Direct single-molecule measurements of phycocyanobilin photophysics in monomeric C-phycocyanin

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Phycobilisomes are highly organized pigment–protein antenna complexes found in the photosynthetic apparatus of cyanobacteria and rhodophyta that harvest solar energy and transport it to the reaction center. A detailed bottom-up model of pigment organization and energy transfer in phycobilisomes is essential to understanding photosynthesis in these organisms and informing rational design of artificial light-harvesting systems. In particular, heterogeneous photophysical behaviors of these proteins, which cannot be predicted de novo, may play an essential role in rapid light adaptation and photoprotection. Furthermore, the delicate architecture of these pigment–protein scaffolds sensitizes them to external perturbations, for example, surface attachment, which can be avoided by study in free solution or in vivo. Here, we present single-molecule characterization of C-phycocyanin (C-PC), a three-pigment biliprotein that self-assembles to form the midantenna rods of cyanobacterial phycobilisomes. Using the Anti-Brownian Electrokinetic (ABEL) trap to counteract Brownian motion of single particles in real time, we directly monitor the changing photophysical states of individual C-PC monomers from *Spirulina platensis* in free solution by simultaneous readout of their brightness, fluorescence anisotropy, fluorescence lifetime, and emission spectra. These include single-chromophore emission states for each of the three covalently bound phycocyanobilins, providing direct measurements of the spectra and photophysics of these chemically identical molecules in their native protein environment. We further show that a simple Förster resonant energy transfer (FRET) network model accurately predicts the observed photophysical states of C-PC and suggests highly variable quenching behavior of one of the chromophores, which should inform future studies of higher-order complexes.

photosynthetic protein | C-phycocyanin | ABEL trap | single-molecule spectroscopy | photodynamics

Photosynthetic antenna structures based on pigment–protein complexes have garnered broad interest as model systems for solar energy harvesting that can directly inform development of alternative energy technologies (1). These systems transfer absorbed energy quickly and efficiently to reaction centers, and must rapidly adapt to changing irradiance to maintain photosynthetic efficiency while protecting against oxidative damage. These macroscopic behaviors of photosynthetic antenna systems are dictated by the nanoscale heterogeneous properties of the individual proteins from which they are assembled; therefore, constructing bottom-up models will be essential to fully understanding these systems. A variety of standard single-molecule techniques have been previously used to optically characterize individual antenna complexes and their substituent components, for example at low concentration (2–4) or immobilized on a surface (5–9) or in a polymer matrix (10). However, studying photosynthetic antenna proteins at the single-molecule level remains challenging (11) due to their relatively low fluorescence yield, variable photostability, and sensitivity to external perturbations of pigment conformation or local environment. Our laboratory has developed an alternative single-molecule confinement strategy, the Anti-Brownian Electrokinetic (ABEL) trap (12), which provides confinement of individual,

untethered particles in free solution while monitoring photophysical parameters over the course of seconds or minutes. The ABEL trap is well-suited to investigate the time-dependent photophysical behavior of single photosynthetic antenna proteins and complexes, for example observing photodynamics (13) and unraveling pigment organization (14) in allophycocyanin, identifying conformational heterogeneity (15), and revealing photoprotective quenched states (16), among others (17, 18).

In this work, we have optimized the ABEL trap for simultaneous detection of brightness, polarization, emission spectrum, and fluorescence lifetime, with which we have performed a single-molecule study of the photosynthetic antenna protein C-phycocyanin (C-PC) from *Spirulina platensis* (19). C-PC is a water-soluble three-pigment protein found in phycobilisomes (20, 21), which are specialized antenna complexes (22) that expand spectral coverage, absorb additional energy, and provide photoprotection in cyanobacteria (23, 24). Recently observed “blinking” in single intact phycobilisomes, of unknown origin and not predicted de novo based on crystal structure, has been proposed to play a key role in photoprotection and light adaptation (25). C-PC self-assembles in stacks of trimers and hexamers to form the main antenna rods (26) that attach to the allophycocyanin core (20, 27). This architecture positions blue-absorbing pigments nearer to the distal ends of the rods such that excitons will be efficiently transferred toward the Photosystem II reaction center (23) in the thylakoid membrane directly below the core. The two subunits of C-PC, α and β (28), covalently bind one (α_{84}) and two (β_{84} and β_{155}) phycocyanobilin pigments (29), organized as shown in its crystal structure [Protein Data Bank (PDB) ID code 1GH0 (30)] (Fig. 1A). All three phycocyanobilins are chemically identical linear tetrapyrroles (31) with distinct photophysical properties directly conferred by their disparate conformations and chemical environments (32). The relative geometry of the three

Significance

The efficiency and adaptability of photosynthetic antenna systems in harvesting solar energy can be ultimately traced to their nanoscale organization. By monitoring the fluorescence emission from single photosynthetic antenna proteins in free solution over long timescales, we directly observed transitions among many distinct photophysical states exhibiting different flows of energy transfer among the embedded photosensitive pigments. We show that these states are well-described by a simple physical model, which allows us to identify and characterize the properties of individual chromophores and reveals surprising heterogeneity and quenching behavior that is not predicted by bulk studies.

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Fig. 1. C-PC structure and confinement in the ABEL trap. (A) Crystal structure of C-PC monomer from *S. platensis* [PDB ID code 1GH0 (30)]. (B) Geometry of the α_{84} , β_{84} , and β_{155} -chromophores, showing pairwise center-to-center and angular separations. (C) Schematic of the ABEL trap. Photons emitted by a diffusing C-PC molecule reveal its position within the laser scan pattern (Left). An electrical field is applied to the trap by fast feedback, creating a restorative electrokinetic force toward the center of the trap (Right) (41). Collected photons are additionally used to monitor brightness, spectrum, fluorescence polarization, and fluorescence lifetime.

chromophores enables significant resonant energy transfer among them (33) (Fig. 1B): Bulk ultrafast studies (34, 35) have shown that β_{155} absorbs strongly near 600 nm, and quickly transfers much of its energy to the neighboring β_{84} ($k \approx 50$ ps), while α_{84} and β_{84} absorb weakly and mutually exchange energy ($k \approx 200$ –300 ps). Energy transfer between α_{84} and β_{155} is slow (>1 ns) (36–38). In trimers and higher aggregates of C-PC, β_{84} serves as the energy sink for absorbed excitons from both α_{84} and β_{155} , and forms a conduit along the central channel of each rod to transfer energy to the phycobilisome core (39, 40).

Here, we directly resolve the multiparameter photophysical properties of each single chromophore of the C-PC monomer in the ABEL trap, along with the photophysical properties of all multichromophore states and the photodynamics of transitions among them. A computational Förster resonant energy transfer (FRET) model based on our data closely predicts these observed states, and reveals a surprising quenched state of the β_{155} -chromophore, consistent with the existence of previously unreported quenching behavior by the β_{84} -chromophore.

Results

Detection of Single C-PC Proteins in the ABEL Trap. The ABEL trap suppresses the Brownian motion of single particles in free solution using electrophoretic or electroosmotic drift forces to continuously restore the particle toward the trap center (41) (Fig. 1C and *SI Appendix*, Fig. S1). Two orthogonal pairs of electrodes immersed in the reservoirs of the microfluidic cell apply a restoring feedback force in x and y in response to the arrival of each photon. The force is based on the analyte particle position as estimated by the known position of a rapidly scanning excitation beam at the instant of photon detection. The microfluidic cell geometry in the trapping region confines the particle's z position to submicrometer range, and the scan pattern is optimized to provide time-averaged constant illumination across the trapping region. The ABEL trap can confine analytes as small as single fluorophores (42) in the trapping

volume for up to several seconds, allowing direct multiparametric observation of photophysical behavior on long timescales.

Trapped C-PC Monomers Transition Among Multiple Photophysical States. Fig. 2 shows sample raw data traces from simultaneous acquisition of the critical C-PC monomer fluorescence parameters of this study: brightness (B), polarization (P), fluorescence lifetime (τ), and emission spectrum along with the corresponding emission spectral center of mass (CM), and these four symbols will be used throughout this paper. The time trace for B (Fig. 2A) shows binned photon arrival times (counts per 5 ms). P (Fig. 2B) and τ are calculated for every 150 photons (yellow circles and cyan triangles, respectively). CM is calculated for each 20-ms frame (red squares). Additional details on data analysis are provided in *SI Appendix*.

Capture of a single molecule is indicated by a stepwise increase in brightness from the background level (Fig. 2A). While trapped, the brightness of each C-PC monomer may transition to other brightness levels, and the molecule eventually either photobleaches or is lost from the trap, marked by a stepwise return to background. The trapping events shown in Fig. 2 each exhibit one or a few distinct brightness levels, identified using a modification of the change-point method of Watkins and Yang (43). Distributions of the number of levels identified per event for different excitation intensities are shown in *SI Appendix*, Fig. S2. Most events begin with a similar, high brightness level, followed by lower brightness levels, but in some instances dim levels may transition back to brighter levels (Fig. 2A, *i*), or briefly enter a dark state before recapture (Fig. 2A, *ii*). Changes in brightness correlate to changes in the other measured parameters (Fig. 2A–E, *i–iii*), and we generally wish to understand these changes by associating all four variables to the “level” defined by constant brightness. We attribute most of these stepwise changes in brightness to photobleaching or recovery of individual chromophores; therefore, the set of observed parameters for each of these brightness levels should reflect a distinct photophysical state of the C-PC monomer, with a unique combination of chromophores participating in absorption and energy transfer.

B , P , τ , and CM Cluster into Distinct Photophysical States. Our multidimensional data for each of thousands of brightness levels contain a wealth of information about the behavior of single C-PC molecules under optical irradiation. Scatter-density plots (Fig. 3A) for B vs. P (Left), B vs. τ (Center), and B vs. CM (Right) show clustering in all projections. Here, the color of each point reflects the normalized local density of points (*SI Appendix*, Fig. S3). Each projection reveals at least six clusters, which exhibit consistent brightness across all projections.

The dimmest clusters in the B - P projection have the maximum observable polarization (44, 45) for a freely rotating single-dipole emitter using our high-N.A. objective (44, 45) (N.A. = 1.3; $P_{\max} = 0.41$; *SI Appendix*, Fig. S4), while brighter states have decreased polarization. We attribute this decrease to energy transfer among one or more nonparallel but coupled pigments. In the B - τ projection, clusters are well-separated in lifetime, with the longest and shortest lifetimes observed for the dimmest clusters. While the CM of the emission spectra shifts by only a few nanometers across all brightness levels, the greatest range of spectral CM s is also observed at the dimmest levels. These features are all consistent with a general model where the dimmer states represent single emitters, which exhibit maximum polarization and maximally diverse lifetimes and spectra, while brighter states involve multiple chromophores, resulting in lower polarization and intermediate fluorescent lifetimes and spectra.

Measured Emission States of C-PC Monomers. The observed clusters can now be quantified, and we will use the term “state” to signify our hypothesis that the level events in a cluster represent a

Fig. 4. Identification of C-PC photophysical emission states based on spectra and transitions. (A) A simple FRET model showing allowed transitions between three-, two-, and one-chromophore states. Triangle outer colors indicate the corresponding observed single-molecule clusters. State S6Q represents quenching of the β_{155} -chromophore by a putative β_{84} -chromophore. (B) The average fluorescence emission spectrum of the single-molecule pristine state S1 closely matches the bulk emission spectrum. (C) Proportion of each population that is immediately preceded by S1. (D) Average fluorescence emission spectra of S2, S3, and S4 show that S4 is the most blue-shifted compared with S1. (E) The peak-normalized change in emission spectra from S1 to S2, S3, and S4 ($\Delta E_m = E_{m_{S1}} - E_{m_{next}}$) shows that S2 and S4 gain emission at ~ 615 nm and lose 640–650 nm emission, while S3 is similar to S1. (F) Probability of transitions from S5, S6, and S7 to each of the other states. (G) Fluorescence emission spectra for S5, S6, and S7, corresponding to α_{84} , β_{84} , and β_{155} , respectively. (H) Proportion of each single-chromophore population that is immediately preceded by S2 ($\alpha_{84} + \beta_{155}$), S3 ($\alpha_{84} + \beta_{84}$), or S4 ($\beta_{84} + \beta_{155}$). All data are assigned to GMM components as described in *Methods* and *SI Appendix*. Spectra are fit with two-component Gaussian models.

turn photobleach into two of the three possible single-chromophore states. If the dark state is irreversible for each chromophore, then we would expect all trapped molecules to exhibit only three states of decreasing brightness, caused by three successive photobleaching events. However, it is clear that C-PC monomers occasionally transition back to higher brightness levels (Fig. 24), indicating that photorecovery is possible for at least some dark states.

The Pristine Monomer (S1). We assign the brightest observed GMM population (S1) to be the “pristine” state of the monomer with all three pigments intact. This population is the most frequently observed initial state within each trapping event ($>70\%$ at intermediate excitation), and its spectrum closely matches the bulk spectrum (35) of C-PC (Fig. 4B). We observe $\tau_{S1} = 1.22$ ns, qualitatively consistent with the bulk fluorescence lifetime [typically best fit by three to four components (48), of which the longest are ~ 0.8 and 1.4 ns]. The polarization of S1, $P_{S1} = 0.23$, also closely matches our measured bulk polarization (*SI Appendix*, Fig. S8).

Two-Chromophore States (S2, S3, and S4). Fig. 4C shows the experimental proportion of each state that is immediately preceded by S1. Large proportions of levels assigned to states S2, S3, and S4 are the result of transitions from S1, suggesting that they represent the two-chromophore states. We assign S2 to be the $\alpha_{84} + \beta_{155}$

state, shown here in green, primarily on the basis of its brightness and spectrum relative to the pristine state. It has been previously shown that β_{155} acts as a sensitizer for β_{84} (32, 49); if β_{84} is dark or photobleached, then β_{155} will emit rather than transfer the bulk of its absorbed energy. Therefore, we expect the $\alpha_{84} + \beta_{155}$ state to be the brightest of the three two-chromophore states, as observed here for S2. It is also known that both the absorption and emission spectra of β_{155} are ~ 20 nm blue-shifted compared with the α_{84} - or β_{84} -spectra (32, 35), so the loss of the β_{84} -pigment should result in a blue-shifted emission spectrum relative to the pristine state, as observed here for S2 (Fig. 4D).

States S3 and S4 are assigned to be the $\alpha_{84} + \beta_{84}$ and $\beta_{84} + \beta_{155}$ states, respectively, as detailed below. The emission spectra for S2, S3, and S4 (Fig. 4D) all appear to be roughly similar to S1, but examination of the change in emission spectrum, ΔE_m , between S1 and each of these states reveals clear differences among them (Fig. 4E): S2 gains blue emission and loses red emission relative to S1, S3 is nearly identical to S1, and S4 gains a small amount of blue emission relative to S1.

Single-Chromophore States (S5, S6, and S7). We assign states S5, S6, and S7 to be single chromophores based upon their low brightness, high polarization, and the observed transitions to and from these states. The polarization of S5 and S7 is consistent with that of a single-dipole emitter (*SI Appendix*, Table S1). Fig. 4F shows the probability of transition from states S5 (Left), S6 (Center), and S7 (Right): the majority of transitions from all three states go to the background level, consistent with photobleaching of a single pigment. In each case, we also observe some transitions to brighter levels, along with a small percentage of self-transitions where a change point was identified, but the next level was assigned to the same population.

The emission spectra for S5, S6, and S7 are shown in Fig. 4G. We assign S6 to be β_{155} on the basis of its blue-shifted spectrum relative to S5 and S7; additional complications regarding S6 are addressed below and in *Quenching of β_{155} by β_{84} in S6*. S5 and S7 are assigned to be α_{84} and β_{84} , respectively, based on their relative brightness: We expect the α_{84} -chromophore to be brighter than the β_{84} -chromophore because α_{84} has been previously shown to have higher quantum yield and absorption at this excitation wavelength (35, 50). This is further supported by the observed transitions from the two-chromophore states to S5, S6, and S7. Fig. 4H shows the proportion of S5, S6, and S7 levels that are immediately preceded by S2 (Left), S3 (Center), or S4 (Right). If S2 is indeed the $\alpha_{84} + \beta_{155}$ state, then S5 must be the α_{84} -chromophore, since S6 has been assigned to the β_{155} -chromophore. By process of elimination, S7 must be the β_{84} -chromophore. The transitions shown in Fig. 4H also allow us to clearly assign S3 and S4 to the $\alpha_{84} + \beta_{84}$ and $\beta_{84} + \beta_{155}$ states, respectively.

Of the identified states, S6 shows unusually high variance in its lifetime and emission spectrum, and has a relatively small population. We have assigned it to β_{155} because it is the only population which shows a blue-shifted spectrum, and because our GMM fits indicate the presence of a statistically significant population at S6. However, we cannot discount the possibility that S6 either (i) does not represent a physically relevant single-chromophore state; that is, our data do not ever capture a long-lived β_{155} -only state, or (ii) arises from multiple rare but distinct photophysical phenomena, which we have incorrectly classified as a single state. The former would require an alternative explanation for the blue-shifted emission of the long-lived levels comprising S6, for example unspecified conformational changes in either the α_{84} - or β_{84} -chromophores, for which we have no direct evidence. Similarly, we have no clear basis for speculation on what processes might result in the latter case. The observation of the long-lived blue-shifted states in S6 can be most parsimoniously explained by emission from the β_{155} -chromophore.

Numerical Modeling to Understand the Observed Clusters. As a check to see whether this simple photobleaching-based model appropriately describes the photophysical behavior of the C-PC monomer, we calculated the predicted B , P , τ , and emission spectrum (CM) for each state in the model, presented in *SI Appendix, Table S2*. Briefly, each chromophore was treated as a member of a FRET network, and the time-dependent probability of exciton residence at each participating chromophore was calculated for the cases of absorption at each participating chromophore to determine the weighted overall photophysical properties of that state (14) (*Methods* and *SI Appendix*). Fig. 5 shows the close agreement between these predictions and the experimentally observed states reported in Fig. 3*B* and *SI Appendix, Table S1*. We find that the optimized FRET model parameters which best fit our experimental data are largely in agreement with literature values (*SI Appendix, Tables S3 and S4*) in terms of relative quantum efficiencies, absorbance, and energy transfer rates. The fastest predicted energy transfer rate is ~ 75 ps, from β_{155} to β_{84} , in keeping with the fastest component fit to bulk lifetime measurements by multiple groups, who also attributed it to this transfer pair. Transfer from α_{84} to either of the other chromophores is slow (~ 500 ps and ~ 14 ns to β_{84} and β_{155} , respectively), also in keeping with the known photophysical behavior of C-PC.

One unexpected aspect of our model is that we have assigned $\tau_{\beta_{84}} = 0.87$ ns based on direct measurements of the population believed to be the β_{84} -only state (S_7). In previous studies of C-PC, most carried out in other species of cyanobacteria, there has been significant disagreement over the origin of the ~ 900 -ps lifetime component, as described in *SI Appendix*. However, we find that assigning a short lifetime to β_{84} is absolutely necessary to explain key aspects of our experimental data for *S. platensis*. First, the observed τ_{S_2} is clearly longer than τ_{S_1} , and there are multiple supporting arguments in favor of S_2 representing the $\alpha_{84} + \beta_{155}$ state, including its blue-shifted spectrum, its relatively high brightness, and the single-chromophore states to which it transitions, as discussed above. Second, the lifetime of the $\alpha_{84} + \beta_{84}$ state (τ_{S_3}) is much shorter than would be expected if $\tau_{\beta_{84}} \approx \tau_{\alpha_{84}}$. Therefore, β_{84} must have the shortest of the three fundamental lifetimes, at least in *S. platensis* monomers. Note that only by the

type of measurements reported here can the lifetimes of individual pigments be directly measured in an intact protein.

Quenching of β_{155} by β_{84} in S_6 . Because of the high variance and low confidence of the observed properties of S_6 , as discussed above, we initially left B_{S_6} and τ_{S_6} as free parameters in our numerical model and let the information from the other states predict state S_6 (light-blue triangle, state S_6b in Fig. 5 and *SI Appendix, Table S2*). However, S_6b did not match our observations, with both a longer lifetime (1.4 ns) and brighter level (0.5) than the S_6 component of the GMM fits to experimental data. We denote the observed S_6 state as S_6a , *vide infra*. Conformational fluctuations or quenching are two possible sources of the observed high variance in lifetime and brightness of S_6 . Furthermore, it has been previously shown in allophycocyanin (APC), a protein with similar structure to C-PC, that its β -pigment (structurally analogous to the β_{84} -pigment in C-PC) may act as a quencher (14). In C-PC, the β_{155} -pigment transfers much of its absorbed energy to the β_{84} -pigment, so any quenching behavior by β_{84} would strongly affect β_{155} . Therefore, we add to the model a scenario where the β_{84} -pigment completely quenches the β_{155} -pigment in C-PC (navy triangle, new state S_6c in Fig. 5 and *SI Appendix, Table S2*). This prediction also does not fit the observed parameters for S_6 , but the predictions for S_6b and S_6c clearly demarcate a linear range of parameters (light-blue ovals, Fig. 5) representing varying degrees of quenching of β_{155} by β_{84} . Although this simple modification to our model does not explain the variation observed in the emission spectrum of S_6 , it does appear to capture much of the observed variation in B_{S_6} and τ_{S_6} . We find that adjusting $k_{\beta_{155} \rightarrow \beta_{84}}$ to roughly 2% of its S_6b (maximum) value closely reproduces the brightness, lifetime, and CM that were experimentally observed for S_6 (bright-blue triangle, presented as state S_6a in Fig. 5 and *SI Appendix, Table S2*).

One might reasonably expect the spectroscopic characteristics of β_{84} to change when it acts as a quencher. Here, we have assumed that β_{84}^Q acts as a quencher with a quantum efficiency of 0, and apply a range of possible transfer rates from β_{155} to β_{84}^Q to represent its altered absorption. If instead β_{84}^Q is assigned a nonzero quantum efficiency, then we expect it to emit weakly and perhaps variably. Even without precise knowledge of the exact spectroscopic characteristics of β_{84}^Q , this is one possible explanation for the observed spectral variation in S_6 . We further expect that a β_{84}^Q -pigment might not only affect β_{155} , but could also produce quenched states of the α_{84} -pigment (S_5) and of the $\alpha_{84} + \beta_{155}$ state (S_2). However, neither of these populations was directly identified in this study. This may be because these states are either short-lived, infrequent, exhibit large variance, or are obscured by or indistinguishable from other observed populations.

Conclusion

Long-timescale simultaneous measurements of the brightness, polarization, lifetime, and emission spectra of single monomeric C-phycocyanin reveal seven distinct photophysical states that can be directly matched to the states predicted by a simple FRET network model. Three of the states correspond to the individual α_{84} -, β_{84} -, and β_{155} -chromophores, which exhibit diverse emission characteristics in the coupled protein environment despite being chemically identical. Numerical calculations of the predicted parameters for each state in our model faithfully reproduce all but one of the experimentally observed photophysical states. Addition of a β_{155} -state that is quenched by the β_{84} -pigment (β_{84}^Q) fully aligns the model predictions with our data.

In the phycobilisome, the β_{84} -pigment is known to serve as the primary acceptor that transfers energy down the central channel of each C-PC rod to the APC phycobilisome core (39, 40). We speculate that the existence of an alternative quenching state of this chromophore, β_{84}^Q , could have biological implications for modulation of energy transfer to the reaction center. Established

Fig. 5. FRET network model fit to experimental data. Triangles show the predicted properties of each photophysical state (triangles) based on global fitting to the FRET network model. Lines indicate the measured values from the GMM. (A) Brightness B vs. fluorescence polarization P . (B) Brightness B vs. fluorescence lifetime τ . (C) Brightness B vs. spectral CM of the fluorescence emission spectrum over the range (615–665 nm). Quenching of the β_{155} -chromophore by the α_{84} -chromophore was modeled both to fit the observed populations (state S_6a , blue), and to determine the range of properties resulting from zero quenching (state S_6b , light blue) to complete quenching (state S_6c , navy) by tuning the forward transfer rate from β_{155} to β_{84} . Modeled properties for S_2 and S_4 both use S_6b (no quenching) as the β_{155} -component. Optimized model values are given in *SI Appendix, Table S2*.

photoprotection mechanisms in the phycobilisome (1) include genetic regulation and structural modification of the phycobilisome, which occur on long timescales, and quenching of the APC core by photoactivated orange carotenoid protein, which responds to high light conditions over the course of several seconds. If β_{84}^Q is also found to exist in higher-order aggregates of C-PC, it could provide a fast-timescale “fuse” to enable dissipation of excess energy in the phycobilisome, a possibility that merits future investigation.

Our goal has been to generate a simple model for the observed single-molecule photophysical states of C-phycocyanin with enough complexity to capture the key photophysical behaviors in this system while avoiding a large number of extraneous parameters. As one might expect, the model we present here may not uniquely describe the behavior of monomeric C-PC, but we hope it will provide a useful framework to guide future thinking.

Methods

Full details of the materials and methods are given in *SI Appendix*. Briefly, the ABEL trap (Fig. 1C and *SI Appendix*, Fig. S1) was implemented as previously described (14, 42), with pulsed polarized excitation at 594 nm. Single-photon arrival times and delays were collected for two polarization channels, and spectra were collected by dispersion on an EMCCD. C-phycocyanin monomers were obtained by dilution to 5 pM of purified C-PC from *Spirulina* sp. (#PB11, Prozyme). For the numerical model, the predicted B , P , τ , and CM for each photophysical state were calculated by directly modeling the time evolution of exciton residence probability at each pigment site under initial conditions of absorption at each pigment site, which were varied under a constrained minimization to optimize agreement between the model and the data. All figure data are available on request.

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- Blankenship RE (2014) *Molecular Mechanisms of Photosynthesis* (Wiley-Blackwell, Oxford), 2nd Ed, pp 41–82, 237–243.
- Chen D, Dovichi NJ (1996) Single-molecule detection in capillary electrophoresis: Molecular shot noise as a fundamental limit to chemical analysis. *Anal Chem* 68: 690–696.
- Mathies RA, Peck K, Stryer L (1990) Optimization of high-sensitivity fluorescence detection. *Anal Chem* 62:1786–1791.
- Keller RA, et al. (1996) Single-molecule fluorescence analysis in solution. *Appl Spectrosc* 50:12A–32A.
- van Oijen AM, Ketelaars M, Kohler J, Aartsma TJ, Schmidt J (1999) Unraveling the electronic structure of individual photosynthetic pigment-protein complexes. *Science* 285:400–402.
- Tietz C, et al. (2001) Single molecule spectroscopy on the light-harvesting complex II of higher plants. *Biophys J* 81:556–562.
- Hildner R, Brinks D, Nieder JB, Cogdell RJ, van Hulst NF (2013) Quantum coherent energy transfer over varying pathways in single light-harvesting complexes. *Science* 340:1448–1451.
- Gerster D, et al. (2012) Photocurrent of a single photosynthetic protein. *Nat Nanotechnol* 7:673–676.
- Saga Y, Shibata Y, Tamiaki H (2010) Spectral properties of single light-harvesting complexes in bacterial photosynthesis. *J Photochem Photobiol Photochem Rev* 11:15–24.
- Kunz R, et al. (2012) Exciton self trapping in photosynthetic pigment-protein complexes studied by single-molecule spectroscopy. *J Phys Chem B* 116:11017–11023.
- Kondo T, Chen WJ, Schlau-Cohen GS (2017) Single-molecule fluorescence spectroscopy of photosynthetic systems. *Chem Rev* 117:860–898.
- Cohen AE, Moerner WE (2005) Method for trapping and manipulating nanoscale objects in solution. *Appl Phys Lett* 86:093109.
- Goldsmith RH, Moerner WE (2010) Watching conformational- and photo-dynamics of single fluorescent proteins in solution. *Nat Chem* 2:179–186.
- Wang Q, Moerner WE (2015) Dissecting pigment architecture of individual photosynthetic antenna complexes in solution. *Proc Natl Acad Sci USA* 112:13880–13885.
- Schlau-Cohen GS, Wang Q, Southall J, Cogdell RJ, Moerner WE (2013) Single-molecule spectroscopy reveals photosynthetic LH2 complexes switch between emissive states. *Proc Natl Acad Sci USA* 110:10899–10903.
- Schlau-Cohen GS, et al. (2015) Single-molecule identification of quenched and unquenched states of LHClI. *J Phys Chem Lett* 6:860–867.
- Bockenhauer SD, Wang Q, Moerner WE (2012) Spectrally resolved anti-Brownian electrokinetic (ABEL) trapping of single peridinin-chlorophyll-proteins in solution. *Proc SPIE* 8427:84274C.
- Bockenhauer SD, Moerner WE (2013) Photo-induced conformational flexibility in single solution-phase peridinin-chlorophyll-proteins. *J Phys Chem A* 117:8399–8406.
- Boussiba S, Richmond AE (1979) Isolation and characterization of phycocyanins from the blue-green alga *Spirulina platensis*. *Arch Microbiol* 120:155–159.
- Adir N (2005) Elucidation of the molecular structures of components of the phycobilisome: Reconstructing a giant. *Photosynth Res* 85:15–32.
- Gantt E (1975) Phycobilisomes: Light-harvesting pigment complexes. *Bioscience* 25:781–788.
- MacColl R (1998) Cyanobacterial phycobilisomes. *J Struct Biol* 124:311–334.
- Glazer AN (1985) Light harvesting by phycobilisomes. *Annu Rev Biophys Biophys Chem* 14:47–77.
- Berns DS, MacColl R (1989) Phycocyanin in physical chemical studies. *Chem Rev* 89: 807–825.
- Gwizdala M, Berera R, Kirilovsky D, van Grondelle R, Krüger TP (2016) Controlling light harvesting with light. *J Am Chem Soc* 138:11616–11622.
- Glazer AN (1982) Phycobilisomes: Structure and dynamics. *Annu Rev Microbiol* 36: 173–198.
- Marx A, Adir N (2013) Allophycocyanin and phycocyanin crystal structures reveal facets of phycobilisome assembly. *Biochim Biophys Acta Bioenerg* 1827:311–318.
- MacColl R, Berns DS, Koven NL (1971) Effect of salts on C-phycocyanin. *Arch Biochem Biophys* 146:477–482.
- Dale RE, Teale FW (1970) Number and distribution of chromophore types in native phycobiliproteins. *Photochem Photobiol* 12:99–117.
- Wang XQ, et al. (2001) Structure of C-phycocyanin from *Spirulina platensis* at 2.2 Å resolution: A novel monoclinic crystal form for phycobiliproteins in phycobilisomes. *Acta Crystallogr D Biol Crystallogr* 57:784–792.
- Glazer AN (1977) Structure and molecular organization of the photosynthetic accessory pigments of cyanobacteria and red algae. *Mol Cell Biochem* 18:125–140.
- Mimuro M, Rübner R, Fuglistaller P, Zuber H (1986) The microenvironment around the chromophores and its changes due to the association states in C-phycocyanin isolated from the cyanobacterium *Mastigocladus laminosus*. *Biochim Biophys Acta Bioenerg* 851:447–456.
- Arnold W, Oppenheimer JR (1950) Internal conversion in the photosynthetic mechanism of blue-green algae. *J Gen Physiol* 33:423–435.
- Xia A, et al. (1993) Time-resolved polarized absorption of C-phycocyanin from the cyanobacterium *Westiellopsis prolifica*. *J Photochem Photobiol B* 19:111–117.
- Debreczeny MP, Sauer K, Zhou J, Bryant DA (1993) Monomeric C-phycocyanin at room temperature and 77 K: Resolution of the absorption and fluorescence spectra of the individual chromophores and the energy-transfer rate constants. *J Phys Chem* 97: 9852–9862.
- Debreczeny MP, Sauer K, Zhou J, Bryant DA (1995) Comparison of calculated and experimentally resolved rate constants for excitation energy transfer in C-phycocyanin. 1. Monomers. *J Phys Chem* 99:8412–8419.
- Sauer K, Scheer H, Sauer P (1987) Förster transfer calculations based on crystal structure data from *Agmenellum quadruplicatum* C-phycocyanin. *Photochem Photobiol* 46:427–440.
- Sauer K, Scheer H (1988) Excitation transfer in C-phycocyanin. Förster transfer rate and exciton calculations based on new crystal structure data for C-phycocyanins from *Agmenellum quadruplicatum* and *Mastigocladus laminosus*. *Biochim Biophys Acta Bioenerg* 936:157–170.
- Schirmer T, et al. (1986) Crystal structure analysis and refinement at 2.5 Å of hexameric C-phycocyanin from the cyanobacterium *Agmenellum quadruplicatum*. The molecular model and its implications for light-harvesting. *J Mol Biol* 188:651–676.
- Holzwarth AR, Bittersmann E, Reuter W, Wehrmeyer W (1990) Studies on chromophore coupling in isolated phycobiliproteins: III. Picosecond excited state kinetics and time-resolved fluorescence spectra of different allophycocyanins from *Mastigocladus laminosus*. *Biophys J* 57:133–145.
- Wang Q, Goldsmith RH, Jiang Y, Bockenhauer SD, Moerner WE (2012) Probing single biomolecules in solution using the anti-Brownian electrokinetic (ABEL) trap. *Acc Chem Res* 45:1955–1964.
- Wang Q, Moerner WE (2013) Lifetime and spectrally resolved characterization of the photodynamics of single fluorophores in solution using the anti-Brownian electrokinetic trap. *J Phys Chem B* 117:4641–4648.
- Watkins LP, Yang H (2005) Detection of intensity change points in time-resolved single-molecule measurements. *J Phys Chem B* 109:617–628.
- Koshioka M, Sasaki K, Masuhara H (1995) Time-dependent fluorescence depolarization analysis in three-dimensional microspectroscopy. *Appl Spectrosc* 49:224–228.
- Axelrod D (1979) Carboxyanine dye orientation in red cell membrane studied by microscopic fluorescence polarization. *Biophys J* 26:557–573.
- Schwarz G (1978) Estimating the dimension of a model. *Ann Stat* 6:461–464.
- Roy R, Hohng S, Ha T (2008) A practical guide to single-molecule FRET. *Nat Methods* 5: 507–516.
- Teale FW, Dale RE (1970) Isolation and spectral characterization of phycobiliproteins. *Biochem J* 116:161–169.
- Glazer AN, Fang S, Brown DM (1973) Spectroscopic properties of C-phycocyanin and of its alpha and beta subunits. *J Biol Chem* 248:5679–5685.
- Grabowski J, Gantt E (1978) Photophysical properties of phycobiliproteins from phycobilisomes: Fluorescence lifetimes, quantum yields, and polarization spectra. *Photochem Photobiol* 28:39–45.