Supporting information for Rothwell *et al*. (2003) *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.0434003100.

Supporting Text

Theory. We describe anisotropy decay parameters within the framework of the "wobbling in a cone" model. For each color, at least two specific rotational correlation times, ρ_1 and ρ_2, can be derived from a refined time-resolved sub-ensemble analysis which displays the anisotropy decay for all the bursts collectively for each of the sub-states P-E and P-P separately.

$$r(t) = r_0(1 - A_\infty)e^{-t\left(\frac{1}{\rho_1}+\frac{1}{\rho_2}\right)} + r_0 A_\infty e^{-\frac{t}{\rho_2}} \qquad \textbf{[8]}$$

Considering rhodamine dyes with a transition dipole moment perpendicular to the axis of rotation, the smaller of the rotational correlation times, ρ_1, describes the local motion of the dye linker arm outside the cone, whereas the larger rotational correlation time, ρ_2, describes the overall tumbling motion of the protein. The cone angle, $\theta_{\min}$, defining the angle of cone not accessible to the wobbling motion of dye, is given by the amplitude $A_\infty = \left[\frac{1}{2}(1-\cos^2(\theta_{\min}))\right]^2$ and is used for subsequent structure determination (1).

In the case that the protein tumbling correlation time, ρ_2, is much longer than the duration of our lifetime measurements ($\rho_2 >> t$), the decay caused by the tumbling of the protein will be too slow to be observed. The two-component decay will be observed as a single-component decay with an offset as given by

$$r(t) = r_0(1 - A_\infty)e^{-\frac{t}{\rho_1}} + r_0 A_\infty . \qquad \textbf{[9]}$$

Results. One set of raw experimental data, corresponding to the RT:Cy5-(5'-dp/dt) sample as described by Fig. 2*A* is shown in Fig. 5. The data has been restricted to bursts with a minimum of 120 photons (combined red and green) and a duration of ≥ 2.5 ms. These criteria remove most of the low intensity bursts (<1 kHz). Otherwise,

there has been no correction or adjustment of the data. Fig. 5*A* displays the total signal intensities, S_G and S_R, in the green and red channels, respectively, as the molecules pass through the detection volume. Signal intensities for the high FRET species were typically 20–30 kHz for both the red and green dyes. The low FRET species showed a green signal of typically 40–50 kHz and a red signal just above the background at about 2 kHz. Fig. 5*B* shows the ratio of the uncorrected signal intensities, S_G/S_R, as a function of the (uncorrected) fitted lifetimes as calculated by the maximum-likelihood estimator. Finally, Fig. 5*C* shows the fluorescence intensity ratio, F_D/F_A, which includes background and detection efficiency corrections, versus the "species weighted" fluorescence lifetime.

Fluorescence lifetime correction. Subensemble measurements of Alexa-488 labeled RT showed that the fluorescence decay of Alexa-488 is shortened and is not single exponential. To correct the data for this effect, we make the following analysis:

For a molecule with two states, characterized by two different lifetimes, τ_1 and τ_2, and switching rapidly between the two states, the overall average lifetime of the molecule will simply be an average of its component lifetimes, weighted by their relative amounts in solution.

$$\bar{\tau}_X = X_1\tau_1 + X_2\tau_2 \qquad \textbf{[10]}$$

The coefficients X_1 and X_2 are the fraction of the molecules in states 1 and 2, respectively, and must sum to unity. However, the maximum-likelihood estimator produces lifetimes which are not "species weighted" with X_1 and X_2, but rather "fluorescence weighted" with $A_1 = X_1\tau_1/\tau_X$ and $A_2 = X_2\tau_2/\tau_X$. The factor τ_n/τ_X describes the effect the different quantum yields will have on the number of photons produced by each species.

$$\bar{\tau}_A = X_1\frac{\tau_1}{X_1\tau_1 + X_2\tau_2}\tau_1 + X_2\frac{\tau_2}{X_1\tau_1 + X_2\tau_2}\tau_2 \qquad \textbf{[11]}$$

Converting the observed $\bar{\tau}_A$ into the desired species-weighted $\bar{\tau}_X$ is accomplished as follows: from the "donor-only" sample, we can fit a double exponential to the sub-ensemble data and determine absolute values in the absence of FRET of X_1, τ_1, X_2, and τ_2 (τ_{D1}= 4.27 ns, X_{D1}= 0.56 and τ_{D2}= 1.11 ns, X_{D2}= 0.44). Next, the effect of a FRET for a set of efficiencies given by $k_{FRET,n}$ is simulated for $\bar{\tau}_X$ and $\bar{\tau}_A$ by using the equations:

$$\bar{\tau}_{X,n} = X_1\left(\frac{1}{\frac{1}{\tau_{D(0),1}} + k_{FRET,n}}\right) + X_2\left(\frac{1}{\frac{1}{\tau_{D(0),2}} + k_{FRET,n}}\right)$$

$$\bar{\tau}_{A,n} = \frac{X_1\left(\frac{1}{\frac{1}{\tau_{D(0),1}} + k_{FRET,n}}\right)^2 + X_2\left(\frac{1}{\frac{1}{\tau_{D(0),2}} + k_{FRET,n}}\right)^2}{X_1\left(\frac{1}{\frac{1}{\tau_{D(0),1}} + k_{FRET,n}}\right) + X_2\left(\frac{1}{\frac{1}{\tau_{D(0),2}} + k_{FRET,n}}\right)} \qquad \textbf{[12]}$$

Finally, for each FRET efficiency, the corresponding $(\bar{\tau}_{A,n}, \bar{\tau}_{X,n})$ pairs are graphed and fitted to a 2nd order polynomial, which is subsequently used to adjust the observed fluorescence-weighted lifetimes into the desired species-weighted lifetimes. For these experiments, the polynomial was

$$\bar{\tau}_X = -0.533\,\bar{\tau}_A^2 + 0.9583\,\bar{\tau}_A + 0.0139 \qquad \textbf{[13]}$$

Anisotropy analysis. ***Acceptor decay.*** Fig. 6 displays the calculated anisotropy decays for the donor dyes (green decays) and acceptor dyes (red decays) in each of the two states P-P and P-E. The acceptor anisotropy decays quickly to zero in both cases, indicating an isotropically mobile acceptor with small rotational correlation times.

Donor decay. The donor anisotropy indicates a confined dye as described by the wobbling in a cone model. The P-P state exhibits a two component decay (ρ_{D1}, ρ_{D2}) as

described by Eqs. **8** and **9**. In contrast, the P-E exhibits at least three rotational correlation times (ρ_{D0}, ρ_{D1}, ρ_{D2}). The smallest rotational correlation time, ρ_{D0}, is too fast to be recorded, reducing the anisotropy from its initial value of 0.4 to the first observed value of 0.2. The remaining two correlation times, ρ_{D1} and ρ_{D2}, describe the motion of the donor within the context of Eqs. **8** and **9**.

κ^2 effects. The value of 2/3 used for κ^2 is based on the assumption that both the donor and the acceptor are isotropically mobile with rotational correlation times lower than the lifetime of the dye. Based on Fig. 6, we claim that at least one of the dyes, the acceptor, meets this condition. If we now assume the worst possible scenario for the donor dye, i.e., that it is restricted to purely linear motion, then κ^2 may assume any value between 1/3 and 4/3 (2). From Eq. **4** in the body of the text, these values for κ^2 can introduce a maximum error of ±11% into the calculation of R_{DA}. Our measurements show an R_{DA} of 46 Å for the P-P state and a ΔR_{DA} of 5 Å between the P-E and P-P states, a difference of >11%. Even though the estimated distance change between species Ia and species Ib is of similar magnitude as the maximum possible error, we conclude that our analysis regarding the positions of the p/t in the P-E and P-P states are valid and cannot be artifacts caused by changes in κ^2, because Fig. 6 shows that the donor dye is also mobile, a fact not included in the error estimation, and the resulting κ^2 error must be <11%.

1. Kinosita, K., Jr., Kawato, S. & Ikegami, A. (1977) *Biophys. J.* **20**, 289–305.

2. van der Meer, B. W., Cooker, G. & Chen, S. Y. (1994) *Resonance Energy Transfer: Theory and Data* (VCH, New York).