Supporting Text

Global and target analysis procedures

In global analysis, a model consisting of independent exponential decays is fitted to the data $\psi(\lambda,t)$

$$\psi(\lambda,t) = \sum_{i=1}^{n_{comp}} c_i(t) DADS_i(\lambda)$$

where n_{comp} is the number of components, $c_i(t)$ corresponds to the convolution of the *i*-th exponential decay with the instrument response function (IRF), and $DADS_i(\lambda)$ is the *i*-th decay-associated difference spectrum (DADS).

The four DADS estimated from pump-probe data of GFPuv in H_2O are shown in Fig. 6. The estimated lifetimes are 2.2 ps (black), 11 ps (red), 400 ps (green), and 3 ns (blue). The DADS with lifetimes of 2.2 and 11 ps both represent the transition $A^* \rightarrow I^*$ and their shapes are practically identical. The absence of spectral differences between the 2.2 and 11 ps components indicates that the biexponentiality in the excited state of GFP is intrinsic, and does not correspond to an evolution from one molecular species to another. The emission from I* (whose difference spectrum resembles the blue DADS) decays in 3 ns. It rises biexponentially from A*, as is evident from the black and red DADS above 510 nm. Finally, the green DADS, which decays in 400 ps, will later be shown to represent the inverse of the I difference spectrum.

In target analysis (1) a compartmental scheme is used to describe the concentrations of the compartments (states). Transitions to and from compartments are described by microscopic rate constants. The model that is fitted to the data now reads

$$\psi(\lambda,t) = \sum_{i=1}^{n_{comp}} c_i(t) SADS_i(\lambda)$$

where $c_i(t)$ now corresponds to the concentration of the *i*-th compartment. $SADS_i(\lambda)$ is the *i*-th species-associated difference spectrum. The concentrations of all compartments are collated in a vector

$$c(t) = \begin{bmatrix} c_1(t) & c_2(t) & c_3(t) & c_4(t) & c_5(t) & c_6(t) \end{bmatrix}^T = \begin{bmatrix} A_1^*(t) & A_2^*(t) & I^*(t) & I_1^{FC}(t) & I_1(t) & I_2(t) \end{bmatrix}^T$$

which obeys the differential equation

$$\frac{d}{dt} c(t) = Kc(t) + j(t)$$

where the transfer matrix K contains off-diagonal elements k_{pq}, representing the microscopic rate constant from compartment *q* to compartment *p*. The diagonal elements contain the total decay rates of each compartment. The input to the compartments is

$j(t) = IRF(t)[x_1 \quad x_2 \quad 0 \quad 0 \quad 0 \quad 0]^T$. Here, x_1 and x_2 are the input fractions of the biexponentially decaying A*. With the dump pulse, the differential equation reads

$$\frac{d}{dt}c(t) = Kc(t) + j(t) + j_{dump}(t)$$

where $j_{dump}(t) = IRF(t - t_{dump})[0 \quad 0 \quad -x_3 \quad x_3 \quad 0 \quad 0]^T$. Here, x_3 represents the dump fraction from I* to I_1^{FC}. The K matrix reads:

$$K = \begin{bmatrix} -k_1 & & & & & \\ & -k_2 & & & & \\ k_1 & k_2 & -k_3 & & & \\ & & k_3 & -k_4 & & \\ & & & k_4 & -k_5 & \\ & & & & k_5 & -k_6 \end{bmatrix}$$

The target model assumes that the SADS of A_1^* and A_2^* are identical, and that the SADS of I_1^{FC}, I_1 and I_2 are zero above 530, 540, and 540 nm, respectively. Imposing these constraints hardly affects the rms error of the fit. The estimated SADS are depicted (with solid lines) in Fig. 9. Note that in this target analysis, 268 time-gated spectra of 240 wavelengths (64320 datapoints) are fitted with only 13 nonlinear parameters [six decay rates, four parameters for IRF location, width, and dispersion, one parameter for the relative fractions x_1 and x_2; two parameters for the dump (magnitude and delay)] as well as five SADS. The rms error of the fit was 0.53 x 10^{-3} units of absorbance, which means that with a maximum signal of 62 x 10^{-3} units of absorbance the signal-to-noise ratio is > 100.

The target analysis includes an intermediate between I* and I_1, termed I_1^{FC}, which was not shown in Fig. 5. Fig. 9 shows the SADS of all species involved including I_1^{FC}, which is indicated with the magenta curve. I_1^{FC} corresponds to a ground-state species in which the nuclear coordinates of the chromophore and its protein environment have the equilibrium positions associated with the excited state I*. Thus, I_1^{FC} corresponds to the region of the ground-state energy-potential surface that shows Franck-Condon overlap with the I* potential-energy surface. It exhibited an absorption maximum at 508 nm and had a lifetime of 100 fs. As expected, its absorption maximum is very similar to the maximum of the GFP emission. The lifetime of I_1^{FC}, 100 fs, can be assigned to rapid nuclear rearrangement to accommodate the newly formed ground state, resulting in the formation of I_1. Extensive time-resolved and molecular dynamics studies on liquids and proteins have shown that on this timescale, only small-amplitude motions of the surrounding protein nuclei take place on (de)excitation of a chromophore, whereby the overall local structure is preserved (2-4). Thus, I_1^{FC} and I_1 are very similar molecular species and cannot be regarded as separate photocycle intermediates. For this reason I_1^{FC} was not discussed in the main text.

Raw multi-pulse transient absorption data

To illustrate that our kinetic modeling is adequate and show the correspondence between the results of the target analysis procedure and the actual measured spectra, raw data at selected points in time are shown below.

Fig. 8*A* shows raw pump-dump-probe spectra of GFP in H_2O at times shortly after the dump at 20 ps. It can be observed that after the dump pulse, the spectra in the region around 480-490 nm shift to the blue on a picosecond timescale (inset, *upper*). At the same time, the stimulated emission feature from 505 to 520 nm shifts to the blue as well, along with an increase of the stimulated emission signal (inset, *lower*). These signals are dump-induced and can be only explained by assuming a spectrodynamic process in the ground state species I. In particular, these signals are consistent with a dynamic blue shift of the I species, which absorbs in the region 480-520 nm, on the picosecond timescale. The apparent increase of stimulated emission in the 500 to 530-nm region follows from a decrease of induced absorption of I in that region. Hence our introduction of two distinct species of I, namely I_1 and I_2 in our kinetic modeling, of which I_2 is formed from I_1 on the picosecond timescale and is blue-shifted with respect to it.

Fig. 8*B* shows the corresponding spectra for GFP dissolved in D_2O. The evolution of the pump-dump probe spectra after the dump is similar to that in H_2O, with a progressive blue shift of the induced absorption around 490 nm and a blue shift and apparent increase of the stimulated emission around 510 nm. However, the dynamic blue shift proceeds slower in GFP in D_2O compared with H_2O: The spectra in H_2O taken at 20 and 35 ps (i.e. 10 and 15 ps after the dump) are almost overlapping, whereas in the spectra in D_2O taken at 110 and 125 ps (i.e. 10 and 25 ps after the dump), the blue shifting is still proceeding. Thus, the dynamic blue shift of I_1 to I_2 is subject to a kinetic isotope effect. The target analysis procedure estimated a time constant of 3 ps for the blue shift in H_2O, whereas a time constant of 7 ps was found for D_2O, leading to a kinetic isotope effect of about 2 (see table 1).

Fig. 7 shows pump-probe and pump-dump-probe spectra of GFP dissolved in D_2O on a longer time scale. Fig. 7 is the equivalent of Fig. 4, and the spectra correspond to the kinetic traces shown in Fig. 3 *D* - *F*. At a delay of 5 ps after the dump pulse (which was set at 100 ps, Fig 7*A*), the dumped spectrum looks very similar to that observed in H_2O shortly after the dump pulse (Fig 4*A*), indicating that a fraction of I* has been dumped to produce the anionic ground state species I. However, the differences with GFP in H_2O become clear at longer time delays: the absorption features of I persist up to 4 ns, whereas the absorption and emission features of I* have decayed away at 4-ns delay. The dumped and undumped spectrum at a delay of 4 ns look very similar to each other and show a main absorption band near 490 nm, which can be attributed to I, and a small residual stimulated emission feature due to a small fraction of remaining I*. These observations demonstrate that in GFP in D_2O, the decay of I is significantly slower than in H_2O, consistent with the kinetic traces shown in Fig. 3 *E* and *F*. The target analysis indicated a lifetime of 5 ns of I_2 for GFP dissolved in D_2O. Thus, the evolution from I_2 to A is subject to a large kinetic isotope effect of a factor of 12, as indicated in Table 1.

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