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FLUORESCENCE PROPERTIES OF THE LIGHT-HARVESTING BACTERIOCHLOROPHYLL PROTEIN FROM *RHODOPSEUDOMONAS SPHAEROIDES* R-26

JOHN D. BOLT * and KENNETH SAUER

Chemistry Department and Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720 (U.S.A.)

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The light-harvesting bacteriochlorophyll-protein (BChl-protein) from *Rhodopseudomonas sphaeroides*, R-26 mutant, exhibits a strong optical absorption peak near 850 nm (Q_y band) and a weaker peak at 590 nm (Q_x band). This pigment-protein appears to contain two BChl molecules per subunit, and previous circular dichroism studies indicated the presence of excitonic interactions between the BChl molecules. The complex exhibits a fluorescence maximum near 870 nm at room temperature. Excitation in the Q_y region results in polarization p values that vary only from +0.12 at 820 nm to +0.14 near 900 nm. These values are appreciably smaller than that for monomeric BChl in viscous solvents ($p > 0.4$). By contrast, using Q_x excitation the p value is −0.25 for the BChl-protein complex, which is close to that observed for the BChl monomer. For the BChl-protein these polarization values do not change greatly at a temperature of 90 K; however, the Stokes' shift of the fluorescence emission increases significantly over that at room temperature.

Introduction

The light-harvesting complexes isolated from photosynthetic bacteria offer the simplest classes of proteins containing bacteriochlorophyll or chlorophyll [1–3]. Typically, two or three BChl and often one or two carotenoid molecules occur stoichiometrically with a small number (one to three) of peptides [4,5]. These simplest subunits usually aggregate in the bacterial intracytoplasmic membranes, and in several cases the 'monomeric' complexes have yet to be identified and characterized. To gain an understanding of the chromophore-chromophore interactions that govern energy transfer and the chromophore-protein interactions that determine the structure of the complex, a better knowledge of the arrangement of the pigment molecules is needed.

Sauer and Austin [4] reported the isolation of a simple light-harvesting complex from *Rhodopseudomonas sphaeroides*, R-26 (carotenoidless mutant). The complex consists of two BChl molecules and two polypeptides, totalling about 20 kdaltons in the presence of SDS. This complex retains the large excitonic circular dichroism (CD) indicative of BChl-BChl interaction and the large red shift of the near infrared (Q_y) absorption of BChl that is characteristic of native membranes [4]. In a previous paper [6] we presented the linear dichroism of this complex from the R-26 mutant, as well as that of the major light-harvesting protein (B800-850) from the parent organism.

We have investigated the fluorescence polarization

* Present address: Department of Chemistry, Havemeyer Hall, Columbia University, New York, NY 10011, U.S.A.

Abbreviations: BChl, bacteriochlorophyll; SDS, sodium dodecyl sulfate.

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of the BChl-protein from the R-26 mutant, and we are able to use this information to refine our previous model for the arrangement of the BChl molecules within the complex.

Materials and Methods

Sample preparation. The light-harvesting protein of *Rps. sphaeroides*, R-26 has been prepared by a new procedure. Intracytoplasmic membranes (chromophores) with an absorbance of 50 cm^{-1} at 862 nm were solubilized with 0.1 vol. of 10% SDS and diluted to 0.2% SDS with 10 mM Tris-HCl, pH 7.5. The solubilized membranes were loaded onto a hydroxyapatite column [7] equilibrated with 0.05 M Na_2HPO_4/NaH_2PO_4, 0.1 M NaCl, 0.1% SDS, pH 7.0. The column was eluted successively with 0.05, 0.10 and 0.15 M phosphate buffers containing NaCl and SDS. A sharp blue band was eluted with the 0.15 M phosphate. Fractions with an absorbance ratio $A_{848}/A_{275} > 2.9$ were pooled, dialyzed and suspended in 10 mM Tris-HCl, 0.1% SDS, pH 7.5. More than 60% of the BChl present in the native membranes of the R-26 mutant is isolated as the intact BChl-protein complex.

The results presented here were also reproduced with the R-26 light-harvesting BChl-protein prepared as described previously using dodecyldimethylamine oxide solubilization, centrifugation and Sephadex chromatography [6]. Comparative measurements were made on a larger aggregate of the light-harvesting protein prepared by Triton X-100 solubilization of chromatophore membranes according to the method of Sauer and Austin [4].

Bacteriochlorophyll was isolated from carotenoidless bacteria by extraction with acetone/methanol (7 : 2, v/v).

Fluorescence measurements. Fluorescence spectra and polarization values were measured using an instrument that was assembled for this purpose. Excitation light from a 500 W xenon arc lamp was passed through a grating monochromator (Jarrell-Ash, Model 82, 0.25 meter, dispersion 3.3 nm/mm). Normally 1 mm slits were used. The output beam passed through appropriate filters for rejection of stray light and second-order diffraction components and was focussed into a 1 cm square cuvette. Fluorescence collected at 90° was passed through optical filters and was dispersed by a second grating monochromator (Bausch and Lomb, 0.5 meter, dispersion 3.5 nm/mm). The detection system, based on photon counting, consisted of a photomultiplier (RCA 31004, S-1 response, cooled by solid CO_2) coupled via an amplifier to a discriminator and pulse counter (Princeton Applied Research, Models 1121 and 1109). Emission spectra were corrected for the wavelength-dependent response of the detection system and for fluorescence reabsorption [8], both minor corrections under our conditions.

For fluorescence polarization measurements the excitation light was passed through a Glan-Thompson prism, emerging with the electric vector oriented vertically. The fluorescent light at 90° was first collimated, then passed through a half-wave plate (optimal for 895 nm wavelength) and a Glan-Thompson prism, also with the electric vector of the transmitted light oriented vertically. Rotation of the half-wave plate by 45° then permitted monitoring of either the vertically or horizontally polarized components of the emitted light (see Ref. 9 for details of this procedure).

Polarization, p, is defined here as

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{1}$$

where $I_{\parallel}$ and $I_{\perp}$ denote intensities of light polarized parallel and perpendicular to the excitation polarization, respectively. At wavelengths other than optimal for the half-wave plate, corrected values of the polarization were calculated using Mueller calculus [10]. Alternatively, the wave plate was removed and polarization was measured by rotation of the polarizing prism, with correction for preferential polarization by the detection system [8].

Positioning of the polarizers and half-wave plate was optimized using Rayleigh scattering from a glycogen solution. The system was checked by measuring the fluorescence polarization of rhodamine 6G. The fluorescence polarization of BChl was measured in cyclohexanol solution at several temperatures. The limiting values of p were determined by plotting $1/p$ against T/η and extrapolating to infinite viscosity (η).

Low-temperature spectra were measured near liquid nitrogen temperature in glycerol- or ethylene glycol-containing glasses. Measurements at liquid helium temperatures were made on the BChl-protein embedded in poly(vinyl alcohol) [6].

Results

Bacteriochlorophyll fluorescence polarization. Our determination of the limiting values of p for BChl in cyclohexanol at room temperature gave +0.42 for Q_y-band excitation (760–780 nm) and −0.23 for Q_x excitation (590 nm). A Stokes' shift of 260 + 30 cm^{-1} was found.

R-26 BChl-protein absorption and fluorescence. The Q_y region absorption and fluorescence emission spectra of the light-harvesting BChl-protein from the R-26 mutant (LH-R26) are shown in Fig. 1 for samples in glycerol/buffer solutions at 295 and at 90 K. At the lower temperature a shoulder on the low-energy side of the absorption band is apparent. We saw this shoulder also in chromatophore membrane suspensions and for LH-R26 in ethylene glycol/buffer glasses. In poly(vinyl alcohol) at 2 K the shoulder is not so prominent, but it is clearly present from the skewed bandshape. At room temperature the Stokes' shift is 245 ± 25 cm^{-1}. At 90 K the Stokes' shift relative to the absorption maximum is 520 ± 35 cm^{-1}. Relative to the absorption shoulder the shift is 345 ± 35 cm^{-1}.

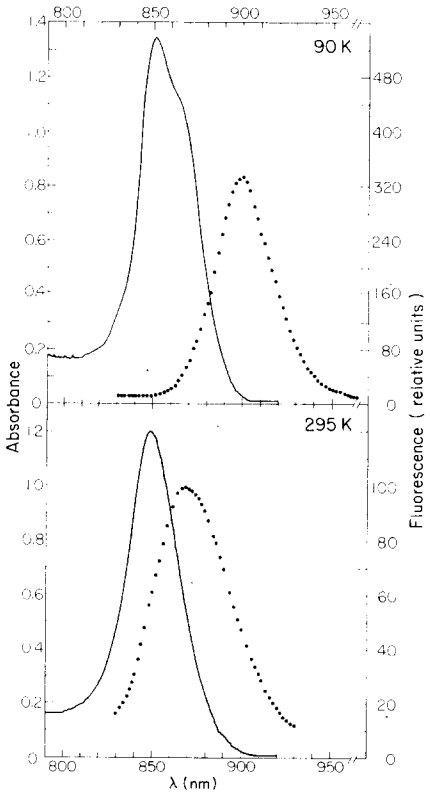

Fig. 1. Absorption and fluorescence of LH-R26 at two temperatures in 10 mM Tris-HCl, 0.1% SDS, 55% glycerol. Measurements at each temperature were made on the same sample. The fluorescence spectra were taken on a sample diluted 10-fold. Fluorescence was excited at 593 nm with excitation monochromator slits removed and with a broadband interference filter (592 nm, bandpass 40 nm) immediately after the monochromator.

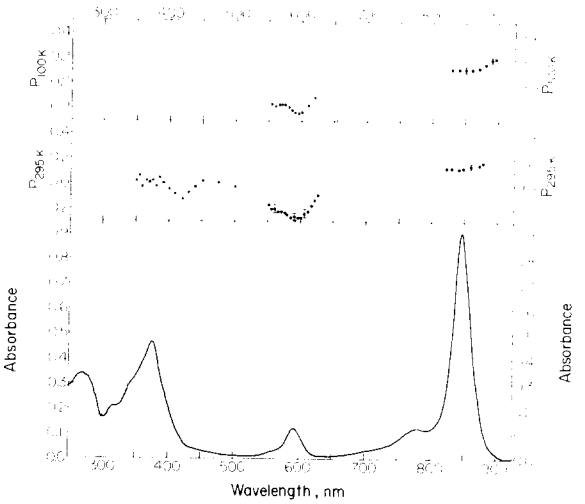

Fig. 2. Absorption at 295 K (bottom) and fluorescence polarization of LH-R26 at 295 K (middle) and 100 K (top). 55% glycerol, 10 mM Tris-HCl, 0.2% SDS.

LH-R26 fluorescence polarization. Fig. 2 shows the absorption spectrum of LH-R26 and the observed fluorescence polarization at various excitation wavelengths. Across the Q_y absorption band the polarization of LH-R26 is significantly smaller than for monomeric BChl in organic solvents. The value of p at room temperature is +0.12 throughout most of the band; it increases to +0.14 at the lower energy edge. At any particular excitation wavelength the fluorescence polarization is independent of emission wavelength. The only exception was for anti-Stokes' fluorescence; excitation at 890 nm and emission at 850 nm gave p = +0.12.

Q_x-band excitation of LH-R26 gives fluorescence polarization of −0.25 at the absorption maximum, 593 nm. The variation of p across the absorption band is quite similar to that found for BChl in organic solvents [9,11]. In the Soret absorption region p is negative on the low-energy side and slightly positive

TABLE I

FLUORESCENCE POLARIZATION OF BChl EXCITED AT THE Q_x BAND AND EFFECTS OF THE STATE OF AGGREGATION

All polarization values were determined with excitation at the Q_x maxima, near 590 nm. LH, light-harvesting complex.

Sample	Estimated number of BChl in aggregate	p	Literature value (Ref.)
BChl in cyclohexanol	1	−0.23	−0.17 (4), −0.23 [a] (11)
LH-R26	2	−0.25	−0.15 (4)
LH inTriton X-100	20	−0.17	−0.07 (4)
Membranes	<200	−0.15	−0.16 (4)
LH in poly(vinyl alcohol)	–	−0.18	

[a] In castor oil.

at the absorption maximum (Fig. 2). The Soret band has absorption shoulders on both sides of the maxima. The light intensity was not sufficient to provide accurate polarization values at excitation wavelengths less than 360 nm. Our room-temperature results are in agreement with those reported recently by Breton et al. [12].

At 100 K only slight changes in fluorescence polarization are observed compared with those at room temperature. The Q_y lower energy edge polarization increases to +0.20 at 895 nm. The Q_x polarization is slightly less negative; $p = -0.23$.

The polarization of the Q_x band was studied in several preparations which have different degrees of BChl aggregation. Table I summarizes the data. The estimated number of BChl molecules within an aggregate represents the number of chromophores over which excitation energy may migrate before fluorescence or nonradiative decay occurs. As noted previously [11] the polarization observed in the larger aggregates suggests significant local order in the BChl arrangement. Quantitatively, our results agree best with those of Ebrey and Clayton [11]. It is probable that earlier polarization values of Goedheer [13] and Sauer and Austin [4] were decreased due to experimental conditions. The lower polarization of the light-harvesting complex in poly(vinyl alcohol) may reflect aggregation in that medium. With 840 nm Q_y excitation p is +0.10 in the polymer matrix.

Discussion

Bacteriochlorophyll. The limiting values of BChl fluorescence polarization support the assignment of roughly orthogonal directions for the transition dipole moments of the Q_x and Q_y transitions. For a random distribution of molecular orientations and no rotational motion within the excited state lifetime (infinite viscosity limit), the polarization is given by:

$$p = \frac{3\cos^2\alpha - 1}{\cos^2\alpha + 3} \qquad (2)$$

where α is the angle between the absorption and fluorescence transition moments. The limiting values are +0.50 for $\alpha = 0$ (parallel transition moments) and −0.33 for $\alpha = 90°$ (perpendicular transition moments). Depolarization owing to energy transfer is considered unlikely at the concentrations used (approx. 10^{-6} M), especially in cyclohexanol where aggregation is unlikely. We attribute incomplete polarization (+0.42 and −0.23) to factors intrinsic to the chromophore. One could calculate effective angles between the absorption and emission transition moment directions, but the experimental values reflect a certain amount of averaging over unresolved vibronic components. For example, this vibronic effect is evident in the vibrational overtones of chlorophyll *a* [14].

LH-R26; Q_y absorption, fluorescence and fluorescence polarization. Our previous CD and linear dichroism experiments [4,6] were interpreted in the strong-coupling molecular exciton limit. In this model the Q_y absorption band is attributed to two orthogonal exciton transition moments, with the higher energy transition possessing greater oscillator strength. The splitting was estimated to be 200–250 cm^{-1} [6,9]. The low-temperature absorption spectrum (Fig. 1) fully supports this model.

The almost constant fluorescence polarization across the Q_y absorption band (approx. +0.12) is unexpected. Trivial explanations due to rotation were ruled out by measurements with and without glycerol present and by measurements at low temperature. Energy transfer to other LH-R26 units is unlikely based on the molecular weight determinations of Sauer and Austin [4] and based on the Q_x polarization of LH-R26 which is nearly identical to that of monomeric BChl. Rationalization of low p values by an unspecified interaction of BChl with protein is unlikely: Q_y fluorescence polarization of the B875 light-harvesting protein of *Rps. sphaeroides*, wild type, is +0.30 at 890 nm [15].

The Q_y value of p for LH-R26 is close to the value expected for planar degenerate emission dipoles and/or absorption dipoles. Based on the simple exciton model, absorption into one exciton band and emission from the other would give a negative p value; and absorption and emission from the same exciton component would give a positive p, approaching +0.5. Even at low temperature (Fig. 2), where emission might originate more from the lower exciton level, p is essentially constant across the band.

Two explanations may be given. (a) The exciton splitting is much less and the relative oscillator strengths are more nearly equal than was suggested by CD and linear dichroism. Absorption dipoles then approach planar degeneracy, and a value of +0.12 for p is acceptable. However, this near degeneracy is not consistent with the low-temperature absorption spectrum. (b) The fluorescence emission originates from a state or states different from those reached by absorption. If the exciton model is correct for absorption, but the exciton coherence decays during the singlet lifetime, emission could originate from a monomeric state of either of the BChl molecules within the complex. Thus, the emission dipole moments would be nearly planar degenerate. The possibility that exciton states may not exist long enough to contribute to the fluorescence has been suggested previously [16,17]. The anomalous Stokes' shift at low temperature may be a consequence of the inequivalence of absorption and emission transition moments.

Q_x fluorescence polarization and a model of the LH-R26 BChl arrangement. Using linear dichroism we were unable to resolve the Q_x absorption band (570–610 nm) into more than one component [6]. Based on the excitonic shape of the Q_x CD [9] we propose that the oscillator strength resides predominantly in one component, resulting from nearly parallel monomer Q_x directions. Given the hypothesis of nearly planar degenerate fluorescing dipoles, the p value for Q_x absorption (−0.23) indicates an angle of 74–90° between Q_x and the plane of Q_y emission (or absorption) transition moments. The smaller angle is based on Eqn. 2 and the larger angle is based on the fluorescence polarization of monomeric BChl (−0.23). This arrangement agrees with the model of BChl arrangement based on linear dichroism [6]. For the Q_x transition to be perpendicular to the Q_y plane, the Q_x transition moment should be at 66°C to the orientation axis of linear dichroism. The angle determined from the linear dichroism measurements was 65°C. (Fig. 7a of Ref. 6 contains an error; the angle from the normal of the plane of the Q_y exciton components to the axis of orientation should be 66°.) A consistent schematic model of the x and y BChl axes in the LH-R26 protein is presented in Fig. 3. The

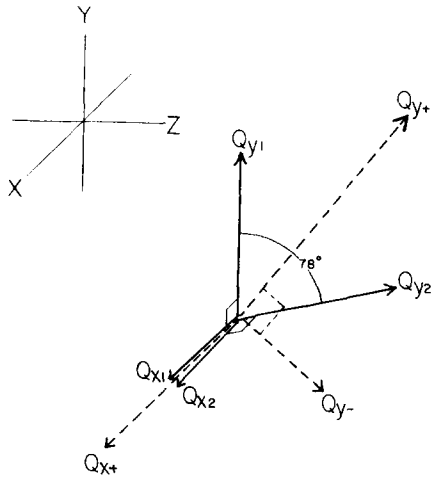

Fig. 3. Schematic view of the mutual orientations of absorption transition dipoles for LH-R26. Solid vectors represent the directions of monomeric BChl transition dipoles. Dashed vectors represent the absorptions in the strong coupling exciton limit. + and − designate relative energy of transitions, not the absolute sign of the wavefunctions. Q_{y+}, Q_{y-}, Q_{y1}, and Q_{y2} are in the yz plane; Q_{x1}, Q_{x2}, and Q_{x+} and Q_{x-} are along the x axis of the reference coordinate system, upper left. The molecules are superimposed for this representation. A complete geometry would specify the position vector from molecule 1 to molecule 2.

angle of 78° was calculated from linear dichroism and reflects nearly planar degenerate fluorescence moments in alternative b, above.

Fluorescence yield and lifetime. The apparent increase in relative fluorescence yield with decreasing temperature (Fig. 1) is a result of two factors. At 90 K the Q_x absorption band narrows and shifts to the blue by several nanometers. This results in additional absorption of light under the conditions of Fig. 1. Secondly, an increase in true quantum yield occurs proportional to the increase in observed fluorescence lifetime (Bolt, J.D. and Nairn, J.A., unpublished results).

Structural similarities with other chlorophyll-proteins may be revealed by applying techniques like these which utilize polarized light. Other approaches to the pigment arrangement of LH-R26 will also prove useful, including magnetic resonance spectroscopy of the triplet state of LH-R26, NMR and resonance Raman spectroscopy. Because of its relative simplicity as a BChl dimer complex, the results will be relevant to the understanding of all chromophore aggregates.

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