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Spectroscopic Characterization of the Light-Harvesting Complex of *Rhodospirillum rubrum* and Its Structural Subunit†

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ABSTRACT: The spectroscopic properties of the light-harvesting complex of *Rhodospirillum rubrum*, B873, and a detergent-isolated subunit form, B820, are presented. Absorption and circular dichroism spectra suggest excitonically interacting bacteriochlorophyll *a* (BChl *a*) molecules give B820 its unique spectroscopic properties. Resonance Raman results indicate that BChl *a* is 5-coordinate in both B820 and B873 but that the interactions with the BChl C2 acetyl in B820 and B873 are different. The reactivity of BChl *a* in B820 in light and oxygen, or $NaBH_4$, suggests that it is exposed to detergent and the aqueous environment. Excited-state lifetimes of the completely dissociated 777-nm-absorbing form [1.98 ns in 4.5% octyl glucoside (OG)], the intermediate subunit B820 (0.72 ns in 0.8% OG), and the in vivo like reassociated B873 (0.39 ns in 0.3% OG) were measured by single-photon counting. The fluorescence decays were exponential when emission was detected at wavelengths longer than 864 nm. An in vivo like B873 complex, as judged by its spectroscopic properties, can be formed from B820 without the presence of a reaction center.

The pigment-protein complexes of photosynthetic bacteria responsible for the initial light-capture and charge-separation events of photosynthesis, namely, the light-harvesting (LH)[1] complexes and reaction centers (RC), absorb light of longer wavelengths (greater than 700 nm) than that absorbed by oxygenic organisms. This ability to utilize light of longer wavelengths is due partly to their use of bacteriochlorophyll (BChl), instead of chlorophyll (Chl), as the chromophore. BChl *a*, in comparison to Chl *a*, contains an acetyl group rather than a vinyl group at position 2 of ring I and a reduced double bond between the β-carbon atoms of ring II which alters its symmetry properties. As a result of these structural differences, about a 100-nm red shift (2000 cm^{-1}) of the major near-infrared (NIR) absorption band (the Q_y transition) is observed for monomeric BChl *a* relative to monomeric Chl *a* in acetone (770 versus 662 nm, respectively). However, an additional 100-nm red shift (1500 cm^{-1}) of the Q_y band is typically observed in vivo for BChl *a* containing LH complexes (van Grondelle, 1985; Thornber, 1986; Cogdell, 1986) and RC (Feher & Okamura, 1978; Gingras, 1978). The furthest red-absorbing BChl *a* containing bacterium known, *Chromatium tepidum*, has a Q_y band at 920 nm (Garcia et al., 1986).

In case of RC, recent X-ray crystallographic results (Deisenhofer et al., 1984; Allen et al., 1986, 1987a,b; Chang et al., 1986; Michel et al., 1986; Yeates et al., 1988) have confirmed that the primary electron donor consists of two BChl

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[1] Abbreviations: BChl, bacteriochlorophyll; B881, light-harvesting complex of wild-type *Rs. rubrum*; B873, light-harvesting complex of G-9 carotenoidless mutant or of benzene-extracted wild-type chromatophores; B820, subunit form of B873; 777(dissoc), 777-nm absorbing material formed by titrating B820 with 4.5-5.0% OG; B873(reassoc), light-harvesting complex formed by reassociation of B820; CD, circular dichroism; Chl, chlorophyll; CTAB, cetyltrimethylammonium bromide; DPG, diphosphatidylglycerol; EDTA, ethylenediaminetetraacetic acid; FWHM, full width at half-maximum; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; LH, light harvesting; LHCII, accessory light-harvesting complex in oxygen-evolving organisms; NIR, near-infrared; OG, *n*-octyl β-D-glucopyranoside; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; RC, reaction center(s); RR, resonance Raman; THF, tetrahydrofuran.

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molecules functioning as an exciton pair (Norris et al., 1971) which may account for much of the red shift. However, even with the known protein conformations and relative chromophore orientations within the RC, debate continues as to whether interaction with unique protein groups (Eccles & Honig, 1983; Brunisholz & Zuber, 1988), exciton coupling (Sauer et al., 1968; Scherz & Parson, 1984, 1986), charge-transfer states (Knapp et al., 1985; Knapp & Fischer, 1985; Parson et al., 1985), or some combination of the above is responsible for the red-shifted absorption bands and unique CD signals. No similar three-dimensional structure has been determined for the purple bacterial LH complexes, nor are the binding sites of the BChl molecules known. Thus, the origin of the red shift of NIR absorption bands and the distinct CD spectra of the LH complexes is even less well understood and is also the subject of much discussion (Kramer et al., 1984; Scherz & Parson, 1986; Zuber et al., 1987; Parkes-Loach et al., 1988; Brunisholz & Zuber, 1988). See Pearlstein (1987) for a recent review of proposed models for the LH complexes.

Although their macromolecular organization remains unresolved, the biochemical composition of many bacterial LH complexes has been determined. The LH complex of *Rhodospirillum rubrum*, B881, consists of two polypeptides, α and β, of about 6 kDa each in a one-to-one ratio, two BChl *a* molecules per $\alpha\beta$ pair (Cogdell et al., 1982; Miller et al., 1987), and one carotenoid per two BChl *a* molecules (Cogdell et al., 1982; Picorel et al., 1983). There are most likely 12 copies of each polypeptide and 24 BChl *a* molecules per RC in the in vivo complex (Loach & Sekura, 1968). There is increasing evidence that these complexes are constructed from smaller fundamental structural units. We previously isolated a dissociated form of B881 (B820) from *Rs. rubrum* wild-type and its G-9 mutant that was stable and could be reassociated into an in vivo like B881 (Loach et al., 1985; Miller et al., 1987). Similar intermediates have also been isolated from the B875 antenna complex of *Rhodobacter sphaeroides* (B825) and *Rhodobacter capsulatas* (B816) (unpublished results). Not only has a smaller structural unit of B881 of *Rs. rubrum* (B820) been isolated and found stable, but also it can be further dissociated to free polypeptides and BChl *a* [777-(dissoc)] and quantitatively reassociated to form B820(reassoc) and B873(reassoc) (Parkes-Loach et al., 1987, 1988). Similar data confirming these observations for the G-9+ mutant have also recently appeared (Ghosh et al., 1988). Thus, it is now possible to conduct reconstitution experiments with selectively modified constituents (unpublished results).

In many oxygen-evolving organisms, an accessory LH complex (LHCII) exists and also seems to have a structural unit, although much larger and rather different from the photosynthetic bacteria. Minimal stable functional units, most likely trimers, of the LHCII complex from pea chloroplasts have also been isolated with the use of the detergent OG (Ide et al., 1987; Butler & Kuhlbrandt, 1988). The LHCII complex from spinach thylakoids has also been reconstituted using chlorophyll *a*, chlorophyll *b*, xanthophylls, and delipidated thylakoid membranes (Plumely & Schmidt, 1987).

In this paper, we present a spectroscopic study of the various dissociated and reassociated forms of B881 from *Rs. rubrum* with the purpose of understanding the structure of the in vivo complex. Resonance Raman (RR), absorption, circular dichroism (CD), and steady-state and single-photon counting fluorescence techniques were selected to examine specific aspects of the complexes such as the coordination number of Mg^{2+} and the acetyl and ketone environments of bound BChl *a*, the extent of pigment–pigment and pigment–protein interactions, and the rate of quenching due to the aggregation state of the various complexes studied. From the results of this study, we begin to distinguish (1) what factors contribute to the red shift of the Q_y absorption band and the complex CD spectra, (2) how BChl *a* molecules are bound to protein, and (3) how the pigment is protected from photochemical reactions.

Materials and Methods

Materials. *n*-Octyl β-D-glucopyranoside (OG), Sephadex G-100, BChl *a*, phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), and phosphatidylethanolamine (PE) were obtained from Sigma Chemical Co. All high-performance liquid chromatography (HPLC) solvents were high-purity HPLC grade.

Isolation of B820. *Rs. rubrum* (wild-type, S.1) was grown anaerobically, and chromatophores were prepared from the whole cells as described in Loach et al. (1963). The B820 complex was isolated as described in Miller et al. (1987). Briefly, the chromatophores were washed with buffer containing EDTA and Triton X-100 to remove loosely bound proteins, leaving only the RC and LH complexes. The washed chromatophores were then lyophilized and extracted with benzene to remove carotenoids and ubiquinones. The carotenoid-depleted material was titrated with the detergent, OG, until the sample's far-red absorption band had shifted from 873 to 820 nm. RC and any free BChl *a* were separated from the LH complex by gel filtration on a Sephadex G-100 column. The resulting B820 complex was stable for at least 3–4 months if stored in the dark at 4 °C. 777(dissoc) was formed by titrating B820 with OG until the 820-nm absorption band was converted to a 777-nm band. Final OG concentration was approximately 4.5%.

Reassociation Assay with Lipids. Samples of B873(reassoc) were prepared by a slightly modified method of that previously reported (Miller et al., 1987). Vesicles of PE, DPG, and PG in a ratio of 2:1:1 were formed by drying the phospholipids on the sides of a conical tube, resuspending them in phosphate buffer, and sonicating. Then an appropriate aliquot of vesicles was added to 5.0 mL of B820 in 0.8% OG in a lipid to BChl *a* weight ratio of 2:1. The solution was chilled overnight at 5 °C, which resulted in the formation of B873(reassoc). To study B873(reassoc) at room temperature, the sample was typically diluted 2–3-fold with cold 50 mM potassium phosphate buffer, pH 7.5, containing 5 mM $MgSO_4$.

Absorption and CD Measurements. Absorption spectra from 1000 to 200 nm were recorded with a Shimadzu UV-160 spectrophotometer. In the range of 1000–350 nm, opal glass was placed after the reference and sample cuvettes to compensate for scatter. The extinction coefficient of BChl *a* in 4.5% OG was measured relative to BChl *a* in ether (ϵ_{770} = 96 mM^{-1} cm^{-1}) (Sauer et al. 1966; Lindsay-Smith & Calvin, 1966; Philipson et al., 1971; Connolly et al., 1973) and acetone (ϵ_{770} = 71.5 mM^{-1} cm^{-1}) (Connolly et al., 1982). Extinction coefficients of B820 and B873(reassoc) were then calculated relative to BChl *a* in 4.5% OG (ϵ_{777} = 55 mM^{-1} cm^{-1}) using the following ratios of peak absorption intensities of the different complexes: B820/777(dissoc) = 1.56 and B873(reassoc)/B820 = 1.38.

CD spectra were recorded with a Jasco J500C spectropolarimeter as previously reported (Parkes-Loach et al., 1988). The extinction coefficients determined above were then used to calculate molar ellipiticities.

In measuring the CD of chromatophores, low concentrations of phenazine methosulfate and ascorbic acid were added to maintain the RC in its reduced state because its CD signal

varies substantially with the oxidation state of the primary electron donor (Sauer & Austin, 1978; Philipson & Sauer, 1973).

Steady-State and Time-Resolved Fluorescence. Steady-state fluorescence measurements were made on a SPEX Fluorolog Series 2 fluorometer interfaced to a DMIB lab coordinator. Slits were set at 1.25 mm, giving a spectral bandwidth of 4.5 nm.

Fluorescence lifetimes were measured on two different time-correlated single-photon counting systems, each of which has been described previously (Chang et al., 1985; Spears & Steinmetz, 1985). For one instrument (Chang et al., 1985), the FWHM of the instrument response function was typically 90 ps, and the excitation wavelength was 590 nm. The visible pulse repetition rate was reduced by a cavity dumper, and detection was observed through a monochromator (1-mm slitwidth, 8-nm bandwidth). The excited-state lifetimes of 777(dissoc) and B820 were detected at wavelengths shorter than 850 nm on this instrument. On the second instrument (Spear & Steinmetz, 1985), the fluorescence lifetimes of B820 and B873(reassoc) were measured at emission wavelengths greater than 864 nm. The excitation wavelength was also 590 nm, and the emission was detected through 10-nm bandwidth interference filters. The FWHM of the instrument response function was approximately 550 ps. The excitation light pulse energies were 1–5 nJ per pulse to avoid annihilation effects. The two instruments were calibrated by measuring the lifetimes of free BChl *a* in 4.5% OG and of oxazine in methanol. Both of these solutions have single fluorescence lifetimes and absorb and emit in the wavelength region of interest.

Data were processed on either a VAX 11/780 or a Celerity C1200 using a nonlinear least-squares program based on the Levenberg–Marquardt algorithm. Fluorescence decay curves detected through a polarizer set at magic angle, 54.7°, were fit to a sum of exponentials (eq 1)

$$K(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) \qquad (1)$$

where $A_1 + A_2 = 1.0$. The quality of the fits was judged according to how well the statistical tests of χ^2 and the number of runs in the set of generated residuals were satisfied [see Cross and Fleming (1984)].

B873(reassoc), B820, 777(dissoc), and BChl *a* samples were placed in a Thunberg anaerobic cuvette and degassed with argon prior to light exposure to prevent formation of a BChl *a* degradation product (absorbing at 690 nm). Samples of BChl *a* used in steady-state fluorescence studies were prepared by first suspending dried BChl *a* in a small volume of acetone and then dispersing the acetone solution in a small volume of 4.0% OG solution. An appropriate aliquot of the BChl *a*, now in 4.0% OG, was used to prepare solutions of lower OG concentrations.

Resonance Raman. The Raman instrumentation consisted of Coherent Ar^+ (Innova 90-5) and Kr^+ (Innova 100) lasers as excitation sources, a Spex Triplemate 1877, and an intensified diode array detector (PARC 1420) coupled to a multichannel analyzer (PARC OMA II) (Callahan & Cotton, 1987). An 1800 groove/mm grating was used in the spectrograph stage for a resolution of 8 cm^{-1}. Indene was used for frequency calibration.

The BChl *a* used in these Raman studies was purified by chromatography on powdered sucrose according to established procedures (Strain & Svec, 1966). For samples in nonaqueous solvents, the BChl *a* was dried by repetitive codistillation from CH_2Cl_2. For aqueous solvents, the BChl *a* pigment was transferred to aqueous/detergent solution by following the procedure described by Fuhrop and Smith (1975). BChl *a* samples were placed in 5-mm Pyrex tubes, vacuum-degassed by using three freeze–pump–thaw cycles, and sealed under vacuum.

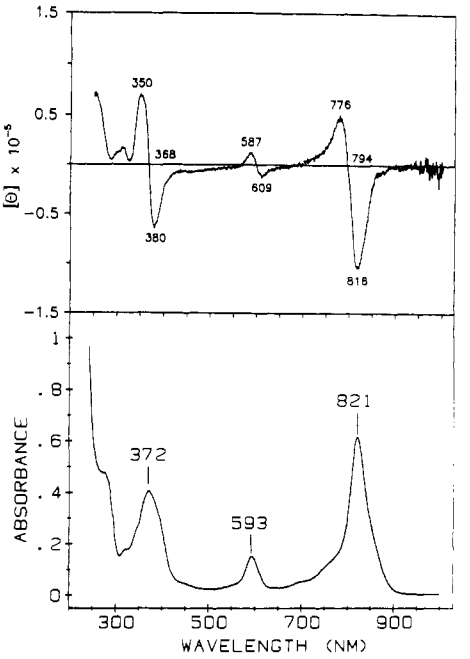

FIGURE 1: Absorption and CD spectra of B820 from *Rs. rubrum* in 0.05 M potassium phosphate buffer, pH 7.5, containing 5 mM $MgSO_4$ and 0.8% OG. BChl *a* concentration in B820 was 7.4×10^{-6} M (1-cm path length, room temperature, in air). Sample was kept dark. About 10% decrease in absorbance occurred during the CD measurement.

In addition to isolated B820 and B873(reassoc) samples, Raman experiments were performed on B820 and B873(reassoc) samples prepared by adding OG to *Rs. rubrum* G-9 chromatophore material that had been washed with EDTA and Triton X-100 and extracted with benzene, but not isolated on a gel filtration column. These samples resulted in spectra with a better signal to noise ratio. All samples for Raman spectroscopy had an absorbance of 20 cm^{-1} at their NIR absorption maximum which was measured in a 0.1-cm pathlength cuvette. The B820 samples for RR were prepared by incubating the Triton/EDTA-washed chromatophores in 1.0% OG for approximately 8 h followed by careful addition of OG to shift the absorption maximum completely to 820 nm. At this high concentration of BChl *a*, the total concentration of OG added to the B820 samples was typically 2.0%. Reassociation to the 873-nm form was accomplished by dilution of the B820 samples with 100 mM potassium phosphate buffer to <0.5% OG. B820 and B873(reassoc) samples were degassed in a custom-designed anaerobic cuvette. The cuvette was repetitively evacuated and purged with water-saturated N_2 gas. Absorption spectra were recorded on a Cary 14 spectrophotometer before and after laser excitation to monitor sample integrity.

Results and Discussion

Absorption and CD Spectra. The absorption and CD spectra of B820 and B873(reassoc) in the wavelength region from 1000 to 250 nm are shown in Figures 1 and 2. The location of the absorption bands and the prominent CD spectral features of BChl *a*, 777(dissoc), B820, and B873(reassoc) are presented in Table I.

As B820 was reassociated to form B873(reassoc), a hyperchromic red shift of the Q_y absorption band and also a 5-nm blue shift of the Q_x band and a slight red shift of the Soret band were observed (Figures 1 and 2, Table I). The extinction coefficient of the Q_y band increased by a factor of 1.4. The

Table I: Absorption and Circular Dichroism Features of BChl *a*, 777(dissoc), B820, and B873(reassoc) from *Rs. rubrum*

sample	% OG	ϵ, (mM^{-1} Q_y cm^{-1})	*A* max (nm) Q_y	Q_x	Soret	CD peaks and troughs (nm)	molar ellipticity (θ) $\times 10^{-5}$
BChl *a*	4.5	55	777	590	375	770	+0.08
777(dissoc)	4.5	55	777	590	375	ND[a]	ND[a]
B820	0.8	86	821	594	372	816	−1.04
						776	+0.50
						609	−0.13
						587	+0.12
						380	−0.64
						350	+0.70
B873(reassoc)	0.3	119	870	589	374	873	−2.23
						841	+0.27
						580	+0.33
						402	+0.48
						375	−1.09

[a] ND = not determined.

location of the Q_y band maximum in B873(reassoc) has varied from about 867 to 873 nm depending on the method of reassociation. The data shown in Figure 2 are typical. A shoulder at 777 nm was always present in the B820 absorption spectrum, which may be due to a vibrational overtone [$Q_y(0,1)$ transition] of BChl *a*, a small population of unbound BChl *a* in equilibrium with the B820 species, and/or a higher energy exciton transition.

Monomeric BChl *a* in 4.5% OG has a very small CD signal ($\theta_{777} = 0.8 \times 10^4$) in the NIR region. It is over an order of magnitude smaller than that observed for B820 or B873-(reassoc) (Table I). Sauer (1972) reported a CD signal for monomeric BChl *a* in ether equivalent to a molar ellipticity of approximately 1.8×10^4.

The CD spectrum of B820 (Figure 1) has a simple peak and trough in the Q_y, Q_x, and Soret regions. While the CD bands in both the Q_x and Soret regions are conservative (the positive and negative rotational strengths sum to zero and the crossover is at the absorption maximum), that in the Q_y region is non-conservative with relatively broad positive and negative bands centered between 776–780 and 816–820 nm, respectively, and a crossover point at 794–798 nm which is far from the absorption maximum (821 nm). These Q_y CD bands may be interpreted as being due to (1) one protein-liganded BChl *a* absorbing at B820 nm and one unbound BChl *a* absorbing at 777 nm; (2) two bound but noninteracting BChl *a* molecules, one absorbing at 780 nm and another at 820 nm, indicating that they are in different environments; or (3) two excitonically coupled BChl *a* molecules giving rise to the 780- and 820-nm absorption bands. The first case seems unlikely because the 776-nm CD signal of B820 is about 6 times larger than that of 777(dissoc) (not shown) or free BChl *a* with a similar absorbance (Table I). Resonance Raman results, as described later, imply that both BChl *a* are liganded because the coordination state of the chromophores remains constant in the differently associated B820 and B873(reassoc) forms. The second interpretation is possible where interactions between a monomeric chromophore and specific protein groups, especially charged groups, have been calculated to cause significant red shifts (Eccles & Honig, 1983) and where protein environment can change chromophore CD signals (Wright & Boxer, 1981). This could account for the asymmetry of the CD signal and the minimum being at the absorption maximum. However, red shifts arising from such specific protein interactions have yet to be proven experimentally. Also, if the relative absorption intensities at 780 and 820 nm of B820 represent equal populations of the two asymmetrically bound BChl *a* as stoichiometry would suggest (2 BChl/$\alpha\beta$), then the extinction coefficient of the 780-nm-absorbing chromophore

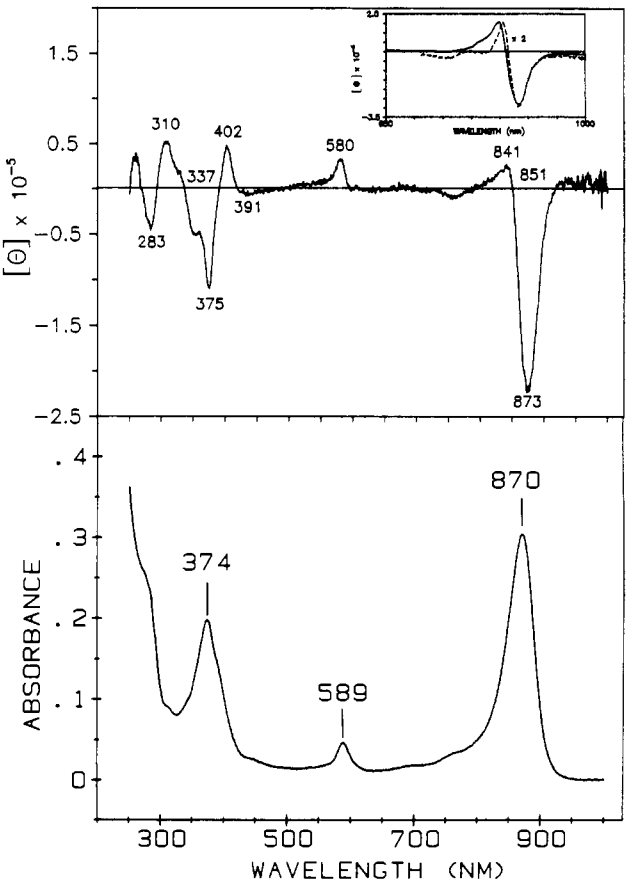

FIGURE 2: Absorption and CD spectra of B873(reassoc) in 0.05 M potassium phosphate buffer, pH 7.5, containing 5 mM $MgSO_4$, 0.3% OG, and lipids. The same B820 sample whose spectra are shown in Figure 1 was used for reassociation here. BChl *a* concentration in B873(reassoc) was 2.5×10^{-6} M (1-cm path length, room temperature, in air). The CD spectrum of B873(reassoc) (—) and the difference CD spectrum of chromatophores minus the presumed RC contribution (- - -) are shown in the inset. The RC CD was arbitrarily red-shifted by 7 nm before subtraction. For easy comparison, the difference CD spectrum was multiplied by a factor of 2. The B873(reassoc) sample used for this comparison is representative of our most red-shifted reassociated species, which also had a higher than average molar ellipticity.

would have to be about 2–3-fold smaller than that of free BChl *a*. Excitonic interaction between two BChl *a* molecules is a more plausible explanation where the positive and negative lobes at 780 and 820 nm of B820 are assigned to the high- and low-energy exciton transitions. Also, charge-transfer states have been calculated to influence the absorption and CD spectra of the RC significantly (Parson et al., 1985) and may

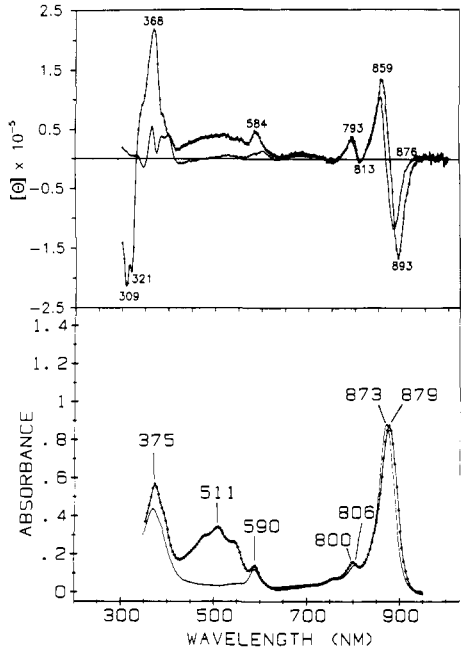

FIGURE 3: Absorption and CD spectra of chromatophores from the wild-type (●—●) and the G-9 mutant (—) of *Rs. rubrum* in 0.05 M potassium phosphate buffer, pH 7.5. The LH BChl *a* concentration in wild-type chromatophores was 4.7×10^{-6} M and in G-9 chromatophores was 7.3×10^{-6} M (1-cm path length, room temperature, in air). The absorption spectra of the wild-type chromatophores and the G-9 mutant have been normalized to the same Q_y absorbance. Both samples contained 1×10^{-4} M ascorbate and 1×10^{-5} M phenazine methosulfate.

play a role here in interpreting the LH absorption and CD spectra.

Whereas the NIR region of the absorption spectrum was most affected when B820 was reassociated to form B873-(reassoc), all regions of the CD spectra were significantly altered. The B873(reassoc) CD bands in the Q_y, Q_x, and Soret regions are all clearly nonconservative where the Q_y and Soret bands have a minimum near their absorption maximum. It should be noted that the NIR CD spectrum does very considerably, depending upon the method of reassociation, from being very nonconservative, as shown in Figure 2, to being somewhat more conservative (inset in Figure 2). It is the intensity of the positive lobe that varies widely while the negative lobe remains fairly constant under most conditions. B873(reassoc) CD spectra similar to the one shown in the inset of Figure 2 were obtained consistently when lipids were added and the reassociation from B820 took place at 5 °C.

The absorption and CD spectra of the in vivo LH complex of wild-type and G-9 *Rs. rubrum* chromatophores are shown in Figure 3 for comparison to the B873(reassoc) spectra (Figure 2). The effects of carotenoids are apparent in Figure 3: a 6-nm red shift in the LH absorption and CD Q_y bands, a 6-nm blue shift in the RC monomeric BChl absorption band (near 800 nm), the appearance of several large absorption bands between 450 and 550 nm, and a red shift of the Soret absorption band in the wild-type spectra. Carotenoids also effect large changes in the Soret region of the wild-type CD spectrum.

Recent crystallographic data have shown that in the *Rb. sphaeroides* carotenoidless mutant (R26) RC, two OG detergent molecules occupy the site to which carotenoid would normally bind in the wild-type RC (Yeates et al., 1988). If carotenoids and OG compete for the same binding site in the LH complex as well, this could explain why carotenoids must first be extracted before successfully titrating chromatophores with OG to form the B820 subunit. Without the prior removal of carotenoids, the B873 in vivo complex is extremely stable to OG. The carotenoid (one carotenoid per two BChl *a* molecules) must lie near the BChl *a* molecules due to the fact that there is efficient energy transfer (30%) from the carotenoid to BChl *a* (Goedheer, 1959; Cogdell & Frank, 1987; Frank et al., 1987) even with poor donor–acceptor spectral overlap. OG, then, could bind to the usual carotenoid binding site and cause dissociation of the closely interacting polypeptides and pigment molecules to form the smaller B820 units. Although they must first be extracted before B873 can be dissociated by OG, carotenoids are not necessary for proper reassociation.

For a more direct comparison of B873(reassoc) and in vivo LH CD spectra, the RC contribution was first accounted for by subtracting the CD spectrum of isolated G-9 RC from that of G-9 chromatophores (Figure 2, inset). Before subtraction, the isolated RC CD spectrum was normalized to the magnitude of the in vivo RC band and then red-shifted by 7 nm, the difference in band position between in vivo and in vitro RC. The NIR chromatophore minus RC and B873(reassoc) (reassociated in the presence of lipids) CD spectra are shown in the inset of Figure 2. The B873(reassoc) spectrum shown here is one of the more conservative ones observed. Two differences are clear. The B873(reassoc) signal is twice the magnitude of and its upper lobe is much broader than the "in vivo" signal. There are also differences with the reported CD spectrum (Cogdell & Scheer, 1985) of the B881 LH complex of *Rs. rubrum* (S.1) isolated by solubilization in LDAO. A strong conservative signal in the Q_y region, centered about its NIR absorption band and interpreted as due to weak exciton coupling of BChl *a* molecules, was observed. However, the "in vivo" absorption spectrum and the double Cotton effect in the NIR CD spectrum as well as the nonconservative Q_x CD band are well reproduced by B873(reassoc). Thus, it is possible to form a B873(reassoc) form which closely resembles the "in vivo" complex as judged by its absorption and CD properties.

From the ultraviolet CD spectra (200–300 nm) of B820 and B873(reassoc) (not shown), the α-helical contents of the two forms are very similar and differ by no more than 10–15% in magnitude. Thus, no major conformational changes occur in the protein structure as B820 is reassembled into B873-(reassoc).

Upon exposure to O_2 and light, B820 is very unstable and easily degraded whereas B873 (reassoc) is relatively inert (Miller et al., 1987). A similar stability pattern is observed when the complexes are exposed to $NaBH_4$ (Callahan et al., 1987). The sensitivity of the chromophores in B820 to these conditions indicates that they are unprotected and readily available to the aqueous environment.

Tryptophyl Fluorescence of B820 and 777(dissoc). The fluorescence yield of Trp in the protein of 777(dissoc) in 4.5% OG (emission λ_{max} = 333 nm, corrected) was a factor of 1.8 times greater than that of Trp in B820 in 0.8% OG. Free Trp in 4.5% OG also had a fluorescence yield that was approximately twice as large as that of free Trp in 0.9% OG. The emission maximum of free Trp in both concentrations of OG was 336 nm which is 3-nm red-shifted from the emission maximum of Trp in B820 and 777(dissoc). Thus, when this is considered together with the results that the near-UV CD spectrum was also relatively unaffected upon dissociation of B820, we conclude that protein structural changes in the vicinity of the Trp residues, as BChl *a* was dissociated from the protein, were negligible.

Table II: Fluorescence Lifetimes and Relative Yields of BChl *a*, 777(dissoc), B820, and B873(reassoc) of *Rs. rubrum*

sample	% OG	*A* max (nm)	λ_{obs} (nm)	τ_1 (ns)	τ_2 (ns)	χ^2	rel yield
BChl *a*[a]	4.5	777	800	2.00		1.00	1.00
777(dissoc)[a]	4.5	777	800	1.98		1.00	1.00
B820[b]	0.8	820	864	0.72 (A_1 = 0.95)[c]	2.45	1.17	0.39
B873(reassoc)[b]	0.3	865	880	0.39 (A_1 = 0.99)[c]	2.91	1.15	0.20

[a] Data from Fleming apparatus. [b] Data from Spears apparatus. [c] Relative weights: $A_1 + A_2 = 1.0$.

Steady-State and Time-Resolved Fluorescence of 777-(dissoc), B820, and B873(reassoc). 777(dissoc) has an emission maximum at 800 nm, and B820 has emission maxima at 800 and 835 nm (uncorrected spectra, not shown) when excited at 590 nm. The 800-nm band may be due either to unbound, monomeric BChl *a* [777(dissoc)] in equilibrium with B820 and/or from a BChl *a* molecule that is bound but in a binding site different than the BChl *a* emitting at 835 nm. The 800-nm-emitting species does not transfer energy to the longer wavelength component as indicated by its high fluorescence yield. B873(reassoc) has emission maxima at 903 nm (van Grondelle, private communication) and 800 nm. Emission maxima of isolated (van Grondelle et al., 1983) and in vivo B880 of *Rs. rubrum* (Olson & Stanton, 1966; Brody & Linschitz, 1961) have been reported at 903 and 906 nm, respectively.

A comparison of the fluorescence decay curves of the three pigment–protein complexes is shown in Figure 4, and their lifetimes and relative yields are presented in Table II. The yields are calculated from the short-lifetime component from time-resolved measurements. This is a valid calculation if two assumptions are made: (1) the minor long-lifetime component is due to free BChl *a* fluorescence, and (2) the fluorescence yield and lifetime are directly proportional. The relative weights of the two lifetime components are wavelength-dependent, indicating that the longer lifetime is due to a separate emitting species (data not shown). It was concluded that BChl *a* in 777(dissoc) is not bound to protein because its spectroscopic properties (including its fluorescence lifetime of 1.98 ns) were identical with those of BChl *a* in 4.5% OG. As BChl *a* became bound to protein in B820, its lifetime decreased by a factor of 2.6 and decreased by nearly another factor of 2 when it was in the aggregated B873(reassoc) complex. In control experiments, it was observed that the fluorescence yield of free BChl *a* decreased with decreasing OG concentration. If the lifetimes of B820 and B873(reassoc) were calculated relative to the lifetime of 777(dissoc) from the ratio of the fluorescence yields of free BChl *a* in the various OG concentrations, they would equal 0.74 and 0.68 ns, respectively, as compared with the observed lifetimes of 0.72 and 0.39 ns. The calculated and observed lifetimes of B820 are nearly the same whereas the observed lifetime of B873(reassoc) is about 40% shorter than that calculated. Therefore, detergent concentration effects cannot completely account for the B873(reassoc) lifetime, and other nonradiative pathways must occur in the more aggregated B873(reassoc) state.

The measured lifetime of B873(reassoc) (0.39 ns) may be compared to the 0.20–0.40-ns reported lifetime of *Rs. rubrum* chromatophores with closed RC (Govindjee et al., 1972; Bakker et al., 1983; Freiberg et al., 1984;). Measured fluorescence lifetimes of *Rb. sphaeroides* R-26 chromatophores having closed RC (Campillo et al., 1977) and isolated B875 complexes (Sebban et al., 1985) range from 0.30 to 0.64 ns.

Resonance Raman. Laser excitation in resonance with the Soret absorption maximum was chosen for the following reasons: detection of fluorescence emission in the red and NIR regions is avoided with near-UV excitation, the large Soret extinction coefficient permits the acquisition of good quality

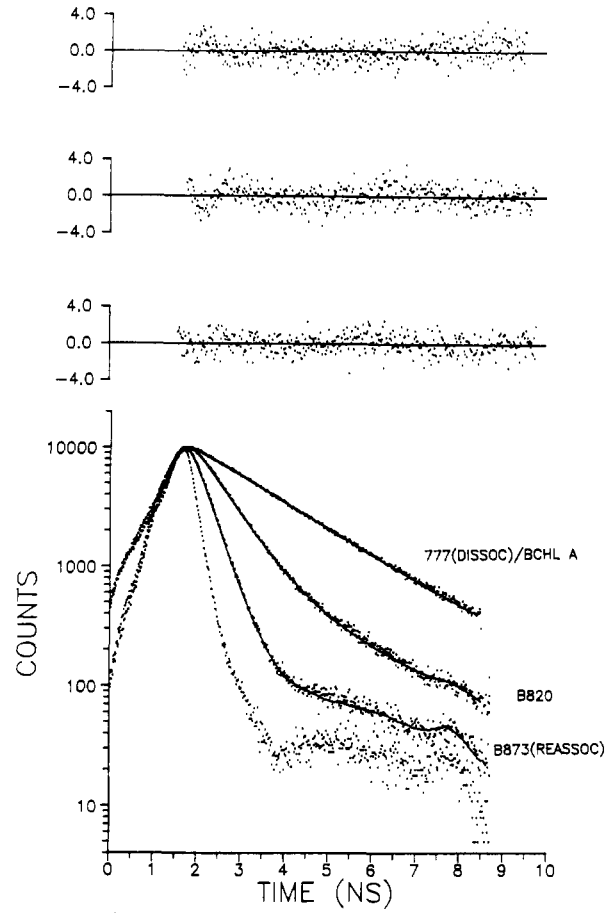

FIGURE 4: Fluorescence decay curves of B873(reassoc), B820, and 777(dissoc). The samples had the respective absorbances 0.63 of 777 nm, 1.25 at 820 nm, and 1.88 at 871 nm and were kept under Ar. The instrument function is also shown. Excitation wavelength is 590 nm, and the detection wavelengths, lifetimes, and χ^2 values from the fits are given in Table II. The weighted residuals of each fit are shown in the same order as the decay curves.

vibrational spectra of samples with an absorbance of 10–20, and the Soret absorption region overlaps with several fixed-frequency ion laser emission lines. Although the dominant detergent-induced absorption changes of B873 occur in the NIR, excitation into the Soret region probed the ground-state vibrations of BChl *a* in the environment that comprises each of the distinct spectral forms.

RR spectra of B873, B820, and B873(reassoc) samples are presented in Figure 5, and their band positions and assignments are given in Table III. These B820 and B873(reassoc) samples were not purified through a gel filtration column (see Materials and Methods). The spectrum of in vivo B873 (Figure 5a) compares well with that previously published by Hayashi et al. (1983) and Robert and Lutz (1985). These spectra are dominated by the intense core-size sensitive band at 1609 cm^{-1} that remains constant in all three samples of Figure 5. The location of this band is indicative of 5-coordinate Mg^{2+} (Cotton & van Duyne, 1981; Callahan & Cotton, 1987). The vibrations >1630 cm^{-1} are assigned to the C2 and C9 ketone vibrations. The differences between B873 and B820

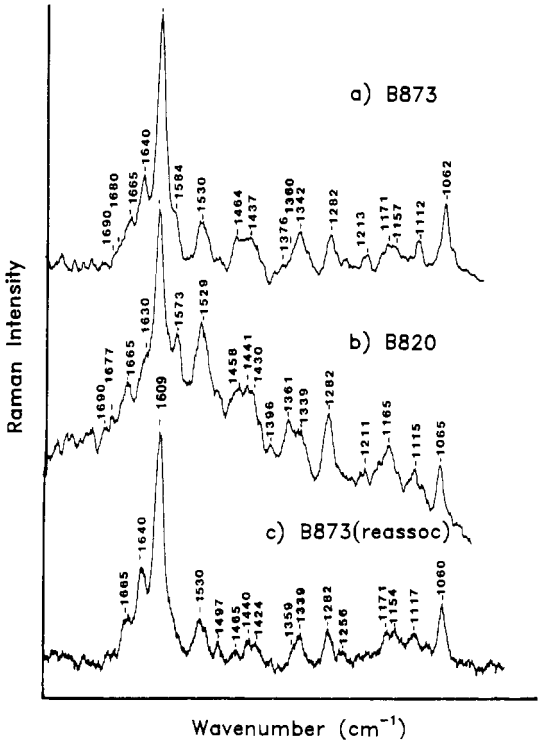

FIGURE 5: Resonance Raman spectra of *Rs. rubrum* G-9 Triton/EDTA-washed chromatophores: (a) B873, (b) B820, and (c) B873(reassoc) in 100 mM potassium phosphate, pH 7.4. BChl *a* concentration was approximately 200 μM for (a) and (b) and 50 μM in (c). The samples were prepared as described under Materials and Methods. Excitation wavelength was 363.8 nm.

Table III: Resonance Raman Vibrational Bands (cm^{-1}) of B820 and B873(reassoc)

B873 frequency (cm^{-1})	B820 frequency (cm^{-1})	assignment
1640	1630	ν(C2=O)
	1573	
1360, 1340	1360, 1340	νCaN, δCmH
1150–1170	1150–1170	νCaN, νCmC10
1060	1065	δCmH
1530	1530	νCaCm
1430–1460	1430–1460	νCaN, δCmH
1282	1282	νCaN, δCmH

are the following: a decrease in intensity of the 1640 cm^{-1} band; a relative intensity change of the 1360 and 1340 cm^{-1} bands (νCaN, δCmH); intensity changes in the 1150–1170 cm^{-1} region (νCaN, νCmC10); and a shift in frequency of the 1060 cm^{-1} band to 1065 cm^{-1} (δCmH). The 1530 cm^{-1} (νCaCm) band, the 1430–1460 cm^{-1} region, and the 1282 cm^{-1} band (νCaN, δCmH) remain constant. In the spectrum of B873(reassoc) shown in Figure 5c, the initial features are recovered upon reassociation from B820, including the distinct acetyl stretching vibration at 1640 cm^{-1}. The spectra from purified samples (not shown) compare quite well to those measured from B820 and B873(reassoc) prepared by titrating chromatophores with OG that were not purified by gel filtration chromatography (Figure 5b,c). A small increase in intensity was observed at 1680 cm^{-1} in the RR spectrum of purified B873(reassoc) and may be an indication of a slightly altered C9 ketone interaction. The only other difference observed was the absence of the 1573 cm^{-1} band in the purified B820 spectrum. No RC contributions were obvious in the nonisolated samples.

The vibrational spectra of isolated BChl *a* in different solvent systems (Figure 6) present the usual range of C2 and C9 acetyl

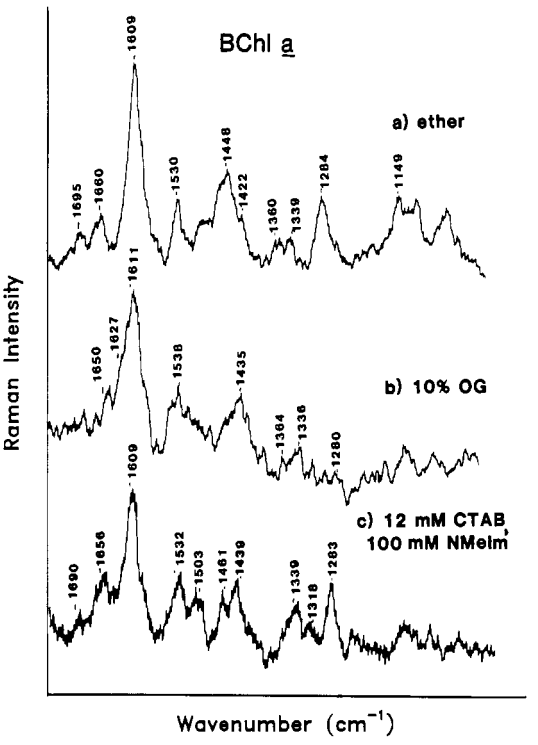

FIGURE 6: Resonance Raman spectra of BChl *a* obtained with 363.8-nm excitation in the following solvents: (a) diethyl ether; (b) 10% OG/50 mM HEPES, pH 7.4; (c) 100 mM *N*-methylimidazole, 12 mM CTAB, and 50 mM HEPES, pH 7.4. BChl *a* concentration was approximately 200 μM for all samples. Laser power, 10 mW; spectral resolution, 8 cm^{-1}.

and ketone stretching frequencies. All of these samples contain 5-coordinate BChl *a*, and monomeric samples are found in Figure 6a,c; ether and *N*-methylimidazole are the respective ligands. BChl *a* in 10% OG (Figure 6b) is in a self-aggregated state at these high concentrations. For BChl *a* in ether, the C9=O and C2=O stretching frequencies occur at 1695 and 1660 cm^{-1}, respectively. In an aqueous/CTAB environment, these vibrations shift to 1690 (with decreased intensity) and 1656 cm^{-1} (Figure 6c). In an aggregated environment (10% OG) (Figure 6b), three bands are found in this region at 1690, 1650, and 1627 cm^{-1}. The 1627 cm^{-1} shoulder may be a strongly interacting C2=O acetyl stretch.

The spectrum in Figure 6b, BChl *a* in 10% OG, also indicates the sensitivity of the 5-coordinate marker band at 1609 cm^{-1} to aggregation. Although located at 1611 cm^{-1} in this figure, this band can shift up to 1613 cm^{-1} in such BChl *a* detergent micelles. The constancy of the 1609 cm^{-1} band position in B873(reassoc) and B820 indicates that although there are most certainly exciton effects involved in the BChl *a*–BChl *a* interactions, large aggregations with random orientation like those found in detergent micelles are not an appropriate model for BChl *a* in either of the LH forms.

The major vibrational difference between the 873- and 820-nm-absorbing forms of the LH protein is the decrease in magnitude of the 1640 cm^{-1} band which was assigned to changes in the C2 acetyl stretching vibration. It is possible to explain the disappearance of this band as a down-shift to 1630 cm^{-1}. (The C9 ketone stretching frequencies at 1665, 1680, and 1690 cm^{-1} are fairly constant.) The acetyl stretching vibration at 1640 cm^{-1} is down-shifted relative to that of BChl *a* in ether by 20 cm^{-1} (compare Figure 5a with Figure 6a), and it occurs at a lower frequency than any IR spectra yet obtained of BChl *a* in vitro. It is difficult to determine what specific interactions (i.e., loss of a hydrogen bond) are responsible for the observed shift. Another possible explanation

for the changes in the ketone and acetyl stretching region that occur when forming B820 from B873(reassoc) is that the 1630 cm^{-1} band is a ring vibration and not associated with the acetyl vibration. The disappearance of the 1640 cm^{-1} band could then be caused by exposure of the pigment to an aqueous environment which results in a decrease in intensity of this acetyl stretching vibration and a shift to 1665 cm^{-1} (Figure 5b). To support an assignment of a ring vibration, there is a 1630 cm^{-1} vibration observed in 2-deoxo-2α-hydroxy-BChl *a* and a correlated vibration is found in copper(II) dihydropheophorbide (data not shown). Both interpretations of the 1630–1640 cm^{-1} region suggest a difference in the environment of the C2 acetyl group in B820 and B873(reassoc).

Conclusions

B820 is a distinct subunit form of the core LH complex of *Rs. rubrum* as indicated by its size, absorption spectrum, and longer fluorescence lifetime. Upon exposure to O_2 and light (Miller et al., 1987) or $NaBH_4$ (Callahan et al., 1987), it is also found to be less stable under these conditions than B873(reassoc). The relative instability of BChl *a* in B820 suggests that the pigment is bound on the surface of the protein and is readily available to water. The shift of the 1640–1630 cm^{-1} RR bands indicates that the C2 acetyl group of BChl *a* is sensitive to changes in environment, such as its degree of exposure to detergent or the aqueous solvent.

It is unlikely that the CD and red-shifted absorption spectra of B820 are the result of bound but monomeric BChl *a* molecules. Model studies by Wright and Boxer (1981) as well as Woody (1985) suggest that while the CD spectrum can be greatly enhanced when a single chromophore is inserted into the heme binding site of apomyoglobin, the absorption spectrum remains relatively unaffected. The largest absorption red shift reported by Wright and Boxer (1985) was 9 nm upon chromophore binding to protein as compared to the 40–50-nm red shift observed for B820 from free BChl *a*. Also, from systematic studies of BChl *a* in organic solvents, hydrogen bonding and interactions with aromatic amino acids would be expected to contribute only about 10 nm to the red shift (Cotton, 1976; Cotton et al., 1978). Thus, this kind of pigment–protein interaction may contribute greatly to the CD spectral features but very little to the red shift of the long-wavelength absorption band. Thus, the resulting CD and absorption spectrum of B820 is most likely due to two or more interacting BChl *a* molecules.

Reassociation of B820 to B873(reassoc) can give a variety of B873-like complexes, depending on the reassociation conditions. Addition of lipids and slowing the rate of aggregation by lowering the temperature seem to have a stabilizing effect on the reassociation process. By carefully controlling these conditions, a reassociated LH complex can be consistently formed with absorption, CD, fluorescence, and RR properties either identical with or closely approaching those found for the in vivo complex. Furthermore, the RC or carotenoid is not required for forming such complexes.

Although several models have been proposed to explain the properties of B873 (Scherz & Parson, 1986; Zuber, 1986; Parkes-Loach et al., 1988; Niederman et al., 1989), none account for all the spectroscopic properties of the complex satisfactorily. Additional structure–function information is required at this juncture. The methods developed in our laboratory for reconstituting B873 from isolated LH polypeptides and pure BChl (Parkes-Loach et al., 1988) are being further employed to systematically examine the role of specific amino acids in the pigment–protein complex. A number of BChl analogues are also being tested to determine the specificity of the BChl binding site.

Other experiments in progress include fluorescence polarization measurements to find relative orientations of the BChl *a* molecules in the different states and the isolation of subunit forms from other photosynthetic bacteria such as *Rb. sphaeroides*, *Rb. capsulatus*, and *Rp. viridis* to understand trends in the macromolecular organization of the core LH complex.

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Registry No. BChl *a*, 17499-98-8.

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