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Does the Reconstitution of RC-LH1 Complexes from *Rhodopseudomonas acidophila* Strain 10050 into a Phospholipid Bilayer Yield the Optimum Environment for Optical Spectroscopy?

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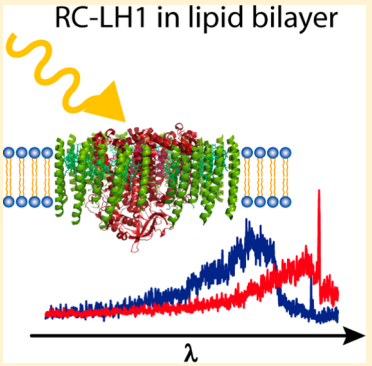
Supporting Information

ABSTRACT: We have investigated reaction-center light-harvesting 1 (RC-LH1) complexes from *Rhodopseudomonas (Rps.) acidophila* in detergent buffer solution and reconstituted into a phospholipid bilayer and compared the results with the outcome of an earlier study conducted on RC-LH1 immobilized in polyvinyl alcohol (PVA). The aim of this study was to test whether the immobilization of the complexes in a PVA matrix might lead to a deterioration of the proteins and thereby limit the accessible information that can be obtained from optical spectroscopy. It has been found that the complexes dissolved in a detergent buffer solution are subject to fast spectral dynamics preventing any meaningful application of single-molecule spectroscopy. In contrast, for the bilayer samples it is revealed that the reconstitution process results in a significantly larger fraction of broken complexes with respect to the preparation of the complexes in a PVA film. Moreover, we find that for the intact complexes the statistics of the key spectral features, such as the spectral separations of the bands and the mutual orientation of their transition-dipole moments, show no variation dependent on using either a bilayer or PVA as a matrix. Given the additional effort involved in the reconstitution process, the lower amount of intact RC-LH1 complexes and, concerning the decisive spectral details, the identical results with respect to embedding the complexes in a PVA matrix, we come to the conclusion that the immobilization of these proteins in a PVA matrix is a good choice for conducting low-temperature experiments on individual light-harvesting complexes.



INTRODUCTION

Single-molecule spectroscopy has proven to be a powerful tool with which to investigate the details of the spectroscopic properties in relation to the structure of photosynthetic pigment−protein complexes.[1−35] However, many of the single-molecule experiments, as well as many ensemble studies, have been performed on detergent-solubilized complexes and involved immobilization of the complexes in a matrix, preferentially in a thin film that was placed onto a surface, usually either directly or by spin coating.[3,5,7,11,14,18,22] It has been questioned whether this then deforms the structure of these antenna complexes and, therefore, introduces, per se, significant changes in their spectroscopic behavior that would not be reflected in their native membrane environment, as it is generally assumed that the light-harvesting (LH) complexes are more stable and fully native when housed in their host biological membrane.[36,37] Supporting evidence for this conjecture was also obtained by single-molecule spectroscopic investigations on the light-harvesting 1 (LH1) complex from the purple bacterium *Rhodospirillum (Rsp.) rubrum*, which revealed significant spectroscopic differences on the single-molecule level between membrane-reconstituted and detergent-solubilized complexes. The results of this study were interpreted in terms of a narrowed statistical distribution of conformational states for the membrane-reconstituted LH1 complex.[38] A similar conclusion has been drawn for photosystem I (PS I) from *Thermosynechoccocus elongatus* that was studied in buffer solution, glycerol-buffer mixtures, and polyvinyl alcohol (PVA).[39] It was found that the degree of observed inhomogeneity for the complexes increased in the matrices in the order glycerol-buffer, buffer solution, and PVA. Moreover, in PVA the optical spectra of the complexes showed a remarkable blueshift with respect to the other two matrices, and it was argued that conclusions drawn from PS I in PVA are of questionable value.

In our group, we have studied the light-harvesting complexes from purple bacteria by single-molecule techniques in great detail.[11,12,18−20,23,31] These bacteria have evolved a modular principle for the light-harvesting apparatus that captures the solar radiation. These modules consist of pairs of hydrophobic, low molecular weight polypeptides, called α and β (usually 50−60 amino acids long) that noncovalently bind a small number

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of bacteriochlorophyll (BChl) *a* and carotenoid (Car) molecules. These modules then oligomerize to produce the peripheral LH2 complex and the core LH1 complex, which encloses the reaction center (RC) to form the RC-LH1 complex. In LH2 the BChl *a* molecules are organized in two concentric rings referred to as B800 and B850, according to the spectral position of their room-temperature Q_y absorption bands. From the intermolecular distances determined from the X-ray structure[40,41] it can be concluded that the dipolar interaction between neighboring BChl *a* molecules in the B850 ring is significant and leads to the formation of exciton states, numbered by the quantum number *k*. Given the circular symmetry of this assembly, in first approximation only the states $k = \pm 1$ carry an appreciable transition-dipole moment that makes them accessible for optical electronic spectroscopy. The basic structural unit of LH1 is an $\alpha\beta$-heterodimer, which binds two molecules of BChl *a* and one or two molecules of carotenoid.[42,43] The pairs of BChl *a* molecules from each dimer interact together to form a strongly coupled assembly of pigments giving rise to a single, strong absorption band in the 870−890 nm spectral region. Also here, the spectroscopic properties of this assembly reflect the strong excitonic coupling among these BChl *a* molecules.

For (most of) the single-molecule experiments that we conducted on light-harvesting complexes from purple bacteria the detergent-solubilized LH complexes were immobilized in a PVA matrix, and thin films were prepared by spin coating a small amount of this solution onto a quartz substrate. Concerning LH2 we addressed the issue whether the sample preparation conditions have a significant influence on the optical spectra some years ago.[44] In that study, we compared the spectroscopic properties of individual LH2 complexes embedded in the usual spin-coated PVA film with those from individual LH2 complexes reconstituted into a 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) lipid bilayer, which was considered as the closest approximation to the natural membrane. A detailed analysis of the spectra showed that the distributions of the key spectral features, that is, the splitting of the $k = \pm 1$ exciton states and the mutual orientation of the corresponding transition-dipole moments, showed no significant differences for the two environments. Moreover, the single complexes immobilized in PVA did not display a more pronounced structural disorder than when in a phospholipid membrane. Generally the RC-LH1 complexes are considered to be more fragile compared to LH2.[45] Indeed, we found that the type of detergent used for stabilizing RC-LH1 in the buffer solution, that is, lauryldimethylamine N-oxide (LDAO) versus dodecyl-β-D-maltoside (DDM), has already a significant influence on the stability of the proteins.[46,47]

In the current paper, we set out to test whether the immobilization of RC-LH1 complexes in a PVA matrix might lead to a deterioration of the proteins and thereby limit the accessible information from optical spectroscopy. This paper is organized into two parts. In the first part, we compare details of the optical spectra from ensembles of RC-LH1 complexes as a function of the matrix and as a function of temperature. For the room-temperature experiments, the complexes were either stabilized in DDM micelles in buffer solution, in lipid vesicles, or in thin films of DDM buffer and DDM/PVA. For the experiments at low temperatures, we compare the experimental results obtained from thin-film samples of RC-LH1 complexes stabilized in DDM micelles in buffer solution, RC-LH1 complexes immobilized in DDM/PVA, and RC-LH1 complexes reconstituted into a DOPC lipid bilayer. The second part of the paper is devoted to the comparison of the results obtained from single-molecule spectroscopy at low temperatures. Therefore, we recorded spectra from individual complexes in thin films of DDM buffer solution and from those reconstituted into the lipid bilayer and compare the results with data from previous work that was conducted on RC-LH1 complexes immobilized in a DDM/PVA thin-film matrix.[47] The general finding is that the distributions of the spectral features, that is, the separation of the absorption bands and their relative polarization properties are identical for the RC-LH1 complexes in DDM/PVA and the lipid bilayer (within the statistical accuracy). Moreover, we argue that the blue shift observed for the ensemble spectra in the DDM/PVA matrix with respect to the other two matrices upon cooling reflects a thermal expansion of the PVA film.

MATERIALS AND METHODS

Materials. For the preparation of lipid vesicles, we used the phospholipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids, Alabaster, U.S.A.), which has a phase transition temperature of $T_c = -20$ °C. The detergent dodecyl-β-D-maltoside (DDM) was purchased from Roth Chemie (Karlsruhe, Germany) and the tris-hydroxymethyl-aminomethane (Tris) buffer from Sigma-Aldrich (St Louis, U.S.A.). The RC-LH1 complexes from *Rps. acidophila* strain 10050 were purified as described previously.[47,48] The pigment−protein complexes were stored in a detergent buffer solution (20 mM Tris HCl, pH 8, 0.02% DDM) at −80 °C until use.

Membrane Reconstitution. In order to create a more native-like environment for the RC-LH1 complexes, they were reconstituted into prefabricated lipid vesicles. For this, a detergent-mediated reconstitution procedure was applied, as described in refs 44, 49, and 50. Briefly, lipid (DOPC) films were resuspended in 20 mM Tris buffer (pH 8) at a concentration of 1 mg/mL and vortexed until a complete hydration of the films was achieved. Then a sequential extrusion of the lipid suspension through polycarbonate membranes (Avanti Polar Lipids) with decreasing pore size 0.8, 0.2, 0.1, and 0.05 μm, respectively (Avanti protocol), was performed. This procedure yielded unilamellar vesicles with a mean diameter of 64 nm, as determined by dynamic light scattering (DLS). Immediately after being prepared, the vesicles were used for membrane reconstitution. In order to do so, 250 μL of detergent-stabilized RC-LH1 complexes were diluted to either 35 pM (single-complex experiments) or to 650 nM (ensemble experiments), mixed with 250 μL of the liposome suspension, and inserted into a Slide-A-Lyser dialysis cassette (Thermo Scientific, Rockford, U.S.A.) with a molecular weight cutoff of 3500 Da. This suspension was then dialyzed against a reservoir of 1.4 L of detergent-free buffer at 5 °C in a dark cold room for 3 days with exchanging the buffer once at half time. Then, the reconstituted RC-LH1 complexes were removed from the dialysis cassette and either used immediately for the spectroscopy experiments or stored at 4 °C in the dark for at most 9 days until use. For the single-complex samples, the average lipid-to-protein ratio was $3.5 \times 10^7/1$ (molar concentration ratio, c/c), which corresponds to about 10^{-3} RC-LH1 complexes per vesicle.[44] This high dilution ensures that the probability for having two complexes in one vesicle is extremely low (0.0001%). For the samples used for ensemble spectroscopy, the average lipid-to-protein ratio was about 1900/1 (c/c) corresponding to an average of 19 RC-LH1

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complexes per vesicle. In order to test for the integrity of the RC-LH1 complexes after the reconstitution process and to verify whether the protocol was successful, the room-temperature absorption spectra were recorded from the ensemble samples using a commercial UV/vis/NIR-Spectrophotometer (Lambda 750, Perkin-Elmer, Waltham, U.S.A.). The stability of the reconstituted samples was checked by taking a second absorption spectrum from the same sample two weeks later. Both spectra were identical and no indication for a dissociation/denaturation of the RC-LH1 complexes was found.

Room-Temperature Absorption Spectroscopy. Ensemble absorption spectra of bulk samples were recorded with a commercial UV/vis/NIR-Spectrophotometer (Lambda 750, Perkin-Elmer, spectral resolution 1 nm) using quartz cuvettes with an optical path length of 1 mm (Hellma, Müllheim, Germany). The optical density of the samples was adjusted to about 1 cm^{-1} at 885 nm (OD_{885}), which corresponds to a concentration of 265 nM.[51]

For ensemble absorption spectroscopy on thin films, the samples were spin-coated under dimmed light onto a quartz substrate. The detergent buffer solution containing the RC-LH1 complexes was either used as is or mixed with 2% (w/w) polyvinyl alcohol (PVA, M_w = 124000−186000 g/mol; Sigma-Aldrich, Milwaukee, U.S.A.). In order to detect a signal from the films with a thickness of about 100 nm, the protein concentration was adjusted to OD_{885} = 25 cm^{-1} corresponding to 6.6 μM.

Sample Preparation for the Low-Temperature Spectroscopy Experiments. A drop (25 μL) of the RC-LH1 solution (either in detergent or in the lipid bilayer) was pipetted under nitrogen atmosphere onto a quartz substrate and left there for adsorption for 10 min. Then the substrate was spun for 10 s at 500 rpm and 60 s at 2000 rpm. In order to minimize the dissociation of the RC-LH1 complexes stabilized in detergent buffer solution[52] these steps were carried out in a dark, cold room at 5 °C. For the samples with the reconstituted RC-LH1 complexes, the dilution and the spin-coating was done at room temperature under dimmed light conditions. Next the sample was plunged into a cryostat that was precooled with liquid nitrogen. After pumping out the nitrogen, the cryostat was filled with liquid helium and cooled down to 1.2 K. The concentration of the RC-LH1 complexes was about 265 nM for the ensemble experiments and 10 pM for the single-molecule experiments.

Low-Temperature (1.2 K) Spectroscopy. *Ensemble.* Both fluorescence-excitation and fluorescence-emission experiments (see Supporting Information) were conducted in a home-built microscope that can be operated either in wide-field or in confocal mode.[53] As excitation source served a continuous-wave tunable titanium-sapphire (Ti:Sa) laser (3900S, Spectra Physics, Mountain View, U.S.A.) that was pumped by a frequency-doubled continuous-wave neodynium/yttrium-vanadate ($Nd:YVO_4$) laser (Millenia Vs, Spectra Physics). Precise changes of the wavelength of the Ti:Sa laser were accomplished by rotating an intracavity birefringent filter with a motorized micrometer screw. The accuracy as well as the reproducibility of the laser frequency were tested with a wavemeter and were about 1 cm^{-1}. Ensemble fluorescence-excitation spectra were recorded by scanning the laser repetitively (60 scans) between 780 and 900 nm and detecting the emitted fluorescence with a single-photon counting avalanche photodiode (APD) (SPCM-AQR-16, Perkin-Elmer, Vaudreuil, Canada) after passing a pair of band-pass filters (BP925/40, AHF Analysetechnik, Tübingen, Germany). Each recorded trace was stored separately in computer memory. The scan speed was 2 nm/s (~26 cm^{-1}/s) and the acquisition time was 10 ms, which yields a nominal resolution of about 0.26 cm^{-1}, ensuring that the spectral resolution is limited by the spectral bandwidth of the laser, that is 1 cm^{-1}. In order to control the polarization of the excitation light a $\lambda/2$-plate is inserted in the excitation path and rotated by 3.1° between successive scans, changing the polarization angle by twice this value. The spectra were corrected for the variation of the incident laser power as a function of the excitation wavelength. The typical excitation intensity for the ensemble fluorescence-excitation measurements was about 5 W/cm^2.

Single-Complexes. First, a wide-field fluorescence image was recorded by exciting the sample at 890 nm with the output from the Ti:Sa laser that passed an excitation filter (BP 875/50, AHF Analysetechnik) to suppress residual laser fluorescence. The excitation light was slightly defocused by a lens to an area of about 45 × 45 μm^2 in the sample plane, and the emitted fluorescence was collected by a microscope objective (Microthek, Hamburg, Germany, NA = 0.85) that was mounted inside the cryostat. The signal passed a set of four band-pass filters (BP 935/40, AHF Analysetechnik) that blocked backscattered excitation light and was registered with a back-illuminated CCD camera (Ikon-M 934 BR-DD, Andor Technology, Belfast, U.K.). Once the individual complexes had been located, the microscope was switched to the confocal mode such that the focal volume coincided with one of the individual complexes from the wide-field image. Fluorescence-excitation spectra were recorded by scanning the laser repetitively between 850 and 910 nm and registering the signal as described above. For the RC-LH1 complexes stabilized in detergent buffer solution, a detection filter with a more blue-shifted transmission range (BP 925/40, AHF Analysetechnik) was used and the scan range of the laser was shifted to 840−900 nm. The excitation intensity was typically 140 W/cm^2.

RESULTS

Ensemble Spectroscopy. A comparison of the room-temperature absorption spectra of ensembles of RC-LH1 complexes from *Rps. acidophila* in detergent buffer solution (DDM, black line) and reconstituted into a lipid bilayer (DOPC, red line) is shown in Figure 1. The main absorption at about 885 nm (B885 band) is due to the Q_y transition of the BChl *a* molecules, whereas the band at around 600 nm reflects the absorption from the Q_x transition of the BChl *a* molecules. These bands are clearly separated from the absorption bands caused by the reaction center (around 800 nm), the carotenoids (around 500 nm), the Soret band of the BChl *a* molecules (around 400 nm), and the protein matrix in the near UV spectral region. Upon reconstitution of the pigment−protein complexes into a lipid bilayer, the B885 absorption band is slightly red shifted by 2 nm with respect to the same band obtained from complexes that were stabilized in detergent buffer solution, see inset Figure 1. This spectral shift indicates that the reconstitution process was successful.[44] In Figure 2, we compare the thin-film absorption/fluorescence-excitation spectra of RC-LH1 in three different matrices at two different temperatures. The top two traces feature the room-temperature B885 absorption band of RC-LH1 sustained either in a film of detergent buffer solution or in a PVA layer. The two spectra

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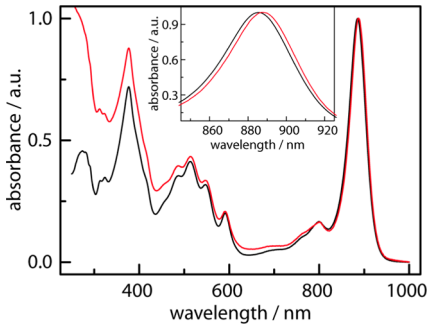

Figure 1. Room-temperature absorption spectra of RC-LH1 complexes from *Rps. acidophila* stabilized in DDM micelles in buffer solution (black line) or reconstituted into a DOPC bilayer (red line). The increase of the absorbance below 500 nm for the reconstituted sample is due to Rayleigh scattering of the vesicles. The inset shows an expanded view of the BChl *a* Q_y absorption bands, peaking at 885.5 nm for the detergent-stabilized complexes and at 887.5 nm for the reconstituted complexes. For better comparison, the spectra were peak normalized to the maximum of their Q_y absorptions.

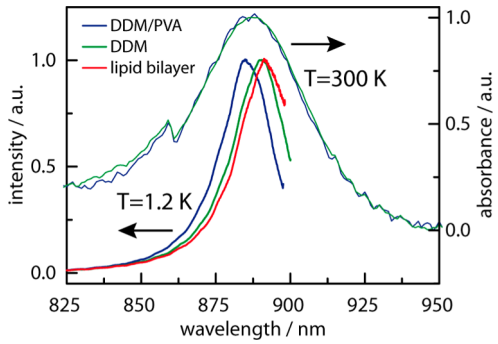

Figure 2. Top, room-temperature absorption spectra of ensembles of RC-LH1 complexes from *Rps. acidophila* (OD_{885} = 25 cm^{-1}) embedded in a thin layer of buffer DDM solution (green) or DDM/PVA (blue). Bottom, low-temperature fluorescence-excitation spectra of RC-LH1 (OD_{885} = 1 cm^{-1}) in buffer DDM solution (green), DDM/PVA (blue) or reconstituted into a DOPC bilayer (red). For clarity, all spectra are peak normalized and correspond to averages over all polarizations of the incident radiation as well as over 40 adjacent data points. The excitation intensity was 5 W/cm^2.

closely overlap and feature about the same spectral peak position and line width.

At low temperatures (1.2 K), we recorded fluorescence-excitation spectra from thin films that contained RC-LH1 complexes (i) in DDM micelles in buffer solution, (ii) in DDM micelles in PVA, and (iii) in a DOPC lipid bilayer. At the bottom of Figure 2, the corresponding fluorescence-excitation spectra are shown for RC-LH1 complexes embedded in the three different environments. The low-temperature fluorescence-emission spectra of the thin-film ensembles are provided in the Supporting Information. All details of the spectral features shown in Figures 1 and 2 are summarized in Table 1.

For the samples embedded in DDM or DDM/PVA, the width (full width at half-maximum, fwhm) of the B885 absorption band decreases from about 560 cm^{-1} at room temperature to about 280 cm^{-1}, upon cooling the samples to low temperatures. Interestingly, the peak position of the absorption band shifts differently for the two matrices. We observe a red shift from 887.0 to 889.9 nm for DDM and a smaller blue shift from 886.5 to 885.5 nm for DDM/PVA,

Table 1. Spectral Properties of the Main Q_y Absorption Band of Bulk/Thin-Film Ensembles of RC-LH1 Complexes from *Rps. acidophila* as a Function of Temperature and Environment

	T (K)	peak of the absorption*/ fluorescence-excitation band (nm)	spectral width (fwhm) (cm^{-1})
		Bulk RC-LH1 Sample (Figure 1)	
DDM	300	885.5*	571
vesicles	300	887.5*	562
		Thin-film RC-LH1 Sample (Figure 2)	
DDM/ PVA	300	886.5*	555
DDM	300	887.0*	565
DDM/ PVA	1.2	885.5	272
DDM	1.2	889.9	287
lipid bilayer	1.2	891.8	293

which is within the resolution of the instrument. For the RC-LH1 complexes reconstituted into a lipid bilayer, we observe a similar temperature-dependent reduction of the line width as for RC-LH1 in the two other environments, here from 562 to 293 cm^{-1}, and a red shift of the peak position from 887.5 nm at room temperature to 891.8 nm at cryogenic temperatures. Hence, the slight difference of 2 nm between the spectral peak positions for RC-LH1 in DDM and reconstituted into a lipid bilayer is conserved when the temperature is lowered.

Single-Complex Spectroscopy. An example for a fluorescence-excitation spectrum from a single RC-LH1 complex stabilized in detergent buffer solution is shown in Figure 3.

Figure 3b displays a stack of 87 consecutively recorded spectra, where the polarization of the incident radiation has been rotated by 6.2° after each individual scan. From this pattern it is obvious that the optical signal strongly fluctuates with extended periods that prohibit the recording of any signal from the complex. But also during the bright periods the signal strength shows strong variations. For some individual scans, the signal level remains sufficiently stable to allow the recording of a fluorescence-excitation spectrum that features the typical RC-LH1 characteristics: a broad band followed by a narrow line on its red side,[18,47] see Figure 3c red line. However, for about half of the individual spectra the signal level is affected significantly by blinking and/or spectral diffusion, which prevents the recording of a spectrum, see Figure 3c blue line. Such a behavior was found for the majority of RC-LH1 complexes in detergent buffer solution. For this preparation method, it was therefore impossible to obtain detailed information from the fluorescence-excitation spectra, such as the number of bands, their energetic separation, or the mutual orientation of the corresponding transition-dipole moments.

The situation is different for RC-LH1 complexes that were reconstituted into a lipid bilayer. For this preparation method the vast majority of the 83 studied complexes featured a stable signal level. Typically the fluorescence-excitation spectra of these complexes featured a few broad bands with widths (fwhm) varying between 100 and 240 cm^{-1}, and at least one narrow absorption line with widths (fwhm) mostly between 1 and 2 cm^{-1}. It is worth noting that the widths of the narrow lines do not reflect the homogeneous linewidths but are determined by unresolved spectral diffusion. In a few exceptional cases with very strong spectral diffusion, the widths

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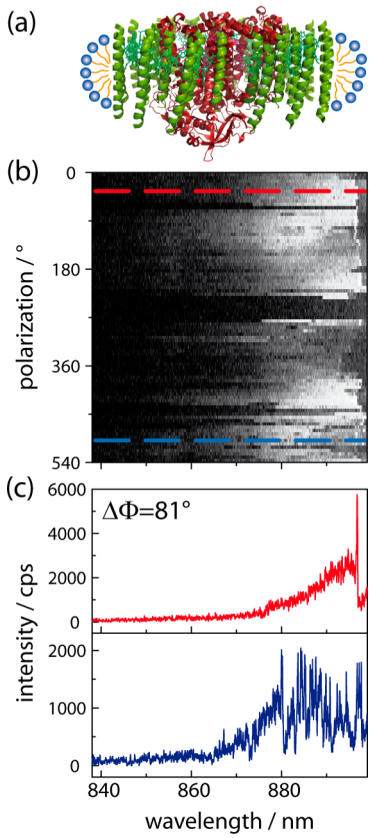

Figure 3. (a) Sketch of a RC-LH1 complex stabilized inside a detergent micelle (red, RC; green, LH1; yellow/blue, hydrophobic tail/hydrophilic headgroup of DDM). (b) Two-dimensional representation of 87 consecutively recorded fluorescence-excitation spectra from an RC-LH1 complex from *Rps. acidophila* stabilized in detergent buffer solution. The horizontal axis corresponds to wavelength, the vertical axis to the polarization of the incident radiation and the fluorescence intensity is given by the gray scale, where white denotes high intensity. Between two successive scans, the polarization of the excitation light has been rotated by 6.2°. (c) Examples of two individual fluorescence-excitation spectra that were recorded for a mutual phase difference of $\Delta\Phi = 81°$ for the polarization of the excitation light. The dashed horizontal lines in (b) indicate the positions of these spectra within the stack. Both spectra have been smoothened by averaging over five adjacent data points. The temperature of the sample was 1.2 K, and the excitation power was 678 W/cm^2.

of these lines increased up to about 7 cm^{-1}. The observed fluorescence-excitation spectra could be grouped into the same three classes as observed previously for the RC-LH1 complexes from *Rps. acidophila* in DDM micelles in PVA.[47]

Briefly, the spectra assigned to class A (49 of 83 complexes) featured two dominant broad bands with distinctly different polarizations and a narrow spectral feature at the low-energy wing, those assigned to class B (4/83) featured (predominantly) a single linear polarization of the whole spectrum, and finally the complexes grouped in class C (30/83) featured multiple narrow lines in the spectrum. A typical example for a fluorescence-excitation spectrum from each class is shown in Figure 4. The patterns in Figure 4b–d display each a stack of 87 consecutively recorded spectra in a two-dimensional representation similar to that of Figure 3. Between two successive scans the polarization of the excitation light was rotated by 6.2°. The spectra underneath the patterns correspond to averages of three consecutive scans and display the spectral features that are characteristic for the class assignment.

The distribution of the complexes across the classes A–C is given in Table 2 together with the distribution obtained in a previous study, where we investigated RC-LH1 complexes from *Rps. acidophila* immobilized in DDM micelles in a PVA matrix.[47] In that study, only the class A spectra were attributed to stem from intact RC-LH1 complexes. The class B spectra were considered as RC-LH1 complexes that were tilted so far that they were oriented with the plane of the BChl *a* ring perpendicular to the substrate, and class C spectra were ascribed to fragments from broken RC-LH1 complexes that were in the process of dissociation/denaturation prior to cooling them to low temperatures. Following the reasoning of the previous paper, the reconstitution process described here leads to a reduction of the fraction of intact complexes from 82 (DDM/PVA) to 59% (lipid bilayer), whereas the fraction of broken complexes increases from 11.5 to 36%. This indicates that the reconstitution procedure is probably a rather harsh treatment for the RC-LH1 complexes.

Accordingly, we will focus the analysis in the following section on the class A type spectra from which we extracted the spectral separations of the observed bands and the mutual orientation of the associated transition-dipole moments. For the example shown in Figure 4b, the energetic separation between the bands B1 and B2, ΔE_{B1B2}, amounts to 35 cm^{-1} and between bands B2 and B3, ΔE_{B2B3}, to 75 cm^{-1}. Integration of the intensity of the bands B2 and B3 over a spectral region that is indicated by the bar on top of the spectral diffusion plot, Figure 4b, yields the modulation of the signal as a function of the polarization of the excitation light. The results are shown next to the pattern in Figure 4b together with cos^2-type fit functions from which we obtain the mutual angle between the respective transition-dipole moments as $\Delta\alpha_{B2B3} = 77°$. Similarly we find $\Delta\alpha_{B1B2} = 2°$ (data not shown).

The distributions of these parameters are shown in Figure 5 for all complexes that featured class A spectra. The red entries refer to the current study; the gray entries are valid for RC-LH1 in DDM/PVA and have been taken from ref 47. The scale for the phase angles has been restricted to values between 0 and 90° by using $\Delta\alpha_{BiBj} = |\alpha_{Bi} - \alpha_{Bj}|$, if the result was less than 90°, or $\Delta\alpha_{BiBj} = 180° - |\alpha_{Bi} - \alpha_{Bj}|$ otherwise. The number of entries for the histograms differs because some spectra did not allow the determination of all parameters. Visual inspection of the histograms reveals already that the distributions for both sample environments are very similar. This is quantified in Table 3, which provides the statistical parameters, mean and standard deviation (SD), for each histogram. This testifies that within statistical accuracy the results of the current study are equivalent to those obtained on RC-LH1 complexes embedded in a PVA matrix.[47]

The distribution of the spectral peak position of the narrow band B1 is shown in Figure 6 (red bars) together with the sum of all individual spectra that were assigned to class A. The histogram is centered at 902 nm (mean) in the red wing of the sum spectrum and has a width (SD) of 40 cm^{-1}. For comparison the gray entries in Figure 6 show the corresponding data for the class A type spectra from RC-LH1 complexes of *Rps. acidophila* embedded in DDM/PVA.[47] For this environment the distribution of the spectral position of the narrow peak (gray bars) is centered at 892 nm (mean) and features a width (SD) of 48 cm^{-1}. Yet, it is obvious from Figure 6 that the narrow spectral feature appears for both sample

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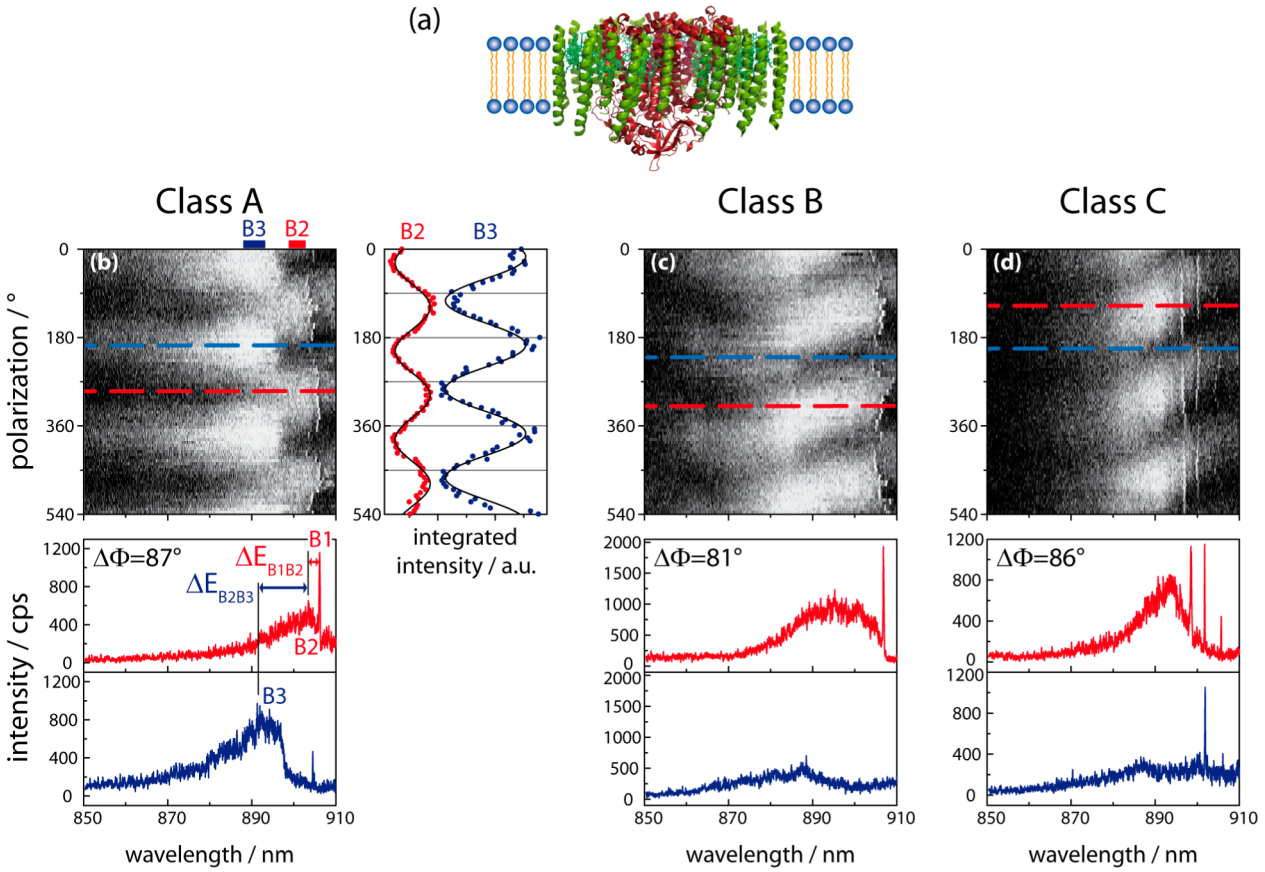

Figure 4. Classes of fluorescence-excitation spectra obtained from different RC-LH1 complexes from *Rps. acidophila* reconstituted into a lipid bilayer. (a) Sketch of a RC-LH1 complex reconstituted into a DOPC bilayer. The color code is the same as in Figure 3. The upper panels in (b–d) show stacks of 87 consecutively recorded fluorescence-excitation spectra. Between two individual fluorescence-excitation spectra, the polarization of the incident radiation has been rotated by 6.2°, and the fluorescence intensity is indicated by the gray scale. The spectra displayed at the bottom of each panel correspond to the average over three adjacent individual spectra (blue/red) that have been recorded for a distinct polarization of the excitation (indicated by the dashed colored lines in the top panels). The relative phase difference between the red/blue example spectra is given by ΔΦ. The excitation intensity was about 140 W/cm^2. For the class A example, the energetic separations between the bands B1 and B2, ΔE_{B1B2}, and between the bands B2 and B3, ΔE_{B2B3}, are indicated in the figure. The graph on the right-hand side of panel (b) shows the variation of the fluorescence intensity that is integrated over the spectral intervals denoted as B2 and B3 at the top of pattern (b), as a function of the polarization of the incident radiation. The dots correspond to the data and the black lines correspond to cos^2-type functions fitted to the experimental data.

Table 2. Relative Fractions (Total Numbers) of RC-LH1 Complexes from *Rps. acidophila* Assigned to the Three Classes A, B, and C in the Lipid Bilayer and in DDM/PVA

	lipid bilayer (this work)	DDM/PVA (data from ref 47)
class	relative fraction (number of complexes)	relative fraction (number of complexes)
A	59% (49)	82% (50)
B	5% (4)	6.5% (4)
C	36% (30)	11.5% (7)

preparation conditions on the low energy wing of the sum spectrum. The slightly broader distribution for the B1 spectral peak position of the RC-LH1 complexes stabilized in detergent micelles inside the PVA matrix with respect to those reconstituted into a lipid bilayer provides a first hint that the packing of the complexes in the polymer matrix is not as tight as in the lipid bilayer, giving rise to more conformational freedom (vide infra). Interestingly, both distributions are slightly narrower with respect to what has been found for the widths of the distributions of the zero-phonon hole (ZPH) action spectra.[54,55] It is likely that this results from the influence of the phonon-side bands on the hole burning action spectra which is excluded here, because the histograms refer exclusively to the peak position of the zero-phonon line.

DISCUSSION

At room temperature, the ensemble absorption spectra from RC-LH1 complexes reconstituted into a DOPC lipid bilayer feature a 2 nm red shift of the B885 band with respect to complexes that are stabilized in DDM detergent and dissolved in buffer solution, see Figure 1. This red shift probably reflects a slight compression of the RC-LH1 complexes in the lipid bilayer (vide infra) and/or an aggregation of the complexes as was shown in refs 36, 56, and 57. The latter is corroborated by the strong Stokes shift that is observed for the reconstituted RC-LH1 complexes with respect to those in the other matrices, see Supporting Information Figure S1, and which is a clear indication for excitation energy transfer toward the lowest energy states within the network of complexes.

In order to compare the influence of temperature on the peak position of the B885 absorption band of RC-LH1 complexes that are embedded in thin-film samples, we used two different setups; namely, a commercial spectrometer for the room-temperature experiments, and our single-molecule

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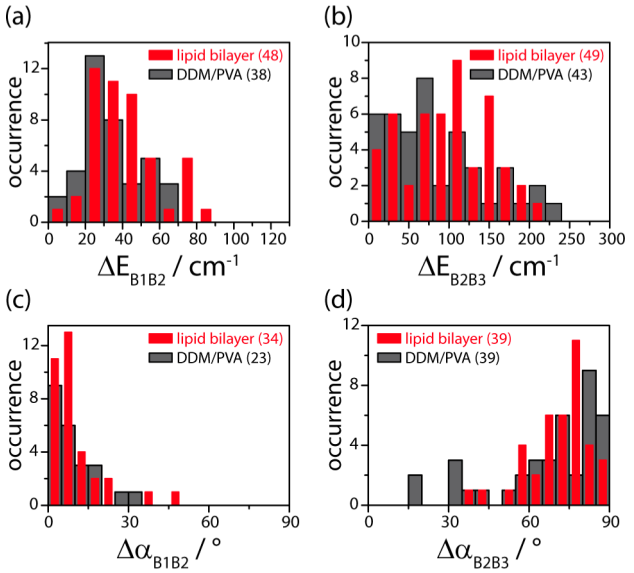

Figure 5. Distributions of the energetic separations between the bands (a) B1 and B2, and (b) B2 and B3. Distributions of the mutual angles between the transition-dipole moments associated with (c) B1 and B2, and (d) B2 and B3. The gray bars refer to RC-LH1 complexes from *Rps. acidophila* that have been embedded in a DDM/PVA matrix and have been taken from ref 47. The red bars refer to the current study. For both cases the number of RC-LH1 complexes that contributed to a histogram is given in the legend.

Table 3. Energetic Separations ΔE_{B1B2} (ΔE_{B2B3}) of the Bands B1 and B2 (B2 and B3) and Mutual Angles $\Delta\alpha_{B1B2}$ ($\Delta\alpha_{B2B3}$) between the Corresponding Transition-Dipole Moments for the Class A RC-LH1 Complexes Stabilized in a Lipid Bilayer and in DDM/PVA

	ΔE_{B1B2} (mean ± SD)/cm^{-1}	ΔE_{B2B3} (mean ± SD)/cm^{-1}	$\Delta\alpha_{B1B2}$ (mean ± SD)/°	$\Delta\alpha_{B2B3}$ (mean ± SD)/°
lipid bilayer (this work)	40 ± 18	98 ± 55	9.4 ± 9.8	71 ± 11
DDM/PVA[47]	33 ± 17	85 ± 60	8.5 ± 7.8	67 ± 20

confocal microscope for the experiments under cryogenic conditions. Because of the different sensitivity of the detectors in the two setups, we had to use a sample with a protein concentration of about 6 μM (corresponding to OD_{885} = 25 cm^{-1}) for the room-temperature experiment. This is in contrast to the low-temperature work where a protein concentration of about 250 nM (OD_{885} = 1 cm^{-1}) was used. Therefore, we first had to verify whether for the high-concentration sample aggregation of the RC-LH1 complexes might play a role.[58,59] Indeed, we observe for the B885 band a red shift of 1.5 nm in the thin-film sample with respect to the monodispersed RC-LH1 complexes in DDM bulk-buffer solution. This shift is small, yet not negligible, which has to be kept in mind for the comparison between room-temperature and low-temperature experiments. Nevertheless, the two top traces in Figure 2 clearly demonstrate that the addition of PVA leaves the spectral position and the shape of the B885 absorption band unaffected.

For RC-LH1 complexes embedded in a lipid bilayer or (thin films of) DDM buffer solution, the peak of the B885 absorption band features a red shift of 4.3 or 2.9 nm, respectively, upon cooling the samples to cryogenic temperatures. This is in contrast to samples immobilized in DDM/PVA where a small

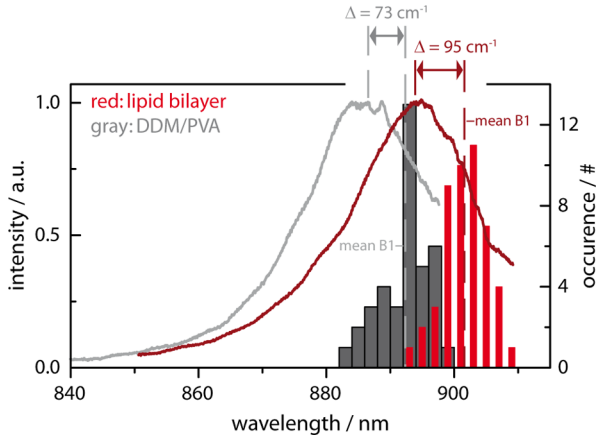

Figure 6. Distribution of the spectral peak position of the band B1 for RC-LH1 complexes from *Rps. acidophila* reconstituted into a lipid bilayer (red bars) or embedded in a DDM/PVA matrix (gray bars). For comparison, the respective sum spectra of all individual complexes assigned to class A are shown by the full lines (for DDM/PVA only those 38 complexes were considered from ref 47 where a narrow absorption line could be detected). The energetic separation between the mean of the histogram and the peak of the sum spectrum amounts to 95 cm^{-1} for the lipid bilayer and to 73 cm^{-1} in DDM/PVA, respectively.

blue shift of 1 nm, which is within the resolution of the spectrometer, is observed. It is well-known that the DOPC bilayer undergoes a phase transition at T_C = −20 °C from a two-dimensional liquid crystalline phase above T_C to a highly ordered and densely packed gel phase below T_C.[60] Hence, it is conceivable that the phase transition leads to a compression of the RC-LH1 complexes that are embedded in the lipid bilayer. In ref 61, the authors ascribed the main contribution of the observed pressure-induced red shift to pigment−pigment interactions, because it was shown that a change of the local pressure had only a minor influence on the protein binding pockets and the conformation of the BChl *a* molecules in LH1 complexes. A putative compression of the LH1 complexes by the lipid bilayer results in an increase of the dispersive interactions that yields a lowering of the BChl *a* site energies.[62,63] At the same time, it causes an increase in the excitonic interaction between the pigments and results in a larger splitting of the exciton states. Because the main oscillator strength is associated with the states at the bottom of the exciton band this yields as well a lowering in the transition energy. Likewise, for the samples in DDM buffer an increase of the local pressure may be induced by crystalline bulk water[39] and similar effects as above may lead to the observed red shift of 2.9 nm (37 cm^{-1}). In contrast, to the other two environments immobilization of the RC-LH1 complexes in the DDM/PVA matrix yields no significant spectral shift of the B885 absorption band upon cooling. This is consistent with the finding that thin polymer films exhibit a negative thermal expansion coefficient below the glass transition temperature T_g.[64] For pure PVA, T_g amounts to 80 °C, and an expansion rather than a contraction of the polymer film upon cooling is conceivable.

Next, we will focus on the single-complex, low-temperature fluorescence-excitation spectra. The complexes have been embedded in three different environments, namely in DDM micelles in buffer, in a DOPC lipid bilayer, and in a DDM/PVA matrix. The data for the latter guest/host configuration have

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been taken from 47. Unfortunately, the RC-LH1 complexes sustained in DDM micelles in buffer feature a pronounced blinking of the emitted intensity that prohibits any quantitative analysis of the spectra. This is in clear contrast to the RC-LH1 complexes embedded in the other two environments, where the majority of complexes showed stable emission characteristics during the more than one hour experimental time. For the RC-LH1 complexes reconstituted into a DOPC bilayer, we find the same characteristic features that led to the grouping of the complexes into three distinct classes, A–C, as observed previously for the complexes immobilized in DDM/PVA.[47] Interestingly, the fraction of complexes that were associated with broken RC-LH1 complexes, that is, those assigned to class C, increases significantly for the lipid bilayer environment with respect to the DDM/PVA matrix. In our opinion, this reflects that the dissociation/denaturation of the RC-LH1 complexes is more pronounced during the reconstitution process. This interpretation is supported by the following observations. For RC-LH1 complexes from *Rsp. rubrum*, it was found that dissociation becomes more effective upon increasing the detergent concentration,[52,65] which is equivalent to a decrease of the protein concentration for the single-complex experiments as in our case. As a matter of fact, for the single-complex experiments on reconstituted RC-LH1 complexes the protein concentration in the detergent buffer solution has been lowered to a detergent-protein-ratio of about $1.1 \times 10^7/1$ (c/c), before it is joined with the vesicle solution. Subsequently, for dialysis this mixture is kept in a cold room at 5 °C for three days. Given the results on *Rsp. rubrum*, it would be expected that some of those RC-LH1 complexes that are still in the detergent environment will tend to dissociate during this time, that is, before they are incorporated into the vesicles. This is consistent with the larger fraction of intact RC-LH1 complexes in the DDM/PVA environment, that is, those assigned to class A, because these samples are cooled down to cryogenic temperatures immediately after the dilution process, which reduces the time interval between preparation and observation tremendously. Yet it is worth to note that once the RC-LH1 complexes have been reconstituted into a lipid bilayer the samples can be kept without a loss of quality in the refrigerator for more than a week, which is impossible for complexes stabilized in a detergent buffer solution at single-complex concentrations. This testifies that the stability of the RC-LH1 complexes once reconstituted into the lipid bilayer is generally higher than in a detergent micelle.[37]

A detailed evaluation of the data was performed only for those spectra that were assigned to class A, which we interpret to stem from intact RC-LH1 complexes. These spectra display the general characteristic features that have been found for (monomeric) RC-LH1 complexes in previous studies.[18,19,46,47] Signatures for an increase of the interaction strength between the BChl *a* molecules in RC-LH1 in the lipid bilayer with respect to those embedded in the DDM/PVA matrix, as have been found in the ensemble spectra, can also be found in the single-molecule data. For example, in Figure 6 the larger spectral separation of 95 cm^{-1} between the lowest exciton state and the maximum of the sum spectrum for the reconstituted complexes with respect to the separation of 73 cm^{-1} for the RC-LH1 complexes embedded in DDM/PVA indicates an increase of the intermolecular interaction strength between the BChl *a* molecules within the RC-LH1 complexes that are reconstituted into the lipid bilayer. Finally, in the lipid bilayer the width of the band B1 is distributed between 1 and 2 cm^{-1}, whereas the same distribution covers the range from 3–12 cm^{-1} in the DDM/PVA matrix.[47] From this observation, it can be concluded that structural fluctuations that give rise to spectral diffusion are more pronounced in the polymer matrix, which might reflect a larger degree of conformational freedom for the RC-LH1 complexes in this matrix.

In summary, we have studied RC-LH1 complexes from *Rps. acidophila* immobilized in different environments. The ensemble absorption spectra show spectral shifts as a function of the matrix as well as a function of temperature. The latter effect can be explained consistently with variations of the local pressure that are induced by the matrix material upon cooling the sample. Application of single-molecule techniques reveals that the reconstitution process results in a significantly larger fraction of broken complexes with respect to embedment in a DDM/PVA film. In single-molecule spectroscopy, these complexes can be treated separately and the intact complexes feature the same spectral signatures as their intact counterparts that have been immobilized in DDM/PVA. However, given the extra effort of reconstitution, the increased fraction of broken complexes, and the fact that the intact complexes feature the same decisive spectral details as those prepared in DDM/PVA, we conclude that, at least for the experiments conducted at low temperatures, using PVA as a matrix is a good choice.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Low-temperature (1.2 K) fluorescence-excitation and -emission spectra of ensembles of RC-LH1 complexes from *Rps. acidophila* stabilized in thin films in three different environments and a table showing the characteristic parameters of the spectra (i.e., peak positions, linewidths, and Stokes shifts). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

RC, reaction center; LH, light-harvesting; PVA, polyvinyl alcohol; BChl, bacteriochlorophyll; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DDM, dodecyl-*β*-D-maltoside; fwhm, full width at half-maximum; SD, standard deviation

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