Competition between Energy and Proton Transfer in Ultrafast Excited-State Dynamics of an Oligomeric Fluorescent Protein Red Kaede

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We investigated femtosecond and picosecond time-resolved fluorescence dynamics of a tetrameric fluorescent protein Kaede with a red chromophore (red Kaede) to examine a relationship between the excited-state dynamics and a quaternary structure of the fluorescent protein. Red Kaede was obtained by photoconversion from green Kaede that was cloned from a stony coral *Trachyphyllia geoffroyi*. In common with other typical fluorescent proteins, a chromophore of red Kaede has two protonation states, the neutral and the anionic forms in equilibrium. Time-resolved fluorescence measurements clarified that excitation of the neutral form gives the anionic excited state with a time constant of 13 ps at pH 7.5. This conversion process was attributed to fluorescence resonance energy transfer (FRET) from the photoexcited neutral form to the ground-state anionic form that is located in an adjacent subunit in the tetramer. The time-resolved fluorescence data measured at different pH revealed that excited-state proton transfer (ESPT) also occurs with a time constant of 300 ps and hence that the FRET and ESPT take place simultaneously in the fluorescent protein as competing processes. The ESPT rate in red Kaede was significantly slower than the rate in *Aequorea* GFP, which highly likely arises from the different hydrogen bond network around the chromophore.

Introduction

A green fluorescent protein from the jellyfish *Aequorea victoria* (*Aequorea* GFP) and GFP variants are extensively studied and widely utilized as fluorescent markers in living cells in the field of cell and molecular biology.[1,2] To receive full benefit of fluorescent proteins, it is very important to understand physicochemical properties of fluorescent proteins and elucidate underlying emission mechanisms.

The fluorescence dynamics of *Aequorea* GFP has been studied in detail.[3,4] The GFP chromophore has two different protonation states owing to the phenol (neutral form, A-from)−phenolate (anionic form, B-form) equilibrium, and two separate absorption bands in the visible region have been assigned to these two forms. However, the excitation of the two forms gives very similar fluorescence spectra which are attributed to the fluorescence from the anionic form. This fluorescence property of GFP has been explained by a three-state model as follows.[1,2] The excitation of the neutral form (A-form) induces an excited-state proton transfer (ESPT) to generate the excited-state anionic form (I*-form) in the picosecond time scale, so that the fluorescence is emitted from the I*-form even when the neutral form is excited. The I*-form is different from the B*-form that is generated by direct excitation of the anionic form, because the I*-form is surrounded by a hydrogen-bond network that stabilizes the neutral A-form, which is different from the network around the anionic B-form. The three-state model is consistent with slightly different fluorescence spectra and lifetimes that are obtained with excitation of the neutral and the anionic forms. Reflecting the environmental difference, the fluorescence from the I*-form is slightly red-shifted (∼5 nm) compared with the fluorescence from the B*-form.[4] The lifetime of the I*-form (3.3 ns) is also different from that of the B*-form (2.8 ns).[5]

In contrast to GFP that usually exists as monomer,[1] a red fluorescent protein from a reef coral *Discosoma* DsRed[6] has a strong tendency to form a homotetramer even at concentrations as low as 1 nM.[7] After protein folding, DsRed initially has the same chromophore as GFP and gives green fluorescence. However, further oxidization of the chromophore automatically takes place in DsRed, and the "mature" chromophore having a longer conjugation shows red fluorescence.[8] Because of the high tendency to form the tetramer, the effect of oligomerization on the excited-state dynamics has been investigated for DsRed. Time-resolved fluorescence studies revealed that fluorescence resonance energy transfer (FRET) between subunits occurs on the picosecond time scale,[7,9,10] instead of ESPT. Unfortunately, coexistence of immature green chromophore in DsRed prevents further investigation.

A fluorescent protein named Kaede was cloned from a stony coral *Trachyphyllia geoffroyi*. Kaede has a very characteristic property that green chromophore is converted to the red one upon UV light irradiation.[11,12] This color-changing property of Kaede makes it a powerful tool for regional optical marking. From the viewpoint of the fluorescence dynamics study, Kaede after photoconversion (red Kaede) has several desirable features. First, in contrast to the unregulated conversion of DsRed, the green to red conversion of Kaede can be completed by UV irradiation, so that red Kaede has no remaining green chromophores. Second, Kaede forms a homotetramer even at a low

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concentration of 1 nM. Therefore, red Kaede is considered to be an ideal system for investigation of the fluorescence dynamics in the fluorescent proteins that form oligomers. In this study, we performed femtosecond and picosecond time-resolved fluorescence measurements of red Kaede and examined a relationship between the excited-state dynamics and a quaternary structure of the fluorescent protein.

Experimental Methods

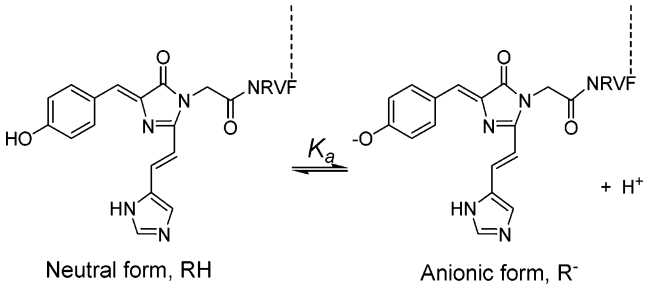
Femtosecond Time-Resolved Fluorescence Measurements. The apparatus for fluorescence up-conversion has been described in detail elsewhere.[13] The second harmonic pulses (400 and 430 nm, 5 mW) of a Ti:sapphire mode-locked oscillator laser (Spectra-Physics, Tsunami) were used for excitation. The residual fundamental pulses after the second harmonic generation were used as gate pulses for the fluorescent up-conversion process. The up-converted signals were separated by a monochromator (HR320, Jobin-Yvon) and detected by a photomultiplier (H585, Hamamatsu) with a photon counter (SR400, Stanford Research Systems). The time resolution of the measurements was 200 fs (fwhm). The sample solution was placed in a 0.5-mm-thick quartz cell. The cell was continuously translated during the measurements so that a fresh part of the solution was always supplied for the excitation. Polarization of excitation and detection light was set at the magic angle for time-resolved intensity measurements. For time-resolved anisotropy measurements, up-converted fluorescence intensities were measured with parallel and perpendicular conditions at each delay time. All measurements were performed at room temperature. Concentration of red Kaede for the femtosecond time-resolved measurements was 200 μM in 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer.

Picosecond Time-Resolved Fluorescence Measurements. Picosecond time-resolved fluorescence measurements were performed with a streak camera (C4334, Hamamatsu). A Ti:sapphire regenerative amplifier (Spitfire, Spectra-Physics) seeded by a Ti:sapphire mode-locked oscillator laser (Tsunami, Spectra-Physics) was used as a light source, and the second harmonic of the amplified pulse (400 nm, 2 μW) was used for excitation. Polarization of excitation and detection light was set at the magic angle. The time resolution of the measurements was evaluated as 20 and 160 ps (fwhm) for the sweep range of 1 and 10 ns, respectively. All measurements were performed at room temperature. Concentration of red Kaede for the picosecond time-resolved measurements was 200 μM in 50 mM HEPES buffer and 9 μM in 50 mM phosphate buffer.

Absorption and Fluorescence Spectra. Absorption spectra were measured by a commercial spectrometer (U-3310, Hitachi). The light path length was 10 mm. Steady-state fluorescence spectra were measured by a polychromator (HR320, Jobin-Yvon) equipped with a CCD (TEA/CCD-1024-EM/1UV, Princeton Instruments). The second harmonic pulses of the femtosecond mode-locked Ti:sapphire laser (400 nm) were used as excitation light. Sensitivity of the detection system was calibrated by a standard lamp (Ushio). Spectral distortion due to self-absorption was corrected. The concentration of red Kaede was 9 μM in 50 mM acetate (pH 4.0−5.5), 50 mM phosphate (pH 6.0−8.0), and 50 mM borate (pH 9.0−10.0) buffers.

Protein Expression, Photoconversion, and Deuteration. Green Kaede protein was produced in *E. coli* and purified as described previously.[11] Photoconversion of green Kaede to red Kaede was performed by illumination of 365 nm using a UV illuminator in 50 mM HEPES (pH 7.5) for 9 h. Deuteration of exchangeable protons in protein was carried out by dissolving the protein with 50 mM HEPES buffer in D_2O at pH 7.5, concentrating to a minimum volume, and repeating the procedure three times. The pH condition is not corrected for the isotope effect. The deuterated sample was kept at room temperature for 3 days to complete the deuteration before measurements. By this treatment, the exchangeable protons of the protein were replaced with deuterons. The GFP solution that was prepared by the same procedure reproduced a reported drastic deuteration effect in femtosecond time-resolved fluorescence.[3,4] This assured the deuteration of the exchangeable proton was accomplished also in red Kaede.

SCHEME 1: Chemical Structure and Acid−Base Equilibrium of Chromophore, 2-[(1*E*)-2-(5-Imidazolyl)-ethenyl]-4-(*p*-hydroxybenzylidene)-5-imidazolinone



Results and Discussion

Acid−Base Equilibrium of Chromophores and Their Absorption Spectra. The chromophore of a typical fluorescent protein has a phenol (or phenolate) group, and it has two protonation states, a neutral and an anionic form. Equilibrium between the neutral and anionic forms of GFP is significantly changed by pH,[1] whereas DsRed is insensitive to pH and the chromophore exists in the anionic form.[14] The chemical structure of the chromophore of red Kaede has been investigated,[11,12] and it is shown in Scheme 1. To check pH sensitivity of red Kaede, we measured absorption spectra at various pH values.

As shown in Figure 1a, absorption spectra significantly change with pH, indicating that the equilibrium of the chromophore of red Kaede is sensitive to pH. Spectra of pH 9.2 and 7.9 were almost identical, indicating that the chromophore is completely deprotonated and exists as the anionic form above pH 7.9. Thus, sharp bands at 530 and 570 nm were assigned to the anionic form. A broad absorption band centered at 450 nm appears under the acidic condition, and it was assigned to the neutral form. An isosbestic point was observed at 490 nm, which implies that the spectra consist of two components: the neutral and anionic forms of the red chromophore. No remaining green chromophore was recognized, indicating that photoconversion from the green to red chromophore was completed.[15]

To quantitatively discuss the acid−base equilibrium of the chromophore, the equilibrium constant K_a was evaluated from the absorption spectra. The equilibrium between the neutral (RH) and the anionic forms (R^-), $RH \rightleftharpoons R^- + H^+$, is assumed with a relationship of $K_a = [R^-][H^+]/[RH]$. The acid−base equilibrium constant K_a was determined as 9.2×10^{-7} as described in Appendix A. The obtained pK_a value of 6.0 is close to the reported value of 5.6.[11]

With use of the K_a value, the population ratio of the neutral form $x_p \equiv [RH]/([RH] + [R^-]) = [H^+]/(K_a + [H^+])$ was calculated for various pH solutions. The x_p values are 0.95 (pH 4.8), 0.73 (pH 5.6), 0.41 (pH 6.2), 0.12 (pH 6.9), 0.03 (pH 7.5), 0.01 (pH 7.9), and 0.0 (pH 9.2). The x_p value of 0.01 at pH 7.9 means that only 1% of the chromophore is the neutral form

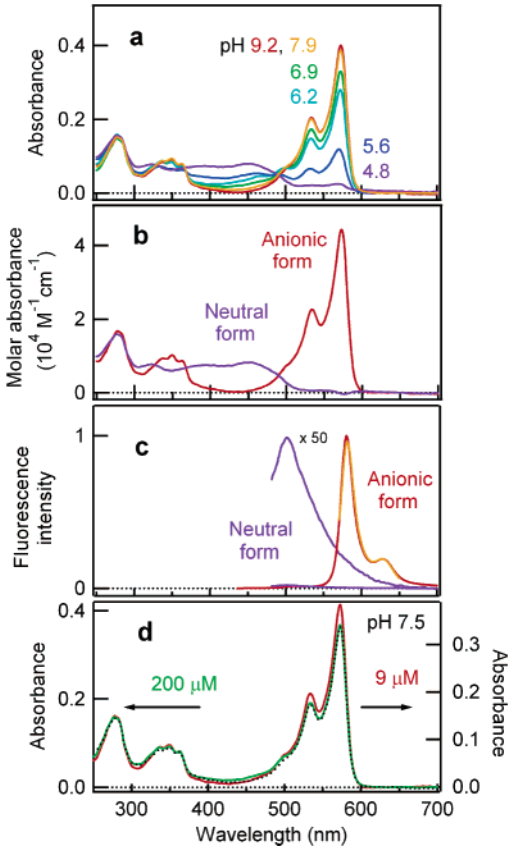


Figure 1. Steady-state spectra of red Kaede. (a) Absorption spectra at various pH values (9 μM). (b) Molar absorption spectra of the neutral form ϵ_N (purple) and the anionic form ϵ_A (red). (c) Fluorescence spectra at pH 7.5 (200 μM, red) and 4.5 (9 μM, purple) measured with 400 nm excitation. The fluorescence spectrum at pH 7.5 measured with 560 nm excitation (orange) is also shown. (d) Absorption spectra of 200 (green) and 9 μM (red) solution at pH 7.5. The cell thickness was 10 mm for the 9 μM solution and 0.5 mm for the 200 μM solution. The calculated spectrum (black dotted line) of the 200 μM solution is also shown.

and the remaining 99% is the anionic form. At pH 7.5 where fluorescent proteins are employed in living cells, the neutral form is a minor component and the anionic form is predominant.

Since x_p is 0.0 at pH 9.2, the spectrum observed at pH 9.2 can be considered as the pure spectrum of the anionic form. At pH 4.8, 5% of the chromophores exist as the anionic form and the remaining 95% as the neutral form. The pure absorption spectrum of the neutral form was then obtained by subtracting the 5% contribution of the anionic form from the spectrum measured at pH 4.8 by using the pure spectrum of the anionic form. The pure absorption spectra of the neutral and anionic forms obtained by this procedure are shown in Figure 1b.

A more concentrated solution was needed for femtosecond time-resolved fluorescence measurements. Figure 1d shows the absorption spectra of a concentrated (200 μM, pH 7.5) and a dilute (9 μM, pH 7.5) solution of red Kaede.[16] It is obvious that the ratio of the neutral form (x_p) in the concentrated solution is larger than that in the dilute solution even at the same pH. This indicates that the equilibrium is changed not only by pH but also by the concentration of the protein, which also has been known for GFP.[17] The obtained pK_a value of 6.0 holds only for the dilute 9 μM solution and the equilibrium constant for the concentrated solution is slightly different. Nevertheless, it is possible to evaluate the concentration of the neutral and anionic forms in the concentrated solution by using the molar absorption spectra of each form. As shown in Figure 1d, the absorption

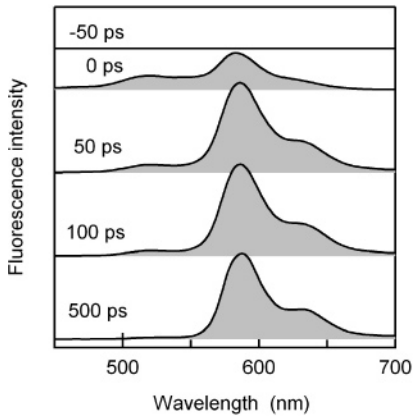


Figure 2. Picosecond time-resolved fluorescence spectra of red Kaede at different delay times after excitation at 400 nm (200 μM, pH 7.5).

spectrum of the 200 μM solution was well reproduced by a linear combination of the pure spectra of the neutral form (ϵ_N) and the anionic form (ϵ_A), $A = cx_p\epsilon_N + c(1 - x_p)\epsilon_A$, and the population ratio x_p was directly determined as 0.13. Therefore, 13% of the chromophore was the neutral form and the remaining 87% was the anionic form in the 200 μM solution at pH 7.5.

Steady-State Fluorescence Spectra. Figure 1c shows the steady-state fluorescence spectra of red Kaede. Fluorescence of the anionic form was measured at pH 7.5 with 560 nm excitation that selectively excites the anionic form in the protein. The fluorescence exhibits two peaks at 580 and 630 nm as shown in the figure. The steady-state fluorescence was also measured with 400 nm excitation. At 400 nm, the molar absorption coefficient of the neutral form ($\epsilon_{N,400} = 7400$) is 7.4 times larger than that of the anionic form ($\epsilon_{A,400} = 1000$). Because the excited-state population generated by photoexcitation is proportional to the product of the ground-state population and the molar absorption coefficient, the amount of neutral excited state created by the 400-nm photoexcitation is comparable to the amount of the anionic excited state, although only 13 % of the chromophore exists in the neutral form in the ground state. As shown in the figure, the fluorescence spectrum observed with 400-nm excitation is indistinguishable from the fluorescence of the anionic form observed with 560-nm excitation. This indicates that the fluorescence is emitted from the anionic form at pH 7.5, regardless of which form is initially photoexcited.

The steady-state fluorescence spectrum of the neutral form could be observed under very acidic conditions (e.g., pH 4.5[18]), as shown in Figure 1c. The neutral fluorescence peaked around 500 nm and exhibits a broad feature extending up to 650 nm.

Picosecond Time-Resolved Fluorescence Spectra. To clarify the excited-state dynamics of red Kaede, picosecond time-resolved fluorescence spectra were measured with 400-nm excitation at pH 7.5 (Figure 2). A remarkable difference from the steady-state fluorescence is that a new fluorescence band was observed around 500 nm immediately after excitation, in addition to the fluorescence of the anionic form. Because the spectral feature is similar to the neutral fluorescence observed at pH 4.5, this new band was assigned to the fluorescence of the neutral form. The major part of the neutral fluorescence disappeared by the delay time of 50 ps, whereas the anionic fluorescence peaked at 580 nm increased up to 50 ps. This temporal fluorescence spectral change indicates that the anionic excited state is produced in accordance with the decay of the neutral excited state in the first few tens of picoseconds. The lifetime of the lowest excited singlet (S_1) state of the anionic

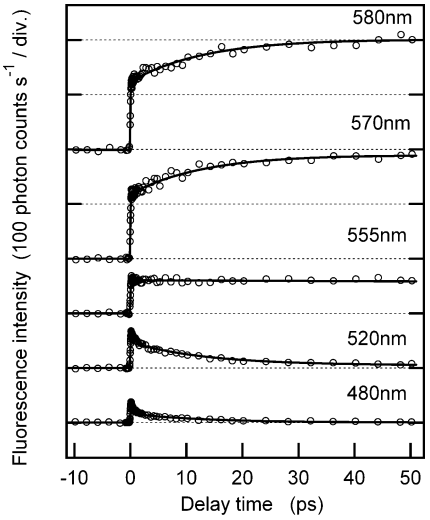


Figure 3. Femtosecond time-resolved fluorescence signals of red Kaede (200 μM, pH 7.5) observed at different wavelengths with excitation at 400 nm (open circles: observed, lines: fitting).

form was determined to be 4.8 ns by slow-sweep measurements of the streak camera. We note that the decay of the neutral fluorescence was actually bimodal and the lifetime of the minor component was 300 ps. The origin of this minor component is discussed later.

Femtosecond Time-Resolved Fluorescence. The conversion process from the neutral excited state to the anionic excited state is so fast that the relevant dynamics was not fully time-resolved with the 20-ps time resolution of the streak camera. Therefore, we carried out fluorescence up-conversion measurements with 200-fs time resolution. The obtained data are shown in Figure 3. The fluorescence trace at 570 nm, where the anionic fluorescence is predominant, exhibits an instantaneous rise and a following finite rise component with a time constant of 13 ps. The 13-ps rise component represents generation of the anionic excited state by the conversion process from the neutral excited state, whereas the instantaneous rise component is attributed to the anionic fluorescence that is induced by direct excitation of the anionic form in the ground state.[19] In contrast, the fluorescence traces at 480 and 520 nm exhibit decays, which were assigned to the decay of the neutral fluorescence. The decay signal at 520 nm was fitted by the three exponential functions having time constants of 590 fs (34%), 13 ps (58%), and 300 ps (8%). The 13-ps decay of the neutral fluorescence coincides very well with the 13-ps rise of the anionic fluorescence. In fact, at 555 nm where both the neutral and anionic fluorescence were observed with the same intensity, the time-resolved trace was almost flat up to 50 ps. This flatness assured that the rise of the anionic fluorescence completely matches the decay of the neutral form. Consequently, it is concluded that the anionic form is generated directly from the excited neutral form with the time constant of 13 ps. The 590-fs decay component observed at 520 nm probably reflects the intramolecular vibrational redistribution process or the vibrational cooling process in the S_1 state of the neutral form. This component was not observed when we excited red Kaede with 430 nm (see Figure S.1 in the Supporting Information).

Mechanism of Fluorescence Dynamics of Red Kaede. The conversion process from the neutral to anionic form in the excited state has been observed for several fluorescent proteins. The conversion process has been assigned to ESPT for GFP[3,4] and FRET for DsRed.[7,9,10] ESPT is a deprotonation reaction of the neutral excited state, and it is a unimolecular reaction taking

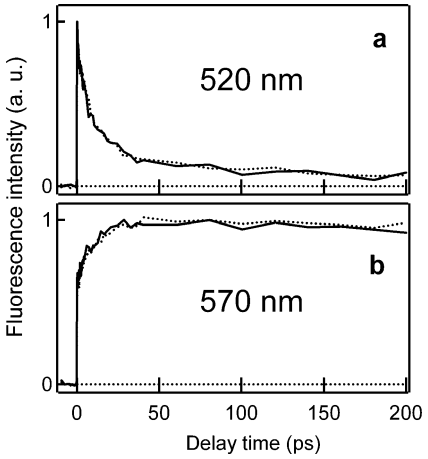


Figure 4. Femtosecond time-resolved fluorescence signals of red Kaede in H_2O (solid line) and D_2O (dotted line) observed at (a) 520 and (b) 570 nm (400 nm excitation).

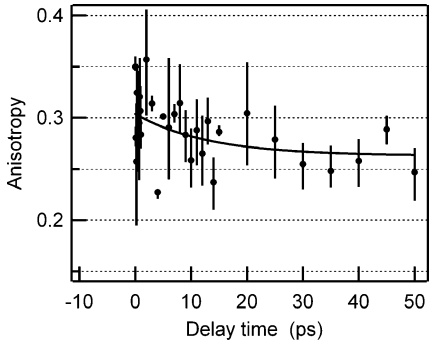


Figure 5. Time-resolved fluorescence anisotropy of red Kaede observed at 680 nm with excitation at 430 nm (circles: observed, line: fitting).

place in a single subunit. On the other hand, FRET occurs between two chromophores, and it is a bimolecular process taking place only when the anionic form exists in a subunit adjacent to the subunit that contains the excited neutral form.

If ESPT is the predominant conversion mechanism in red Kaede, the fluorescence dynamics is expected to change with deuteration, because it affects the hydrogen-bond network surrounding the chromophore. In fact, it is known that the fluorescence dynamics of GFP is drastically changed by deuteration:[3,4] the decay of the neutral excited state (A*-state) and the rise of the anionic excited state (I*-state) become five times slower by deuteration.[3] We deuterated exchangeable protons in red Kaede and performed up-conversion measurements in D_2O. As shown in Figure 4, the time-resolved fluorescence observed for deuterated red Kaede was indistinguishable from that of the native one. Especially, the time constant of 13 ps characteristic of the conversion dynamics remained unchanged.

To examine the possibility of FRET between subunits in the tetramer, we carried out time-resolved fluorescence anisotropy measurements with 430-nm photoexcitation. The anisotropy value of 0.35 was observed at 520 nm just after photoexcitation. This anisotropy value is close to 0.4, which ensures that the S_1 state of the neutral form is generated directly by photoexcitation at 430 nm. The time-resolved anisotropy data observed at 680 nm are shown in Figure 5. The fluorescence at 680 nm contains two components, anionic fluorescence induced by direct photoexcitation (the instantaneous rise component) and that generated by the conversion process (the finite rise component).[20] The anisotropy value just after photoexcitation was 0.30 and it

decreased down to 0.26 at the delay time of 50 ps. The initial value of 0.30 deviates from the theoretical value of 0.4, implying that the transition dipole moment of the anionic fluorescence induced by direct excitation is somewhat tilted, compared to the transition moment of the absorption. It indicates that the excitation at 430 nm generates not only the S_1 state but also the higher S_n state of the anionic form.[21] Actually, the excitation wavelength of 430 nm corresponds to the valley between the blue-side tail of the second lowest absorption band and the red-side tail of the lowest absorption band.

The fluorescence anisotropy value exhibits a small but significant decrease as the contribution of the fluorescence generated by the conversion process increases, and it becomes 0.26 at the delay time of 50 ps. This means that the anisotropy value of the anionic fluorescence induced by the conversion process is less than 0.26. (Note that the anisotropy change due to the rotational diffusion of the protein is negligible for the first 50 ps.) This means that the S_1-S_0 transition dipole moment of the anionic form generated from the neutral excited state is significantly tilted from that of the original neutral form, because the anisotropy value of neutral fluorescence observed at 520 nm was 0.35. The S_1-S_0 transition moments of the neutral and anionic forms are considered to be almost parallel as reported for GFP.[22] Therefore, the depolarization of this anionic fluorescence indicates that the anionic excited state is generated in a different subunit from the subunit that contains the neutral form excited. X-ray crystallography has revealed that the chromophores in four subunits of typical tetrameric fluorescent proteins are not oriented parallel,[23−27] and it is also true for red Kaede.[28] Therefore, the depolarization of the anionic fluorescence is expected to occur when FRET takes place, because the transition dipole moments of the neutral and the anionic forms in the different subunits are tilted. Consequently, it can be safely concluded that the 13-ps fluorescence dynamics is assignable to the FRET process from the neutral to the anionic form that are located in different subunits. This means that FRET is the predominant conversion process in red Kaede.

Concerning FRET, it may be worth discussing the possibility of FRET between the anionic forms located in two different subunits (HOMO FRET). This process is thought possible especially when the Stokes shift is small. We directly excited the anionic form with 570-nm excitation and found that the anisotropy of the anionic fluorescence was not depolarized (~0.4) in the measured time range up to 100 ps. The result clearly demonstrated that HOMO FRET between the anionic forms does not occur in red Kaede.

The fluorescence dynamics observed in DsRed also has been assigned to FRET based on the similar fluorescence depolarization.[7,29] However, an unambiguous discussion could not be made for DsRed because of the coexistence of the mature red and the immature green chromophores. Actually, this complicated situation of DsRed left ambiguity in conclusions of previous studies, where the FRET donor was assigned to the different species, i.e., the red anionic form[29,30] (HOMO FRET), the green anionic form,[7,9,10] and the red neutral form of the chromophore.[7] We note that red Kaede is free from such complexity and hence it is very suitable for the comprehensive study.

It was concluded that FRET is the main conversion mechanism in red Kaede at pH 7.5. This conclusion was further supported by the fluorescent lifetime measurements. The lifetime of the anionic fluorescence excited by 400 nm was 4.8 ns, which was the same as that of the anionic fluorescence excited with 570 nm. The exact same fluorescence lifetime implies that the anionic excited state converted from the neutral excited state is identical with that generated by direct excitation. This is a contrasting result to GFP[5] where the anionic excited state generated by ESPT (I*-form) has a different lifetime from the anionic excited state generated by direct photoexcitation (B*-form). Obviously, an intermediate species corresponding to the I*-form is not involved in the FRET process.

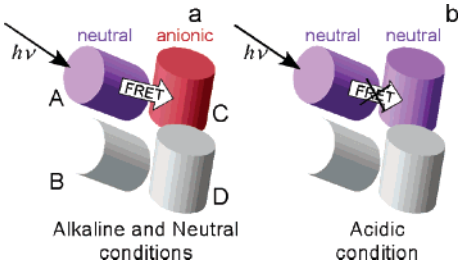


Figure 6. Schematic illustration of the conditions of the tetrameric fluorescent protein at (a) alkaline and neutral pH and (b) acidic pH.

Energy Donor−Acceptor Pair in the Tetrameric Structure. By using the anisotropy value of the FRET component of the anionic fluorescence, the angle between the transition dipole moments of the neutral and anionic forms can be evaluated. As already described, the anionic fluorescence arising from the direct excitation and the FRET process contribute to the anisotropy value of 0.26 at the delay time of 50 ps (Figure 5). We subtracted the direct excitation component and determined the anisotropy value of the FRET component as $r_{FRET} = 0.17 \pm 0.08$ (details are provided in Appendix B). The angle between the transition dipole moments of the neutral and the anionic forms was evaluated as $\beta = 38° \pm 8°$ by the equation $r_{FRET} = (2/5)[(3\cos^2\beta - 1)/2]$.[31]

It is worth discussing which subunits in the tetramer are the predominant energy donor−acceptor pair of the FRET process in red Kaede. Because preliminary X-ray crystallography data indicate that the structure of red Kaede is very similar to that of DsRed,[28] we can discuss this issue with referring to the crystal structure of DsRed.[23,24] The subunits in typical tetrameric fluorescent proteins are usually called A, B, C, and D (Figure 6a). The crystal structure of DsRed shows that A and B subunits are positioned parallel and that the relative configuration of the A and B subunits is similar to that of C and D subunits. In a similar way, A and C subunits are tilted against each other and their relative configuration is similar to that of B and D subunits. Thus, if we neglect the small difference in the relative configuration between the AB and CD subunits and that between AC and BD subunits, we can discuss the FRET process in red Kaede with a simplified structure in which the three energy donor−acceptor pairs, AB (parallel), AC (tilted), and AD (distant), represent all six combinations among four subunits in the tetramer.

According to the energy transfer model by Förster,[31] the energy transfer rate is proportional to $1/r^6$ and κ^2, where r is the distance between the donor and acceptor whereas κ^2 is the factor describing the orientational dependence of the dipole−dipole interaction. For AB, AC, and AD pairs, r and κ^2 are different and other parameters are in common. In the simplified tetrameric structure, the distances r of AB, AC, and AD pairs are 33.6, 27.4, and 42.9 Å,[32] respectively. Although it is difficult to determine the κ^2 value for the donor−acceptor pairs in general, the κ^2 values have been estimated for DsRed[29] ($\kappa^2_{AB} = 0.57$; $\kappa^2_{AC} = 1.64$; $\kappa^2_{AD} = 0.01$[33]). If we adopt these values for discussion about red Kaede, relative FRET rates for AB, AC, and AD pairs are calculated as 250, 2400, and 1,

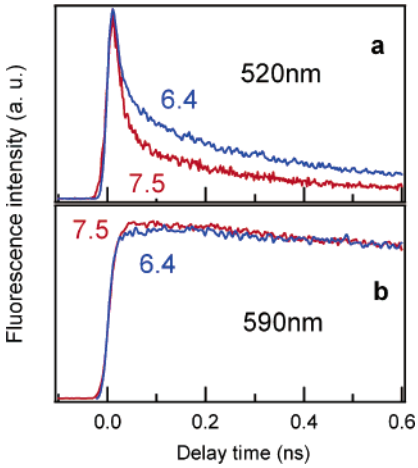


Figure 7. Picosecond time-resolved fluorescence signals of red Kaede at pH 7.5 (red) and 6.4 (blue) observed at (a) 520 and (b) 590 nm (400 nm excitation).

respectively. This consideration indicates that the energy transfer predominantly occurs between the AC pair. Consequently, the transition moment angle of 38° ± 8° evaluated from the fluorescence anisotropy of the FRET component practically corresponds to the angle between the neutral and anionic forms in the A and C subunits.

Proton-Transfer Dynamics of Red Kaede. It was revealed that the FRET process is the main conversion mechanism in red Kaede at pH 7.5. The observed 13-ps dynamics was assigned to the FRET process. Although the time constant of the FRET process in red Kaede is similar to that of the ESPT process in GFP,[3] the ESPT process was hardly recognized at pH 7.5 in red Kaede. This means that the ESPT process occurs much more slowly in red Kaede, even if it occurs. For the energy transfer to occur, it is necessary for the anionic ground state to exist in a subunit adjacent to the subunit that contains the excited neutral form (Figure 6a). This condition is realized with high probability at pH 7.5, because 87% of the chromophore exists in the anionic form. Under the acidic condition, however, the population of the anionic form decreases and the probability that the neutral form exists in both subunits of the AC pair increases (Figure 6b). In such a tetramer, the energy transfer is prohibited and hence we have a chance to observe the slow proton-transfer process. To know the ESPT process in red Kaede, we measured fluorescence dynamics of an acidic solution (pH 6.4) and compared it with that at pH 7.5.

The experiment at pH 6.4 was performed for a dilute solution (9 μM) because rapid degradation of the protein occurred in a concentrated solution. (Thus, we could perform only picosecond time-resolved measurements.) At pH 6.4, 31% of the chromophore exists in the neutral form ($x_p = 0.31$), which is significantly higher than the population at pH 7.5 ($x_p = 0.13$). In Figure 7, time-resolved fluorescence signals measured at pH 6.4 are compared with those at pH 7.5. Figure 7a depicts the temporal change of the neutral fluorescence observed at 520 nm. It is shown that the time constant attributed to the FRET dynamics (13 ps) remains unchanged by the pH change, which confirms our assignment because the FRET rate is determined by the configuration of the donor and acceptor in space, but not affected by pH. A remarkable change induced by the pH change is a significant increase of the amplitude of the 300-ps component at pH 6.4. This indicates that the 300-ps component is ascribed to the neutral form that does not undergo FRET because the adjacent subunit contains also the neutral form. Correspondingly, the rise of the anionic fluorescence measured

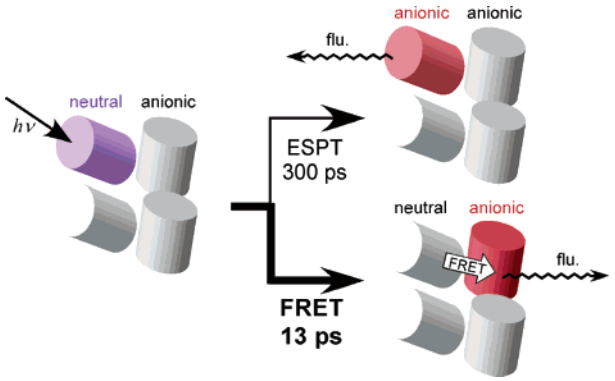


Figure 8. The fluorescence mechanism of red Kaede.

at 590 nm exhibits small but significant change upon the change of pH (Figure 7b). The comparison between the time-resolved fluorescence traces measured at two different pH values revealed that a slowly rising component exists and its amplitude is significantly larger at pH 6.4. This slow rise of the anionic fluorescence corresponds to the 300-ps decay of the neutral fluorescence, implying that the conversion process other than FRET occurs with the time constant of 300 ps. This slow conversion process is assignable to the ESPT process in red Kaede, which is hidden by the efficient FRET process. We note that this is the first observation that the proton transfer and the energy transfer take place simultaneously in the fluorescent protein.

Fluorescence Mechanism and Quaternary Structure. Figure 8 summarizes the photodynamics of the tetrameric fluorescent protein, red Kaede. Although only ESPT takes place from the neutral excited state in the monomeric fluorescent protein like GFP, the FRET process occurs in oligomeric fluorescent protein like red Kaede because the anionic form can exist in the vicinity of the neutral form with oligomer formation. In this situation, the competition between the proton transfer and energy transfer occurs and the decay pathway of the excited neutral form is governed by the faster process. Since the FRET process (13 ps) takes place much faster than the ESPT process (300 ps), the observed fluorescence dynamics is controlled by the energy transfer process in red Kaede under the neutral condition.

The time constant characteristic for the ESPT process in red Kaede is significantly large compared with that of GFP. There are two possible reasons for the difference. One is the modified chemical structure of the chromophore of red Kaede that has a longer conjugation. This intramolecular effect can alter the ESPT rate. The other possibility is an "intermolecular effect". The change of the ESPT rate may also arise from the different hydrogen bond network around the chromophore. It is known that a well-developed hydrogen bond network of GFP achieves the effective proton-transfer reaction.[34,35] In red Kaede, the network seems undeveloped, which can make proton transfer unfavorable. We consider that both effects are relevant. These two factors are also the origin of the different neutral/anionic form ratio between GFP and red Kaede; the neutral form is dominant for GFP while the anionic form is dominant for red Kaede even under the same neutral condition. We note that the same discussion can be made for DsRed. The chromophore of DsRed is different from either GFP or red Kaede, and DsRed has a significantly different hydrogen-band network from that of GFP.[24,36] Probably, these differences make the chromophore of DsRed exist exclusively in the anionic form in the protein.

In this study, we revealed that both FRET and ESPT take place as competing processes in red Kaede. The hidden ESPT

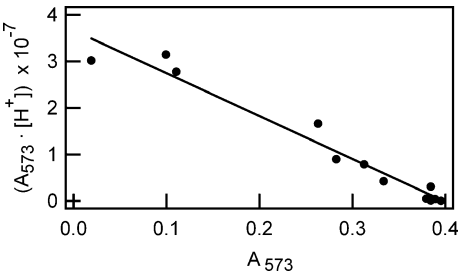


Figure 9. Plot of $A_{573}[H^+]$ against A_{573}, where A_{573} is the absorbance at 573 nm. The circles are the experimental data points measured in the pH range of 4.8−9.2. The straight line is the best fit.

process with a variety of time scale also might be observed for other fluorescent proteins. Although ESPT is the most well-known ultrafast excited-state process for the fluorescent protein family, FRET is also important, as revealed for red Kaede as well as DsRed. The results of the present study demonstrate that the excited-state dynamics of fluorescent proteins depends not only on the chromophore but also on the environment within the β-can structures and the quaternary structure.

Appendix A. Estimation of the Acid−Base Equilibrium Constant

To quantitatively discuss the acid−base equilibrium of the chromophore, the equilibrium constant K_a was evaluated from the absorption spectra.[37] The equilibrium between the neutral (RH) and the anionic forms (R^-), $RH \rightleftharpoons R^- + H^+$, is described with a relationship of $K_a = [R^-][H^+]/[RH]$. The concentration of the anionic form can be related to the absorbance at 573 nm as $A_{573} = \epsilon_{A,573}[R^-]$, because the spectra do not show any absorption at this wavelength below pH 4.5, where the population of the anionic form is negligible. Then, we obtain the following equation,

$$A_{573}[H^+] = \epsilon_{A,573} c K_a - A_{573} K_a \qquad (A.1)$$

where $\epsilon_{A,573}$ is the molar absorption coefficient of the anionic form at 573 nm. The total concentration of the protein c is the sum of the concentration of the anionic and neutral forms, $c = [R^-] + [RH] = 9\ \mu M$. Equation A.1 means that $A_{573}[H^+]$ is a linear function of A_{573}. The values of $A_{573}[H^+]$ were obtained from the absorption spectra measured in the pH range from 4.8 to 9.2, and it was plotted against A_{573} in Figure 9. From the slope and the intercept of the best-fitted line, the acid−base equilibrium constant K_a was determined as 9.2×10^{-7} and the molar absorption coefficient of the anionic form at 573 nm ($\epsilon_{A,573}$) as 43000.

Appendix B. Evaluation of the Anisotropy Value of the FRET Component of the Anionic Fluorescence

With use of the anisotropy value of the FRET component of the anionic fluorescence, the angle between the transition moments of the neutral and anionic form was determined. Because both the direct excitation and the FRET components contribute to the observed anisotropy value of 0.26 at the delay time of 50 ps (Figure 5), we need to separate these two components. The observed anisotropy r_{obs} can be represented as follows,

$$r_{obs}(t) = \frac{I_{||}^{obs}(t) - I_{\perp}^{obs}(t)}{I_{||}^{obs}(t) + 2I_{\perp}^{obs}} = \frac{(I_{||}^{direct}(t) + I_{||}^{FRET}(t)) - (I_{\perp}^{direct}(t) + I_{\perp}^{FRET}(t))}{(I_{||}^{direct}(t) + I_{||}^{FRET}(t)) + 2(I_{\perp}^{direct}(t) + I_{\perp}^{FRET}(t))} \qquad (A.2)$$

where I^{obs}, I^{direct}, and I^{FRET} mean the total anionic fluorescence intensity observed, the direct excitation component of anionic fluorescence, and the FRET component of anionic fluorescence, respectively. To obtain the anisotropy of the FRET component, r_{FRET}, the fluorescence amplitude of the 13-ps rise component in $I_{||}^{obs}$ and $I_{\perp}^{obs}$ was extracted. The observed fluorescence intensities ($I_{||}^{obs}$ and $I_{\perp}^{obs}$) at 680 nm were fitted by a function,

$$I^{obs}(t) = a_1 \exp(-t/\tau_1) + a_2[\exp(-t/\tau_1) - \exp(-t/\tau_2)] \qquad (A.3)$$

where τ_1 and τ_2 are fixed at 4.8 ns and 13 ps, respectively. The coefficients $a_{1,||}$, $a_{2,||}$, $a_{1,\perp}$, and $a_{2,\perp}$ were determined as 0.51, 0.16, 0.23, and 0.1, respectively. The first term of eq A.3 represents the direct excitation component whereas the second term describes the FRET component that exhibits the 13-ps rise. Finally r_{FRET} can be obtained by

$$r_{FRET}(t) = \frac{I_{||}^{FRET}(t) - I_{\perp}^{FRET}(t)}{I_{||}^{FRET}(t) - 2I_{\perp}^{FRET}(t)} = \frac{a_{2,||}(\exp(-t/\tau_1) - \exp(-t/\tau_2)) - a_{2,\perp}(\exp(-t/\tau_1) - \exp(-t/\tau_2))}{a_{2,||}(\exp(-t/\tau_1) - \exp(-t/\tau_2)) - 2a_{2,\perp}(\exp(-t/\tau_1) - \exp(-t/\tau_2))} = \frac{a_{2,||} - a_{2,\perp}}{a_{2,||} - 2a_{2,\perp}} \qquad (A.4)$$

The anisotropy value of the FRET component was determined as 0.17 ± 0.08.

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Supporting Information Available: Femtosecond time-resolved fluorescence signals of red Kaede at 520 nm observed with excitation at 430 nm. This material is available free of charge via the Internet at http://pubs.acs.org.

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(19) Although the fluorescence observed at 570 nm is predominantly due to the anionic fluorescence, we note that a small contribution from the neutral fluorescence cannot be completely neglected, because the neutral fluorescence is broad and extends to the red region (see Figure 1c).

(20) The contribution of the broad neutral form fluorescence is negligible in this longer wavelength region (see Figure 1c).

(21) The initial anisotropy value decreased down to 0.20 in the case of 400 nm excitation. The larger deviation from 0.4 with shorter wavelength photoexcitation is consistent with the involvement of the S_n-S_0 excitation of the anionic form.

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