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The origin of the intrinsic fluorescence of the Green Fluorescent Protein

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Abstract

The Green Fluorescent Protein, GFP, has revolutionized biology, owing to its use in bio-imaging. It is widely accepted that the protein environment makes its chromophore fluoresce, whereas the fluorescence is completely lost when the native chromophore is taken out of GFP. By the use of a new fs pump-probe scheme, based on time-resolved action spectroscopy, we demonstrate that the isolated deprotonated GFP chromophore can be trapped in the first excited state, when cooled to 100K. The trapping is shown to last for 1.2 ns, which is long enough to establish conditions for fluorescence and consistent with calculated trapping barriers in the electronically excited state. Thus, GFP fluorescence is traced back to an intrinsic chromophore property, and by improving excited-state trapping, protein interactions enhance the molecular fluorescence.

Introduction

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*^{1,2} has a tremendous impact on life sciences, where GFP-like proteins are used extensively as fluorescent markers.^{3,4} In GFP, the light-emitting molecular unit, the chromophore, is *p*-hydroxybenzylidene-2,3-dimethylimidazolinone (HBDI) in its anionic form - see Fig. 1. In the protein, the fluo-

rescence quantum yield of the chromophore is close to unity.⁵ However, upon denaturation of the protein, fluorescence is quenched, thereby revealing that the protein environment greatly influences the photophysical properties of the chromophore, yet with little effect for the absorption wavelength (S_0 - S_1 band gap).⁶ This has spurred many theoretical and experimental studies, aiming at revealing the detailed mechanism of the change in fluorescence induced by the protein.⁷

Prevailing paradigms ascribe the fluorescence of the GFP protein to environmental effects,⁸ which suppress faster, non-radiative excited-state decay channels of its non-fluorescent native chromophore by orders of magnitude. A fundamental challenge is to understand the relationship between the properties of the entire protein and its much smaller active chromophore at the heart of the molecular machinery in GFP. In a wider perspective, the question is to what extent new properties in complex biosystems may be predicted from properties of their individual blocks.

In solution, the electronically excited chromophore relaxes to the electronic ground state, S_0 , in a $\sim$ ps non-radiative decay at room temperature.⁹⁻¹² The relaxation involves the rotation about one or two bonds along the link between the two rings (see Fig. 1), a motion which directs the excited state, S_1 , towards a conical intersection with the electronic ground state, and results in a rapid radiation-less tran-

sition to the ground state (internal conversion). However, when the solution is cooled to liquid-nitrogen temperatures (77 K) fluorescence is recovered,^{9,10} implying that internal conversion is suppressed. This is believed to be caused by a friction controlled mechanism introduced by the solvent.¹⁰

The fluorescence is also lost for isolated chromophores in the gas phase, void of any solvent effects.¹³ Instead, the electronically excited anions relax via internal conversion or electron detachment, as revealed by experiment^{6,13–15} and theory.^{16,17} Time-resolved photo-electron spectroscopy has shown that the excited-state population of chromophore anions in the gas-phase decays bi-exponentially with lifetimes of 330 fs and 1.4 ps.^{15,18} As in solution, fluorescence of the gaseous anionic HBDI chromophore thus seems quenched by ultrafast internal conversion.

Here, we investigate intrinsic properties of the GFP chromophore which are important for the protein’s ability to fluoresce. By introducing a new combination of a fs pump-probe scheme and a time-resolved action technique, we address both the excited-state and the ground-state recovery dynamics of isolated, deprotonated GFP chromophores at various temperatures (Fig. 1). Our new time-resolved action scheme allows us to study photo-initiated dynamics, which results in a particular molecular decay within a selected time window. The corresponding lifetimes are registered in the excited-state decay as well as in the ground-state recovery as probed through different decay modes of ions following absorption of fs pump and probe pulses. The technique enables us to differentiate between non-radiative and radiative transitions, since radiative transitions do not yield significantly heated ground-state ions unlike those of non-radiative transitions.

The combined informations reveal that the gas-phase GFP chromophore anion may indeed live long enough in the first excited state to fluoresce when the internal motion is frozen out. Furthermore, we find that the timescales of non-radiative relaxation exhibit a significant temperature dependence, indicating that internal conversion is a thermally activated process.

These results are rationalized through successful modeling of the experimental data together with high-level quantum chemistry calculations of the excited state potential energy surface, which provide information about the barrier heights that define conditions for trapping the isolated GFP chromophore in the excited state to enable the slow radiative (fluorescent) channel.

Methods

Experimental

The measurements were performed at the new electrostatic storage ring, SAPHIRA¹⁹ equipped with an electrospray ion source and an ion trap. The setup is shown schematically in Fig. 2. The HBDI anions were brought into the gas phase by electrospraying a sample containing the chromophore dissolved in methanol. The produced ions were accumulated for 20 ms in a multipole radio-frequency ion trap with a He-buffer gas. The ion trap could be operated at room temperature or cooled to 100 K with liquid nitrogen. The ions were cooled, translationally as well as internally, to the trap temperature by collisions with the cold He-buffer gas (internal energy distributions are given in Supplementary Discussion A). They were then ejected from the trap during 20 μ s, accelerated to a kinetic energy of 4 keV, and mass-to-charge selected in a magnetic field before injection into SAPHIRA. The ions circulated in SAPHIRA with a revolution time of ~ 64 μ s throughout the entire storage time of 5 ms. After 2 ms of storage, the excited-state dynamics was initiated and probed by overlapping the stored ions with two femtosecond laser pulses in the third arm of the storage ring.

The pump and probe laser pulses were derived from the fundamental 800 nm output delivered by a Ti:Sapphire oscillator and amplifier system (Coherent Libra-HE). The 800 nm (50 fs) output was split into two parts (3:1) of which the major part was used to pump an Optical Parametric Amplifier (TOPAS-C) which produced

480 nm light to drive the $S_0 - S_1$ electronic transition in the chromophore. To probe the ground state recovery, the 480 nm light pulse was further split into two parts of which one was passed through an optical delay stage to delay the two 480 nm pulses relative to each other. The pulses were overlapped with the ions and each other in an inclined-beams configuration. To probe the excited-state decay, the 480 nm pump pulse was overlapped with the minor part of the 800 nm fundamental, and the light pulses were merged at a dichroic mirror and subsequently overlapped with the ions in a merged-beams configuration. The typical pulse energy for the pump pulse was 60–80 μJ , while the probe pulse energy was 1 mJ for the 800 nm probe pulse and 120 μJ for the 480 nm probe pulse. The cross-correlation between the two 480 nm pulses (individual pulse width of 56 fs) was measured to be 80 fs (full-width at half-maximum, FWHM) using a β -barium borate crystal. The overall experiment was running at a repetition rate of 50 Hz. Laser-pulse energies were measured behind the exit window of the ring. The pulse energy was recorded for each experimental cycle and used for normalization to the photon flux.

Neutral particles, created in the third and fourth straight section of the ring, left the ring unaffected by the electrical fields and impinged on detectors (DET 1 or DET 2) behind the corners (see Fig. 2). The first detector, DET 1, was a channeltron detector facing a glass plate which allowed for simultaneous transmission of the laser light and detection of impinging neutral particles.²⁰ The second detector, DET 2, was a multi-channel plate (MCP) detector. Both detectors registered the timing information for each neutral particle impact. Figure 3 shows the time distributions of the impacts in the cases where ions were exposed to either the pump pulse alone, or the pump as well as a probe pulse. The number of collisional induced background neutralization events was proportional to the ion-beam intensity and the data was thus also normalized to the ion-beam intensity by considering the yield of counts with the laser blocked.

There were only minor single-photon con-

tributions to the signal because the 800 nm pulse was off-resonance with the $S_0 - S_1$ transition and the 480 nm single-pulse excitation mostly resulted in internal conversion back to a single-photon heated ground state, which was stable on the time scales of the experiment²¹ (discussed in Supplementary Discussion B). In any case, single-photon contributions were subtracted throughout the data collection.

Computational

Equilibrium geometry parameters and the topography around the minimum energy structure of the HBDI anion in the first excited state were obtained using the extended multi-configuration quasi-degenerate perturbation theory XMCQDPT2.²² The zeroth-order wavefunctions were constructed within the complete active space self-consistent field method CASSCF(14,13), where almost all valence π -type orbitals of the chromophore were included in the active space, except for the lone pair of electrons localized at the nitrogen atom of the imidazolinone ring. The cc-pVDZ basis set, augmented with diffuse functions on the oxygen atoms, (aug)-cc-pVDZ, was used. S_1 potential-energy curves as a function of the two twist angles in the bridge moiety of the chromophore were obtained as XMCQDPT2/SA(2)-CASSCF(14,13) relaxed geometry scans, using state-specific nuclear gradients computed over state-averaged orbitals. S_0 potential-energy curves were constructed based on ground-state energies calculated at the corresponding optimized geometries in the first excited state. The normal mode analysis was carried out for the ground-state chromophore anion at the optimized structure within the MP2/(aug)-cc-pVTZ level of theory. Internal energy distributions of the HBDI anion at various temperatures were calculated based on these frequencies. For all electronic structure calculations the Firefly package,²³ version 8.2, partially based on the GAMESS (US) source code,²⁴ was used.

In the GFP chromophore anion, there are two distinct types of conical intersections (CIs) as obtained at the XMCQDPT2/CASSCF(14,13)

level of theory.¹⁷ One of the CI structures lies 0.2 eV below the fluorescent state, *i.e.* the planar minimum in S_1 , and is associated with twisting about the bridge bond adjacent to the imidazolinone ring. The other CI structure exhibits a hula-twist geometry and lies ~ 0.2 eV above the fluorescent state. It is associated with pyramidalization of the central part of the anion and simultaneous twisting about the two bridge bonds. In the present work, we consider only the lowest-energy CI as a funnel to the non-radiative decay and use the corresponding coordinates of its branching plane to map the minimum-energy parthway for internal conversion.

Results and discussion

To monitor both excited and ground-state populations, we report in the present work on two different fs pump-probe schemes, where we make use of different probe wavelengths, while keeping the pump pulse fixed at the $S_0 - S_1$ transition (480 nm). We take advantage of the storage ring technique which allows us to differentiate between the molecular response (action) which may be prompt (electron emission) or statistical (dissociation and delayed thermionic emission from the hot ground state), the decay time of which carry information about the number of photons that were absorbed. The technique also enables us to register if energy is re-emitted by photons since the lack of energy would result in an extraordinary long decay time, as discussed below.

To record the time evolution of the *excited state* population, the ions were exposed to a 800 nm probe pulse which was delayed up to 600 ps with respect to the $S_0 - S_1$ (480 nm) pump pulse. The only effect of the probe pulse was to cause rapid electron detachment of electronically excited ions that would have relaxed by other channels, such as internal conversion or fluorescence, in the absence of the probe pulse. The 800 nm probe did not cause detachment of ground state ions by direct two-photon absorption, as no detachment signal was observed in absence of the 480 nm pump pulse.

The additional number of detachment events caused by the probe pulse as recorded by DET 1 (signal appearing in the blue area of Fig. 3 (a)) was directly proportional to the excited state population. The time evolution of the excited-state population was recorded by monitoring the events as a function of time delay between the pump and probe pulses.

To record the time evolution of the *ground state* (re)population, the excited ions were exposed to 480 nm probe pulses, which were delayed up to 900 ps with respect to the $S_0 - S_1$ (480 nm) pump pulse. In this case, the probe pulse caused electron detachment of excited ions as well as ground-state ions as revealed by the additional events appearing in the first peak after laser excitation (Fig. 3 (c)). In addition, ions that underwent internal conversion back to S_0 following excitation by the pump pulse could be re-excited by the 480 nm probe pulse. Following a second return to the ground state, these ions were so hot that they dissociated on the sub-millisecond time scale thereby producing neutral fragments during many revolutions in the ring, as highlighted by the light red areas in Fig. 3 (c+d), and discussed in details in Supplementary Discussion B. The signal appearing in the red areas is directly proportional to the number of ions that underwent internal conversion after excitation by the pump pulse, and the ground state recovery was therefore measured by monitoring the number of events in this area as a function of the time delay between the two laser pulses.

Figure 4 shows the decay of the excited state and the corresponding recovery of the ground state, recorded with the ion trap at room temperature (a+b), and cooled to 100 K (c+d). At room temperature, the excited-state population exhibits a bi-exponential decay with lifetimes in the one to tens of picoseconds range, which are also the lifetimes characterizing the bi-exponential recovery of the ground state. When cooled to 100 K (c+d), the two components become longer, and a new long-lived ns component appears in the excited state decay, which is *not* visible in the ground-state recovery data. A very fast sub-ps component was also observed, as shown in Supplementary Dis-

cussion C and also discussed later.

To interpret the experimentally observed temperature dependence, we use the accurately calculated potential-energy curves (see Fig. 5). They show that rotation of the imidazolinone-ring angle (θ - see Fig. 1) brings the two electronic states together, potentially resulting in fast internal conversion (IC) through a conical intersection (CI),¹⁷ whereas a rotation of the phenol-ring angle (ϕ - see Fig. 1) does not. Moreover, two small potential-energy barriers in S_1 create two minima, corresponding to a planar, fluorescent structure and a highly twisted (ϕ) non-fluorescent structure, capable of trapping a population temporarily in S_1 . As a result, the excited-state decay out of the planar minimum in S_1 exhibits two branches – a direct non-radiative pathway back to the hot ground state and a non-reactive pathway, along which a transient structure is formed that subsequently repopulates the planar minimum in S_1 .

To account for the experimental observations (Fig. 4), we discuss in the following four time components, which span several orders of magnitude, from a few hundreds of femtoseconds^{18,25} to nanoseconds.

First, we ascribe the short 330 fs component observed previously by electron detection^{15,18} to ultrafast 'over-the barrier' trajectories along the imidazolinone-ring twist of hot ions in the ion-beam ensemble. We see a similar fast component (see Supplementary Discussion C), but with less intensity. Differences may be explained by a different amount of collisional heating during beam-transport of the anionic chromophores in the experimental setups. The component is found to be slightly temperature dependent (300 fs at 300K and 500 fs at 100K). No temperature variation was considered in the previous experiments.^{15,18}

The two ps components seen in Fig. 4 are highly dependent on temperature and hence of particular interest, since they provide information about the energy barriers in S_1 . Based on the potential-energy curves and with reference to labels in Fig. 5, we model the population as a function of time after initial excitation to S_1 at 480 nm through the involved states:

The excited-state decay, as well as the ground-state recovery can be derived from the following equations:

$$dN_{A^*}/dt = -(k_1 + k_2)N_{A^*} + k_{-2}N_{B^*} \quad (2)$$

$$dN_{B^*}/dt = k_2N_{A^*} - k_{-2}N_{B^*} \quad (3)$$

$$dN_C/dt = k_1N_{A^*} \quad (4)$$

These equations have analytical solutions, given by the rate constants k_1 , k_2 , and k_{-2} as discussed in Supplementary Discussion D.

Inspired by the theoretically predicted almost equal barriers (Fig. 5), and to keep the dimensionality of the problem as low as possible, we simplify the solutions by letting $k_1=k_2=k(T)$ and $k_{-2}=\alpha(T)k(T)$. Thus, the two barrier heights around the fluorescent state are assumed identical and the well depths associated with the two trapped states are allowed to be different. We explicitly allow k and α to be temperature dependent.

The simplified solutions for the excited-state population ($N_{A^*} + N_{B^*}$), as revealed by 480 nm pump and 800 nm probe pulses, are bi-exponential in time with rate constants γ_1 and γ_2 :

$$N_{A^*}(t) + N_{B^*}(t) = C_1 e^{-\gamma_1 t} + C_2 e^{-\gamma_2 t} \quad (5)$$

where the coefficients are solely functions of α and k :

$$\gamma_1 = \frac{k}{2}(2 + \alpha - \sqrt{4 + \alpha^2}) \quad (6)$$

$$\gamma_2 = \frac{k}{2}(2 + \alpha + \sqrt{4 + \alpha^2}) \quad (7)$$

$$C_1 = \frac{1}{2} \left(1 + \frac{\alpha}{\sqrt{4 + \alpha^2}} \right) \quad (8)$$

$$C_2 = \frac{1}{2} \left(1 - \frac{\alpha}{\sqrt{4 + \alpha^2}} \right) \quad (9)$$

The ground state re-population, as detected by delayed action following absorption of both a 480 nm pump and a 480 nm probe pulse, is

given by:

$$N_C(t) = N_{A^*}(t_0) - (N_{A^*}(t) + N_{B^*}(t)) \quad (10)$$

The three-level model describes the data in the 1-25 ps region very well (Fig. 6) through the decay with two characteristic lifetimes $1/\gamma_1$ and $1/\gamma_2$, which provides evidence for the validity of the model. From fits to the excited-state population $N_{A^*} + N_{B^*}$, we obtain the rate constant k as well as the scaling parameter α - see Fig. 6. The obtained rate constant k shows a strong T -dependence due to the presence of the energy barriers. At the same time, α is almost T -independent with a value of about 0.6, thus providing conditions for temporary trapping in the non-fluorescent B^* state.

Assuming that internal energy distributions in S_1 are the same as those in S_0 at a given temperature (*i.e.* pump excitation at 480 nm, being close to the adiabatic transition, merely shifts an internal energy distribution from S_0 to S_1 , except for the lowest temperature considered in this work), we use Transition State Theory with an explicit temperature dependence of the pre-exponential factor to model $k(T)$ (for details, see Supplementary Discussion E). The fit to the experimental values of $k(T)$ is shown as green line in Fig. 6. From this, we obtain that the energy-barrier in S_1 equals 0.043 ± 0.002 eV as defined from the bottom of the potential-energy curve. This value agrees well with the calculated value of 0.05 eV from the XMCQDPT2 electronic-structure theory.

Importantly, we identify a long ns component, seen in Fig. 4, which we ascribe to cold ions, trapped in the planar fluorescent configuration A^* . In support of this, there are several observations:

- i) It only appears when the ions are cooled to 100 K, hence not observed before in gas phase.
- ii) It is only visible in the decay out of the excited state and not in the return to the ground-state data (green data, Fig. 4). The reason is that energy is radiated away through fluorescence, and therefore the chromophore returns to the ground state without enough energy to fragment sufficiently fast to contribute to the signal used to monitor the ground-state recovery.

This is due to the fact that molecular anions, which are exposed to [480 nm (pump) + 480 nm (probe)] versus [480 nm (pump) + fluorescence + 480 nm (probe)], have immensely different ground-state fragmentation rates. In the latter case, the fragmentation rate is exceedingly small, and hence it does not contribute to the signal as discussed in Supplementary Discussion B.

iii) The measured long decay time of 1.2 ns corresponds to the observed ~ 3 ns fluorescence lifetime in the GFP protein.²⁶

iv) The calculated energy-barrier heights and internal energy distributions at various temperatures (Supplementary Discussion A) support the experimental findings and provide evidence that trapping conditions are indeed obtained at temperatures of 100 K and below.

Conclusions

Our work outlines new perspectives for pump-probe experiments in combination with ion-storage techniques, by introducing time-resolved action spectroscopy in two dimensions (pump-probe delay time and decay time of a particular action). This technique can be used to resolve the ultrafast photo-initiated dynamics of biological chromophores as it happens in different decay channels, or through states that are distinguished by the characteristic timescales of the corresponding molecular action. The new time-resolved method is highly advantageous for mapping the excited-state decay as well as the ground-state recovery, and it is applicable also for positively charged ions.

The work shows that native GFP chromophores are intrinsically capable to fluoresce outside the protein environment with a nanosecond lifetime. We traced the fluorescent key property to the presence of intrinsic energy barriers in the S_1 excited state of the chromophore, which prevent the HBDI anion in S_1 from returning to the S_0 ground state through fast, non-radiative internal conversion. In the gas-phase, fluorescence is obtained only when the chromophore is cooled to such an extent that the energy barriers becomes effi-

cient in preventing the otherwise fast internal conversion, whereas in the protein, the energy barriers are enlarged by the coupling to the protein environment and also efficient in preventing internal conversion at room temperature. Beyond solving the question about the origin of the intrinsic fluorescence of GFP, the work shows that detailed molecular knowledge, obtained for example through high-level quantum chemistry calculations, may be used together with molecular engineering in the quest for new functional proteins^{27,28} and RNA-fluorophore architectures.²⁹

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Additional information

The authors declare no competing financial interests. Supplementary information accompanies this paper. Correspondence and requests for materials should be addressed to L.H.A or A.V.B.

Supporting Information Available: Additional information on the experimental techniques and theoretical methods are available in the Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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Figure 1: The chromophore structure and the employed scheme for probing internal dynamics. Top: The structure of the deprotonated *p*-hydroxybenzylidene-2,3-dimethylimidazolinone (HBDI) anion and the torsional angles relevant for the excited-state dynamics. Bottom: Schematic representation of the two approaches to probe the decay of the population in the electronic excited state, S_1 , (left) and the recovery of the population in the electronic ground state, S_0 , (right) along a reaction coordinate. D_0 represents the ground state of the neutral HBDI radical. Fragmentation in the ground state after internal conversion is only observed after absorption of *both* a pump and a probe pulse from the ground state and allows us to register when, and only when, the system has returned to the ground state after the pump pulse. The influence of the internal temperature on the internal energy distribution in the excited state is shown schematically – see Supplementary Discussion A for details.

Figure 2: **The experiment.** Schematic of the experimental setup comprising the electro-spray ion source with the cooled ion trap, the SAPHIRA storage ring, and the femtosecond laser system.

Figure 3: **Number of detected neutral particles as a function of time.** Number of neutral particles impinging on DET 1 (a+c) or DET 2 (b+d) as a function of time after laser excitation when ions are exposed to 480 nm pump pulses alone (black traces), to 480 nm pump as well as 800 nm probe pulses (red traces), or to 480 nm pump as well as 480 nm probe pulses (green traces). Time zero is defined as the time at which the ions were irradiated by the light pulses. Adjacent peaks are separated by the revolution time in the ring ($\sim 64 \mu\text{s}$). The grey dashed lines represent the background level due to neutralization by residual-gas collisions. This level is used for normalization to the ion density. The blue area indicates the signal-time window in which events stemming from electron detachment of the excited-state ions appear. Very hot ions that have been excited, and have returned to the ground state in a sequential two-photon absorption process, dissociate on the sub-millisecond time scale, and these events appear in the areas highlighted in light red.

Figure 4: **Time dependence of the excited state and ground-state populations at two different temperatures.** The time-dependent ground state (open green circles) and excited state (open purple squares) populations were recorded while keeping the ion trap at room temperature, $T = 300$ K (panels a+b) and while cooling the ion trap to $T = 100$ K (panels c+d). The chromophores were excited to S_1 by a 480 nm pump pulse (see Fig. 1). The S_1 excited-state population was then monitored by a 800 nm probe pulse, and the ground-state re-population by a 480 nm probe pulse. (a+b) Fits to the data at 300 K by a bi-exponential decay function (purple line) or bi-exponential recovery function (green line) with lifetimes $\tau_1 = 1.3 \pm 0.2$ ps and $\tau_2 = 11.5 \pm 0.5$ ps. (c+d) Fits to the data at 100 K by a tri-exponential decay function (full purple line) with lifetimes of $\tau_1 = 4.6 \pm 2.1$ ps, $\tau_2 = 27 \pm 2$ ps, and $\tau_3 = 1.2 \pm 0.1$ ns, and the corresponding bi-exponential ($\tau_1 = 4.6 \pm 2.1$ ps, $\tau_2 = 27 \pm 2$ ps) recovery function (green line). The dashed purple line represents the long ns lifetime component, τ_3 , and the dashed green line is the expected ground-state recovery without fluorescence in which case a two-photon heated repopulated ground state would appear on the τ_3 timescale.

Figure 5: **S_0 and S_1 potential-energy curves as a function of the two twist angles θ and ϕ associated with the imidazolinone and phenol ring, respectively.** The electronic energy gap at the planar structure was adjusted to match the predicted 0-0 transition energy.¹⁷ At 480 nm pump wavelength we may disregard the contribution of vibrationally assisted autodetachment,¹⁷ which is therefore not included in the model. IC/CI denotes internal conversion through a conical intersection.

Figure 6: **Temperature dependence of the excited state decay dynamics, measured with 480 nm pump and 800 nm probe.** The upper graph shows the experimental data along with fits based on the model with only two parameters ($k=k_1=k_2$ and $\alpha=k_{-2}/k$), yielding two exponential functions (data at ultrashort times (<1 ps) were not used in the fit - see Supplementary Discussion C). The strong temperature dependence is clearly seen with increasing lifetimes at low temperatures. The lower graphs show the obtained fit parameters, α and k , the rate constants γ_1 and γ_2 , and the coefficients C_1 and C_2 as a function of trap temperature.

Graphical TOC Entry