,79]

It can be seen from the above that significant progress has been made in describing the mechanism of radiationless decay in HBDI. However the critical question of how the protein suppresses radiationless decay in the chromophore is still to some extent unresolved. Geometric constraint of the isomerization coordinate is an obvious possibility, and is consistent with the observed enhancement of HBDI fluorescence in low temperature glasses.[43,54,60] Calculations suggest that constraints of the single bond rotation of the phenyl ring in the protein may be significant,[86] although it is not certain that this coordinate is a major contributor to the IC (see above). However, such simple packing arguments are not adequate to account for the full range of photophysics exhibited by FPs. For example some proteins with quite similar structures have rather different quantum yields.[91] Further, the fact that the same protein can be optically switched between fluorescent and non-fluorescent states suggests that some additional factors operate.[45] For example Coulombic effect may be important in the charged protein environment. Quantum chemical calculations suggest that the HBDI excited state has a degree of intramolecular charge transfer character.[87] Thus charged residues may interact to stabilise or destabilise the excited state (depending on their charge and location). If the isomerization is accompanied by further intramolecular charge transfer (reminiscent of the twisted intramolecular charge transfer, or TICT, mechanism[92]) then the effect of an adjacent charged residue may be to alter the shape of the excited state potential energy surface. There are as yet no conclusive calculations concerning this possibility, although charge effects have been considered in some cases.[93] An additional factor likely to be significant in controlling the excited state chemistry of the chromophore in the protein is the nature and extent of the hydrogen bonding interactions. The anionic form of the chromophore, which dominates the fluorescence in wtGFP (see below) may form as many as five H-bonds with surrounding amino acid residues and structural water molecules. These will influence the friction experienced by the isomerization coordinate, since some of the H-bonds must be broken during rotation about the bridging bond. In addition such strong interactions can modify the electronic structure of the chromophore, and may be implicated in the large shift observed in the absorption spectra in the protein compared to HBDI in solution. Changes in electronic structure could themselves lead either to the appearance of barriers in the isomerization pathway, or to changes in minimum energy pathways, perhaps directing the excited state away from conical intersections with the ground electronic state.

3. Photophysics and proton transfer in wtGFP

The electronic absorption spectrum of wtGFP consists of two bands at *ca*. 395 nm and 480 nm, named the A and B bands respectively (Fig. 4). Excitation of either results in intense green emission with a peak at around 510 nm. Although wtGFP itself is relatively insensitive to pH, a study of the S65T mutant showed that these two bands are associated with neutral and anionic forms of the chromophore.[94] The protonated (A) and deprotonated (B) nature of these transitions was confirmed by structural studies,[94] spectroscopic measurements[47,95] and studies of the effects of pH on the model chromophore.

Boxer and co-workers reported the time resolved fluorescence of wtGFP with ultrafast time resolution.[96] The directly excited

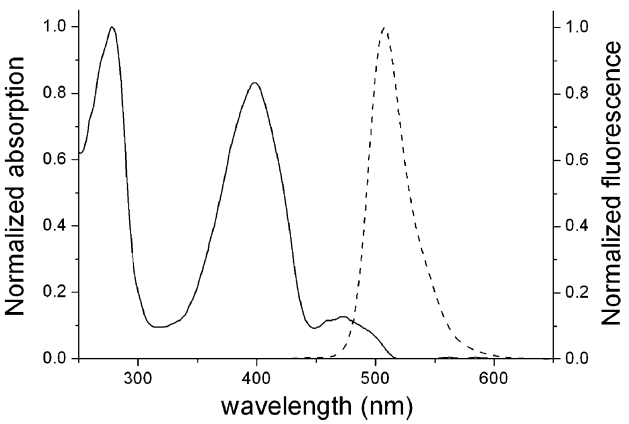


Fig. 4 The absorption spectrum of wtGFP, with the A and B bands clearly shown and the emission dominated by the anionic I* state. (Concentration 0.1 mM, absorbance uncorrected for solvent background and sample scatter.)

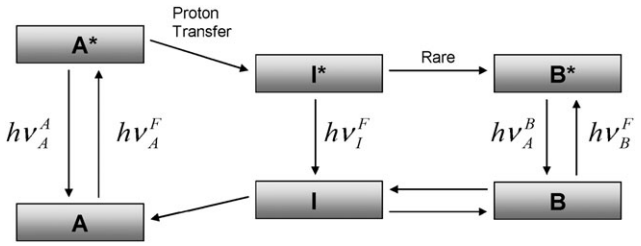


Fig. 5 The scheme for interconverting states of wtGFP introduced by Chattoraj *et al.*[96] This seminal but illustrative scheme has proved extremely useful in GFP research, although more detailed assignments may be obtained from low temperature measurements,[100] and the rate or existence of the reverse reaction B → A is much debated.

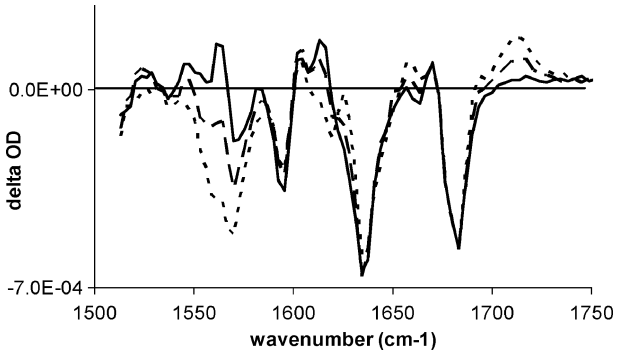


Fig. 6 Transient IR difference spectra of wtGFP in D_2O at 4 (solid), 30 (dash) and 200 (dotted) ps after excitation.

A* state decays in a non-single exponential fashion (up to three exponential terms are required to fit the data) with a mean lifetime of 18 ps. Significantly the 510 nm fluorescence was observed to grow-in on a similar picosecond timescale. This result points to the occurrence of an excited state proton transfer (ESPT) reaction, which is unique in biology (though extremely well characterised in simpler chemical systems[97]). This assignment was confirmed by the observation of a large deuterium isotope effect, which extended the A* state lifetime and correspondingly increased the risetime for the green emission.[96] Similar observations were made combining ultrafast fluorescence with transient absorption spectroscopy.[98]

Since the population of the anionic (B) ground state does not increase rapidly as a result of irradiation it is evident that the main fate of the deprotonated excited state is fluorescence followed by re-protonation to yield the A state. Chattoraj and co-workers proposed a model which incorporates this behaviour (Fig. 5), where the emissive (deprotonated) state (I*) is in the geometry of the original ground state, and following relaxation by fluorescence returns to the A state.[96] Subsequently ultrafast pump–dump–probe spectroscopy revealed the I → A proton transfer dynamics on the ground state surface.[99] It was proposed that the B state is populated by a reorganisation of the protein matrix about I* occurring with a low probability.[96] The X-ray structures of the A and B states suggested that the reorganisation involves T203 reorientation.[94] Low temperature spectral hole burning measurements gave further details on the energies of these electronic states and the transformations between them.[94,100,101]

The location of the proton acceptor was investigated by time resolved vibrational spectroscopy, as described in more detail elsewhere in this themed issue by van Thor. The A state of wtGFP was optically excited and the transient vibrational spectrum monitored with picosecond time resolution between 1500 and 1800 cm^{-1}.[102–105] The data are displayed as IR difference spectra (pump on minus pump off) as a function of time after excitation (Fig. 6). The instantaneous appearance of 4 strong bleach bands (negative ΔOD) is associated with excitation of the chromophore, and these are accompanied by some positive ΔOD signals, formed within the sub-picosecond experimental time resolution, which can be associated with chromophore excited state absorption. These bands were assigned by analogy with HBDI and through polarisation spectroscopy and isotopic labelling of both HBDI and the protein.[103] Although the main bands can be assigned to the chromophore the temporal evolution of their shape does suggest additional underlying contributions from the protein.[103] The origin of some of these protein modes is revealed by mutagenesis.[105] The proton transfer dynamics are evident in the two time dependent bands—a bleach becoming apparent at 1560 cm^{-1} and a new transient absorption above 1700 cm^{-1}. These bands, which evolve on the same picosecond timescale, may be assigned to the conversion of a carboxylate to a carboxylic acid.[106] Inspection of the structure of wtGFP suggests that this can be ascribed to protonation of the residue E222, which, as first shown by Brejc *et al.*,[94] is connected to the proton donor by a proton wire *via* a structural water molecule and the S205 residue. Thus, transient IR spectroscopy confirms the proposed assignment of the E222 residue as the proton acceptor. A comparison of the fluorescence decay with the vibrational dynamics shows that donor decay and acceptor protonation occur simultaneously, suggesting a concerted mechanism for proton motion, or at least that any intermediates are very short lived.[102] The assignment to protonation of E222 has been supported by studies of mutants, polarisation resolved measurements,[107,108] isotopic labelling, mutagenesis[105] and observations over a wider spectral range.[103,108] The non-exponential dynamics observed in both fluorescence and transient IR suggest dispersive kinetics, which observations over a wider spectral range were able to assign to side chain disorder leading to different proton transfer rates.[108]

Although accurate quantum chemical calculations of excited states in proteins remain challenging there has been some significant progress in modelling the ESPT in wtGFP. In an early work Lill and Helms employed classical MD to simulate the proton transfer along the three step proton wire.[109] They concluded that after the transfer was triggered by ejection of the proton from the chromophore the steps leading to protonation of E222 occur on the tens of femtosecond timescale. Subsequently quantum chemical calculations have been reported by two groups using the geometry of the proton transfer chain suggested by the protein structure, but in the absence of surrounding residues.[110–112] Both groups calculated that the proton transfer occurred in a single concerted step along a single low barrier potential surface, with no stable intermediate states. The calculations also suggested that the first proton to move in the concerted process was the last in the chain (*i.e.* the step S205 to E222).

Zhang *et al*. found that the H148 residue, which is not part of the proton transfer chain but is H-bonded to the donor O atom, had a significant impact on the potential surface, suggesting an important role for the surrounding residues.[112] This in turn suggests that modelling the proton transfer in the protein on a potential energy surface based on only a few residues may not lead to quantitatively correct reaction dynamics.

More recently, Lluch and co-workers[113–115] used molecular dynamics to study the structure and stability of the proton relay chain in wtGFP, and then performed quantum chemical calculations on a reduced set of residues, using the geometries obtained from MD simulations.[113] This is expected to give a more realistic structure than adoption of the minimum geometry required to create the proton transfer chain from crystallographic data. Importantly they investigated the proton transfer surface for both ground and excited states of the chromophore, and found that the photoactive state was $\pi\pi^*$ rather than the $\pi\sigma^*$ implicated in some other proton transfer reactions.[116] The potential energy surfaces were calculated to have minima for the proton localized on the chromophore in the ground state and on E222 in the excited state, in agreement with experiment. The excited state proton transfer has a small barrier (*ca*. 2 kcal mol^{-1}) and is strongly downhill for the S203 to E222 step, as was also found in earlier calculations. This is consistent with a concerted but asynchronous proton transfer, with the last proton 'leading'.[113] These data certainly show the potential for the application of quantum chemical calculations in modelling ESPT in GFP, but many challenges remain, particularly in accounting for the measured picosecond timescale of the dynamics, the isotope effects and the influence of mutagenesis. However, these preliminary results and recent progress in modelling excited electronic states is encouraging.

4. Manipulating the proton wire through mutagenesis

The concerted three step ESPT reaction in wtGFP presents a unique example of photoactivated proton transport on a proton wire in a protein. Thus, in addition to the intrinsic interest in GFP it affords experimentalists the opportunity of studying the dynamics of proton wires, an area of interest in both protein and materials science.[117] In terms of protein science, proton wires are proposed to have prominent roles in transmembrane proton transport[118] and in allowing information to be transferred from site to site within a protein.[119] In an effort to study the dynamics of such protein proton transfers a number of mutants have been constructed which modify the proton wire compared to wtGFP. These mutants have been investigated through X-ray structure determination, ultrafast fluorescence and time resolved vibrational spectroscopy. This data set will, especially when complemented by high level quantum chemical calculations, provide a unique insight into proton transfer in proteins.

The mutation of residues directly involved in the proton relay chain is expected to have the largest effect on the ESPT dynamics. Shu *et al*. studied the S205V mutant and showed

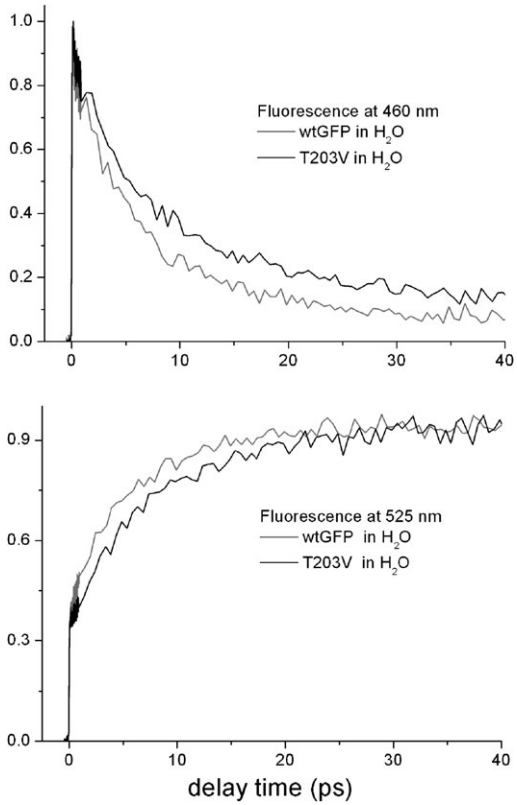


Fig. 7 Time resolved fluorescence of the A* (upper) and B* (lower) emission from T203V following excitation at 410 nm (grey) compared with wtGFP (black).

that the proton transfer reaction is slowed considerably with respect to wtGFP, and becomes more sensitive to deuteration.[120] The structural changes resulting from this mutation involve changes to the position and orientation of the E222 acceptor and the T203 residue, which is H-bonded to the structural water in the proton wire in wtGFP; this H-bond appears to be considerably weakened in S205V.[120] Unfortunately transient IR data have not yet been reported for this mutant. Interestingly the double mutant S205V/T203V essentially blocks the ESPT reaction, such that the chromophore is largely trapped in the A* state—a blue fluorescent protein.[120] This shows that mutations are certainly not additive in their effect since the single T203V mutation is known to have a relatively small effect on the ESPT reaction,[91] only slightly slowing the fluorescence decay compared to wtGFP (Fig. 7).[121]

The S65T mutation has been very significant in applications of GFP. In particular S65T GFP mutants are much more sensitive to pH. This arises because the modification of the connection between the S65 side chain and E222 causes E222 to translate and reorient compared with wtGFP.[122] The structure data (and the electronic spectra) show that at pH < 6 both the E222 acceptor and the chromophore phenyl group are protonated. This clearly has the effect of blocking transfer down the proton wire of wtGFP (since much of the driving force is associated with the final transfer to E222[113]) and the electronic spectra are consistent with this; excitation of the neutral form (390 nm) results in a blue shifted A state emission.[123]

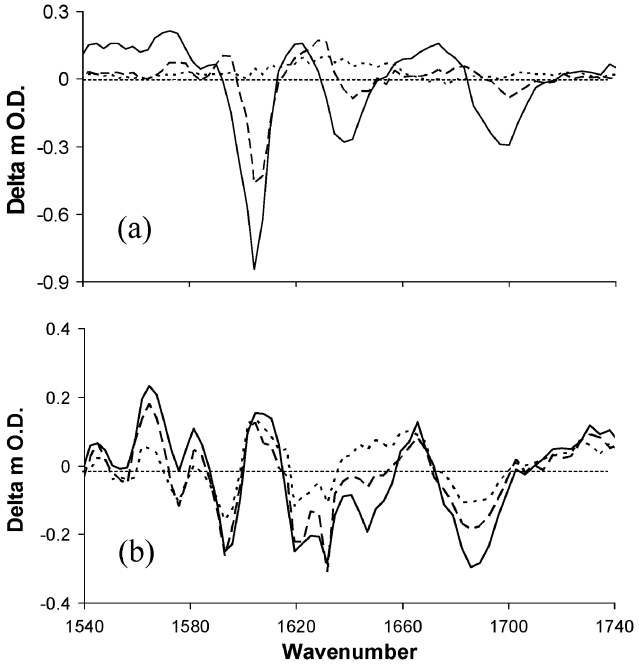


Fig. 8 Transient IR difference spectra of (a) HBDI in DMSO at 2 (solid), 6 (dash) and 30 (dotted) ps after excitation and (b) S65T GFP at pH 5.5 at 6 (solid), 30 (dash) and 100 (dotted) ps after excitation.

The photophysics of S65T GFP mutants trapped in the A state have been reported.[124–127] Common features are a very broad and asymmetric blue shifted emission spectrum, with an excited state decay time of only a few hundred picoseconds. This is in sharp contrast to the narrow slightly structured emission of I* which is associated with a fluorescence lifetime of a few nanoseconds. The short lifetime of these blue FPs is reminiscent of (though still much longer than) that found for the HBDI chromophore in solution. The broad emission spectrum of blue FP is consistent with a change in the equilibrium structure between ground and excited electronic states.

Two blue FP mutants have been investigated by time resolved vibrational spectroscopy, namely S65T GFP and the triple mutant S65G/T203V/E222Q (called blGFP) both at pH 5.5.[127] Since the ESPT reaction is blocked, a similar transient IR spectrum to that observed for neutral HBDI might be predicted. This is not the case (Fig. 8). The spectra for HBDI show three clear ground state bleach modes which were assigned to the carbonyl stretch, the bridging double bond and a phenyl ring mode.[103] The equivalent excited state modes can also be seen slightly red shifted, broadened and with lower intensity. The time evolution is ultrafast (as expected for HBDI, see above) with the ground state bleach being filled on a picosecond timescale and a complicated lineshape for the transient absorption, reflecting relaxation from the excited state surface to generate a vibrationally hot ground state.[103] Comparing this with the spectra for S65T GFP (Fig. 8) the carbonyl and phenyl ring bleach modes can still be identified, assignments which were confirmed by polarisation spectroscopy.[127] However, between these two modes, where a single bleach is seen in HBDI, a broad and complex bleach pattern emerges, with at least three overlapping peaks apparent. In addition a new transient absorption appears between 1560 cm^{-1} and 1580 cm^{-1}. The final noteworthy feature in Fig. 8 is the lack of a time dependent evolution in the spectral profile—all that is observed is an induced bleach/absorption followed by a recovery, which is consistent with the ESPT reaction being suppressed, resulting in a dominant A* to A relaxation channel. Very similar behaviour was seen for the blGFP mutant, although the excited state lifetime (and so bleach recovery time) is longer.[127]

Neither the transient absorption nor the complex multi-component bleach have an equivalent in wtGFP (Fig. 6) even at early times, before ESPT has developed and the A* state is expected to dominate the spectrum. In the absence of equivalent modes in neutral HBDI these new transitions in blue FPs were assigned to perturbations of the protein matrix by the excited electronic state of A*. Specifically the excitation appeared to shift the protein modes to lower frequency (*i.e.* from 1620 cm^{-1}–1660 cm^{-1} to near 1570 cm^{-1}). A number of modes may contribute in this region of the spectrum, including the amide I modes and NH_3^+ deformation.[106] The spectral shift observed suggested a strong interaction between the excited electronic state and the matrix in the blue fluorescent mutants, perhaps due to frustrated attempts at proton transfer, or to electrostatic interactions between the amino acid residues and the excited state dipole moment of A*.

The following pattern emerges in the excited state decay times. For all charge states of HBDI in solution an ultrafast excited state decay is observed. The neutral A* state in the protein has a lifetime hundreds of times longer than neutral HBDI, but still 10–100 times shorter than for I*. Further, at least in the mutants in which ESPT is blocked, there appears to be a strong interaction between the A* state and the protein, causing some reorganisation of the protein vibrational structure. This is in contrast to direct excitation of the I* state, which does not lead to intense new modes in the transient vibrational spectrum.[102,105] It appears then that the wtGFP matrix is well adapted for accommodating the emissive I* state, while the A* state need only be stabilized for times long enough for the proton transfer to take place.

The S205 and T203 mutations allow a perturbation of the GFP proton wire, while the S65T and related mutants allow, under some circumstances, the breaking of that wire and consequent blocking of the ESPT. It has also proved possible to add an additional mutation to redirect the proton transfer. Specifically the mutation H148D places a new proton acceptor in the vicinity of the phenolic proton donor of the chromophore, suggesting the possibility of redirecting proton transfer to a D148 acceptor.[122,128–130]

The spectroscopic consequences of this S65T/H148D double mutation are large. The A → A* absorption is shifted to the red by 20 nm, appearing at 411 nm, while the emission is at 508 nm, *i.e.* characteristic of emission from the I* state.[128–130] This suggests that a new route for proton transfer has indeed been created, overcoming the disruption due to S65T and low pH. The structure data for the double mutant are significant. They show that the proton wire found in wtGFP has been reformed, but also that the carboxylate

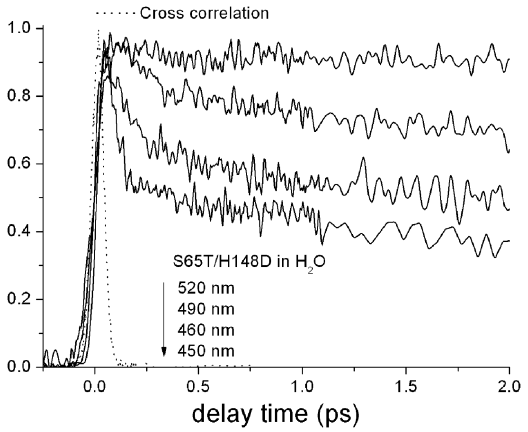


Fig. 9 Time resolved emission of H148D/S65T GFP on the blue and red edges of the emission spectrum. Time resolution indicated by the cross correlation (dotted).

oxygen of D148 lies very close (0.23 nm) to the phenolic oxygen of the chromophore.[129] This corresponds to the formation (at least potentially) of a short strong H-bond. Such short H-bonds are of particular interest in proteins since they have been proposed (albeit controversially[131,132]) to be involved in a number of enzyme catalysed reactions.[133,134] Whether or not this is the case, it is evident that short H-bonds are a common motif in a number of enzymes. Thus S65T/H148D GFP has the potential to act as a model for proton transfer dynamics on such strong short H-bonds.

When the donor and acceptor distance is short, and the donor and acceptor pK_a are similar, the barrier to proton transfer is predicted to be low.[135] Boxer and co-workers investigated the time resolved fluorescence of S65T/H148D GFP and found that the green fluorescence from the anion was formed within the 175 fs time resolution of their experiment.[128] Recently our group refined the ultrafast fluorescence experiment to achieve a sub 70 fs time resolution.[136] In that case it has proved possible to time resolve the proton transfer, which does indeed evolve on a sub 100 fs timescale (Fig. 9).[137] The observed kinetics do not follow those expected for a two state mechanism, but rather suggest temporal evolution on a single, complex and essentially barrierless potential energy surface.[137] The S65T/H148D GFP has also been investigated by transient vibrational spectroscopy.[130] The formation of a distinct carbonyl mode associated with the proton acceptor was not observed. Rather the transient data suggested a simple excited to ground state relaxation. This is also consistent with the consequences of formation of a low barrier H-bond, where donor and acceptor are not really distinct, and the differences between ground and excited state of this system are mainly due to translational movement of the shared proton.

5. Second generation fluorescent proteins

In the past ten years a rich and varied photochemistry has been uncovered for GFP and (especially) the wider family of FPs.[138] Typically photochemical reactions are associated with photobleaching, the loss of fluorescence under prolonged irradiation, and are thus regarded as a negative feature in a fluorophore for bioimaging. However, photochemistry in FPs has recently been shown to have a number of extremely important benefits, and underlies some of the most exciting developments in imaging applications. Among the first examples of GFP photochemistry was the photoconversion of the A to B state in wtGFP on prolonged irradiation around 400 nm (A* excitation) and, with greater efficiency, in the ultra-violet. It was shown that this photoconversion arises from a low yield photoexcited electron transfer reaction in GFP which leads to photodecarboxylation of the E222 residue.[95,139] This creates an unstable intermediate which can extract a proton from its surroundings, which then allows the chromophore to permanently donate its proton and thus form the B state. The overall effect is to create photochemically the B state, which has a higher fluorescence yield than A, and a more convenient wavelength for laser excitation. Thus, arguably, the A → B photoconversion is a positive benefit in fluorescence imaging.

Much more efficient photochemistry has been reported in FPs derived from coral. The protein kaede undergoes an efficient green to red emission transformation on irradiation of its protonated form, thus creating a new colour not present in the unirradiated sample.[140] This has been used as an 'optical highlighter' in bioimaging, allowing a spatially localised area of the sample to be converted from green to red emission. The subsequent spatial evolution of this specific population can be followed in real time. The mechanism operating in the kaede photoconversion was investigated by Miyawaki and co-workers.[141,142] It involves a light initiated C–N bond cleavage in the original (protonated) chromophore followed by a secondary reaction which results in an extended π-electron system, leading to the observed red shift. Subsequent experiments demonstrated the design of an improved optical highlighter (kikGR) through mutagenesis and structural studies.[44,143]

A second important class of photochemically active fluorescent proteins are photoactivatable FPs. It was known that a number of naturally occurring proteins have the basic β-barrel structure of FPs and form the chromophore, but are nevertheless non-fluorescent.[139,144] It has been found that some of these proteins (called chromoproteins, CPs) can, under continuous irradiation, be converted to a fluorescent form.[145,146] This is a particularly important development, and indeed underpins a number of new single molecule imaging methods.[147] In these methods a single (or few proteins) in a sample where many protein labels are present can be photo-activated and imaged without interference from out of focus fluorophores. By taking advantage of the spatially localised nature of two-photon excitation for the activation step it is possible to photoselect single or few-molecule emission from a spatially localised point in the sample.[148] Such measurements would have been difficult or impossible with conventional FPs.

At least two photoactivation mechanisms appear to operate. The first, *e.g.* in a T203H GFP is essentially a more effective variant of the photodecarboxylation process reported for wtGFP, which on irradiation yields a strongly fluorescent anionic form of the chromophore.[149] The second involves a *cis–trans* isomerization in the chromophore. This is the

mechanism proposed for the so-called 'kindling' proteins, where a non-fluorescent but chromophore-forming protein converts to a fluorescent form under irradiation.[150,151] Structural studies and calculations suggest that this is a result of a *cis–trans* isomerization. There is now quite extensive evidence from crystallographic studies of switched and un-switched forms that this mechanism operates in many cases,[45] although it is possible that the isomerization is also coupled to an ESPT reaction.[152] Remarkably a number of the photoactivatable proteins also exhibit the reverse (bright to dark) process on irradiation at a second wavelength, allowing fluorescence to be turned both on and off. An early example was the coral protein dronpa (the nomenclature of FPs now requires a familiarity with topics as diverse as Japanese theatre and the colouration of soft fruits); in this case *cis–trans* isomerization is a quite natural candidate for a reversible photoprocess, being well established and widely exploited in photochromic devices.[36,153] However, in all of these cases, the mechanistic question to be addressed is why some structures undergo *cis–trans* isomerizations while others do not; this will require kinetic studies, careful structural studies of light and dark states and theoretical calculations; some progress is being made in this area.[45,154,155] This is clearly a key question to be addressed if more efficient photoactivation processes are to be developed.

6. Summary

The growing family of fluorescent proteins are firmly embedded as one of the key technologies in the life sciences. In addition they reveal a rich and complex excited state chemistry. The study of this excited state behaviour has led on to new applications. The photophysics of the basic chromophore of GFP, HBDI, have been studied in detail. The ultrafast IC which dominates its photophysics has been quite fully characterised. The mechanism promoting IC is dramatically suppressed when the chromophore is fixed in the wtGFP protein matrix. The mechanism by which this suppression occurs is not fully understood, but probably involves a combination of spatial confinement and specific chromophore–protein interactions, which both tether the chromophore and modify its electronic structure. Although the radiationless decay pathways in HBDI are suppressed in wtGFP they may underpin the mechanism of decay in the non-fluorescent CP members of the family, and be important in understanding the mechanism of photoactivation.

The wild type protein exhibits an ESPT reaction which is unique in biology. Structural and spectroscopic considerations show that the ESPT involves concerted proton transfer along a three step proton wire. This suggests that GFP may, in addition to its role as marker molecule of choice for bioimaging, also act as a nanoscale laboratory in which to investigate proton relay reactions in proteins. Mutants which modify, block and redirect the proton transfer have been structurally characterised and their excited state and structural dynamics have been recorded.

A new generation of FPs has been characterised, in which the optical properties can themselves be manipulated by optical excitation. A number of these second generation FPs have already stimulated important progress in single molecule imaging. The mechanism of the photoprocesses appears to involve a mixture of photocleavage, photoisomerization and ESPT. The detailed characterisation of the photophysics underlying these photoprocesses is a challenging, exciting and vital undertaking if the range of application of FPs is to continue to grow.

Acknowledgements

I would like to thank the students and postdocs past and present at UEA who have worked on this project. I gratefully acknowledge Peter Tonge and his group at Stony Brook University, NY, for their invaluable collaboration over many years of enlightening FP research. We have benefited enormously from access to the Laser Support Facility of the CCLRC Rutherford Appleton laboratories and the expertise of its staff. I would also like to thank Tahei Tahara and Atsushi Miyawaki, RIKEN, Japan, for many helpful conversations.

References

1 R. Y. Tsien, *Annual Review of Biochemistry*, 1998, **67**, 509.
2 A. B. Cubitt, R. Heim, S. R. Adams, A. E. Boyd, L. A. Gross and R. Y. Tsien, *Trends in Biochemical Sciences*, 1995, **20**, 448.
3 A. Miyawaki, *Developmental Cell*, 2003, **4**, 295.
4 D. Prasher, R. O. McCann and M. J. Cormier, *Biochemical and Biophysical Research Communications*, 1985, **126**, 1259.
5 M. Chalfie, Y. Tu, G. Euskirchen, W. W. Ward and D. C. Prasher, *Science*, 1994, **263**, 802.
6 O. Shimomura, F. H. Johnson and Y. Saiga, *Journal of Cellular and Comparative Physiology*, 1962, **59**, 223.
7 O. Shimomura and F. H. Johnson, *Biochemistry*, 1969, **8**, 3991.
8 J. G. Morin and J. W. Hastings, *Journal of Cellular Physiology*, 1971, **77**, 313.
9 R. M. Wachter, *Accounts of Chemical Research*, 2007, **40**, 120.
10 C. W. Cody, D. C. Prasher, W. M. Westler, F. G. Prendergast and W. W. Ward, *Biochemistry*, 1993, **32**, 1212.
11 B. G. Reid and G. C. Flynn, *Biochemistry*, 1997, **36**, 6786.
12 R. Heim, D. C. Prasher and R. Y. Tsien, *Proceedings of the National Academy of Sciences of the United States of America*, 1994, **91**, 12501.
13 D. C. Prasher, V. K. Eckenrode, W. W. Ward, F. G. Prendergast and M. J. Cormier, *Gene*, 1992, **111**, 229.
14 N. P. Lemay, A. L. Morgan, E. J. Archer, L. A. Dickson, C. M. Megley and M. Zimmer, *Chemical Physics*, 2008, **348**, 152.
15 R. M. Wachter, M. A. Elsliger, K. Kallio, G. T. Hanson and S. J. Remington, *Structure with Folding & Design*, 1998, **6**, 1267.
16 L. P. Zhang, H. N. Patel, J. W. Lappe and R. M. Wachter, *Journal of the American Chemical Society*, 2006, **128**, 4766.
17 D. P. Barondeau, C. J. Kassmann, J. A. Tainer and E. D. Getzoff, *Journal of the American Chemical Society*, 2006, **128**, 4685.
18 D. P. Barondeau, J. A. Tainer and E. D. Getzoff, *Journal of the American Chemical Society*, 2006, **128**, 3166.
19 D. P. Barondeau, C. J. Kassmann, J. A. Tainer and E. D. Getzoff, *Biochemistry*, 2005, **44**, 1960.
20 H. Niwa, S. Inouye, T. Hirano, T. Matsuno, S. Kojima, M. Kubota, M. Ohashi and F. I. Tsuji, *Proceedings of the National Academy of Sciences of the United States of America*, 1996, **93**, 13617.
21 M. Ormo, A. B. Cubitt, K. Kallio, L. A. Gross, R. Y. Tsien and S. J. Remington, *Science*, 1996, **273**, 1392.
22 N. C. Shaner, M. Z. Lin, M. R. McKeown, P. A. Steinbach, K. L. Hazelwood, M. W. Davidson and R. Y. Tsien, *Nature Methods*, 2008, **5**, 545.
23 N. C. Shaner, P. A. Steinbach and R. Y. Tsien, *Nature Methods*, 2005, **2**, 905.

24 M. V. Matz, A. F. Fradkov, Y. A. Labas, A. P. Savitsky, A. G. Zaraisky, M. L. Markelov and S. A. Lukyanov, *Nature Biotechnology*, 1999, **17**, 969.
25 M. V. Matz, K. A. Lukyanov and S. A. Lukyanov, *Bioessays*, 2002, **24**, 953.
26 V. V. Verkhusha and K. A. Lukyanov, *Nature Biotechnology*, 2004, **22**, 289.
27 S. Karasawa, T. Araki, M. Yamamoto-Hino and A. Miyawaki, *Journal of Biological Chemistry*, 2003, **278**, 34167.
28 A. Miyawaki, *Cell Structure and Function*, 2002, **27**, 343.
29 D. A. Shagin, E. V. Barsova, Y. G. Yanushevich, A. F. Fradkov, K. A. Lukyanov, Y. A. Labas, T. N. Semenova, J. A. Ugalde, A. Meyers, J. M. Nunez, E. A. Widder, S. A. Lukyanov and M. V. Matz, *Molecular Biology and Evolution*, 2004, **21**, 841.
30 Y. A. Labas, N. G. Gurskaya, Y. G. Yanushevich, A. F. Fradkov, K. A. Lukyanov, S. A. Lukyanov and M. V. Matz, *Proceedings of the National Academy of Sciences of the United States of America*, 2002, **99**, 4256.
31 S. G. Dove, O. Hoegh-Guldberg and S. Ranganathan, *Coral Reefs*, 2001, **19**, 197.
32 M. V. Matz, N. J. Marshall and M. Vorobyev, *Photochemistry and Photobiology*, 2006, **82**, 345.
33 A. M. Bogdanov, A. S. Mishin, I. V. Yampolsky, V. V. Belousov, D. M. Chudakov, F. V. Subach, V. V. Verkhusha, S. Lukyanov and K. A. Lukyanov, *Nature Chemical Biology*, 2009, **5**, 459.
34 L. A. Gross, G. S. Baird, R. C. Hoffman, K. K. Baldridge and R. Y. Tsien, *Proceedings of the National Academy of Sciences of the United States of America*, 2000, **97**, 11990.
35 R. M. Wachter, B. A. King, R. Heim, K. Kallio, R. Y. Tsien, S. G. Boxer and S. J. Remington, *Biochemistry*, 1997, **36**, 9759.
36 G. T. Hanson, T. B. McAnaney, E. S. Park, M. E. P. Rendell, D. K. Yarbrough, S. Y. Chu, L. X. Xi, S. G. Boxer, M. H. Montrose and S. J. Remington, *Biochemistry*, 2002, **41**, 15477.
37 N. C. Shaner, G. H. Patterson and M. W. Davidson, *Journal of Cell Science*, 2007, **120**, 4247.
38 X. K. Shu, A. Royant, M. Z. Lin, T. A. Aguilera, V. Lev-Ram, P. A. Steinbach and R. Y. Tsien, *Science*, 2009, **324**, 804.
39 K. R. Finley, A. E. Davidson and S. C. Ekker, *Biotechniques*, 2001, **31**, 66.
40 A. Miyawaki, O. Griesbeck, R. Heim and R. Y. Tsien, *Proceedings of the National Academy of Sciences of the United States of America*, 1999, **96**, 2135.
41 A. Miyawaki, J. Llopis, R. Heim, J. M. McCaffery, J. A. Adams, M. Ikura and R. Y. Tsien, *Nature*, 1997, **388**, 882.
42 M. Kneen, J. Farinas, Y. X. Li and A. S. Verkman, *Biophysical Journal*, 1998, **74**, 1591.
43 S. Kojima, H. Ohkawa, T. Hirano, S. Maki, H. Niwa, M. Ohashi, S. Inouye and F.I. Tsuji, *Tetrahedron Letters*, 1998, **39**, 5239.
44 M. C. Y. Chan, S. Karasawa, H. Mizuno, I. Bosanac, D. Ho, G. G. Prive, A. Miyawaki and M. Ikura, *Journal of Biological Chemistry*, 2006, **281**, 37813.
45 J. N. Henderson, H. W. Ai, R. E. Campbell and S. J. Remington, *Proceedings of the National Academy of Sciences of the United States of America*, 2007, **104**, 6672.
46 X. He, A. F. Bell and P. J. Tonge, *Journal of Physical Chemistry B*, 2002, **106**, 6056.
47 A. F. Bell, X. He, R. M. Wachter and P. J. Tonge, *Biochemistry*, 2000, **39**, 4423.
48 N. M. Webber and S. R. Meech, *Photochemical & Photobiological Sciences*, 2007, **6**, 976.
49 C. Scharnagl and R. A. Raupp-Kossmann, *Journal of Physical Chemistry B*, 2004, **108**, 477.
50 A. A. Voityuk, M. E. Michel-Beyerle and N. Rosch, *Chemical Physics Letters*, 1997, **272**, 162.
51 K. L. Litvinenko, N. M. Webber and S. R. Meech, *Bulletin of the Chemical Society of Japan*, 2002, **75**, 1065.
52 K. L. Litvinenko, N. M. Webber and S. R. Meech, *Chemical Physics Letters*, 2001, **346**, 47.
53 K. L. Litvinenko, N. M. Webber and S. R. Meech, *Journal of Physical Chemistry A*, 2003, **107**, 2616.
54 N. M. Webber, K. L. Litvinenko and S. R. Meech, *Journal of Physical Chemistry B*, 2001, **105**, 8036.
55 A. D. Kummer, C. Kompa, H. Niwa, T. Hirano, S. Kojima and M. E. Michel-Beyerle, *Journal of Physical Chemistry B*, 2002, **106**, 7554.
56 M. Vengris, I. H. M. van Stokkum, X. He, A. F. Bell, P. J. Tonge, R. van Grondelle and D. S. Larsen, *Journal of Physical Chemistry A*, 2004, **108**, 4587.
57 D. Mandal, T. Tahara and S. R. Meech, *Journal of Physical Chemistry B*, 2004, **108**, 1102.
58 D. Mandal, T. Tahara, N. M. Webber and S. R. Meech, *Chemical Physics Letters*, 2002, **358**, 495.
59 J. Dong, K. M. Solntsev and L. M. Tolbert, *Journal of the American Chemical Society*, 2006, **128**, 12038.
60 R. Gepshtein, D. Huppert and N. Agmon, *Journal of Physical Chemistry B*, 2006, **110**, 4434.
61 S. S. Stavrov, K. M. Solntsev, L. M. Tolbert and D. Huppert, *Journal of the American Chemical Society*, 2006, **128**, 1540.
62 S. K. Kim and G. R. Fleming, *Journal of Physical Chemistry*, 1988, **92**, 2168.
63 R. J. Sension, S. T. Repinec, A. Z. Szarka and R. M. Hochstrasser, *Journal of Chemical Physics*, 1993, **98**, 6291.
64 T. Nagele, R. Hoche, W. Zinth and J. Wachtveitl, *Chemical Physics Letters*, 1997, **272**, 489.
65 V. Sundstrom and T. Gillbro, *Journal of Physical Chemistry*, 1982, **86**, 1788.
66 B. J. Loughnane, A. Scodinu, R. A. Farrer, J. T. Fourkas and U. Mohanty, *Journal of Chemical Physics*, 1999, **111**, 2686.
67 D. C. Todd and G. R. Fleming, *Journal of Chemical Physics*, 1993, **98**, 269.
68 D. C. Todd, J. M. Jean, S. J. Rosenthal, A. J. Ruggiero, D. Yang and G. R. Fleming, *Journal of Chemical Physics*, 1990, **93**, 8658.
69 S. Takeuchi, S. Ruhman, T. Tsuneda, M. Chiba, T. Taketsugu and T. Tahara, *Science*, 2008, **322**, 1073.
70 D. Ben-Amotz and C. B. Harris, *Journal of Chemical Physics*, 1987, **86**, 4856.
71 F. D. Lewis, D. M. Bassani, R. A. Caldwell and D. J. Unett, *Journal of the American Chemical Society*, 1994, **116**, 10477.
72 L. X. Wu and K. Burgess, *Journal of the American Chemical Society*, 2008, **130**, 4089.
73 J. Dong, K. M. Solntsev and L. M. Tolbert, *Journal of the American Chemical Society*, 2009, **131**, 662.
74 K. Y. Chen, Y. M. Cheng, C. H. Lai, C. C. Hsu, M. L. Ho, G. H. Lee and P. T. Chou, *Journal of the American Chemical Society*, 2007, **129**, 4534.
75 J. Dong, K. M. Solntsev, O. Poizat and L. M. Tolbert, *Journal of the American Chemical Society*, 2007, **129**, 10084.
76 K. M. Solntsev, O. Poizat, J. Dong, J. Rehault, Y. B. Lou, C. Burda and L. M. Tolbert, *Journal of Physical Chemistry B*, 2008, **112**, 2700.
77 J. S. Yang, G. J. Huang, Y. H. Liu and S. M. Peng, *Chemical Communications*, 2008, 1344.
78 X. He, A. F. Bell and P. J. Tonge, *FEBS Letters*, 2003, **549**, 35.
79 J. Dong, F. Abulwerdi, A. Baldridge, J. Kowalik, K. M. Solntsev and L. M. Tolbert, *Journal of the American Chemical Society*, 2008, **130**, 14096.
80 W. Weber, V. Helms, J. A. McCammon and P. W. Langhoff, *Proceedings of the National Academy of Sciences of the United States of America*, 1999, **96**, 6177.
81 R. S. H. Liu and G. S. Hammond, *Chemistry–A European Journal*, 2001, **7**, 4536.
82 A. A. Voityuk, M. E. Michel-Beyerle and N. Rosch, *Chemical Physics Letters*, 1998, **296**, 269.
83 P. Altoe, F. Bernardi, M. Garavelli, G. Orlandi and F. Negri, *Journal of the American Chemical Society*, 2005, **127**, 3952.
84 B. G. Levine and T. J. Martinez, *Annual Review of Physical Chemistry*, 2007, **58**, 613.
85 M. E. Martin, F. Negri and M. Olivucci, *Journal of the American Chemical Society*, 2004, **126**, 5452.
86 S. Olsen, L. Manohar and T. J. Martinez, *Biophysical Journal*, 2002, **82**, 359A.
87 A. Toniolo, G. Granucci and T. J. Martinez, *Journal of Physical Chemistry A*, 2003, **107**, 3822.
88 A. Toniolo, S. Olsen, L. Manohar and T. J. Martinez, *Faraday Discussions*, 2004, **127**, 149.
89 C. M. Megley, L. A. Dickson, S. L. Maddalo, G. J. Chandler and M. Zimmer, *Journal of Physical Chemistry B*, 2009, **113**, 302.
90 S. S. Patnaik, S. Trohalaki and R. Pachter, *Biopolymers*, 2004, **75**, 441.

91 A. D. Kummer, J. Wiehler, H. Rehaber, C. Kompa, B. Steipe and M. E. Michel-Beyerle, *Journal of Physical Chemistry B*, 2000, **104**, 4791.
92 W. Rettig, 'Photoinduced Charge Separation Via Twisted Intramolecular Charge-Transfer States', *Topics in Current Chemistry*, 1994, **169**, 253.
93 A. Sinicropi, T. Andruniow, N. Ferre, R. Basosi and M. Olivucci, *Journal of the American Chemical Society*, 2005, **127**, 11534.
94 K. Brejc, T. K. Sixma, P. A. Kitts, S. R. Kain, R. Y. Tsien, M. Ormo and S. J. Remington, *Proceedings of the National Academy of Sciences of the United States of America*, 1997, **94**, 2306.
95 A. F. Bell, D. Stoner-Ma, R. M. Wachter and P. J. Tonge, *Journal of the American Chemical Society*, 2003, **125**, 6919.
96 M. Chattoraj, B. A. King, G. U. Bublitz and S. G. Boxer, *Proceedings of the National Academy of Sciences of the United States of America*, 1996, **93**, 8362.
97 L. G. Arnaut and S. J. Formosinho, *Journal of Photochemistry and Photobiology, A: Chemistry*, 1993, **75**, 1.
98 H. Lossau, A. Kummer, R. Heinecke, F. Pollinger-Dammer, C. Kompa, G. Bieser, T. Jonsson, C. M. Silva, M. M. Yang, D. C. Youvan and M. E. Michel-Beyerle, *Chemical Physics*, 1996, **213**, 1.
99 J. T. M. Kennis, D. S. Larsen, N. H. M. van Stokkum, M. Vengris, J. J. van Thor and R. van Grondelle, *Proceedings of the National Academy of Sciences of the United States of America*, 2004, **101**, 17988.
100 T. M. H. Creemers, A. J. Lock, V. Subramaniam, T. M. Jovin and S. Volker, *Nature Structural Biology*, 1999, **6**, 557.
101 C. Seebacher, F. W. Deeg, C. Brauchle, J. Wiehler and B. Steipe, *Journal of Physical Chemistry B*, 1999, **103**, 7728.
102 D. Stoner-Ma, A. A. Jaye, P. Matousek, M. Towrie, S. R. Meech and P. J. Tonge, *Journal of the American Chemical Society*, 2005, **127**, 2864.
103 D. Stoner-Ma, E. H. Melief, J. Nappa, K. L. Ronayne, P. J. Tonge and S. R. Meech, *Journal of Physical Chemistry B*, 2006, **110**, 22009.
104 J. J. van Thor, G. Y. Georgiev, M. Towrie and J. T. Sage, *Journal of Biological Chemistry*, 2005, **280**, 33652.
105 J. J. van Thor, G. Zanetti, K. L. Ronayne and M. Towrie, *Journal of Physical Chemistry B*, 2005, **109**, 16099.
106 K. Rahmelow, W. Hubner and T. Ackermann, *Analytical Biochemistry*, 1998, **257**, 1.
107 X. H. Shi, J. Basran, H. E. Seward, W. Childs, C. R. Bagshaw and S. G. Boxer, *Biochemistry*, 2007, **46**, 14403.
108 J. J. van Thor, K. L. Ronayne, M. Towrie and J. T. Sage, *Biophysical Journal*, 2008, **95**, 1902.
109 M. A. Lill and V. Helms, *Proceedings of the National Academy of Sciences of the United States of America*, 2002, **99**, 2778.
110 S. F. Wang and S. C. Smith, *Physical Chemistry Chemical Physics*, 2007, **9**, 452.
111 H. Zhang and S. C. Smith, *Journal of Theoretical & Computational Chemistry*, 2007, **6**, 789.
112 R. B. Zhang, M. T. Nguyen and A. Ceulemans, *Chemical Physics Letters*, 2005, **404**, 250.
113 O. Vendrell, R. Gelabert, M. Moreno and J. M. Lluch, *Journal of Chemical Theory and Computation*, 2008, **4**, 1138.
114 O. Vendrell, R. Gelabert, M. Moreno and J. M. Lluch, *Journal of Physical Chemistry B*, 2008, **112**, 13443.
115 O. Vendrell, R. Gelabert, M. Moreno and J. M. Lluch, *Journal of Physical Chemistry B*, 2008, **112**, 5500.
116 O. Vendrell, R. Gelabert, M. Moreno and J. M. Lluch, *Journal of the American Chemical Society*, 2006, **128**, 3564.
117 Q. Cui and M. Karplus, *Journal of Physical Chemistry B*, 2003, **107**, 1071.
118 K. J. Schweighofer and A. Pohorille, *Biophysical Journal*, 2000, **78**, 150.
119 R. A. W. Frank, C. M. Titman, J. V. Pratap, B. F. Luisi and R. N. Perham, *Science*, 2004, **306**, 872.
120 X. Shu, P. Leiderman, R. Gepshtein, N. R. Smith, K. Kallio, D. Huppert and S. J. Remington, *Protein Science*, 2007, **16**, 2703.
121 D. Stoner-Ma and *et al.*, in preparation.
122 M. A. Elsliger, R. M. Wachter, G. T. Hanson, K. Kallio and S. J. Remington, *Biochemistry*, 1999, **38**, 5296.
123 A. A. Jaye, D. Stoner-Ma, P. Matousek, M. Towrie, P. J. Tonge and S. R. Meech, *Photochemistry and Photobiology*, 2006, **82**, 373.
124 T. B. McAnaney, E. S. Park, G. T. Hanson, S. J. Remington and S. G. Boxer, *Biochemistry*, 2002, **41**, 15489.
125 T. B. McAnaney, E. S. Park, G. T. Hanson, M. E. P. Rende, S. J. Remington and S. G. Boxer, *Biophysical Journal*, 2002, **82**, 314A.
126 T. B. McAnaney, X. H. Shi, P. Abbyad, H. Jung, S. J. Remington and S. G. Boxer, *Biochemistry*, 2005, **44**, 8701.
127 D. Stoner-Ma, A. A. Jaye, K. L. Ronayne, J. Nappa, P. J. Tonge and S. R. Meech, *Chemical Physics*, 2008, **350**, 193.
128 X. Shi, P. Abbyad, X. Shu, K. Kallio, P. Kanchanawong, W. Childs, S. J. Remington and S. G. Boxer, *Biochemistry*, 2007, **46**, 12014.
129 X. Shu, K. Kallio, X. Shi, P. Abbyad, P. Kanchanawong, W. Childs, S. G. Boxer and S. J. Remington, *Biochemistry*, 2007, **46**, 12005.
130 D. Stoner-Ma, A. A. Jaye, K. L. Ronayne, J. Nappa, S. R. Meech and P. J. Tonge, *Journal of the American Chemical Society*, 2008, **130**, 1227.
131 C. N. Schutz and A. Warshel, *Proteins: Structure Function and Bioinformatics*, 2004, **55**, 711.
132 A. Warshel and A. Papazyan, *Proceedings of the National Academy of Sciences of the United States of America*, 1996, **93**, 13665.
133 W. W. Cleland and M. M. Kreevoy, *Science*, 1994, **264**, 1887.
134 M. E. Tuckerman, D. Marx, M. L. Klein and M. Parrinello, *Science*, 1997, **275**, 817.
135 G. A. Kumar and M. A. McAllister, *Journal of the American Chemical Society*, 1998, **120**, 3159.
136 I. A. Heisler, M. Kondo and S. R. Meech, *Journal of Physical Chemistry B*, 2009, **113**, 1623.
137 M. Kondo and *et al.*, submitted for publication.
138 S. J. Remington, *Current Opinion in Structural Biology*, 2006, **16**, 714.
139 J. J. van Thor, T. Gensch, K. J. Hellingwerf and L. N. Johnson, *Nature Structural Biology*, 2002, **9**, 37.
140 R. Ando, H. Hama, M. Yamamoto-Hino, H. Mizuno and A. Miyawaki, *Proceedings of the National Academy of Sciences of the United States of America*, 2002, **99**, 12651.
141 I. Hayashi, H. Mizuno, K. I. Tong, T. Furuta, F. Tanaka, M. Yoshimura, A. Miyawaki and M. Ikura, *Journal of Molecular Biology*, 2007, **372**, 918.
142 H. Mizuno, T. K. Mal, K. I. Tong, R. Ando, T. Furuta, M. Ikura and A. Miyawaki, *Molecular Cell*, 2003, **12**, 1051.
143 H. Tsutsui, S. Karasawa, H. Shimizu, N. Nukina and A. Miyawaki, *EMBO Reports*, 2005, **6**, 233.
144 V. I. Martynov, B. I. Maksimov, N. Y. Martynova, A. A. Pakhomov, N. G. Gurskaya and S. A. Lukyanov, *Journal of Biological Chemistry*, 2003, **278**, 46288.
145 D. M. Chudakov, V. V. Belousov, A. G. Zaraisky, V. V. Novoselov, D. B. Staroverov, D. B. Zorov, S. Lukyanov and K. A. Lukyanov, *Nature Biotechnology*, 2003, **21**, 191.
146 G. H. Patterson and J. Lippincott-Schwartz, *Science*, 2002, **297**, 1873.
147 A. Vaziri, J. Y. Tang, H. Shroff and C. V. Shank, *Proceedings of the National Academy of Sciences of the United States of America*, 2008, **105**, 20221.
148 M. Schneider, S. Barozzi, I. Testa, M. Faretta and A. Diaspro, *Biophysical Journal*, 2005, **89**, 1346.
149 J. N. Henderson, R. Gepshtein, J. R. Heenan, K. Kallio, D. Huppert and S. J. Remington, *Journal of the American Chemical Society*, 2009, **131**, 4176.
150 J. M. Battad, P. G. Wilmann, S. Olsen, E. Byres, S. C. Smith, S. G. Dove, K. N. Turcic, R. J. Devenish, J. Rossjohn and M. Prescott, *Journal of Molecular Biology*, 2007, **368**, 998.
151 M. L. Quillin, D. A. Anstrom, X. K. Shu, S. O'Leary, K. Kallio, D. A. Chudakov and S. J. Remington, *Biochemistry*, 2005, **44**, 5774.
152 T. A. Schuttrigkeit, T. von Feilitzsch, C. K. Kompa, K. A. Lukyanov, A. P. Savitsky, A. A. Voityuk and M. E. Michel-Beyerle, *Chemical Physics*, 2006, **323**, 149.
153 S. Habuchi, R. Ando, P. Dedecker, W. Verheijen, H. Mizuno, A. Miyawaki and J. Hofkens, *Proceedings of the National Academy of Sciences of the United States of America*, 2005, **102**, 9511.
154 H. Mizuno, T. K. Mal, M. Walchli, A. Kikuchi, T. Fukano, R. Ando, J. Jeyakanthan, J. Taka, Y. Shiro, M. Ikura and A. Miyawaki, *Proceedings of the National Academy of Sciences of the United States of America*, 2008, **105**, 9227.
155 L. V. Schafer, G. Groenhof, M. Boggio-Pasqua, M. A. Robb and H. Grubmuller, *PLoS Computational Biology*, 2008, **4**, e1000034.

