The effect of internal voids in membrane proteins: high-pressure study of two photochemical reaction centres from *Rhodobacter sphaeroides*

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Abstract The effect of application of high pressure on the carotenoid-containing bacterial reaction centre from *Rhodobacter sphaeroides* strain 2.4.1 was studied, and compared to recent experiments performed on its carotenoid-less counterpart, isolated from strain R26.1. Our results indicate that the cavity created by the absence of carotenoid contributes to localised differences in protein compressibility when using the intrinsic chromophores as molecular probes. Differential stability of the electronic transitions of the primary electron donor under high hydrostatic pressure is observed, dependent on the presence of the carotenoid cofactor. This suggests that the transition intensity loss is induced by a slight change of the primary electron donor structure, allowed by the void created by the absence of the carotenoid molecule.

Key words: Absorption spectroscopy; Bacteriochlorophyll; Membrane protein; Photosynthesis; Pressure; Protein structure; Raman spectroscopy

1. Introduction

The bacterial photochemical reaction centre (RC) from *Rhodobacter sphaeroides* constitutes an ideal model for understanding how the structure affects the stability of membrane proteins as a number of X-ray structures exist where ordered internal 'holes' or voids have been created [1–5]. Furthermore, the RC protein contains several different pigmented molecules, or chromophores. These individual chromophores, which are located throughout the volume of the RC protein, constitute a series of intrinsic molecular probes, therefore, are able to monitor localised structural changes (or changes in relative stability), when examined by spectroscopic methods [6,7] including Raman spectroscopy [8–10] as a function of external stresses such as applied hydrostatic pressure [11,12]. Some of the induced voids in the RC protein are the result of loss of a specific chromophore [1–3] or the exchange of a voluminous amino acid residue for a smaller one [4,5]. This latter class of holes is the result of specific site-directed mutagenesis studies [13].

The structure of the wild-type photochemical RC from *Rb. sphaeroides* has three transmembrane-spanning polypeptides two of which (L and M) possess very similar tertiary structures. Each of these polypeptides has five transmembrane-spanning helices that are linked by a series of small helices and loop regions. The H subunit is much less hydrophobic, it contains only a single membrane-protruding helix located at the N-terminus of its sequence, and the rest of its structure is located at the membrane interface on the cytoplasmic side of the membrane. Together, the 10 membrane-spanning α-helices of L and M form a protective scaffold for the cofactors. This scaffold non-covalently binds six bacteriochlorin chromophores (four bacteriochlorophylls (Bchl) and two bacteriopheophytins (Bpheo)), two quinones, one carotenoid and one non-haem iron. The bacteriochlorin molecules and quinones are arranged in pairs around a pseudo-C_2 axis of symmetry, which runs from the centre of the primary donor of electrons (P, a pair of closely interacting Bchls) to the non-haem iron [14]. Despite this apparent symmetry, energy transduction and electron transfer within the RC is highly asymmetric, occurring along the branch of chromophores most closely associated with the L subunit (the so-called L branch). A carotenoid molecule, which is non-covalently attached to the M subunit, protects the protein from photo-oxidative damage [15,16]. In strain 2.4.1 the carotenoid present is spheroidene; a trace amount of the oxygenated form (spheroidenone) may be present. It is noteworthy that the X-ray crystal structures of the spheroidene-containing and spheroidenone-containing RC proteins do not show any structural deviations, except for very minor ones in the immediate vicinity around the keto end group of the carotenoid [17]. *Rb. sphaeroides* strain R26.1 is unable to synthesise coloured carotenoids. There is crystallographic evidence that suggests that in the carotenoid-free structure from strain R26.1 there is a nicely shaped partial electron density distribution for a detergent (*N*,*N*-dimethyldodecylamine-*N*-oxide, LDAO) molecule sitting in the middle of the carotenoid binding site [18] and presumably replaces the in vivo water molecules. The RC is an interesting membrane protein to study at high pressure because the presence/absence of the carotenoid cofactors permits spectroscopic methods to probe the influence of this molecule when the polypeptides are physically stressed. For exam-

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Abbreviations: Bchl, bacteriochlorophyll *a*; Bpheo, bacteriopheophytin *a*; Crt, carotenoid; FT, Fourier transform; LDAO, *N*,*N*-dimethyldodecylamine-*N*-oxide

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ple, recent high-pressure experiments on the carotenoid-less R26.1 mutant showed that the Q_y transition of P was greatly reduced as pressure increased to about 0.6 GPa but was restored upon returning to atmospheric pressure [12].

In this work, the properties of the carotenoid-containing (Crt^+) RC from *Rb. sphaeroides*, strain 2.4.1, has been studied at high hydrostatic pressures and compared to a previous investigation of the carotenoid-less (Crt^-) RC found in strain R26.1 [12], in order to study how a large internal void in the RC alters the stability of neighbouring proteotic sub-structures.

2. Materials and methods

Liquid cultures of *Rb. sphaeroides* strain 2.4.1 were grown anaerobically in the light at 30°C in Böse medium [19] then harvested by centrifugation. Photosynthetic membranes were prepared and the RC proteins purified as previously described [12,20]. Polyacrylamide gel electrophoresis was used to confirm the polypeptide composition of the sample [21]. For use in the pressure cell samples were prepared in a buffer containing 0.035% LDAO, 10 mM Tris–HCl, 150 mM NaCl, pH 8.0 [12]. RC samples were poised in their reduced (P) state using sodium ascorbate as previously described [22].

The high-pressure optical cell used in this work has been described previously [23]. Pressure was generated inside the cell by a hydraulic press, and was transmitted to the sample in a separate cell by a liquid phase (hexane). Increases (and decreases) in pressure occurred at an average rate of 0.1 GPa/min. Room temperature absorption spectra were recorded using either a V570 spectrophotometer (Jasco, Tokyo, Japan) or a Cary 5E spectrophotometer (Varian, Sydney, Australia). Spectra were corrected by subtracting, at each applied pressure, a reference spectrum corresponding to that of the detergent-containing buffer alone in the pressure cell. Prior to data analysis the absorption spectra were converted from nanometres to an energy scale (wavenumbers, cm^{-1}). Fourier transform (FT)-Raman spectra were recorded, using a Bruker IFS 66 interferometer coupled to a Bruker FRA 106 Raman module equipped with a continuous Nd:YAG laser, as described previously [24]. Depending on sample conditions, spectra were the result of up to 9000 co-added interferograms. This corresponded to a maximum of 225 min of data acquisition. Reversibility of the pressure-induced effects on the proteins was checked systematically by measuring absorption and/or FT-Raman spectra upon pressure release, after the pressure maximum had been applied. In order to avoid any possibility of mixing the pressure-transmitting medium with the protein sample, a minimum pressure has to be applied when the reversibility of the pressure-induced effects was tested.

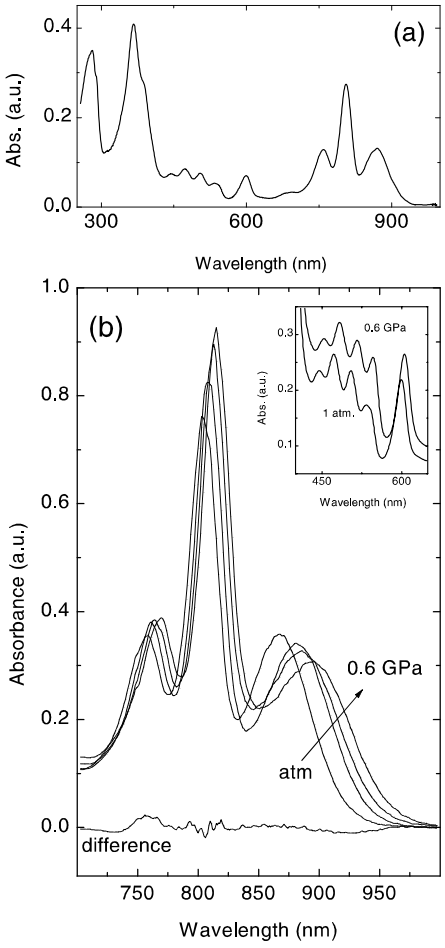

Fig. 1. Electronic absorption spectra of isolated the RC from *Rb. sphaeroides* 2.4.1 measured at room temperature. a: UV-Vis near-infrared absorption spectrum of isolated RC protein in a quartz cuvette. b: The effect of pressure on the near-infrared electronic absorption spectrum: atmospheric, 0.2, 0.4 and 0.6 GPa which show a red shift in peak position as pressure increases (arrow). The process is reversible as evidenced by the difference spectrum at minimal pressure before and after the applied pressure cycle. Inset: The carotenoid and Q_x transitions also show a pressure-dependent red shift between atmospheric pressure and 0.6 G Pa.

3. Results and discussion

Shown in Fig. 1a is the room temperature absorption spectrum of the RC from *Rb. sphaeroides* strain 2.4.1; it is characteristic of a detergent-isolated RC. Bchl and Bpheo molecules exhibit electronic transitions located in the near-infrared region (ca. 750–860 nm) of the spectrum (the so-called Q_y transition), which are usually used to probe the structure and function of the RC [6]. Their Soret and Q_x transitions also contribute at ca. 350–380 nm and ca. 540–610 nm, respectively. The remaining absorption transitions at ca. 420–530 nm arise from the vibronic transitions of the carotenoid spheroidene.

Fig. 1b shows the effect of increasing pressure on the near-infrared absorption spectrum of the RC protein. The absorption peaks at about 760, 800 and 860 nm arise from the Q_y electronic transitions of the Bpheo (H), the accessory Bchl (B) molecules and of the primary electron donor (P), respectively. All the electronic transitions experience a red shift when raising the hydrostatic pressure. A similar phenomenon is observed for the electronic transitions of the carotenoid molecule (Fig. 1b, inset).

The difference absorption spectrum shown in Fig. 1b demonstrates the reversibility of the pressure-induced effects on the RCs upon pressure release, after the maximum pressure had been applied. The reversibility indicates that this multi-subunit membrane protein exhibits a full elastic response to pressure application up to 0.6 GPa, and that it is not irreversibly denatured during the experiment, in harmony with recent pressure studies on native multi-subunit LH1 and LH2 antennae [24,25].

It is apparent from the plots in Fig. 2 that the pressure-induced shift rates, defined as $\Delta\nu/\Delta p$ (cm^{-1} GPa^{-1}), of the energies of the different Bchl, Bpheo and carotenoid electronic transitions are different; there is no evidence of hysteresis. Most of these responses, as a function of applied pressure below $\sim$0.2 GPa, may reasonably be fitted with a linear dependence (see Table 1 and Fig. 2). This results in pressure sensitivities of between 808 and 1012 cm^{-1} GPa^{-1} for the three vibronic transitions of the carotenoid molecule (Fig.

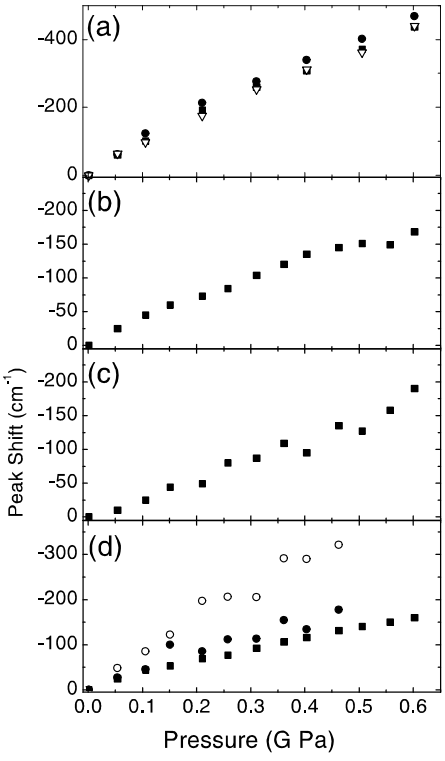

Fig. 2. Room temperature pressure sensitivity of selected electronic transitions. a: The three absorption maxima ascribed to the carotenoid spheroidene. b: The Q_y electronic transitions of the monomeric accessory Bchls, B. c: the Q_y electronic transitions of the monomeric Bpheo, H. d: The Q_x transitions of Bchl (solid squares), H_L (closed circles), H_M (open circles).

2a): 350 cm^{-1} GPa^{-1} and 254 cm^{-1} GPa^{-1} for the Q_y transitions of the accessory Bchls (Fig. 2b) and Bpheos (Fig. 2c), respectively. These numbers are very close to those measured for the carotenoid-less R26.1 mutant (see Table 1).

The pressure sensitivities of the Q_x electronic transitions of the Bchl (ca. 600 nm) and the Bpheo (H_M and H_L, ca. 540 nm) molecules are shown in Fig. 2d. It is known that at atmospheric pressure the Q_x electronic transitions of H_M and H_L have slightly different absorption maxima, which results in a flattened absorption peak (Fig. 1a and [26]). Applying Gaussian distributions to these bands measured at atmospheric pressure, we obtained the curves that approximate the electronic absorption bands of H_M and H_L with absorption maxima at about 530 and 543 nm, respectively (not shown). Indeed, the pressure sensitivity of the H_M molecule is about twice as much (703 ± 63 cm^{-1} GPa^{-1}) than that of the H_L chromophore (320 ± 53 cm^{-1} GPa^{-1}) for the pressure range up to 0.4 GPa. This analysis is in reasonable agreement with previous results, including high-pressure studies on Crt^- RCs [12,26]. This remarkably different pressure sensitivity of the Q_x electronic transition energies of the two Bpheo molecules underscores the inherent inhomogeneity of the RC protein interior around the pseudo-C_2 symmetry axis. Experimentally, at pressures higher than 0.45 GPa it was impossible to separate the two peaks. The energy shifts of the Q_x transitions of the Bchl molecules in the 2.4.1 protein up to 0.4 GPa was calculated to be 254 ± 7 cm^{-1} GPa^{-1} and found to be similar to the RC sample from strain R26.1 (307 ± 9 cm^{-1} GPa^{-1} [12]). Thus, in this pressure range the averaged degrees of compressibility of the two RC structures around the chromophores B_M, B_L, P_M and P_L are very similar. Furthermore, the positions of the final energy shift at 0.6 GPa is remarkably similar for both RC complexes.

Upon the application of high pressure perhaps the most striking difference between the Crt^+ and Crt^- RCs is the pressure sensitivity profile of the Q_y transition of the primary electron donor (P). It is clear from Fig. 3 that at intermediate pressures the rate of the red shift of the Q_y transition of P reduces in both membrane proteins. This occurs at about 0.2 GPa for the Crt^- RC (Fig. 3, open triangles) and at a slightly higher pressure for the Crt^+ complex (Fig. 3, solid squares). It is of note that this pressure range corresponds to that where the P protein binding site stops reorganising measurably according to pressure, as evidenced by FT-Raman spectroscopy (see below and [12]). Furthermore, the plateau-like feature in the pressure sensitivity of P is distinctly less pronounced in the Crt^+ membrane protein, which lacks the large internal cavity. The final energy shift of the primary electron donor at 0.6 GPa is virtually identical for both RC complexes indicating that at high pressures the compressibility of both multi-subunit integral membrane proteins is, in effect, the same.

It was previously shown in strain R26.1, and confirmed in this work with strain 2.4.1, that the electronic transitions of the different chromophores indicate a possible change in protein structure between 0.2 and 0.4 GPa. FT-Raman spectroscopy was thus employed to verify this hypothesis. Raman spectroscopy is a vibrational technique that provides informa-

Table 1
Comparison of the linear pressure sensitivities ($\Delta\nu/\Delta p$ in cm^{-1} GPa^{-1}) of the electronic transitions of the carotenoid, Bpheo and accessory Bchl molecules in RCs from strains 2.4.1 (Crt^+) and R26.1 (Crt^-), determined for pressures below 0.2 GPa

Electronic transition	Pressure sensitivity (cm^{-1} GPa^{-1})	
	2.4.1	R26.1
Crt_1	900 ± 41	n/a
Crt_2	1012 ± 62	n/a
Crt_3	808 ± 72	n/a
Q_y-Bchl (B_L and B_M)	350 ± 30	312 ± 45
Q_y-Bpheo (H_L and H_M)	254 ± 28	276 ± 26
Q_x-Bchl (all Bchl molecules)	323 ± 29	263 ± 14

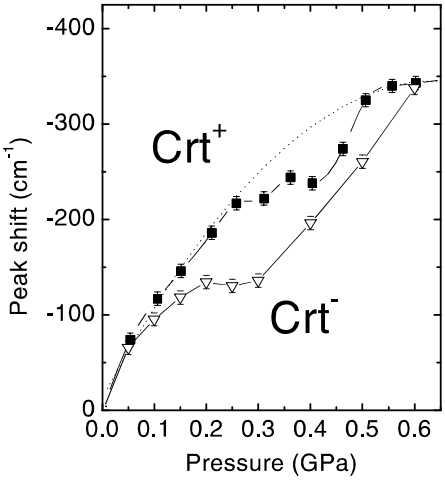

Fig. 3. Comparison of the room temperature pressure sensitivity of the Q_y absorption transition of the primary electron donor, P, for RCs containing (solid squares) and lacking (open triangles) a carotenoid molecule. The dotted line is present only to aid the reader.

tion on the conformation of and intermolecular interactions assumed by the chromophores under study, which are influenced by the surrounding protein structure [9]. When using an excitation wavelength of 1064 nm, FT-Raman spectra of RCs from purple bacteria produce highly selective information on the strongly excitonically coupled primary electron donor molecules due to the pre-resonant signal enhancement effect [27,28].

Prior to using Raman spectroscopy that requires up to many tens of minutes of data collection a further high-pressure absorption measurement was performed. The goal of this experiment was to evaluate the long-term stability of the Q_y transition of the primary electron donor (P) at 0.6 GPa pressure. In agreement with the first pressure experiment (see Fig. 1b) there was no apparent loss of intensity of P (Fig. 4) or integrated area (not shown). This is in contrast to that of R26.1 where, at elevated pressures, a progressive loss of the intensity of this electronic transition was observed (see [12] and Fig. 4).

An in-depth analysis of the Raman signature of the RCs from *Rb. sphaeroides* R26.1 has been discussed previously [12]. Fig. 5 displays the high-frequency carbonyl region of the FT-Raman spectra of the carotenoid-containing RC as a function of applied hydrostatic pressure. In this region, at atmospheric pressure, the R_1 mode (1607 cm^{-1}) arises from the stretching modes of the methine bridges of both P_L and P_M chromophores. Four stretching modes, other than the R_1 mode, have been identified as follows [22,28–30]. The two bands at 1620 and 1652 cm^{-1} arise from the stretching modes of the acetyl carbonyl groups of P_L and P_M, respectively. The last two bands at 1679 and 1692 cm^{-1} are due to the stretching modes of the keto carbonyl groups of P_M and P_L, respectively. All these vibrational modes have been extensively studied both in vitro and in vivo and it is well established that their frequencies primarily depend on the strength of the intermolecular interactions that the carbonyl groups are involved in [8,9,31]. As with the carotenoid-less RC, upon applying external pressure, the frequencies of three out of four bands in Fig. 5 remain relatively unchanged. However, the acetyl mode of

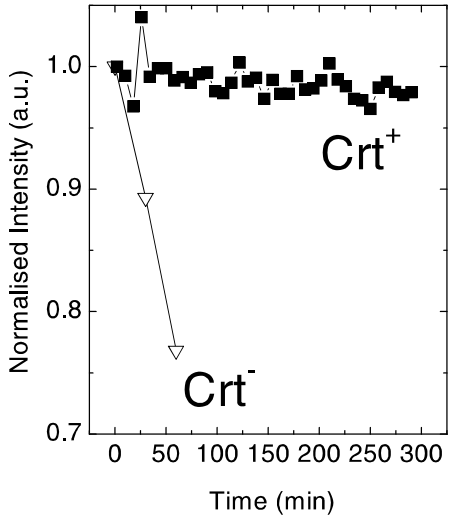

Fig. 4. Comparison of the temporal evolution of the peak intensity of the Q_y electronic transition of P at 0.6 GPa (normalised at $t = 0$ min) for RCs containing (solid squares) and lacking (open triangles) a carotenoid molecule.

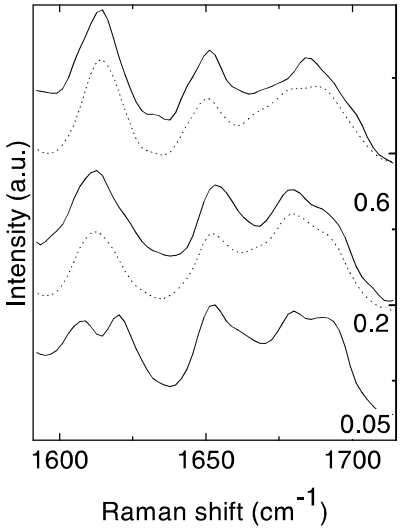

Fig. 5. The effect of pressure on the carbonyl high-frequency region of the room temperature FT-Raman spectrum of bacteriochlorin molecules in the RC complex. The numbers at each curve indicate the pressure in GPa at which the different spectra were recorded. For comparison, the FT-Raman spectra of Crt^- RCs under pressure (dotted spectra) are also plotted, see also [12]. The excitation wavelength was 1064 nm.

P_L, the only one of the above-mentioned four modes that is directly involved in hydrogen bond with the protein surroundings in the RC structures under study, rapidly (at pressure of c. 0.2 GPa) downshifts from 1620 to 1616 cm^{-1}. This reflects an increase in the intermolecular interaction between the acetyl of P_L and its histidine (His_{L168}) partner. The FT-Raman signatures of the compressed RC proteins from strains 2.4.1 and R26.1 [12] are thus nearly identical. One may hence conclude that based on the Raman spectra, the structure surrounding the primary electron donor in each protein displays a similar behaviour as a function of applied pressure. It is worth noticing, however, that the long integration time of the FT-Raman measurements may smear out some of the differences. So, the physical reasons of the spectral changes of the primary electron donor under compression await further investigations.

It is clear that the chromophore molecules in the bacterial RC can be successfully employed to study local responses of proteins to external high hydrostatic pressure. Based on this present work, we may conclude that the decrease of the intensity of the P electronic transition in the RC from strain R26.1 is directly due to the void in the structure caused by the absence of the carotenoid molecules which probably allows a limited reorganisation of the structure of P, which in turn affects its electronic structure. This indicates that any water and/or detergent molecules which are found in this cavity do not provide a degree of equivalent compactness as that provided by the natural carotenoid (spheroidene) cofactor. There are many other RC mutants: some have smaller internal cavities but sometimes closer to P. In the future, the use of these mutants will help in further characterising the role of internal cavities with respect to protein and electronic structure/stability in the hydrophobic phase.

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