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Estimating Hydrogen Bond Energy in Integral Membrane Chromoproteins by High Hydrostatic Pressure Optical Spectroscopy

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1. Introduction

Proteins are biological macromolecules that participate in virtually every process in live organisms. By nature they are divided into three categories: globular, membrane, and fibrous proteins. The globular proteins are present in the cytosol of cells and in body fluids such as blood; the membrane proteins are “solubilized” in two-dimensional lipid membranes that organize the macroscopic body space; the, usually, large fibrous proteins reinforce membranes and maintain the structure of cells and tissues. In the cell the membrane proteins are responsible for structural, catalytic, transport, signalling, control, and other crucial life-supporting functions (Palazzo 2006). Protein function is defined by its folded structure (Rose and Wolfenden 1993) and the self-assembly into the folded structure is largely governed by a manifold of generally weak, spatially oriented hydrogen (H) bonds that also confer distinct quantum properties (Li, Walker et al. 2011). Multiple H-bond interactions are responsible for binding the strands of RNA, DNA, and other biopolymers together, as well as for elasticity of skeletal and cardiac muscles. Cooperativity of the H-bond interactions appears to be a defining feature at all levels of biomolecular folding and function (Lu, Isralewitz et al. 1998; Finkelstein and Ptit syn 2002; Lin, Mohammed et al. 2011). Proteins are only functional if associated with water, but unlike the dynamic network of H-bonds in the bulk solvent, the interactions with biomolecules are short-range, affecting only one or two layers of waters (Ball 2008). Although there is no doubt that H-bonds are extremely important structural elements of proteins, their role in stabilizing proteins is still a matter of debate. Studying H-bonds within proteins, particularly membrane bound proteins, therefore, permits insights into fundamental biological phenomena, indeed all of life on earth.

Hydrogen bond energies in simple model compounds and small peptides have been investigated in great detail (Sheu, Yang et al. 2003; Wendler, Thar et al. 2010). Hydrogen bonds in folded globular proteins, and especially in membrane proteins, are, however, much more difficult to characterize. The most frequently used methods are scanning calorimetry
and titration with chemical denaturants such as urea. Both these methods probe unfolding of the whole protein and provide integrated rather than bond-specific information. Moreover, changing the temperature at constant pressure causes simultaneous changes of the system’s kinetic energy and its volume/potential energy that are difficult to separate. In this work we show that in certain cases the use of pressure, another fundamental thermodynamic variable, offers attractive advantages. Continuous and reversible tuning of the protein density can be achieved over a wide range without changing its primary structure and chemical potential of the solute. Hydrogen bonds are controversially reported to be either widely insensitive to pressure (Phelps and Hesterberg 2007) or promoted by it (Boonyaratanakornkit, Park et al. 2002). We use native protein co-factors as intrinsic probes and optical spectroscopy as a sensitive tool for identifying specific H-bonds that undergo major changes under external compression as well as for studying the energetics of these bonds.

The light harvesting (LH) antenna pigment-protein complexes of purple photosynthetic bacteria are one of the best-characterized membrane chromoproteins (Blankenship, Madigan et al. 1995; Hunter, Daldal et al. 2008). They absorb solar photons and transfer the resulting excitations to the reaction center (RC) special pair sites, where the excitation energy is transformed into potential chemical energy. In the wild type (wt) purple bacterium Rhodobacter (Rb.) sphaeroides the photosynthetic apparatus is organized into spherical chromatophore vesicles of ~50 nm diameter (Hu, Ritz et al. 2002; Cogdell, Gall et al. 2006; Sener, Olsen et al. 2007; Sener, Strümpfer et al. 2010). The reaction center light-harvesting 1 core complex and peripheral light-harvesting 2 protein complexes (RC-LH1 and LH2, respectively) form the majority of the proteins in these vesicles, where the self-assembled photosystems of LH complexes are reminiscent of ordered two-dimensional crystals. Low resolution electron microscope and full atomic resolution crystal structures of the protein complexes have also been known for some time for a number of species (Karrasch, Bullough et al. 1995; McDermott, Prince et al. 1995; Koepke, Hu et al. 1996; Roszak, Howard et al. 2003; Qian, Hunter et al. 2005). The basic building block of these antenna structures is a heterodimer of α-helical polypeptides designated α and β (coloured yellow and magenta in Fig. 1), each non-covalently binding either two (LH1) or three (LH2) bacteriochlorophyll a (Bchl) chromophore co-factors along with carotenoid molecules. The number of heterodimers in the structures of bacterial core complexes vary from 15 in the bacterium Rhodopseudomonas palustris (Roszak, Howard et al. 2003) or 16 in Rhodopseudillum rubrum (Karrasch, Bullough et al. 1995) to 28 in the dimeric core complex of Rb. sphaeroides (Qian, Hunter et al. 2005; Bullough, Qian et al. 2009). In contrast, only two forms of the peripheral antenna complex are known, including 8 (as in Rhodospirillum molicianum (Koepke, Hu et al. 1996)) or 9 heterodimers (Rb. sphaeroides, Rhodopseudomonas (Rps.) acidophila, and Rubrivivax gelatinosus (McDermott, Prince et al. 1995; Walz, Jamieson et al. 1998; Ranck, Ruiz et al. 2001)). In LH2 from Rb. sphaeroides each polypeptide pair binds two Bchl molecules at the outer membrane surface and one molecule on its cytoplasmic side, forming two concentric pigment circles (B850 and B800, respectively) with Cs symmetry, see Fig. 1.

The spectroscopic properties of LH1 and LH2 chromoproteins have been extensively studied (Van Amerongen, Valkunas et al. 2000; Hu, Ritz et al. 2002; Cogdell, Gall et al. 2006). While free in organic solvents, the lowest singlet (Qy) electronic transition of Bchl is located in the near infrared region at ~775 nm (Grimm, Porra et al. 2006; Rätsep, Cai et al. 2011).
Significant red shifts of this transition are observed in the antenna systems, for example, the major absorption band in the LH2 complex from Rb. sphaeroides peaks at 850 nm (B850 band, see Fig. 2) (Van Dorssen, Hunter et al. 1988; Freiberg, Rätsep et al. 2011), while the spectroscopic equivalent of the α-(Bchl)₂ heterodimer subunit called B820, obtained by breaking down the LH1 complex, has a maximum at 820 nm (Loach and Parkes-Loach 2008). These large spectral shifts are primarily related to unique arrangements of the Bchls in the LH proteins, promoting strong inter-pigment (exciton) interactions. The B850 absorption band is the product of the 18 tightly packed and overlapping Bchl molecules on the periplasmic side of the complex, in a waterwheel arrangement, having intermolecular distances of less than 1 nm. The 9 monomeric Bchls of the other ring on the cytoplasmic side of the complex, which are widely separated (~2 nm), give rise to an absorption band at around 800 nm (B800 band). The B800 band shift from the molecular 775-nm absorption is mainly determined by interactions between the chromophores and the surrounding protein. Universal dispersion interactions aside, the factors that contribute most to the solvent/protein shifts are H-bonds to the C₃-acetyl carbonyl of the Bchl chromophores (Fowler, Sockalingum et al. 1994; He, Sundstrom et al. 2002; Uyeda, Williams et al. 2010) and various conformational interactions (Gudowska-Nowak, Newton et al. 1990).

The absorption spectra of Bchl molecules, being sensitive to even minor structural rearrangements in the LH complexes, have been taken advantage of in the present work. According to Lesch et al. (Lesch, Schlichter et al. 2004) the integrity of protein complexes can be monitored with sub-nanometer spatial resolution by the so-called molecular probe method.

**Fig. 1.** Different representations of the LH2 pigment-protein complex using the same colour code for structural elements. The space fill model on the right clearly shows how densely packed the molecule actually is; the ribbon representation on left allows easy reference to the individual pigment cofactors; placed in the middle is an overlapped pair. The lipid bilayer covering hydrophobic parts of the complex is also schematically presented. The green B850 and blue B800 Bchl cofactors are sandwiched between the inner yellow ring of α-polypeptides and the outer magenta ring of β-polypeptides. The carotenoid molecules closely attached to the B850 and B800 Bchl rings are omitted for clarity.

To date mostly only water-soluble globular proteins have been studied under high pressures, with the result that functional protein structures cover just a narrow region in the pressure-temperature phase diagram close to physiological temperatures (Silva and Weber 1993; Boonyaratankornkit, Park et al. 2002; Scharnagl, Reif et al. 2005; Meersman, Dobson et al. 2006). The photosynthetic LH membrane complexes were first investigated under high

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Pressure in (Freiberg, Ellervee et al. 1993). This and subsequent studies (Sturgis, Gall et al. 1998; Gall, Ellervee et al. 2001; Timpmann, Ellervee et al. 2001; Gall, Ellervee et al. 2003) have shown that increasing the pressure at laboratory temperatures causes a smooth red (i.e., lower-energy) shift and broadening of the near-infrared absorption bands of Bchl. In the wt LH2 complexes from \textit{Rh. sphaeroides} and \textit{Rps. acidiphila} extracted with mild detergents into a detergent-buffer environment a non-monotonic behaviour was observed (Kangur, Timpmann et al. 2008). With reference to genetic engineering results (Fowler, Visschers et al. 1992; Fowler, Sockalingum et al. 1994) this observation was qualitatively interpreted as due to rupture of the H-bonds at the binding sites of the excitonically coupled B850 chromophores (Kangur, Leiger et al. 2008).

In this chapter, we shall concentrate on a quantitative evaluation of H-bond energies that stabilize the strongly coupled Bchl co-factors in the B850 ring of the peripheral LH2 complex from \textit{Rh. sphaeroides} by means of high-pressure optical spectroscopy. The availability of different genetically-modified preparations is an important advantage of this bacterium, besides its known spatial structure (Walz, Jameson et al. 1998). The mutants constructed to support and widen our findings on wt complexes include a LH2 complex with a modified carotenoid background (neurosporene instead of a mixture of spheroidene and spheroidenone in the wt complex) and two B850-only LH complexes devoid of the B800 ring of Bchl molecules, one with wt carotenoids and another with neurosporene. To confirm that studies of purified membrane proteins have relevance to the intact membrane, the measurements have been performed both on detergent-isolated and native membrane-bound complexes. As a result, not only pressure-induced breakage of the H-bonds to the B850 chromophores have been demonstrated in all the samples, confirming the previous results on wt complexes (Kangur, Leiger et al. 2008; Kangur, Timpmann et al. 2008), but also their energies have been determined for the first time. The breakage appears to be a cooperative, “all-or-none” type transition, apparently triggered by the rupture of the weakest bond. Furthermore, the energies derived for the membrane-embedded and detergent-isolated complexes match each other within experimental uncertainty.

2. Thermodynamic aspects of protein stability against pressure

The phenomenological basis of protein stability against pressure is well established (Silva and Weber 1993; Boonyaratanakornkit, Park et al. 2002; Scharnagl, Reif et al. 2005; Meersman, Dobson et al. 2006). In a minimalist version of thermodynamic modelling just two global protein states, native (N) and denatured (D), are assumed. The equilibrium constant of the two-state denaturation reaction is given by Eq. (1), where \([N]\) and \([D]\) indicate the equilibrium concentrations of native and denatured protein, respectively, \(R\) is the universal gas constant, \(T\) is the thermodynamic temperature, and \(P\) is the pressure.

\[ K(P) = [D] / [N] = \exp[-\Delta G(P) / RT] \]  

(1)

The pressure dependence of the free energy change associated with protein denaturation, in the lowest (linear) order of approximation, can be represented as

\[ \Delta G(P) = \Delta G^0 + \Delta V^0 P, \]  

(2)

\[ \Delta V^0 \]
where $\Delta G^0 = G_D^0 - G_N^0$ is the standard free energy difference between the denatured and the native state and $\Delta V^0 = V_D - V_N$ is the partial molar volume change in going from the native to the denatured state measured at standard conditions. $\Delta G^0$ has to be positive in order for the protein to be stable at standard conditions. If the volume of the denatured state is smaller than the volume of the native state (i.e., $\Delta V^0$ is negative), the free energy change decreases as pressure is increased. The phase boundary (midpoint) pressure is determined as $P_{1/2} = |\Delta G^0 / \Delta V^0|$. At pressure $P_{1/2}$, native and denatured structures have equal probabilities of existence. Past this pressure, the denatured state has lower free energy and is stabilized against the native state.

3. Absorption spectra of LH2 complexes under ambient conditions

Figure 2 presents the overview optical absorption spectra of the LH2 complexes in the spectral range from 240 nm to 1000 nm, recorded at ambient temperature and pressure. For solubilization and purification of the complexes from their native membrane environment the detergent dodecyl-dimethyl-amine oxide, commonly known as LDAO, was used. As seen, the spectra of the detergent-isolated and native membrane-bound complexes are similar. Peak positions of the key bands observed in the spectra are presented in Table 1.

![Absorption spectra of the wt and mutant LH2 complexes studied, recorded at ambient temperature and pressure. The spectra of detergent-isolated (drawn with coloured lines) and native membrane-embedded (black lines) complexes are normalized relative to the B850 absorption band peak. The reference spectrum of Bchl in diethyl ether is shown in magenta. Neuro designates the mutant containing neurosporene, while B850-only+neuro is the double mutant with no B800 molecules and containing neurosporene carotenoids.](image)
Table 1. Peak positions in nanometers (±0.5 nm) in the absorption spectra of the membrane-bound (m) and detergent-isolated (i) LH2 complexes recorded at ambient conditions. The bands are classified according to the related Bchl transitions as described in the text.

A comparison with the spectrum of Bchl in diethyl ether implies that the bands of the cofactor chromophores peaking around 850 (B850 band), 800 (B800 band), 590, and 380 nm are related to, respectively, the $Q_y$, $Q_x$, and Soret electronic transitions of the Bchl molecule. The bands evident between the $Q_x$ and Soret transitions are from carotenoid molecules, being a mixture of spheroidene and spheroidenone in wt and neurosporene in mutant complexes. Compared with the structure-less spectrum of the native mixture, the neurosporene spectrum is clear-cut and blue shifted, showing three sharp peaks between 430 and 490 nm. Replacement of the wt carotenoids with neurosporene does not significantly influence the electronic states of the Bchl co-factors. The blue-shifted position of the neurosporene spectrum allows easier observation of the $Q_x$ transitions of the protein-bound Bchls. The ultraviolet part of the spectrum, including the peak at 270 nm, is characteristic of the bulk protein scaffold.

The $Q_y$ transitions of LH2 Bchls, which give rise to the B800 and B850 bands, are shifted toward longer (red) wavelengths and are associated with strongly excitonically coupled Bchls in the case of B850. In contrast, the $Q_x$ transitions of the Bchl molecules belonging to the B800 and B850 arrangements apparently overlap. This can be interpreted as arising from the relatively weak oscillator strength of the $Q_x$ transitions, leaving the transitions in all participating molecules almost localized. The stronger B850 exciton coupling compared with B800 clarifies not only the splitting between these bands but also the larger width of the B850 band (Freiberg, Timpmann et al. 1999). A peculiar triple structure in the Soret range of the complexes suggests considerable interactions between the respective strong molecular transitions and related large splitting of the bands. A greater red shift of the B850 band in the B850-only mutant as compared with the wt complex has been noted. It was explained by
a somewhat enhanced exciton coupling in this complex, presumably because the missing B800 molecules allow more compact packing of the protein (Timpmann, Trinkunas et al. 2004). A weak shoulder around 795 nm in the spectrum of the B850-only mutant is most probably due to overlapping transitions of the B850 (vibronic) excitons and possibly the presence of some “free” Bchl molecules rather than residual B800 molecules (Rätsep, Hunter et al. 2005). More details about spectral properties of the LH2 complexes and their temperature dependencies can be found in (Freiberg, Rätsep et al. 2011).

In the following sections we shall mainly focus on the lowest-energy B850 absorption band. The particular interest toward this absorption feature is explained by the central role the respective transitions play in native photosynthesis by mediating excitation energy funnelling into the RC. Since Bchl molecules from both the B850 and B800 rings contribute to the Qy band at 590 nm, we shall briefly characterize the influence of pressure on this band too. The detailed behaviour of the quasi-monomeric B800 band under high pressure will be described elsewhere.

4. Variations of the B850 absorption spectra induced by externally applied high hydrostatic pressure

4.1 Detergent-isolated complexes

As noted above, a non-trivial dependence of the B850 absorption band was observed upon application of high hydrostatic pressure to isolated wt LH2 complexes from Rb. sphaeroides. This behaviour was related to the rupture of H-bonds at binding sites of the excitonically coupled B850 chromophores (Kangur, Leiger et al. 2008; Kangur, Timpmann et al. 2008). A comparable situation is demonstrated in Fig. 3 for the mutant LH2 complexes, implying the general applicability of this phenomenon. At low pressures, below ~0.4 GPa, the spectra gradually red shift and broaden. The initial shift and broadening rates of the B850 band, which are similar in wt and mutant species, are a few times greater than that of the B800 band or the Qy absorption band of isolated Bchl molecules in normal solutions. This is a consequence of the Bchl excited states in antenna complexes having excitonic origin. A contribution of short-range electron overlap effects into the advanced shift rate cannot be excluded (Freiberg, Ellervee et al. 1993; Wu, Rätsep et al. 1997; Timpmann, Ellervee et al. 2001). Toward higher pressures the continuous red shift is interrupted and abruptly reversed to a blue shift, best seen in Fig. 3c, where the B850 band maximum is plotted as a function of pressure. The reverse trend occurs at different pressures in individual samples: ~0.4 GPa in the neurosporene mutant, ~0.5 GPa in the wt, and ~0.6 GPa in the B850-only mutant. Only at about 0.1 GPa higher pressures the red shift is restored, albeit generally with a different slope. The bandwidths (defined as full width at half maximum, FWHM) show correlated changes (see Fig. 3d).

Enhanced stability of isolated complexes was observed (Kangur, Timpmann et al. 2008) at high protein/low detergent concentration, which resulted in aggregation, as well as when a cosolvent (e.g., glycerol) was added into the buffer solution. Aggregation occurred when the molar ratio (D/P) of the detergent (D) and protein (P) dropped below a few hundred (see Kangur, Timpmann et al. 2008) for determination of this ratio). The combined glycerol-aggregation effect of stabilization is demonstrated in Figs. 3c and 3d on the mutant complexes incorporating neurosporene, where D/P=100 and the complex is solubilized in a
buffer that contains 60 % (by volume) glycerol. As seen, the discontinuities of the shift and width dependences move toward significantly higher pressures, and their amplitudes are considerably reduced.

![Graphs showing absorption spectra and pressure dependence](image)

Fig. 3. (a, b) Absorption spectra of the detergent-solubilized neurosporene (a) and B800-only (b) mutant LH2 complexes recorded at the indicated pressures in MPa. Arrowed lines connect the selected absorption maxima. (c, d) Pressure dependence of the B850 absorption peak position (c) and width (d). Solid lines are added as guides to the reader. D/P denotes the ratio of detergent and protein molecules.

Figure 4 demonstrates the recovery of the absorption spectra of isolated complexes, upon pressure release, at the end of the cycle of measurements at elevated pressures. Two major effects following decompression are a change of the spectral shape and overall decrease of intensity. While the latter decrease is unavoidable due to plasticity of the gasket and resulting leakage of the solution (see Experimental section), the transformation of the spectrum is a signature of a partial degradation of the sample. A difference spectrum obtained by subtracting the normalized final spectrum from initial spectrum provides an approximate spectrum of the degradation products. The deformed long red tail aside, this spectrum shown in green in Fig. 4b and peaking at 776 nm is referred to in the literature as the B777 band. It has been identified as a mixture of monomeric α- and β-apoproteins that non-covalently bind just a single Bchl molecule (Loach and Parkes-Loach 2008) (see also Introduction). Reasonable sample homogeneity can be derived from the fact that the spectrum of the surviving complexes almost overlaps with the initial spectrum (not shown). Pressure effects appear to be fully elastic for this sub-population of the ‘fittest’ complexes, while representatives of the ‘less healthy’ population(s) fall apart entirely upon decompression.
4.2 Native membrane-bound complexes

Having established that the LH2 complexes purified from the native membrane with mild detergents exhibit clear signs of volatility with respect to high pressure, we undertook a series of experiments on membrane-bound LH2 complexes. It was concluded earlier that the wt membrane-protected complexes are rather resilient to damage by high-pressure compression compared with the isolated complexes (Kangur, Timpmann et al. 2008).

The results of the B850 absorption band shift measurements on wt and mutant membrane-bound LH2 complexes are shown in Fig. 5. Apart from the neurosporene mutant, the remaining two membrane samples in pure buffer solution behave rather uniformly, the main pressure effect being a gradual red shift (and broadening) of the band. These dependences, which demonstrate essential stabilization of the complexes in the native membrane environment, can be reasonably well approximated by two linear components, which have a greater low-pressure slope and a smaller high-pressure slope. The break point between the slopes is at ~0.7 GPa.

In contrast, the neurosporene mutant membrane complexes in buffer solution perform in every sense like their isolated counterparts in Fig. 3c, except that the turning point is moved by ~0.2 GPa toward higher pressures, indicating a degree of stabilization. Since the stability variations of proteins against externally applied pressure generally correlate with the extent of water molecules penetrating into the hydrophobic interior (Chryssomallis, Torgerson et al. 1981; Collins, Hummer et al. 2005; Harano, Yoshidome et al. 2008), it appears plausible that altering the native mixture of spheroidene/spheroidenone carotenoids to neurosporene results in less compact structure, which is more accessible to water than the wt protein.
Glycerol is known as an effective cryoprotectant; its ability to protect against high-pressure denaturation of detergent-isolated LH2 complexes has also been confirmed (Fig. 3, see also (Kangur, Timpmann et al. 2008)). However, the stabilizing effect of glycerol on native membrane-embedded LH2 complexes demonstrated in Fig. 5 has not been observed before and comes as a surprise. While in the case of the wt membrane adding the glycerol only slightly modifies the dependence, it almost neutralizes the destabilizing effects of neurosporene on the mutant complex in the membrane.

4.3 Comparison of the high-pressure effects on isolated and native membrane-bound LH2 complexes

Figure 6 shows a comprehensive summary of the B850 absorption band relative peak shift and broadening data for the complexes studied, using the peak position as well as the width of wt membrane complexes in glycerol as reference points. In this “free from background” representation we first compensated for the initial, ambient pressure positional differences (see Table 1). Thereafter, the relative shifts were calculated as \( \Delta \nu = \nu_i - \nu_r \), where \( \nu_i \) and \( \nu_r \) denote the peak frequencies of the studied sample (i) and the reference (r) membrane sample. A blue shift of the studied spectrum with respect to the reference spectrum thus results in a positive-valued \( \Delta \nu \), while a red-shift, results in a negative-valued \( \Delta \nu \).

The analytical line shape functions greatly simplify bandwidth analysis. We have found that Gaussians reasonably fit the experimental absorption spectra of LH2 complexes at ambient temperatures. In this approximation the relative widths have been evaluated as

\[
\Delta \delta = \left( \Gamma_i^2 - \Gamma_r^2 \right)^{1/2},
\]

where \( \Gamma_i \) and \( \Gamma_r \) are the corresponding FWHM of the B850 absorption bands. The fact that \( \Gamma_i \) is invariably greater than \( \Gamma_r \) implies that the membrane complexes are more ordered than those in detergent micelles.
Fig. 6. Relative peak shifts (a, b) and widths (c, d) of the B850 band in various isolated (a, c) and membrane (b, d) LH2 complexes indicated. The shifts and widths are calculated against the wt membrane in glycerol reference values. In isolated samples, similar molar ratio of the detergent and protein molecules (D/P \( \approx 2000 \)) has been applied for proper comparison. Solid lines are added as guides to the reader. See text for further details.

Characteristic step-like dependences resembling titration curves of the relative shifts and widths are obtained for all the complexes studied. The data in Fig. 6 that correspond to wt and mutant complexes demonstrate variable thresholds and step heights. In terms of the transition midpoint pressure, explained in paragraph 2, the neurosporene mutant complex appears least resistant to applied pressure, followed by the wt and B850-only mutant complexes. It also demonstrates the largest steps. Similar general trends can be observed for isolated and membrane complexes. However, the midpoint pressures that characterize individual membrane complexes are significantly shifted toward higher pressures and the step heights are generally lower compared to those of their respective isolated complexes. The dependences corresponding to membrane samples are also less noisy than for isolated complexes. This is to be expected as native membranes provide a more protective and less heterogeneous environment for antenna complexes than detergent micelles.

Spectral bandwidths are considered as sensitive reporters of static as well as of dynamic disorder present in antenna complexes. As can be seen in Fig. 6, the steps of relative shift and width correlate with each other, implying a common physical origin. The relative broadening of the spectra indicates an increased freedom of movement of the Bchl molecules in the binding sites of isolated complexes compared with the situation in the glycerol-enhanced membrane environment taken as a reference. Since the pressure-induced denaturation of proteins is driven by a decrease in volume, resulting in penetration of the buffer solvent (essentially of electrically polar water molecules) into the protein interior,
rather than swelling of the protein (Chryssomallis, Torgerson et al. 1981; Collins, Hummer et al. 2005; Harano, Yoshidome et al. 2008), the extra freedom of intra-protein movements can only be caused by breaking the bonds that help bind the Bchl molecules in their protein pocket. Given that axial ligation has a minimal effect on the Bchl Qy band position (Ellervee and Freiberg 2008), the loss of H-bonds in isolated complexes is the most likely explanation of the observed abrupt changes of the absorption bands with pressure.

This interpretation, first provided by Kangur et al. for the wt LH2 complexes from *Rb. sphaeroides* (Kangur, Leiger et al. 2008), is based on the observation that a blue shift of the B850 absorption band can be achieved by genetic manipulation of the H-bonds anchoring the Bchl molecules to the αβ-protein heterodimers (Fowler, Visschers et al. 1992; Fowler, Sockalingum et al. 1994). The α44-Tyr and α45-Tyr residues normally form H-bonds to the C3-acetyl carbonyls of the α- and β-Bchls, respectively, in the B850 ring. It was shown that removal bonds to the α45-Tyr residues by replacing Tyr to non-H-boding residues in the LH2 complex correlates with the blue shift of the B850 absorption band by 11 nm (or ~150 cm⁻¹). The blue shift corresponding to removal of all H-bonds is 26 nm (~360 cm⁻¹). The latter blue shift of the double H-bond mutant almost coincides with the relative shift obtained in Fig. 5a for the wt isolated complexes (~367 cm⁻¹), implying the high-pressure induced breakage of every C3-acetyl carbonyl H-bond in the B850 array.

The accompanying broadening of the spectra, an indication of increased freedom of movements of the Bchl probe molecules in their binding sites, supports this explanation. When bond ruptures increase the disorder of the system, they simultaneously widen the spectrum (Fowler, Visschers et al. 1992; Fowler, Sockalingum et al. 1994), thus the broadening effects are to be expected. Due to macroscopic heterogeneity of the samples (see discussion concerning Fig. 4) the broadening generally begins at somewhat lower pressures than the shift.

The step heights of relative shifts measured for the three isolated complexes in Fig. 5a vary between 410 and 292 cm⁻¹. The step height of about 410 cm⁻¹ in the neurosporene membrane complex (Fig. 5b) fits the same range. The broad, >100-cm⁻¹ variation (410-292=118 cm⁻¹), may be considered as too large to be related to the same shift/broadening mechanism in all these samples. However, taking into consideration the vast diversity of structural adjustments available upon compression, such as rotations of the acetyl carbonyl groups or turning the Bchl planes relative to the vector connecting the centres of the adjacent molecules in the B850 array, this figure is hardly exceptional. Moreover, we will show that the discrepancy between step heights in the LH2 complexes from the same species but having different D/P ratios is almost as great (~90 cm⁻¹). We thus argue that the relative shifts measured for the three (wt, B850-only, and neuro) isolated complexes as well as for the neurosporene membrane complex are not only quantitatively similar but also most probably due to the same mechanism. A similar conclusion, however, cannot be drawn with respect to other membrane complexes (wt, B850-only, B850-only+neuro, and glycerol-enhanced neurosporenc), which show step heights that are less than half the size. According to genetic engineering results (Fowler, Visschers et al. 1992; Fowler, Sockalingum et al. 1994), this might imply breakage of every second H-bond rather than all of those to the C3-acetyl carbonyls of the Bchl molecules in the B850 ring.
5. Evaluation the B850 chromophore-binding hydrogen bond energies

The use of the equilibrium thermodynamic stability model described in paragraph 2 to the present high-pressure experiment is justified by the observed reversibility of the pressure-induced effects. We define the $N$ state as corresponding to the protein at ambient pressure, while the $D$ state, to its compressed phase with broken H-bonds. The connection with the spectroscopic experiment is established by calculating the pressure-dependent equilibrium constant for a reversible transition between the $N$ and $D$ phases as

$$K(P) = \frac{\Delta \nu(P) - \Delta \nu_i}{\Delta \nu_f - \Delta \nu(P)}$$,

where $\Delta \nu(P)$ is the relative absorption peak frequency shift at pressure $P$ (see Figs. 6a and 6b), and $\Delta \nu_i$ and $\Delta \nu_f$ are the relative shifts of the B850 absorption band measured at initial, pre-transition (i) and final, post-transition (f) pressures, respectively. Inserting Eq. (3) into Eq. (1) provides Eq. (4):

$$-RT \ln K(P) = \Delta \nu^0 + \Delta \nu^0 P$$.

Assuming $\Delta \nu_i = 0$, one gets a still simpler Eq. (5):

$$RT \ln \left( \frac{\Delta \nu_f}{\Delta \nu(P)} - 1 \right) = \Delta \nu^0 + \Delta \nu^0 P$$.

The linear pressure functions, Eqs. (4) and (5), allow easy determination of the key model parameters that characterize the $N \leftrightarrow D$ phase equilibrium in LH2 proteins: $\Delta \nu^0$ as the initial ($P = 0$) value, $\Delta \nu^0$ as the slope, and $P_{1/2}$ as the crossing point with the y-axis zero.

Experimental plots of $-RT \ln K$ as a function of pressure for the B850 absorption band in all the LH2 samples studied are shown in Fig. 7. The fairly linear dependences obtained around respective phase transition regions justify the applied linear approximation model. The fitting parameters evaluated from these dependences are presented in Table 2.

As we have already noted, the transition midpoint pressures, $P_{1/2}$, are usually larger in membrane bound (where they vary between 641 and 995 MPa) than in isolated complexes (445–621 MPa). The volume changes, $\Delta V^0$, which broadly follow the published volume changes of protein unfolding (Scharnagl, Reif et al. 2005), are generally greater for isolated (-51 to -71 ml/mol) than for membrane (-14 - -39 ml/mol) samples. Both the pressure and volume features agree with the general notion that membranes protect and stabilize integral membrane proteins. The volume changes are negative, meaning that compressed states are stabilized by high pressure. A straightforward explanation of the negative volume effect in connection with H-bonds is as follows: since the states with broken bonds are more compressible, their volume under high pressure is smaller than the volume with intact H-bonds.

Single H-bond energies in proteins between 2 and 25 kJ/mol (167-2090 cm$^{-1}$) have been reported (Sheu, Yang et al. 2003; Wendler, Thar et al. 2010), depending on the H-bond donor and acceptor as well as their environment. Most of the transition midpoint energies, $\Delta \nu^0$, in Table 2 fall in this range. Yet a few complexes (wt, B850-only) demonstrate significantly
Fig. 7. Pressure dependences of $-RT \ln K(P)$ (see Eq. (4)) for the samples indicated. Continuous lines represent linear fits of the scattered experimental data. The prefix i and m denotes the data for isolated and membrane complexes, respectively.

larger energies, reaching 41 kJ/mol. A closer inspection of the data in Table 2 reveals several notable details about the free energy change related to breakage of the H-bonds in the LH2 complexes: (i) its value for isolated complexes is greater than for membrane/glycerol-enhanced membrane complexes; (ii) it is greater in complexes with native carotenoid background (wt and B850-only) than in complexes, which contain neurosporene (neuro and B850+neuro); (iii) irrespective of the sample, its value in membrane/glycerol-enhanced membrane complexes is similar ($\leq$19 kJ/mol). The findings (i) and (ii) once again underline the importance of local environment and/or structure of the binding site, specifically the carotenoid background, in determining H-bond energies in the B850 ring.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Delta G^0$</th>
<th>$\Delta V^0$</th>
<th>$P_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kJ/mol</td>
<td>ml/mol</td>
<td>MPa</td>
</tr>
<tr>
<td>wt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i</td>
<td>39±2</td>
<td>-71±10</td>
<td>519±10</td>
</tr>
<tr>
<td>m</td>
<td>19±3</td>
<td>-23±10</td>
<td>815±15</td>
</tr>
<tr>
<td>B850-only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i</td>
<td>41±6</td>
<td>-67±10</td>
<td>621±10</td>
</tr>
<tr>
<td>m</td>
<td>12±5</td>
<td>-16±10</td>
<td>785±10</td>
</tr>
<tr>
<td>neuro</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m+g</td>
<td>24±5</td>
<td>-36±10</td>
<td>641±15</td>
</tr>
<tr>
<td>i</td>
<td>24±5</td>
<td>-51±10</td>
<td>445±10</td>
</tr>
<tr>
<td>B850-only+neuro</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m</td>
<td>23±7</td>
<td>-23±10</td>
<td>983±10</td>
</tr>
<tr>
<td>i</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2. Thermodynamic parameters characterizing phase transitions in isolated (D/P≈2000) and membrane LH2 complexes related to breakage of H-bonds in the B850 ring. m+g indicates the membrane in 60% glycerol; N/A means not available.
The neurosporene mutant complex appears to be unique. Only in this case do the energetic and volume effects that characterize the transitions in isolated and membrane complexes match each other within experimental uncertainty. However, when the neurosporene membrane complexes are stabilized by glycerol, their behaviour becomes similar to other membrane samples (see also Figs. 6 and 7).

6. Dependence on detergent-to-protein ratio

For sake of standardized comparison, a fixed D/P ratio equal to about 2000 has been previously used in the case of isolated complexes. Here, we investigate the possible dependence on this ratio, between 100 and 5000, of the phase transition energetics associated with the breakage of H-bonds in the B850 ring. The results of this study, in terms of the relative absorption peak frequency shift, are presented in Fig. 8 and Table 3. Also included for completeness are mutant membrane data, which effectively correspond to D/P=0.

A systematic decrease of the step height and shift of the midpoint pressure toward higher pressures is observed with decreasing D/P ratio. Nonetheless, the corresponding \( \Delta G^0 \) values are similar, within experimental uncertainty, due to compensating effects of volume and pressure (see Table 3). In the two membrane samples the step heights differ drastically. While in the m neuro case the height is comparable with that in isolated complexes, in the m neuro glycerol case, it is less than half the size. In terms of the free energy change, m is again similar to the isolated complexes, whereas m+g is very different.

![Fig. 8. Dependence on D/P ratio of the relative peak shifts of the B850 band in the spectra of isolated (i) and membrane (m) neurosporene mutant LH2 complexes. The shifts are calculated against the neurosporene mutant membrane in glycerol (in case of isolated complexes) or wt membrane in glycerol (in case of neurosporene mutant membrane in glycerol). Solid lines are added as guides to the reader.](www.intechopen.com)
Table 3. Thermodynamic parameters characterizing phase transitions in the B850 ring of isolated neurosporene mutant LH2 light-harvesting complexes as a function of D/P ratio. As in Table 2, m+g indicates the membrane in 60% glycerol.

<table>
<thead>
<tr>
<th>D/P</th>
<th>ΔG°</th>
<th>ΔV°</th>
<th>P_{1/2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>m+g</td>
<td>14±2</td>
<td>-14±5</td>
<td>995±15</td>
</tr>
<tr>
<td>m</td>
<td>28±5</td>
<td>-39±10</td>
<td>700±10</td>
</tr>
<tr>
<td>100</td>
<td>34±6</td>
<td>-65±10</td>
<td>528±10</td>
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<td>500</td>
<td>35±11</td>
<td>-75±25</td>
<td>461±10</td>
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<td>24±4</td>
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<td>445±10</td>
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<td>24±5</td>
<td>-51±10</td>
<td>445±10</td>
</tr>
<tr>
<td>5000</td>
<td>24±4</td>
<td>-56±10</td>
<td>425±10</td>
</tr>
</tbody>
</table>

Based on these carotenoid mutant studies, one may conclude that H-bond energies, as determined by high-pressure spectroscopy, do not depend significantly on detergent concentration over a broad (three orders of magnitude) D/P ratio. An analysis based on data published in (Kangur, Timpmann et al. 2008) leads to a similar conclusion regarding wt complexes. This is important as it provides evidence that the studies on isolated membrane proteins have direct relevance to the native membrane situation.

It is finally worth noting that the fairly sharp Q\textsubscript{x} band at around 590 nm shows no splitting upon high-pressure compression, despite being contributed to by Bchl molecules from both B850 and B800 origin. Yet, as seen in Fig. 9, there is a small blue shift and broadening of this band in correlation with the large blue shift and broadening of the Q\textsubscript{y} band, which is related to the B850 Bchl molecules. The Q\textsubscript{x} transition is known to be sensitive to axial coordination of chlorophylls (Rätsep, Linnanto et al. 2009; Rätsep, Cai et al. 2011). Pressure-induced formation of an extra (i.e. sixth) coordination bond to the central Mg atom would, however, lead to a spectral red shift (Ellervee, Linnanto et al. 2004; Ellervee and Freiberg 2008). We thus conclude that the small changes in the Q\textsubscript{x} spectrum are a reflection of the H-bond breakage in the B850 system.

7. Summary and conclusions

Protein function is governed by its folded structure, whereas denatured states are characterized by disordered conformations. Since the folded state is largely defined by H-bonds, their properties are profoundly important in our understanding of structure, stability, and also function of proteins. In the present chapter, experimental evidence is provided for an externally applied pressure induced rupture of the H-bonds that coordinate the Bchl chromophore cofactors with the protein scaffold in wt and various mutant peripheral LH2 light harvesting complexes from the photosynthetic bacterium Rb. sphaeroides. We have taken advantage of the Bchl cofactors which act as sensitive spectroscopic nano-probes of their protein binding sites. The mutant LH2 complexes with a modified carotenoid background and/or with a missing B800 ring of Bchls were constructed to support and extend the relevance of the data obtained on wt complexes. To confirm that the studies of purified membrane proteins have relevance to intact membrane situations, the measurements have been performed both on detergent-isolated and native membrane-bound complexes.
Abrupt spectral blue shifts have been observed in the pressure range of 0.45-1.00 GPa instead of usual gradual pressure-induced red shifts of the absorption and fluorescence emission spectra arising from the lowest-energy optical $Q_y$ and $Q_x$ transitions in the B850 ring of 18 Bchl molecules. The shifts are correlated with similar abrupt broadening of the spectra. For a number of complexes the spectra recover perfectly upon the release of the pressure, demonstrating reversibility of these pressure effects.

The most remarkable quantitative correspondence between the removal of single or double H-bonds in the basic unit of LH2 complexes by genetic engineering, on the one hand, and spectral shifts in membrane or isolated complexes induced by pressure, on the other, has been noted. This suggests a cooperative (“all-or-none” type) rupture of the H-bonds that bind the cofactors to the surrounding protein as the prime source of the observed spectral shifts. The increasing freedom of movements upon removal of the H-bonds to Bchls in their binding sites is the foremost cause of the spectral line broadening. A stepwise (“unit-by-unit”) mechanism predicts clear correlations between the energy/volume changes and the number of H-bonds involved, but these have not been observed. The cooperative process is most probably triggered by significant weakening and finally disruption of the so-called weak-link H-bonds. While concerted disruption of the H-bond network of water under pressure is well documented (Jonas, DeFries et al. 1976; Cunsolo, Formisano et al. 2009), evidence of similar reversible effects in proteins, particularly in membrane proteins has so far been scarce (Kangur, Leiger et al. 2008; Kangur, Timpmann et al. 2008).

![Fig. 9. Relative shifts of the $Q_y$ (B850) and $Q_x$ absorption spectra for neurosporene mutant membrane LH2 complexes. The shifts are calculated against the reference values of wt membranes in glycerol. Solid lines are added as guides for the reader.](www.intechopen.com)
2). The bond ruptures are accompanied by a decrease of the partial molar volume amounting 23±10 ml/mol and 71±10 ml/mol, respectively. Replacement of the native mixture of spheroidene/spheroidenone carotenoids with neurosporene significantly destabilizes the protein, so that the single and double bond energies are reduced to 14±2 kJ/mol and 24±5 kJ/mol, respectively. These results highlight the important role carotenoids play in reinforcement of the photosynthetic light-harvesting protein structures. The H-bond energies determined for the wt photosynthetic bacterial LH complexes are an order of magnitude larger than thermal energy under physiological conditions, explaining the great stability of these proteins both against temperature and pressure.

Comparable data are not available to the best of our knowledge; thus these results validate high-pressure optical spectroscopy as an effective non-invasive tool for the studies of bonding energetic and related structural integrity of integral membrane proteins with individual bond-mapping selectivity.

8. Experimental section

8.1 Samples

The LH2 membranes and complexes were prepared according to (Dawkins, Ferguson et al. 1988; Fowler, Visschers et al. 1992), respectively, and stored at liquid nitrogen temperature. The complexes have been removed from their native membrane environment and solubilized in micelles of the detergent (LDAO) that, above the critical micelle concentration, mimic the embedding of the proteins in native membranes. The detergent concentrations used, which have been elaborated above, resulted in no degradation of the complexes at ambient pressure. The experimental design also ensured that the detergent-solubilized complexes generally remained stable under elevated pressures for at least 10 hours, long enough for the present trials. The samples were thawed before the experiments and diluted with an appropriate buffer to obtain an optical density of about 0.3 of the B850 absorption band maximum in the assembled pressure cell. For complexes in their natural membrane environment no detergent was added. Colloidal properties of membrane proteins depend on the solvent acidity (Palazzo 2006). Therefore, for LH2 samples a 20 mM HEPES buffer, pH 7.5, was utilized since its buffering ability is preserved over a broad pressure range (Samaranayake and Sastry 2010). In some cases glycerol was added to stabilize the samples, to push the solvent solidification limit towards higher pressures, and to improve the solvent hydrostaticity in the solid phase.

8.2 High pressure spectroscopy

The absorption spectra of isolated and membranes complexes under ambient conditions were taken using a Jasco V-570 spectrophotometer (Jasco Corporation). The high-pressure spectroscopy system, based on a commercial diamond anvil cell (DAC D-02, Diacell Products Ltd.) has been previously described (Kangur, Timpmann et al. 2008). It allows absorption, fluorescence, fluorescence excitation, and Raman measurements to be performed in the same set-up. The DAC cell was equipped with a 0.35-mm thick stainless steel gasket, pre-indentened between the anvils under small pressure. A ruby-microbead pressure sensor (RSA Le Rubis SA), mounted directly in the sample volume, was used to determine the pressure inside the DAC. The temperature of the cell was maintained at 20±
0.5 °C during the experiment. No essential variations of the results were observed when the temperature was varied between 18 and 27 °C. The accuracy of the pressure measurements (defined as the pressure needed to shift the emission line at the output of the spectrograph by one pixel of the recording CCD camera) was 6.6 MPa. The pressure was increased stepwise with an average rate of 25-30 MPa per minute. Recurrent measurements ensured reproducibility of the data. Reversibility of the measurements was regularly checked by way of releasing pressure from its maximum value down to ambient pressure.

9. Acknowledgments

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10. References


Since the dawn of recorded history, and probably even before, men and women have been grasping at the mechanisms by which they themselves exist. Only relatively recently, did this grasp yield anything of substance, and only within the last several decades did the proteins play a pivotal role in this existence. In this expose on the topic of protein structure some of the current issues in this scientific field are discussed. The aim is that a non-expert can gain some appreciation for the intricacies involved, and in the current state of affairs. The expert meanwhile, we hope, can gain a deeper understanding of the topic.

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