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Phycocyanin: One Complex, Two States, Two Functions

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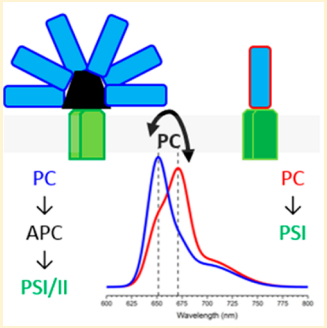
Supporting Information

ABSTRACT: Solar energy captured by pigments embedded in light-harvesting complexes can be transferred to neighboring pigments, dissipated, or emitted as fluorescence. Only when it reaches a reaction center is the excitation energy stabilized in the form of a charge separation and converted into chemical energy. Well-directed and regulated energy transfer within the network of pigments is therefore of crucial importance for the success of the photosynthetic processes. Using single-molecule spectroscopy, we show that phycocyanin can dynamically switch between two spectrally distinct states originating from two different conformations. Unexpectedly, one of the two states has a red-shifted emission spectrum. This state is not involved in energy dissipation; instead, we propose that it is involved in direct energy transfer to photosystem I. Finally, our findings suggest that the function of linker proteins in phycobilisomes is to stabilize one state or the other, thus controlling the light-harvesting functions of phycocyanin.



The photosynthetic apparatus efficiently stabilizes solar energy even under low light intensity thanks to numerous light-absorbing pigments embedded in the light-harvesting complexes. The energy harvested by these complexes is transferred in a highly controlled manner[1] to the photosynthetic reaction centers, where it is converted by a few specialized pigments into a physical separation of charges accessible for other biochemical processes.[2] The diversity of light-harvesting complexes, which allows various photosynthetic organisms to occupy different environmental niches, derives particularly from their protein and pigment composition, size, and cellular location.

The handlike structure of *Synechocystis* PCC6803 (hereafter *Synechocystis*) phycobilisomes (PBs) covalently binds nearly 400 chemically identical linear tetrapyrrole pigments, namely phycocyanobilins.[3−8] Up to 324 of them are embedded in the most blue-shifted part of the PB complexes, the rods composed of phycocyanin (PC). Upon illumination, excitation energy from the rods is transferred to the central core. Besides 68 pigments characterized by an emission maximum at ∼660 nm, the core carries a few pigments with emission shifted an additional 20 nm into the red, the so-called terminal emitters. The terminal emitters are usually responsible for funneling excitation energy to the neighboring photosystems, located inside the thylakoid membranes.[3,9−11] Their red-shifted emission provides a large overlap between the emission from a PB and the absorption of the photosystems, ensuring efficient excitation energy transfer from PB to the photosystems. However, since the majority of PB's pigments are bound to the rods, the rods play the most significant role in solar energy harvesting. With a room-temperature emission maximum at ∼650 nm, PC is well-tuned to transfer excitation energy to the core of PB, which has an absorption maximum at the same wavelength (i.e., 650 nm).[10−13]

Because of the distances separating individual phycocyanobilins in the PB complexes,[14] their spectroscopic properties are predominantly determined by the protein matrix forming pigment binding sites. The protein matrix is responsible for tuning the absorption and emission maxima over a range of 40 and 30 nm, respectively, for different components of PBs (i.e., comparing the rods and terminal emitters).[3−5,7] On top of the heterogeneity in the steady-state spectral properties of PB, our recent spectroscopic characterization at the level of single complexes has identified the dynamics of emission and a new intrinsic light-activated nonradiative energy decay channel from PB, related to protein conformational changes in the complex.[15] Other reports confirm that phycobiliproteins, and in particular PC, can dynamically respond to changes in the environment in extreme conditions.[16,17] Here, we are reporting another type of spectral dynamics of PC rods, isolated from the ΔAB mutant[18] of *Synechocystis*, and its potential physiological importance for the recently reported light-harvesting processes in photosystem I (PSI).[19]

The genes encoding for the core of PB are not present in the ΔAB mutant;[18] hence, only the rods made up of PC or the subunits of PC rods can be isolated from this strain. In our study, we isolated a homogeneous fraction of PC rods, characterized by an absorption spectrum with the main peak

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near 620 nm and a clear blue shoulder (Figure S1), identical to the one reported previously for the PC rods.[18] Moreover, rods isolated from this mutant bound all linker proteins but two: CpcG1 or CpcG2.[18] The former is the rod−core linker protein involved in binding of the rods to the core, necessary to form a complete PB complex.[20] Notably, recent reports showed that PC-CpcG2, instead of being a component of a PB complex, is bound directly to PSI as a rod vertically protruding from the plane of the thylakoid membrane.[19,21−23] PC-CpcG2 was proposed to function as a light-harvesting complex for PSI, transferring excitation energy directly from PC to PSI; however, the ensemble emission maximum of PC at ∼650 nm made us question the efficiency of such a transfer due to limited spectral overlap. Although previous analysis of the fluorescence emitted from mutants lacking the individual CpcG genes showed shifts in the fluorescence peak position,[23] our single-molecule spectroscopy analysis clearly shows a remarkable spectral flexibility of PC missing both CpcGs, which strongly suggests distinct physiological roles of the two newly identified conformations of PC characterized by different spectra.

Two spectrally different types of phycocyanobilin pigments are embedded in PC, partly explaining the broad ensemble fluorescence emission peak (Figure 1). While the pigments

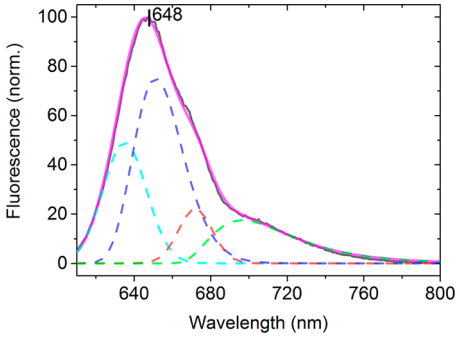

Figure 1. Steady-state ensemble fluorescence emission spectrum of PC isolated from the ΔAB mutant of *Synechocystis* upon excitation at 590 nm at room temperature (black). The spectrum was normalized to 100 at the emission maximum of 648 nm. For the fit (magenta), the same peak positions at 651 (dashed blue), 672 (dashed red), and 697 nm (dashed green); widths; and skewness were used as for the single-molecule spectra in Figure 2B,C, together with a band representing the blue pool of pigments emitting at 635 nm (dashed cyan). PC rods were dissolved in 0.8 M K-phosphate buffer at pH 7.5, containing ∼0.5 M sucrose from a sucrose gradient.

bound to Cys155 in the β-subunits of the PC monomers are characterized by steady-state emission at ∼635 nm, the emission from the remaining two-thirds of pigments is expected close to the main fluorescence peak at 648 nm.[11,13] The spectral broadening on the red side of the main peak cannot be assigned to any specific pool of pigments and is poorly understood, in particular in the range between 650 and 685 nm. Emission shifted further to the red (> ∼695 nm) has been proposed to be correlated with light-activated quenched states.[15]

Using single-molecule spectroscopy, we explored the spectroscopic properties of individual PC rods at room temperature, with the goal to explain the origin of the broadening between 650 and 685 nm in the bulk emission spectrum at room temperature. To our surprise, apart from the narrow "blue" spectra emitted by single PC rods, with peak position characteristic for the bulk emission of PC rods at ∼650 nm (Figure 2A), we identified two other quasistable spectral

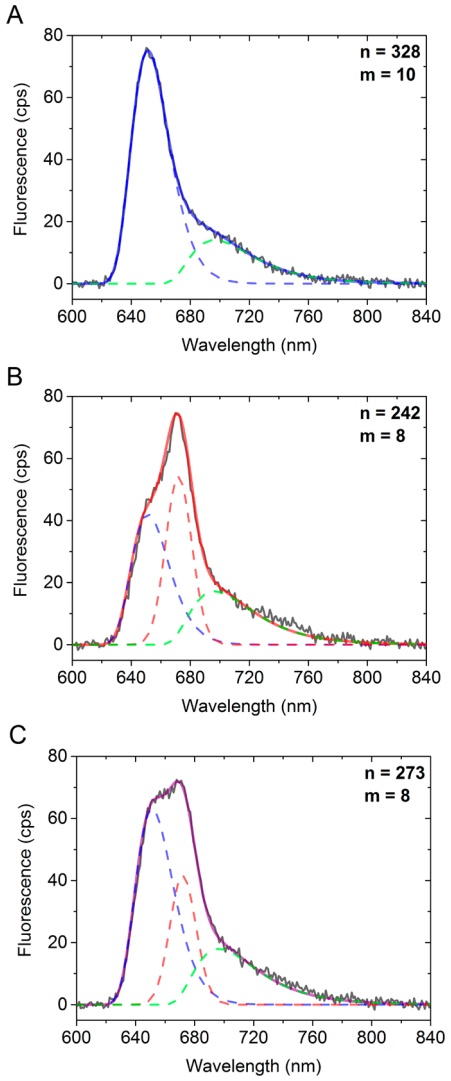

Figure 2. Average single-molecule florescence emission spectra (black) of isolated PC at room temperature upon excitation at 594 nm. Three typical spectral shapes were identified: (A) "blue", (B) "red", and (C) "double". First, the spectrum in panel A was fitted with two skewed Gaussians. Thereafter, the spectra in panels B and C were fitted simultaneously, using the same peak positions, widths, and skewnesses of the bands resolved in panel A (blue and green dashed), and an additional skewed Gaussian (dashed red) with the same peak position, width, and skewness in panels B and C. Three skewed Gaussian functions were required for a satisfactory fit, with peak positions at 651, 672, and 697 nm. The spectra are averages over *n* spectra, each integrated over 1 s, from *m* individually measured complexes.

shapes, all of similar intensities (Figure 2B,C). Displayed in Figure 2B is an average single-molecule "red" spectrum, characterized by the main peak near 670 nm and a clear shoulder near 650 nm. The third spectral shape, shown in Figure 2C, is broader across its whole amplitude and seems to derive from the combination of the two other spectral shapes. We therefore refer to it as "double". While the "blue" average spectrum can be fitted very well using only two skewed Gaussians, the "red" and "double" spectral shapes required a third skewed Gaussian. All fitting parameters were the same across the three spectra, except the relative amplitudes of the skewed Gaussian functions, and one less Gaussian function was used to fit the "blue" spectral shape. It is noteworthy that the

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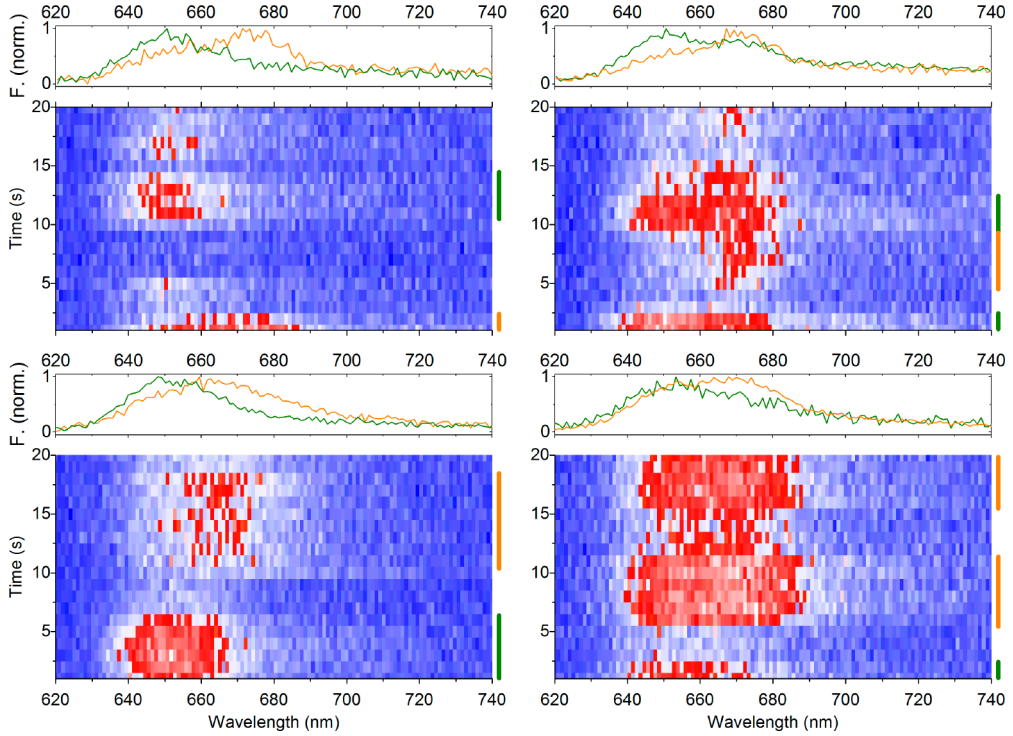

Figure 3. Four examples of single PC complexes showing spectral switching during the measurement. For clarity, a window of 20 s, showing 20 consecutive spectra, is displayed. Normalized spectral shapes are shown on top, averaged over the time indicated by the color-coded bars on the right.

amplitude of the 697 nm band was the same for the three spectral shapes; only the relative amplitude of the 651 and 672 nm bands changed. Specifically, in the blue spectrum (Figure 2A), the 672 nm band appears to be absent, in the red spectrum (Figure 2B), the 672 nm band dominates the spectrum while the 651 nm band appears as a blue shoulder, and in the double spectrum (Figure 2C), the 651 nm band is dominant again while the 671 nm band features as a clear additional peak.

The intensity ratio of the resolved 651 and 672 nm bands is 53%:47% and 70%:30% for the red and double spectral shape, respectively. Curiously, according to the Boltzmann distribution at 23 °C, where the measurements were taken, identical ratios are obtained when considering a pool of 24 pigments of which 2 and 1 emit at 672 nm and the rest at 651 nm, respectively. This suggests that the red and double spectral shapes can be quantitatively explained by 1 red pigment per hexamer and 1 red pigment per 2 hexamers, respectively. Since the presence of the 672 nm band was correlated with the decrease in intensity of the 651 nm band, the red-emitting pigment was assumed to derive from the two-thirds of PC pigments that are responsible for emission near 651 nm.

The bulk emission spectrum was fitted well with the same three skewed Gaussians as for the single-molecule spectra, i.e., using the same peak positions, widths, and skewnesses (Figure 1). This shows that the red-shifted emission with a maximum at 672 nm is a component contributing to the broad bulk spectrum at room temperature. In addition, the weighted average of all three spectral shapes closely matches the bulk emission spectrum from PC rods (Figure S2), confirming that the presence of red spectra was not artificially induced by factors stemming from the experimental technique. Since 74% of the complexes displayed the blue spectral form shown in Figure 2A, its shape dominates the average spectrum.

Fluorescence spectral sequences measured from individual complexes revealed that PC rods switch reversibly between the red, blue, and double spectral forms (Figure 3). The switching, taking place instantaneously on the time scales accessible by our measurements, signifies that the distinct spectral forms are not deriving from a static heterogeneity in the sample and that different spectral states are available for any PC rod. Interestingly, in the presence of 0.5% and 1% (v/v) glutaraldehyde, a chemical cross-linker, the number of observed switching events dropped by 36% and 70%, respectively. As reported previously,[15] the decrease in switching rates in single-molecule spectroscopy measurements in the presence of a cross-linker indicates the involvement of conformational changes. A cross-linker creates a scaffold on the structure of a protein complex, like a plaster on an injured limb, limiting the conformational freedom of the complexes. Therefore, the three spectral shapes discussed here derive from different conformations that PC can assume reversibly. Since each conformation stabilizes a number of pigments in the states responsible for the red or blue emission we will refer to them as the blue and red conformations.

One possible molecular mechanism behind the switching between the conformations could involve minor alterations of the assembly state of PC in the rods. According to a previous study, the assembly state impacts the emission properties of PC; however, none of the previously reported spectra of different assembly states of PC were similar to the red spectrum revealed in this study.[24] It is more likely that the conformational changes behind the spectral switching could induce reversible protonation of 1 or 2 pigments in the PC rods.[25] Protonation of linear tetrapyrroles is expected to enhance absorption in the far-red part of the spectrum,[26] presumably also leading to red-shifted emission.

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A clear double-band shape of the emission spectrum collected at 77 K (Figure S3) confirms the presence of two conformational forms of PC. Since the emission takes place predominantly from the lowest-energy pigments, due to little available thermal energy at 77 K, the occurrence of two well-resolved, distinct bands corroborates the presence of two different populations of weakly connected or disconnected emitters in the sample. At 77 K these populations were trapped in two conformations, giving rise to two bands. The fact that the peak position of the red band is at 664 nm and not at 672 nm like at room temperature suggests that lowering of the temperature causes a spectral shift of emission in PC, in agreement with a previous report on PC monomers.[27]

Since the average blue and red spectra at room temperature represent two distinct blue and red conformations of PC, we aimed at characterizing them further by fitting all single-molecule emission spectra of PC rods with two skewed Gaussians in the spectral window between 640 and 700 nm and with an additional skewed Gaussian accounting for the far-red states at ~695 nm.[15] Due to the characteristics of the optical components in the utilized experimental setup, fitting of individual spectra did not require an additional band for the blue pool of PC pigments emitting at 635−640 nm. In contrast to a previous report,[28] in which pigments were intentionally photobleached, under the experimental conditions used here, in particular because of a relatively low laser intensity of 4.3 W/cm^2, photobleaching of the pigments in this blue pool was unlikely. Instead, most emission from the blue pigment pool was removed to fully filter out excitation light from the emission signal. The remaining contribution from the blue-shifted pigments is included in the main blue peak with maximum at ~650 nm. Overall, this approach led to a satisfactory fit of all analyzed spectra of PC. Keeping all spectral parameters free, the fits provided a broad distribution of the spectral properties, and we focused in particular on the two bands assigned as dominating in the conformational forms of the PC rods. Figure 4A shows the distribution of fluorescence peak positions for these two bands, with average values at 650.8 and 676.8 nm, respectively. The average fluorescence peak position of the blue band matches well the maximum of the blue skewed Gaussian fitted to the average single-molecule emission spectra (Figure 2). For the red band, however, the difference between the values is larger and can be explained by the contribution of the far-red emission, shifting the fluorescence peak position into the red. In particular, for complexes in a quenched state, the reduced signal-to-noise ratio prevented the red band (at ~670 nm) from being unambiguously separated from the far-red band (at ~695 nm). Hence, a single skewed Gaussian fitting both bands together will be red-shifted. Indeed, when only the bands with an amplitude within 70% of the maximum are considered, the peak position of the red band shifted to 672.9 nm (Figure S4), while the peak position of the blue band was only slightly altered. Nevertheless, both bands, deriving from different conformations, are clearly distinguishable in the analysis of all single-molecule spectra. The large separation between the peak positions of the two bands in Figure 4A further demonstrates that the bands discussed here derive from two well-defined structural states and not from one disordered state.

A >20 nm shift of emission (Figure 4A) not only points to a change in conformation and possibly in configuration of the pigments but also makes us question the physiological relevance or function of the two conformations. With the

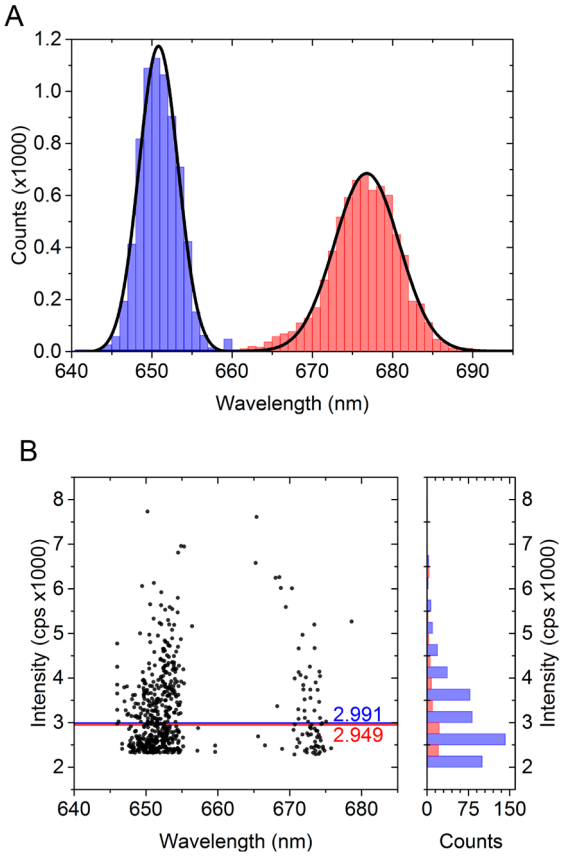

Figure 4. Properties of individual PC rods in this study. (A) Spectral peak distributions when two bands were fitted to the single-molecule spectra. The average peak position of the blue band is at 650.82 nm (SE = 0.06), as derived from a Gaussian fit (black). The average peak position of the red band is at 676.78 nm (SE = 0.03), as given by a Gaussian fit (black). The analysis included 7121 single-molecule spectra from 130 PC rods. (B) Fluorescence intensity of the single-molecule spectral bands as a function of the peak position. The median fluorescence intensity of the two bands is indicated with horizontal lines: blue for the blue form, and red for the red form. On the right, a histogram of intensity distributions of the blue bands. Only the top 70% of the brightest bands were included in order to exclude the spectra reflecting the quenched states (as for Figure S4).

emission at 651 nm, the bulklike blue form of the PC rods matches well the cascade of energy in an intact PB, transferring excitation energy to the PB core. However, the previously unknown emission of the red form (near 672 nm) is uphill from the absorption maximum of the PB core, thereby limiting excitation energy transfer to the PB core. Instead, the emission of the red form at 672 nm matches well the absorption of photosynthetic photosystems. Indeed, it was previously reported that in *Synechocystis*, PC can directly bind to PSI in the form of a single rod.[19] While the spectral overlap between the emission of the blue form of PC and the absorption of PSI makes the relevance of PC as a light-harvesting complex of PSI doubtful, in the case of the red form, this overlap is increased by 40% (Figure 5). Since the efficiency of excitation energy transfer increases proportionally with the increase of spectral overlap, the red conformation of PC is a good candidate to be responsible for direct energy transfer from PC to PSI in the recently described PC-CpcG2-PSI complex.[19]

We propose that in both spectral states described here, PC rods have a discrete light-harvesting function; therefore, none

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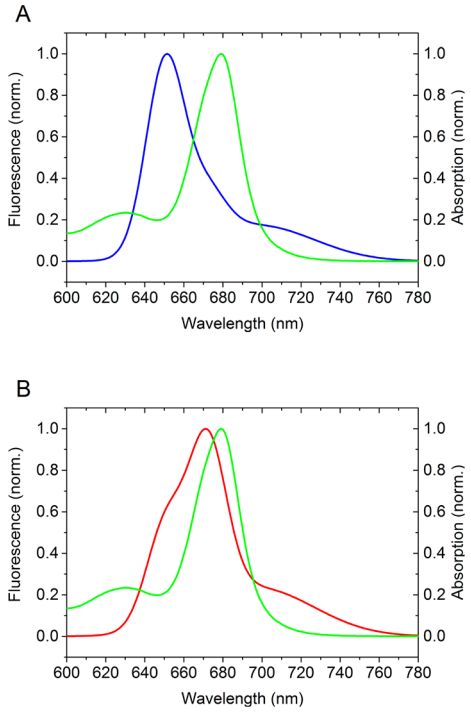

Figure 5. Overlaps between the fits to the single-molecule emission spectra of PC from Figure 2 and absorption spectrum of PSI. (A) Overlap for the blue form of PC. (B) Overlap for the red form of PC.

of the spectral states are expected to be quenched, in line with our observations. Previously it was reported that red-shifted states are often mildly quenched and associated with the quenching processes in different photosynthetic systems (e.g., in refs 29−32). However, after the intensities of fluorescence for the red and blue bands from fitting the individual single-molecule spectra were compared, it was seen that the medians of these distributions are virtually identical (Figure 4B). This indicates that PC rods in the blue and the red conformations are intrinsically capable of transferring excitation energy instead of dissipating it. Identical fluorescence intensities for the blue and the red conformations, apart from pointing at their light-harvesting function, suggest that the photophysical properties of the excited states of the pigments in those two forms are similar.

Linker proteins that bind PC rods, either to the PB core or PSI in *Synechocystis*, CpcG1 or CpcG2, respectively, were not present in the PC complexes used in these measurements.[18] A previous study on the PC-binding CpcG1 or CpcG2, isolated from mutants of *Synechocystis*, showed that the absence of any of these linkers causes shifts in the fluorescence emitted from PC.[23] Therefore, we propose that the presence of a linker stabilizes a specific conformation of PC rods and promotes certain spectral properties as identified in this work. In the PC-CpcG1-PB-core complex, the blue conformation would be stabilized by the CpcG1 linker protein for efficient excitation energy transfer to the PB core. In the PC-CpcG2-PSI complex, the red conformation would be promoted to stabilize one or more pigments in their red-emitting state and allow for direct excitation energy transfer to the photosystem. Interestingly, our study shows that different conformations signified by different spectral forms are intrinsically available for PC and that the function of linker proteins is to tune the equilibrium between the states, or, in other words, to choose and stabilize the conformation desired for a specific function. This concept redefines the previously proposed function of linker proteins, namely, (1) to keep the components of PB bound together[33] and (2) to tune their spectral properties.[34] Our study shows that CpcGs do not tune the optical properties of PC but select from the catalogue of available ones. This could be also the case for all other linker proteins present in PBs that were suggested to be involved in spectral tuning.[24,33,34]

The astonishing spectral flexibility of PC complexes, which allows them to perform at least two functions in *Synechocystis*, i.e., as a part of PB and as a single light-harvester attached to PSI, can also shed new light on light-harvesting complexes from other cyanobacterial strains, like *Acaryochloris marina*. In the latter strain, the light-harvesting complexes of photosystem II (PSII) were described as single rods composed mainly of hexamers of PC. However, the hexamer in the proximity to PSII was proposed to contain both PC and red-shifted allophycocyanin (APC).[35] While the spectroscopic analysis was pointing to the necessity of the presence of APC (1) to describe the spectroscopic properties of these light-harvesting complexes and (2) to permit efficient excitation energy transfer to the photosystem,[35] convincing biochemical evidence of APC in these complexes was absent. Our single-molecule spectroscopy results, showing that PC can enter red, nonquenched states, which most likely can be stabilized by interaction with a linker protein, allow us to propose that PC could be the only phycobiliprotein comprising *A. marina*'s PB. According to our results, the presence of one red pigment in PC of *A. marina*'s PB would allow for efficient energy transfer directly from PC to the photosystems. This hypothesis awaits experimental evidence.

The heterogeneity of emission from PC rods at room temperature with two dominating unquenched spectral forms is hidden in the broad ensemble fluorescence emission spectra of these complexes. Clearly and directly observed in single-molecule spectroscopy measurements, PC rods can enter two forms that differ in energy of emitted fluorescence but not in intensity. While the blue spectra match well the energy gradient of the PB complex, the red form is tuned for transferring energy directly to the photosystems. Switching between these two forms involves conformational changes in the protein matrix and the formation of 1 or 2 red-emitting pigments per complex. The presence of any CpcG protein very likely stabilizes one or the other conformation. The molecular flexibility of PC rods, as revealed by this single-molecule study, allows these complexes to perform two distinct physiological functions and sheds new light on the role of linker proteins in the functioning of PB complexes. Thanks to the fascinating ability of single-molecule spectroscopy to directly reveal the heterogeneous nature of emissive states of light-harvesting complexes, we have pushed the boundaries of our understanding of the flexibility and robustness of the early stages of photosynthesis as well as the mechanisms governing the dynamic properties of complexes performing solar energy conversion.

EXPERIMENTAL METHODS

Sample Preparation. Phycocyanin was isolated from ΔAB mutant[18] of *Synechocystis* as described before.[36] Briefly, the cells were broken in a 0.8 M K-phosphate buffer at pH 7.5, and after 2 h incubation with 2% (v/v) Triton X-100 (Sigma) at room temperature, the blue liquid supernatant was loaded onto a sucrose gradient. After ultracentrifugation the blue fraction at

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~0.5 M sucrose was collected. The PC rods were flash frozen in liquid nitrogen and stored at −80 °C before the analysis.

Bulk Measurements. Steady-state absorption spectra were collected using a Specord S600 (Analyticjena) spectrophotometer at room temperature.

Steady-state fluorescence emission spectra at room temperature were collected in a CARY Eclipse fluorescence spectrophotometer fluorometer (Varian) upon excitation at 590 nm as reported previously.[36]

For the fluorescence emission measurements at 77 K, the PC rods in K-phosphate buffer pH 7.5, containing ~0.5 M sucrose, were flash frozen in a custom glass cuvette immersed in the liquid N_2 chamber of an Oxford cryostat (DN1704). Steady-state spectra were collected upon excitation at 590 nm using a custom fluorescence setup built as described elsewhere.[37]

Single-Molecule Spectroscopy and Data Analysis. Directly before the measurement PC rods were thawed, diluted to 5−10 pmol with 0.8 M K-phosphate buffer at pH 7.5, and immobilized onto the surface of a poly-L-lysine treated coverslip, as described before.[15] A custom single-molecule spectroscopy setup introduced previously,[38] with sample-specific modifications including the dichroic beam splitter (605dcxt, Chroma Technology, Rockingham, VT) and fluorescence filter (KC13, Utrex Cryostats, Estland), was used to perform measurements similar to those reported before.[15] For each complex, emission spectra were collected in consecutive 1 s intervals for a total duration of 60 s, using continuous illumination at an excitation intensity of 4.3 W/cm^2 at 594 nm, i.e., the equivalent of (2.5−4) × 10^{-13} μmole of photons absorbed per complex per second. From all presented spectra of single molecules, a 60 s averaged background spectrum was subtracted. Only complexes that showed stable emission and did not photodegrade, i.e., did not undergo an irreversible drop of fluorescence intensity, were considered. Data was analyzed using a custom MATLAB script described in detail previously,[15] and figures were prepared in Origin 9.1.

Only in the measurements involving chemical cross-linking was glutaraldehyde (Sigma) added after the immobilization of the PC rods on the microscope glass as described previously.[15]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.8b00621.

Absorption spectrum and normalized bulk fluorescence emission spectrum of PC rods, steady-state fluorescence emission spectrum at 77 K, and distribution of peak positions like in Figure 4 (PDF)

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Notes

The authors declare no competing financial interest.

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