Stochastic dynamic study of optical transition properties of single GFP-like molecules

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Abstract Due to high fluctuations and quantum uncertainty, the processes of single-molecules should be treated by stochastic methods. To study fluorescence time series and their statistical properties, we have applied two stochastic methods, one of which is an analytic method to study the off-time distributions of certain fluorescence transitions and the other is Gillespie's method of stochastic simulations. These methods have been applied to study the optical transition properties of two single-molecule systems, GFPmut2 and a Dronpa-like molecule, to yield results in approximate agreement with experimental observations on these systems. Rigorous oscillatory time series of GFPmut2 before it unfolds in the presence of denaturants have not been obtained based on the stochastic method used, but, on the other hand, the stochastic treatment puts constraints on the conditions under which such oscillatory behavior is possible. Furthermore, a sensitivity analysis is carried out on GFPmut2 to assess the effects of transition rates on the observables, such as fluorescence intensities.

Keywords Stochastic methods · Single molecule processes · Off-time distribution

1 Introduction

Single-molecule fluorescence spectroscopy has emerged as a powerful tool for characterizing the properties and interactions of biomolecules. Crucial information is lost in conventional methods, which measure the average properties of an ensemble of many molecules. In contrast, single-molecule methods permit detection and characterization of individual members of an ensemble, revealing details of a distribution [1, 2]. For instance,

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single-molecule fluorescence spectroscopy reveals fluctuations that are obscured by conventional measurements. Correspondingly, due to these fluctuations and quantum uncertainties associated with optical transitions, we need to develop stochastic dynamic methods to analyze the time series of single-molecule fluorescence experiments. Several approaches have been developed to study single-bio-molecular processes [2, 3]; stochastic methods [4–7] are one type of such approaches. As for the processes observed in the bulk, experimental observations correspond to the results averaged over a large number of molecules, and in such cases we may describe them in terms of rate equations. Among a wealth of single-bio-molecular processes, we mention here two systems of interest. The green fluorescent protein (GFP), composed of 238 amino acid residues, has become a preferred marker for gene expression and protein targeting in cells and organisms *in vivo*, because of its amazing ability to generate a highly visible fluorescence [8]. The GFP from *Aequorea victoria* has a major excitation peak at a wavelength of 395 nm and a minor one at 475 nm, which indicates the existence of at least two chemically distinct substates. Its emission peak is at 509 nm, which is in the lower green portion of the visible spectrum. pH value of the solution has a strong influence on the amplitudes of the excitation peaks. A simple explanation of these facts is that there are a neutral and an anionic substate [9]. GFPmut2 is a fluorescent GFP mutant (S65A, V68L, S72A) that has enhanced fluorescence emission with respect to wild type GFP and high yield of protein due to a more efficient folding at 37°C [10]. Baldini et al. [11, 12] reported, in single-molecule fluorescence experiments in the presence of denaturants, the observation of periodic millisecond oscillations in green and blue fluorescences immediately before GFPmut2 unfolded. They recorded both the blue and green fluorescences from single GFPmut2 molecules trapped in silica gels simultaneously. In the native state of GFP, the off-time of green fluorescence decays exponentially with an average around 0.14 ms. However, with denaturants added and right before it unfolds, the off-time follows a tilted Gaussian distribution with an average off-time around 0.64 ms [11]. For the potential applications in high-resolution cell imaging, GFP-like proteins with photo-switching properties have been designed and studied in recent years [13, 14]. Repeatedly photo-activatable GFP-like proteins like Dronpa [14] represent a new generation of fluorescent tools for studying trafficking inside cells through selective markings. In view of their importance, photoswitching properties of GFP-like proteins are interesting subjects to be investigated.

In the present article, we analyze the optical transition properties of these two single GFP-like molecules using two different stochastic approaches. In one approach, we apply Gillespie's stochastic dynamic methods for such analysis. Gillespie had introduced and developed systematic methods to study coupled chemical reactions [6, 15, 16]. These methods can be easily adopted for the present study. In another approach, we apply the method of the waiting time distributions of Kou et al., first introduced to study the enzyme kinetic reactions of a two-state enzyme [17], an enzyme which can exist in two interconverting conformations. This method based on Laplace transformation gives analytical results. In the GFPmut2 case, we shall analyze the system using a four-state and a six-state model. In the statistical sense, the six-state model does yield results in better agreement with the experiment than the four-state model. However, we have not in either case obtained the oscillatory behavior observed experimentally before GFPmut2 unfolds in the presence of denaturants, because the matrix associated with the master equations for the transitions and transformations has only real eigenvalues (not complex [18]) and the branching options having roughly equal probabilities. On the other hand, we discuss below that, based on the stochastic treatments, highly oscillatory behavior put constraints on the experimental conditions and local

environment of single molecules for which such oscillatory behavior is possible. The organization of the article is as follows: In Section 2, we briefly summarize several existing photophysical models of GFP-like molecules in the literature. In Section 3, we use simple four-state and six-state models to study the photophysical properties of single GFPmut2 system, using both analytical and computational stochastic methods. In Section 4, we carry out a sensitivity analysis of our model, studying the effects of the rate constants on the fluorescence intensities. The theoretical methods developed in previous sections are applied to study the photoswitching properties of a single Dronpa-like molecule. This is done in Section 5, which is followed by discussion in Section 6.

2 Existing models

In this section, we briefly review our current knowledge of the energy diagram of the GFP chromophore. It has been proposed that the GFP chromophore can adopt three distinct forms: anionic A (deprotonated), neutral N (protonated), and intermediate I (chemically similar to A, but with a different conformation) [19]. When the protein is folded, the ground state of I has a higher energy level than the ground states of both N and A [20]. Based on available experimental data, Creemers et al. proposed an energy level diagram for the ground and excited states of the three forms of wt-GFP. A schematic plot is shown in Fig. 1, where the vertical energy axis is not drawn to scale, and the thinner lines represent radiationless processes. It stated that the only transition between excited states is from the neutral form N^* to intermediate I^* , and the conversions from A^* to I, I^* to A, and I^* to N all occur through radiationless processes [20]. Several other models for the photophysical behaviors of GFP have been proposed with the suggestion that there might be a dark state Z (Zwitterionic) at which GFP does not emit visible light [21], among which is the scheme by Weber et al. [22]. A simplified version of it is shown in Fig. 2. Photo-excitations have been omitted for simplicity in this figure and the energy spacings are roughly sketched. The ground state energy levels depend on the environment, such as pH value of the solution. The thinner lines represent NAC (non-adiabatic crossing). It suggests that Z^* prefers NAC, and N^* , I^* and A^* undergo radiationless decay through NAC. The relative energy between the ground states depends on the environment and might vary with mutants as well. Some of the elements of the 3- and 4-chemical species models are supported by experimental results, but some are not, for example, there is no direct experimental evidence of the existence of the zwitterionic species [21]. Baldini et al. [11] proposed an energy diagram for GFPmut2 shown in Fig. 3. They deduced the two-photon excitation and emission wavelengths for N, A and I species from excitation and emission spectra. Again here our scheme is not drawn to the correct energy scale. The key difference of this diagram from that of Weber

Fig. 1 The model proposed by Creemers et al. [20]

Fig. 2 The 4-chemical substate model of Weber et al. [22]

et al. is that the dark state is between the A and I states, instead of being on the side. All these models proposed sequential interconversions between the ground states and sequential interconversions between the excited states, in other words, the protonation and deprotonation processes can take place in both the ground-state and excited-state chromophores. The ground-state switching involves external proton transfer, which is between the chromophore and a nearby water molecule. This rate of external proton transfer strongly depends on the environment and is usually slow, but the deprotonation of the N^* state chromophore, which is internal proton transfer, is ultra-fast [23]. Excited-state proton transfer (ESPT) of the N form chromophore, the $N^* \rightarrow I^*$ transition in Figs. 1 and 2, or the $N^* \rightarrow A^*$ transition in Fig. 3, happens at the ps time scale while $N^* \rightarrow N$ decay is at the ns scale [23, 24], which is why weak blue fluorescences are detected. In high pH environment, for EGFP (F64L, S65T), the excited anionic state, A^* , under 488-nm illumination, emits fluorescence at a rate of $3.5 \times 10^8 \text{ s}^{-1}$, for wt-GFP and for GFP-S65T variants, the rates are $3.12 \times 10^8 \text{ s}^{-1}$ and $3.36 \times 10^8 \text{ s}^{-1}$, respectively [25]. In a low pH environment, a slow protonation process from the anionic to the neutral form of the chromophore in EGFP can occur following an external proton transfer from low-pH buffer to the embodied chromophore. The time scale of this proton exchange is approximately 45–340 μs [26]. In wt-GFP and mutants, conversion from the neutral to the anionic form of the chromophore, in solution, takes place via an excited-state internal proton transfer, at the time scale of around $10 \pm 5 \text{ ps}$ [25]. Approximate lifetimes of N^* and I^* are around 1 ns and 3.3 ns [24].

Fig. 3 The photoconversion scheme proposed by Baldini et al. [11]

3 Our approach

The steady switching behavior between neutral and anionic forms of a single GFPmut2 molecule right before it unfolds under the action of denaturants is characterized as a near Gaussian distribution of the off-times of the green emissions in the data of Baldini et al. [11]. To investigate such dynamic behavior, we assume that in the process of unfolding, the basic scheme for photo-conversions among different species remains the same, but some of these rates will change. These changes are motivated by the experimental setup, in which a protein molecule is in the process of unfolding, induced by 4.45 M guanidium hydrochloride (GuHCl) added an hour earlier. Due to this harsh environment, we assume, for example, (1) The conformation differences between the A and I states are being reduced by the presence of denaturants, thus we expect the transformation between I and A states to speed up significantly. (2) The internal proton transfer rate might go down as the protein is unfolding, because the proton network being perturbed by denaturants. (3) The external proton transfer rate might change as the chromophore becomes more exposed to water and denaturant molecules. To find simple models that can be used to interpret the experimental results, we start with a 4-state system shown in Fig. 4, and then we investigate how results can be improved in a 6-level system shown in Fig. 9. Again energy is not drawn to the correct scale in these figures. Different mutants of GFP have been designed for various reasons. There are GFP mutants and GFP-like fluorescent proteins which have been discovered to display similar reversible photoswitching behaviors as GFP [14, 27, 28]. For example, red-fluorescent protein (dsRed) from a coral of the *Discosoma* genus and yellow fluorescent protein mutant Citrine (with mutations S65G/V68L/Q69M/S72A/T203Y) both undergo photoconversion between bright and dark states [29]. While the energy levels and transition rates of each protein may be different from those of GFP mutants, we can easily modify our model and apply our methods to study their photoswitching properties.

3.1 The 4-state model

In this section we study the 4-state system shown in Fig. 4, where the four states are: N (neutral), N* (excited neutral state), I (intermediate), and I* (excited intermediate state). We note

Fig. 4 The 4-state model

that here we could have used A^* and A in place of I^* and I . In mathematics they are equivalent and just represent labels which may not denote real states. Also shown in Fig. 4 are rate constants: k_1 and k_8 being the two photon absorption rates, and k_7 and k_9 being the blue and green emission rates for the N and I GFP forms, respectively. We assume the deprotonation rate of the N^* chromophore state and the protonation rate of the I chromophore state are much faster than those of the reversed reactions to simplify the system.

3.1.1 Analytical approach

We introduce in this section an analytical stochastic method to analyze the optical transitions of single GFPmut2 molecules. In this method, we calculate the off-time distribution of the green emission, which is an adaption of the waiting-time distribution method for single-molecule Michaelis-Menten equations developed by Kou et al. [17] in their treatment of the two-state enzyme kinetic model. Their method is very general and can be applied to other single-molecule dynamic processes. We here apply it to derive the off-time distribution of green emissions. We define an off-time, t_{off} , of the green emission as the time from one green emission to the next with at least one blue emission in between, that is, we take the blue emissions as the sign that green emissions have been turned off. Let us assume the protein is initially in the N state, then to get to the I^* state, it may either go through $N \rightarrow N^* \rightarrow I^*$ directly or go through that after a number of the $N \rightarrow N^* \rightarrow N$ loops. If we define T_N as the time to get to the I^* state from the N state, then by following the method of Kou et al. [17], we can write down the probability of the transition from N to I^* happening within time t , $P(T_N < t)$, as

$$P(T_N < t) = P(T_{k_2} < T_{k_7})P(T_{k_1} + T_{k_2} < t) + P(T_{k_2} > T_{k_7})P(T_{k_1} + T_{k_7} + T_N < t) \quad (3.1)$$

where T_{k_i} is the time it takes to complete the transition with rate k_i , which we assume follows the Poisson distribution $f_{T_{k_i}}(t) = k_i e^{-k_i t}$. In this equation, the $P()$ are probability symbols, for example, $P(T_{k_2} < T_{k_7})$ and $P(T_{k_1} + T_{k_2} < t)$ are probabilities that the T_{k_2} transition time is shorter than the T_{k_7} transition and that the sum of the $T_{k_1} + T_{k_2}$ transitions times is shorter than t .

For any random variable, X , the probability density of a distribution $P(X < x)$ is given by $f_X(x) = \frac{dP(X < x)}{dx}$. Therefore differentiating (3.1) with respect to t gives us the expression of the distribution for T_N :

$$\begin{aligned} f_{T_N}(t) &= \frac{dP(T_N < t)}{dt} \\ &= P(T_{k_2} < T_{k_7})f_{T_{k_1}+T_{k_2}}(t) + P(T_{k_7} < T_{k_2})f_{T_{k_1}+T_{k_7}+T_N}(t). \end{aligned} \quad (3.2)$$

Since the distribution of the sum of random variables is the convolution of the distributions of the individual random variables, (3.2) can be expressed as

$$\begin{aligned} f_{T_N}(t) &= \int_0^t \int_0^{t_1} dt_1 P(t_1 < T_{k_7}) f_{T_{k_1}}(t - t_1) f_{T_{k_2}}(t_1) \\ &\quad + \int_0^t \int_0^{t_1} dt_1 dt_2 P(t_2 < T_{k_2}) f_{T_{k_1}}(t_1 - t_2) f_{T_{k_7}}(t_2 - t_2) f_{T_N}(t - t_1). \end{aligned} \quad (3.3)$$

Based on the assumption that the time it takes for each step follows an exponential distribution, the probability of a transition with a rate of k_i taking longer than a time t , $P(t < T_{k_i})$, can be written as,

$$\begin{aligned} P(t < T_{k_i}) &= \int_t^\infty k_i e^{-k_i t_1} dt_1 \\ &= e^{-k_i t}. \end{aligned} \quad (3.4)$$

Applying (3.4) and again the assumption that the time it takes for each transition follows an exponential distribution, the distribution of T_N can then be written as,

$$\begin{aligned} f_{T_N}(t) &= \frac{k_1 k_7}{k_2 + k_7 - k_1} \int_0^t dt_1 (e^{-k_1 t_1} - e^{-k_2 t_1 - k_7 t_1}) f_{T_N}(t - t_1) \\ &\quad + \frac{k_1 k_2}{-k_1 + k_2 + k_7} (e^{-k_1 t} - e^{-k_2 t - k_7 t}). \end{aligned} \quad (3.5)$$

Because the Laplace transform of a convolution is the product of Laplace transforms, (3.5) after the Laplace Transform reads,

$$\begin{aligned} \tilde{f}_{T_N}(s) &= \mathcal{L} f_{T_N}(t) \\ &= \frac{k_1 k_7}{k_2 + k_7 - k_1} \left(\frac{1}{s + k_1} - \frac{1}{s + k_2 + k_7} \right) \tilde{f}_{T_N}(s) \\ &\quad + \frac{k_1 k_2}{-k_1 + k_2 + k_7} \left(\frac{1}{s + k_1} - \frac{1}{s + k_2 + k_7} \right) \end{aligned} \quad (3.6)$$

therefore

$$\tilde{f}_{T_N}(s) = \frac{k_1 k_2}{(s + k_1)(s + k_2 + k_7) - k_1 k_7}. \quad (3.7)$$

The off-time of green emission is defined as the time from one green emission to the next with at least one blue emission in between. The probability of the off-time less than t can be expressed as:

$$P(t_{off} < t) = P(T_{k_6} < T_{k_8}) P(T_{k_7} < T_{k_2}) P(T_{k_6} + T_{k_1} + T_{k_7} + T_N < t) \quad (3.8)$$

where the presence of $P(T_{k_6} < T_{k_8}) P(T_{k_7} < T_{k_2})$ is to ensure there will at least be one blue emission during the off-time. The probability density of the off-time is

$$\begin{aligned} f_{t_{off}}(t) &= \frac{dP(t_{off} < t)}{dt} \\ &= \frac{k_1 k_6 k_7}{-k_6 + k_1 - k_8} \int_0^t dt_1 \left(\frac{e^{-k_6 t_1 - k_8 t_1} - e^{-k_7 t_1 - k_2 t_1}}{-k_6 - k_8 + k_2 + k_7} \right. \\ &\quad \left. - \frac{e^{-k_1 t_1} - e^{-k_7 t_1 - k_2 t_1}}{-k_1 + k_2 + k_7} \right) f_{T_N}(t - t_1). \end{aligned} \quad (3.9)$$

The Laplace transform of $f_{t_{off}}(t)$ yields

$$\begin{aligned} \tilde{f}_{t_{off}}(s) &= \frac{k_1 k_6 k_7}{-k_6 + k_1 - k_8} \left(\frac{\frac{1}{s + k_6 + k_8} - \frac{1}{s + k_2 + k_7}}{-k_6 - k_8 + k_2 + k_7} - \frac{\frac{1}{s + k_1} - \frac{1}{s + k_2 + k_7}}{-k_1 + k_2 + k_7} \right) \tilde{f}_{T_N}(s) \\ &= \frac{k_6 k_1^2 k_7 k_2}{(s^2 + s k_2 + s k_7 + k_1 s + k_1 k_2)(s + k_8 + k_6)(s + k_1)(s + k_2 + k_7)}. \end{aligned} \quad (3.10)$$

To investigate how different rates affect the off-time distribution, we need to solve for $f_{t_{off}}(t)$ as a function of t and the transition rates, which can be achieved by applying the inverse Laplace transform on (3.10). The expression that we obtain is rather complicated, so

we will not present it here. It is made mainly of exponential terms of the form of $C_1 e^{-C_2 t}$, where both C_1 and C_2 are combinations of the rate constants. However, we can obtain other important information before applying the inverse Laplace transform. Because the Laplace transform of $f_{t_{off}}(t)$ is defined as:

$$\tilde{f}_{t_{off}}(s) = \int_0^\infty e^{-st} f_{t_{off}}(t) dt \quad (3.11)$$

the mean off-time, which is defined as $\int_0^\infty t f_{t_{off}}(t) dt$, can be written as:

$$\langle t_{off} \rangle = - \frac{d \tilde{f}_{t_{off}}(s)}{ds} \Big|_{s=0}. \quad (3.12)$$

Therefore we can get the mean off-time based on $\tilde{f}_{t_{off}}(s)$ alone from (3.12) and see how each rate k_i affects the mean off-time, by calculating $\frac{d \langle t_{off} \rangle}{dk_i}$. This yields

$$\begin{aligned} \langle t_{off} \rangle &= \frac{k_6 k_7 (k_2 + k_7 + k_1)}{k_1 k_2 (k_6 + k_8) (k_2 + k_7)} + \frac{k_6 k_7}{k_1 (k_6 + k_8) (k_2 + k_7)} + \frac{k_6 k_7}{(k_6 + k_8)^2 (k_2 + k_7)} \\ &\quad + \frac{k_6 k_7}{(k_6 + k_8) (k_2 + k_7)^2}. \end{aligned} \quad (3.13)$$

As mentioned before, when the protein unfolds in the presence of denaturants, the rate constants might change, which causes the off-time to increase, as observed by the experimentalists [11]. To verify that, we first look at how the rates affect the mean off-time. For instance, the effect of k_1 on $\langle t_{off} \rangle$ is given by:

$$\frac{d \langle t_{off} \rangle}{dk_1} = - \frac{k_6 k_7 (2k_2 + k_7)}{k_2 k_1^2 (k_6 + k_8) (k_2 + k_7)}. \quad (3.14)$$

It says that increasing k_1 will reduce the mean off-time, as expected according to Fig. 4. Similarly, $\frac{d \langle t_{off} \rangle}{dk_2}$ and $\frac{d \langle t_{off} \rangle}{dk_8}$ are also always negative, while $\frac{d \langle t_{off} \rangle}{dk_7}$ is always positive. The influence of k_6 depends on other rates, especially k_8 . With small k_8 , increasing k_6 might decrease the mean off-time. However, when $k_8 \gg k_6$, changing k_6 will not affect the mean off-time by much. In order to obtain the off-time distribution $f_{t_{off}}(t)$, we have to take the inverse Laplace transform of (3.10). Plots of $f_{t_{off}}(t)$ with different sets of rates confirm the trends mentioned above. For example, Fig. 5 shows how k_2 , k_6 and k_8 affect the off-time distributions when k_1 , k_7 and k_9 are fixed. Smaller k_2 shifts the peak toward the right (see Fig. 5c and d), so does smaller k_8 (see Fig. 5b and d), while increasing k_6 from 10^3 to 10^5 with the given rates will also shorten the off-time (see Fig. 5a and c), which all agree with our conclusions based on the mean off-time change. Reducing k_6 to increase the off-time only works effectively when k_6 is comparable to or greater than k_8 .

At the end of this subsection, we should mention that besides the method used here the methods based on the master equations for transitions and transformations (for example, (3.16) introduced later) may also be used to derive analytical expressions for the off-time distributions.

3.1.2 Stochastic simulations

Besides deriving analytical result for the off-time distribution, we have carried out numerical simulations of the GFP emission processes. For biological processes involving a small number of molecules, we should use a stochastic approach, because of the large fluctuations

Fig. 5 The off-time distributions based on our 4-state model with varying excited state proton transfer rates, k_2 , and different ground state proton transfer rates, k_6 , as well as the absorption rate, k_8 . In all four plots, $k_1 = 3 \times 10^4$, $k_7 = 10^9$, $k_9 = 3 \times 10^8$. (a) $k_2 = 10^8$, $k_6 = 10^3$, $k_8 = 10^4$, (b) $k_2 = 10^{11}$, $k_6 = 10^5$, $k_8 = 10^6$, (c) $k_2 = 10^8$, $k_6 = 10^5$, $k_8 = 10^4$, (d) $k_2 = 10^{11}$, $k_6 = 10^5$, $k_8 = 10^4$. For (a) and (c), the only difference is k_6 , for (b) and (d), the only difference is k_8 , while (c) and (d), the only difference is k_2

involved. This is certainly true for single-molecule processes. One of the standard algorithms for simulating stochastic kinetics is the “Gillespie algorithm”, which was published over 30 years ago [15]. The basic steps of the Gillespie algorithm [15, 16] are:

1. Initiation: Initialize the numbers of molecules in the system; calculate h_μ , which is the number of distinct molecular reactant combinations available for the μ th reaction, denoted by R_μ ; define the stochastic reaction rate constant, c_μ , and the total number of reactions M and initialize the random number generators.
2. The Monte Carlo step: Generate random numbers using random number generators to determine which reaction will occur as well as the time interval. The probability of each reaction is proportional to the numbers of the reactants and also depends on the reaction rate. The time interval τ is generated according to the probability density function $P_1(\tau) = a_0 \exp(-a_0 \tau)$, where $a_0 \equiv \sum_{v=1}^M h_v c_v$. Two random numbers r_1 and

- r_2 need to be generated. r_1 is used to determine the time interval $\tau = (\frac{1}{a_0}) \ln(\frac{1}{r_1})$ and r_2 is used to determine the probability that the μ th reaction takes place next, where μ satisfies $\sum_{v=1}^{\mu-1} a_v < r_2 a_0 \leq \sum_{v=1}^{\mu} a_v$, where $a_v = h_v c_v$.
3. Updating: Increase the time by the randomly generated time step in Step 2. Update the molecule counts based on the reaction that occurred.
 4. Iteration: Go back to Step 2 unless the number of reactants is zero or the simulation time has been exceeded.

In our simulations, instead of different “molecules”, we have different chemical states of the chromophore of GFP, and “the number of molecules” mentioned above is either 1 or 0, corresponding to whether the chromophore is at that specific state or not. Given the initial state of the protein and all the reaction rates, we can obtain the photon counts for the green and blue emissions as functions of time and at the same time calculate off-time distributions. Figure 6 presents the blue and green emission intensities and the off-time distributions obtained from such stochastic dynamic simulations for the set of rate parameters: $k_1 = 5 \times 10^5$, $k_2 = 5 \times 10^{10}$, $k_6 = 10^3$, $k_7 = 10^9$, $k_8 = 10^4$, $k_9 = 3 \times 10^8$. This set of rates is chosen to simulate the case when the protein is folded. The off-time distribution matches the experimental results quite well, but the emission intensities are lower than those observed in the experiment [11]. To simulate an unfolding protein, a set of transition rates ($k_1 = 5 \times 10^4$, $k_2 = 10^8$, $k_6 = 10^3$, $k_7 = 10^9$, $k_8 = 7 \times 10^4$, $k_9 = 3 \times 10^8$) is used, for which the blue and green emission intensities and the off-time distribution obtained are

Fig. 6 Stochastic simulation results for folded GFPs based on our 4-state model. It shows the blue emission intensity (the *top* plot), the green emission intensity (the *middle* plot) and the off-time distribution (the *bottom* plot) where rates are set at: $k_1 = 5 \times 10^5$, $k_2 = 5 \times 10^{10}$, $k_6 = 10^3$, $k_7 = 10^9$, $k_8 = 10^4$, $k_9 = 3 \times 10^8$

Fig. 7 Stochastic simulation results for pre-unfolding GFP based on our 4-state model. It shows the blue emission intensity (the *top* plot), the green emission intensity (the *middle* plot) and the off-time distribution (the *bottom* plot) where transition rates are set at: $k_1 = 5 \times 10^4$, $k_2 = 10^8$, $k_6 = 10^3$, $k_7 = 10^9$, $k_8 = 7 \times 10^4$, $k_9 = 3 \times 10^8$

plotted in Fig. 7. With this set of rates, the emission intensities in the simulations are close to experimental values of an unfolding protein [11], and the off-time distribution has a finite peak, but the peak value 0.2 ms is lower than that observed in the experiment, 0.6 ms [11]. With reduced k_1 and k_8 rates, other rates remain the same, the blue and green emission intensities and the off-time distribution are shown in Fig. 8. As shown there, we are able to increase the off-time peak value to be closer to the experimental result [11], but the emission intensities are lower than experimental values [11]. With the 4-state model, we are able to show that, by varying rates, the off-time distribution can be changed from an exponential decay curve to a distribution which peaks at a finite value of t_{off} . However, we have not been able to find a set of rate constants that yield the results of both the intensities and off-time distribution to match well with the experimental values.

3.2 The 6-state model

The 4-state model reproduces some of the important features observed, such as the off-time distribution peaks at ms values, but not all the features observed with a single set of transition rates. Therefore we continue to study a 6-state system. The system studied is shown in Fig. 9, where the six states are: N (neutral), N* (excited neutral state), I (intermediate), I* (excited intermediate state), A (anionic), and A* (excited anionic state). The transition rates

Fig. 8 Stochastic simulation results for pre-unfolding GFP based on our 4-state model. It shows the blue emission intensity (the *top* plot), the green emission intensity (the *middle* plot) and the off-time distribution (the *bottom* plot) when $k_1 = 7 \times 10^3$, $k_2 = 10^8$, $k_6 = 10^3$, $k_7 = 10^9$, $k_8 = 7 \times 10^3$, $k_9 = 3 \times 10^8$

k_1 , k_{10} and k_8 are the two-photon absorption rates and k_7 , k_4 and k_9 are the emission rates for the N, A and I forms of GFP, respectively. The emission from N^* is in the blue spectrum and those from I^* and A^* are both in the green. We assume the transitions from $N^* \rightarrow I^*$, $I^* \rightarrow A^*$, $A \rightarrow I$ and $I \rightarrow N$ are irreversible to simplify the model.

Fig. 9 The 6-state model

3.2.1 Analytical approach

If the protein is initially in state N, to get to the next green emission, it may either go through $N \rightarrow N^* \rightarrow I^* \rightarrow I$ or $N \rightarrow N^* \rightarrow I^* \rightarrow A^* \rightarrow A$ directly or go through that after a number of $N \rightarrow N^* \rightarrow N$ transitions. Therefore the probability of the transition from N to the next green emission within time t is given by

$$\begin{aligned} P(T_N < t) = & P(T_{k_2} < T_{k_7})P(T_{k_9} < T_{k_3})P(T_{k_1} + T_{k_2} + T_{k_9} < t) \\ & + P(T_{k_2} < T_{k_7})P(T_{k_3} < T_{k_9})P(T_{k_1} + T_{k_2} + T_{k_3} + T_{k_4} < t) \\ & + P(T_{k_7} < T_{k_2})P(T_{k_1} + T_{k_7} + T_N < t). \end{aligned} \quad (3.15)$$

As before, for the 6-state model, we define the off-time as the time between two non-successive green emissions, but with the time the second green emission takes included. We include the time for the second green emission for the purpose of simplifying the calculation. This emission time should be relatively short, so the effect of including it on the off-time distribution should be negligible. As stated above, the green emissions in this model can come from either the $A^* \rightarrow A$ transitions or $I^* \rightarrow I$ transitions. Therefore, when we calculate the off-time distribution, we have to consider two cases. We assume the probability of the last green emission before it turns off being caused by the $A^* \rightarrow A$ transition

is $\frac{P_{A^*}k_4}{P_{I^*}\frac{k_9^2}{k_9+k_3}+P_{A^*}k_4}$ and that of it being caused by the $I^* \rightarrow I$ transition is $\frac{P_{I^*}\frac{k_9^2}{k_9+k_3}}{P_{I^*}\frac{k_9^2}{k_9+k_3}+P_{A^*}k_4}$, where P_{I^*} and P_{A^*} are the steady-state probabilities of the protein being in state I^* and A^* , respectively. The probabilities are the steady-state solutions to the master equations:

$$\begin{aligned} \frac{dP_N}{dt} &= -k_1P_N + k_7P_{N^*} + k_6P_I, \\ \frac{dP_{N^*}}{dt} &= k_1P_N - (k_2 + k_7)P_{N^*}, \\ \frac{dP_{I^*}}{dt} &= k_2P_{N^*} - (k_3 + k_9)P_{I^*} + k_8P_I, \\ \frac{dP_{A^*}}{dt} &= k_3P_{I^*} - k_4P_{A^*} + k_{10}P_A, \\ \frac{dP_A}{dt} &= k_4P_{A^*} - (k_5 + k_{10})P_A, \\ \frac{dP_I}{dt} &= k_9P_{I^*} + k_5P_A - (k_8 + k_6)P_I. \end{aligned} \quad (3.16)$$

The probability of the off-time shorter than a time t is given by

$$\begin{aligned} P(t_{off} < t) = & \frac{P_{I^*}\frac{k_9^2}{k_9+k_3}}{P_{I^*}\frac{k_9^2}{k_9+k_3} + P_{A^*}k_4} P(T_{k_7} < T_{k_2})P(T_{k_6} < T_{k_8})P(T_{k_6} + T_{k_1} + T_{k_7} + T_N < t) \\ & + \frac{P_{A^*}k_4}{P_{I^*}\frac{k_9^2}{k_9+k_3} + P_{A^*}k_4} \\ & \times P(T_{k_5} < T_{k_{10}})P(T_{k_6} < T_{k_8})P(T_{k_5} + T_{k_6} + T_{k_1} + T_{k_7} + T_N < t). \end{aligned} \quad (3.17)$$

We follow a similar procedure as used in the 3- and 4-state model to derive the off-time probability distribution from (3.16). The details are shown in the [Appendix](#). Some examples

of the analytic $f_{i_{off}}$ results (expressions not shown) obtained are plotted in Fig. 10. These figures show that reducing the values of k_2 and k_6 shifts the peak values of the off-time distributions to higher values, thus peaking values around 0.6 ms can be reached (not shown here). Our results also show, just as in the 4-state model, if $k_6 \ll k_8$, then decreasing k_6 will not increase off-time as effectively. When k_3 and k_5 are small, that is, the transitions between the intermediate and anionic state are slow, the results are reduced to the case of the 4-state model.

3.2.2 Stochastic simulations

Figure 11 shows the green and blue emission intensities and the off-time distribution from our stochastic simulations of the behaviors of the native GFPmut2. The results are in qualitative agreement with experimental observations [11]. To simulate the non-native,

Fig. 10 The off-time distributions based on our 6-state model, when $k_1 = 3 \times 10^4$, $k_3 = 10^5$, $k_4 = 3 \times 10^8$, $k_5 = 5 \times 10^4$, $k_7 = 10^9$, $k_8 = 10^4$, $k_9 = 3 \times 10^9$, $k_{10} = 10^4$ are fixed. **a** $k_2 = 10^{11}$, $k_6 = 10^5$, **b** $k_2 = 10^8$, $k_6 = 10^5$, **c** $k_2 = 10^{11}$, $k_6 = 10^3$, **d** $k_2 = 10^8$, $k_6 = 10^3$

Fig. 11 The stochastic simulation results for folded GFPs based on our 6-state model. It shows the blue emission intensity (the *top* plot), the *green* emission intensity (the *middle* plot) and the off-time distribution (the *bottom* plot) with transition rates set at: $k_1 = 5 \times 10^5$, $k_2 = 5 \times 10^{10}$, $k_3 = 3 \times 10^8$, $k_4 = 3 \times 10^8$, $k_5 = 1 \times 10^5$, $k_6 = 2 \times 10^3$, $k_7 = 10^9$, $k_8 = 1 \times 10^4$, $k_9 = 3 \times 10^8$, $k_{10} = 3 \times 10^5$

pre-unfolding GFP protein, we set the transition rates to be: $k_1 = 3 \times 10^4$, $k_2 = 10^8$, $k_3 = 10^8$, $k_4 = 5 \times 10^8$, $k_5 = 3 \times 10^4$, $k_6 = 10^3$, $k_7 = 3 \times 10^9$, $k_8 = 10^4$, $k_9 = 3 \times 10^8$, $k_{10} = 10^5$. The blue and green emissions and the off-time distribution are shown in Fig. 12. Compared to the experimental results, our off-time distribution peaks in the correct range, but it has a longer tail than the measured curve [11]. This might be due to the fact that it is a dynamic process in the experiment, with the rates changing, while in our simulation rates are fixed. The off-time distributions in our stochastic simulation agree with our analytical results (data not shown). When GFPmut2 is unfolding, the off-time of green emission increases compared to that of the folded proteins [11]. At first glance, the easiest way to achieve longer off-time will be reducing the rate of the slowest reaction. If the slowest one is $N \rightarrow N^*$ or $A^* \rightarrow A$, it will work. However, it will not work as well if the slowest reaction faces “competition”. For example, when the chromophore is at the I state, it can go to the N state or I^* state; in other words, the $I \rightarrow N$ competes with the $I \rightarrow I^*$ transition. If k_6 is the lowest rate, reducing k_6 will lower the probability of the $I \rightarrow N$ transition, while the timestep will be constrained by k_8 . With the Gillespie algorithm, when the protein is in the I state, the next timestep is taken as $\frac{1}{k_6 + k_8} \times (\text{a random number})$. Therefore, the off-time depends strongly on the rate of the competitor of the slowest transition. In our 6-state model, the $N \rightarrow N^*$ and $A^* \rightarrow A$ transitions do not have competitors, so adjusting these two rates appears to be the most effective way of changing the off-time. However, the blue and green emission intensities are also very sensitive to the changing of these two. The advantage of the 6-state model

Fig. 12 The stochastic simulation results for unfolding GFPs based on our 6-state model. It shows the blue emission intensity (the *top* plot), the *green* emission intensity (the *middle* plot) and the off-time distribution (the *bottom* plot) with transition rates set at $k_1 = 3 \times 10^4$, $k_2 = 10^8$, $k_3 = 10^8$, $k_4 = 5 \times 10^8$, $k_5 = 3 \times 10^4$, $k_6 = 10^3$, $k_7 = 3 \times 10^9$, $k_8 = 10^4$, $k_9 = 3 \times 10^8$, $k_{10} = 10^5$

is that both intermediate and anionic state GFPmut2 emit green light. It has more adjustable parameters when it comes to fitting the photon counts of the green emission. These are the reasons why that the 6-state model gives better results than the 4-state model. As shown in Fig. 12, the peak position, the intensity and the width of the off-time distribution of the green emissions are in approximately agreement with the experiment [11], except that the shape of the simulated distribution tilts toward the long-time side and the observed shape is more Gaussian. Of course, we do not expect the simulated trajectories to be sinusoidal, corresponding to an oscillatory behavior, because of the stochasticity and the reasons mentioned in Introduction. We will discuss in the discussion section the implications of high oscillatory behavior as observed by Baldini et al. based on stochastic dynamics.

4 Sensitivity analysis

The rates in our models are not known precisely and some of them depend strongly on the environment. In order to study how the system responds to the changes in rates, we have carried out a sensitivity analysis [30] and ranked the rates in the order of their influences on a certain state [31]. The rate causing the largest change in the probability of each state will be the most important one to change in parameter fitting. The sensitivity parameter S_{ij}

is defined as the derivative of the probability of the i th state with respect to the j th rate constant:

$$S_{ij} = \frac{\partial P_i}{\partial k_j}. \quad (4.1)$$

By using the chain rule of differentiation, we get

$$\frac{dS_{ij}}{dt} = \sum_{k=1}^N \frac{\partial f_i}{\partial P_k} \frac{\partial P_k}{\partial k_j} + \frac{\partial f_i}{\partial k_j} \quad (4.2)$$

where N is the number of states, and we express the master equations as

$$\frac{dP_i}{dt} = f_i. \quad (4.3)$$

For the 6-state model, if we label N, N*, I*, A*, A and I states by number 1 to 6, then the master equations can be written as:

$$\begin{aligned} \frac{dP_1}{dt} &= k_7 P_2 + k_6 P_6 - k_1 P_1 \\ \frac{dP_2}{dt} &= k_1 P_1 - k_2 P_2 - k_7 P_2 \\ \frac{dP_3}{dt} &= k_2 P_2 + k_8 P_6 - k_9 P_3 - k_3 P_3 \\ \frac{dP_4}{dt} &= k_3 P_3 + k_{10} P_5 - k_4 P_4 \\ \frac{dP_5}{dt} &= k_4 P_4 - k_5 P_5 - k_{10} P_5 \\ \frac{dP_6}{dt} &= k_9 P_3 - k_8 P_6 - k_6 P_6 + k_5 P_5 \end{aligned} \quad (4.4)$$

and sensitivities, $S_{i,j}$, satisfy the following inhomogeneous linear differential equations:

$$\begin{aligned} \frac{dS_{1j}}{dt} &= -k_1 S_{1j} + k_7 S_{2j} + k_6 S_{6j} + \delta_{j,7} P_2 + \delta_{j,6} P_6 - \delta_{j,1} P_1 \\ \frac{dS_{2j}}{dt} &= k_1 S_{1j} - (k_2 + k_7) S_{2j} + \delta_{j,1} P_1 - \delta_{j,2} P_2 - \delta_{j,7} P_2 \\ \frac{dS_{3j}}{dt} &= k_2 S_{2j} - (k_3 + k_9) S_{3j} + k_8 S_{6j} + \delta_{j,2} P_2 - \delta_{j,3} P_3 - \delta_{j,9} P_3 + \delta_{j,8} P_6 \\ \frac{dS_{4j}}{dt} &= k_3 S_{3j} + k_{10} S_{5j} - k_4 S_{4j} + \delta_{j,3} P_3 + \delta_{j,10} P_5 - \delta_{j,4} P_4 \\ \frac{dS_{5j}}{dt} &= k_4 S_{4j} - (k_5 + k_{10}) S_{5j} + \delta_{4,j} P_4 - \delta_{j,5} P_5 - \delta_{j,10} P_5 \\ \frac{dS_{6j}}{dt} &= k_9 S_{3j} + k_5 S_{5j} - (k_6 + k_8) S_{6j} + \delta_{j,9} S_3 + \delta_{j,5} S_5 - \delta_{j,6} S_6 - \delta_{j,8} S_6 \end{aligned} \quad (4.5)$$

where $\delta_{i,j}=0$ if $i = j$ or 1 if $i \neq j$. The subscript “ j ” goes from 1 to 10 for all 10 transition rates. Sensitivity analysis on biological systems is often difficult, because the differential equations involved are usually stiff. The system parameters can span several orders of magnitude. In our case, internal proton transfer occurs at ps time scale, while external proton transfer can take 0.1 ms or longer, depending on the environment [23–25]. Sensitivity equations can be solved with the master equations or separately. With the coupled direct method or simply the direct method (DM), sensitivity equations are solved along with the model equations [32, 33], while with the decoupled direct method (DDM), these equations

are solved separately [34]. The direct method provides error controls on both sensitivities and probabilities of states, which is important for the integrations of stiff differential equations like ours. Therefore, we choose to solve the sensitivity equations coupled with the master equations numerically with the direct method using an adaptive Runge-Kuta integrator. In order to compare the impact of the transition rates, instead of the sensitivity S_{ij} , we calculate the dimensionless sensitivity $S_{ij} \times \frac{k_j}{P_i}$. For the 6-state model, with the set of rates we use to simulate GFPmut2 proteins when they are folded in Section 3.3.2, that is, $k_1 = 5 \times 10^5$, $k_2 = 5 \times 10^{10}$, $k_3 = 3 \times 10^8$, $k_4 = 3 \times 10^8$, $k_5 = 10^5$, $k_6 = 2 \times 10^3$, $k_7 = 10^9$, $k_8 = 10^4$, $k_9 = 3 \times 10^8$, $k_{10} = 3 \times 10^5$, the probability of the protein being in state I^* depends most strongly on the absorption rate of the intermediate form, k_8 . The excited conformation change rate from state I^* to state A^* , k_3 , and the emission rate of the intermediate form, k_9 , are the next in line, ahead of the ground state proton transfer rate, k_6 . Among all the rates, the probability of the state A^* depends strongly on the emission rate of the anionic form, k_4 , as well as the absorption rates of the intermediate form, k_8 , and that of the anionic form, k_{10} . The rate of the ground state conformation change from state A to state I , k_5 , also plays a significant role. After these are the emission rate of the intermediate form, k_9 , and the rate of the excited state conformation change, k_3 . The probability of the protein being in state N^* is not as sensitive as the other two above toward changes in the transition rates except the excited and ground state proton transfer rates, k_2 and k_6 . In view of the importance of fluorescence intensities in, for example, *in vivo* imaging, we focus below on the influence of transition rates on the emission intensities. In the 6-state model, the green emission intensity is proportional to $P_{I^*} \frac{k_9^2}{k_3+k_9} + P_{A^*} k_4$ and the blue emission intensity is proportional to $P_{N^*} \frac{k_7^2}{k_2+k_7}$. Again, in order to compare the impacts of different transition rates, we scale the sensitivities. For the green emission intensity, we calculate the dimensionless sensitivities as

$$S_j = \frac{\partial(P_{I^*} \frac{k_9^2}{k_3+k_9} + P_{A^*} k_4)}{\partial k_j} \times \frac{k_j}{P_{I^*} \frac{k_9^2}{k_3+k_9} + P_{A^*} k_4} \quad (4.6)$$

while for the blue emission intensity the dimensionless sensitivities are

$$S_j = \frac{\partial(P_{N^*} \frac{k_7^2}{k_2+k_7})}{\partial k_j} \times \frac{k_j}{P_{N^*} \frac{k_7^2}{k_2+k_7}}. \quad (4.7)$$

With the set of rates given above, the sensitivities of the blue emission intensity to the transition rates are shown in Table 1. The blue emission intensity is most sensitive to the internal proton transfer rate k_2 and the blue emission rate k_7 . While increasing k_7 will drive the intensity up, increasing k_2 will achieve the opposite. Increasing the ground state proton transfer rate k_6 will also increase the blue emission intensity. With the same set of rates, the sensitivities of the green emission intensity are shown in Table 2. The green emission intensity is most sensitive to the absorption rates k_8 and k_{10} , and the ground state conformation change rate k_5 . Increasing these two absorption rates will both result in higher emission intensities, while increasing k_5 will reduce the green emission intensity.

Table 1 Sensitivities of the blue emission intensity for 6-state model

Rate	Sensitivities	Meaning of the rate
k_2	-2.0	The rate of the internal proton transfer
k_7	1.98	The emission rate of the neutral form GFPmut2
k_6	0.99	The rate of the ground state proton transfer
k_5	5.6×10^{-2}	The rate of the ground state conformation change
k_8	-4.7×10^{-2}	The absorption rate of the intermediate form GFPmut2
k_3	-2.8×10^{-2}	The rate of the excited state conformation change
k_9	2.8×10^{-2}	The emission rate of the intermediate form GFPmut2
k_1	3.8×10^{-3}	The absorption rate of the neutral form GFPmut2
k_4	7.5×10^{-5}	The emission rate of the anionic form GFPmut2
k_{10}	-5.6×10^{-5}	The absorption rate of the anionic form GFPmut2

5 Photo-switching properties of a Dronpa-like molecule

Reversibly switchable fluorescent proteins (RSFPs) can be repeatedly photoswitched between a fluorescent and a nonfluorescent state by irradiation with light of two different wavelengths. One such protein is Dronpa, a GFP-like photoswitchable protein that can be turned on with irradiation around 400 nm and switched off with light around 490 nm [14]. Furthermore, Dronpa is monomeric, can be switched on and off repeatedly many times and its off-state is thermally stable [14]. In the nonfluorescent off-state Dronpa is protonated, thus in a neutral state, while in the fluorescent on-state it is deprotonated, thus in an anionic state [35]. Some models have been proposed for the switching scheme [35, 36]. A simplified schematic version [36] is shown in Fig. 13, where N stands for the protonated form resulting from photoswitching, I is a nonfluorescent intermediate form, A is the deprotonated form and Z is a dark state. The transition from the excited intermediate form to the excited anionic form is supposed to have a very low efficiency, and the ground state transition between these two forms is slow as well. The ground state transitions between the protonated form and intermediate form should be pH dependent. In both single-color excitation at 488 nm ($A \rightarrow A^*$) and dual-color excitation at 488 nm and 405 nm ($N \rightarrow N^*$) experiments,

Table 2 Sensitivities of the green emission intensity for 6-state model

Rate	Sensitivities	Meaning of the rate
k_8	0.79	The absorption rate of the intermediate form GFPmut2
k_{10}	0.67	The absorption rate of the anionic form GFPmut2
k_5	-0.61	The rate of the ground state conformation change
k_3	0.31	The rate of the excited state conformation change
k_9	-0.31	The emission rate of the intermediate form GFPmut2
k_6	0.15	The rate of the ground state proton transfer
k_1	3.8×10^{-3}	The absorption rate of the neutral form GFPmut2
k_2	7.5×10^{-5}	The rate of the internal proton transfer
k_4	7.5×10^{-5}	The emission rate of anionic form GFPmut2
k_7	-7.5×10^{-5}	The emission rate of the neutral form GFPmut2

Fig. 13 A photoswitching state diagram for Dronpa

the off-time distribution of the green fluorescence is measured to be an exponential distribution with a time constant around 65 ms. A short off-time with a Gaussian distribution is also observed in both experiments. In the dual-color experiments, the average value of the short off-time is around 1.3 ms, and it is around 1.2 ms in the single-color one [36]. As a second example, we apply the two stochastic methods to study the switching behavior of a Dronpa-like molecule based on the energy-level diagram outlined in Fig. 13. This is not a real Dronpa system, for which the cis-trans isomerization seems to play a role [35] and other energy schemes have been proposed, which will not be explored here. First, we carry out the stochastic simulations according to Gillespie's method [15, 16] and the rates are chosen so as to make comparison with experimental results possible [36]. The fluorescence on-time at 518 nm depends on the absorption rate, k_{10} , emission rate, k_4 , and switching-off rates of A^* , k_{11} and k_{12} . Faster absorption rates lead to shorter on-time, as do faster switching-off rates. How the emission rate k_4 affects the on-time distribution depends on its relative value to the switching rates, k_{11} and k_{12} . When only the anionic form is excited, i.e., $k_1 = 0$, off-time might correspond to the time it takes to finish the transition $A^* \rightarrow N \rightarrow I \rightarrow A$ or $A^* \rightarrow Z \rightarrow A$. As the three plots on the left of Fig. 14 show, we have found a set of transition rates ($k_1 = 0$, $k_2 = 3 \times 10^{11}$, $k_3 = 9 \times 10^{-2}$, $k_4 = 3 \times 10^8$, $k_{10} = 1.1 \times 10^5$, $k_{-6} = 9.1 \times 10^{-2}$, $k_7 = 5 \times 10^{11}$, $k_{-5} = 15$, $k_9 = 3 \times 10^8$, $k_{11} = 5.5 \times 10^4$, $k_{12} = 3 \times 10^4$, $k_{13} = 15$) which yields simulation results in reasonable agreement with experimental data. The top and middle panels show the off-time distributions at different time scales. The off-time distribution shown in the top panel can be fit with a single exponential function with a decay time of about 73 ms. The larger time scale plot shown in the middle panel reveals a multi-exponential decay which involves both short and long off-times. The on-time distribution of the bottom panel can be fit with a single exponential function with a decay time of about 38 ms. These decay times are fairly close to the experimental values of 65 ms and 38 ms. As was done by Habuchi et al. [36] we use the auto-correlation method to obtain an even shorter off-time, around 1.4 ms. This shorter off-time was also mentioned in the experimental results. The three plots on the right show the results from the double excitation simulation where both the anionic and neutral forms are excited and k_1 is set to be 1.85×10^4 . In this case, the decay time for the short off-time distribution is about 69 ms and that for the on-time distribution is around 37 ms, while they are 65 ms and 52 ms, respectively, in the experiment. The shortest off-time we get from the auto-correlation method is around 1.3 ms in this case. The long off-times on the scale of seconds are almost non-existent in this case, because the absorption rate of the neutral form is much faster than the transition rate from the ground

Fig. 14 Simulation results for a Dronpa-like molecule, when it is singly and doubly photo-excited. **a–c** are for the case when only the anionic form is excited. Transition rates are set at: $k_1 = 0$, $k_2 = 3 \times 10^{11}$, $k_3 = 9 \times 10^{-2}$, $k_4 = 3 \times 10^8$, $k_{10} = 1.1 \times 10^5$, $k_{-6} = 9.1 \times 10^{-2}$, $k_7 = 5 \times 10^{11}$, $k_{-5} = 15$, $k_9 = 3 \times 10^8$, $k_{11} = 5.5 \times 10^4$, $k_{12} = 3 \times 10^4$, $k_{13} = 15$. **a** the off-time distribution with a bin size of 30 ms, **b** the off-time distribution with a bin size of 5 s, **c** the on-time distribution. **d–f** are for the case when both the neutral and anionic forms are excited. k_1 is changed to 1.85×10^4 . **d** the off-time distribution with a bin size of 30 ms, **e** the off-time distribution with a bin size of 5 s, **f** the on-time distribution

state N to I, which makes the probability of the transition $N \rightarrow I \rightarrow A$ very low. Our simulations yield the on-time and off-time distributions which agree reasonably well with experiments, but the fluorescence intensities are higher than those in the experiment by a factor of 2 to 8 depending on the time resolution with the chosen rates. One contributing factor is the fluorescence quantum yield which is given experimentally at about 85%, but it is close to 100% with the chosen rates. Analytically, the probability of the short off-time, t_{so} , shorter than t is given by

$$P(t_{so} < t) = P(T_{k_{12}} < T_{k_4})P(T_{k_{12}} + T_{k_{13}} < t), \quad (5.1)$$

therefore the distribution of the short off-time becomes

$$\begin{aligned} f_{t_{so}}(t) &= \int_0^t dt_1 e^{-k_4 t_1} k_{12} e^{-k_{12} t_1} k_{13} e^{-k_{13}(t-t_1)} \\ &= \frac{k_{12} k_{13} (e^{-(k_4+k_{12})t} - e^{-k_{13}t})}{-(k_4 + k_{12}) + k_{13}}. \end{aligned} \quad (5.2)$$

With our chosen rates, (5.2) yields

$$f_{t_{so}}(t) = -1.5 \times 10^{-3} \times (e^{-\frac{t}{3.3 \times 10^{-9}}} - e^{-\frac{t}{6.7 \times 10^{-2}}}) \quad (5.3)$$

which gives a dominating decay time of about 67 ms. Similarly, the probability of the long off-time, t_{lo} , shorter than t is given by

$$P(t_{lo} < t) = P(T_{k_{11}} < T_{k_4})P(T_{k_{11}} + T_{k_{-6}} + T_{k_{-5}} < t) \quad (5.4)$$

and the distribution of t_{lo} becomes

$$\begin{aligned} f_{t_{lo}}(t) &= \int_0^t \int_0^{t_1} dt_1 dt_2 e^{-k_4 t_2} k_{11} e^{-k_{11} t_2} k_{-6} e^{-k_{-6}(t_1-t_2)} k_{-5} e^{-k_{-5}(t-t_1)} \\ &= \frac{k_{11} k_{-6} k_{-5}}{k_{-6} - (k_4 + k_{11})} \left(\frac{e^{-(k_4+k_{11})t} - e^{-k_{-5}t}}{-(k_4 + k_{11}) + k_{-5}} - \frac{e^{-k_{-6}t} - e^{-k_{-5}t}}{-k_{-6} + k_{-5}} \right). \end{aligned} \quad (5.5)$$

With our rates,

$$f_{t_{lo}}(t) = 8.3 \times 10^{-13} \times e^{-\frac{t}{3.3 \times 10^{-9}}} - 1.7 \times 10^{-5} \times e^{-\frac{t}{6.7 \times 10^{-2}}} + 1.7 \times 10^{-5} \times e^{-\frac{t}{11}}. \quad (5.6)$$

It shows a dominating decay time of about 11 s, which agrees with the experimental result.

6 Discussion

The main purpose of the article is to introduce stochastic methods to study single-molecular processes. The two methods that we have used are the method of off-time distribution and Gillespie's stochastic method for chemical kinetics. The former method is mainly an analytic method based on Laplace transforms, adapted from the method of waiting-time distributions of Kou et al. [17]. These methods are applied to study optical transitions of two systems: GFPmut2 and a Dronpa-like protein. For both systems the results of calculations and simulations show certain degree of success, when compared to experimental data.

Therefore, stochastic methods are indeed useful for the study of single molecule processes. We give some more comments on the results of GFPmut2 below. As we mentioned earlier, because of the nature of the stochastic treatment the time series obtained in simulations representing blue and green emissions will not be periodic as observed in the experiment [11]. Therefore we focus more on the off-time distribution and its moments. The first moment is the average value (approximated by its peak value), the second moment is its width and the third moment is its skewness. By adjusting the transition rates, we can get the peak value and the width close to the experimental values, but the shape of the distributions tilts towards the long-time side.

The long-time tail of the off-time distribution may be partly due to the fact that proteins have reached the unfolded stage, which does not fluoresce, close to the end of the experiment. As shown in the experiment [11, 12], when GFPmut2 is unfolding, blue emission intensity is higher than that in the native state and comparable to the intensity of green emissions. This is most possibly due to the excited-state proton transfer being slowed down from a rate several orders of magnitude faster than that of the green emission to a rate much closer to it. However, that alone is not enough to cause the off-time to increase as much as observed in the experiments [11, 37]. In reality, the observed oscillatory transformations between N and A forms are transient behaviors. It is possible that the kinetic processes involving denaturants may play a role as well. There are certainly limitations of a treatment like ours where constant transition rates are assumed. This may partly explain the long-tail deviation. One possible remedy is to use time-dependent rates, which can be explored in a future study. We next come back to the problem that the stochastic simulations based on the models that we have used do not yield highly oscillatory behavior. We can actually turn the problem around and ask the following question: accepting the experimental fact that pre-unfolding oscillatory behavior exists and the fact that stochastic dynamics is the proper way to study single-molecular processes, what would the energy scheme (Fig. 9) suggest for a highly oscillatory behavior [2, 18, 38] consistent with the experiments? There are two possibilities: 1) $k_1 \rightarrow k_2 \rightarrow k_3 \rightarrow k_4 \rightarrow k_5 \rightarrow k_6 \rightarrow k_1$. 2) $k_1 \rightarrow k_2 \rightarrow k_9 \rightarrow k_6 \rightarrow k_1$. For these two pure cyclic trajectories the cycle time is about 1 ms so it is in the right order of magnitude. Therefore the question now becomes, right before GFPmut2 unfolds in the presence of denaturants, what factors contribute to switch off the branching possibilities, that is, making k_7 and k_{10} vanishingly small in case (1) or k_7 and k_8 vanishingly small in case (2). This is now more a question concerning the experimental conditions and the effects of local environments on the single molecules and a question perhaps experimentalists can answer better.

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Appendix: The off-time distribution for the 4-state and 6-state models

For the 6-state model, the probability that it takes less time than t to reach the next green emission from state N is:

$$\begin{aligned}
 P(T_N < t) = & P(T_{k_2} < T_{k_7})P(T_{k_9} < T_{k_3})P(T_{k_1} + T_{k_2} + T_{k_9} < t) \\
 & + P(T_{k_2} < T_{k_7})P(T_3 < T_{k_9})P(T_{k_1} + T_{k_2} + T_{k_3} + T_{k_4} < t) \\
 & + P(T_{k_7} < T_{k_2})P(T_{k_1} + T_{k_7} + T_N < t).
 \end{aligned} \tag{A.1}$$

The probability distribution of T_N can be shown to be

$$\begin{aligned}
 f_{T_N}(t) &= \frac{dP(T_N < t)}{dt} \\
 &= \int_0^t \int_0^{t_1} dt_1 dt_2 e^{-k_7(t_1-t_2)} e^{-k_3(t-t_1)} k_1 e^{-k_1 t_2} k_2 e^{-k_2(t_1-t_2)} k_9 e^{-k_9(t-t_1)} \\
 &\quad + \int_0^t \int_0^{t_1} \int_0^{t_2} dt_1 dt_2 dt_3 e^{-k_7(t_2-t_3)} e^{-k_9(t_1-t_2)} k_1 e^{-k_1 t_3} k_2 e^{-k_2(t_2-t_3)} k_3 e^{-k_3(t_1-t_2)} \\
 &\quad \times k_4 e^{-k_4(t-t_1)} \\
 &\quad + \int_0^t \int_0^{t_1} dt_1 dt_2 e^{-k_2(t_1-t_2)} k_1 e^{-k_1 t_2} k_7 e^{-k_7(t_1-t_2)} f_{T_N}(t-t_1) \\
 &= \frac{k_1 k_2 k_9}{-k_1 + k_2 + k_7} \left(\frac{e^{-k_1 t} - e^{-k_3 t - k_9 t}}{-k_1 + k_3 + k_9} - \frac{e^{-k_2 t_1 - k_7 t_1} - e^{-k_3 t - k_9 t}}{-k_2 - k_7 + k_3 + k_9} \right) + \frac{k_1 k_2 k_3 k_4}{k_7 - k_1 + k_2} \\
 &\quad \times \left(\frac{\frac{e^{-k_1 t} - e^{-k_4 t}}{k_4 - k_1} - \frac{e^{-k_9 t - k_3 t} - e^{-k_4 t}}{-k_9 - k_3 + k_4}}{k_9 - k_1 + k_3} - \frac{\frac{e^{-k_7 t - k_2 t} - e^{-k_4 t}}{k_4 - k_7 - k_2} - \frac{e^{-k_9 t_1 - k_3 t_1} - e^{-k_4 t}}{-k_9 - k_3 + k_4}}{k_9 - k_7 - k_2 + k_3} \right) \\
 &\quad + \frac{k_1 k_7}{k_2 - k_1 + k_7} \int_0^t dt_1 (e^{-k_1 t_1} - e^{-k_2 t_1 - k_7 t_1}) f_{T_N}(t-t_1). \tag{A.2}
 \end{aligned}$$

After applying the rule for the Laplace transform of a convolution, (A.2) yields

$$\begin{aligned}
 \tilde{f}_{T_N}(s) &= \frac{k_1 k_2 k_9}{-k_1 + k_2 + k_7} \left(\frac{\frac{1}{s+k_1} - \frac{1}{s+k_3+k_9}}{-k_1 + k_3 + k_9} - \frac{\frac{1}{s+k_2+k_7} - \frac{1}{s+k_3+k_9}}{-k_2 - k_7 + k_3 + k_9} \right) \\
 &\quad + \frac{k_1 k_2 k_3 k_4}{k_7 - k_1 + k_2} \left(\frac{\frac{\frac{1}{s+k_1} - \frac{1}{s+k_4}}{k_4 - k_1} - \frac{\frac{1}{s+k_3+k_9} - \frac{1}{s+k_4}}{-k_9 - k_3 + k_4}}{k_9 - k_1 + k_3} - \frac{\frac{\frac{1}{s+k_2+k_7} - \frac{1}{s+k_4}}{k_4 - k_7 - k_2} - \frac{\frac{1}{s+k_3+k_9} - \frac{1}{s+k_4}}{-k_9 - k_3 + k_4}}{k_9 - k_7 - k_2 + k_3} \right) \\
 &\quad + \frac{k_1 k_7}{k_2 - k_1 + k_7} \left(\frac{1}{s+k_1} - \frac{1}{s+k_2+k_7} \right) \tilde{f}_{T_N}(s). \tag{A.3}
 \end{aligned}$$

Therefore,

$$\tilde{f}_{T_N}(s) = \frac{(k_9 s + k_4 k_9 + k_3 k_4) k_1 k_2}{(s^2 + s k_2 + s k_7 + s k_1 + k_1 k_2)(s + k_4)(s + k_3 + k_9)}. \tag{A.4}$$

The probability of off-time less than t is

$$\begin{aligned}
 P(t_{off} < t) &= \frac{P_{I*} \frac{k_9^2}{k_9 + k_3}}{P_{I*} \frac{k_9^2}{k_9 + k_3} + P_{A*} k_4} P(T_{k_7} < T_{k_2}) P(T_{k_6} < T_{k_8}) P(T_{k_6} + T_{k_1} + T_{k_7} + T_N < t) \\
 &\quad + \frac{P_{A*} k_4}{P_{I*} \frac{k_9^2}{k_9 + k_3} + P_{A*} k_4} P(T_{k_5} < T_{k_{10}}) P(T_{k_6} < T_{k_8}) \\
 &\quad \times P(T_{k_5} + T_{k_6} + T_{k_1} + T_{k_7} + T_N < t) \tag{A.5}
 \end{aligned}$$

where P_{I^*} , P_{A^*} are the probabilities of the protein being in states I^* and A^* , and T_{k_i} is the time it takes to complete the transition with rate k_i . The distribution of the off-time can be shown as:

$$\begin{aligned}
 f_{t_{off}}(t) &= \frac{dP(t_{off}(t) < t)}{dt} \\
 &= \frac{P_{I^*} \frac{k_9^2}{k_9 + k_3}}{P_{I^*} \frac{k_9^2}{k_9 + k_3} + P_{A^*} k_4} \\
 &\quad \times \int_0^t \int_0^{t_1} \int_0^{t_2} dt_1 dt_2 dt_3 e^{-k_2(t_1 - t_2)} e^{-k_8 t_3} k_6 e^{-k_6 t_3} k_1 e^{-k_1(t_2 - t_3)} k_7 e^{-k_7(t_1 - t_2)} \\
 &\quad \times f_{T_N}(t - t_1) \\
 &\quad + \frac{P_{A^*} k_4}{P_{I^*} \frac{k_9^2}{k_9 + k_3} + P_{A^*} k_4} \int_0^t \int_0^{t_1} \int_0^{t_2} \int_0^{t_3} dt_1 dt_2 dt_3 dt_4 e^{-k_{10} t_4} e^{-k_8(t_3 - t_4)} e^{-k_2(t_1 - t_2)} \\
 &\quad \times k_5 e^{-k_5 t_4} k_6 e^{-k_6(t_3 - t_4)} k_1 e^{-k_1(t_2 - t_3)} k_7 e^{-k_7(t_1 - t_2)} f_{T_N}(t - t_1). \quad (A.6)
 \end{aligned}$$

We rewrite (A.6) as:

$$f_{t_{off}}(t) = \frac{P_{I^*} \frac{k_9^2}{k_9 + k_3}}{P_{I^*} \frac{k_9^2}{k_9 + k_3} + P_{A^*} k_4} k_1 k_6 k_7 I_1 + \frac{P_{A^*} k_4}{P_{I^*} \frac{k_9^2}{k_9 + k_3} + P_{A^*} k_4} k_5 k_6 k_1 k_7 I_2 \quad (A.7)$$

where

$$\begin{aligned}
 I_1 &= \int_0^t \int_0^{t_1} \int_0^{t_2} dt_1 dt_2 dt_3 e^{-k_2(t_1 - t_2)} e^{-k_8 t_3} e^{-k_6 t_3} e^{-k_1(t_2 - t_3)} e^{-k_7(t_1 - t_2)} f_{T_N}(t - t_1) \\
 &= \int_0^t dt_1 \frac{\frac{e^{-k_6 t_1 - k_8 t_1} - e^{-k_2 t_1 - k_7 t_1}}{-k_6 - k_8 + k_2 + k_7} - \frac{e^{-k_1 t_1} - e^{-k_2 t_1 - k_7 t_1}}{k_2 + k_7 - k_1}}{k_1 - k_6 - k_8} f_{T_N}(t - t_1) \quad (A.8)
 \end{aligned}$$

and

$$\begin{aligned}
 I_2 &= \int_0^t \int_0^{t_1} \int_0^{t_2} \int_0^{t_3} dt_1 dt_2 dt_3 dt_4 e^{-k_{10} t_4} e^{-k_8(t_3 - t_4)} e^{-k_2(t_1 - t_2)} \\
 &\quad \times e^{-k_5 t_4} e^{-k_6(t_3 - t_4)} e^{-k_1(t_2 - t_3)} e^{-k_7(t_1 - t_2)} f_{T_N}(t - t_1) \\
 &\quad \times \int_0^t dt_1 \frac{\frac{e^{-k_{10} t_1 - k_5 t_1} - e^{-k_2 t_1 - k_7 t_1}}{-k_{10} - k_5 + k_2 + k_7} - \frac{e^{-k_1 t_1} - e^{-k_2 t_1 - k_7 t_1}}{k_2 + k_7 - k_1}}{(-k_{10} - k_5 + k_6 + k_8)(-k_{10} - k_5 + k_1)} f_{T_N} \\
 &\quad + \int_0^t dt_1 \frac{\frac{e^{-k_6 t_1 - k_8 t_1} - e^{-k_2 t_1 - k_7 t_1}}{-k_6 - k_8 + k_2 + k_7} - \frac{e^{-k_1 t_1} - e^{-k_2 t_1 - k_7 t_1}}{k_2 + k_7 - k_1}}{(-k_{10} - k_5 + k_6 + k_8)(-k_6 - k_8 + k_1)} f_{T_N}(t - t_1). \quad (A.9)
 \end{aligned}$$

The Laplace transform of the off-time distribution yields:

$$\begin{aligned} \tilde{f}_{t_{off}}(s) = & \frac{(sk_9^2 + k_5k_9^2 + k_9^2k_{10} + k_3k_5k_9 + k_3^2k_{10} + k_3^2k_5 + k_3k_9k_{10})(sk_9 + k_4k_9 + k_3k_4)}{(s + k_4)(s + k_3 + k_9)(s^2 + k_2s + k_7s + k_1s + k_1k_2)(s + k_6 + k_8)(s + k_1)} \\ & \times \frac{1}{(s + k_5 + k_{10})(s + k_2 + k_7)} \\ & \times \frac{k_1^2k_2k_5k_6k_7}{k_3k_5k_9 + k_3^2k_5 + k_3k_9k_{10} + k_3^2k_{10} + k_5k_9^2}. \end{aligned} \quad (\text{A.10})$$

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