Spectroscopy of Single Phycoerythrocyanin Monomers: Dark State Identification and Observation of Energy Transfer Heterogeneities

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ABSTRACT Phycoerythrocyanin (PEC) is part of the light harvesting system of cyanobacteria. The PEC monomer contains one phycoviolobilin chromophore, which transfers excitation energy onto two phycocyanobilin chromophores. Many spectroscopical methods have been used in the past to study the bulk properties of PEC. These methods average over many molecules. Therefore, differences in the behavior of individual molecules remain hidden. The energy transfer within photosynthetic complexes is however sensitive to changes in the spectroscopic properties of the participating subunits. Knowledge about heterogeneities is therefore important for the description of the energy transfer in photosynthetic systems. Here, the recording of the fluorescence emission of single PEC molecules is used as a tool to obtain such information. Spectrally resolved detection as well as double resonance excitation of single PEC molecules is used to investigate their bleaching behavior. The *trans* isomer of the phycoviolobilin chromophore is identified as a short-lived dark state of monomeric PEC. Polarization sensitive single molecule detection is used for the direct observation of the energy transfer in individual PEC molecules. The experiments reveal that more than one-half of the PEC molecules exhibit an energy transfer behavior significantly different from the bulk. These heterogeneities persist on a time scale of several seconds. Model calculations lead to the conclusion that they are caused by minor shifts in the spectra of the chromophores.

INTRODUCTION

Light harvesting systems of photosynthetic organisms consist of protein assemblies in which structurally divers chromophores are held in well-defined geometries. They are electronically coupled, and their absorptions cover a wide spectral range. A subtle interplay between different electronic coupling mechanisms is responsible for an efficient transfer of the absorbed energy to the reaction center. During the last two decades, results from ultrafast laser spectroscopy in combination with structural data provided an increasingly detailed knowledge of the energy transfer processes in photosynthetic pigments (Fleming and van Grondelle, 1994; Sundström et al., 1999). Fluorescence techniques are well suited for investigations of such complexes, because most of them exhibit strong fluorescence when being uncoupled from the reaction centers. The ultrafast techniques were recently complemented by optical single molecule spectroscopy of light harvesting complexes of a variety of organisms (Wu et al., 1996; Bopp et al., 1999; van Oijen et al., 1999; Tietz et al., 1999; Jelezko et al., 2000; Ying and Xie, 1998).

Phycobiliproteins are an important class of antenna pigments. In contrast to chlorophyll carrying proteins, their fluorophores fluoresce only in the intact protein environment. Denaturation of the protein matrix consequently leads to the extinction of the fluorescence emission. This emission behavior allows the isolation of intact subunits from phycobiliprotein assemblies for systematic aggregation effect studies (Scheer, 1981; Maccoll and Guard-Friar, 1987; Glazer, 1994). In this work we present investigations of the photobleaching and the energy transfer of single phycoerythrocyanin (PEC) monomers. PEC is a short wavelength-absorbing unit of the light-harvesting complex of certain cyanobacteria (Bryant et al., 1982). In its native form, it aggregates to trimers and hexamers (Duerring et al., 1990). The PEC monomer contains two different chromophores, phycoviolobilin (PVB), and phycocyanobilin (PCB) (Bishop et al., 1987; Zhao et al., 1995). Based on the x-ray structure of the trimer, it is an $\alpha\beta$ heterodimer in which the single PVB is bound at position α-84 and the two PCBs at positions β-84 and β-155 (Duerring et al., 1990). The distances between the centers of the chromophores are 47 Å (α-84 $\leftrightarrow$ β-84), 48 Å (α-84 $\leftrightarrow$ β-155), and 35 Å (β-84 $\leftrightarrow$ β-155) (Fig. 1). In the native state (15Z, reduced Cys-98, -99; see below), the PVB carrying α-subunit has its absorption maximum at 566 nm and the emission maximum at 588 nm, whereas the β-subunit with the two PCBs has absorption and emission maxima with centers at 593 and 630 nm, respectively. The main biological function of PEC is to transfer absorbed energy to the reaction center. Structural and spectral data indicate that in PEC, this energy transfer is of Förster type (Förster, 1968; Hucke et al., 1993; Palsson et al., 1993; Schneider et al., 1996; Parbel et al., 1997). The coefficients for the Förster transfer of excitation energy from α-84 to β-84 and β-155 are sensitive to the spectral overlap and the relative orientation of the transition dipole moments. Spectral shifts of the chromophores and varying orientations of the transition dipole moments in PEC should

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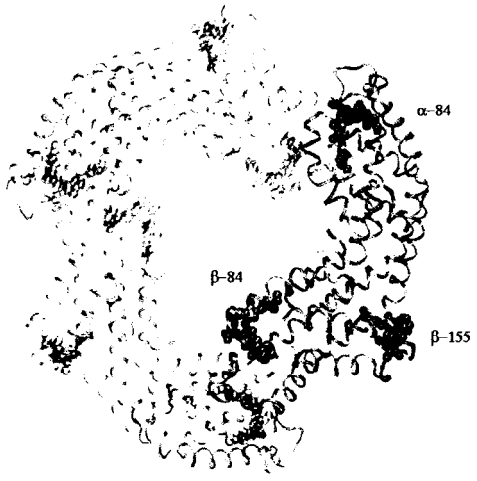

FIGURE 1 Schematic representation of the chromophore position in PEC based on the x-ray crystallographic structure (Duerring et al., 1990) with the protein backbone as a ribbon and the chromophores as calottes. The PEC monomer is an $\alpha\beta$-heterodimer (*dark gray*). The positions of the chromophores in monomeric PEC used in the calculations are derived from the trimer structure assuming that no structural change of the monomer occurs upon dissociation.

therefore give rise to variations in the energy transfer efficiencies between individual PEC molecules.

The spectroscopy of single PEC molecules avoids the ensemble averaging encountered in bulk spectroscopy. It can thus be used to investigate heterogeneities in the energy transfer coefficients of individual PEC molecules. In the first part of this contribution, we use spectrally resolved single molecule detection to investigate the photobleaching behavior of individual PEC monomers at room and cryogenic temperatures. A two-color experiment gives evidence that the photoisomerization of the absorbing PVB chromophore produces the 15*E* form as a dark state of monomeric PEC. The second part describes studies of the energy transfer from the absorbing PVB chromophore to each of the two emitting PCB chromophores in the PEC monomer. The emission spectra of the two PCB chromophores are nearly indistinguishable, making it difficult to determine the transfer rates with bulk measurements. Instead we used polarization sensitive single molecule spectroscopy (Ha et al., 1999) of PEC monomers at room temperature to determine the fraction of excitation energy, which is transferred to each of the two PCB chromophores. Our results reveal that a large part of the individual PEC molecules possess energy transfer coefficients that differ significantly from those calculated on basis of the bulk spectroscopic data. They exhibit a heterogeneity, which is stable on a time scale of seconds. Model calculations show that small changes in the absorption and emission spectra of the chromophores of PEC most likely are the source for differences in the energy transfer coefficients. We suggest that changes in the degree of conjugation of the π system of the chromophores are the cause of these spectral shifts. If similar processes operate in the phycobilisome containing a large number of chromophores, our data lead to the conclusion that also the heterogeneity between chromophores has to be taken into account for a description of the energy transfer process in photosynthetic systems (van Oijen et al., 2000).

MATERIALS AND METHODS

The spectroscopic setup is based on a homebuilt confocal microscope. The sample was mounted on a piezo driven translation stage (P-731, Physik Instrumente, Karlsruhe, Germany) on top of an inverted microscope (TE 300 equipped with a 60×, 1.2 NA Plan Apochromat water immersion lens, Nikon, Tokyo, Japan). Light from a ring dye laser (CR-699, Coherent Inc., Santa Clara, CA) or from an Ar^+-ion laser (Innova 90-6, Coherent Inc.) was focused onto the sample. For imaging single molecules fluorescence light was collected in a back reflection geometry and separated from the excitation light by means of a dichroic mirror (575 DCXR, Chroma, Brattleboro, VT). Residual excitation light was removed with a notch filter (HNPF-568-10, Kaiser Optical Systems, Inc., Ecully, France). The fluorescence emission was detected through a confocal 100-μm pinhole with a single photon counting avalanche photodiode (SPCM-AQR-16, EG&G Inc., Gaithersburg, MD). Sample images were generated by raster scanning the sample over the fixed focus. Experiments at cryogenic temperatures were performed using a beam scanning confocal microscope (Mais, 2000).

For spectral selection experiments, a dichroic mirror with an edge at 595 nm (595 DCXR, Chroma) was inserted after the notch filter for dividing the emission signal. This resulted in one detection channel with 575 nm $< \lambda_{DET} <$ 595 nm and a second channel with $\lambda_{DET} >$ 595 nm. A second identical single photon avalanche photodiode was used to detect the signal on this channel. Both channels were read out simultaneously. Using this setup, one-half of the signal from the α-chromophores was detected in channel one with the other half being detected in channel two. Emission from the β-subunits was only detected in the latter channel and was completely suppressed in channel one. All spectrally resolved experiments were done with circularly polarized excitation light.

For the polarization experiments, we used linearly polarized excitation light. It is crucial for the determination of the energy transfer rates between the chromophores in the monomer that only the PVB chromophore on the α-subunit is excited. We therefore recorded data from the isolated α- and β-subunits at different excitation wavelengths (data not shown) with the same setup as described above. Excitation at 568 nm was well suited for recording single molecule images of the isolated α-subunit, whereas no signal was detected from the isolated β-subunit. We therefore replaced the 575-nm dichroic mirror used in the spectral selection experiment with the 595-nm dichroic mirror. The notch filter was replaced by a bandpass filter (HQ 620/60, Chroma). Behind the bandpass filter, a polarizing beamsplitter was inserted in the detection path to accomplish the separation of the two polarization components of the fluorescence emission. The polarization of the exciting laser beam was quickly rotated between 0° and 180° by passing it through an electro optic modulator (LM 0202, Gsänger Optoelektronik GmbH, Planegg, Germany) and a $\lambda/4$ waveplate.

The preparation of monomeric PEC from *Mastigocladus laminosus* has been described elsewhere (Parbel et al., 1997). Concentrated solutions of PEC in water/glycerol (2:3) buffered at pH 7 with K_2HPO_4 were diluted with the same solution additionally containing 4 M urea. For the isolation of single PEC monomers, a drop of diluted solution was deposited onto a glass coverslip (Marienfeld No. 1, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). Immobilization was achieved by nonspecific electrostatic interaction between the proteins and the glass surface (Ying and Xie, 1998). To check for immobilization, we rotated the polarization of the exciting laser beam with a constant speed over 180° and recorded the integral fluorescence signal. With this excitation scheme, the signal intensity follows the excitation polarization modulation with a cos^2 law, no detectable phase shift, and a modulation depth down to the

background level (data not shown). This indicates that the molecules were immobilized under these conditions. Because an ordered orientation of the molecules on the sample can be excluded, this experiment also serves as a proof for successful single molecule detection. During all measurements the samples were kept humid by covering them with a lid.

RESULTS AND DISCUSSION

Bleaching behavior of single PEC monomers

Single PEC molecules were located on images from samples either at room temperature or at 4 K (Fig. 2). Subsequently, trajectories of the fluorescence emission of single PEC monomers were recorded to determine their photobleaching stability. Fig. 3 shows a comparison between the emission times of single PEC molecules before the occurrence of a final photobleaching step at $T = 4$ K and at room temperature for an excitation intensity of 0.4 kW/cm^2. Fitting a monoexponential decay curve to the histogram yields a decay constant of $t_{1/2} = 20$ s at 4 K. This value reduces to 4 s at room temperature. The most likely explanation for the increased stability at low temperatures is the restricted access of molecular oxygen due to the freezing of the sample. Experiments with the isolated α-subunit show a sevenfold increase in stability when oxygen is removed from the sample with the glucose oxidase/catalase system (Bopp et al., 1999). Due to the necessary presence of urea, which inhibits the glucose oxidase, this depletion method cannot be used in investigations of monomeric PEC, whereas commercially available antifade kits are known not to work with phycobilin proteins (Molecular Probes Inc., Eugene, Oregon. Product Information. SlowFade). Attempts to record excitation spectra of single PEC monomers at cryogenic temperatures proved futile. We attribute this to fast spectral jumps of the PEC molecules leading to broad spectra even at cryogenic temperatures. Linear tetrapyrrole chromophores are highly flexible in solution and much more rigid in the native protein. Even in the protein however, the α-84 PVB chromophore has some conformational flexibility. This can be seen from its unusual 15*Z/E* isomerization. Although this photochemistry is inhibited below liquid nitrogen temperatures (Zhao and Scheer, 1999), there is apparently still some residual motion of the chromophore possible. A strong coupling of the electronic transitions of the chromophore to the environment dynamics is thus expected.

Spectrally resolved emission of single PEC monomers are used to investigate the bleaching behavior in more detail. Although it is possible to distinguish between the emission from the α- and the β-subunits, one cannot discriminate the emission of the two PCB chromophores. The upper channel in the graphs of Fig. 4 shows the emission signal of single PEC molecules with $\lambda_{det} > 595$ nm, which originates almost exclusively from the β-subunit. In the simultaneously recorded lower channel only emission light was recorded with 575 nm $< \lambda_{det} <$ 595 nm, corresponding

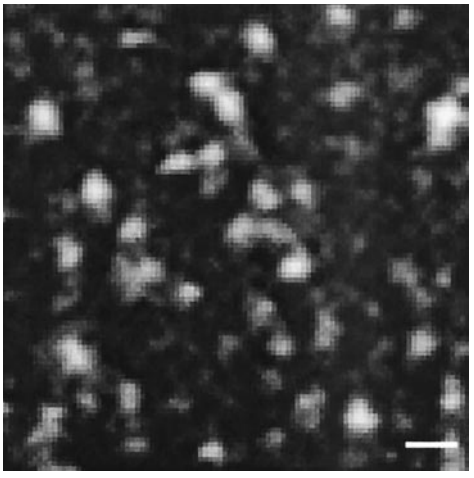

FIGURE 2 Image of single PEC monomers from a H_2O/glycerol solution (3:2) at room temperature, immobilized on a glass surface. Excitation with 0.4 kW/cm^2 at 568 nm. Scale bar = 1 μm.

to the emission of the α-subunit. The typical behavior, which was observed for 113 of 130 molecules, is represented in the top part of Fig. 4. Here, the emission detected on either of the channels ceases after the emission of the α-chromophore vanishes. Obviously in these cases photobleaching of the whole PEC monomer is caused by photobleaching of the PVB chromophore. Only three molecules showed a behavior as depicted in the bottom part of Fig. 4. In these cases, the emission of the α-subunit continues after the disappearance of the β-subunit emission. These findings are not surprising, as the PVB chromophore is primarily excited. For 11 molecules we observed intensity fluctuations in the β-channel that did not have any influence on the

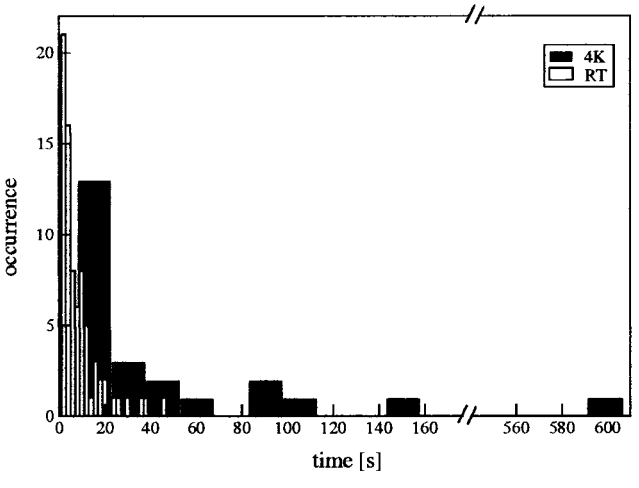

FIGURE 3 Histogram of on-times before the final photobleaching step of single PEC monomers at room temperature (*gray*) and at 4 K (*black*). A single exponential fit to the data yields $t_{1/2} = 4$ s at room temperature and $t_{1/2} = 20$ s at 4 K. Excitation intensities are 0.4 kW/cm^2 at 568 nm.

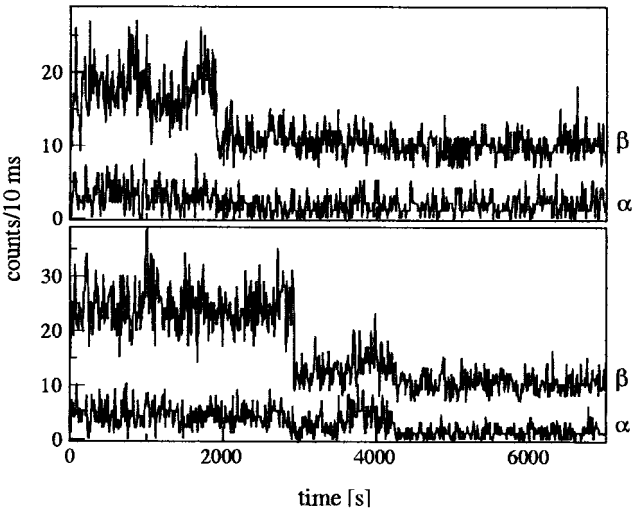

FIGURE 4 Two simultaneously recorded fluorescence trajectories of the α- and the β-subunits of single PEC monomers. The lower channel labeled "α" shows only emission from the α-subunit with 575 nm $< \lambda_{det} <$ 595 nm, whereas in the upper channel labeled "β" with $\lambda_{det} >$ 595 nm, mainly emission from the β-subunit is seen. A leakage signal of the α-subunit emission is detected in the upper "β" channel. λ_{exc} = 568 nm with 0.4 kW/cm^2. The upper channel is offset by 14 counts/20 ms.

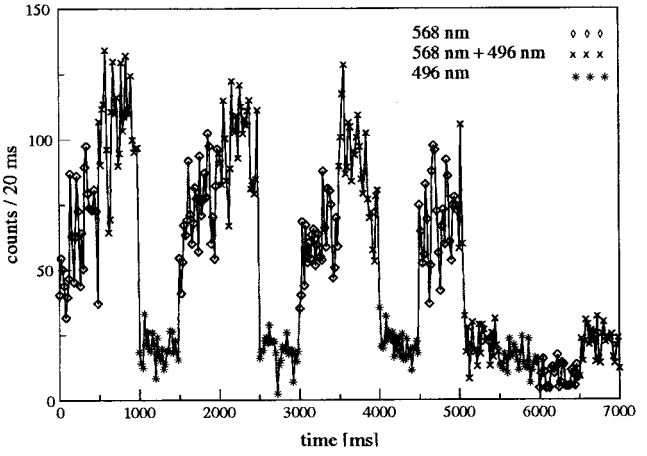

FIGURE 5 Alternating one and two color excitation of single PEC monomers. Illumination (500 ms) at 568 nm (*light gray*) were followed by 500 ms of simultaneous illumination at 496 and 568 nm (*dark gray*), then by 500 ms at 496 nm only (*black*). The intensities used were 0.5 kW/cm^2 at 496 nm and 0.6 kW/cm^2 at 568 nm. The average count rate/20 ms over three cycles was 98 counts/20 ms for simultaneous excitation with the two excitation colors, 20 counts/20 ms for 496 nm only, and 65 counts/20 ms for 568 nm only.

emission intensity detected on the α-channel before photobleaching occurred. This behavior is explicable by changes in the fluorescence quantum yields of the PCB chromophores or by fluctuations in the energy transfer between them, whereas the transfer from the α- to the β-subunit remains constant. Yet a different behavior was observed for three other molecules that only exhibited emission on the α-channel. This can be due to a PEC monomer, which is either dissociated or which contains damaged PCB chromophores. The spectrally sensitive experiments thus indicate that using 4 M urea as a dissociation agent, disintegration of the PEC monomers into the subunits is a rare event.

Dark state identification of PEC monomers

Dark states, responsible for the blinking behavior encountered in single molecule spectroscopy, are commonly not identified. PEC is known to have a rich photochemistry (Zhao and Scheer, 1995). This involves the *cis*/*trans* interconversion of the PVB chromophore between the 15*Z* and the 15*E* isomers, as well as the reduction and oxidation of the two thiol groups at Cys-98, -99. In the isolated α-subunit, the 15*Z* form exhibits strong fluorescence with an absorption maximum at 566 nm, whereas the 15*E* form with an absorption maximum at ≈505 nm is very weakly fluorescent. The bulk spectroscopic data show that the 15*E* form is produced with a low quantum yield after excitation of the 15*Z* form (Zhao et al., 1995) and that no thermal repopulation occurs. It can, however, be converted back to the fluorescing 15*Z* form by excitation with $\lambda_{exc} \approx$ 500 nm. We recently used a simultaneous two-color excitation scheme to characterize a dark state in the green fluorescent protein (Jung et al., 2001). Here, this method has been adapted to check whether the 15*E* form of the PVB chromophore is a dark state of the PEC monomer. If the 15*E* form does not absorb at 568 nm and no thermal repopulation of the 15*Z* form occurs, then 15*E* will be a long lived dark state of the PEC monomer. In a typical experiment, transitions into this state would not be distinguishable from photobleaching. If, however, the 15*E* form absorbs weakly at 568 nm, then it would be a short-lived dark state reducing the observable emission count rate.

An alternating excitation scheme was used in the experiment. For 500 ms, a molecule is excited only at λ_{exc} = 568 nm, then for 500 ms simultaneously at λ_{exc} = 496 nm and λ_{exc} = 568 nm, and finally for 500 ms only at λ_{exc} = 496 nm. This scheme is then repeated. In the example shown in Fig. 5, a molecule emits for three of these excitation cycles and bleaches during the fourth. The fluorescence count rates were averaged over the three cycles during which the molecules emitted. The average count rate of 98 counts/20 ms for simultaneous two color excitation is 15% higher than the sum of 20 counts/20 ms and 65 counts/20 ms of the signals for one color excitation at 496 nm and 568 nm, respectively. Excitation of a single PEC molecule at 568 nm yields a fluorescence count rate reflecting the average occupation of the molecule's emissive 15*Z* state during the integration time. Simultaneous excitation with λ_{exc} = 496 nm and λ_{exc} = 568 nm shifts the *cis*/*trans* equilibrium toward the 15*Z* form compared with single color illumination at 568 nm. This leads to fluorescence count rates, which are higher than

the sum of count rates at 496 and 568 nm. Our results indicate the repopulation of the emitting 15*Z* form from the dark state 15*E* with the second color illumination. They show that the 15*E* form of the PEC monomer is not a long-lived dark state. Instead, the residence time of the PEC monomers in the 15*E* state is on a submillisecond time scale under the experimental conditions chosen. Intensity-dependent experiments can be used to unravel the kinetics of the population of the dark states (Garcia-Parajo et al., 2000). In the case of PEC, the trajectories are however not sufficiently long for this kind of analysis. Even with simultaneous two-color illumination, high fluctuations in the fluorescence intensities are observed. These are due to the existence of multiple dark states, which are not depopulated by the additional excitation color.

Observation of the energy transfer of single PEC monomers

Polarization modulated excitation and polarization sensitive detection have been used extensively in connection with the spectroscopy of single molecules to investigate molecular orientation (Güttler et al., 1993), molecular movement (Ha et al., 1999; Häsler et al., 1998; Warshaw et al., 1998), structural dynamics (Bopp et al., 1999; Tietz et al., 1999), and excited state electronic structure (van Oijen et al., 1999). Here, the polarization properties of the fluorescence emission of single PEC molecules are used to determine the energy transfer coefficients from α-84 onto β-84 and β-155. In PEC, the excitation energy is transferred from the short wavelength absorbing PVB chromophore onto the two PCB chromophores. The energy transfer is of Förster type. On this basis, the energy transfer rates between the respective chromophores of the monomer are calculated using the x-ray structure coordinates of the trimer (Duerring et al., 1990). The values are given in the schematic model in Fig. 6. Whereas most rates are much faster than the fluorescence decay rate, some rates are of the same order of magnitude. The energy transfer between the chromophores therefore does not equilibrate completely before fluorescence emission occurs. We performed Monte-Carlo simulations to obtain the fluorescence yields from each chromophore. The results are 0.06 (α-84), 0.69 (β-84), and 0.25 (β-155). These data are calculated on basis of the measured bulk absorption and emission spectra. A fluorescence lifetime of 1 ns and a quantum yield of 0.5 were assumed. In our experiment, only emission from the two β-chromophores is observed. The fraction of intensity emitted by β-155 is given by the energy transfer coefficient γ, whereas the emission intensity from β-84 is $(1-\gamma)$. γ can be obtained by multiplying the fluorescence yield for β-155 by 1.06.

Subsequently it is shown how the energy transfer coefficients γ for individual molecules are obtained from the polarization spectroscopic data of single PEC monomers. We proceed as follows. First, the angle ϕ of the projection

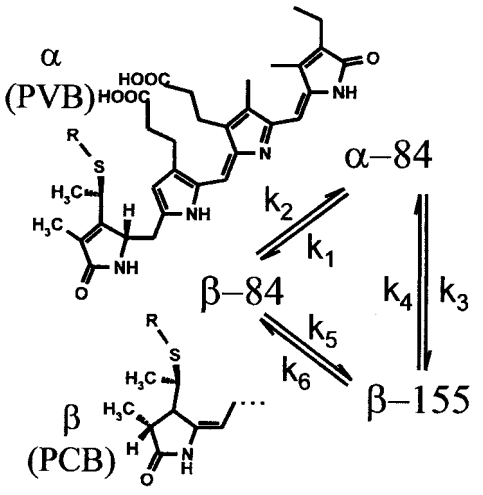

FIGURE 6 Diagram depicting the energy transfer in a PEC monomer and the molecular structures of the phycoviolobilin (PVB) and the phycocyanobilin (PCB) chromophores. The Förster transfer rates calculated on basis of the x-ray structure and the measured spectra are $k_1 = 22.1\ \text{ns}^{-1}$, $k_2 = 0.7\ \text{ns}^{-1}$, $k_3 = 2.6\ \text{ns}^{-1}$, $k_4 = 0.3\ \text{ns}^{-1}$, $k_5 = 13.0\ \text{ns}^{-1}$, $k_6 = 35.1\ \text{ns}^{-1}$.

of the absorption dipole moment $\vec{\mu}_{\text{abs}}$ of the α-84 PVB chromophore onto the *xy* plane in the laboratory coordinate system (compare Fig. 7 for the definition of all angles) is measured. This gives us information on the orientation of the PVB chromophore in the *xy* plane. Second, the intensities of the orthogonally polarized emission components *P* and *S* are recorded simultaneously. Their ratio depends on the orientation of the two emitting PCB chromophores and on the distribution of excitation energy transferred onto each of these. Third, the x-ray structural data are used to calculate the emission rate from each PCB chromophore for every possible orientation of the PEC monomer. The measured values for ϕ and the ratio $V = \frac{P}{S}$ restrict the possible orientations of the PEC monomer and lead to a limited range of energy transfer coefficients γ reconcilable with the experimental data. We thus obtain minimal energy transfer coefficients for individual PEC molecules.

For the analysis it is necessary to first regard the emission of a single chromophore with parallel absorption and emission dipole moments. The excitation probability of a single immobilized chromophore is proportional to $|\vec{\mu}_{\text{abs}}\vec{E}(t)|^2$, in which $\vec{E}(t)$ is the exciting field. The tight focusing of the excitation light leads to the presence of a small *z* component. It has been shown that its influence on the absorption can be neglected for focusing with an NA 1.4 objective (Ha et al., 1999). To minimize the possible depolarization effect, we chose to not fully illuminate the back aperture of the microscope objective. This yields

$$|\vec{\mu}_{\text{abs}}\vec{E}(t)|^2 \propto \sin^2\theta\cos^2\phi \qquad (1)$$

with θ being the angle between $\vec{\mu}_{\text{abs}}$ and the *z* axis. The expression above allows us to determine ϕ, the angle be-

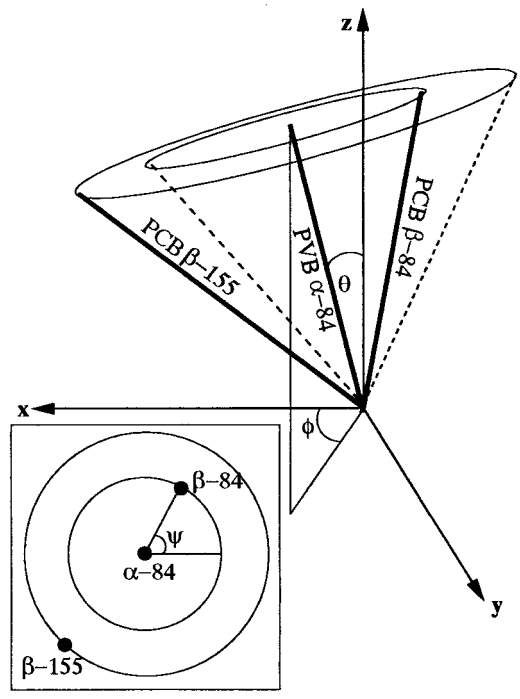

FIGURE 7 Angles ϕ and θ are describing the position of the absorber (PVB) in the laboratory system (x, y, z). ψ is defined as the rotation angle of β-84 around α-84. With a given angle ψ and the angles between the chromophores one absolute orientation of the PEC monomer is described. The inset shows the top view onto the cones.

tween $\vec{\mu}_{abs}$ and the x axis, by recording the integral emission intensity while rotating the polarization axis of the excitation light over π. Having determined $\vec{\mu}_{abs}$, the excitation polarization was set to be parallel to the xy component of $\vec{\mu}_{abs}$.

While the integral emission signal is used to determine the orientation of the absorption dipole within the xy plane, information about the emission dipoles' orientation can only be inferred from the two orthogonally polarized emission components P and S. For a single chromophore, the collected emission intensities P and S are given by (Axelrod, 1979)

$$P = I_{tot}\,(K_1x^2 + K_2y^2 + K_3z^2)$$
$$S = I_{tot}\,(K_2x^2 + K_1y^2 + K_3z^2) \qquad (2)$$

Here x, y, and z are the components of the emission dipole moment along the respective coordinate axis and I_{tot} is the integral fluorescence count rate. We recalculated the normalized values K_n for a NA 1.2 objective and obtained $K_1 = 0.3208$, $K_2 = 0.0056$, and $K_3 = 0.097$ (Ha et al., 1999). This result shows that the ratio $V = \frac{P}{S}$ is dominated by the orientation of the chromophore in the xy plane. K_3 however has a nonnegligible value, which gives a depolarization contribution if the emission dipole has a significant z component. In the limiting case of an emission dipole oriented parallel to the xy plane, we obtain $V = \frac{P}{S} = \tan^2(\phi + \delta)$ in which δ is an apparatus-dependent parameter that was determined independently in a calibration measurement with single Terrylene molecules.

In the case of PEC one has to consider a molecule in which the PVB chromophore acts as the absorber and the two PCB chromophores as the emitters. The angles between the respective transition dipole moments in the PEC monomer are obtained from the x-ray structure assuming that no significant structural deviation from that of the trimer occurs. It is commonly assumed that the relative orientations of the dipole moments are the same as those of the long axis of the respective tetrapyrrole π-systems (Duerring et al., 1990). For the PVB chromophore, only the three conjugated rings are included. The angle ϕ is determined experimentally in the manner described above. The main difficulty is the determination of the absorption dipole's orientation along the z axis. Recently proposed methods (Sick et al., 2000; Novotny et al., 2001) are not applicable here due to the poor photostability of the PEC monomers. Instead, we use the integral emission count rate as a measure for the z orientation of the absorption dipole. The statistics of 101 observed single PEC molecules allow us to assign the highest detected count rate to a molecule with its absorption dipole oriented perpendicular to the z axis. This maximal count rate is used to determine θ for all observed molecules according to the $\sin^2\theta$ dependence of the integral count rate. An uncertainty of $\pm 20\%$ arises because of the emission originates from two emitters. This means that a range of possible z orientations of $\vec{\mu}_{abs}$ has to be considered.

Even with the knowledge of the absolute orientation of the PVB chromophore, many orientations of the whole PEC monomer can lead to the observed values for V. This is due to the fact that also the energy transfer coefficient γ for the transfer of excitation energy from α-84 onto β-155 has to be considered. We therefore determined all possible orientations of the emission dipole moments, which can be reconciled with the experimental data for ϕ, θ, and V. These calculations were performed by applying the proper rotation matrices onto the coordinate matrix $C = (\vec{\alpha}^{84}, \vec{\beta}^{84}, \vec{\beta}^{155})$ of the chromophores as taken from the x-ray structure. The α-84 chromophore in C is oriented along the z axis and the β-84 chromophore lies in the xz plane. The two β chromophores were rotated around α-84 by an angle ψ by operating $D_{z,\psi}$ on C. $D_{z,\psi}$ rotates by an angle ψ along the z axis. The chromophores' coordinates were rotated by the experimentally determined angles ϕ and θ around the y and z axis by operating $D_{y,\theta}$ and $D_{z,\phi}$ on the coordinate matrix. By repeating this procedure stepwise for all ψ values from 0° to 360°, the complete configuration space for the PEC molecule was sampled. The matrix multiplication for one step is given by $D_{z,\phi}D_{y,\theta}D_{z,\psi}C = C'$. Thus, new coordinates C' are obtained. The squares of the components x', y', and z' are directly related to the emission intensity of each emitter on both detection channels. To calculate the emis-

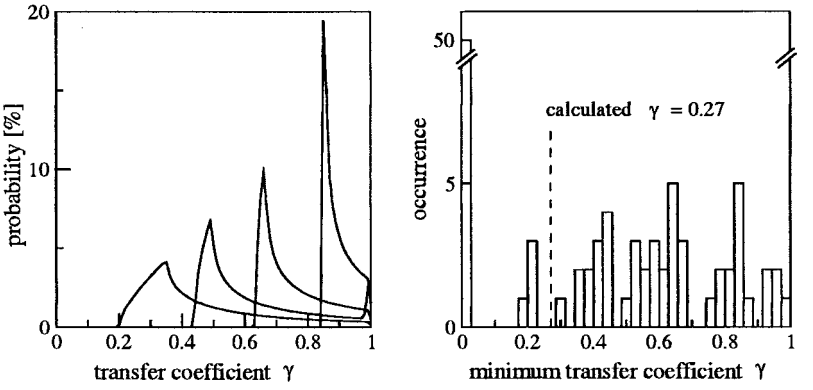

FIGURE 8 (*Left*) Energy transfer coefficient γ from α-84 to β-155 for selected molecules. The probabilities for γ are obtained from the measured values for ϕ and V as described in the text. (*Right*) Histogram of minimal values of γ for different individual molecules. Molecules for which the experimental data can be reconciled with any energy transfer coefficient between 0 and 1 are grouped under the 0 value in the histogram. Molecules as those shown on the left possess a minimal energy transfer coefficient larger than 0.

sion intensities P and S expected for each orientation, also the collection characteristics as given in Eq. 2 and the degree of energy transfer from the absorber to the emitters have to be taken into account. One obtains:

$$V = \frac{P}{S} = \frac{(1-\gamma)P_{\beta^{\prime}84} + \gamma P_{\beta^{\prime}155}}{(1-\gamma)S_{\beta^{\prime}84} + \gamma S_{\beta^{\prime}155}} \tag{3}$$

Each specific orientation now leads to a certain transfer coefficient γ. By considering all orientations for an individual molecule, the possible values for γ can be determined. For 50 of the 101 molecules under investigation, it is not possible to ascribe a discrete minimal value to γ. In these cases, the specific orientation of the molecules does not allow us to ascribe a certain minimal value to γ. These molecules are grouped under the $\gamma = 0$ value in the histogram in Fig. 8. For the remaining molecules, a minimal value of γ can be assigned that is represented in the histogram in Fig. 8.

The energy transfer coefficient calculated with Förster theory is $\gamma = 0.27$. Thus, the γ values that were determined for individual molecules deviate significantly from the calculated value. Apart from the differences in the absolute values, the single molecule data also reveal a pronounced heterogeneity of the sample in the energy transfer coefficients. The energy transfer coefficients were determined only for molecules exhibiting intensity ratios for the two perpendicular polarization components, which remained stable over the course of several seconds. This means that also the calculated energy transfer coefficients were stable over this time range.

The energy transfer coefficients are influenced by the distances between the chromophores, their relative orientation toward each other, and the spectral overlap between the emission spectra of the donors and the absorption spectra of the acceptors (Förster, 1968). The preparation of the samples could influence the spectra of the chromophores and the orientation of their transition dipole moments by interaction with surface charges on the glass coverslip. Using the same preparation method, Ying and Xie (1998) however observed no differences in the spectra and fluorescence lifetimes of immobilized single allophycocyanin molecules. In PEC, the transition dipole moments are oriented along the long axis of the nearly linear structure of the chromophores. It therefore seems unlikely that a polarization component perpendicular to the long axis of the chromophore can be induced, which would be large enough to have a significant influence on the energy transfer coefficients (see below). The energy transfer calculations for the monomer are based on the x-ray structural data of the trimer. This procedure is justified for the following reasons. All molecules observed absorb at 568 nm and emit at $\lambda_{det} > 595$ nm. Because PVB and PCB only fluoresce when being embedded in the protein, only intact PEC monomers are detected in which the x-ray structure is largely preserved. The spectra of biliproteins are exquisitely sensitive to conformational changes (Braslavsky et al., 1983; Falk, 1989; Scharnagl and Schneider, 1991; Scheer, 1982). Because the spectra of the trimer differ only little from those of the monomer (Parbel et al., 1997), we conclude that there are only minor changes in the conformation of the chromophores in PEC monomers compared with PEC trimers. Significant changes in the distances between the chromophores are therefore not considered as a cause of the variations in the energy transfer coefficients. The different chromophores in PEC are coupled in the way depicted in Fig. 6. Monte-Carlo simulations were used to investigate the influence of changes of the absorption and emission dipole moments' orientation on the transfer rates. Geometrical changes can lead to substantially altered absolute values of the rates. The transfer rates from one chromophore to the other are however always affected in the same way as the reverse rates. The maximal increase in γ for a variation of all relevant angles by $\pm 20°$ is 3%. This leads to the conclusion that geometrical changes do not account for the large γ values observed experimentally. In contrast to geometrical changes, spectral shifts influence the ratio of the transfer rates more strongly. Recently, spectral jumps of more than 1000 cm^{-1} have been reported for amino substituted Perylenes (Blum et al., 2001). Before and after the jump, the spectra remained stable for tens of seconds. The authors attribute the appearance of different

spectra to two states in which the lone pair of the amino group is either in or off resonance with the chromophore's π-system. Apart from shifts in the spectral positions, also changes in the line shape and the fluorescence quantum yield were observed. Keeping the latter parameters and the Stokes shift constant, we calculated the influence of spectral shifts of $\pm 600\ cm^{-1}$ for the β-chromophores. The calculations show that changing the spectral positions of the two β-chromophores relative to each other by $330\ cm^{-1}$ already results in a 100% change in the energy transfer coefficient. A spectral heterogeneity of the chromophores therefore seems the most likely source for differences in the energy transfer between individual PEC monomers. The molecular origin for the spectral shifts remains speculative, but might include different protonation states of the chromophores or varying degrees of conjugation of lone pairs on their nitrogen atoms. The latter could be caused by slight geometrical distortions of the chromophores in the protein pocket. The biologically active forms of the PEC are trimers and hexamers. In these, one α- and one β-chromophore of each monomeric subunit are in close proximity to each other. It has been shown for the closely related phycocyanin that in monomers as well as in trimers, the dominant energy transfer processes are well described by Förster theory (Debreczeny et al., 1995). We therefore expect that also in the naturally occurring oligomers of PEC, the observed heterogeneity of the energy transfer coefficients will be of significance for the transduction of excitation energy.

SUMMARY

Single molecule images of phycoerythrocyanin monomers were recorded. Their photobleaching behavior was studied using spectrally resolved emission spectroscopy. The PVB chromophore was found to be responsible for the photobleaching of PEC. Using simultaneous two-color illumination, the 15*E* form of PVB was identified as one of the short-lived dark states of PEC. Polarization sensitive detection was used to determine the energy transfer coefficients of individual PEC monomers. A surprisingly large, hitherto unknown static heterogeneity was observed. This finding is attributed to chromophores, which are spectrally shifted with respect to the bulk spectra.

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