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Investigations of intermediates appearing in the reassociation of the light-harvesting 1 complex of *Rhodospirillum rubrum*

Anjali Pandit^{1,*}, Ivo H. M. van Stokkum², Sofia Georgakopoulou², Gert van der Zwan² & Rienk van Grondelle²

¹Department of Structural Biology, Faculty of Earth and Life Sciences and Division of Physics and Astronomy, ²Faculty of Sciences, Vrije Universiteit Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands; *Author for correspondence (e-mail: pandit@bio.vu.nl; fax +31-20-4447999)

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Abstract

We investigated the temperature-mediated reassociation of the B820 subunit of *Rs. rubrum* to form a lightharvesting 1 complex (LH 1). By combining several spectroscopic techniques with global spectral data analysis fitting, we present evidence for the occurence of two spectral intermediates that appear during the reassociation process. At high temperatures, halfway the reassociation reaction, a prominent intermediate appears that has an absorption maximum around 850 nm, a fluorescence maximum around 860–867 nm, a high anisotropy (0.3 to 0.4) and a circular dichroism spectrum with three or four bands with alternating signs. At lower temperatures, more towards the end of the reassociation process, a second intermediate tends to appear that has an absorption maximum around 860 nm, a fluorescence maximum around 885 nm, a medium to high anisotropy (0.1 to 0.3) and a circular dichroism spectrum with two bands with alternating signs. The latter circular dichroism spectrum has a blueshifted zero-crossing compared to the spectrum of the LH 1 complex. Both intermediates have the spectroscopic features of a small oligomer. In the Qy region, the fluorescence anisotropy of both intermediates slightly increases at longer excitation wavelengths, indicative for energy transfer among the pigments within the intermediate oligomers.

Abbreviations: BChl-bacteriochlorophyll; CD-circular dichroism; CMC-critical micelle concentration; LDAO-lauryldimethylamine *N*-oxide; LH 1-the core light-harvesting complex; LH 2-the peripheral light-harvesting complex; OG-*n*-octyl- β -D-glucopyranoside; RC-photosynthetic reaction center

Introduction

In purple non-sulfur bacteria the capture of light and charge separation for conversion into chemical energy is performed by two highly organized transmembrane pigment-protein complexes. These are the peripheral antenna (LH 2) and the core antenna (LH 1) that surrounds the reaction center (RC) where charge separation takes place (van Grondelle et al. 1994). For LH 2 the structure has been resolved to high resolution for the strains *Rhodopseudomonas acidophila* (McDermott et al. 1995) and *Rhodospirillum molis*- *chianum* (Koepke et al. 1996). This structure appears to consist of a ring of nine or eight pigment-protein subunits respectively. Its subunits are heterodimers of the so called α and β polypeptides and form ligands to three bacteriochiorophyll a pigments: two BChls absorbing at 850 nm and oriented perpendicular to the membrane plane, and a third BChl absorbing around 800 nm and oriented parallel to the membrane plane. For LH 1 only a low resolution structure exists that has been resolved for the strain *Rs. rubrum* (Karrasch et al. 1995). Knowledge of the low-resolution strucure of LH I combined with the high-resolution of LH 2 has stimulated detailed modelling (Hu and Schulten 1998). According to these models, the LH 1 consists of a ring of 16 pigment-protein subunits, which encircles the RC. Its subunits, called B820, are also heterodimers of α and β polypeptides, but each binds one BChl *a* pigment. It is still under discussion whether the LH 1 complex completely encircles the RC *in vivo*. Hypotheses are that *in vivo* the LH 1–RC complex is shaped like a 'horseshoe', or that it consists of stacked LH 1–RC dimers (Frese et al. 2000; Jungas et al. 1999; Francia et al. 1999).

The LH 1 complexes of the purple bacteria strains Rs. rubrum, Rps. marina, Rps. viridis and Rb. sphaeroides can be dissociated into their subunits by treatment with detergent and extraction of the carotenoids (Miller et al. 1987; Visschers et al. 1992; Meckenstock et al. 1992). For Rs. rubrum a mutant strain, called G9, exists that produces LH 1 antenna complexes lacking carotenoids: isolated LH complexes of this strain can be reversibly dissociated into the B820 subunits, absorbing at 820 nm, and further into monomeric pigment-ligated α or β polypeptides, called B777 and absorbing at 777 nm (Ghosh et al. 1988; Miller et al. 1987; Chang et al. 1990; Loach and Parkes-Loach 1995; Pandit et al. 2001). The LH 1 complexes from this strain form a suitable model system for the study of the B820 antenna building blocks, for reconstitution experiments and for study of the assembly of photosynthetic antenna complexes or, more general, assembly properties of transmembrane protein complexes. Its bacteriochlorophyll pigments are intrinsic spectroscopic probes that sense the state of assembly, which in its turn can be modulated by varying such parameters as detergent concentration, temperature or protein concentration.

The B820 antenna subunit has been studied intensively. It appears to contain one dimer of BChl a molecules that are excitonically coupled (van Mourik et al. 1991). The Q_{y} absorbance band of B820 is inhomogeneously broadened and no energy transfer occurs among the pigments within the B820 dimers, resulting in a high anisotropy over the whole Q_v region (Visschers et al. 1991, 1993). Compared to LH 1, the B820 subunit has a small Stark signal and a large non-conservative CD signal (Beekman et al. 1997; Koolhaas et al. 1997). The B820 subunit can reversibly be dissociated into monomeric B777 and for this reaction the thermodynamics have been determined (Sturgis and Robert 1994; Pandit et al. 2001). Several studies have been conducted to find the requirements for in vitro reconstitution of the B820

subunit from its separated components. It appears that, whereas *in vivo* the B820 subunit consists of an $\alpha\beta$ heterodimer, the subunit can also be formed from only β -polypeptides but not from α -peptides only (Meadows et al. 1995; Parkes-Loach 1988). Stability of the subunit originates from ion-pairing and/or hydrogen bonding interactions between the polypeptides in the core region (Bustamante and Loach 1994; Meadows et al. 1995) and it is proposed that the BChl pigment bound to the α -peptide is hydrogen-bonded to the His residue of the β -polypeptide and vice versa (Olsen et al. 1997). Furthermore, the central Mg atom in the bacteriochlorophyll rings is a structural requirement for BChl binding to form the subunit complex and LH 1 of Rs. rubrum and Rb. sphaeroides (Davis et al. 1996). Successful reconstitution of B820 from separated BChl as and α - and β -polypeptides has been demonstrated by absorption and CD spectroscopy of reconstituted subunits, which show spectra similar to spectra of isolated B820 subunits (Meadows et al. 1995; Davis et al. 1997; Parkes-Loach et al. 1988). Recently it has been demonstrated that the B820 subunit in octylglucoside can exist not only as a dimeric, but also a tetrameric form dependent on the detergent concentration (Arluison et al. 2002).

Other studies have been conducted to find the requirements for reconstitution of the full LH 1 complex. For reconstitution of the LH 1 complex from separated components the α -peptide is required, in contrast to requirements for reconstitution of the B820 subunit (Parkes-Loach et al. 1988). For each subunit, the C31 carbonyl groups of the pigments are hydrogen-bonded with the Trp 11 residue of the α -peptides of the preceding subunit (Davis et al. 1997; Parkes-Loach et al. 1990; Sturgis et al. 1997). Spectroscopy of reconstituted LH 1 complexes shows native-like complexes with absorption maxima equal to or slightly blueshifted compared to native complexes and CD spectra that either resemble native CD spectra or are inverted (Bustamante and Loach 1994; Chang et al. 1988; Miller et al. 1987; Ghosh et al. 1988; Parkes-Loach et al. 1988). The LH 1 complex has been reconstituted together with isolated RCs, demonstrating that photosynthetic units can be reassembled in which excitation energy is successfully transferred to the RC (Bustamante and Loach 1994). In the latter study, reconstitution of the RC-LH 1 complex was carried out in the presence of liposomes, proving that the whole photosynthetic unit can be reconstituted from separated components in a native-like environment.

In several reconstitution experiments, it has been demonstrated that the dissociation reaction of the LH 1 complex into subunits and further into B777 monomers is reversible (Chang et al. 1990; Miller et al. 1987; Loach et al. 1985; Ghosh et al. 1988). Upon treatment with OG, direct dissociation of B873 into B820 subunits has been observed (Loach et al. 1985; Miller et al. 1987). Lowering the detergent concentration again (from 0.8% OG to 0.36% OG) showed a B873-like complex absorbing at 865 nm, but with an inverted CD signal compared to the native complex in chromatophores (Miller et al. 1987). Similarly, isolated B873 complexes also showed reversible dissociation into B777 monomers upon treatment with LDAO. Upon addition of Na-Cholate to the LDAOtreated protein a B873-like complex was reformed that absorbed at 860 nm and that had an inverted CD signal compared to native LH 1 (Ghosh et al. 1988). In the latter reassociation experiment the 860 nm-absorbing complex was formed within hours via discrete transitory states. In other studies similar intermediates have also been observed upon reassociation of the LH 1 from B820 subunits (Loach and Parkes-Loach 1995; Visschers and van Grondelle 1992; Beekman et al. 1997; van Mourik et al. 1992; Pandit et al. 2001). Van Mourik et al. (1992) investigated for the first time the fast kinetics of the B820-B873 reassociation reaction, by using a diode-array coupled stopped-flow apparatus. They could fit the B820-B873 reassociation kinetics assuming a two-step reaction, but structured residuals and a blueshifted spectrum for reassociated B873 compared to native LH 1 suggested that more intermediates might be present in the reaction.

In an earlier study we investigated the reversible dissociation of the LH 1 of Rs. rubrum G9 into B820 subunits as a function of temperature and detergent concentration (Pandit et al. 2001). We found that, whereas the dissociation of LH 1 into B820 subunits by temperature always seemed to be a one-step process, the reassociation of B820 into LH 1 would always occur via one or more intermediate states (Pandit et al. 2001). The most characteristic intermediate form occurred as an absorption band around 850 nm, halfway through the reassociation process. On the basis of simulated absorption spectra of 'arcs' of the LH 1 ring of varying size, it was proposed that this intermediate state most likely corresponds to a tetrameric aggregate consisting of two B820 subunits. Furthermore we observed intermediates with absorption bands a few nanometers blueshifted from B873 that might represent large incomplete rings.

This study is a further characterization of the intermediates occuring during the B820–B873 reassociation reaction. By combining several spectroscopic techniques such as absorbance, polarized fluorescence and circular dichroism (CD) more information is obtained about the oligomeric states of these complexes and the possible organization of their pigments.

Materials and methods

n-Octyl- β -D-glucopyranoside (OG) was obtained from Calbiochem (San Diego, California). The purified B820(OG) complexes were prepared from the carotenoidless strain Rs. rubrum G9 as described previously (Pandit et al. 2001). Samples were prepared by diluting stock solutions in buffer containing 50 mM potassium phosphate, pH 7.5, and 0.8% OG. Buffers were flushed with nitrogen and cuvettes tightly sealed to prevent oxidation of samples. Absorption spectra were measured on a Perkin Elmer Lambda 40 UV/VIS Spectrophotometer using a resolution of 4 nm. For fluorescence emission spectra, either a tunable Ti:Sa laser (excitation power 200 μ W) pumped by an 8W Arglaser or a halogen tungsten light bulb with a 595 nm interference filter was used as an excitation source. Emission light was detected by a spectrograph (Chromex). For anisotropy measurements, a Glan-Thompson polarizer was used in the excitation path and an IR sheet polarizer in the detection path. All fluorescence spectra have been corrected for the detector sensitivity. For CD measurements, a home-built CD spectrophotometer was used containing an optoacoustic modulator (Hinds) and two lock-in amplifiers (EG&G and Ithaco). All samples were measured in 1×1 cm quartz cuvettes in a temperature-controlled cuvette holder.

Global analysis fitting of the CD, absorption and polarized fluorescence spectra was performed using a three-component and four-component spectral model (van Stokkum et al. 1995) with constraints on some of the amplitudes. CD and absorption spectra as a function of temperature were fitted simultaneously using the measured CD and absorption spectrum of B820, B873 and one or two intermediate fitspectra, each consisting of two skewed gaussians with variable skewness, widths and peak locations. Polarized fluorescence spectra as a function of temperature were fitted using a three- or four-component spectral model,



Figure 1. Magic-angle fluorescence spectra upon excitation of the Q_x band (595 nm) of a sample thermostated at stepwise lowered temperatures.

according to:

$$\begin{pmatrix} I_{VV}(T) \\ I_{HV}(T) \end{pmatrix} = \begin{pmatrix} \Sigma_{i=1}^{N} (1+2r_{i})I_{MA,i}(T) \\ \Sigma_{i=1}^{N} (1-r_{i})I_{MA,i}(T) \end{pmatrix}$$
(1)

In this equation $I_{\rm VV}$ and $I_{\rm HV}$ are the parallelly and perpendicularly polarized fluorescence intensities, Tis the temperature, N is the number of spectral components, and $I_{\rm MA,i}$ is the magic-angle fluorescence and r_i the anisotropy of the *i*-th component. Spectral components consisted of the fluorescence spectrum of B820, B873 and one or two intermediates, each formed by a skewed gaussian with variable skewness, width, peak location and anisotropy.

Polarized fluorescence spectra as a function of the excitation wavelength (exciting in the Q_y region) were analyzed using the global-analysis results from fitting of the temperature-dependent polarized fluorescence spectra. The latter spectra were obtained upon exciting at 830 nm. Fluorescence emission spectra as a function of excitation wavelength were assumed to consist of a linear combination of the fluorescence components revealed from the temperature-analysis (excited at 830 nm), correcting for the change in extinction for each component upon scanning through the absorption band:

$$\begin{pmatrix} I_{VV}(\lambda_{exc}) \\ I_{HV}(\lambda_{exc}) \end{pmatrix} = \\ \begin{pmatrix} \sum_{i=1}^{N} \frac{\epsilon_{i}(\lambda_{exc})}{\epsilon_{i}(830)} [1 + r_{i}(\lambda_{exc})] I_{MA,i}(830) \\ \sum_{i=1}^{N} \frac{\epsilon_{i}(\lambda_{exc})}{\epsilon_{i}(830)} [1 - r_{i}(\lambda_{exc})] I_{MA,i}(830) \end{pmatrix}$$
(2)



Figure 2. Magic-angle fluorescence spectra upon blue-edge excitation of the Q_y band (830 nm) of a sample thermostated at stepwise lowered temperatures. The sharp peak at 830 nm is scattered excitation light. Inset: the fluorescence emission anisotropy at 880 nm vs temperature.

In which λ_{exc} is the excitation wavelength and ϵ_i is the extinction coefficient of the *i*-th component. For the shapes of the intermediate spectra, the fitspectra results from the temperature analysis were taken. The anisotropies of B820 and B873 were considered to be constant over the excitation range ($r_{B820}(\lambda_{exc}) = r_{B820}$ and $r_{B873}(\lambda_{exc}) = r_{B873}$). For a three-component model, the amplitude and anisotropy of the intermediate component at each excitation wavelength were obtained by straightforward calculation (subtracting the fractional contributions of B820 and B873 from each measured spectrum). For a four-component model, the amplitudes and anisotropies of the two intermediate components were resolved using MatLab (MathWorks Inc., Natic, Massachusetts).

Results

Temperature-dependent fluorescence polarization

Polarized or magic-angle fluorescence emission spectra were obtained from samples prepared in 0.8% OG and thermostated at temperatures from 301 K down to 281 K. Figure 1 shows the magic-angle emission spectra upon excitation in the Q_x region (595 nm) upon lowering the temperature from 293 to 281 K. The fluorescence spectra show the same trend as described earlier for temperature-dependent absorption measurements (Pandit et al. 2001): upon lowering the temperature, the fluorescence peak shifts from the fluorescence maximum of B820 (830 nm) to the maximum of B873 (890 nm) without an isosbestic point,



Figure 3. (A) The absorption spectrum of an LH 1 sample equilibrated half-way the B820–B873 reassociation process, with arrows indicating the excitation wavelength. Dotted spectra are the absorption spectra of B820 (peak 820 nm) and B873 (peak 873 nm). (B) The magic-angle fluorescence spectra, with in the inset the fluorescence emission anisotropy spectrum upon excitation at 848 nm. The spikes are due to scattered excitation light.



Figure 4. (A) The absorption spectrum of an LH 1 sample equilibrated towards the end of the B820–B873 reassociation process, with arrows indicating the excitation wavelength. Dotted spectra are the absorption spectra of B820 (peak 820 nm) and B873 (peak 873 nm). (B) The magic-angle fluorescence spectra, with in the inset the fluorescence emission anisotropy spectrum upon excitation at 848 nm. The spikes are due to scattered excitation light.

indicating the appearance of one or more intermediate forms during the reassociation. Figure 2 shows the magic-angle emission spectra upon excitation in the Q_y region (830 nm) and successively lowering the temperature from 301 K to 281 K. The inset shows the anisotropy detected at 880 nm versus the temperature. Upon lowering the temperature, the anisotropy of the emission decreased from 0.4 to 0.05. The fluorescence emission maximum shows a redshift of the emission maximum from 830 nm towards 895 nm. Taking the spectrum at 296 K, it is clear that the spectral shape deviates from the spectrum of B820. The corresponding anisotropy spectrum has an anisotropy around 0.32, which is much higher than the anisotropy of B873 (around 0.05). Hence, the shoulder around 870 nm in this polarized fluorescence spectrum probably originates from one or more intermediate structures that have a high anisotropy.

Excitation wavelength-dependent fluorescence polarization was measured for two types of samples. The first type (further referred to as sample 1) consisted of a sample prepared in 0.8% OG the temperature of which was lowered until a clear band around 850 nm appeared in the absorption spectrum. The second



Figure 5. (A) Absorption spectra of an LH 1 sample thermostated at stepwise lowered temperatures and (B) the corresponding CD spectra.

type (further referred to as sample 2) consisted of a sample prepared in 0.8% OG the temperature of which was further lowered than for the first type, until the absorption peak had shifted to 870 nm. This way two samples were prepared that were equilibrated in different stages of the reassociation reaction. Both samples were excited at different locations within the Q_v absorption band. Figure 3A shows the absorption spectrum of the sample 1, with arrows indicating the excitation wavelengths. For comparison, the spectra of B820 and B873 are also drawn in this figure (dotted spectra), showing that the sample has the absorption features of an intermediate half-way the reassociation process. Figure 3B shows the corresponding magicangle emission spectra. The inset shows a representative example of an anisotropy spectrum. The spikes in the emission spectra are due to scattered excitation light. Figure 3B shows that upon exciting more to the red, selectively more of the intermediate structure or structures are excited. The anisotropy spectrum is non-flat because of heterogeneity of the sample; at the left side mainly B820 and/or the intermediate(s) are emitting (having an anisotropy around 0.3) and on the right side emission of B873 is contributing (having an anisotropy around 0.05). Figure 4A shows the absorption spectrum of the sample 2, with arrows indicating the excitation wavelengths. For comparison, the spectra of B820 and B873 are also drawn in this figure (dotted spectra), showing that the sample contains protein in an intermediate state or states towards the end of the reassociation process. Figure 4B shows the corresponding magic-angle emission spectra, with in the inset a typical anisotropy spectrum. Again the anisotropy spectrum is non-flat due to heterogeneity of the sample. Upon exciting more to the red, selectively less B820 and more intermediate(s) and/or B873 are excited, demonstrated by redshifted emission spectra.

Temperature-dependent CD

In three independent temperature variation experiments, samples were prepared with 0.8% OG and the CD and absorption spectra recorded while the temperature was stepwise lowered from 303 K to 278 K. Figure 5 shows an example of such an experiment, containing the absorption spectra (5A) and corresponding CD spectra (5B) recorded at different temperatures. The CD spectrum at 291 K, corresponding to a state of the sample where the absorption clearly displays an intermediate band around 850 nm, has a CD signal in which a negative shoulder around 850 nm and a positive shoulder around 870 nm appears. In all cases the sign of the CD spectrum of the reassociated complexes was opposite to that of Rs. rubrum G9 chromatophores and to the CD spectrum of isolated LH 1 of wild-type Rs. rubrum. Leaving samples for 24 or 48 h at 40 °C did not change the spectra, so no slow conformational change was taking place.

Fitting of data assuming one intermediate

In a first attempt both the temperature-dependent polarized fluorescence spectra and the temperaturedependent CD spectra were analyzed using a threecomponent global analysis spectral fitting model. Fitting of the set of temperature-dependent polarized fluorescence data, assuming one intermediate spectrum with variable width and location, yielded an



Figure 6. Intermediate magic-angle fluorescence spectrum, resulting from a three-component spectral global analysis on a series of temperature-dependent polarized fluorescence spectra. The inset shows the residual of the fit. The anisotropy associated with the intermediate is 0.35.



Figure 7. Intermediate CD spectrum, resulting from a three-component spectral global analysis on a series of temperature-dependent absorption and CD spectra. The inset shows the residual of the fit.

intermediate fluorescence spectrum located at 867 nm with an anisotropy of 0.35. Figure 6 shows the intermediate magic-angle fluorescence spectrum with in the inset the residual of the fit. Note that the spectrum is broad (full width at half maximum of 60 nm) and that the residual has some structure. Simultaneous fitting of the CD and absorption spectra yielded an intermediate absorption spectrum with a maximum at 850 nm and an intermediate CD spectrum with four alternating bands. Figure 7 shows a result for an intermediate CD spectrum with in the inset the residual of the fit. The fit residuals had similarly structured shapes for the three independent experiments and they had a negative contribution to the fits at high temperatures and a positive contribution at low temperatures. This



Figure 8. The resolved magic-angle fluorescence spectra of the intermediate structure(s) in the LH 1 sample equilibrated half-way the B820–B873 reassociation, for excitation at different wavelengths in the Q_y region. The insets shows a the fluorescence emission anisotropy spectrum upon excitation at 848 nm.

strongly suggests that in the CD spectra actually two spectral intermediates appear during the reassociation instead of one, which are linear combinations of the intermediate found and the residual.

The two sets of excitation-dependent polarized fluorescence spectra were linked to the temperaturedependent fluorescence spectra as described in 'Materials and methods' (see above). Taking the fractional contributions of B820 and B873 to the fluorescence spectra excited at 830 nm, the fractional contributions of B820 and B873 to fluorescence spectra upon excitation at other wavelengths were calculated, correcting for the change in extinction upon scanning through the excitation band. Subtraction of the contributions of B820 and B873 from each spectrum unraveled the vertically and horizontally polarized fluorescence spectra of the intermediate at each excitation wavelength. Figure 8 shows some of the thus obtained intermediate magic-angle fluorescence spectra of sample 1, with in the inset an example of an anisotropy spectrum. Upon exciting more to the red, the maxima of the intermediate spectra shift to the red, suggesting that in reality more than one intermediate is present. Also the anisotropy spectra are again not flat, suggesting heterogeneity instead of one intermediate species.

Fitting of data assuming two intermediates

The fitresults obtained with the three-component spectral analysis, assuming one intermediate, strongly indicate that more intermediates are indeed present during the reassociation of B873, because of the struc-



Figure 9. The two intermediate magic-angle fluorescence spectra resulting from a four-component spectral global analysis on a series of temperature-dependent fluorescence spectra. Dotted lines: the fluorescence spectrum of B873 and the shoulder of the fluorescence spectrum of B820. Solid line: the most blueshifted intermediate, appearing at high temperatures. Dash-dotted line: the second intermediate appearing at lower temperatures. The inset shows the residual of the fit. The associated anisotropy values of the intermediate spectra are 0.34 (solid spectrum) and 0.27 (dash-dotted spectrum).



Figure 10. The two intermediate CD spectra resulting from a four-component spectral global analysis on a series of temperature-dependent absorption and CD spectra. Dotted lines: the CD signal of B820 and of B873. Solid line: the most blueshifted intermediate, appearing at high temperatures. Dash-dotted line: the second intermediate appearing at lower temperatures. The inset shows the residual of the fit.

tured shapes of the residuals of the CD and polarized fluorescence fits, the broad width of the intermediate fluorescence fitspectrum, the redshift of the intermediate fluorescence maximum upon scanning through the excitation band and the non-flat anisotropy fluorescence emission spectra of the intermediate. Therefore, in a second step, the data was analyzed as-



Figure 11. The fractions of the B820 (crosses), B873 (triangles), the most blueshifted intermediate (closed circles) and the second intermediate (open circles) as a function of the temperature.

suming two intermediates instead of one. Both the temperature-dependent polarized fluorescence spectra and the temperature-dependent CD spectra were analyzed using a four-component global analysis spectral fitting model as described in 'Materials and methods'. Fitting of the temperature-dependent polarized fluorescence spectra yielded two intermediate fluorescence spectra with maxima at 859 nm and at 885 nm and anisotropy values of 0.34 and 0.27, respectively. The results are shown in Figure 9. Note that the solid fitspectrum is still relatively broad, but the residual of the fit is less structured. Fitting with two intermediates instead of one further improved the fit by 15%, reducing the standard deviation (rms) from 45.6 to 38.0. The signal to noise ratio, defined as the the absolute maxima of the polarized fluorescence spectra divided by the rms, was in the order of 50 to 100.

Simultaneous fitting of the temperature-dependent CD and absorption series of three independent experiments showed some variation in the fitting results. Fits yielded two intermediate CD spectra of which the one occuring at high temperatures typically displayed four bands with alternating signs, and the second one, occuring at lower temperatures, had two bands with alternating signs. Figure 10 shows an example of the estimated intermediate CD spectra, with in the inset the residual of the fit. The two corresponding intermediate absorption fitspectra have maxima located around 853 nm (corresponding with the CD spectrum with four bands) and around 859 nm (corresponding with the CD spectrum with two bands). The residual still shows structure. In contrast to the three-component fitting, the residuals of four-component fits of the three



Figure 12. The fractions of B820 (crosses), B873 (triangles), the most blueshifted intermediate (closed circles) and the second intermediate (open circles) as a function of the excitation wavelength, in a sample equilibrated halfway the B820–B873 reassociation process (see Figure 3).

different sets of CD experiments had different shapes (not shown). Fitting with two intermediates instead of one did not significantly improve the fits and the rms in both cases was in the order of $1 \cdot 10^{-5}$, giving a signal to noise ratio of 10 to 20. Figure 11 shows the calculated fractional contributions of B820, B873 and of the two intermediates as a function of temperature.

The two sets of excitation-dependent polarized fluorescence spectra were again linked to the temperature-dependent fluorescence spectra, now using the results of the four-component global-analysis fitting of the temperature-dependent polarized fluorescence series: vertically and horizontally polarized fluorescence spectra upon exciting at 830 nm were assumed to consist of a linear combination of the polarized B820 spectrum, polarized B873 spectrum, and the two polarized intermediate spectra resulting from the global spectral analysis on the set of temperaturedependent fluorescence spectra. The amplitudes of the four components were calculated by MatLab (see 'Materials and methods'). Using the fractional contributions of B820 and B873 to the fluorescence spectra at 830 nm, the fractional contributions of B820 and B873 to fluorescence spectra upon excitation at other wavelengths were calculated, correcting for the change in extinction upon scanning through the excitation band. Subtraction of the contributions of B820 and B873 from each spectrum left the vertically and horizontally polarized fluorescence spectra of the combination of the two intermediates at each excitation wavelength. The relative contribution of each intermediate to these spectra and corresponding anisotropy was resolved using MatLab. Figure 12 shows the fractional contributions of the four components as a function of the excitation wavelength for sample 1. According to Figure 12, the 859-nm fluorescing intermediate has an absorption maximum around 850 nm and the 885-nm fluorescing intermediate has an absorption maximum located around 866 nm. The anisotropy values of the 859-nm fluorescing intermediate increased from 0.27 to 0.37 over the excitation range of 820–886 nm and the anisotropy values of the 885nm fluorescing intermediate increased from 0.09 to 0.29 (not shown).

Discussion

Characterization of two intermediates in the B820–B873 reassociation process

The measured polarized fluorescence, CD and absorption spectra as a function of temperature all show the appearance of a spectral intermediate during the recooling process. Whereas the appearance of this prominent intermediate, which features are described by the results of the three-component analysis fitting, is clear by eye looking at the measured spectra, the appearance of a second intermediate is more speculative. Motives that more than one intermediate appears during the reassociation process are given by the results of the three-component spectral analysis fitting and by analyses of the excitation-dependent polarized fluorescence spectra. First, analyses of excitationwavelength dependent fluorescence spectra from two samples, equilibrated in different stages of the B820-B873 reassociation process, both yield an intermediate fluorescence spectrum with a redshifting peak for increasing excitation wavelengths and non-flat anisotropy spectra, when only one intermediate is taken into account. Second, the residuals of global analysis of three temperature-dependent CD experiments have pronounced structures with similar shapes for the three independent experiments, when only one intermediate is taken into account. Third, also the three-component global-analysis residuals of the temperature-dependent polarized fluorescence experiment show some structure. These arguments strongly suggest that the sets of spectra contain spectral contributions of more intermediates, that can be analyzed spectroscopically by introducing more spectral fit components. Indeed, a second intermediate component could be extracted by expanding the fitting analyis to a four-component fit. The spectroscopic features of this intermediate in terms of absorption, fluorescence, polarization and CD consistently show the features of a second small oligomer, that is somewhat larger than the prominent intermediate. To some extent the introduction of a second intermediate improves the fitting. The global-analysis residuals of the temperature-dependent polarized fluorescence spectra show less structure and the rms is reduced, while the residuals of the temperature-dependent CD spectra seem to contain more random fluctuations than pronounced structures. It is not unlikely that in reality a range of differently sized aggregates coexists in the different equilibria. In this paper, however, we attempted to get an overview of the spectroscopic parameters of the intermediate(s). Therefore our fitting approach, in which the shapes of the fitspectra in terms of widths and peak locations are fitting parameters, is limited by a small number of components to reduce the number of free parameters. In an alternative approach, this problem could be handled by fitting a distribution of intermediates with defined spectral shapes to the sets of spectra to focus on the relative contributions of each form as a function of temperature.

Because of their relatively blueshifted absorption and high anisotropy, both intermediates most likely represent small oligomers. The increase of anisotropy for longer excitation wavelengths (from 0.3 to 0.4 for the most blueshifted and from 0.09 to 0.29 for the second) suggests that energy transfer takes place between the pigments within the intermediate oligomers. The second intermediate has a steeper increase of anisotropy for increased excitation wavelengths and a more redshifted absorption and fluorescence spectrum than the first one. This suggests that the second intermediate, appearing at lower temperatures (further in the reassociation process), is a larger oligomer, consistent with the idea of a stepwise oligomerization process. In the modelling study of Westerhuis et al. (1999) who computated polarized fluorescence Q_v excitation spectra for diferently-sized 'arcs' of an LH 1 ring under low temperature conditions, an increase of polarization upon exciting more to the red also was found, that was a more steep increase for larger oligomers.

Earlier we presented a reaction model in which the reassociation of B820 into B873 could be described by a moderately cooperative association reaction (Pandit et al. 2001). Such a reaction would yield equilibria in which B873 and B820 can coexist together with



Figure 13. A schematic picture of the two BChls within one dimer with the coordinate basis used in the CD/absorption simulation.

very small or very large aggregates of B820. This agrees with our findings of two intermediates in this study, if both are considered small aggregates. As the most blueshifted intermediate (assumed dimerized B820) coexists mainly with B820 in equilibrium (Figure 3A shows a clear absorption band of B820), is seems likely that further in the reassociation reaction this intermediate will associate with B820 subunits or will dimerize, forming structures that might be represented by the second intermediate. The results here describe intermediates that are formed in a thermodynamic equilibrium and might differ from intermediates that are temporarily formed during a kinetic reassociation process. The results of van Mourik et al. (1992), who followed the B820-B873 reasociation process kinetically, could distinguish a kinetic intennediate step that was partly attributed to proteindetergent interactions. Their fitting analysis shows fitresiduals that have structure around 850 nm, suggesting the formation an 850-nm intermediate during the kinetic reassociation reaction. Complementary to equilibrium experiments, more expanded kinetic experiments on the B820-B873 association/dissociation process should be performed to adress the question whether the here presented spectral intermediates also appear in a kinetic reassociation process.

The CD spectra of reassociated B873 in our experiments have an opposite sign compared to the CD spectrum of *Rs. rubrum* G9 chromatophores and

to the CD spectrum of isolated wildtype LH 1 (not shown). Also in other reassociation studies sometimes inverted CD spectra are observed for the reassociated LH 1 complexes (Miller et al. 1987; Ghosh et al. 1988). Parkes-Loach et al. showed that the sign of the CD spectrum strongly depends on BChla binding site (Parkes-Loach et al. 1990) and observed that B873 complexes reconstituted with BChla ethyl or phytyl derivatives had inverted CD spectra. We did not observe any slow reconformation towards a 'nativelike' CD spectrum. However, we have some evidence that reconstituted LH 1 complexes in Rs. rubrum G9 chromatophores have an inverted CD signal that in some cases slowly (within days) will change sign. This might indicate that reassociated B873 complexes with inverted CD signal are intermediates of which the pigments slowly refold towards the native conformation. It is also possible that, depending upon the conditions for reassociation, both native-like and non-native aggregates are formed.

Simulated CD and absorption spectra of arcs of an LH 1 ring

In a preliminary modelling study (Georgakopoulou et al., in preparation) the CD and absorption spectrum of an LH 1 ring are simulated, using the electron-density map structure of Rs. rubrum as a template and using the $\alpha\beta$ -heterodimer structure of *Rs. molischianum* for positioning of the α - and β -BChls. In this model two transitions $(Q_y \text{ and } Q_x)$ of each chromophore are allowed. The template for the LH 1 ring consists in fact of two rings about 0.1 Å separated: the α -BChls are positioned on the upper ring and the β -BChls on the lower ring. Figure 13 shows a schematic picture of the two BChls within one dimer in this model. The dipole moments of each chromophore are positioned on the template and each given a certain rotation in the plane of the BChl (i.e., the Q_x dipole moments are not perpendicular to the radius of the LH 1 ring and the Q_y , dipole moments of the α - and β -BChl within one dimer are not parallel and not in the plane of the α , resp. β ring). For simulation of the CD and absorption spectrum, the Hamiltonian for this system is calculated, taking into account all excitonic interactions between the chromophores. Diagonal disorder is added to include inhomogeneous broadening. In the next step, the Hamiltonion is diagonalized and the absorption and CD spectra are calculated.

In all our reassociation experiments, the CD spectra of reassociated B873 are inverted compared to

'native' CD spectra of isolated wild-type LH 1 and LH 1 in chromatophores. We found that in the simulation, inversion of the CD signal was achieved by switching the orientation of the μ_v -dipole moments of the α and β -BChls in the dimers. For α - and β -BChls positioned on one ring, inversion of signal would simply be induced by rotation of the α - or β -BChl μ_v -dipole moment through the plane of the ring. In our model it is less easy to give a geometrical explanation, because presumably two planes have to be taken into acount if interaction between neighbouring dimers contributes to the CD. In that case one plane is spanned by the vector connecting the centers of the two BChls within the dimer and the $\mu_{\rm v}$ -dipole moments, and a different plane is spanned by the vector connecting the β - and α -BChls of two neighbouring dimers.

Although we did not investigate the full parameter space of possible dipole orientations that lead to an inversion of CD, some interesting remarks can be made about this finding. Firstly, this demonstrates that small changes of the direction of the dipole moments can cause an inversion of CD. This means that easily reassociated LH 1 complexes can be formed of which the CD signal differs from the native CD spectrum, by small differences in pigment orientation. Secondly, this finding suggests that in our reassociation experiments B873 complexes are formed in which the orientation of the α - and β -BChls within the dimers is a mirror image of the orientation in native dimers. Possibly, more stable complexes are formed by mirrored orientations of the and β -BChls.

We applied the above explained model to calculate CD and absorption spectra of increased-sized 'arcs' of an LH 1 ring, for comparison with the intermediate absorption and CD spectra found in this study. Because CD spectra of reassociated B873 had an inverted CD compared to native LH 1, LH 1 spectra were simulated in which the CD spectrum of LH 1 either resembled the native CD signal, or had this inverted sign. The BCh1 site-energies of the Qy were set to 800 nm, the site-energies of the Q_x to 600 nm, a homogeneous broadening of 350 cm^{-1} and inhomogeneous broadening of $550 \,\mathrm{cm}^{-1}$ was assumed and the energy difference between the α and β BChls was set to 300 cm⁻¹. The Q_y and Q_x dipole moments were set to 6.3 and 1.9 Debye, respectively. For simulation of a native-like CD spectrum, the α and $\beta \mu_y$ dipole moments were rotated 7° and 5° out of the plane of the ring respectively, and for an inverted CD spectrum the rotation angles were switched to 5° and 7° respectively. The α -BCh1 was rotated 19.18 degrees around



Figure 14. Simulated absorption and CD spectra of increased-sized arcs of an LH 1 ring with inverted sign compared to native LH 1. Spectra are drawn of B820, $(B820)_2$ (2N), $(B820)_4$ (4N), $(B820)_8$ (8N) and of the LH 1 ring (B873). Dotted line: CD spectrum of $(B820)_2$, which is part of a native-like LH 1 ring.

the x-axis. Simulations would yield an interaction energy around 255 cm⁻¹ between the α - and β -BChl within the dimers and an energy around 214 cm⁻¹ between the BChls on adjacent dimers. The CD spectrum of the dimer would have alternating a positive and and negative peak, as observed experimentally, for both types of simulations and only for larger aggregates the signs would be inverted by switching the α - and β -BCh1 dipole orientations.

Figure 14 shows the obtained simulated absorption and CD spectra of arcs of an 'inverted' LH 1 ring. The dotted spectrum is the simulated CD signal of (B820)2 from part of a native-like LH 1 ring. Note that the absorption of B873 is around 844 nm in this simulation, instead of 873 nm, and that the CD signal of B820 is conservative, instead of non-conservative as observed experimentally. In both types of simulations (series of inverted and non-inverted CD spectra compared to wild-type LH 1) the CD spectrum of B820 had the same sign as observed experimentally, although its relative size compared to CD spectra of larger aggregates was changed if rotations of the α - and β -BChls were inverted. The higher excitonic band at 777 nm is more pronounced in both the absorption and CD simulations than in measured spectra. Probably in reality, the site energies and the angles of the dipole moments change for each intermediate, i.e., by increasing the angle between the Q_v dipole moments. Such changes were not introduced in the modelling to keep the process simple and get a clear view of the effect that only ring parts of different size have on the absorption and CD spectra without interference of other parameters.

Figure 14A shows a rapid redshift of the absorption upon increasing the aggregation size. The most blueshifted intermediate we found, appearing half-way the reassociation process, therefore matches best with the absorption characteristics of a B820 dimer ((B820)₂). An interesting result from the CD simulations is that for arcs of an LH 1 with inverted CD, all aggregates have only two bands (plus the higher excitonic band around 777 nm). For arcs of a native LH 1 ring, this was also the case for aggregates larger than $(B820)_2$. In fact, the CD spectrum of $(B820)_2$ in that simulation shows four alternating bands reminding ona of the CD intermediate fit spectra obtained by global-analysis fiting of our temperature-dependent CD series. This suggests that the B820 subunits can dimerize, having their pigments oriented as in native LHI, whereas larger aggregates are formed with an inverted pigment orientation.

The simulations show how the CD spectral shapes might change with increased oligomeric states. According to the simulated spectra, a transition from B820 to (B820)₂ is accompanied by the rise of two or four alternating bands (of which the signs are reversed compared to the nearest B820 peaks) whereas a transition from (B820)₂ towards larger aggregates (including the full LH 1 ring) induces a redshift of the spectra. Although the absorption spectra show only a very small redshift for aggregates larger than (B820)₂ upon increase of the aggregate size, the CD signals shift significantly to the red as the zero-crossings are less blueshifted compared to the absorption maxima for larger aggregates. The simulations demonstrate that inverting rotation of the α and β pigments in the BCh1 plane results in a similarly shaped, but inverted, CD spectrum for the whole ring and for large aggregates, but in differently shaped CD spectra for (B820)₂.

Conclusion

We applied a combination of spectroscopic techniques to investigate the spectral properties of intermediates appearing in the B820-B873 association reaction. Independently, results from different spectroscopic experiments could be explained consistently assuming that at least one, but probably two, intermediates are present in the reassociation process. In case data was fitted assuming one intermediate component, this spectral intermediate appears to have an absorption maximum around 850 nm, a fluorescence maximum around 867 nm, an anisotropy of 0.3 to 0.4 and a CD spectrum with four bands with alternating signs. In case data was fitted with two intermediates, the fluorescence spectrum of the prominent intermediate is more blueshifted with a maximum around 860 nm. The second, more redshifted, intermediate appears further on in the B820-B873 reassociation reaction, and has an absorption maximum around 866 nm, fluorescence maximum around 885 nm, an anisotropy of 0.1 to 0.3 and two alternating peaks in the CD. Both intermediates display a slow rise of anisotropy as function of the excitation wavelength, indicating that energy transfer takes place between the pigments within the oligomers. The spectral features of both intermediates match best with the spectroscopic properties of a small oligomers, for instance a B820 dimer and B820 trimer respectively. This suggests that the first steps towards formation of the LH 1 complex from B820 subunits in vitro might consist of the stepwise aggregation of B820 into small complexes that have excitonic interaction and energy transfer among the pigments, leading to the formation of a functional light-harvesting antenna complex.

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