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Hydrogen Bond Flexibility Correlates with Stokes Shift in mPlum Variants

Patrick Konold^{1,2}, Chola K. Regmi³, Prem P. Chapagain³, Bernard S. Gerstman³, and Ralph Jimenez^{1,2}

¹JILA, University of Colorado and NIST, Boulder CO 80309

²Department of Chemistry & Biochemistry, University of Colorado, Boulder CO 80309

³Department of Physics, Florida International University, Miami FL 33199

Abstract

Fluorescent proteins have revolutionized molecular biology research and provide a means of tracking subcellular processes with extraordinary spatial and temporal precision. Species with emission beyond 650 nm offer the potential for deeper tissue penetration and lengthened imaging times, however, the origin of their extended Stokes shift is not fully understood. We employed spectrally resolved transient grating spectroscopy and molecular dynamics simulations to investigate the relationship between the flexibility of the chromophore environment and Stokes shift in mPlum. We examined excited state solvation dynamics in a panel of strategic point mutants of residues E16 and I65 proposed to participate in a hydrogen bonding interaction thought responsible for its red-shifted emission. We observed two characteristic relaxation constants of a few ps and tens of ps that were assigned to survival times of direct and water-mediated hydrogen bonds at the 16-65 position. Moreover, variants of the largest Stokes shift (mPlum, I65V) exhibited significant decay on both timescales indicating the bathochromic shift correlates with a facile switching between a direct and water-mediated hydrogen bond. This dynamic model underscores the role of environmental flexibility in the mechanism of excited state solvation and provides a template for engineering next generation red fluorescent proteins.

Keywords

Ultrafast spectroscopy; Fluorescent proteins; Solvation dynamics

Introduction

Fluorescent proteins (FPs) derived from marine organisms, including the green fluorescent protein extracted from the jellyfish *Aequorea victoria*, have revolutionized cellular imaging experiments and become an invaluable tool in molecular biology research¹. Their utility is owed to several favorable intrinsic properties, namely, tunable excitation and emission, genetic encodability, and targetability. Next generation far-red emitting species with

Supporting Information Available: mPlum transient absorption spectrum, water occupancy, E16L time trajectory derived from MD simulations, and remaining fitting data are provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

enhanced fluorescence quantum yields and high photostability are eagerly sought. These improvements would enable deeper tissue penetration and lengthened imaging times given a lower autofluorescence background, lower light scattering, and higher transmission beyond 650 nm².

The fluorescent chromophore of FPs is autocatalytically formed in the presence of molecular oxygen, and is contained within an eleven-stranded β -barrel. Electronic excitation in the chromophore occurs with electron density transferred from the phenoxy ring towards the acylimine across the π -conjugated system^{3,4}. Several strategies have been used to engineer further red-emitting species^{2,5}. One approach is to extend conjugation within the chromophore itself or with nearby sidechains *via* selective mutagenesis. Alternatively, one can modify the surrounding chromophore environment leading to direct stabilization (destabilization) of the excited (ground) state by electrostatic effects, hydrogen bonding (H-bond) interactions, or isomerization. Examples of these interactions are found in several red FP species, though in most cases, interpretation is derived from crystal structures and neglects a possible dynamic element⁵⁻⁸.

The fluorescent protein mPlum, developed through iterative somatic hypermutation of mRFP (from the progenitor DsRed), has a peak excitation of 589 nm and a Stokes shift of 59 nm⁹. A H-bond between the E16 sidechain and N-acylimine oxygen of the I65 residue at the base of the chromophore as depicted in Figure 1 is thought to be the key feature leading to its large Stokes shift. This hypothesis evolved from a few striking observations: first, as illustrated in the original report by Wang *et al.*, steady state emission values vary widely with point mutations at these positions and all reported variants show a nearly 20 nm blue shift relative to the native E16 configuration. The Stokes shifts fall within 3 distinct groups around 25 nm, 35 nm, and 50 nm, which may imply common dynamic behavior within mutant subgroups of similar hypsochromic shift. Moreover, these perturbations occur primarily on the excited state given the sensitivity of the emission wavelength. Second, Abbyad *et al.* identified a large dynamic Stokes shift with timescales of 4 and 71 ps in mPlum using fluorescence upconversion spectroscopy¹⁰. These dynamics differed from mRaspberry and mRFP, which showed very little solvation over the 1 ns probe window. They explained this shift in terms of a specific interaction between E16 and I65 on the basis of their influence on the steady state Stokes shift. This was the first report of a time dependent interaction leading to an extended red shift in FPs. These authors proposed that the solvation process resulted from an excited state rearrangement of a specific interaction between residues 16 and 65, yet their evidence did not discount the possibility that general flexibility of side-chains or water around the chromophore may be responsible. More recently, crystal structures of mPlum together with E16Q and I65L variants reaffirmed the hydrogen bond between E16 and I65 in mPlum and identified a similar interaction in the E16Q mutant¹¹. They also implicated the steric influence of I65 and predict it plays a role in strengthening this hydrogen bond in the excited state.

Several different femtosecond spectroscopy techniques are capable of resolving the time dependent red-shift due to the excited state solvation dynamics¹². Here, we employ spectrally resolved transient grating spectroscopy (SRTG) as applied by Joo *et al.* to observe nuclear wavepacket dynamics in ground and excited electronic states and solvation of dyes

in bulk solvent^{13, 14}. In this four-wave mixing technique, two pump pulses (with wavevectors $\mathbf{k}_1$ and $\mathbf{k}_2$) interfere imprinting a spatial grating on the sample which is later probed by a third pulse (with wavevector $\mathbf{k}_3$) at a controllable delay that diffracts into a spatially isolated phase matching direction ($\mathbf{k}_1 - \mathbf{k}_2 + \mathbf{k}_3$). For a chromophore with a large Stokes shift together with a sufficient excitation window, this signal can be spectrally resolved into stimulated emission (SE) and ground-state bleach (GSB) contributions. Time evolution of the GSB and SE bands represent ground and excited state solvation dynamics respectively.

In this report, we employ SRTG spectroscopy and molecular dynamics (MD) simulations to explore the relationship between flexibility of the chromophore environment and the large Stokes shift in mPlum. Here, we investigate relaxation in a panel of strategic point mutants of the participating 16 and 65 residues. The experimental results and simulations indicate the red-shifted emission in mPlum is correlated with a facile switching between a direct and water-mediated hydrogen bond between the E16 sidechain and the N-acylimine oxygen at position 65. All discussions about the role of a H-bond network in earlier papers were derived from a static crystal structure in the ground state. This paper represents an advance in that it explores H-bond dynamics around the chromophore rather than the H-bond network derived from an average ground state structure.

Experimental Methods

Time-resolved Measurements

Transient grating measurements were performed in the traditional BOXCARS geometry as established in the previously described JILA MONSTR nonlinear optical platform¹⁵. Upon irradiation with 3 excitation pulses (20 fs, ~ 10 nJ/beam), generated from a noncollinear optical parametric amplifier pumped with a Ti:Sapphire regenerative amplifier at 20 KHz, the spatially isolated four-wave mixing signal emitted in the prescribed $\mathbf{k}_s = -\mathbf{k}_1 + \mathbf{k}_2 + \mathbf{k}_3$ phase matching direction. Excitation wavelength was chosen to maximize contrast between ground state bleach and stimulated emission components. The sample was refreshed with a spinning cell to mitigate photobleaching and accumulation of laser-induced photoproducts. Sample concentration and path length were carefully tuned to avoid aggregation and reabsorption of the nonlinear signal. Concentrations of ~ 50 μM and a path length of 0.5 mm were used for all samples (OD 0.15 at peak absorption). Spectrally resolved detection was carried out with a liquid N₂-cooled CCD camera. Data was collected as a function of the T time delay ($\mathbf{k}_1$ and $\mathbf{k}_2$ temporally overlapped) from a range of 0 to 1.3 ns, spaced logarithmically, with a background spectrum collected at -500 fs delay to eliminate contributions from excitation scatter and spontaneous emission.

Protein Expression and Purification

Fluorescent proteins were expressed with a pBAD expression vector containing the mPlum gene. Point mutations were accomplished with QuikChange mutagenesis from commercial primers. Upon sequencing confirmation, plasmids were transformed into Top 10 *E. coli* followed by induction with 0.02% arabinose for 24 hours at 30 °C. Samples were purified via 6X-His tag/Ni-NTA chromatography and the crude specimens were buffer-exchanged

with 15 mM pH 8.0 TRIS buffer, 100 mM KCl. Emission spectra of each mutant, excited at 532 nm, were detected with a diode array spectrometer.

Molecular Dynamics Simulations

Time series trajectories were obtained from explicit solvent, all-atom simulations using the NAMD molecular dynamics package with the CHARMM27 force field¹⁶. The initial X-ray crystallographic structures were obtained from the Protein Data Bank ((mPlum pdb code 2QLG, E16Q pdb code 2QLI, I65L pdb code 2QLH). The other four mutants do not have PDB structures, so we used CHARMM to make the amino acid substitution in the mPlum pdb 2QLG file. Force field parameters for the mature chromophore were adopted from the anionic GFP chromophore developed by Reuter *et al.* and from CHARMM27 parameters for acylimine nitrogen¹⁷. Throughout the simulation, the deprotonated anionic form of the chromophore in the ground state was used. In addition, E215 was protonated using a patch.

The VMD package was used to setup the system for simulations¹⁸. The initial structure of mPlum with crystallographic water molecules was solvated by using the *solvate* plugin in VMD. For all mutants, the box cutoff was set to 10 Å. For mPlum, this resulted in a simulation box of dimensions $65.1 \times 62.6 \times 71.7 \text{ Å}^3$, with similar dimensions for the other mutants. The solvated system was electrically neutralized by adding five Na⁺ ions randomly in the bulk water using the VMD autoionize plugin. The final system contained a total of 27188 atoms. All water molecules overlapping with the protein were removed. The particle mesh Ewald method was used to treat long-range interactions with a 12 Å nonbonded cutoff¹⁹. Energy minimization was performed using the conjugate gradient and line search algorithm. The system was then heated for 90 ps with a linear gradient of 20K/6ps from 20 to 300 K. At 300 K, the system was equilibrated for 910 ps with a 2 fs integration time step in the NVT (constant number, volume, and temperature) ensemble. Langevin dynamics was used to maintain the temperature at 300 K. The production run was 50 ns using NVT dynamics with 2 fs time steps. The last 40 ns of the production run was used for analysis.

Results

Steady State Spectroscopy

Linear absorption and emission peaks for each mutant are shown in Table 1. The absorption peaks fall within a narrow range around 590 nm aside from E16L, E16H, and I65L, which exhibit ~5 nm blue and red shifts respectively. The Stokes shifts range from 1600 cm⁻¹ and 778 cm⁻¹ with mPlum and E16L being the largest and smallest respectively.

Time-resolved Measurements

To differentiate the solvation dynamics of the variants, we analyzed both the spectral shape and time evolution of the transient grating signals. SRTG spectra of mPlum and the I65L for various time slices are displayed in Figure 2a. The results for these mutants were plotted to illustrate the range of behavior among all species.

In each case, we observed two distinct bands corresponding to the GSB (high energy) and SE (low energy) responses. The absence of signal between bands for mPlum and on the red

extreme for I65L suggests a strong excited state absorption (ESA). To test this effect, we evaluated our signal in the case of infinite decay. For a heterodyne detected signal (*e.g.* transient absorption), one would expect the signal spectrum upon complete relaxation to reflect the excitation-weighted sum of steady state absorption and emission. To account for this discrepancy in our homodyne experiment, we divided the SRTG data by the probe intensity spectrum as explained previously by Lee and coworkers¹⁴. This test confirms the presence of ESA to the red of ω_{eg} , likely overlapping the SE band. Furthermore, the transient absorption spectrum of mPlum (Figure S1) also shows a negative band located at wavelengths both red-shifted and blue-shifted of the GSB indicative of ESA. We assume the ESA does not spectrally evolve on the experimental timescale.

Transient grating spectra at each time increment were fit to a sum of two Gaussians and the center positions of each band were subsequently fit to a multiexponential decay. Data at early and later delays were omitted due to finite pulse effects and poor signal-to-noise, respectively. Example SE peak position fits for mPlum and I65L are displayed in Figure 2b and the remaining parameters are presented in Table 2.

The solvation responses can be categorized in terms of either timescale or amplitude. Timescales ranged from around a hundred ps (E16Q, I65A), tens of ps (mPlum, E16H, E16L, I65L), and a few ps (mPlum, I65V). It should be noted that the mPlum time constants of 3 and 51 ps are consistent with those measured with fluorescence upconversion by Abbyad *et al.* (4.3 and 71 ps)¹⁰. The shift magnitudes fell into two groups: 22-37 cm^{-1} (E16Q, E16L, I65L, I65A) and 65-112 cm^{-1} (mPlum, I65V, E16H). The mutants with the largest Stokes shifts (mPlum, I65V) have multiple timescales of relaxation. The remaining show only a single timescale of tens of ps. Longer decay times cannot be ruled out given the 500 ps experimental time window. Timescales of the GSB component are in qualitative agreement suggesting similar ground and excited state dynamics in accordance with a linear response. The magnitudes of the spectral shifts in all cases are smaller than observed by Abbyad *et al.* (793 cm^{-1}). In principle, this can be attributed to either inadequate time or spectral resolution. The former is unlikely since the time resolution in the previous study was on the order of 100s of fs, whereas SRTG was performed with 20 fs excitation pulses. On the other hand, reductions in the measured spectral shifts are inevitable given a finite excitation bandwidth of approximately 1000 cm^{-1} along with an overlapping ESA.

The SRTG response was modeled to reconcile the disparity in shift magnitude between our measurements and upconversion spectroscopy. We consider the influence of a finite laser bandwidth and ESA on the SE band position. For three electronic levels, there are three possible resonant time ordered contributions to the third order optical response to consider for our experimental phase-matching direction²⁰. To simplify fitting, the GSB response was omitted. This simplification will not affect the results since this band is blue-shifted relative to the SE band. An initial center position of 16178 cm^{-1} was chosen for the SE component with an asymmetric Gaussian shape replicating the steady state emission spectrum. The excitation pulse center and width mimicked that used in our experiment (16000 cm^{-1} , 1025 cm^{-1}). An asymmetric Gaussian with a shape closely matched to the ground state absorption spectra was used to model ESA. The intensity of the ESA transition was assumed equal to 0.4 of the GSB consistent and centered at 16667 cm^{-1} , consistent with the transient

absorption spectrum. The SE and ESA signal responses were added according to the signs of their respective optical responses. A red shift was assumed to follow the measured upconversion time constants and shift magnitudes for mPlum of 4.3 (301 cm^{-1}) and 71 ps (492 cm^{-1}). Figure 3 illustrates the modeled data with and without application of a finite excitation bandwidth and ESA. Inclusion of these effects yields a total shift magnitude of 137 cm^{-1} , in close agreement with our experimentally measured value. Moreover, the shift time constants of 5 and 72 ps for the filtered signal closely match the inputted values. By proportionally decreasing the shift magnitude relative to mPlum in Table 1, we also find the calculated shift magnitudes similarly agree with the experimental values for the mutants. This analysis demonstrates that accounting for a finite excitation bandwidth and ESA explains the decreased shift magnitudes relative to upconversion spectroscopy and that SRTG is sensitive to the underlying constants of the excited state reorganization.

The amplitude decays of each band reveal predictable photophysical behavior (Table S3). In the case of SE, the dominant component closely matches the measured excited state lifetime of 800 ps for mPlum after correcting twofold due to the effect of homodyne detection¹⁰. In some mutants, minor contributions exist on both shorter and longer timescales consistent with complex photophysics known to FPs. The GSB band intensity decay reflects the ground state recovery process, which is expected to follow both directly from the excited state, or more slowly from a third level, as FPs are known to undergo varying degrees of dark state conversion with repopulation occurring on microsecond and longer timescales²¹. This effect would yield a disparity between rates of ground state repopulation and excited state depopulation. The amplitude decays are also slightly influenced by bands shifting within the finite laser bandwidth. We will not further consider the GSB recovery timescales, but instead focus on the excited state dynamics revealed by the timescales of SE band shifts.

Molecular Dynamics Simulations

MD trajectories of each mutant were used to examine the chromophore flexibility and the interconversion between hydrogen bond states. First we examined the dynamics of the interaction between positions 16 and 65 in mPlum and found two distinct conformations, displayed in Figure 4. The important difference between Figure 4 (a) and (b) is the hydroxyl group orientation (OE2 and HE2 in the CHARMM file) of E16. In Figure 4a, the OH group is positioned so that it can make a direct hydrogen bond with I65, whereas in Figure 4b, it reorients to accommodate a water-mediated H-bond. The simulations reveal the presence of approximately 5 water molecules within 3 Å of the chromophore for all mutants (Figure S2).

In the mPlum variants, we found that some can also form two distinct 16-65 H-bonds (E16Q, I65V), whereas other mutants can only form one type of H-bond (I65A, I65L, E16H) or none at all (E16L). Figure 5 presents times series trajectories from MD simulations from 0-40 ns. The relative 16-65 positioning is expressed as r , and is defined as the distance between the participating atom in the 16 sidechain and the N-acylimine oxygen of residue 65.

The capability to switch between substates is correlated with Stokes shift. For the three proteins with the largest Stokes shift, mPlum, E16Q, and I65V, there are distinct, ns-timescale periods in which the separation is characteristic of relatively stable direct H-bonds

(<3.5 Å), as well as switching to distinct periods in which the separation is characteristic of water-mediated H-bonds (>4 Å). This is reflected in the first two columns of Table 3, which shows that for these proteins, conformational interconversion allows each type of H-bond to occur for a significant fraction of the MD simulation. For the other four mutants, with smaller Stokes shifts, the time series trajectories of Figure 5 do not reveal conversion between two relatively stable types of H-bonds. Mutants I65L and I65A spend most of their trajectories in a direct H-bond, while E16H fluctuates primarily within the water-mediated H-bond conformation. Finally, the E16L variant cannot form a stable H-bond of either variety due to its aliphatic nature and hence, exhibits random fluctuations throughout the trajectory (Figure S3). The 16-15 distance for E16Q shows an additional substate with $r \sim 6.5$ Å, caused by a unique rotation of Q16 as shown in Movie S1. A histogram of the 16-65 distance r for each FP similarly reveals the presence of these distinct H-bond configurations described above (Figure S4).

We next analyzed the MD results to determine if the lifetimes of the various H-bond configurations are consistent with their characteristic experimental timescales. Simulations were carried out for time intervals long enough to ensure exhaustive sampling of all molecular subconformations²². We extracted H-bond survival times from the simulations by calculating the H-bond survival function in the direct substate, and separately in the water-mediated substate. The survival function gives the conditional probability that a hydrogen bond intact at time t_{i-1} will also remain intact at time t_i and is given by $p(t) = 1 - f(t)$, where $f(t)$ is the fraction of hydrogen bonds broken at time t . The average survival time was estimated by fitting the survival function to an exponential: $p(t) \sim \exp(-t/\tau)$ ²³. The survival time functions show decays on a few ps and tens of ps timescales for most mutants (Figure S6). Both sets of decays show qualitative agreement with the experimental time constants shown in Table 2, with the 30° criteria being proportionally shorter for the direct H-bond. For variants with significant populations in both substates, it is observed that direct H-bonds survive for much longer times than do the water-mediated H-bonds. This implies that the shorter experimental time constants may be due to 16-65 water-mediated H-bond dynamics, whereas the longer experimental time constants may be due to 16-65 direct H-bond dynamics. If the percentage of a type of H-bond is very small, we are not able to calculate survival times, which might explain why mutants of smaller Stokes shift have only one experimentally observed time constant. In general, the biggest disagreements fall among the E16 mutants. The absence of shorter time component in E16Q is likely a result of poor signal-to-noise given the small shift magnitudes. A strong ESA overlap with the SE band would have an effect similar to a finite excitation bandwidth mentioned above in that it would decrease the magnitudes of the spectral shifts. The longer time constant observed in this mutant can be explained in terms of a unique rotation of Q16 with a calculated correlation time of 91 ps, which closely matches the experimental value of 112 ps. For E16L and E16H, the origin of this discrepancy is less obvious, although we find an elevated chance of a water-I65 H-bond (without a simultaneous water-16 H-bond) in these mutants (Table S4). The survival time of this hydrogen bond is only a few picoseconds, and therefore the observed relaxation is clearly not the result of the dynamics of this bond. It is possible that water motion in this region near the chromophore could facilitate solvation on the timescales observed. In general, the mutations at position 16 yield less predictable responses

than those at position 65, probably because these mutations replace a charged side chain with uncharged, polar or nonpolar sidechains, which are likely induce greater electrostatic perturbation of the chromophore environment versus the position 65 mutations. Further analysis will require quantum mechanical calculations.

To connect the dynamics observed in the MD trajectories with Stokes shift, we examined the influence of the 16-65 interaction on H-bonds made to the chromophore with the surrounding environment. In particular, interactions with S146, R95, E215, and Q109 are known to be important for spectral tuning in other green and red FPs⁶. Table 4 summarizes this analysis. The first two columns show the fraction of time spent H-bonded, direct or water-mediated, at positions 16 and 65. The remaining columns tabulate the time a chromophore-sidechain H-bond occurs, partitioned by 16-65 substate, and normalized by its respective occupancy. Residue 65 is considered to be part of the extended chromophore and therefore hydrogen bonding with 65 can directly influence the chromophore environment¹⁰. Chromophore-H-bond interactions at other sidechains appear independent of whether there is a direct or water-mediated interaction at 16-65. We conclude that 16-65 switching constitutes the largest observable structural change within the chromophore environment and must be integral for the bathochromic shift observed in mPlum.

The chromophore flexibility was analyzed by extracting a root mean square fluctuation (RMSF) on an atomic basis from the MD simulations. We observe no correlation between chromophore flexibility and Stokes shift among these mutants (Figure S7).

Discussion

The time dependent shifts of the SE band observed in SRTG measurements are directly related to the relaxation of the excited state energy leading to the Stokes shift of the steady state emission spectrum. Classical MD simulations are not capable of estimating the magnitude of electronic energy relaxation since they only model dynamics on the ground electronic state. However, within linear response theory, these dynamics approximate motions leading to excited state solvation and thus, serve as a means of modeling the effect of variations in chromophore-environment interactions on molecular dynamics and their timescales²⁴. Our results provide new insight into the dynamics of the interaction between the 16 and 65 sidechains in mPlum and an explanation for its large Stokes shift relative to other DsRed-derived FPs. These conclusions follow from two key observations. First, the two timescales observed experimentally and in the simulations represent survival times of direct and water-mediated H-bond substates between the 16 and 65 sidechains. Second, there is a direct correlation between the Stokes shift of each mutant and to the extent to which it undergoes H-bond interconversion. Cases in which no direct H-bond is possible (E16L, E16H) or where a direct H-bond forms almost exclusively (I65L, I65A) show the smallest Stokes shifts of mutants tested. Conversely, species with the greatest propensity to interconvert between H-bond states (mPlum, E16Q, and I65V) have the largest Stokes shift. This dynamic model of the chromophore solvation stands in contrast with previous studies of mPlum mentioned above, which primarily attributed its red-shifted emission to stabilization of the excited state through a direct H-bond between E16 and the N-acylimine oxygen of I65.

The influence of confined water molecules on the solvation of probes buried within protein environments following photoexcitation has brought about intense debate²⁵. In particular, it is questioned whether confined water or protein sidechains are responsible for the slow (relative to bulk water) ps solvation observed in these environments. For GFP, Xu and coworkers found 10 water molecules in close vicinity to the chromophore with predicted solvation dynamics timescales ranging from tens of fs to several hundred ps²⁶. While our simulations indicate a similar presence of water, we think it is unlikely that these solvent molecules are responsible for the variation in dynamics. First, we observe similar water occupancy among all variants indicating any contribution is likely consistent regardless of mutation (Figure S2). Moreover, we do not observe the slowest 333 ps time constant predicted by Xu *et al.* in any sample. It is possible that the anomalous responses of the E16 mutants, noted above, is related to a water-I65 H-bond not observed in the I65 variants. These observations suggest water may play a minor role in the observable dynamic Stokes shift, but the dominant response is governed by internal H-bond dynamics as reflected by the above survival time calculations for most species.

It is clear that the presence of neither a direct or water-mediated H-bond is the sole determinant of a large Stokes shift. It is possible that the 16-65 interaction modulates chromophore flexibility. Flexibility about the methyldene bridge would disrupt coplanarity between the imidazole and phenoxy moieties, inhibit electron delocalization, and lead to a smaller Stokes shift^{7, 27}. A similar effect was proposed in quantum mechanical simulations of DsRed where a noted torsion of the terminal carbonyl of I65 leads to a blue shift due to loss of conjugation as observed in mOrange^{3, 6}. It is plausible H-bonding to this position serves as an anchor that preserves coplanarity either at the carbonyl or more broadly across the ring system. Among our series of mutants, this may be manifest in slightly different ways given the precise nature of the 16-65 interaction. These can be broken down into four cases: 1. forms no H-bond (E16L), 2. forms a direct H-bond (I65A, I65L), 3. forms a water-mediated H-bond (E16H), 4. demonstrates interconversion between multiple H-bond states (mPlum, E16Q, I65V). Assuming E16L as a baseline, those with a single interaction (I65A, I65L, and E16H) represent the middle grouping of observed Stokes shifts, whereas those in the fourth category (mPlum, E16Q, I65V) hold the largest Stokes shifts. The influence of switching is less obvious, but it may be true that, on average, coplanarity is higher among those interconverting between conformations. Alternatively, the most coplanar configuration (local energetic minimum) may lie along the switching coordinate and remain unpopulated by those not able to interconvert. Lastly, switching between states may act to suppress flexibility of the chromophore in dimensions other than ring torsion, which would also help drive stabilization of the excited state. It is interesting to note that of the I65 mutants, only the valine derivative undergoes H-bond switching, while I65A and I65L demonstrate more rigid conformations. We suggest this may be a steric effect and possibly essential to controlling H-bond interconversion.

Chromophore-sidechain H-bonding interactions are observed in crystal structures of mCherry, mStrawberry, mNeptune, mRojoA, mRouge, eqFP650, and eqFP670⁵⁻⁸. Our findings highlight the importance of the dynamics, not the mere presence of these interactions, and suggest that Stokes shift is related to the capability of interconversion

between multiple H-bonded states. It seems reasonable to hypothesize that this behavior scales with the number of weak, interconvertible chromophore H-bond interactions. Consistent with this idea, a recent publication describing the structure and properties of the farthest red emitting FP engineered to date, TagRFP675, which has a 75 nm Stokes shift²⁸. They attribute its red emission to several H-bonding contacts involving Q41 and S28 at the N-acylimine position with additional H-bonds at the phenoxy end of the chromophore with N143, N158, and R197.

Our results have significant implications in the context of engineering an ideal red fluorescent protein, *i.e.* one with both red-shifted emission and high quantum yield desired for cellular imaging applications. Previous studies recognized an apparent tradeoff between these properties, with brightness hindered by fast nonradiative deactivation, whereas red-shifted emission results from environmental solvation of the excited state²⁷. The implication is that chromophores of the brightest species, with the smallest Stokes shifts, are contained within rigid environments unable to deactivate excitation through thermal fluctuations, phenolic rotation, or cis-trans isomerization, while the reddest emission requires local flexibility to facilitate solvation of the excited state electronic distribution. We hypothesize that the H-bond switching phenomenon observed here acts to selectively tether the chromophore in such a manner that provides a degree of transient flexibility combined with stabilization of the excited state. A similar effect is observed in mNeptune where the guanidinium group of R197 runs parallel along the chromophore and is thought to provide stability across the ring system⁷. This “transient flexibility” may provide a mechanism for simultaneous optimization of these two photophysical properties.

Conclusions

Time-resolved spectroscopy was carried out on mPlum and a panel of strategic point mutants. Our results, supported by MD simulations, provide new insight into the dynamics of the interaction between the 16 and 65 sidechains in mPlum and a possible explanation for its large Stokes shift relative to other DsRed-derived FPs. We arrive at our conclusions following two key observations. First, the two timescales observed experimentally and in the simulations represent interconversion between direct and water-mediated hydrogen bonding substates between the 16 and 65 sidechains. Second, there is a direct correlation between the Stokes shift of each mutant and to the extent to which it undergoes H-bond interconversion. Cases in which no direct H-bond is possible (E16L, E16H) or where a direct H-bond forms almost exclusively (I65L, I65A) show the smallest Stokes shifts of mutants tested. Conversely, species with the greatest propensity to interconvert between H-bond states (mPlum, E16Q, and I65V) have the largest Stokes shift. This dynamic model of the chromophore stands in contrast with previous studies of mPlum, which primarily attributed its red-shifted emission to direct stabilization of the excited state through a H-bond between E16 and the N-acylimine oxygen of I65. We hypothesize such dynamics facilitate a bathochromic shift through a combination of direct excited state solvation and regulation of chromophore flexibility. This model may provide a guide for further development of highly desirable red-emitting FPs. Moreover, our findings demonstrate the utility of ultrafast spectroscopy in measuring functionally relevant sidechain fluctuations, not typically resolvable with steady state methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Chemical structure of mature mPlum chromophore. The hydrogen bond thought responsible for its large Stokes shift is shown as a dashed line between E16 and the chromophore terminus of residue I65.

Figure 2.

(a) Transient grating data for mPlum and I65L mutant with excitation spectrum (EXC) overlaid, (b) Center peak positions for each band obtained from transient grating data for mPlum and I65L mutant (black circles). A best-fit curve derived from nonlinear least squares fitting is overlaid (solid red line).

Figure 3. Simulated SRTG responses with and without application of a finite excitation bandwidth (EXC). (a) Hypothetical SRTG data without excitation filter and ESA. (b) With excitation filter and ESA.

Figure 4.

Two different 16-65 H-bonds in mPlum. (a) The E16 hydroxyl group orientation provides a small enough separation from I65 to allow a direct H-bond. (b) The E16 hydroxyl group orientation results in a larger separation from I65 and a water-mediated H-bond.

Figure 5. MD time series trajectories for each mutant. r refers to the distance between the sidechain of 16 and the N-acylimine oxygen of I65.

Table 1

Steady state absorption and emission data for each mutant.

Mutant	Absorption Peak (nm)	Emission Peak (nm)	Stokes Shift (cm ⁻¹)
mPlum	589	648	1593
E16Q	588	630	1134
E16H	586	620	936
E16L	586	614	778
I65L	596	629	881
I65A	591	630	1047
I65V	591	641	1319

Table 2

Summary of SRTG fitting results following fitting the raw data to a sum of two Gaussians.

Mutant	SE Red Shift		GSB Blue Shift	
	Tau (ps)	Shift Magnitude (cm ⁻¹)	Tau (ps)	Shift Magnitude (cm ⁻¹)
mPlum	3	17	--	--
	51	95	58	46
E16Q	107	26	86	21
E16H	49	69	--	--
E16L	13	23	34	7
I65L	44	24	237	19
I65A	--	--	9	16
	100	37	173	29
I65V	2	19	19	13
	51	84	142	18

Table 3

Survival times calculated for the direct H-bond molecular configuration and for the water-mediated H-bond configurations following two H-bond criteria. The survival times listed are obtained from the single exponential fits to the time correlation function. Calculation of survival times was not possible for cases with negligible H-bond percentage. Hydrogen bond survival times are given using two different sets of criteria for determining H-bonds (angle cut-off of 30° versus 40° in VMD).

Mutant	Survival Time (ps) 3.5 Å/30°		Survival Time (ps) 3.5 Å/40°	
	Direct	H ₂ O mediated	Direct	H ₂ O mediated
mPlum	10	2	29	4
E16Q	3	2	5	3
E16H	--	2	--	4
E16L	--	--	--	--
I65L	13	--	44	--
I65A	29	--	167	--
I65V	8	2	17	3

H-bonds to chromophore from neighboring sidechains are presented in terms of time fraction as calculated from MD simulations. For each mutant, bonds with sidechains S146, R95, E215, and Q109 are reported for each 16-65 substate, direct and H₂O-mediated, normalized by their respective occupancy. For all entries, 3.5 Å distance and 30° angle cutoff values were used for determining the H-bonds.

Table 4

Mutant	16-65 H-Bond (%)		S146-CRO (%)		R95-CRO (%)		E215-CRO(%)		Q109-CRO (%)	
	Direct	H ₂ O mediated	Direct	H ₂ O mediated	Direct	H ₂ O mediated	Direct	H ₂ O mediated	Direct	H ₂ O mediated
mPlum	31.3	18.4	72.8	49.9	88.9	89.7	82.3	79.6	38.7	10.9
E16Q	18.4	14.3	80.6	85.9	93.4	95.1	79.3	80.1	43.6	58.5
E16H	0.0	25.2	85.7	43.6	85.7	87.2	71.4	80.9	28.6	6.2
E16L	0.3	0.2	84.0	93.0	94.0	84.0	74.5	66.6	20.3	26.7
I65L	76.8	0.0	3.0	0.0	85.8	83.3	86.5	83.3	34.9	50.0
I65A	89.9	0.5	83.5	95.6	91.9	89.1	86.4	86.9	58.7	45.7
I65V	28.2	14.8	27.6	38.9	64.9	66.6	82.2	81.8	11.1	6.3