Probing dimerization and intraprotein fluorescence resonance energy transfer in a far-red fluorescent protein from the sea anemone *Heteractis crispa*

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Abstract. Proteins from *Anthozoa* species are homologous to the green fluorescent protein (GFP) from *Aequorea victoria* but with absorption/emission properties extended to longer wavelengths. HcRed is a far-red fluorescent protein originating from the sea anemone *Heteractis crispa* with absorption and emission maxima at 590 and 650 nm, respectively. We use ultrasensitive fluorescence spectroscopic methods to demonstrate that HcRed occurs as a dimer in solution and to explore the interaction between chromophores within such a dimer. We show that red chromophores within a dimer interact through a Förster-type fluorescence resonance energy transfer (FRET) mechanism. We present spectroscopic evidence for the presence of a yellow chromophore, an immature form of HcRed. This yellow chromophore is involved in directional FRET with the red chromophore when both types of chromophores are part of one dimer. We show that by combining ensemble and single molecule methods in the investigation of HcRed, we are able to sort out subpopulations of chromophores with different photophysical properties and to understand the mechanism of interaction between such chromophores. This study will help in future quantitative microscopy investigations that use HcRed as a fluorescent marker. © *2008 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.2937477]

Keywords: green fluorescent proteins; HcRed; dimer; fluorescence resonance energy transfer; fluorescence microscopy; single-molecule methods.

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1 Introduction

Intrinsically fluorescent proteins such as those originating from the *Aequorea victoria* green fluorescent protein (GFP) are popular reporters for intracellular localization, protein folding, protein expression, and protein-protein interaction studies using fluorescence microscopy.[1] GFPs have been the subject of detailed biochemical, biophysical, and mutational studies, with the mutational work having extended the color range from blue to yellow-green.[1] Red intrinsic fluorescent proteins with origins in *Anthozoa* species complement the emission color palette of GFPs,[2–5] and they are in high demand for protein-protein interaction studies based on fluorescence resonance energy transfer (FRET), for *in vivo* multicolor tracking studies, and for cellular investigations where autofluorescence poses a problem. Of these, HcRed is a novel far-red fluorescent protein generated by coupled site-directed and random mutagenesis of a chromoprotein cloned from the sea anemone *Heteractis crispa*.[3] Although used as a marker in fluorescence-based investigations,[6,7] little is known about its biophysical properties. In particular, an improved understanding of the spectroscopic behavior of HcRed is necessary for a correct interpretation of FRET results in which it is used as an acceptor. In this paper, we report on the biophysics of HcRed. Through a combination of single molecule and ensemble spectroscopic methods, we demonstrate that HcRed exists as a stable dimer while in a dilute solution and elucidate the mechanism of interaction between two spectrally distinct chromophores present in HcRed dimers. The information provided here is essential in the eventual use of such fluorescent reporters in quantitative microscopy studies. Our study builds upon the general effort to understand how intrinsic fluorescent proteins work, thus helping to define future avenues to improve the photophysical properties of such popular fluorescent markers.

2 Materials and Methods

Cloning, expression, and purification of HcRed is detailed in Ref. 3. The monomeric GFP used in these studies is the super folder GFP.[8] In our solution experiments, samples were pre-

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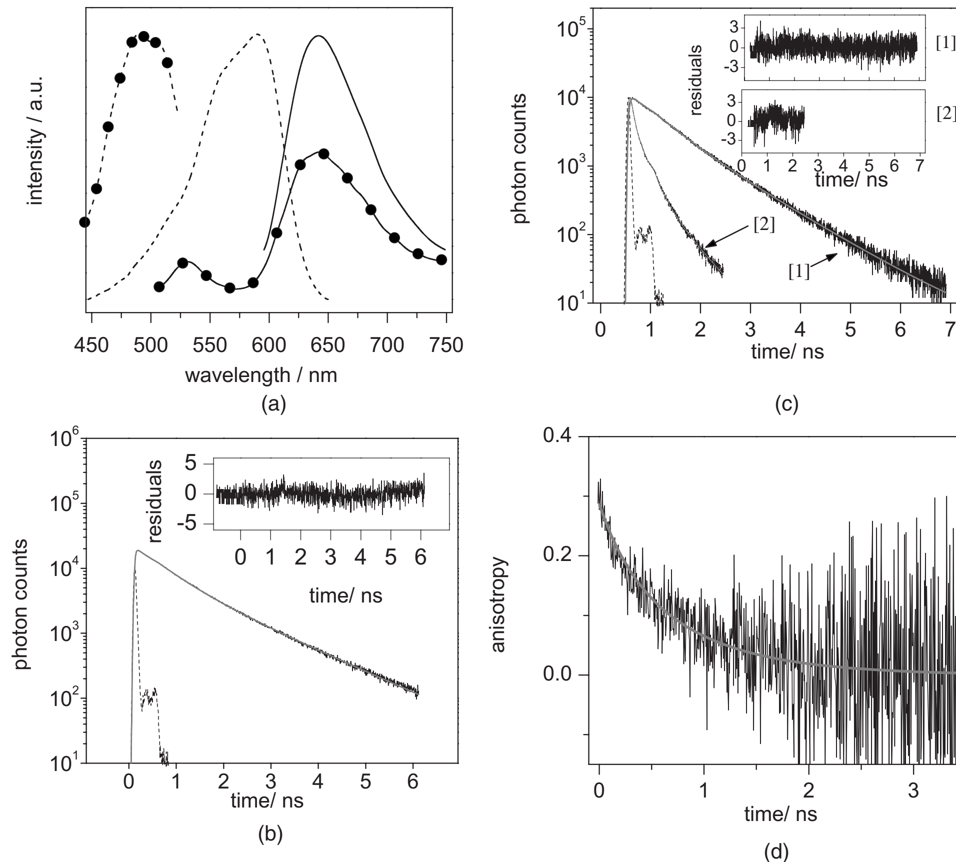

Fig. 1 (a) Absorption (dashed line), fluorescence emission (solid line, excitation at 590 nm, and solid line plus dots, excitation at 470 nm), and fluorescence excitation (dashed line plus dots, detection at 530 nm) spectra of HcRed in phosphate buffered saline (PBS). (b) Fluorescence decay of HcRed in PBS excited at 570 nm and detected at 645 nm. Shown in gray is a biexponential fit with decay time constants of 1.47 and 0.51 ns. The inset is the residuals of the fit from the main panel. (c) Fluorescence decays of HcRed in PBS excited at 470 nm and detected at 645 nm (curve 1) and 530 nm (curve 2). Shown in gray are multiexponential fits (see main text). The inset is the residuals for the fits from the main panel. (d) Decay of the fluorescence anisotropy of HcRed (570-nm excitation) and biexponential fit with time constants of 0.3 and 50 ns.

pared by diluting HcRed in phosphate buffered saline (PBS, pH 7.4, Dulbecco) to concentrations of 10^{-9} M [for fluorescence correlation spectroscopy (FCS) experiments] and 10^{-6} M (for steady-state spectroscopy and time-correlated single-photon counting). For the single-molecule spectroscopy experiments, we immobilized HcRed proteins in water-filled pores of polyacrylamide gels, as described in Ref. 9. Briefly, a 10^{-11} M solution of HcRed in PBS was mixed with a PBS solution containing 2% by weight of polyacrylamine (Agfa, MW 25000) and then sandwiched between two clean cover glasses. A similar procedure was used for the preparation of GFP samples.

2.1 *Ensemble Spectroscopy*

UV-VIS absorption spectra were recorded with a Lambda 6 spectrophotometer (Perkin-Elmer). Fluorescence spectra were recorded with a Spex Fluorolog 1500 fluorimeter (Spex Industries). The fluorescence quantum yield of HcRed in PBS was measured relative to that of Texas Red (Sigma Aldrich) in water with using 590-nm excitation. Time-correlated single-photon counting (TCSPC) experiments were performed using either 470-nm or 570-nm pulsed excitation from the frequency-double output of a Titanium:Sapphire laser (Mira 900 from Coherent, pulse width 150 fs, repetition rate 76 MHz) pumped by an Ar-ion laser (Spectra Physics 2080 Beamlok). For these experiments, a pulse picker (Conoptics, Inc.) was used to reduce the repetition rate of the laser to 7.6 MHz. Isotropic and anisotropic fluorescence decays were detected using a microchannel plate photomultiplier (Hamamatsu, response time of 23 ps) coupled to a monochromator (Acton Research). Fluorescence decays were collected with a TCSPC board (SPC630, Becker & Hickl GmbH) in 4096 channels with 10,000 photon counts in the peak and analyzed by a reweighted iterative reconvolution method based on the Levenberg-Marquardt algorithm. Details on the TCSPC setup and the fluorescence lifetime analysis have been published previously.[9]

2.2 *Fluorescence Correlation Spectroscopy*

For FCS experiments, we excited HcRed with the 594-nm line of a He-Ne laser (Uniphase). The excitation power at the sample was adjusted using a polarizer combined with a half-wave plate. The laser light was steered into an inverted confocal microscope (IX-71, Olympus-Japan) by a dichroic mirror (590DRLP, Chroma Technologies) and focused into the sample by a water-immersion objective (Olympus Japan, 1.2 NA, 60×). Fluorescence was collected from the sample through the same lens, spectrally filtered by the dichroic mir-

ror and a bandpass filter (650/50HQBP, Chroma Technologies), spatially filtered by a 75 μm pinhole, split by a 50/50 beamsplitter, and last imaged onto two single-photon avalanche photodiodes (SPAD, SPCM AQR 14, Perkin-Elmer). The signals from the SPADs were cross-correlated by a fast hardware correlator (ALV5000/E-FAST, ALV-Laser GmbH) whose lag-time resolution is 12.5 ps. Curve fitting of the FCS data was performed using Igor Pro 5.2 (WaveMetrics, Inc.).

2.3 *Single-Molecule Spectroscopy*

Samples containing individual immobilized HcRed or GFP proteins were imaged with the inverted confocal microscope described earlier, now fitted with a closed-loop piezo-electric scanning stage (NanoH100, Mad City Labs, Inc.). The same objective lens as earlier was used for optical excitation and fluorescence collection. For HcRed, we used the 594-nm line of a He-Ne laser and a similar detection scheme as for the FCS experiments, except that the beamsplitter was removed so that the collected fluorescence was imaged into a single SPAD. For GFP, optical excitation was accomplished with the 470-nm frequency-doubled output of our Ti:Sapphire laser, with the collected fluorescence filtered in this case by a combination of a dichroic mirror (DRLP505, Chroma Technologies) and a bandpass filter (HQ535/50, Chroma Technologies). Time trajectories for both HcRed and GFP proteins were recorded with the ALV5000 hardware correlator, with a dwell time of 1 ms.

3 Results and Discussion

3.1 *Dimerization of HcRed*

According to the crystal structure,[5] HcRed is a dimer comprised of two 25-kDa GFP-like protomers. Each protomer contains a chromophore formed from a Glu64-Tyr65-Gly66 tri-peptide. HcRed absorbs maximally at 590 nm [Fig. 1(a) dashed line], with an extinction coefficient of 120,000 M^{-1} cm^{-1}. HcRed fluorescence emission has its peak at 645 nm [Fig. 1(a), line, excitation at 590 nm], with a quantum yield of 0.05. This fluorescence decays bi-exponentially with lifetimes of 1.47 ns (75% contribution) and 0.51 ns (25%) [Fig. 1(b)]. HcRed owes its far-red absorption and emission properties to an interaction[5] between the aromatic ring of the chromophore and a nearby amino acid residue, Hisl74. Increased π-stacking interactions with the chromophore are known to red-shift the spectral properties of GFP.[1]

We probed the oligomerization state of HcRed with two methods: fluorescence correlation spectroscopy (FCS) and single-molecule fluorescence spectroscopy (SMS). Both methods require a low protein concentration (1 to 100 pM) at which aggregation may be excluded.

FCS measures the fluorescence fluctuations arising from molecules or molecular complexes that diffuse freely into and out of a diffraction-limited laser focus. The time dependence of these fluctuations can yield information about the translational and rotational diffusion coefficients of the molecules and, therefore, about their size.[10] These coefficients are obtained from the autocorrelation of the fluorescence intensity:

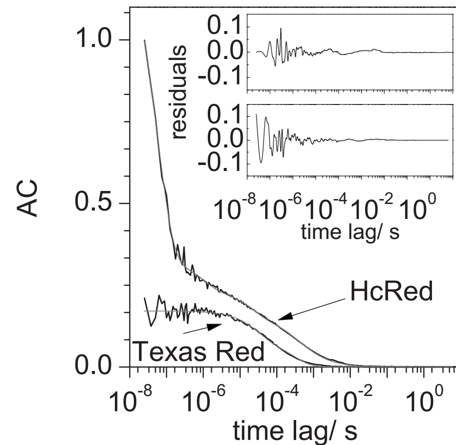

Fig. 2 Autocorrelations of the fluorescence intensity of HcRed and Texas Red in PBS (570-nm excitation). Also shown are fits (gray lines, main panel; see text for details) and residuals of the fits (inset panels; upper, HcRed; lower, Texas Red).

$$AC(\tau) = \frac{\delta I(t) - \delta I(t+\tau)}{\langle I^2(t)\rangle}, \quad (1)$$

where $\delta I = I - \langle I \rangle$ is the time-dependent fluctuation of the fluorescence intensity signal. For three-dimensional (3-D) diffusion, the AC is modeled by[10]:

$$AC(\tau) = N^{-1}\frac{1}{(1+\tau/\tau_D)[1+\tau/\tau_D(z_0/\omega_0)^2]^{1/2}} \times \prod_i \left(1+\frac{F_i}{1+Fi}\right)\exp(-\tau/\tau_i). \quad (2)$$

Here N is the average number of molecules in the detection volume, τ_D is the translational diffusion time, z_0 and ω_0 are, respectively, the axial and radial dimensions of the probe volume, and F_i and τ_i are the fraction contribution and lifetime of the fluorescence flickers present in addition to translational diffusion. Figure 2 shows the AC of HcRed in phosphate buffered saline (PBS, pH 7.4, excitation at 594 nm, with an average power at the sample of 50 μW). In addition to a translational diffusion component (τ_D=285 μs), the AC of HcRed contains flicker lifetimes of 0.05 μs (57% contribution), 3 μs (18%), and 23 μs (25%).[11] Monomeric GFP in PBS excited at 470 nm has a translational diffusion time of 140 μs (FCS data not shown here: see Ref. 12). The translational diffusion time, τ_D, and the diffusion coefficient, D, of a protein relate to each other by $\tau_D=\omega_0^2/4D$. D is dependent on the hydrodynamic radius of the protein through the Stokes-Einstein equation: $D=k_B T/6\pi\eta R_h$, where η is the viscosity of the solvent, and R_h is the hydrodynamic radius of the protein. Assuming diffraction-limited probe volumes in these FCS experiments, the ratio $\omega_0(594\text{ nm})/\omega_0(470\text{ nm})$ is $\sim$1.3, and the ratio of the hydrodynamic radii of HcRed and monomeric GFP is $R_h(\text{HcRed})/R_h(\text{GFP})\sim 1.3$. If we assume that the molecular weight (MW) of the protein scales with the third power of its molecular hydrodynamic radius, then MW(HcRed)/MW(GFP) is approximately 2.0, indicating that HcRed is a dimer in solution.

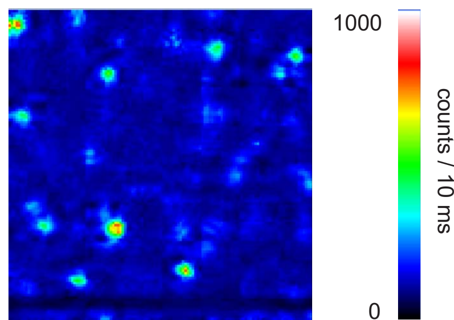

Fig. 3 Confocal fluorescence image of single HcRed proteins (5 $\times 5$ μm, 125×125 pixels, 10-ms integration time per pixel, average excitation power of 570 nm at the sample 2 μW).

Rotational diffusion of the protein induces the fast fluorescence flicker in the *AC* of HcRed (here τ_{rot}=0.05 μs). For a comparison, Fig. 2 shows the *AC* of Texas Red in PBS (excitation at 594 nm with an average power of 50 μW), which is flat at short time delays. This organic dye has a rotational correlation time of ~250 ps, which is below the minimum lag-time resolution of the FCS experiment. The rotational correlation time τ_{rot} of a protein and its molecular hydrodynamic volume V_h relate to each other[13] by $\tau_{rot} = 1/(6D_{rot} = k_B T/(36V_h)$, where D_{rot} is the rotational diffusion coefficient. Monomeric GFP has a rotational correlation time of 16 ns.[13] The ratio of the hydrodynamic volumes for HcRed and monomeric GFP will then be $V_h(\text{HcRed})/V_h(\text{GFP}) = \tau_{rot}(\text{HcRed})/\tau_{rot}(\text{GFP}) \sim 3.1$, once again suggesting the presence of dimeric HcRed.

We show evidence for the dimerization of HcRed by fluorescence spectroscopy of immobilized single molecules. Figure 3 is a confocal fluorescence image (5×5 μm) of individual HcRed proteins immobilized in water-filled pores of polyacrylamide gel. The immobilized proteins were excited with a 2-μW, 594-nm laser light, and their fluorescence was detected for wavelengths above 610 nm. The diffraction-limited fluorescence spots in this image correspond to single proteins and are comprised of as many as 700 photon counts per spot. Figure 4(a) shows the fluorescence intensity trajectory recorded from an individual HcRed molecule located by confocal imaging. This particular trajectory shows two discernible intensity levels, with stepwise intensity changes prior to photobleaching [Fig. 4(a)]. This behavior is characteristic of multichromophoric entities when observed individually.[14–16] For this particular molecule, the background corrected histogram of the detected photon counts [Fig. 4(b)] is bimodal with peaks at 49 counts/ms and 29 counts/ms. As a comparison, Fig. 4(c) shows a fluorescence intensity trajectory recorded from a single monomeric GFP immobilized in polyacrylamide gel, under a 1-μW excitation at 470 nm. The GFP trajectory shows one intensity level above the dark state, with a background-corrected histogram of the counts [Fig. 4(d)] displaying a single-mode distribution. The GFP trajectory is interrupted by dark periods, a phenomenon known as "blinking."[1,15,16] The combination of blinking and of a single-mode intensity histogram is a fingerprint of a single-chromophore single protein. Among the population of individual HcRed proteins we have probed, the majority of the recorded single-molecule trajectories (43 out of 60) show two levels of intensity [as seen in Fig. 4(b)], while the rest show one level. The observation of two levels of intensity in single-molecule fluorescence signals is indicative of the presence of two absorbing chromophores, that is, of the presence of dimeric HcRed.

3.2 *Intraprotein Energy Transfer in Dimers of HcRed*

The center-to-center distance[5] between chromophores within a dimer of HcRed is ~3.2 nm. Assuming a molar extinction

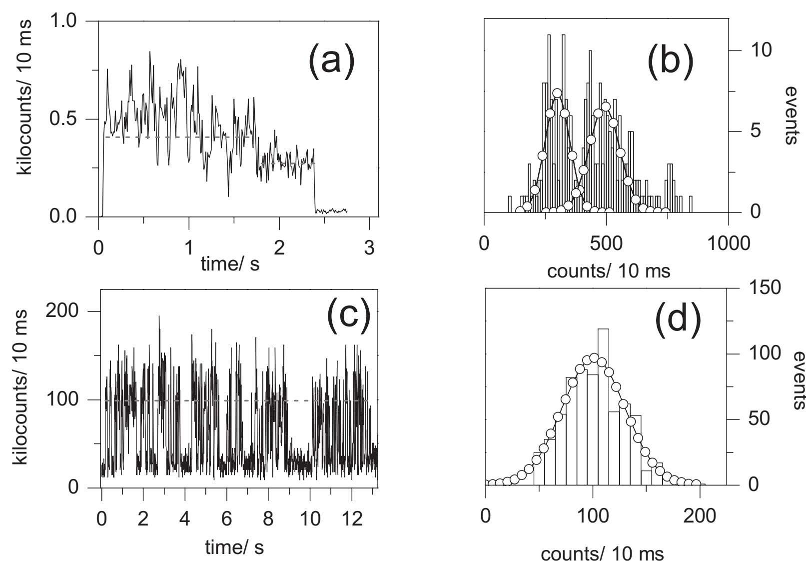

Fig. 4 (a) Fluorescence intensity signal detected from a single immobilized HcRed protein and featuring two levels. (b) histogram of photon count data from panel (a) and bimodal Gaussian fit (peaks at 290 and 490 counts/10 ms), clearly showing that two levels are present in the intensity signal from the left panel. (c) Fluorescence intensity signal detected from a single immobilized GFP and featuring one intensity level. (d) Histogram of photon count data from panel (c) and monomodal Gaussian fit (peak at 102 counts/10 ms).

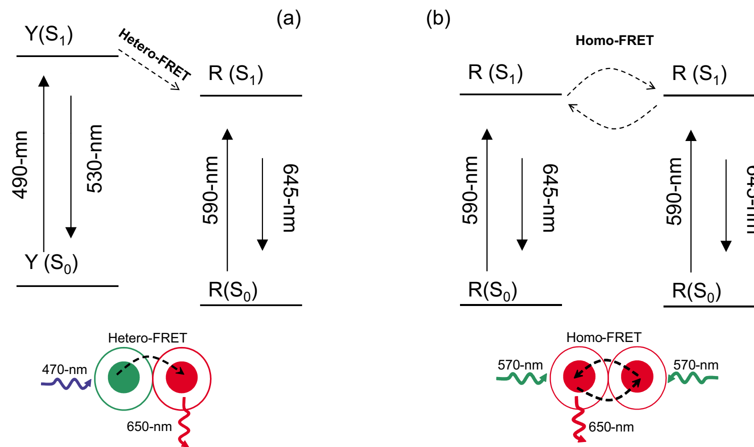

Fig. 5 (a) Scheme accounting for directional intraprotein directional FRET in HcRed. Y, yellow chromophore; R, red chromophore; S_0, ground state; S_1, singlet excited state. (b) Scheme accounting for intraprotein energy hopping (homo-FRET) in HcRed.

coefficient per red chromophore of 60,000 $M^{-1} cm^1$ and a quantum yield of fluorescence of 0.05, we estimate[17] a Förster radius for energy hopping (FRET between identical chromophores) of $R_0 \sim 3.5$ nm. This suggests that energy hopping between identical chromophores within a single HcRed dimer, illustrated in Fig. 5(b), takes place with an efficiency[17] $E = R_0^6/(R_0^6 + R^6) \sim 63\%$.

We show evidence of intraprotein energy hopping in HcRed dimers by time-resolved fluorescence anisotropy experiments. Figure 1(d) shows the decay of the fluorescence anisotropy of HcRed in PBS (excitation at 590 nm, detection at 645 nm). For a simple fluorophore, rotational diffusion is the dominant cause of fluorescence depolarization: following vertically polarized pulsed excitation, the fluorescence anisotropy $r(t)$ of a simple fluorophore decays as a single exponential,[17] $r(t) = r_0 \exp(-t/\tau_{rot})$, where τ_{rot} is the rotational correlation time of the fluorophore. For example, the fluorescence anisotropies of Texas Red and GFP decay as single exponentials with rotational correlation times of $\sim$0.25 ns and 16 ns, respectively, values reflecting their hydrodynamic volumes. For HcRed, the fluorescence anisotropy decays biexponentially, dominated by a fast (0.3 ns) decay component in addition to a long (>20 ns) decay component that contributes more weakly. The long component is the rotational correlation time characteristic of the rotation of the whole HcRed dimer. Because of the relatively short fluorescence lifetime of this protein ($\sim$1.5 ns), an accurate determination of the long rotational correlation lifetime is difficult. We assign the short, dominant component to the energy hopping between identical (red) chromophores within an HcRed dimer. This process will induce additional depolarization in the fluorescence emitted by HcRed.[17] We exclude the possibility that this fast component is due to rapid rotation of the chromophore inside the protein's β-can, because chromophores in GFP-like proteins are surrounded by a complex and relatively rigid hydrogen bond network involving several amino acids and water molecules.[1] This rigid network can be expected to forbid rotation of the chromophore on a short time scale similar to that of a small dye molecule rotating freely in solution (e.g., $\tau_{rot} \sim 250$ ps for Texas Red).

The red chromophore is not the only species present in HcRed dimers. Under 470-nm excitation, the fluorescence spectrum of HcRed features a peak at 530 nm, apart from the main peak at 645 nm [Fig. 1(a), line and dots spectrum]. When monitored at this wavelength (530 nm), the excitation spectrum of HcRed shows a main peak at 490 nm [Fig. 1(a), dashed line and dots spectrum]. It is noteworthy that this 490-nm excitation peak has no corresponding peak in the absorption spectrum of HcRed. On 470-nm excitation, the fluorescence of HcRed detected at 530 nm [Fig. 2(c), curve 2] decays as a multiexponential with a major contributing lifetime of 0.04 ns (63% contribution) and two additional lifetimes of 0.14 ns (33%) and 0.51 ns (4%). For the same excitation wavelength (470 nm), the fluorescence decay detected at the main peak of the emission spectrum [645 nm; Fig. 1(c), curve 1] contains a rise time of 0.04 ns (−21% contribution) and two lifetimes with similar values to those detected in the 640-nm fluorescence decay recorded upon 570-nm excitation. The stationary and time-resolved fluorescence data reported here [Figs. 1(a)–1(c)] clearly show that the yellow species absorbing/emitting at 490/530-nm is connected with the red chromophore through an excited state pathway, as depicted in Fig. 5(a). Given the overlap between the emission spectrum of the yellow chromophore [Fig. 1(a), line plus dots] and the absorption spectrum of the red chromophore [Fig. 1(a), dotted line], when both chromophores are part of the same HcRed dimer,[5] conditions are satisfied for directional intraprotein energy transfer [hetero-FRET; Fig. 5(a)] to occur upon direct excitation of the yellow chromophore. FRET is demonstrated here by the match between the major decay time component detected at 530 nm (yellow emission, 0.04 ns) and the rise time component detected at 645 nm (red emission, 0.04 ns). The 0.04-ns decay time detected at 530 nm is the quenched fluorescence of the yellow chromophore due to FRET interaction with the red chromophore, while the 0.04-ns rise time detected at 645 nm is due to the delayed buildup in the excited state population of the red chromophore, as it is excited indirectly via FRET from the yellow chromophore.

The time-resolved fluorescence data we report here also show that a fraction of the yellow chromophores optically excited at 470 nm are not involved in directional intraprotein FRET toward red chromophores. A large subpopulation of yellow chromophores fluoresces with a lifetime of about 0.5 ns (37% of the total population of yellow species excited at 470 nm). These chromophores are part of "yellow" HcRed dimers, i.e., dimers that contain a pair of yellow chromophores. Assuming that 0.51 and 0.04 ns are the fluorescence lifetimes of the yellow (donor) chromophore in the absence and presence of the red (acceptor) chromophore, directional intraprotein FRET from a yellow to a red chromophore takes place with an efficiency $E=R_0^6/(R_0^6+R^6)=1-\tau_{DA}/\tau_D\sim 92\%$, thus yielding a Förster radius of $R_0=4.8$ nm.

We believe that the yellow chromophore of HcRed is an immature form of the red chromophore. In another red fluorescent protein, DsRed from *Discosoma genus*, the red chromophore maturates through an intermediate, GFP-like green absorbing/emitting chromophore, a step that includes an additional oxidation step at one of the residues involved in the chromophore formation.[9,15] In DsRed, this maturation does not complete for every chromophore, leading to a distribution of green and red chromophores within individual oligomers, similar to what we see here in HcRed: yellow dimers (two yellow chromophores), mixed dimers (one yellow and one red chromophore), and fully matured dimers (two red chromophores). The absorption and emission properties of the yellow chromophore of HcRed are expected to be red-shifted in comparison to those of immature chromophores of DsRed because of the π-stacking with the nearby His174 residue.[5]

4 Conclusion

We have investigated the oligomerization status of HcRed and the interaction among chromophores within HcRed oligomers by a combination of ensemble and single-molecule measurements of fluorescence. We have shown evidence from spectroscopic data that HcRed forms a dimer, as suggested by the recently published crystal structure for this protein.[5] We have found HcRed in dimer form even at the very low concentrations used in single-molecule experiments. This suggests that the HcRed dimer is very strong and stable. We have found that dimers of HcRed may contain red chromophores only ("red" dimers), yellow chromophores only ("yellow" dimers) or one of each species of chromophore. In the "red" dimers, chromophores interact through energy hopping, a process characterized by an efficiency of 63%. In "mixed" dimers containing both yellow and red chromophores, we detect a very efficient (92%) directional energy transfer from the yellow to the red chromophore.

The yellow chromophore is an immature form of the red chromophore of HcRed, a species encountered in many proteins derived from *Anthozoa* species. Our results show that care must be taken when using HcRed in combination with other yellow or green fluorescent proteins, such as EGFP or EYFP, especially when seeking quantitative data in FRET-based applications.[6]

Details about the distribution of the different types of HcRed dimers would require separate and simultaneous detection of the yellow and red chromophores of HcRed, similar to a method used recently to study DsRed.[15] Unfortunately, such methods would be difficult if not impossible, given that the very low brightness of the yellow chromophore makes single-molecule detection of the yellow chromophore difficult.

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