Journal of Photochemistry and Photobiology A: Chemistry 205 (2009) 1–11

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology A: Chemistry

journal homepage: www.elsevier.com/locate/jphotochem

Invited Feature Article

Excited state dynamics in the green fluorescent protein

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ARTICLE INFO

Article history:
Received 17 February 2009
Received in revised form 23 March 2009
Accepted 24 March 2009
Available online 5 April 2009

Keywords:
Photophysics
Photochemistry
Green fluorescent protein
Proton transfer
Proton wire
Low barrier hydrogen bond
Internal conversion
Isomerization

ABSTRACT

The photophysical properties of the wild type green fluorescent protein (wtGFP), the isolated GFP chromophore in solution, and a number of GFP mutants are described. The mechanism of the very efficient radiationless decay of the isolated chromophore is discussed in the light of experimental measurements and theoretical calculations. The dramatic switch in photophysics, from radiationless decay dominated to proton transfer dominated, between the isolated chromophore and wtGFP is illustrated and discussed. The excited state dynamics and their manipulation through mutagenesis are described. The potential for utilizing GFP as a model system for studying the dynamics of proton transfer reactions in proteins, including low barrier hydrogen bonds and proton transport on proton wires, is discussed.

1. Background

The green fluorescent protein (GFP) was first isolated from the jellyfish *Aequorea victoria* more than 30 years ago, and its novel fluorescence properties noted [1,2]. Some years later it was shown that GFP could be cloned and expressed in other organisms [3,4]. The beautiful fluorescence images obtained opened the way to the use of GFP as a fluorescent marker protein [4]. Further developments through mutagenesis enhanced the stability and broadened the available spectral range, allowing the full potential of GFP and related fluorescent proteins (FPs) to be realised [5–7]. A testament to the importance of these developments is the near ubiquitous application of FPs as markers for fluorescence imaging, single molecule dynamics, protein–protein interactions and very many other applications in life sciences. It is difficult to imagine that there is a significant biological sciences department anywhere in the world which does not make use of GFP based methods. If further evidence for the importance of these developments were needed it was surely supplied by the award of the 2008 Nobel Prize in chemistry to the founders of the field [8].

The enormously important man made uses of GFP may somewhat obscure its photobiological significance. However, this deserves some attention, not least because new FPs are ultimately derived from natural sources. When GFP was first isolated it was found as part of the bioluminescence apparatus (aequorin) of *A. victoria*, and it was shown that GFP could be excited by resonant energy transfer from the bioluminescent excited state [9]. Presumably then in this specific instance GFP is associated with signalling, and *A. victoria* obtained some advantage from a green rather than blue emission. One of the unique features of GFP, which enabled the reliable performance of this signalling function (and is also critical for bioimaging), is that the chromophore is an intrinsic part of the amino acid chain, and is thus irreversibly bound to the protein. The mechanism of chromophore formation in GFP has been studied in detail. It involves the autocatalytic cyclisation and subsequent oxidation reactions between three amino acid residues, Ser65-Tyr66-Gly67 [10]. The structural features enabling this reaction have been discussed in a number of publications [11–18]. For some years this neat trick was believed to be restricted to this one obscure (albeit alluringly emissive) jellyfish. However, with the growing importance of GFP in bioimaging the origin of the colouring of other marine organisms was reinvestigated. 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 [19–23]. The lack of fluorescence in some of the FPs derived from coral argues against a signalling mechanism, and a number of other functions have been proposed. For example the co-location of FP and photosynthetic apparatus in some organisms, notably coral, is strongly suggestive of a photoprotection role [23–25], while others have proposed that the colouring is involved in a kind of camouflage [26].

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doi:10.1016/j.jphotochem.2009.03.021

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 [27]. This has the effect of broadening the palette of colours available for imaging research [21,28–30]. Even the new FPs that possess a chromophore with the same structure as wtGFP may have quite different spectra. Changes in the charge state and degree of protonation of the chromophore can have a dramatic effect on both the intensity and the frequency of the fluorescence, while more subtle spectral shifts can be achieved by the modification of adjacent amino acid residues [16]. The range of photophysical properties can be further extended through mutagenesis, particularly concerning the residues which make up or surround the chromophore [5,31–33].

Even more striking is the variation in photochemistry among the various FPs. One very exciting development is the discovery of photoswitchable proteins [34–37], in which the protein can be optically switched between fluorescent and non-fluorescent (or green and red fluorescent) states, and in some cases switched back again by irradiation at a second wavelength [38,39]. There is structural evidence that this may result from structural flexibility around the chromophore [40], or switching between *cis* and *trans* isomers of the chromophore [41,42], although this need not be the mechanism in every case [43]. Of course such photochromic behavior is well established for molecules in solution and has found many potential uses in materials science [44], but its observation in FPs suggest a number of very important potential applications in bioimaging [34–36]. Accordingly the potential mechanisms for photoswitching have recently been the topic of some quite detailed quantum chemical or molecular dynamics calculations [45–47].

2. Photophysics in the GFP chromophore

It is clear from the foregoing that the deliberate design of FPs with a range of properties (photoswitchable, photosensing, phototoxic ...) will require a detailed understanding of FP photophysics, and how it is coupled to the protein environment. From the point of view of the photophysicist a key starting point is the isolated chromophore in solution. For GFP this is 4′-hydroxybenzylidene-2,3-dimethylimidazolinone [48,49] (HBDI) whose structure is shown in Fig. 1. One of the most striking features of GFP photophysics is that both HBDI and the denatured GFP in aqueous solution are essentially *non-fluorescent* at room temperature [50]. This is in sharp contrast to GFP itself which has a quantum yield in excess of 0.8, but accords with the low or negligible fluorescence observed for some other natural FPs [51]. Evidently the protein matrix exercises a high degree of control over the chromophore quantum yield.

The mechanism of radiationless decay in HBDI has been studied in some detail. Ultrafast polarization spectroscopy showed that following electronic excitation the ground state is repopulated on

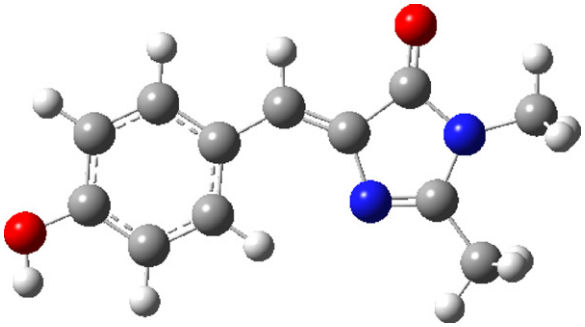

Fig. 1. The structure of the HBDI chromophore in its neutral protonated form.

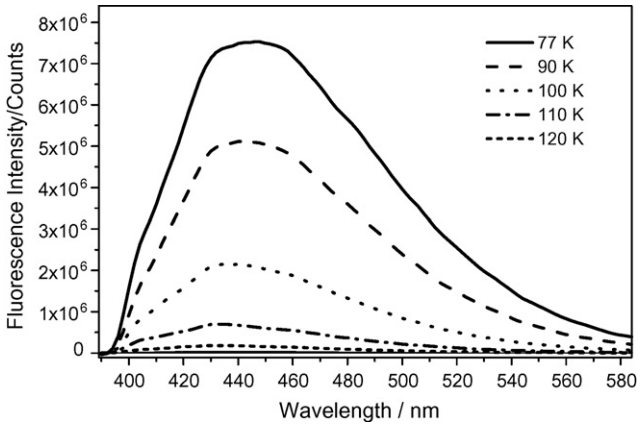

Fig. 2. Temperature dependence of the fluorescence spectrum of HBDI in ethanol.

the picosecond timescale, suggesting an internal conversion (IC) process [52–54]. The same rapid internal conversion was seen for neutral, cationic and anionic forms of HBDI, although the neutral form has a somewhat faster decay time [53]. The high fluorescence quantum yield characteristic of GFP is only recovered in HBDI when the temperature is reduced close to or below the solvent glass transition temperature [50,54,55], suggesting that IC is either (or both) thermally activated or sensitive to the viscosity of the medium (Fig. 2). Detailed studies of the temperature and viscosity dependence of the excited state decay and ground state recovery suggested that the coordinate promoting IC in HBDI is nearly barrierless and is a weak function of medium viscosity (Fig. 3). The latter result in turn suggests that the coordinate promoting IC is volume conserving, i.e. one which does not require the displacement of a significant volume of solvent [56,57]. Analysis of the temperature dependent emission suggested that two coordinates are coupled in the mode promoting IC [58].

Identification of this coordinate is important for understanding the fluorescence enhancement mechanism operating in GFP, and also the variation in fluorescence yield among different FPs with essentially the same chromophore. Its identification may make possible a degree of control over the photochemistry behind photoswitchable GFPs. Comparison of HBDI with structurally similar molecules which also undergo IC (e.g. cyanine dyes [59]) suggests that a coordinate involving excited state isomerization about the single–double bond bridging motif may be important. This notion was supported by early quantum chemical calculations [60]. Essentially, electronic excitation reduces the bond order in the methylene double bond, allowing near barrierless rotation about that bond. This leads to a large increase in the ground state energy resulting in an intersection between ground and excited state surfaces and

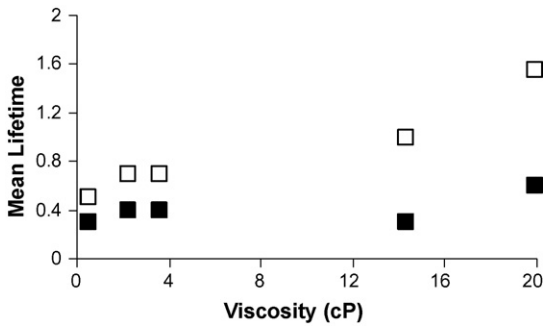

Fig. 3. Plot of the mean lifetime of the non-single exponential fluorescence decay of HBDI in alcohol solvents as a function of the solvent viscosity (adapted from ref. [56]). Filled symbols are for the neutral form, open for the anion.

consequently fast internal conversion. Weber et al. considered a 90° rotation about three possible coordinates for different charge states of the model chromophore [60]. They found that only the twisting about the exocyclic double bond was barrierless for both neutral and ionic states, although it did not lead to a crossing of ground and excited states for the anion. They also considered the so called 'Hula twist' coordinate introduced by Liu and Hammond which requires a much lower volume of rotation [61], consistent with the low effect of solvent friction found experimentally [53]. However, this coordinate only leads to S_0/S_1 crossing in the anion via a significant energy barrier, inconsistent with experimental observations [60]. Voityuk et al. considered the single bond twist coordinates for three different charge states and for the zwitterion, 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 [62].

Subsequent and increasingly sophisticated theoretical calculations have built on this early work, including a variety of plausible reaction coordinates and searches for minimum energy pathways and conical intersections between ground and excited states (at which IC is expected to be very efficient) [63–67]. 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 like motion also lead to fast IC but only via a significant energy barrier.

However, there is not yet conclusive agreement on which single bond rotation is most significant in the excited state isomerization, with different calculations finding phenoxy or imidazolinone bond rotation to be the most strongly downhill. Olivucci and co-workers found in their calculations (which correspond to the chromophore in the gas phase) that two coordinates were important in achieving close approach of S_0 and S_1—a stretching coordinate and rotation about the phenolic single bond [66]. In contrast Altoe et al. included a polarizable continuum model of the solvent and found in that case that rotation about the imidazolinone double bond was most effective in promoting IC [67]. Martinez and co-workers also identified a significant role for the medium in their study of the model chromophore [64,65,68]. In vacuum, the dynamics involved primarily a twist about the bridging double bond but with a large excursion in the phenyl torsion and a significant lifetime on the excited state surface. In contrast, in the calculation for the solvated chromophore they found fast downhill rotation about the double bond leading to a conical intersection, resulting in a sub-picosecond excited state lifetime, in accord with experiment. 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 a means by which the protein can modulate chromophore photophysics.

More recently Olsen and co-workers investigated the effect on the excited state dynamics of different substituents on the model chromophore, and the effect of different initial isomers in the ground electronic state, specifically the Z and E isomers formed by rotation about the imidazolinone double bond (the barrier to isomerization in the ground state is high) [69,70]. Such calculations are directly relevant to the wider family of FPs, including, for example, DsRed which has a substituent which extends the conjugation of the basic chromophore, and FP595 a so-called 'kindling protein' which exists in both Z and E isomeric forms (a change which can only be achieved by double bond isomerization). Olsen and co-workers found that both the initial isomer and the substituent can modify the excited state chemistry to the extent that different single bond rotations may be favoured in the excited electronic state. This result suggests that FP chromophores may undergo a variety of excited state processes, which could potentially be exploited, both in nature and through mutagenesis, to tune the function of the protein. The extension of these calculations to investigate solvent tuning effects on these pathways will be important.

Neither of the barrierless single bond rotation pathways predicted to lead to a 90° twisted geometry and fast IC are truly volume conserving. Both require significant solvent displacement, making the sub-picosecond internal conversion and the weak viscosity dependence observed surprising [53,56,57]. This result suggests that either the potential energy surface for the isomerisation provides a sufficiently strong driving force to overcome the solvent friction, or that IC back to the ground state surface occurs much earlier along the twisting coordinate, before the 90° twist has occurred. There have been only a few calculations of the dynamics of the transfer from the excited to the ground state potential energy surface. Martinez and co-workers showed in their calculations that population decay in a model aqueous solvent occurred in 200 fs for a model GFP chromophore, in accord with experiment [65]. However, it is not clear how to introduce solvent friction into this calculation.

Solvent dynamics may be one important factor driving the excited state chemistry. This may be particularly significant for the single bond twist about the imidazolinone double bond, which is calculated to be accompanied by a significant degree of intramolecular charge transfer in the twisted excited electronic state [68]. It is thus conceivable that solvent dynamics in response to the intramolecular charge transfer are a driving force for motion along the twisting coordinate. The role of solvation dynamics in promoting outer sphere electron transfer reactions is well established [71]. If such solvent reorganization is important in the potential energy surface leading to IC it may offer a route to the observed weak dependence on solvent friction. In polar solvents the fastest components of solvent reorganization occur on a sub-picosecond timescale and are relatively insensitive to the macroscopic friction [72]. Thus, stabilisation of the twisted internal charge transfer state by fast, librational, solvent dynamics, followed by fast IC, may be consistent with both calculated and observed excited state dynamics. However, two factors should be noted: first, the fluorescence spectrum of HBDI does not show a significant time dependent Stokes loss, which is characteristic of such solvation dynamics [56]; second, a number of other examples of excited state single bond rotation, also leading to nonradiative twisted internal charge transfer states, have been reported, and these *do* exhibit a significant dependence on solvent friction [73,74], unlike the model GFP chromophore. Thus a complete understanding of the precise details of the excited state dynamics of GFP and related model compounds requires continued investigation.

However, even the existing incomplete data already suggest a number of ways in which the protein matrix can modify the chromophore's photophysics, leading to the dramatic fluorescence enhancement observed. Most obviously the geometrical constraint of internal motion by the surrounding protein residues might suppress the IC promoted through excited isomerization, and thus render the chromophore fluorescent (analogous to the effects observed on freezing a solution of the chromophore below the glass transition temperature). It is not however clear that such simple packing effects alone are sufficient to explain the observed enhancement of florescence in the protein [75,76]. Indeed, the protein matrix would favour a twisted form of the chromophore were it not for conjugation stabilizing a planar structure [77]. Thus steric effects might be expected to *promote* IC in some cases. However, recent molecular mechanics calculations suggest a correlation between increased (dihedral) rotational freedom for the chromophore in the protein and a decreased quantum yield [78].

As described above, both experiment and theory suggest that medium polarity and charge effects might be important in determining the shape of the excited state potential energy surface and the dynamics upon it. This is a second route through which the surrounding residues can influence chromophore excited state

dynamics. For example the charge on residues adjacent to the chromophore might influence excited state pathways leading to intramolecular charge transfer, through Coulombic interactions [79]. This could have either an enhancing or suppressing effect on IC, depending on the sign and location of the charge. Finally the chromophore is held in the protein not only by the covalent bonds which anchor it to the polypeptide backbone, but also by a range of hydrogen bonds. H-bond formation can both hinder excited state structural reorganisation, and also modify the electronic structure of the chromophore (and thus, presumably, the shapes of the excited state potential energy surfaces) [40,75]. Indeed the electronic spectrum of the chromophore in the protein is markedly different to that of the HBDI chromophore in a range of solvents [80,81]. In particular the anionic form is rather red shifted in the protein; we return to discuss H-bonded structures in the next section. The pivotal role that the protein environment has in controlling the structure and photophysics of FPs is nicely illustrated in the combined structural and spectroscopic study of cyan FPs from Henderson, Remington and co-workers. They observed that mutations in residues adjacent to the chromophore could control the spectrum, quantum yield and the population of the Z or E isomers [82,83].

There have been a number of reports of structural modifications in the isolated HBDI chromophore in an effort to modify its photophysics. Litvinenko et al. [53] studied the phenyl derivative, in which the OH group is replaced by a H atom, 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 [80]). In an elegant synthetic approach Chen et al. [84] showed that the *o*-hydroxy derivative had a strongly red shifted emission spectrum compared to the *p*-hydroxy form. They showed that this new emission arose from 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 chromophore in solution. Very recently Solntsev et al. reported that the *m*-OH derivative of HBDI exhibited a longer fluorescent lifetime than *p*-HBDI, and was able to support intermolecular proton transfer [85,86]. These studies of proton transfer in HBDI are interesting in their own right. However, still more striking is that considerable synthetic effort is required to stimulate proton transfer in HBDI. This stands in stark contrast to the known photophysics of wtGFP, which are dominated by an excited state proton transfer reaction which is unique in biology; this topic forms the second subject of this review.

3. Excited state proton transfer: wild type GFP

The UV and visible electronic absorption spectrum of wtGFP consists of two bands at *ca* 395 nm and 480 nm, named the A and B bands respectively. Excitation of either results in intense green emission with a peak at around 510 nm. Although wtGFP itself is insensitive to pH, a study of the S65T mutant suggested that these two bands are associated with neutral and anionic forms of the chromophore [87]. Subsequently, the protonated (A) and deprotonated (B) nature of these bands was confirmed by structural studies [87], spectroscopic measurements and studies of the effects of pH on the model chromophore [88,89]. The observation of excited state proton transfer in a protein is unexpected, but the phenomenon is of course well known and characterised in solution phase, and these early data provide important background information [90,91].

In 1996 Boxer and co-workers measured the time resolved fluorescence of wtGFP with ultrafast time resolution [92]. The directly excited A^* state was observed to decay in a non-single exponential fashion with a mean lifetime of 18 ps. Crucially the 508 nm fluorescence was observed to grow in with a ca 8 ps mean lifetime. This result immediately suggested the occurrence of an excited state proton transfer reaction. This 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. Similar observations were made combining ultrafast fluorescence with transient absorption spectroscopy [93].

Chattoraj et al. noted that the very fast excited state proton transfer reaction was at odds with the fact that the population of the B ground state did not grow rapidly under irradiation, even though B itself was apparently stable. They proposed a model which is essentially that still in use today, illustrated in Fig. 4 [92]. Excitation to A^* initiates a fast excited state proton transfer to form an intermediate state I^*, which is the deprotonated chromophore in the geometry of the neutral ground state. The primary fate of I^* is decay back to the I ground state, from which A is repopulated by reverse proton transfer. Occasionally during the excited state lifetime of I^* a reorganisation in the protein matrix occurs which traps the molecule in the B form. Supporting evidence was found for this model from the observation of small differences between the I^* and B^* emission spectra, presumably reflecting their different environments [92]; structural studies have suggested that the reorganisation involved Thr203 reorientation [87].

Subsequent studies have added some details to this model. Ultrafast transient absorption revealed a barrier in the $A^* \rightarrow I^*$ proton transfer process and permitted detailed analysis of the energy dependent kinetics [94]. Ultrafast fluorescence emission spectroscopy showed that there is little evolution in the emission spectra as a function of time after excitation, with the A^* directly feeding the final I^* state [95]. Transient hole burning spectroscopy at low temperatures identified spectra of the three forms (A, B and I) and clarified pathways of photoconversion between them [96]. Further low temperature measurements suggested that these pathways may be quite complex [97]. The ultrafast dynamics of proton transfer on the ground state surface for the $I \rightarrow A$ transformation were studied through multipulse ultrafast pump-dump-probe

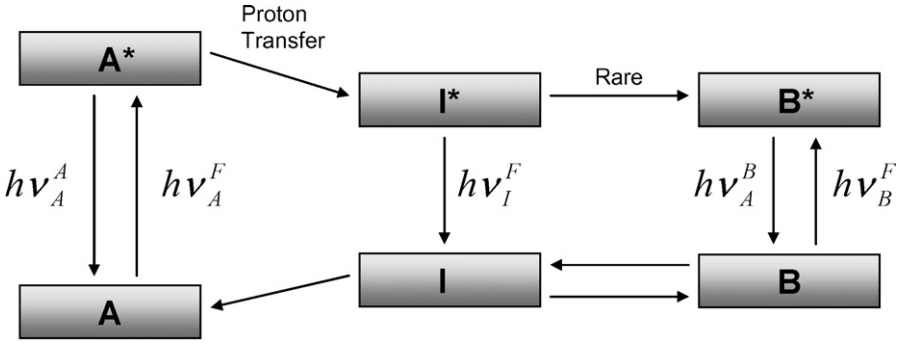

Fig. 4. Schematic diagram of the interconversion of A, I and B states and the transitions observed in electronic spectroscopy. Adapted from the original proposal in ref. [92].

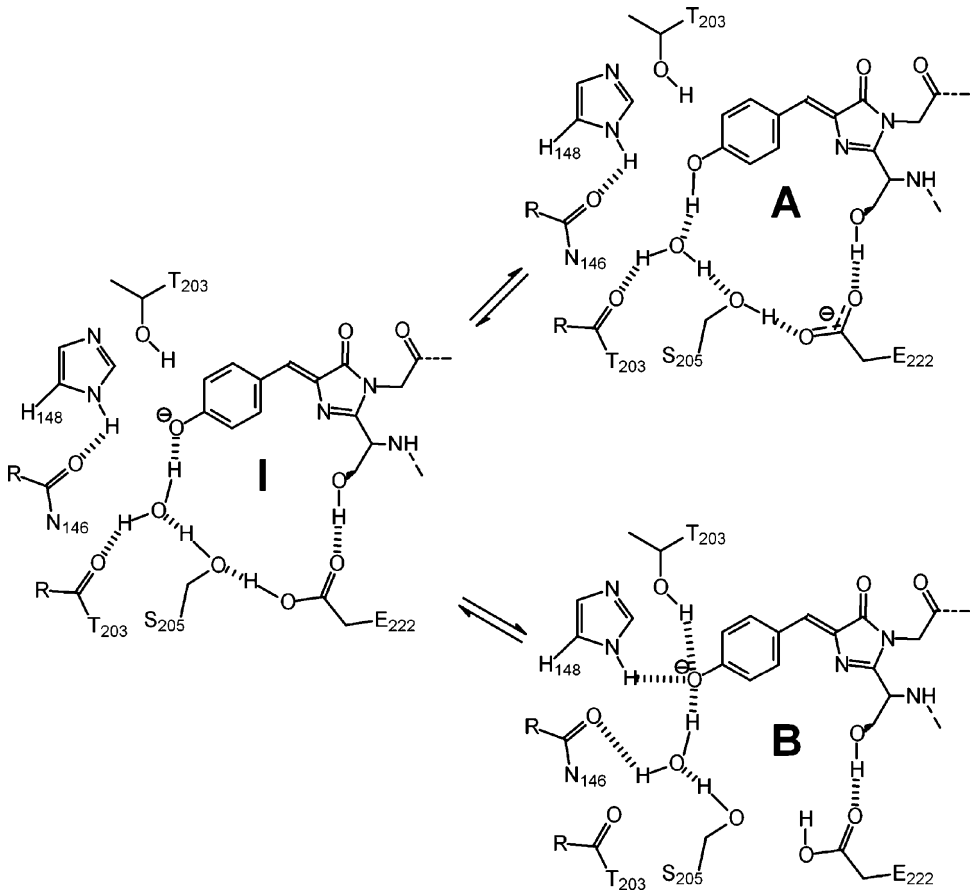

Fig. 5. The structure of the three forms of GFP showing the proposed H-bonds between the chromophore and the protein matrix, and identifying the potential proton wire to E222. Adapted from ref. [87].

spectroscopy [98]. The recovery of A was described by multiexponential kinetics, and some intermediate steps were shown to be very sensitive to deuteration, consistent with a proton tunnelling mechanism.

The evidence for the occurrence of an excited state ultrafast chromophore deprotonation reaction from electronic spectroscopy is thus very convincing. However, the site of the proton acceptor in the protein remained to be determined. In their structural studies Brejc et al. [87] showed that the OH group of the chromophore was H-bonded to a structural water molecule, itself H-bonded to residues Thr203 and Ser205, and Ser205 was further H-bonded to Glu222 (Fig. 5). Their result thus suggests that Glu222 may be the ultimate acceptor, and reveals a potential pathway to it. However the formal identification of the acceptor and the rate at which it is protonated are not available from structural studies but can be obtained from time resolved vibrational spectroscopy.

Stoner-Ma et al. addressed this question through ultrafast time resolved infra-red spectroscopy [99]. wtGFP in D_2O was excited to the A^* state at 400 nm and the transient IR spectra measured between 1500 cm^{-1} and 1750 cm^{-1}. The transient IR difference spectra (excited–unexcited) revealed a number of vibrational bands (Fig. 6). The negative (bleach) bands formed instantaneously on excitation can be assigned, on the basis of studies of the model HBDI (and its ^{13}C isotopes [100]) and DFT calculations, to chromophore modes, the three of highest frequency being stretching modes localised on the carbonyl, the exocyclic C=C bond and the phenyl ring respectively [100,101]. The most striking time dependence in the transient IR spectra can be seen around 1710 cm^{-1} where a new band appears on the timescale of a few tens of picoseconds. Simultaneously, a bleach appears in the spectrum at 1565 cm^{-1}. Neither band is observed in HBDI, and a bleach increasing with time can only be understood as a reaction between the photoexcited state and a residue in the protein host matrix. The product of this reaction evidently has its absorption at 1710 cm^{-1}.

This pair of absorption and bleach modes appearing simultaneously at 1565 cm^{-1} and 1710 cm^{-1} suggests an assignment to a carboxylate to carboxcylic acid transformation, with the time resolved bleach corresponding to loss of the asymmetric stretch of the carboxylate, and the transient absorption to the appearance of a carbonyl stretch. This is consistent with the proton acceptor being the Glu222 residue [99,101]. This residue is quite remote from the chromophore hydroxyl group (~12 Å), suggesting that it is protonated through proton translation along the proton wire char-

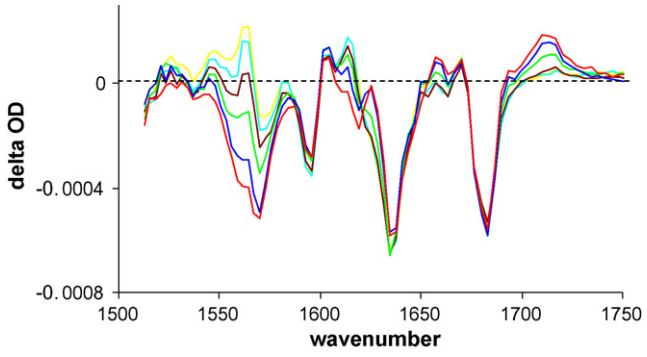

Fig. 6. Time resolved IR spectra of wtGFP recorded as a function of time after excitation of A^*. 2 ps (yellow), 4 ps (turquoise), 10 ps (brown), 30 ps (green), 100 ps (blue), and 200 ps (red). Adapted from ref. [101] (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article)

acterised in the structure of Brejc et al. (Fig. 5) [87]. Confirmation that the transient absorption was not associated with, for example, the C=O mode of the chromophore in the I^* state was made through isotopic labelling of the tyrosines in the protein leading to incorporation of ^{13}C into the imidazolinone carbonyl, a procedure which left the 1710 cm^{-1} transient assigned to Glu222 unshifted [101]. Interestingly, the isotope measurements actually suggested a significant downshift in the frequency of the chromophore carbonyl mode in the excited electronic state.

Further assignment of the transient vibrational spectrum is achieved through polarisation resolved experiments. From the ratio of the difference spectra measured for pump radiation polarised parallel and perpendicular to the polarisation of the IR probe it is possible to calculate the angle between the electronic and vibrational transition dipole moments, the latter being calculated by density functional theory (for the HBDI chromophore) [101–103]. These measurements are consistent with the orientation of Glu222 from structural studies [87].

The transient IR data reveal additional details on the protein response to the proton transfer reaction, not accessible through electronic spectroscopy. For example an instantaneous transient absorption is found in the carbonyl region around 1690 cm^{-1}. This mode was characterised and assigned by van Thor and co-workers in their ultrafast transient IR study of wtGFP and the E222D mutant as arising from Q69 perturbed by the optical excitation of the A state [104]. This residue is adjacent to the chromophore, so may respond to the new dipole moment of the excited state. A further protein mode at around 1640 cm^{-1} was found to evolve on the same timescale as the proton transfer reaction. This may be associated with a perturbation of amide I modes by the evolving charge distribution [104]. Significantly, van Thor and co-workers extended the transient IR measurements below 1500 cm^{-1} and observed the symmetric stretch of the E222 carboxylate group [103]. This band was observed to be unusually broad, suggesting an inhomogeneity in the H-bonding of the E222 proton acceptor, which was also observed in the kinetics. They found that distinct kinetics were associated with the different H-bonded populations, in a fashion that was consistent with the observed distribution of lifetimes in the time resolved fluorescence [103].

Despite these quite detailed studies of the time dependent vibrational spectrum of wt GFP, no convincing evidence for the existence of intermediates in the proton transfer chain has yet been obtained (e.g. H_3O^+, S205 anion). Stoner-Ma et al. [99] compared the time resolved fluorescence decay of the A^* proton donor reported by Chattoraj et al. [92] with the kinetics extracted from the transient IR studies of the disappearance/formation of the carboxylate/carboxcylic acid on the acceptor. The kinetics were quite well fit by a common set of time constants, leading to the proposal that the proton motion along the H-bonded chain, once initiated, proceeds in a cooperative manner with negligible population on any intermediate [99].

The dynamics of the proton transfer chain have been investigated by theoretical calculations. In a very early paper Lill and Helms employed MD to simulate the proton transfer [105]. After the transfer was triggered by ejection of the proton from the chromophore the subsequent two steps leading to E222 protonation were found to occur on the femtosecond timescale. Subsequently quantum chemical calculations have been made, typically on the isolated proton transfer chain fixed in the geometry suggested by the protein structure, but in the absence of surrounding residues. Zhang et al. [106] and Smith and co-workers [107,108] calculated that the proton transfer occurred in a single concerted step along an essentially barrierless potential, but suggested that the 'leading proton' was the last in the chain (S205 to E222). Significantly, 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 [106]. This suggests that modelling the proton transfer in the protein on a potential energy surface based on only a few residues may be problematic, although probably computationally essential.

More recently, Lluch and co-workers [109–113] 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 from MD simulations. Importantly they investigated the proton transfer surface for both ground and excited states of the chromophore. They found that the photoactive state was $\pi\pi^*$ rather than the $\pi\sigma^*$ implicated in some other proton transfer reactions [111]. The calculated energy surfaces 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 again strongly downhill for the S203 to E222 step. This is consistent with a concerted but asynchronous proton transfer, with the last proton 'leading' [110].

The proton transfer in wtGFP is a unique example of an excited state proton transfer reaction in a protein, and this lends it an added significance. Proton transfer is believed to be important in a wide range of processes in biology, and often proton transfer takes place over long distances (proton wires) and across small or negligible energy barriers (low barrier H-bonds). One of the key features of wtGFP is that it affords experimentalists the opportunity of photoinitiating such a proton transfer process, allowing interrogation of the subsequent dynamics with ultrafast time and vibrational energy resolution. Such measurements can be tested against molecular dynamics and quantum chemical calculations, and thus have the potential to provide new insights into proton transfer reactions in proteins. A second feature of GFP is that many stable mutants can be prepared, and their structures determined. This suggests the possibility of modifying the structure of the proton relay chain and monitoring the effects of those modifications on the mechanism and the dynamics of the proton transfer. This is the topic of the next section.

4. Excited state proton transfer in GFP: manipulation through mutagenesis

A number of mutants of GFP have been produced which favour exclusive population of either the A or B ground states. In particular numerous mutants with the key S65T mutation retain the basic structure of the chromophore π electron system but render the A/B ratio and the rate of proton transfer highly pH dependent [31]. The photophysics of a number of these mutants have been characterised in detail [114–116]. Both the ground state population and the barrier to proton transfer were found to be pH dependent. A particularly interesting case is the S65T mutants at lower pH, where the chromophore is trapped in the A state, and the excited state proton transfer reaction does not occur. These proteins allow the possibility of studying the A^* state in isolation. It was established on the basis of electronic spectroscopy that mutants trapped in the A state (the blue fluorescent mutants) generally have a low fluorescence quantum yield [5].

The dynamics associated with the A^* state have been studied through time resolved fluorescence and transient vibrational spectroscopy for both S65T GFP (pH 5.5) and a related mutant, S65G/T203V/E222Q [117]. The latter is also trapped in the A form, but has a higher emission quantum yield; this mutant is called for convenience blGFP. The time resolved emission spectra are shown in Fig. 7 for both mutants. The emission spectra are very broad and unstructured, which is typically an indication of a significant difference between the equilibrium structure in ground and excited states (a feature which is also evident in wtGFP(95)). Interestingly

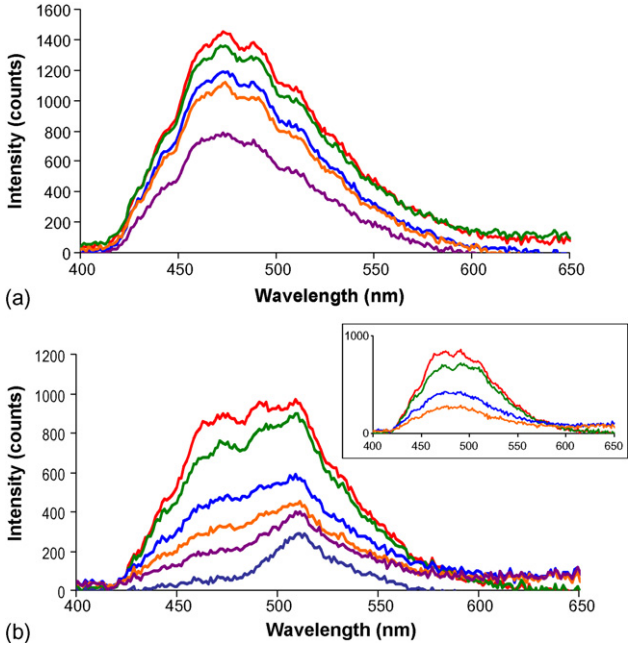

Fig. 7. Time resolved fluorescence spectra (a) blGFP 3 ps (red), 10 ps (green), 50 ps (blue), 100 ps (orange), 300 ps (violet) after excitation. (b) S65T at pH 5.5 2 ps (red), 10 ps (green), 20 ps (blue), 50 ps (orange), 150 ps (violet), 400 ps (indigo) after excitation. Inset, the same spectra as (b) with the long lived directly excited I^* emission component subtracted [117] (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

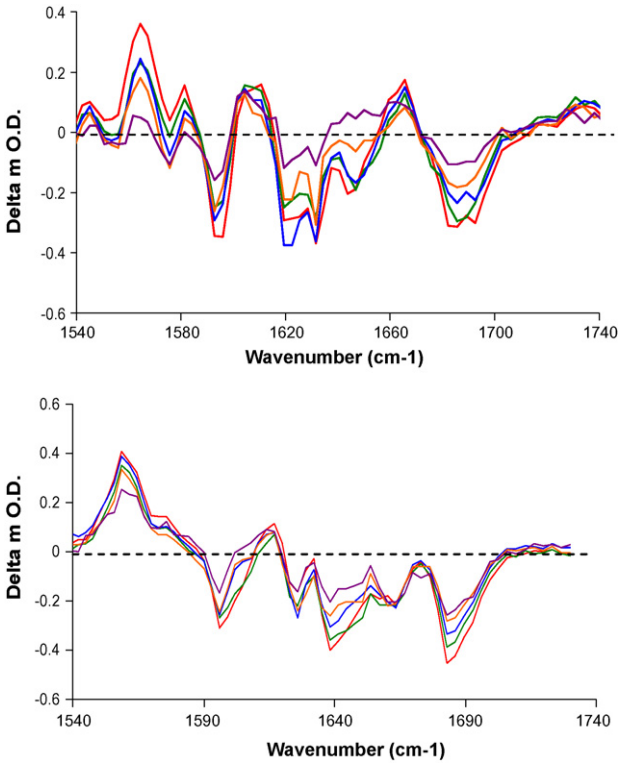

Fig. 8. Time resolved IR spectra of mutatnts of GFP trapped in the A state. (a) S65T at pD 5.5: 2 ps (red), 6 ps (green), 10 ps (blue), 30 ps (orange), 100 ps (violet) after excitation (b) blGFP: 2 ps (red), 4 ps (green), 10 ps (blue), 30 ps (orange), 200 ps (violet) after excitation (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

there is no evidence for a significant time dependent Stokes shift on the timescale of 1–500 ps. This in turn suggests that any environmental relaxation in the protein matrix to accommodate the newly formed excited state proceeds either on a sub-picosecond timescale or is too slow to contribute on the fluorescence timescale. In addition there is no evidence in either fluorescence spectrum for an excited state proton transfer reaction (the 510 nm emission seen in the S65T emission (Fig. 7) arises from direct excitation of a small fraction of the B form which exists at pH 5.5, and appears in emission only because of its high quantum yield).

Having trapped the chromophore in the A form the effect of electronic excitation on the protein can be investigated through transient IR spectroscopy [117]. The transient spectra for blGFP and S65T are shown in Fig. 8. The spectra are dominated by a number of strong bleach modes formed promptly on excitation and a strong transient absorption at ca 1560 cm^{-1}. These spectra are markedly different to those of wtGFP and surprisingly complex given that the proton transfer reaction, which contributed much of the complexity in the wtGFP spectra, has been blocked. The carbonyl (1685 cm^{-1}) and phenyl modes (1595 cm^{-1}) associated with the chromophore are still apparent, assignments which were confirmed by polarisation resolved measurements [117]. In addition one of the bands in the complex bleach profile between 1620 cm^{-1} and 1670 cm^{-1} is probably associated with the chromophore C=C stretch. The remaining bleach modes in this region are not seen in either HBDI or wt GFP. It seems likely that they are associated with a perturbation to the protein structure occurring promptly on electronic excitation. That such a perturbation is not seen in wtGFP suggests that the structural modifications brought about to stabilise the A state and block the proton transfer have increased the interaction between the A^* state of the chromophore and the protein. As a result electronic excitation perturbs the vibrational spectrum of the protein matrix. The interaction between the chromophore and its protein matrix and the relatively short lifetime of the A^* state both suggest that wtGFP has evolved to stabilize the emissive I^* state, with A^* surviving only long enough for the excited state proton transfer to occur. The structures for wt and S65T GFP (pH 4.6) were determined by Remington and co-workers [118]. While the structures are quite similar the S65T has a new H-bond between the chromophore hydroxyl and T203. Possibly the strength of this H-bond is modified upon excitation causing a shift in the residue vibrational modes.

Finally, an intense transient absorption appears at 1560 cm^{-1} (Fig. 8). Polarisation resolved measurements show that this band comprises at least two modes. These may include contributions from the protein modes, shifted from around 1650 cm^{-1}, and from the excited state of the chromophore—for example the A^* C=O occurs in this region for wtGFP [101]. Further resolution of this band will require isotopic substitution.

An alternative possibility for GFP mutagenesis is the modification or redirection of the proton transfer chain. It was established by Remington and co-workers that introducing the mutation H148D in mutants where the proton relay was suppressed restored the emission associated with the anionic I^* form [118]. The interesting proposal was made, that this may be caused by the formation of a new H-bond between chromophore donor and the D148 acceptor, effectively short circuiting the proton relay characteristic of wt GFP. The crystal structure of S65T H148D GFP was determined and the O atom of D148 was found to be unusually close to the chromophore O atom (2.4 Å), strongly suggesting the formation of a short strong H-bond between them [119].

The electronic spectroscopy of the H148D mutant is consistent with a distinct and specific chromophore protein interaction. Although the pH can be adjusted to populate the neutral A form almost exclusively, the resulting $A \rightarrow A^*$ absorption is red-shifted by some 20 nm compared to the usual A absorption. However, excitation of this red-shifted A band results exclusively in emission

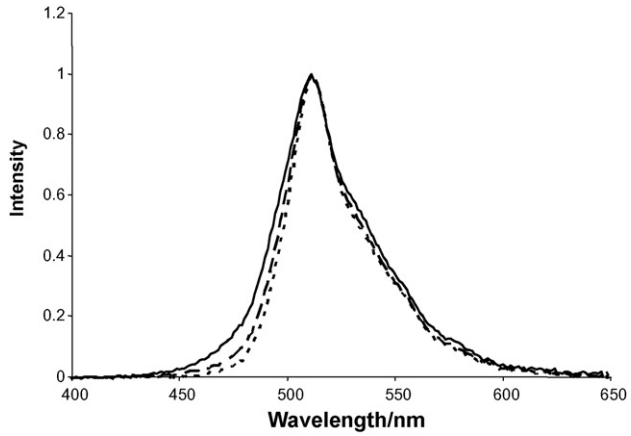

Fig. 9. Emission spectra of S65T/H148D GFP recorded 6 ps (solid), 20 ps (dash) and 100 ps (dot) after excitation at 400 nm.

from the anionic state of the chromophore, suggesting an efficient proton transfer reaction (Fig. 9). The time resolved emission following A state excitation was studied by Boxer and co-workers with 170 fs time resolution, and it was found that the green emission was formed within the instrument response time, i.e. orders of magnitude faster than in wtGFP [119,120]. Further, also unlike wtGFP, the ultrafast proton transfer was independent of deuteration, and insensitive to temperature. However, the electronic spectra, and particularly their temperature dependence were thought to show the existence of a range of conformations not resolved in structural studies [121]. Analysis of the gated fluorescence spectra also showed prompt emission from the anionic form of the chromophore. There was no evidence for a distinct A^* emission, with only a slight narrowing of the spectrum of the anionic form in the first few ps after excitation [120].

The transient IR spectra shed further light on this new proton transfer pathway. The spectra for S65T/H148D (Fig. 10) may be compared with those of the single mutant S65T in its neutral form (pD 5.1) in Fig. 8a [122]. There are a number of quite striking differences induced by the H148D mutant. The intense transient absorption at 1565 cm^{-1} in S65T is absent in the double mutant, consistent with this mode being associated with a perturbation to the protein brought about by blocking the excited state proton transfer reaction. Of the three characteristic chromophore bleach modes the phenyl ring mode around 1600 cm^{-1} has the lowest ΔOD in S65T/H148D in contrast to S65T alone. This is more similar to the wtGFP spectrum. In addition this mode is broad and asymmetric in S65T/H148D, whereas in wtGFP and all other mutants it is observed to be sharp and well defined. This is consistent with a strong interaction between the phenyl ring and the aspartate residue reported in the X-ray structure.

The most surprising feature in the S65T H148D time resolved IR spectrum is the absence of any transient that can be associated with a carbonyl absorption in the aspartic acid residue, which was assumed to be the proton acceptor. On the basis of ultrafast appearance of the anionic (I^*) emission the prompt appearance of a carbonyl band above 1710 cm^{-1} was expected. There is no evidence for such a proton acceptor mode in the transient spectrum, nor is their evidence for the corresponding disappearance of a carboxylate group around 1560 cm^{-1}. In fact the S65T H148D spectrum shares with the S65T spectrum the appearance of a simple combination of ground and excited state absorption, with isosbestic points observed at 1620 cm^{-1}, 1655 cm^{-1} and 1673 cm^{-1}.

This presents an interesting challenge to interpretation—the electronic spectroscopy points to a four level system involving ultrafast proton transfer from the (perturbed) A^* state to I^* and its reverse in the ground state, while the transient IR data suggest only excitation followed by ground state recovery on the hundreds of picoseconds timescale. One rationalisation of these results is that the interaction between the chromophore and the newly located aspartate acceptor is so strong that they can no longer be considered as distinct proton donor and acceptor. Rather the proton is shared in the ground state, with the result that the vibrational spectrum of the aspartate is already quite similar to that of the protonated form. Such a strong interaction is also consistent with the perturbed form of the A state absorption. The result of electronic excitation is then for the equilibrium position of the proton to be shifted somewhat towards the aspartate to yield the observed green emission. Thus the excited state dynamics can be viewed as essentially an intramolecular vibrational cooling in a reactive proton transfer coordinate involving the strongly interacting donor and acceptor. These ideas are illustrated in Fig. 11 [122].

This interpretation has the merit of being consistent with the observed structure and with most of the available spectroscopic data, provided it is accepted that the location of the proton on the potential surface can influence the vibrational and electronic spectra to different extents. Specifically in the ground state the 'acceptor' vibrational modes are assumed to resemble those of the protonated form while the electronic spectrum is that of a perturbed neutral chromophore 'donor'. The evolution on the upper surface apparently influences the emission spectrum more than the vibrational one. Appropriate tests of this model will require both high level quantum mechanical calculations on the S65T H148D structure,

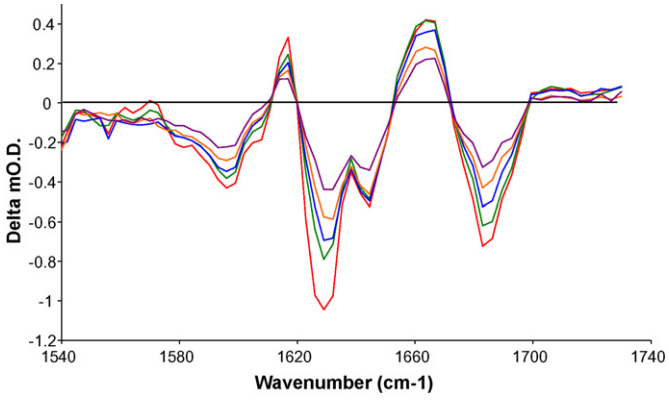

Fig. 10. Transient IR spectra of S65T H148H GFP recorded, 2 ps (red), 10 ps (green), 100 ps (blue), 200 ps (orange) and 500 ps (violet) after excitation at 400 nm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

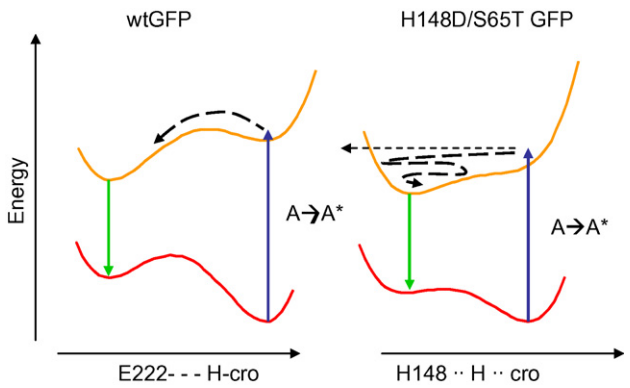

Fig. 11. Proposed potential energy surfaces for the proton transfer coordinate in wt GFP and H148D GFP. The latter has a barrierless potential in the excited state so the proton translates on an ultrafast timescale to the acceptor.

while isotopic substitution on D148 will help to further analyse the transient vibrational spectra.

The unusual photophysical properties of the S65T H148D mutant present another example whereby studies of GFP can potentially illuminate a wider problem in protein proton transfer. Both the structure and dynamics point to the existence of a proton shared between the oxygen atoms of the donor and acceptor. This is analogous to the case of a short low barrier hydrogen bond (LBHB). Such hydrogen bonds have been implicated in a number of enzyme processes [123,124]. In the present case the LBHB is an asymmetric one, and electronic excitation of the chromophore changes the degree of asymmetry. Thus, once again, combinations of structural and ultrafast spectroscopic studies of GFP allow us to investigate a potentially important proton transfer process in biology in a way which has not hitherto been possible.

5. Summary

Considerable progress has been made in understanding the photophysics of FPs, but much remains to be done. The efficient radiationless decay of the isolated chromophore is rather well characterised by both experiment and theory. The subsequent enhancement of the fluorescence yield for the chromophore in the protein matrix has been widely studied, and a number of enhancement mechanisms have been described. Further studies including ultrafast spectroscopy, mutagenesis, structure determination and more detailed quantum chemical calculations will serve to distinguish between these possible mechanisms. It is likely that different mechanisms operate in different FPs. Much more research is required to understand in detail the photophysics of the newly discovered photoactivateable FPs, which appear to have a number of extremely important applications in bioimaging. Progress will be dependent on correlating high resolution structural studies with ultrafast electronic and vibrational spectroscopy.

The photophysics of GFP are dominated by a proton relay reaction. The characterisation of this reaction through ultrafast electronic and vibrational spectroscopy was described. Considerable progress has been made recently in reproducing the proton relay dynamics through quantum chemical calculations. The ability to combine structure, dynamics and theory suggests that FPs may provide unique insights into proton transfer in proteins quite independent of their role in bioimaging. In particular, the studies of the H148D mutant show that the proton transfer pathway can be redirected through mutagenesis, and the combination of structure and dynamics suggest that this protein may present an example of proton transfer on a LBHB potential energy surface. A particularly exciting opportunity for the future is the possibility of including unnatural amino acids in the residues that make up the chromophore. This would yield fine control over the proton relay potential energy surface. Such measurements are planned.

Acknowledgements

S.R.M. and P.J.T. would like to thank past and current members of their research groups for their essential contributions to this work and Drs Kate Ronayne, Mike Towrie and Pavel Matousek of the CCLRC Central Laser Facility for their help and support in the TRIR measurements. S.R.M. thanks the EPSRC for financial support, while P.J.T. thanks NIH for financial support (GM66818).

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