Excitation Trap Approach to Analyze Size and Pigment−Pigment Coupling: Reconstitution of LH1 Antenna of *Rhodobacter sphaeroides* with Ni-Substituted Bacteriochlorophyll†

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Received September 26, 2000; Revised Manuscript Received January 12, 2001

ABSTRACT: Replacement of the central Mg in chlorophylls by Ni opens an ultrafast (tens of femtoseconds time range) radiationless de-excitation path, while the principal ground-state absorption and coordination properties of the pigment are retained. A method has been developed for substituting the native bacteriochlorophyll a by Ni-bacteriochlorophyll a ([Ni]-BChl) in the light harvesting antenna of the core complex (LH1) from the purple bacterium, *Rhodobacter* (*Rb.*) *sphaeroides*, to investigate its unit size and excited state properties. The components of the complex have been extracted with an organic solvent from freeze-dried membranes of an LH1-only strain of *Rb. sphaeroides* and transferred into the micelles of *n*-octyl-*β*-glucopyranoside (OG). Reconstitution was achieved by solubilization in 3.4% OG, followed by dilution, yielding a complex nearly identical to the native one, in terms of absorption, fluorescence, and circular dichroism spectra as well as energy transfer efficiency from carotenoid to bacteriochlorophyll. By adding increasing amounts of [Ni]-BChl to the reconstitution mixture, a series of LH1 complexes was obtained that contain increasing levels of this efficient excitation trap. In contrast to the nearly unchanged absorption, the presence of [Ni]-BChl in LH1 markedly affects the emission properties. Incorporation of only 3.2 and 20% [Ni]-BChl reduces the emission by 50% and nearly 100%, respectively. The subnanosecond fluorescence kinetics of the complexes were monoexponential, with the lifetime identical to that of the native complex, and its amplitude decreasing in parallel with the steady-state fluorescence yield. Quantitative analysis of the data, based on a Poisson distribution of the modified pigment in the reconstituted complex, suggests that the presence of a single excitation trap per LH1 unit suffices for efficient emission quenching and that this unit contains 20 ± 1 BChl molecules.

The photosynthetic apparatus of purple bacteria consists of highly specialized integral membrane pigment−protein complexes that enable them to efficiently utilize low-energy light quanta, in a wavelength range where oxygenic photosystems are ineffective (*1*). The photons are captured by a system of light harvesting antennae that efficiently absorb the light and transfer the excitation energy to the reaction centers, where it is converted into an electrochemical potential by ultrafast and long-lived charge separation across the photosynthetic membrane. *Rb. sphaeroides*,[1] like most purple bacteria, has two types of antennae, functioning in sequence. According to their near-infrared absorption maxima, they are termed B875 (or LH1), with its absorption maximum around 875 nm, and B800-B850 (or LH2), with absorption maxima at 800 and 850 nm. LH1 is closely associated with the reaction center to form the so-called core complex, while the LH2 antenna is located more peripherally and is synthesized by the bacterium in a variable ratio to the core complex, depending on the growth conditions (leading recent reviews on bacterial photosynthesis: refs *2*−*4*).

Following the high-resolution structures of native and modified RC (*5*−*9*), the structures of several LH2 (peripheral) antenna complexes have been solved at atomic resolution (*10*, *11*). The high-resolution structure of the LH1 antenna is not yet available but has been modeled (*12*, *13*) on the basis of existing low resolution images (*14*) and

† The project was supported by the Deutsche Forschungsgemeinschaft (Sche 140/9-3 and SFB 533). L.F. acknowledges the postdoctoral fellowship provided by the Alexander von Humboldt Foundation.

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[1] Abbreviations: BChl = bacteriochlorophyll a; CD = circular dichroism; EM = electron microscopy; LH1 = light-harvesting complex 1, also termed B875; LH2 = light-harvesting complex 2, also termed B800-850; [Ni]-BChl = nickel-bacteriochlorophyll (= BChl with Mg replaced by Ni); OD = optical density; OG = *n*-octyl-*β*-glucopyranoside; PAGE = polyacrylamide gel electrophoresis; *Rb.* = *Rhodobacter;* RC = reaction center; *Rs.* = *Rhodospirillum*; SDS = sodium dodecyl sulfate; t_r = retention time.

10.1021/bi002257f CCC: $20.00 © 2001 American Chemical Society
Published on Web 03/02/2001

spectroscopic data (*15*−*17*) as well as sequence homologies to LH2 (*18*). Accordingly, its organization is assumed to be very similar to that of the B850-only complex but with a larger ring size containing, for example, in *Rhodospirillum* (*Rs.*) *rubrum* 16 $\alpha\beta$-units and an intercalated set of 32 closely spaced BChls (*14*, *19*). The reaction center snugly fits into the central area of such an LH1 ring, and electron micrographs of core complexes from several species lacking the LH2 antenna have been interpreted this way (*14*, *20*, *21*).

This arrangement, however, poses functional problems because it impedes shuttling of the (hydro)quinone between the RC and the cytochromes b/c complex within the membrane (*22*). In fact, recent electron microscopic (*23*) as well as biochemical studies (*24*) have indicated that smaller LH1 units comprising only sections of a ring cannot only exist in vitro but are relevant to the in vivo situation. Such sections, but also snugly fitting rings and even enlarged rings, have been sugggested on the basis of BChl/BPhe ratios for several LH1-only species (*25*). Various size estimates gave numbers of 12 to 16 ($\alpha\beta$BChl$_2$) subunits per isolated LH1 complex (*14*, *25*−*30*), which seem however to depend on the species and on the isolation method (*24*). "Curved complexes" with a wide distribution of sizes have been prepared by Westerhuis et al. (*24*), and McGlynn et al. (*31*) showed that C-terminal deletion of the LH1α polypeptide gives rise to a variety of LH aggregation states in vivo. Francia et al. (*32*) and Jungas et al. (*23*) provide evidence that there are situations in vivo where the LH1 antenna of *Rb. sphaeroides* does not form closed rings around the RCs but rather supercomplexes of pseudo-2-fold symmetry, which are composed of two RCs enclosed by a pair of C-shaped LH1 antennae, facing each other on the open side. Here, only about 24 BChl molecules, the number similar to the early estimates of the LH1 ring size, are expected in each of the C-shaped LH1-sections [a somewhat larger number of 29 BChls has been estimated by McGlynn et al. (*31*)]. The noncircular arrangement around the RC is related to the presence of a small protein, PufX, which enables the quinone exchange between the RC and the cytochrome bc$_1$ complex. This rationalizes earlier observations that PufX facilitates quinone shuttling between the RC and the ubiquinone pool in the membrane (*22*, *33*). It is concluded that the PufX protein plays an important role in the structure of the functional photosynthetic unit, and its absence results in loss of efficient electron transfer from the Q_B site of the reaction center to the Q_Z site of the cytochrome bc$_1$ complex.

The high resolution structures have triggered numerous attempts to evaluate the electronic structures of the closely packed B850 BChls in LH2, as well as of the B875 pigments in LH1, which are believed to be arranged in a similar fashion. One goal of such work is to determine the consequences of these structures for energy transfer dynamics within these antenna complexes. Two extreme models are currently discussed. In the "dimer model" proposed first by Scherz et al. (*34*), the B850 and B875 complexes are considered as rings of tightly packed BChl dimers which, in turn, seem similar to the special pair (P870) in the RC. In fact, the LH1 antennae of carotenoid-free mutants are known to dissociate into such subunits (B820) containing dimeric BChl (*35*). Most of the key spectroscopic features, including strong spectral shifts, are rationalized as originating from interactions within these dimers, while excitonic interactions between neighboring dimers are relatively weak. The excitation is therefore localized at any time on a pair of the excitonically coupled dimers (delocalization length $N = 4$), and energy transfer proceeds by hopping between such dimers (*17*, *36*−*38*). In the "extreme delocalization model", the spectral features are accounted for by excitonic eigenstates that comprise the entire set of 16/18 (LH2) or ≤ 32 (LH1) closely spaced BChls (delocalization length $N = 16/18$ and ≤ 32, respectively) (*39*, *40*). Here, the red shift of the Q_Y absorption band and the circular dichroism features, observed upon ring formation, originate completely from excitonic interactions among all BChl molecules of the supercomplex. In a perfectly cyclic aggregate, the oscillator strength would be concentrated in two degenerate orthogonal transitions, which energetically lie slightly above the lowest, optically forbidden transition (*17*, *36*, *37*). Evidence for the occurrence of such a state at early times after excitation in LH2 has been found by Leupold et al. (*41*) using the nonlinear absorption technique and by van Oijen et al. (*42*) in single-molecule spectroscopy polarization-dependent experiments.

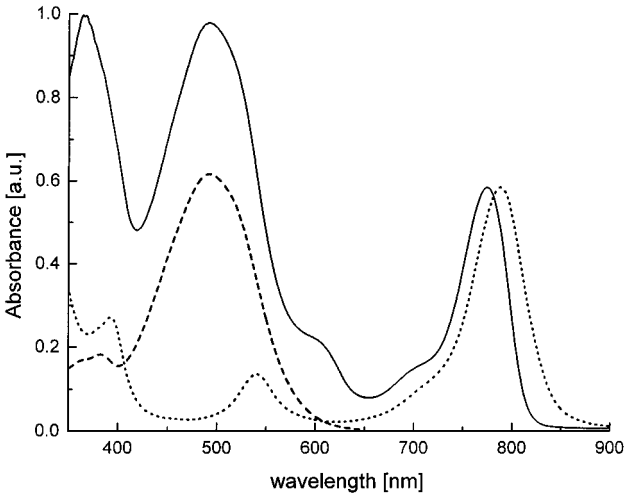

FIGURE 1: Absorption spectrum of methanol/chloroform extract of LH1-only membranes of *Rb. sphaeroides* strain RC$^-$LH2$^-$ DD13 RKEH (—) as compared with the absorption spectra of [Ni]-BChl (···) and spheroidenone (− − −) in the same solvent.

Both models describe ideal, limiting situations. Their applicability rests not only on the relative sizes of the inter- and intradimer excitonic interactions, the electron exchange contribution, intrinsic energetic disorder (*42*, *43*) (different site energies and inhomogeneous line-widths), and electron−phonon coupling (*17*, *36*, *37*), but also on deviations of the ring geometry from the cyclic one (*42*). Moreover, the delocalization length is expected to decrease with time, and the above factors strongly contribute to the kinetics of this process (*16*, *43*, *44*). A variety of methods has been and is currently applied to quantitatively evaluate these factors, but there is a considerable disagreement in interpreting the results obtained with different methods and in different time-regimes.

It is known that introduction of metals other than Mg into the central cavity of the porphyrin macrocycle can strongly modify its excited-state properties (*45*). In particular, [Ni]-BChl (absorption spectrum shown in Figure 1) shows extremely rapid radationless excited-state decay with lifetimes well below 100 fs, due to a cascade of π- and metal-

centered excited states of intermediate energies (*46*, *47*), while its ground-state and coordination properties are similar to those of BChl (*48*). In exploiting these properties, we have taken a new approach to spectroscopically study the (physical and delocalization) unit size of photosynthetic antennae and their kinetics of energy transfer and relaxation. It is based on introducing [Ni]-BChl into the antenna as a relaxation channel that is fast enough to compete with intracomplex excited state dynamics and energy transfer. We have developed a method for the reassembly of LH1 in OG micelles and used it to incorporate the modified pigment into this antenna. Thus, a series of reconstituted LH1 complexes has been prepared in which an increasing fraction of the native BChl is replaced with [Ni]-BChl. Using a femtosecond pump/probe technique, we have studied the initial processes of ultrafast energy transfer and relaxation in these modified antennae, indicating a large delocalization of the excited state (*49*). Here, we present the results of our complementary investigations carried out to further characterize this model system. We show that the approach is useful in particular for the investigation of structure−function relationships and energy transfer in antenna complexes.

EXPERIMENTAL PROCEDURES

Bacterial Cultures and Membrane Isolation. The LH1-only ($RC^{-}LH2^{-}$) mutant strain DD13 [pRKEK] of *Rb. sphaeroides* (*50*) was grown under aerobic conditions in the dark in M22+ medium, and its LH1-containing membranes were isolated as described previously (*51*). The dark-red cells were harvested after 2−3 days and disrupted by French press treatment (2 × 15 000 psi). The membrane-bound LH1 fraction was isolated by ultracentrifugation in a sucrose density step gradient (15%/40%).

Isolation of Native LH1 Complex. The LH1 containing membranes (OD_{875} ∼40 cm^{-1}) were incubated with OG (3%) in Tris-HCl buffer (20 mM, pH 7.6) for 90 min at room temperature while stirring. The mixture was centrifuged (1 h, 230000*g*) to remove nonsoluble material. The dark pink supernatant was loaded on a DEAE-Sepharose column (Fast Flow, Pharmacia, 2 × 25 cm), equilibrated in Tris-HCl buffer (20 mM, pH 7.6) containing OG (1%). The column was eluted with a linear gradient of 0.05 to 0.45 M NaCl in the same buffer at a flow rate of 3 mL/min. The fraction eluting with 0.225−0.250 M NaCl was collected, NaCl was removed, and the volume was reduced by membrane filtration. The concentrate was subjected to a Superdex-200 column (2 × 60 cm), equilibrated in 1% OG in the same buffer as above. The column was eluted at a flow rate of 1 mL/min, and the center fraction of the dark red band of isolated LH1, which eluted with ∼80 mL of the buffer, was collected. The quality of the fractions (see Results) was checked spectrophotometrically.

LH1 Reconstitution via Organic Solvent Extraction of LH1-Only Membranes. The freeze-dried LH1-only membranes were extracted with chloroform/methanol (1:1) containing ammonium acetate (0.1 M), following the method of Theiler et al. (*52*). Usually, 100 mL of the solvent was used to extract a portion of membranes equivalent to ∼1.3 mg of pure BChl (corresponding to 100 "OD × V units"). The extract (absorption spectrum in Figure 1) could be stored for up to one week in the dark at −20 °C. The solvent was then evaporated under vacuum. To remove the excess ammonium acetate, the solids were re-suspended in 2−3 mL of Tris-HCl buffer (100 mM, pH 7.6) and centrifuged at low speed (10 min, 10000*g*). The pellet could be stored for 5−6 weeks under Ar at −20 °C without noticeable alteration.

A portion of the pellet, containing ∼0.13 mg of BChl, was suspended in 200 μL Tris-HCl buffer (100 mM, pH 7.6) containing 20% OG (w/v) and solubilized by vigorous stirring for 5 min, followed by 5 min sonication on ice. Then three 200-μL and three 400-μL portions of Tris-HCl buffer (20 mM, pH 7.6) were added to the suspension to reduce the detergent concentration to 1.5%, each addition followed by the stirring/sonication steps (method 1). Alternatively, the LH1 membrane pellet was first suspended in an appropriate volume (usually 600−800 μL) of Tris-HCl buffer (100 mM, pH 7.6), and then, while stirring, a 20% OG aqueous solution was added dropwise to a final OG concentration of 3.4%. The resulting suspension was sonicated on ice, stirred for 15 min at ambient temperature, and diluted stepwise with 20 mM Tris-HCl buffer (pH 7.6) to reduce the detergent concentration to 1.5% (method 2). The reconstitution progress was monitored by absorption spectroscopy of the suspension (increase at 875 nm). In each case, the final mixture was centrifuged for 10 min at 17 °C at low speed (5000*g*) to remove nonsolubilized material. If still colored, the pellet was re-extracted with the 1% OG solution. The combined supernatants were loaded on a small DEAE-Sepharose column preequilibrated in Tris-HCl buffer (20 mM, pH 7.6) containing OG (0.8%) and NaCl (25 mM). The fraction of free pigments was removed with the low salt 0.8% OG Tris-HCl buffer (100−150 mM NaCl). A band of the B820 subcomplex (carotenoid-less) was eluted with 200−225 mM NaCl in the same buffer, and a band of the LH1 complex (B875) with 280−300 mM NaCl. All separations on the column were monitored by absorption spectroscopy.

BChl Isolation and Central Metal Replacement. BChl was isolated from the carotenoid-less strain R26.1 of *Rb. sphaeroides* according to a method of Omata et al. (*53*), with some modifications. The freeze-dried cells were extracted with methanol, the pigments transferred to acetone and separated on a DEAE-Sepharose column, preequilibrated in acetone. The fraction of pure BChl was eluted with 20% methanol in acetone.

The central Mg^{2+} ion of BChl was replaced by Ni^{2+} using the transmetalation method via the Cd-BChl intermediate according to Hartwich et al. (*48*). The purity of the final product (absorption spectrum in Figure 1) was confirmed by TLC and HPLC analysis. All preparative steps were carried out under dim light.

UV/VIS Absorption and CD Spectra. The electronic absorption spectra were measured using a Perkin-Elmer Lambda 2 UV/VIS spectrophotometer, usually in 1-mm quartz cells. Circular dichroism spectra were recorded at room temperature in 1-cm quartz cells with a CD 6 spectropolarimeter (Jobin Yvon). The samples' optical densities were kept near 0.6 at the Q_y maximum, and to reduce noise the spectra were averaged over 8 to 10 scans.

HPLC Analyses. Pigment analysis was done on an HPLC system consisting of a Pharmacia LKB LCC 2252 HPLC controller, two 2248 HPLC pumps, a TIDAS (J & M) diode array detector and a silica gel Alltech Econosphere Si 5U column (4.6 × 150 mm). For pigment separations, solvent

A (99.85% toluene/0.1% 2-propanol/0.05% methanol) was mixed with increasing proportions of solvent B (95.9% toluene/0.1% 2-propanol/4% methanol). After injection in pure solvent A, the following linear gradient was applied: from 0 to 10% B in 8 min, from 10 to 50% B in 3 min, from 50 to 100% B in 4 min. The identity of pigments was confirmed by online measurements of absorption spectra as well as by coinjections of samples of isolated BChl and [Ni]-BChl.

The extractions of the LH1 pigments were done by loading an appropriate aliquot of the analyzed complex on a small DEAE-Sepharose (fast flow) column, equilibrated in 0.8% OG Tris-HCl buffer (25 mM NaCl). The buffer was replaced by distilled water, and the colored band was eluted to completion with acetone. Before the sample was subjected to the HPLC, the extracted pigments were transferred to the solvent A.

Solvents. All solvents were of analytical grade and used without any further purification. The solvents for HPLC were filtered (0.22 μm) and degassed prior to use.

Ultracentrifugation in Sucrose Density Gradient. The ultracentrifugation was done on a Sorvall Discovery 90 ultracentrifuge using a RPS40T-767 swing-out rotor. Two samples of reconstituted LH1 (300 μL, equivalent to ca. 5 μg of BChl), one containing no modified pigment and the other containing 3.2% [Ni]-BChl, were loaded on a sucrose density step gradient (seven layers, 1.5 mL each, of sucrose solutions increasing from 10 to 40% by 5% steps) in 20 mM Tris-HCl buffer containing 0.9% OG. The separation was carried out at 12 °C for 24 h at 20 000 rpm (49200*g*). Afterwards, the colored fractions were collected from the centrifuge tubes through a glass capillary, carefully lowered to beneath the band. Using a peristaltic pump (0.2 mL/min), the bands were divided into eight subfractions of ~0.1 mL each. They were then diluted with the buffer containing 0.8% OG to 1.3 mL, and their absorption and emission spectra (λ_{ex} = 500 nm) were measured immediately.

Steady-State Emission Measurements. The steady-state emission spectra were recorded at room temperature on a Spex Fluorolog 1680 spectrofluorimeter (0.22 m double monochromators) using 1-cm quartz cells. The samples were diluted to an optical density of 0.07 at the excitation wavelength of 500 nm.

Time-Resolved Fluorescence Measurements. The measurements of LH1 emission decay were carried out using a home-built multidecay spectrometer operating in boxcar mode, which was described in detail elsewhere (*54*). The samples (in 0.8% OG) were excited at 850 nm with laser pulses of 400 ps duration, using a dye laser, pumped with a nitrogen laser (MSG 850/LTB Berlin). The measurements were done in 1-cm cuvettes utilizing a right-angle configuration of the excitation and emission beams. To avoid reabsorption effects, the optical density of the sample was adjusted to $OD_{500\ nm} < 0.05$. The recorded emission traces were numerically analyzed by deconvolution with a sum of exponentials as the modeling function.

RESULTS

Isolation of Native LH1. The isolation of LH1 from wild type *Rb. sphaeroides* is difficult. It leads to considerable losses, and the preparations are often contaminated with reaction centers. Therefore, a mutant lacking both RC and LH2 has been used to avoid these complications. After solubilization of the LH1-only membranes in OG, the complex was purified by ion-exchange chromatography, followed by gel filtration. The purified LH1 complex was collected from the gel filtration column as the center fraction of a dark-red band. With respect to the starting material (LH1-only membranes), the procedure gives 30−40% yield of purified LH1 complex. Its absorption spectrum (Figure 2, dotted line) shows a relatively low level of impurities, as judged by the absence of 770 and 820 nm bands and a high A_{875} to A_{275} absorption ratio (~2.4). Upon excitation of the complex in the carotenoid region at 500 nm (or at 400 nm into the BChl Soret transition), the emission (not shown) has a maximum near 895 nm, which is typical for LH1.

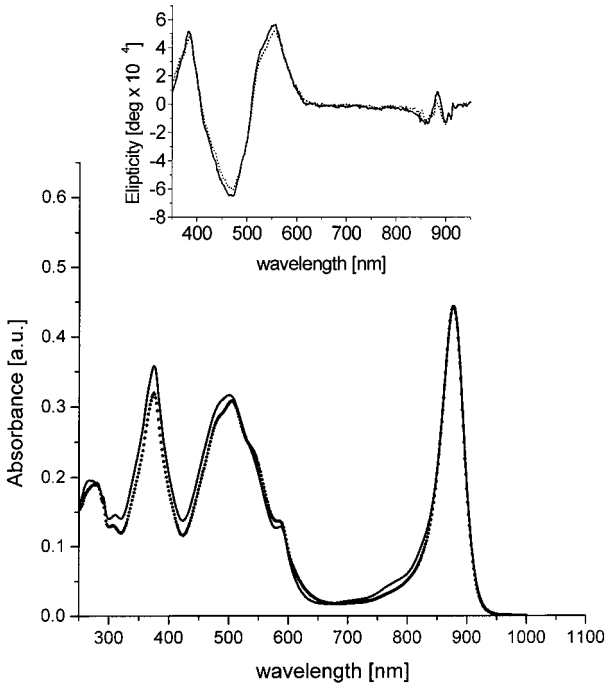

FIGURE 2: Absorption (main panel) and CD (inset) spectra of native (···) and reconstituted (—) LH1 complexes in Tris-HCl buffer containing 0.8% OG.

LH1 Reconstitution. The extraction of freeze-dried LH1-only membranes with methanol/chloroform yields an extract containing the pigments and the apoproteins of the LH1 complex and allows for a partial elimination of impurities insoluble in organic solvent. The absorption spectrum of this extract (Figure 1) shows maxima nearly identical to those of the free pigments in the same solvent.

For resolubilization of the extracted LH1 (membrane) components, they were first treated with high concentrations of OG (20% or alternatively 3.4%), followed by dilution to 1.5−1.1%. The procedure leads to re-formation of an LH1 complex, containing both BChl and carotenoids, as can be judged from the absorption and CD spectra (Figure 2). Generally, the reconstitutions using the lower detergent concentration (3.4%) give higher yields (up to 40%). The reconstituted complex was purified and isolated from the other soluble components of the membrane extract on a DEAE-Sepharose column. With increasing NaCl, the following fractions were eluted: the first contains free pigments (carotenoids, BChl and its degradation products, with absorption maxima at 500, 750, and 695 nm, respectively). This

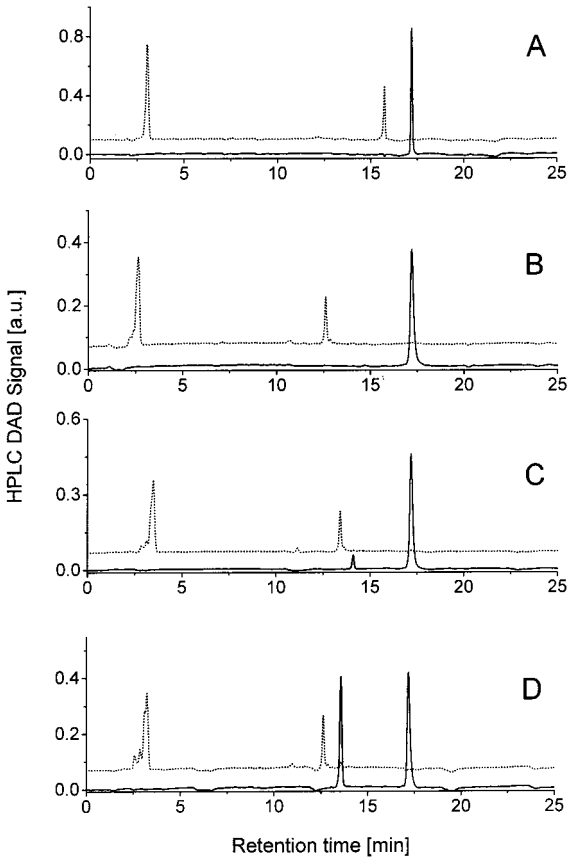

FIGURE 3: HPLC-chromatograms recorded at 500 nm (upper traces) and 770 nm (lower traces), obtained for four types of the LH1 complexes: (A) OG-isolated native complex; (B) reconstituted complex with native pigment composition; (C) complex reconstituted with 11.6% Ni-BChl; and (D) complex reconstituted with 57% Ni-BChl. The scatter of retention times is due to varying amounts of residual detergent in the samples. Traces have been normalized such that the retention time of BChl is 17.2 min.

fraction was not analyzed further. It was followed by the fraction devoid of carotenoids (λ_{max} = 820 nm), which contains the carotenoid-less B820 subcomplex (*55*), and finally by the reconstituted LH1 complex whose absorption spectrum is shown in Figure 2. On the basis of (i) the ratio of the absorbance at 875 nm (Q_y) to the one at 275 nm (A_{875}/A_{275}, see Figure 2), which is as high (2.3−2.5) as that of the isolated (nonreconstituted) complex, and (ii) indications of only minor contaminations by free BChl and B820 fractions; just one ion-exchange chromatography step was sufficient to obtain fairly pure preparations of the reconstituted LH1 complex. These purity criteria, however, do not exclude the presence of colorless nonproteinaceous contaminations. The purified complex seems to be quite stable at ambient temperatures at low OG concentrations (0.8−1.0%), as no apparent degradation has been observed, and neither a loss of pigments nor a decreased yield of pure LH1 has been observed during repurification or concentration on the ion-exchange column.

In preliminary experiments, the same protocol has been applied to the freeze-dried chromatophores isolated from *Rs. rubrum* (wild type). The reconstitution in OG, followed by purification on the DEAE-Sepharose column, yielded a complex with the absorption spectrum (data not shown) closely resembling one of the native complex (*55*), with typical band positions peaking at 376 and 882 nm (the Soret

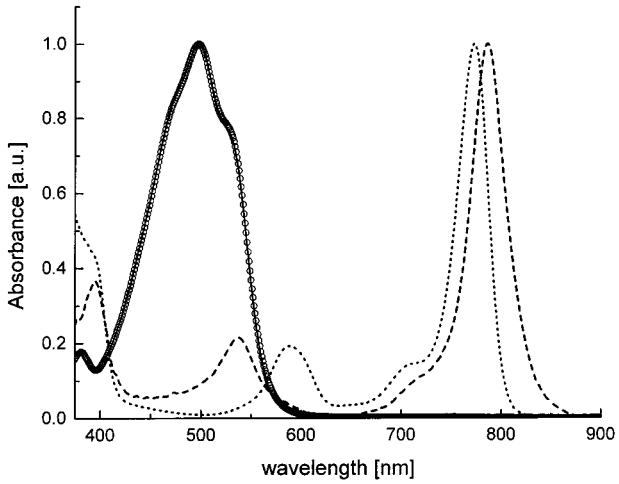

FIGURE 4: Absorption spectra of the four fractions seen in the HPLC-chromatogram C of Figure 3 (LH1 complex reconstituted with 11.6% [Ni]-BChl), recorded online using diode array detection. BChl (t_r = 17.2 min, - - -), NiBChl (t_r = 14.1 min, − − −), spheroidenone 1 (t_r = 3.5 min, −), and spheroidenone 2 (t_r = 13.4 min, ···). The respective peaks in the other chromatograms show identical spectra despite variations of the retention time due to varying amounts of residual detergent in the samples.

and Q_y transitions, respectively) and a broader carotenoid absorption with a maximum at 503 nm.

The pigment composition of the isolated and reconstituted complexes has been analyzed by normal-phase HPLC and shows that nonmodified complexes (Figure 3, panels A and B) contain two major carotenoids and BChl, and the modified LH1 also [Ni]-BChl (Figure 3, panels C and D). As an example, Figure 4 shows the absorption spectra, recorded online during the chromatographic separation, of the pigments found in the LH1 complex reconstituted with 11.6% [Ni]-BChl. Bchl and [Ni]-BChl were identified by coinjections of pure pigments while the carotenoids of identical absorption spectra, closely resembling the one of spheroidenone (Figure 1, dashed line) were not analyzed further. The pigments show some retention time drift (Figure 3), probably due to varying amounts of coextracted detergent (OG), but their identities were verified in all cases by their absorption spectra. However, as seen in the chromatograms, very similar ratios of the components (BChl and carotenoids) were found in all types of the complexes. Accordingly, the absorption spectra of the LH1 complexes, obtained by the two different methods, are nearly identical (Figure 2). The SDS−PAGE analysis of the native (isolated) and reconstituted LH1 complexes confirms the presence of only the two polypeptides (α, β) of low molecular weight in the preparations (data not shown).

In both preparations, viz. the isolated LH1 and the reconstituted complex, the BChl near-IR absorption band (Q_y) is red-shifted to 876 nm, and the carotenoids have characteristic broad, structured absorption bands located between 450 and 550 nm, with a maximum at 500 nm. The complexes also show similar emission maxima and yield. If excited at 500 nm, the complex shows an emission band in the near-IR, with the maximum at 897 nm (see Figure 6, uppermost trace). For samples of identical optical densities at the excitation wavelength (A_{500} = 0.07), the peak positions and emission intensities are identical to the values obtained for the LH1-only membranes or native LH1 (data not shown),

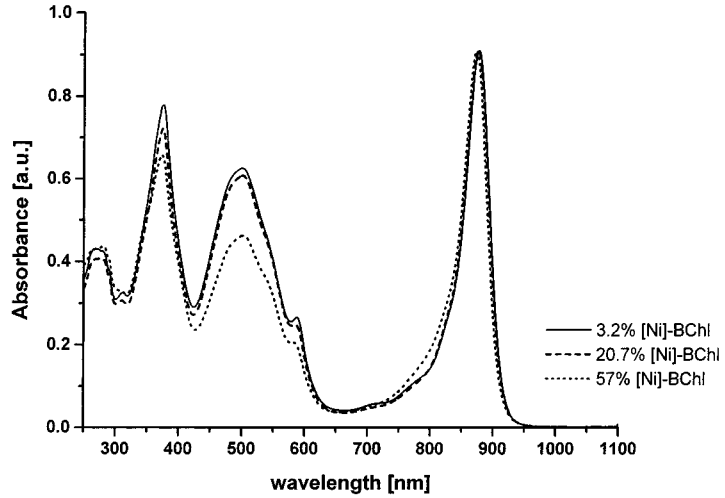

FIGURE 5: Absorption spectra of reconstituted LH1 complexes in Tris-HCl buffer containing 0.8% OG, in which part of the native BChl was substituted by [Ni]-BChl. Modified pigment contents were 3.2% (—), 20.7% (− − −) and 57% (···). The spectra were normalized to the maximum absorption of the Q_y band.

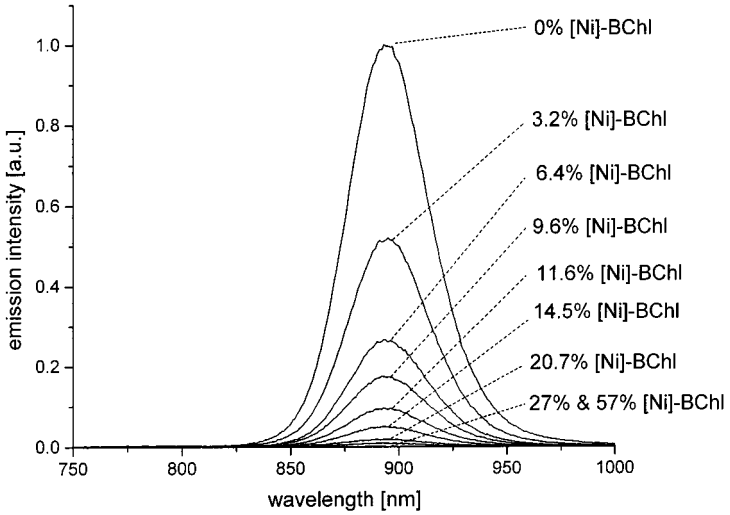

FIGURE 6: Steady-state emission spectra obtained for reconstituted LH1 complexes in Tris-HCl buffer containing 0.8% OG, into which varying amounts of [Ni]-BChl were incorporated. All samples were of the same optical density (OD = 0.07 cm^{-1}) at the excitation wavelength (λ_{ex} = 500 nm).

indicating a complete reconstitution of energy transfer from the carotenoid to BChl. Spectral similarities between the preparations also extend to the circular dichroism spectra, which show features that are typical of LH1 complexes (*56*), i.e., an intense S-shaped signal in the carotenoid range (400−600 nm) and a relatively weak, W-shaped signal in the Q_y region (850−900 nm) (inset of Figure 2). It should be noted that they are also very similar to the CD spectrum of LH1-only membranes in the same buffer but lacking the detergent (data not shown).

Pigment Exchange in LH1 and HPLC Pigment Analysis. Addition of [Ni]-BChl to the LH1 organic extract, followed by solvent removal and detergent solubilization under the same conditions as above, results in partial replacement of native BChl in the reconstituted LH1 complexes by the externally added pigment. The pigment exchange in LH1 was confirmed by pigment analysis by reverse-phase HPLC and in-stream diode array detection (Figure 3, results summarized in Table 1).

The HPLC analyses of the samples showed, besides the original carotenoids and BChl, only one additional pigment,

Table 1: Pigment Composition of LH1 Complexes Reconstituted with Varying Amounts of [Ni]-BChl in the Initial Chloroform/Methanol Reconstitution Mixture, Determined by HPLC Analysis

[Ni]-BChl content in reconstitution mixture [% of total tetrapyrroles]	[Ni]-BChl content in reconstituted LH1 complexes [% of total tetrapyrroles]
0	0
1	3.2
2	6.4
3	9.6
4	11.6
5	14.5
7	20.7
10	27.0
30	57.0

which by its retention time (t_r ~14 min, Figure 3, panels C and D) and absorption spectrum (Figure 4, dashed line) is identified as [Ni]-BChl. The amount of [Ni]-BChl in LH1 increases with the amount added externally, but it is consistently higher than it was initially present in the reconstitution mixture. At low [Ni]-BChl concentrations, the enrichment factor (viz. the ratio of the [Ni]-BChl percentage

in the added pigment mixture, versus the percentage of [Ni]-BChl incorporated into the complex) is as high as 3.2. Even at the highest concentrations used for reconstitution (30% [Ni]-BChl), it amounts to 1.9. Such a preferential binding of [Ni]-BChl versus native BChl had already been observed in LH1 of another bacterium, *Rs. rubrum*, for one of the two BChl-binding sites (*57*, *58*).

The incorporation of [Ni]-BChl into LH1 influences only slightly the ground-state absorption (Figure 5) and CD properties (not shown) of the reconstituted LH1 complex. At low [Ni]-BChl contents (3−20%), the most significant effect is a 2−3 nanometer blue-shift of the Q_y transition from 876 to 873 nm. At the highest [Ni]-BChl content, viz. 57%, there is a relative absorption decrease of the BChl-Soret and the carotenoid bands by 16 and 26%, respectively, as compared to the Q_y band, while the position of the Q_y maximum remains nearly constant at 872 nm.

Steady-State Fluorescence. In contrast to the minor changes in optical absorption, the incorporation of [Ni]-BChl into LH1 has dramatic effects on the emission properties of the modified complexes. Figure 6 shows the emission spectra obtained for a series of LH1 preparations containing increasing amounts of incorporated [Ni]-BChl, excited at 500 nm. All spectra were taken under the same conditions, and the samples were of equal optical density (0.07) at the excitation wavelength. In fact, no further corrections were required, at low-to-moderate [Ni]-BChl concentrations, since the carotenoid/BChl absorbance ratios remain nearly constant in samples with contents up to 21% of [Ni]-BChl (see Figure 5). Qualitatively, the spectra are identical, all showing a single emission band at 897 nm. However, the emission intensities strongly decrease in the presence of even small amounts of [Ni]-BChl. Already the incorporation of 3.2% [Ni]-BChl into reconstituted LH1 complex causes a nearly 50% decrease in the emission intensity, and at [Ni]-BChl contents ≥20% the emission is practically quenched to zero. The same quenching efficiencies of the incorporated [Ni]-BChl on the LH1 emission have been observed upon excitation in the Soret region (data not shown).

Time-Resolved Emission Measurements. The kinetic results in the subnanosecond domain parallel the static ones. The incorporation of [Ni]-BChl into LH1 only effects on the intensites (amplitudes) of the fluorescence decays, but not their kinetics. The nonmodified (native pigment composition) LH1 complex shows a monoexponential emission decay with a characteristic lifetime of 850 ± 100 ps (Figure 7), which agrees well with literature data (*59*). These kinetics remain unaffected in the presence of increasing amounts of [Ni]-BChl: all decays can be fitted well monoexponentially with this time constant (Figure 7, note that these curves have been normalized for better comparison, see the figure legend).

However, the amplitudes of the emission signals decrease strongly upon incorporation of [Ni]-BChl (Table 2), and this decrease in amplitude is quantitatively in excellent agreement with the reduction of fluorescence yield. Both results support a two-component model in which two subpopulations are present among the reconstituted complexes. One subpopulation shows the fluorescence properties of the native LH1 and the other one, due to the presence of [Ni]-BChl, shows no fluorescence at all on a time scale >10 ps. This model will be evaluated in detail in the discussion section.

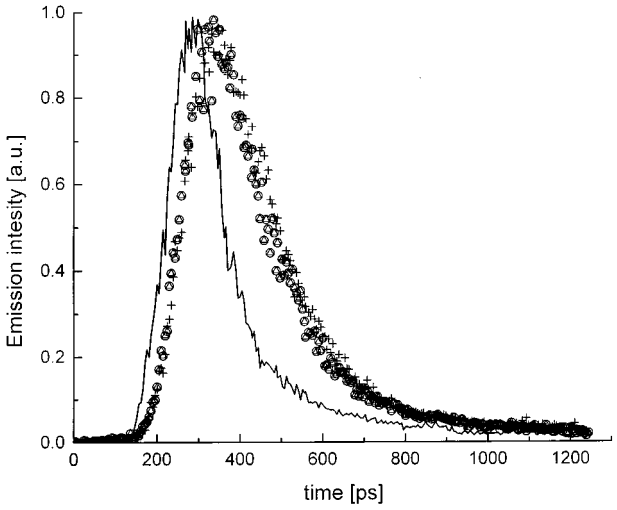

FIGURE 7: Emission decay profiles recorded for the LH1-only membranes (open triangles) and LH1 complexes reconstituted with native pigments (= 0% [Ni]-BChl, crosses) and with 11.6% [Ni]-BChl (open circles); upon excitation at 850 nm with dye laser pulses (solid line). For better comparison, all traces have been normalized to the maximum. Note, however, that the absolute intensities decrease with increasing contents of [Ni]-BChl (see Table 2 and Results).

Table 2: Fluorescence Emission Lifetimes[a] and Relative Emission Intensities ("amplitudes") of the Native and [Ni]-BChl-Substituted LH1 Complexes[a]

type of complex	emission lifetime[b] [ps]	relative amplitude $I_{\text{modified LH1}}/I_{\text{nonmodified LH1}}$
LH1-only membrane	850 ± 100	0.57
reconstituted LH1-complexes containing:		
0% [Ni]-BChl	850 ± 100	1
3.2% [Ni]-BChl	850 ± 100	0.49
6.4% [Ni]-BChl	850 ± 100	0.31
11.7% [Ni]-BChl	850 ± 100	0.14

[a] The normalized decay traces are shown in Figure 7. [b] In all cases, using neutral glass filters, the measured signals were adjusted to be on a similar level; therefore, the error of the measurement remains constant (although the emission intensity decreases with increasing content of the trap).

DISCUSSION

LH1 Reconstitution and Pigment Exchange. The modularity of the LH1 structure, with a repeating protomer consisting of one α- and one β-subunit each binding one BChl and (most likely) one carotenoid, has facilitated the complete reconstitution of this type of antenna from several strains of purple bacteria. Most of these studies have focused on carotenoid-less mutants of *Rs. rubrum* and *Rb. sphaeroides*, where the assembly of B875 in vitro proceeds via two intermediates, one absorbing at 770 nm (similar to free BChl) and the other [$\alpha\beta$BChl$_2$ or its dimer, (*60*)] at 820 nm. These studies included the use of hybrid and mutant apoproteins (*60*−*63*) as well as of modified pigments (*58*, *60*, *64*, *65*).

The B820 intermediate is formed without incorporation of carotenoids (*60*). Our results show that this is true even if carotenoids are present in the reconstitution mixture: a small amount of B820 fraction has often been separated from the integral B875 by ion-exchange chromatography, which unlike the B875 was devoid of carotenoids; this supports

the results obtained by Westerhuis et al. (*24*) on LH1 antenna from M21 mutant strain of *Rb. sphaeroides*. Moskalenko et al. have also reported a similar behavior of LH1 preparations from several other strains of purple bacteria in which carotenoid synthesis was partially inhibited (*66*). Previous reconstitutions have therefore focused on carotenoid-less complexes, including the kinetics and thermodynamics of the assembly process and the structures of the intermediates (*57*, *67*, *68*) and of the partially dissociated antenna (*24*). The spectroscopic studies were commonly carried out with reconstitution mixtures without further purification (*60*) as the yield of the resulting preparations is limited by the necessity of polypeptide and pigment purification and by their low stability.

Starting with LH1-only membranes devoid of RC and LH2 complexes, we have shown that a carotenoid-containing LH1 complex can be reconstituted in good yield from a crude methanol/chloroform extract. Membrane extraction with organic solvents has been used previously in preparation of the LH1 polypeptides from wild-type organisms (*52*, *60*, *69*), and it was shown by 2-D NMR spectroscopy that these short polypeptides retain nearly native conformations in the organic solvent (*70*). The extraction of the membranes even shows some selectivity toward the components of the LH1 antenna, and it can be regarded as a preliminary purification step. The effect is further amplified by the spontaneous formation of LH1 complexes in the crude reconstitution mixture, obviously driven by high affinities between the pigments, the α/β polypeptides, and perhaps lipids as well. The latter are certainly present in the extract and therefore also in the reconstitution mixture, but no lipids have been analyzed at this stage.

Pigment analyses of the reconstituted LH1 complexes gave no indication of the presence of BChl degradation products such as BPhe, 13^2-OH-BChl, or 13^2-OH-[3-acetyl]-Chl, which all form readily upon solubilization of the LH1 extracts in detergent where the interactions are disrupted. This points to stabilizing interactions not only in the detergent-solubilized LH1 but also in the organic extract. It is well-known that chlorophylls can strongly interact with cosolutes in organic solvents (*71*), and that these interactions affect their reactivities (*72*). However, these interactions are not sufficient to prevent degradation of the pigment.[2] The selective and mutually stabilizing interactions with the polypeptides in the extract thus require more specific interactions, which points to the formation of some sort of complex between the pigment and the other components even in the organic solvent, but this has not been studied in the current context.

Reconstitution from the dried methanol/chloroform extract of LH1-only membranes, followed by purification via ion-exchange chromatography, therefore, provides a convenient and rapid method for preparing pure, reconstituted LH1 complexes, both with (this paper) and without carotenoids (unpublished), in quantities suitable for spectroscopic (and structural) investigations. At the same time, the method provides a means for introducing modified pigments into the complex, enabling us in particular to partly replace the native BChl with its Ni-substituted analogue, [Ni]-BChl. Our preliminary results with *Rs. rubrum* show that the same technique is applicable for the reconstitution of LH1 complexes from other species of purple bacteria.

Pigment binding into LH1 has also been shown to be selective in a series of LH1 reconstitution experiments using BChl analogues (*58*, *64*, *65*). It had previously been shown that in the carotenoid-less LH1 complex from *Rs. rubrum*, the native BChl could be replaced quantitatively by Zn- and [Ni]-BChl (*57*, *58*), with even an increase in stability of the B820 subcomplex. The preferential binding[3] of [Ni]-BChl is retained in the carotenoid containing complex, at least up to the exchange of nearly 60% BChl, which was maximum studied here. Seemingly, the presence or absence of carotenoid has little effect on the formation of the pigment-modified holo-complex B875, while carotenoids are reproducibly absent from the B820 intermediate, even if they are present in the incubation mixture (not shown).

Comparison of Isolated and Reconstituted LH1. Spectroscopically, the reconstituted complexes containing the native BChl were almost indistinguishable from the freshly isolated ones, and even the incorporation of [Ni]-BChl produced only minor spectral changes. These criteria include absorption, circular dichroism, and (in the absence of [Ni]-BChl) fluorescence spectroscopy. However, above a certain size, the spectra of complexes formed by repeating units become quite insensitive to their size, and the latter therefore needs to be determined by other means (*40*, *75*). The uniform size distribution (see below) of the isolated, reconstituted, and pigment-modified complexes was ascertained by gel-filtration and sucrose-density centrifugation. Gel-filtration gave narrow bands of the same mobility for all three types of complexes (results not shown). In sucrose-density centrifugation, we focused on the reconstituted complexes with little to no [Ni]-BChl, which are most relevant for the analyses. Both LH1 which contains no modified pigment, and the reconstituted complex containing 3.2% [Ni]-BChl, yielded single, narrow bands located at a position corresponding to 15−20% sucrose. These results point to the conclusion that (i) the pigment arrangement within the subunits, the pigment interactions among the subunit, and the size (distribution) of the reconstituted complexes are the same as in the native ones and that (ii) this is true as well for the complexes modified by incorporation of [Ni]-BChl.

LH1 Size Estimation from Fluorescence Quenching. As anticipated (*46*−*48*), [Ni]-BChl incorporated into LH1 acts as an extremely efficient deactivation channel. The fluorescence of LH1 is quenched very rapidly with an increasing fraction of [Ni]-BChl, and the decrease of both steady-state fluorescence and of the amplitude of the 850 ps fluorescence, as a function of the amount of [Ni]-BChl being incorporated, shows the same behavior (Figure 8). Such a close correlation between the [Ni]-BChl content and the LH1 emission amplitudes can be rationalized well by a two-component model, comprising only (i) a subpopulation of complexes with high fluorescence quantum yield and a decay-time of

[2] Oxidation at, for example, C-13^2 is even possible in the interior of the bacterial RC (*73*).

[3] The Q_x band of the reconstituted complex does not shift upon [Ni]-BChl incorporation (Figure 5), which is indicative of a five-coordinated central Ni^{2+} ion, viz. one extra ligand (*48*). This unusual coordination number has, in fact, been confirmed by EXAFs spectroscopy in another photosynthetic complex, i.e., the $B_{A,B}$ sites of the RC (*79*). This is probably the reason for the preferential binding of [Ni]-BChl versus native Bchl in LH1, as it is known that distortions stabilize the low-spin d^8 complexes [Jahn−Teller theorem (*74*)].

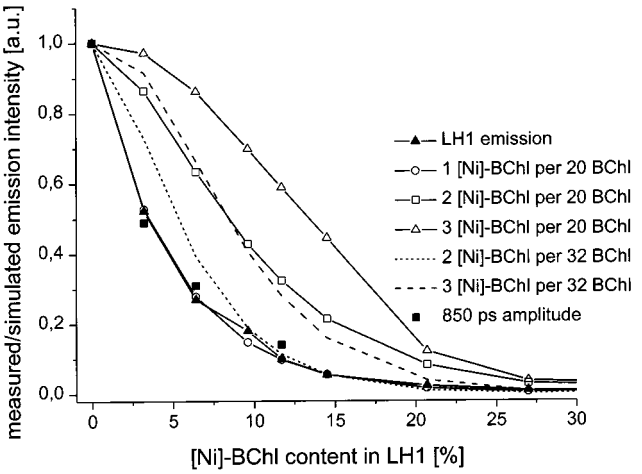

FIGURE 8: Experimental and calculated emission intensities of LH1 in which part of the native BChl has been replaced by [Ni]-BChl, as a function of the fraction of the modified pigment. Calculations are based on Poisson distribution assuming different unit sizes (N) and minimum numbers of modified pigments required for quenching (m), see text (Discussion) for details. Solid triangles: experimentally determined emission intensities taken from the data shown in Figure 6. Open symbols/solid lines: calculated emission intensities based on a unit size of the complex of $N = 20$, and dashed lines: $N = 32$ BChl molecules and assuming that a minimum of $m = 1$, 2, or 3 [Ni]-BChl molecules per unit are required for complete emission quenching. A two-component model has been assumed, see text for details. Also shown (solid squares) are the relative amplitudes of the 850 ps component of the LH1 emission decay (see Table 2).

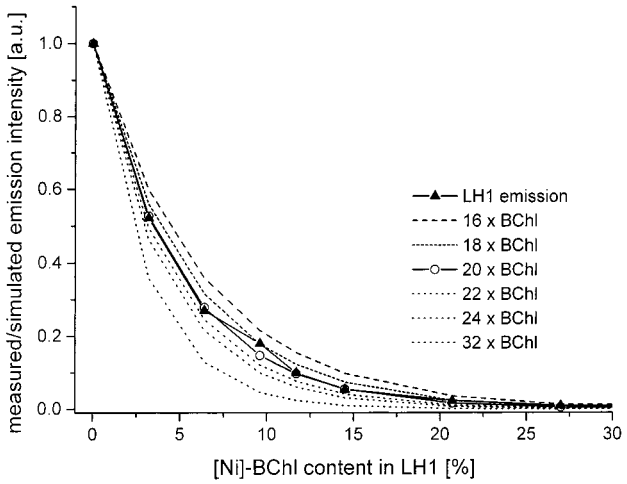

FIGURE 9: Experimental and calculated (Poisson distribution) emission intensities of LH1 in which part of the native BChl has been replaced by [Ni]-BChl, as a function of the fraction of the modified pigment. Solid triangles: experimentally determined emission intensities taken from the data shown in Figure 6. The simulated curves were computed for unit sizes of $N = 16-32$ tetrapyrroles per LH1 (see inset legend), and assuming that $m = 1$, viz. one [Ni]-BChl molecule per LH1 unit causes complete quenching of the emission and only the population of nonsubstituted LH1 antennae contributes to the emission (see text for details). The best fit (empty circles) was obtained for 20 BChl molecules comprising such a unit.

about 850 ps, and (ii) a second subpopulation of complexes, which are practically nonfluorescent on the time-scale of several tens of picoseconds. In the following quantitative analysis, we assume that the fluorescence quenching is due to the incorporation of a certain minimum number (m) of [Ni]-BChl molecules that have to be present within the interacting unit composed of N tetrapyrroles (BChl and [Ni]-BChl). The values of both m and N could be evaluated by a quantitative analysis based on Poisson statistics, assuming a random incorporation of the modified pigment.

The value of m describes the degree of cooperativity for the quenching, as shown by the set of curves with m varying from 1 to 3 (Figure 8). Irrespective of the value of N, their shape is determined by m: it is sigmoidal for all $m > 1$. From the fit to the experimental values of both steady state and time-resolved emissions, it is obvious that $m = 1$, i.e., a single molecule of [Ni]-BChl is sufficient to completely quench the fluorescence of the entire LH1 unit. In a somewhat similar approach, Law and Cogdell (*76*) have studied the quenching of fluorescence in LH1 from *Rhodobium marinum* by another trap, viz. the chemically induced BChl cation radical. It was concluded, too, that oxidation of a single BChl within an LH1 complex was sufficient to quench completely the fluorescence of the ensemble. Next, the number of pigments within the LH1 unit was obtained by comparison of the experimental quenching curve with a series of theoretical ones obtained by varying N and maintaining $m = 1$ (Figure 9). These data give a nearly perfect fit for $N = 20$, and already the next best fits ($N = 18$ or $N = 22$) deviate significantly. Although similarly good fits can be obtained under the assumption of a bimodal size-distribution, this possibility can be discarded on the basis of the gel-filtration and sucrose-density gradient data discussed above. We therefore conclude that the unit-size of reconstituted LH1 is 20, corresponding to 10 protomeric subunits, $(\alpha\beta BChl_2 \cdot Car)_{10}$.

The analysis shows that the model is sensitive enough to the parameters of the simulation (and to the assumptions) to determine a unique set of m and N for reconstituted LH1. The resulting unit size of $N = 20$ differs from the range of sizes (24−32) obtained by pigment analyses of core complexes and by electron microscopy of a variety of LH1 (*14*, *20*, *23*, *25*−*30*). However, an even smaller number, i.e., $N = 16$ BChl per LH1 unit, has been obtained by numerical modeling of the annihilation process in the isolated LH1 antenna, observed in ultrafast fluorescence depolarization studies by Bradforth et al. (*36*). Great care has been taken to avoid pigment degradation, in particular of BChl, during the experiments with the reconstituted LH1. In particular, all spectroscopic and HPLC analyses have been performed on fresh samples (the samples, showing the emission of free BChl near 780 nm upon excitation at 400 nm, were not considered any further). Therefore, even if one assumes losses as high as 10% BChl during analytics (which is much beyond the losses observed with BChl samples under the conditions used), the upper size limit would just be brought close to the lowest number of 24 BChls per unit obtained from previous biochemical studies.

Considering the same physical size of the [Ni]-BChl containing complex and the one reconstituted with BChl only, we have neither an indication that the presence of the modified pigment is responsible for the unusually low aggregation number, nor that it results in a more heterogeneous size distribution. However, the reconstituted LH1 complex may be different in size from the freshly isolated one, due, for example, to the loss of certain lipids or the PufX protein, while the spectral features are not affected by this difference. Moreover, in view of the relatively labile

LH1 from *Rb. sphaeroides* (as compared, for example, to that of *Rs. rubrum*), the aggregate size may already change during its isolation from the membrane. More work is needed to clarify these questions, and more native conditions of [Ni]-BChl incorporation may provide an approach to this.

The experiments reported here were primarily aimed at verifying the size (viz. the number of BChl molecules) of the complexes bearing modified BChl, irrespective of the mechanism of energy transfer as long as it is fast on the time-scale of the time-resolved fluorescence experiment (several tens of picoseconds). Hence, they bear no information about the ultrafast excitation relaxation and possible intraunit energy transfer, because, for example, even Förster transfer with discrete hopping among BChl monomers or dimers is expected to take place on the subpicosecond time scale. In another complementary study, we have addressed this question by using ultrafast pump−probe absorption spectroscopy (∼50 fs time resolution) (*49*). The major conclusions from the latter work were that trapping occurs on a time-scale comparable to or even faster than the resolution of the apparatus (ground state recovery in 60 fs) and that it occurs already in the nonrelaxed, nonfluorescent state of the antenna. The femtosecond spectroscopy findings suggest that even on this ultrafast time scale the excitation is delocalized over >10 BChl molecules, presumably even over the entire LH1 unit of 20 BChls of the reconstituted antenna (*49*). This result would also point to the validity of the "extreme delocalization model" in the description of the B875 electronic structure.

The possibility to expand into this time-scale renders the replacement method of BChl by its Ni-analogue a unique tool to study unit size, excited-state properties of the ensemble, and energy transfer kinetics on all available time scales, information that is otherwise very difficult to obtain. Since [Ni]-BChl can replace BChl in all cases studied hitherto (*57*, *65*, *77*, *78*), and there is evidence that [Ni]-Chl can replace Chl, too, in some systems (unpublished), the technique can principally be extended to other systems as well, including supercomplexes comprising more than one type of light-harvesting complex and other photochemical systems involving chlorophylls such as the protochlorophyllide-NADPH-oxidoreductase (POR).

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BI002257F