Bacteriochlorin-protein interactions in native B800-B850, B800 deficient and B800-Bchla_p-reconstituted complexes from *Rhodopseudomonas acidophila*, strain 10050

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Received 1 February 1999; received in revised form 16 March 1999

Abstract Recently, a method which allows the selective release and removal of the 800 nm absorbing bacteriochlorophyll *a* (B800) molecules from the LH2 complex of *Rhodopseudomonas acidophila* strain 10050 has been described [Fraser, N.J. (1999) Ph.D. Thesis, University of Glasgow, UK]. This procedure also allows the reconstitution of empty binding sites with the native pigment Bchla_p, esterified with phytol. We have investigated the bacteriochlorophyll*a*-protein interactions in native, B800 deficient (or B850) and in B800-bacteriochlorophylla_p-reconstituted LH2 complexes by resonance Raman spectroscopy. We present the first direct structural evidence which shows that the reconstituted pigments are correctly bound within their binding pockets.

Key words: Bacteriochlorophyll; Light harvesting complex; Photosynthesis; Pigment extraction

1. Introduction

The primary events in the purple bacteria photosynthesis consist of the absorption of photons of vis-NIR light by light harvesting (LH) antenna complexes followed by their rapid and efficient transfer to a reaction centre (RC) where they are 'trapped' [1]. In most species of purple bacteria, there are two types of antenna complexes [2]. The first type, LH1, is intimately associated with the RC and forms the so-called 'core' complex. Arranged more peripherally to this, and present in variable amounts, is the second type of antenna complex, called LH2. The spectral properties of these antenna complexes are precisely tuned to ensure the efficient funnelling of excitation energy towards the RC.

In both types of LH complexes, the LH pigments (bacteriochlorophyll (Bchl)*a* and carotenoid molecules) are non-covalently attached to a protein scaffold. The determination of the crystal structure of the LH2 complex from *Rhodopseudomonas acidophila* 10050 to a resolution of 2.5 Å has revealed the arrangement of the pigments within that complex [3]. It has a nonameric ring structure. Each monomeric unit contains an α- and β-apoprotein, three Bchl*a* and one carotenoid (rhodopin glucoside) molecules. The nine α-apoproteins form a hollow cylinder with the nine β-apoproteins arranged radially outside. The complex contains two discrete pools of Bchl*a* molecules. Eighteen of the Bchl*a* molecules are sandwiched between the α- and β-apoproteins and form a continuous overlapping ring. They are each coordinated to conserved His residues on either the α- or β-apoproteins via their central Mg^{2+} ion. These molecules absorb prominently at –850 nm and are known as the Bchl-B850 molecules. The other nine Bchl*a* molecules lie towards the cytoplasmic side of the membrane and are located between the transmembrane helices of the β-apoproteins. They are liganded to an extension of the N-terminal methionine residue of the α-apoprotein. These pigments absorb at 800 nm and most often exhibit spectroscopic properties typical of monomeric BChls not involved in strong excitonic interactions [4] and are denoted Bchl-B800.

In free solution, the Q_y absorption maximum of monomeric Bchl*a* is located at –770 nm and is red-shifted to –800 nm or –850 nm when bound to the LH2 complexes. Recent calculations have shown that the wavelength of absorption by any given bound Bchl*a* molecule is determined by its specific micro-environment and is affected by both pigment-pigment and pigment-protein interactions [5]. A molecular description of the parameters which control where a given Bchl*a* molecule absorbs requires that the relative roles of Bchl*a*-Bchl*a* and Bchl*a*-protein interactions are delineated. The contribution of protein-Bchl*a* interactions to the red shift of the Bchl*a* Q_y transition observed in the LH2 complexes has been extensively studied by resonance Raman spectroscopy [6]. In this regard, it has been especially useful in combination with the technique site-directed mutagenesis, which has been used to create LH complexes with altered spectral properties [7,8].

From these studies, for example, it has been shown that each H-bond breakage between the apoproteins and the acetyl carbonyl oxygens of the B850 molecules correlates with a 10 nm blue shift of the absorption maximum [9]. A similar effect also occurs with the B800 molecules. The conserved Arg residue at position −12 on the β-apoprotein interacts with the acetyl carbonyl oxygen of the B800 molecules. Breakage of this H-bond causes a –10 nm blue shift in their Q_y absorption band [10]. Moreover, it was suggested that the local dielectric

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Abbreviations: Bchl*a*, bacteriochlorophyll *a*, the esterifying alcohol is indicated by the subscript (p = phytol); cm^{-1}, wavenumber; H-bond, hydrogen bond; LH, light harvesting; RR, resonance Raman; B800, B500, binding sites for bacteriochlorophyll a_p absorbing at 800 and 850 nm, respectively

PII: S0014-5793(99)00410-X

properties of the B800 binding pocket could account for an additional 10 nm blue shift in the Q_y transition of these molecules.

The roles of the various pigment-protein interactions which control the absorption properties of the Bchl*a* molecules within the LH2 complex can also be investigated by selective removal and reconstitution of pigments into their binding sites. Recently, it has been shown that the B800 molecules from the LH2 complex from *Rhodobacter sphaeroides* can be released by an acid treatment [11]. This study also showed that the extracted pigments can be replaced with both native and modified (bacterio)chlorin molecules. Since then, we have developed a similar procedure which allows the selective release and removal of the B800 molecules from the LH2 complex of *R. acidophila* 10050 [12]. At a later stage, the empty binding sites can be reconstituted with native Bchla_p, *vis*. Bchl*a* esterified with phytol. In this type of study, however, it is very important to determine whether or not the reconstitution procedure produces a native-like complex. Here, we have compared the Bchl*a*-protein interactions in native and Bchl*a*-reconstituted complexes by resonance Raman spectroscopy. The Raman spectra of LH2 complexes have previously been shown to be very sensitive to the environment of the Bchl*a* molecules [6,9,10]. This allowed us to use this technique to assess whether or not the Bchl*a* molecules are correctly bound within the reconstituted complex.

2. Materials and methods

2.1. Isolation of LH2 complexes

Liquid cultures of *R. acidophila* 10050 were grown anaerobically at 30°C in Pfenning media [13]. Cells were harvested by centrifugation. Photosynthetic membranes were then isolated by rupturing the whole cells in a French press. Finally, the membranes were solubilised using LDAO and LH2 complexes purified as described by Fraser [12].

2.2. Pigment exchange procedure

The pigment exchange protocol has been extensively described elsewhere [12]. In short, all of the B800 molecules were released from their binding sites by incubating a LH2 sample in buffer containing Triton BG-10 at a pH of 4.75 at 30°C for 1 h. B850 complexes lacking Bchl-B800 were then purified by ion exchange chromatography using phosphocellulose as the absorbent. Optimal reconstitution of the B800 sites was achieved by incubating a B850 sample with a 3-fold excess of phytol Bchl*a* for 2 h at a pH of 8 and at room temperature.

2.3. Absorption spectroscopy

Low temperature absorption spectra were collected at 10 K in a SMC-TBT flow cryostat (Air Liquide, Sassenage, France) cooled with liquid helium using a Varian Cary E5 double-beam scanning spectrophotometer.

2.4. Resonance Raman spectroscopy

The Bchl*a* molecules were selectively excited at 363.8 nm using a Coherent Innova 100 Argon laser. Spectra were recorded with a 90 degree geometry using a Jobin Yvon U1000 spectrophotometer equipped with a back-thinned charge-coupled device detector (Jobin Yvon Spectrum ONE). Samples were maintained at 10 K in a SMC-TBT flow cryostat (Air Liquide, Sassenage, France) cooled with liquid helium. Absorption spectra were taken before and after Raman measurements to verify the sample integrity. The background fluorescence was removed and spectra were normalised to the methine bridges using GRAMS32 Spectral Notebase (Galactic Industries).

3. Results

The low temperature (10 K) absorption spectrum of the LH2 complex is shown in Fig. 1a. At this temperature, the

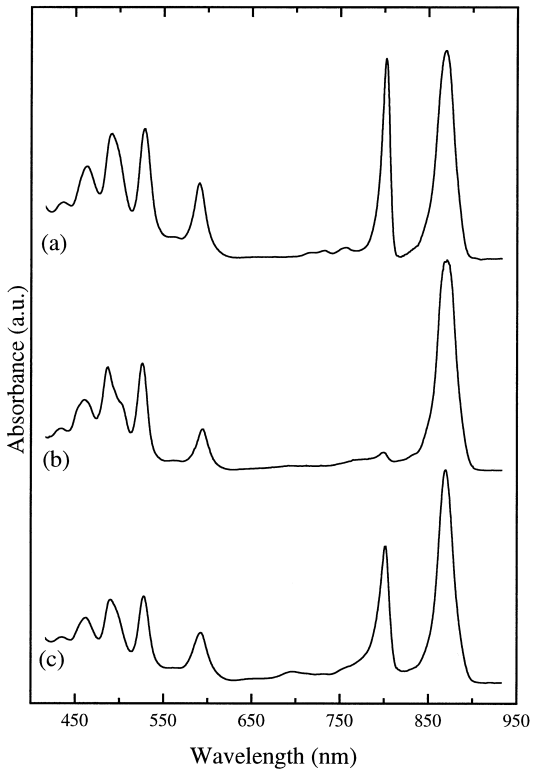

Fig. 1. Low temperature absorption spectra of (a) native LH2 (b) B850-only and (c) Bchla_p-reconstituted complexes from *R. acidophila* 10050. All spectra were recorded at 10 K.

Q_y absorption transitions of the B800 and B850 molecules have maxima at 803 and 869 nm, respectively. The absorption band at 590 nm comes from the Q_x transitions of the three Bchl*a* molecules. The peaks at 434, 463, 491 and 529 nm belong to the rhodopin glucoside molecules. The absorption spectrum of the B850 complex is recorded in Fig. 1b. The position of the Q_y absorption band of the Bchl-B850 molecules is the same as in the native complex. A small residual peak at 800 nm can be seen. This may arise either from any remaining native Bchl-B800 and/or from the upper exciton component of the Bchl-B850 absorption [11,14,15]. The Bchl*a* Q_x absorption maximum is also red-shifted by 3–4 nm. The crystal structure of the LH2 complex shows that the carotenoid and B800 molecules interact closely [16]. It is not surprising, therefore, that the removal of the B800 molecules from the complex causes spectral shifts in the region of the carotenoid absorption. These consist of a splitting of the central absorption band to give a peak at 486 nm and a shoulder at 499 nm and a general blue shift in the absorption maxima by 2 nm. This can be attributed to a change in the conformation/configuration affecting one end of the conjugated polyenic chain of the carotenoid molecule [17].

The absorption spectrum of the Bchla_p-reconstituted complex is essentially the same as that of the native complex (see Fig. 1c). The most important spectral features can be summarised as follows. The Q_y absorption band, associated with the B800 molecules, is restored. By comparing the integrated area of this peak with that in the native complex, we estimate that the average occupancy of the B800 sites in the reconstituted complex is $\approx 80\%$. In addition, the splitting and blue shift, associated with the carotenoid absorption and the red

shift of the Bchl*a* Q_x absorption band which occur in the B850 complex, are fully reversed.

The tertiary structure of the LH2 complex from *R. acidophila* contains many carotenoid-Bchl interactions [3,16] and HPLC analysis has shown that during the pigment removal/reconstitution protocols, some carotenoid loss does occur [12]. Therefore, the reduction in the overall rhodopin glucoside intensity in the absorption spectrum of the reconstituted B800-850 complex (Fig. 1c) is a direct result of its reduced carotenoid content. Removal of some of the carotenoids will allow, at least in part, the spatial re-orientation of some of the remaining Bchl molecules. Combined with the $\approx 80\%$ occupancy of the B800 sites, the reduction in the carotenoid content may also explain why one may observe slight spectral differences between the B850 Q_y absorption bands of the native (Fig. 1a) and reconstituted (Fig. 1c) LH2 complexes.

In order to probe the vibrational contributions of the different Bchl*a* molecules in the various protein complexes, resonance Raman spectra were recorded. All samples were excited under resonance conditions with the Soret electronic transition of the bound Bchl*a* molecules. Fig. 2 shows the induced Raman shift in the high frequency carbonyl region (1585–1735 cm^{-1}) for the native, B850-only and Bchla_p-reconstituted complexes.

In these conditions of excitation, all of the spectra are dominated by a strong signal at 1610 cm^{-1}, arising from the stretching modes of the Bchl*a* methine bridges. In comparison, the contribution of the Bchl*a* carbonyl stretching modes are rather weak and are partially obscured by the intense 1610 cm^{-1} band. The RR spectrum of the native LH2 complex (see Fig. 2a) is similar to those previously reported [18] and three bands at 1623, 1669 and 1696 cm^{-1}, which are due to the stretching modes of the Bchl*a* carbonyl groups. These modes have been attributed to H-bonded acetyl, H-bonded keto and zfree from interaction keto carbonyl groups, respectively [6,18].

With respect to that of the native complex, the RR spectrum of the B850 complex shows an attenuation of the 1610 cm^{-1} signal. There is also a shift in the frequency (linked to a net loss of intensity) of the mode at 1623 cm $^{-1}$ to 1626 cm^{-1} and disappearance of the 1696 cm^{-1} band (see Fig. 2b). To a first approximation, and within the limitation of our experimental set-up, the Raman signatures arising from the methine bridges of the Bchl-B800 and Bchl-B850 molecules are very similar. Therefore, the normalised spectrum of the B850-only complex was divided by 2/3 (resulting in Fig. 2b) to account for the lack of the Bchl-B800 pigments. Following a simple subtraction of the normalised Raman spectrum of the native LH2 complex (Fig. 2a), one arrives at Fig. 2c which corresponds to the net loss associated with removal of the Bchl-B800 molecules. Two main features in the difference spectrum are apparent. Firslty, the lack of any H-bonded Bchl (there is no intensity in the 1650–1670 cm^{-1} region) indicates that this difference spectrum contains no significant contributions from either Bchl-B850 molecules or free Bchl molecules in solution. The second feature relates to the intensities at 1620 and 1699 cm^{-1}. These two Raman modes are in agreement with previous assignments of B800 sites having a full Bchl occupancy [6]. As a result, one may conclude, to a first approximation, that Fig. 2c is essentially that of the Bchl molecules that occupy the B800 site. The signal associated with the C3 acetyl carbonyl group has a maximum value at 1620 cm^{-1}, compared to the corresponding signal of the B850-only complex which peaks at 1626 cm^{-1}. This may suggest that the H-bond between the protein and C3 acetyl carbonyl group of the B800 molecules is stronger than that which is made to the Bchl-B850 molecules. Alternatively, these different signals may reflect local differences in the polarity of the environment surrounding the Bchl-B800 and Bchl-B850 molecules [10]. In addition, the RR spectrum of the Bchl-B800 molecules has a signal at 1699 cm^{-1} but not at 1669 cm^{-1}. This indicates that the C13 keto groups of the B800 molecules are free.

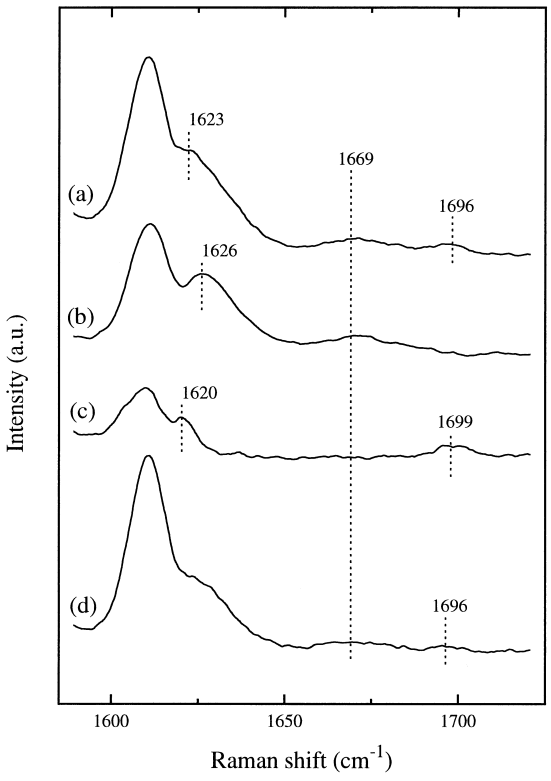

Fig. 2. The high frequency carbonyl stretching modes of the Bchl*a* molecules in the (a) native, (b) B850-only and (d) Bchla_p-reconstituted complexes. The RR spectrum of the B800 molecules (c) was obtained by subtracting the RR spectrum for the B850-only complex from that for the native complex. All spectra were recorded at 10 K. The Bchl*a* molecules were excited in their Soret transition at 363.8 nm.

The RR spectrum of the Bchl*a*-reconstituted complex is essentially the same as that for the native complex with a shoulder at 1623 cm^{-1} and weak bands at 1669 and 1696 cm^{-1} (see Fig. 2d). The intensities of these signals, however, are weaker than those which occur in the native complex. This attenuation, and the slight distortions observed between the RR spectra of native and reconstituted LH2 in the 1640–1660 cm^{-1} region, are probably due to incomplete occupancy of the B800 sites (see Fig. 1c). The re-appearance of the shoulder at 1623 cm^{-1} shows that the C3 acetyl carbonyl groups of the Bchl-B800 molecules in the reconstituted complex are H-bonded.

By extending the measured range to 350–1750 cm^{-1}, we include the vast majority of the vibrational modes of Bchl*a*. After allowing for the small quantity of non-specifically bound phytol Bchl*a* (see above) in reconstituted sample, the spectra are near identical (data not shown).

4. Discussion

The Q_y absorption transitions of the Bchl*a* molecules in the LH2 complex are red-shifted with respect to that in free solution. This is, at least in part, due to interactions between the pigments and the polypeptides. The best way of investigating the underlying molecular mechanisms which give rise to the observed red shift is to modulate those pigment-protein interactions which occur. This can be done in one of two ways. Firstly, the amino acid sequence of the protein can be changed by site-directed mutagenesis. Alternatively, the Bchl*a* binding pockets can be reconstituted with (bacterio)chlorin molecules which have modified chemical structures. We have developed a technique which allows the selective release and removal of the Bchl-B800 molecules from the LH2 complex of *R. acidophila* 10050. The empty binding sites can later be reconstituted with the native Bchla_p. At this stage in developing our method, it was necessary to test whether or not the reconstituted complexes have native-like structures. Here, we have shown that the vibrational modes of the Bchl*a* molecules in the Bchla_p-reconstituted complex have essentially the same frequency as those in the native complex. If the reconstituted pigments were bound differently from those in the native complex, the vibrational modes of the Bchl*a* molecules in the two complexes would have significantly different frequencies. The similarity of the RR spectra of the two complexes would suggest that our reconstitution method produces a structure which is very similar to the native one. By comparing the B850-only with the B800-850 complex, it was possible to separate the RR spectra of the Bchl in the two pigment binding sites.

More recently, we have shown that the B800 binding sites can be reconstituted with a range of modified (bacterio)-chlorin molecules [12]. Using these complexes, we intend to use resonance Raman spectroscopy as a tool to investigate those features of the bacteriochlorin molecule which are required for binding and how the interactions between the protein and bacteriochlorin molecules affect both the spectral and energy transfer properties of the LH2 complex.

Acknowledgements: We would like to acknowledge the generous financial support from the BBSRC (RJC), the Gatsby Charitable Trust (NJF), the Royal Society (AG) and the DFG (HS, Sche140/13).

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