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Observation of robust energy transfer in the photosynthetic protein allophycocyanin using single-molecule pump-probe spectroscopy

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Photosynthetic organisms convert sunlight to electricity with near unity quantum efficiency. Absorbed photoenergy transfers through a network of chromophores positioned within protein scaffolds, which fluctuate due to thermal motion. The resultant variation in the individual energy transfer steps has not yet been measured, and so how the efficiency is robust to this variation has not been determined. Here, we describe single-molecule pump–probe spectroscopy with facile spectral tuning and its application to the ultrafast dynamics of single allophycocyanin, a light-harvesting protein from cyanobacteria. We disentangled the energy transfer and energetic relaxation from nuclear motion using the spectral dependence of the dynamics. We observed an asymmetric distribution of timescales for energy transfer and a slower and more heterogeneous distribution of timescales for energetic relaxation, which was due to the impact of the protein environment. Collectively, these results suggest that energy transfer is robust to protein fluctuations, a prerequisite for efficient light harvesting.

Photosynthetic light-harvesting systems power most life on earth by capturing and directing absorbed energy through networks of protein-scaffolded chromophores. Rapid transfer of the absorbed energy is driven by coupling between the transition dipole moments of the chromophores. Fluctuations of the protein scaffold induce changes in the distances and orientations of the transition dipole moments that can, in turn, change the timescales of energy transfer. Despite these fluctuations, energy travels through the light-harvesting systems to reach the reaction centre with near unity quantum efficiency. Heterogeneity in the timescales of energy transfer, that is, the impact of protein fluctuations on light harvesting, has not yet been characterized.

Over the past decades, single-molecule methods have been a powerful approach to characterize heterogeneity in biological, chemical and material systems, including photosynthetic light-harvesting proteins. More recently, single-molecule pump–probe spectroscopy (SM2P) has emerged as a technique to resolve femtosecond processes, such as energy transfer. SM2P maps femtosecond dynamics onto fluorescence intensity using two ultrafast isoenergetic pulses to generate a pump–probe-like excitation, where the temporal resolution is from the delay time between pulses (Fig. 1a). This technique has been applied to various ultrafast processes in single molecules, including coherent wave-packet oscillations and relaxation among the excitation states of light-harvesting proteins from purple bacteria. Comparison of the exciton relaxation measurements with theoretical calculations found a narrow distribution of chromophore transition energies, enabling robust exciton delocalization and, therefore, robust energy transfer. The complexity of the ultrafast dynamics of the measured light-harvesting protein has, however, obfuscated the heterogeneity specific to an individual energy transfer step. Furthermore, most of the previous measurements lacked spectral dependence, which provides an additional axis to help disentangle the contributions associated with each process.

The cyanobacterial light-harvesting protein, allophycocyanin (APC), contains strongly coupled dimers of chromophores that serve as a minimal system to examine photosynthetic energy transfer (Fig. 1b). Ensemble ultrafast measurements found complex kinetics of energy transfer within APC, potentially due to heterogeneous timescales. Consistently, single-molecule fluorescence measurements identified heterogeneous photophysical states that arose from fluctuations of the protein scaffold, yet their impact on energy transfer could not be resolved due to the limited time resolution of fluorescence measurements.

Here, we report SM2P with facile spectral tuning across the visible region and perform SM2P measurements on APC, the homologous protein C-phycocyanin (CPC) and a chromophore in solution. Based on the spectral dependence of the dynamics and the concomitantly measured fluorescence lifetimes, distributions were constructed for APC in which the populations were dominated by proteins with energy transfer or by proteins with only energetic relaxation due to nuclear motion. For the distribution with energy transfer, the median of the distribution was ~100 fs shorter than the mean, suggesting that the ensemble values may be lengthened or non-mono-exponential due to slow subpopulations. Broad distributions of energetic relaxation timescales were observed for both APC and CPC, which a comparison to chromophores in solution indicated arises from the protein structure. Furthermore, slow energetic relaxation was observed in APC, enabling energy transfer to precede, and thus likely be unaffected by, the heterogeneity in the relaxation timescales. Together, these results demonstrate that rapid energy transfer is maintained despite fluctuations of the protein, which may play a role in the high quantum efficiency of photosynthetic light harvesting.

Results

Ultrafast dynamics in APC. We used SM2P to determine the distribution of ultrafast energetic relaxation timescales in APC. APC forms a trimer where each monomer contains two protein subunits, known as α and β, that each bind a phycocyanobilin chromophore, which are positioned on distal ends of the monomer
The structural model of trimeric APC is shown in Fig. 1b. Upon trimerization, complementary chromophores on different monomers are brought close together (~2 nm apart) to generate the dimer shown in Fig. 1b, inset. The trimers assemble into the central core of the primary cyanobacterial light-harvesting structure, known as the phycobilisome. The linear absorption spectrum of APC is shown in Fig. 1c with the calculated absorption profiles for the underlying states. The higher energy state has a broad absorption, and the lower energy state has a narrow absorption with a vibrational mode at ~1,600 cm$^{-1}$ above the primary transition. Although the electronic coupling between the $\alpha$ and $\beta$ chromophores leads to excited states that are a linear combination of the excited states of the individual chromophores, the large energy gap means that the higher energy state is dominated by the $\beta$ chromophore and the lower energy one by the $\alpha$ chromophore. Thus, we refer to the states based on their dominant contribution.

The SM2P apparatus was constructed with a tunable excitation laser and a single-axis prism compressor for straightforward
wavelength changes across the visible range, which was used to investigate the dynamics of the individual chromophores. SM2P experiments on APC were performed with an excitation laser centered at 610 nm and 645 nm, which were selected to predominantly excite the β or α chromophores, respectively (Fig. 1c), as in previous ensemble ultrafast measurements\(^{22,24,26,38}\). In SM2P, the first saturating pulse drives damped Rabi oscillations between the ground state \((|0\rangle\)\) and the initially excited state \((|1\rangle\)\), resulting in an equal probability of population in both states after interaction with the pulse. The population in \(|1\rangle\) relaxes to the off-resonance state \(|2\rangle\) with a timescale determined by the microscopic properties of the sample. The second saturating pulse drives the same Rabi oscillations between \(|0\rangle\) and \(|1\rangle\), but does not interfere with the population out of resonance (that is, in \(|2\rangle\)\). On timescales longer than the relaxation time (Fig. 1a, right), the second Rabi oscillation provides another opportunity to populate \(|1\rangle\) and subsequently transfer to \(|2\rangle\), increasing the population and thus fluorescence from \(|2\rangle\). By detecting fluorescence from \(|2\rangle\) and scanning the delay from negative to positive times, an SM2P trace is recorded that shows a dip-like shape, which can be fit to extract the timescale of energy relaxation between states.

Representative SM2P traces are shown for primarily β excitation at 610 nm (Fig. 1d,e) and primarily α excitation at 645 nm (Fig. 1f,g). While these β excitation traces exhibit timescales that are not statistically different (148 ± 15 and 225 ± 70 fs in Fig. 1d,e, respectively), the α excitation traces differ significantly (371 ± 46 and 185 ± 39 fs in Fig. 1f,g, respectively), providing an initial demonstration of the ability of SM2P to uncover heterogeneity in ultrafast dynamics.

**Energy transfer in APC.** In APC, rapid energy transfer occurs within the chromophore dimers\(^2\). Energy transfers from the higher energy β chromophore to the lower energy α chromophore and so can be studied using the β excitation data. While slower (picosecond) energy transfer also occurs between dimers, it is outside the timescale measured here. Along with energy transfer, both chromophores undergo energetic relaxation on a similar timescale due to nuclear motion. SM2P measures the overall energy relaxation timescale, which includes both energy transfer and energetic relaxation out of the bandwidth of the laser. Under the high excitation fluences of SM2P, the chromophores photodegrade into quenching radical cations that decrease the fluorescence lifetime\(^{30,31}\). The negligible oscillator strength and/or spectral shifts of the photodegraded chromophore eliminate the rapid β-to-α energy transfer, and so only energetic relaxation due to nuclear motion remains\(^{33,34,35}\).

To separate and characterize energy transfer and energetic relaxation, four histograms were constructed from the measured timescales, divided by the excitation wavelength and the concomitantly measured fluorescence lifetime. The histograms are shown in Fig. 2 for β excitation (Fig. 2a,b) and α excitation (Fig. 2c,d) for bright (fluorescence lifetime, \(\tau_F \geq 1.50\) ns) and quenched (\(\tau_F < 1.50\) ns) populations, respectively, with their primary photophysical pathways illustrated. The quenching properties of the photodegraded β chromophore lead to quenched populations enriched in proteins without energy transfer and vice versa. Following previous work\(^{31,32}\), the photophysics were simulated (Supplementary Fig. 20 and Supplementary Section 8). The simulations showed that the enrichment of the bright population in proteins with energy transfer reached a maximum of 86% at a cut-off value of \(\tau_F \geq 1.50\) ns (Supplementary Fig. 19). Statistical parameters for the histograms are given in Supplementary Section 6.

In the β excitation data, the histograms have medians of 168 fs for bright APC and 286 fs for quenched APC (Fig. 2a,b). Comparison of the two distributions with a permutation test yielded a \(P\) value of 0.0001 (Supplementary Section 6.4), which establishes with a high probability (99.99%) that the two distributions are different. By contrast, in the α excitation data, the histograms have medians of 242 fs for bright APC and 242 fs for quenched APC (Fig. 2c,d), and comparison of the two distributions showed that they are the same (\(P > 0.05\); Supplementary Section 6.4). Similarly, the quenched populations for β excitation and α excitation were the same (\(P > 0.05\); Supplementary Section 6.4). In agreement with these results, energetic relaxation is expected to dominate in all three of these populations. The statistically significant shorter median timescale for the bright population of the β excitation data, the only histogram in which energy transfer is expected, is consistent with this picture.

The β excitation data can also be further analysed to quantify the dynamics. Each of the histograms contains APC with intact chromophore dimers, which undergo energetic relaxation and energy transfer simultaneously, and photodegraded ones, which undergo energetic relaxation alone. Rate histograms were constructed for both the bright and quenched populations and fit with a two-component Gaussian mixture model (Supplementary Section 6.5 and Extended Data Fig. 2). While similar rates were extracted from both distributions (Supplementary Table 4), the faster component of ~0.007 fs\(^{-1}\) (140 fs) was dominant in the bright population, whereas the slower component of ~0.003 fs\(^{-1}\) (330 fs) was dominant in the quenched population. The simulations of APC predicted that 86% and 32% of the photophysical states can exhibit energy transfer in the bright and quenched populations, respectively (Supplementary Section 8). Thus, the faster component was assigned to energetic relaxation and energy transfer and the slower component was assigned to energetic relaxation alone.

To explore the ultrafast dynamics, a kinetic model of energy transfer and energetic relaxation was constructed (Supplementary Section 9). From the additivity of rates, the two components of the Gaussian mixture model give a rate of ~0.004 fs\(^{-1}\) (250 fs) for energy transfer alone. Using the experimental rates in the kinetic model, >90% of energy transfer was complete by 600 fs, when only ~20% of the energetic relaxation had occurred, as illustrated in Fig. 2e. The relative amplitudes indicate energy transfer generally precedes energetic relaxation.

The distribution of timescales that primarily includes APC with both energy transfer and energetic relaxation, shown in Fig. 2a, has a median of 168 fs and a mean of 249 fs, where the large difference between these two values is due to the asymmetric profile of the distribution. In previous ensemble measurements, which are more accurately compared to the mean of single-molecule distributions, energy transfer between the two chromophores was found to occur on 220 fs and 280 fs timescales. The two timescales were ascribed to different conformational states of the protein backbone\(^{32,35}\). Analysis using rates determined through a Gaussian mixture model, which is expected to recover the mean, found an energy transfer timescale of ~250 fs, consistent with the ensemble values. However, the asymmetric nature of the measured distribution suggests that ensemble measurements are lengthened by small subpopulations with slow transfer. This observation of an asymmetric distribution suggests that traditional measurements of photosynthetic energy transfer, which were primarily developed based on mean values\(^2,7,39\), may be distorted by these slow subpopulations.

An asymmetric distribution of energy transfer timescales can not only lengthen the average value but also influence the functional form of measurements. Whereas a Gaussian distribution gives rise to an energy transfer process well described by a single-exponential function, an asymmetric distribution can lead to more complex behaviour, such as a stretched exponential or the bi-exponential observed previously for APC (Supplementary Section 7.4 and Supplementary Fig. 17)\(^33\). Thus, instead of characterizing distinct subpopulations, ensemble measurements of multi-exponential dynamics could instead arise from non-Gaussian microscopic heterogeneity, which was observed theoretically for another photosynthetic light-harvesting protein in previous work and experimentally for APC here\(^40\).
The full distribution of timescales that primarily includes APC with both energy transfer and energetic relaxation spans ~65 fs to ~800 fs. In ensemble measurements with high temporal resolution, sub-50 fs energy transfer was also observed. This population is absent here, which may be due to a smaller amplitude of this component and/or the challenges associated with characterizing this timescale due to the pulse duration (100–200 fs; Supplementary Sections 7.1 and 7.2)\(^3,\)\(^4\). The few measured timescales above ~400 fs likely arise from the combination of the asymmetric distribution and the effect of Poissonian noise, which elongates the tail of SM2P molecules. The collective reorganization of solvent molecules (diffusive solvation) occurs on longer timescales than the picosecond regime. In Atto 647N, the timescale due to the pulse duration (100–200 fs; Supplementary Section 7.3)\(^5\) is due to the contribution of energy transfer to the measured timescales, whereas the other three distributions are dominated by energetic relaxation. A kinetic model of energetic relaxation and energy transfer with the experimentally determined rates (Supplementary Section 9) was used to calculate the percent of population transferred (blue) and of energetic relaxation due to nuclear motion (red) as a function of time. The faster rate enables energy transfer to precede the heterogeneous energetic relaxation. Median values are 159 \(\alpha\), 308 \(\beta\), 265 \(\gamma\), and 257 \(\delta\) fs.

### Environment-dependent heterogeneity in energetic relaxation.

The influence of the protein environment on the distribution of energetic relaxation timescales was investigated by comparing the distributions for APC, CPC and Atto 647N. CPC is a cyanobacterial light-harvesting protein that is homologous to an APC monomer with an additional peripheral chromophore (Extended Data Fig. 1)\(^2\)\(^3\)\(^5\)\(^6\). There are large distances between the chromophores in CPC, and so energy transfer is slower than the time window measured here. Therefore, only energetic relaxation is present in the distribution. Atto 647N is a widely used single-molecule chromophore with sufficient photostability for SM2P, which the chromophores in APC and CPC lack. Histograms of the measured energetic relaxation timescales are shown in Fig. 3a–c for APC with \(\alpha\) excitation, CPC and Atto 647N, respectively. Statistical parameters for all three distributions are given in Supplementary Table 2, which show differences in both the peak locations and widths.

The distribution for Atto 647N was compared to the distributions for APC and CPC in order to investigate the contribution of a protein scaffold, as Atto 647N was embedded in a polymer matrix with a local aqueous environment. The asymmetric distribution and median timescale (148 fs) for energetic relaxation of Atto 647N was similar to previous measurements of other chromophores in solution. Based on the previous work, we assign the measured energetic relaxation in Atto 647N to IVR with a contribution on the short timescales from electronic dephasing. The median timescales of energetic relaxation for both chromophores, although two components are present, the shorter one is absent. This may be due to the coherent excitation scheme used in the ensemble measurements, or the second component may be hidden in the width of the SM2P distribution.

### Energetic relaxation in APC.

Upon photoexcitation, the chromophores in APC undergo energetic relaxation (redshifting) due to nuclear motion including intramolecular vibrational relaxation (IVR) and solvation within the protein pocket. The ultrafast solvation, known as inertial solvation, involves coupling to the short-range motions of nearby amino acid side chains and solvent molecules. The collective reorganization of solvent molecules (diffusive solvation) occurs on longer timescales than the picosecond window investigated here. The combined effects of IVR and solvation give rise to the Stokes shift, which moves the population out of resonance with the laser pulse. This energetic relaxation can be investigated using the histograms where no energy transfer is present. For the \(\alpha\) excitation data, the histograms of timescales from bright (Fig. 2c) and quenched (Fig. 2d) APC have similar median values (286 fs and 242 fs, respectively), which is consistent with previous pump–probe experiments that measured a 250 fs Stokes shift.

The spectral dependence of the energetic relaxation can be examined by comparing the \(\alpha\) excitation data to the \(\beta\) excitation data for quenched APC. Similar median timescales of 308 fs for the \(\beta\) excitation data (330 fs from the Gaussian mixture model, which finds mean values) and ~260 fs for the \(\alpha\) excitation data were determined. Previous studies reported comparable timescales of energetic relaxation for both chromophores, although two components at 120 fs and 230 fs were observed. While the median timescales are approximately consistent with the longer of the two components, the shorter one is absent. This may be due to the coherent excitation scheme used in the ensemble measurements, or the second component may be hidden in the width of the SM2P distribution.

### Fig. 2 Distributions of energetic relaxation and energy transfer timescales for APC.

- **a**. Histograms of the energy relaxation timescales were constructed from the \(\beta\) excitation data (excitation wavelength, \(\lambda_{\text{ex}} = 610\) nm, blue) for the bright \((\tau > 1.50\) ns\) population (\(\alpha\)) and quenched \((\tau < 1.50\) ns\) population (\(\beta\)) and from the \(\alpha\) excitation data (\(\lambda_{\text{ex}} = 645\) nm, red) for the bright population (\(\gamma\)) and quenched population (\(\delta\)). The median values of the distributions are indicated by the dashed lines. The dominant photophysical pathways are illustrated in schematics on the right. As shown in **a**, right, excitation of the \(\beta\) chromophore (blue) leads to energy transfer to the \(\alpha\) chromophore (red). As shown in **b** and **d**, right, photobleached chromophores (dark grey) lead to a loss of photoactivity and/or conversion into quenchers. The shorter median value in **a** is due to the contribution of energy transfer to the measured timescales, whereas the other three distributions are dominated by energetic relaxation. **e**. A kinetic model of energetic relaxation and energy transfer with the experimentally determined rates (Supplementary Section 9) was used to calculate the percent of population transferred (blue) and of energetic relaxation due to nuclear motion (red) as a function of time. The faster rate enables energy transfer to precede the heterogeneous energetic relaxation. Median values are 159 \(\alpha\), 308 \(\beta\), 265 \(\gamma\), and 257 \(\delta\) fs.
CPC are nearly tenfold faster than those of the chromophores in APC. The binding pocket of the α chromophore of APC is more polar than the other pockets, which could stabilize the excited state and give rise to the slightly slower relaxation. Furthermore, in CPC, the torsional angle of the chromophore is ~25° larger than in APC, and the more strained configuration could be less stable, that is, exhibit faster nuclear motion. The more buried position of the chromophores in trimeric APC (Fig. 1b) than in monomeric CPC (Extended Data Fig. 1) could also slow energetic relaxation. The solvent-accessible surface area for chromophores in CPC (300 Å²) is twice that for chromophores in APC (150 Å²). The increased solvent accessibility in CPC may create a more solution-like environment that speeds up energetic relaxation, as observed for Atto 647N. Ensemble ultrafast measurements compared the relaxation timescales of chromophore-containing photosynthetic proteins from cryptophytes with open and closed protein scaffolds, which have correspondingly more and less solvent accessibility. These measurements showed faster relaxation for the open scaffolds, consistent with the results described here. Other studies found that the dynamics of CPC were independent of bulk solvent, initially in contradiction with a dependence of relaxation on solvent accessibility. However, the local chromophore environment, that is, the first solvation shell, may not fully reflect changes to the bulk due to interactions such as hydrogen bonding between the protein and water molecules.

Along with differences in the median, the distributions of timescales can be used to compare the heterogeneity in energetic relaxation. Each distribution was smoothed using a kernel density estimation (Fig. 3d), and the full width at half maximum values (FWHM) of the smoothed curves were calculated, which describe the widths of the main peaks (Supplementary Fig. 11). The distribution for Atto 647N (FWHM = 179 fs) is much narrower than the distributions for APC (FWHM = 462 fs) and CPC (FWHM = 295 fs). The width for Atto 647N is similar to that measured previously for a series of other chromophores in solution, where the width was ascribed to a bimodal profile from the electronic dephasing and IVR contributions.

The wider distributions for APC and CPC suggest that, in addition to slowing energetic relaxation, the protein matrix introduces more heterogeneity in the relaxation timescale than is present in an aqueous solution.

The distribution of energetic relaxation timescales is also broader for APC (FWHM = 462 fs) than for CPC (FWHM = 295 fs). The breadth for APC may arise from increased interaction with the protein due to the more buried position of their chromophores, although the differences in polarity and torsional angle could also play a role. The distribution for APC has signatures of a bimodal structure with peaks at ~175 fs and ~375 fs, along with an elongated tail as discussed above. By contrast, the distribution for CPC has only a single broad peak with a tail. The two peaks in the distribution for APC may correspond to the previously hypothesized two conformations responsible for the 220 fs and 280 fs timescales of energy transfer from ensemble measurements. Alternatively, previous measurements found a 400 fs component of energetic relaxation exclusively associated with the α chromophore in APC. Although this component appeared clearly only upon excitation of the vibronic transition in ensemble measurements, which we do not excite here, signatures of this pathway may be contributing to the width of the measured distribution.

**Discussion**

In this work, we described spectrally tunable SM2P and the use of this tunability to disentangle the ultrafast dynamics of APC at the single-protein level. The distribution that primarily contains populations with energy transfer is clustered around the median at ~150 fs, whereas the mean—and potentially ensemble values—were lengthened due to the influence of small subpopulations. Previous
assignments of complex dynamics could instead arise from the microscopic heterogeneity, that is, non-Gaussian distribution, observed here.

Slow and heterogeneous energetic relaxation timescales for APC and CPC indicate that the protein matrix introduces variation in the photophysical processes. Because energetic relaxation is slower than energy transfer, the states involved in energy transfer are the initially excited ones, which may be one mechanism for how rapid energy transfer is maintained despite the effects of heterogeneous energetic relaxation. In this picture, the reorganization of the environment, which can lead to trapping, is preceded by energy transfer among the electronic excited states. It may also be that slow energetic relaxation in the protein helps maintain vibronic coupling during an energy transfer event, which is thought to mediate rapid transfer.

While previous work established that the electronic structure, and consequently energy transfer, was robust to protein-induced static heterogeneity, here we found that the heterogeneity also impacts the timescales of the dynamics. These studies illustrate that the ability of the protein to influence the timescales of energetic relaxation means the protein structure can control the excited states involved in energy transfer, and even the mechanism of energy transfer itself.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at [https://doi.org/10.1038/s41557-021-00841-9](https://doi.org/10.1038/s41557-021-00841-9).

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Methods

SM2P spectroscopy. A schematic of the experimental set-up is shown in Supplementary Fig. 2 and is described in detail in ref. 41. In brief, a tunable fibre laser (FemtoFiber pro, Toptica; 120 fs pulse duration, ~4 nm bandwidth, 80 MHz repetition rate) was used as the excitation source, and the laser repetition rate was reduced from 80 MHz to 2.5 MHz or 312.5 kHz with an acousto-optic pulse picker (Brimrose Corporation, FSSP-400-80-BR-800). The prism compressor was designed with single-axis translation for easy optimization with spectral maximum. The compressor was translated to minimize dispersion for each centre wavelength as measured at the sample position with an interferometric autocorrelation using a GaP photodiode (Marktecht, MTDP3650D-1.4) 

Representative intensity autocorrelations for different laser excitations are shown in Supplementary Fig. 3 with the FWHM determined assuming a Gaussian pulse. The laser pulses were split by a set of 50/50 beam splitters (Thorlabs, UBF55050) and a delay stage in a Mach–Zehnder interferometer. The polarization was converted from horizontal to circular polarization using a quartz wave plate (Newlight Photonics, WPM03-Q-VIS). The excitation was coupled into a custom-designed inverted confocal microscope (Mad City Labs, RM21) and focused onto the sample with an objective (UPLSAPO100XO, Olympus; numerical aperture, 1.4). Fluorescence emission was collected through the same objective and separated from the excitation by use of dichroic mirrors optimized for excitation filtering (Chroma, T635spxr-UF3; Chroma, ZT647rdc-UF2) and a pair of band-pass filters (Semrock, FF02-675/67-25; Chroma, ET690/120x). Fluorescence photons were detected on an avalanche photodiode (SPCM-AQRH-15, Excalibur) with a time-correlated single photon counting module (TimeFagger20, Swabian Instruments). SSMF lifetimes were fit to a single-exponential decay convolved with an experimentally determined instrument response function (~0.5 ns) as described in Supplementary Section 5.

For SM2P experiments, the power was set to ~1 pJ per pulse before the objective to 700 μJ cm⁻² per pulse at the sample plane. The saturation power was experimentally determined by monitoring the fluorescence intensity of single APC (Supplementary Section 3). The centre wavelength of the laser was tuned to either 610 nm or 645 nm. For the 610 nm measurements, experiments were performed with both near-Fourier-transform-limited temporal compression (118 fs) and no temporal compression (300 fs) due to power restraints. The delay time between the two pulses was scanned from ~1.5 ps to 1.5 ps at 100 μm s⁻¹ (0.33 ps/s). Fluorescence emission was binned into 50 ms bins before being fitted with maximum likelihood estimation to extract energy relaxation timescales. The fit function was the convolution of the measured intensity autocorrelation with an exponential rise function for energy relaxation as detailed in Supplementary Section 1 (ref. 41). The standard error was estimated using the Fisher information matrix. Single-molecule blinking and on–off transitions in SM2P traces were identified by eye and omitted from the data analysis.

Energy relaxation timescales were used to construct histograms with the bin width determined by the Freedman–Diaconis rule. The generated distributions were compared using a permutation test, which determines the likelihood of randomly allocating the complete dataset into two groups and obtaining the experimentally observed difference (Supplementary Section 6.2). Solvent-accessible surface area values were determined using the Protein interfaces, surfaces and assemblies’ service (PISA) at the European Bioinformatics Institute located online (http://www.ebi.ac.uk/prot_int/pistart.html).

Sample preparation. APC (Sigma Aldrich, S868), CPC (Agilent, PB11) and Atto 647N (ThermoFisher, 04507-1MG-F) were purchased and diluted to ~1 nM in pH 7.4 phosphate buffer solution (ThermoFisher, AM9624). An enzymatic oxygen scavenging system was added to the solution at final concentrations of 25 nM protocatechuate-3,4-dioxygenase and 2.5 mM protocatechuate acid (ref. 41). The solution was spin-coated in 1% polyvinyl alcohol onto glass coverslips, which were placed on a piezoelectric stage (Mad City Labs, Nano-LP100) on the microscope.

Data availability

The raw photon stream used to construct the single-molecule pump–probe traces and the corresponding fluorescence lifetime histograms are available at https://doi.org/10.5281/zenodo.5541825. Source data are provided with this paper.

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Author contributions

R.M., T.K. and G.S.S.-C. conceived and designed the experiments. R.M. and A.C.N. performed the experiments. R.M. and G.S.S.-C. analysed the data. R.M., T.K. and G.S.S.-C. co-wrote the paper. All authors discussed the results and commented on the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | Single-molecule pump-probe experiments on C-phycocyanin. (a) The structure of C-phycocyanin (Protein Data Bank ID Code 1GHO) is shown with a callout of a tetrapyrrole chromophore (purple). (b) The corresponding absorption (solid) and emission (dashed) spectra are shown with the 610 nm excitation shown in blue. (c–f) Representative traces for C-phycocyanin with 610 nm excitation are with values of 125 ± 2, 503 ± 124, 113 ± 29, and 270 ± 51 fs, respectively. Errors given are the standard error of the maximum likelihood estimate.
Extended Data Fig. 2 | Gaussian mixture model extracts two rate components. The distributions of energy relaxation rates from the bright (top) and quenched (bottom) populations were fit to a two-component Gaussian mixture model, which is shown in dashed lines. All parameters of the Gaussian mixture model fit are given in Supplementary Table 4.