The Effect of Pressure on the Bacteriochlorophyll *a* Binding Sites of the Core Antenna Complex from *Rhodospirillum rubrum*

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ABSTRACT: In this paper we examine the effect of pressure on the absorption spectrum and binding site of the core antenna complex from the photosynthetic bacterium *Rhodospirillum rubrum*. Absorption spectra and Raman spectra in preresonance with the Q_y transition of the bacteriochlorophyll *a* were studied at pressures up to 625 MPa. In agreement with previous work we observe a pressure-induced red shift and broadening of the absorption spectrum. We show that at these pressures the pigments within the protein matrix at room temperature experience little if any distortion, and the hydrogen-bonding network involving the C_2 and C_9 carbonyl groups of the pigment molecules are undisturbed. Having shown the lack of sensitivity to pressure of the binding site interactions, which are known to modulate the absorption spectrum, we feel that it is relatively safe to attribute the pressure-induced red shift broadly to solvatochromic effects and, in particular, to the modulation of the pigment−pigment interactions by the pressure. This paper represents the first vibrational study of photosynthetic complexes at high pressure and the first application of FT Raman spectroscopy to biological molecules at high pressure.

In purple photosynthetic bacteria, the energy of the light is funneled to the reaction centers by a series of interconnected antenna complexes. Most of these organisms synthesize two types of such proteins, the peripheral and core antennae (or LH2[1] and LH1, respectively), with the LH1 lying closer to the reaction center both physically and functionally (*1*). These complexes are composed of repetitions of a basic unit, itself containing two small (∼5 kDa) polypeptides, α and β. These polypeptides have been sequenced from a large number of species (*2*). Recently, the X-ray structures of the LH2 complex of *Rhodopseudomonas* (*Rps.*) *acidophila* 10050 (*3*) and *Rhodospirillum* (*Rsp.*) *molischianum* (*4*) were determined, and it was revealed that the LH2 complex of *Rps. acidophila* consists of 18 α-helical, membrane-spanning polypeptides. In this case, the 850 nm absorption arises from 18 strongly interacting bacteriochlorophyll *a* (Bchl) molecules within the transmembrane region, while the 800 nm absorption is due to nine weakly interacting, monomeric Bchl molecules, bound to the α polypeptide and located toward the cytoplasmic surface of the membrane (*3*). The electron crystallographic structure of *Rsp rubrum* LH1 (*5*) does not provide detailed information at an atomic level about this type of complex: however, it shows clearly that LH1 is composed of a larger ring-like structure consisting of 16 αβ pairs. The about 880 nm absorption band of this protein is thus arising from a ring of 32 interacting Bchl molecules.

The control of the absorption wavelength of light-harvesting proteins from purple bacteria has been the subject of many studies. While isolated Bchl, when monomeric, absorbs in most solvents at about 770 nm, the Bchl within LH complexes from purple bacteria exhibits absorption maxima between 800 and 890 nm, that is, with a near-infrared absorption maximum which may be red-shifted relative to the isolated pigment by as much as 1750 cm^{-1}. Moreover, the precise tuning of the absorption properties of these complexes seems to be involved both in the funneling of the energy toward the reaction centers (*1*, *6*) and in the adaptation of some of these photosynthetic organisms (*7*, *8*). It is now generally accepted that two different types of mechanisms underlie the red shift of the Bchl absorption in these proteins, namely the pigment−pigment interactions and pigment−protein interactions that tune the individual monomer absorption.

Various molecular mechanisms have been proposed as contributing to the protein-induced red shift of the Q_y absorption band in individual monomers, namely, distortions of the bacteriochlorophyll macrocycle; neighboring charged residues; hydrogen bonds between pigment functional groups and the protein; and effects of binding pocket polarizability (for review see ref *9*). Among these, the effects of hydrogen bonding between the protein carbonyl groups and pigments have been well documented experimentally in light-harvesting complexes (*10*−*15*) as well as the influence of the polarizability in the vicinity of the carbonyl groups (*16*). Unfortunately the importance of the remaining effects remains unclear, the assessment of their role relying on calculation and extrapolation from model compounds.

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[1] Abbreviations: BChl, bacteriochlorophyll *a*; FT, Fourier Transform; fwhm, full width at half-maximum; GPa, gigaPascal; kDa, kiloDalton; LH, light harvesting; MPa, megapascal; nm, nanometer; OD, optical density; *Rb*., *Rhodobacter*; *Rps*., *Rhodopseudomonas*; *Rsp*., *Rhodospirillum*; WT, wild type.

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Modulation of the Bchl absorption by altering pigment−pigment interactions within these complexes, although expected theoretically (*17*−*19*), has not yet been clearly demonstrated experimentally. Nevertheless, there is evidence that such interactions play an important role in determining the absorption of antenna complexes (*1*).

Various antenna pigment−protein complexes have been previously studied at high pressure, both at ambient temperature (*20*, *21*) and at cryogenic temperatures (*20*, *22*, *23*). These studies have shown that increasing the pressure causes a substantial red shift and broadening of the long wavelength Q_y band both in LH1 and in LH2. This red shift is generally much larger than that observed for monomeric Bchl molecules (*24*), as large as 725 cm^{-1} for LH1 brought to about 0.8 GPa (*21*). Indeed, in general, aggregated and red-shifted BChl molecules appear to show a greater pressure sensitivity than isolated pigment molecules.

In the work reported here, we have tried to understand whether this large pressure-induced shift of the absorption of the LH1 complexes is due to changes in the Bchl binding site structure or to alterations of BChl−protein and BChl−BChl interactions. For this we have used Fourier transform (FT) preresonance Raman spectroscopy, which yields information on both the conformation of the Bchl molecules (*25*) and intermolecular interactions assumed within antenna complexes (*26*). This paper constitutes the first vibrational study on photosynthetic complexes at high pressure, and the first application of FT Raman spectroscopy to study biological molecules at high pressure.

MATERIALS AND METHODS

Rsp. rubrum chromatophores of the carotenoidless strain $G9^+$ were prepared according to Cogdell et al. (*27*). Room-temperature absorption spectra of the membrane samples were determined for samples that had been diluted such that the maximum absorption of the LH1 complex lay in the range of 0.2−0.8 OD/cm. For FT Raman measurements, samples were prepared such that their maximum absorption reached about 100 OD/cm.

The pressure cell used in this work has already been described previously by Freiberg et al. (*22*). It consisted of a piston-cylinder type high-pressure optical cell with three sapphire windows. The sample under study was inserted into a separate sample cell. The sample cell has two windows, 2−3 mm apart, with one window fixed and another movable like a piston to adapt to the pressure variations. Pressure was generated inside the cell by a small hydraulic press and was transmitted to the sample by a liquid (glycerol−water) phase. This system has been shown to be able to reproducibly generate pressures up to about 1 GPa (*22*). Samples were allowed to equilibrate for at least 30 min after an increase in the pressure. Reversibility of the pressure-induced effects on LH1 proteins was checked systematically by measuring absorption and/or FT Raman spectra upon pressure release, after the pressure maximum had been applied.

FT Raman spectra were recorded at 4 cm^{-1} resolution using a Bruker IFS 66 interferometer coupled to a Bruker FRA 106 Raman module equipped with a continuous Nd:YAG laser. The setup, laser powers, and sample behavior are extensively described in Mattioli et al. (*28*). For pressure measurements, all spectra were recorded at room temperature with back-scattering geometry. Depending on the samples, spectra were the result of 1000−10000 co-added interferograms. Room-temperature absorption spectra, at atmospheric pressure and high pressure, were recorded on a Cary 5 spectrophotometer (Varian plc, Sidney). Peak widths were estimated from the difference between the positions of the absorption maximum and the low-energy half-absorption maximum.

RESULTS

The effect of increasing pressure on the room-temperature absorption spectrum of *Rsp. rubrum* $G9^+$ chromatophores is shown in Figure 1. These spectra are similar to those reported by Freiberg et al. (*20*) and Tars et al. (*21*). Also in agreement with previous studies, a large quasi-linear pressure-induced red shift and broadening of the absorption band can be seen. From these spectra, it is possible to estimate the pressure sensitivity (slope) of the position of the absorption maximum and the bandwidth (fwhm) as −916 ± 75 and 260 ± 43 cm^{-1} GPa^{-1}, respectively (see inserts in Figure 1; the errors indicate the 95% confidence limits). These values are again close to those previously published (*20*, *21*). It should be noticed that the this slope is strongly temperature dependent, being about two times smaller at 77 K when compared to the slope at ambient temperature (*20*, *23*).

To investigate more closely the mechanisms behind this effect, we obtained FT Raman spectra of the chromatophores at various pressures. This technique provides an accurate probe to the structure of the chromophore and its binding site (*26*); in particular it provides information on various pigment−protein interactions believed to be important for the absorption properties of the BChl molecules.

FT Raman spectra of LH1 complexes from *Rsp. rubrum* $G9^+$ were recorded at atmospheric pressure: 0.084, 0.195, 0.300, 0.410, 0.515, and 0.625 GPa. Table 1 displays the frequencies observed in these spectra at each pressure used. In Figure 2 we show the 400−1750 cm^{-1} region of the FT Raman spectra obtained at 84 MPa (lower trace) and 625 MPa (upper trace). It should be noted that the increase in pressure results in an increase in the resonance enhancement since the absorption band moves significantly closer to the laser excitation wavelength at 1064 nm. This absorption band displacement also results in a stronger fluorescence background, which in the spectra shown in Figure 2 was removed by fitting polynomial functions to the background signal and subtracting.

As may be observed in Figure 2, in FT Raman spectra of LH1 from *Rsp. rubrum* $G9^+$ the majority of the bands show small, but significant, pressure-induced displacements. These are summarized in Table 1, the last column of which indicates the pressure sensitivity of each observed mode together with a 95% confidence range for this value. It can be seen that all but the 1664 cm^{-1} C_9 keto carbonyl stretching mode show frequency upshifts of less than 10 cm^{-1} GPa^{-1}, while the C_9 keto carbonyl mode frequency shows a insignificant downshift.

Both upshifts and downshifts of vibrational frequencies upon application of hydrostatic pressure have been observed for organic molecules (*29*). A theoretical description of such

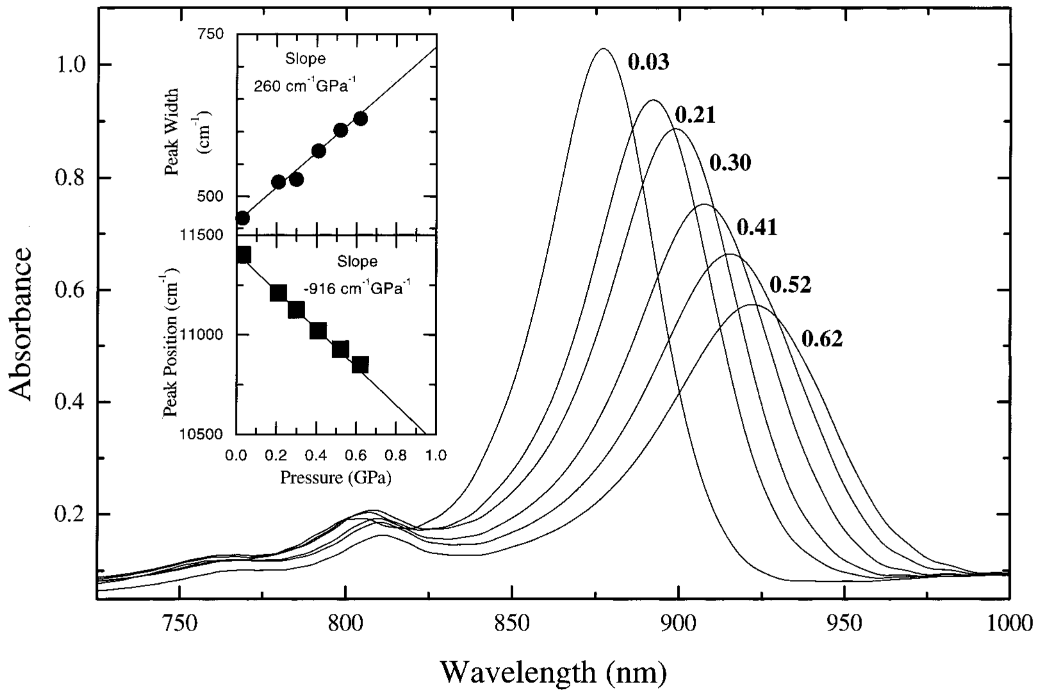

FIGURE 1: The effect of pressure on the Q_y absorption spectrum of chromatophores from *Rhodospirillum rubrum* strain G9$^+$. Main panel: absorption spectra of chromatophores at the different pressures used in this study. The figures indicate the pressure in gigaPascals at which the different spectra were recorded. Insets: pressure dependence of peak position and fwhm for the Q_y transition.

Table 1: FT Raman Frequencies Observed for LH1 Proteins from *Rsp. Rubrum* G9$^+$ at Various Pressures

pressure (GPa)							slope (cm^{-1} GPa^{-1})	slope error
0.0001	0.084	0.195	0.300	0.410	0.515	0.625		
183	184	185	185	186	187	188	7.44	1.18
290	291	291	292	294	295	295	8.81	2.16
344	346	346	347	347	348	348	5.69	2.22
418	418	418	418	418	419	419	1.71	1.26
567	568	568	569	569	569	569	3.01	1.65
682	683	684	684	685	685	685	4.71	1.83
729	729	730	730	731	731	732	4.76	1.06
752	751	751	751	752	752	753	2.10	2.50
775	775	776	777	777	778	778	5.42	1.29
794	795	796	797	797	797	797	4.69	2.46
897	897	898	899	900	900	901	6.79	1.21
949	950	950	951	951	951	953	5.07	2.08
1017	1018	1019	1019	1020	1020	1020	4.71	1.83
1028	1029	1030	1031	1031	1031	1031	4.69	2.46
1116	1116	1117	1117	1117	1117	1117	1.68	1.29
1141	1142	1144	1145	1145	1145	1146	7.41	2.97
1175	1175	1175	1176	1177	1178	1178	5.79	1.74
1255	1256	1257	1257	1257	1257	1256	1.63	2.96
1371	1372	1372	1373	1373	1373	1373	3.01	1.65
1385	1385	1385	1388	1388	1388	1388	6.10	3.41
1447	1447	1448	1449	1449	1449	1450	4.75	1.45
1539	1539	1540	1541	1541	1541	1542	4.75	1.45
1607	1606	1606	1607	1608	1610	1611	7.55	3.94
1643	1645	1644	1644	1644	1644	1644	0.27	2.45
1664	1665	1664	1664	1664	1664	1664	−0.71	1.46

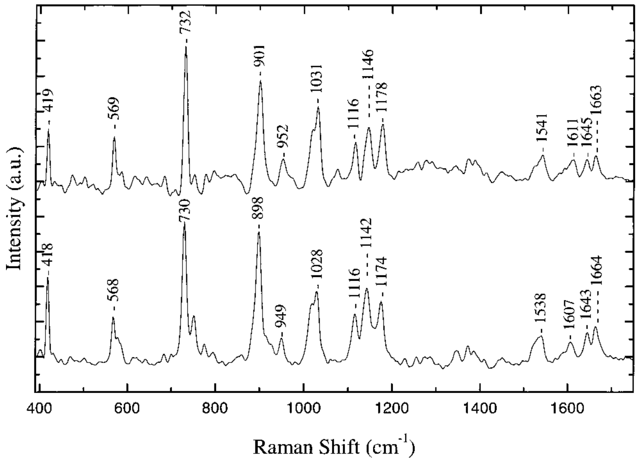

FIGURE 2: The effect of pressure on the FT Raman spectrum of the bacteriochlorophyll *a* molecules in the core antenna complex, obtained in pre-resonancce with the Q_y transition. The 400−1750 cm^{-1} region is shown at the lowest and highest pressures studied: bottom trace, 0.084 GPa; top, 0.625 GPa.

shifts for intramolecular vibrations (*30*) identifies two terms of importance: the first due to the effect of pressure on the harmonic force constant and the second due to the anharmonicity of the oscillator. If the first term is dominant, then an upshift/downshift of the vibrational frequency under pressure reflects a strengthening/weakening of the intramolecular bonds. Since the frequency shifts observed are small, less than 1% of the frequency of the relevant modes, the pressure-induced force constant changes are probably sufficient to explain them. A number of minor intensity changes are also observed with varying pressure. However, these are probably associated with changes in the resonance condition that result from the pressure-induced shift of the Q_y transition rather than direct effects of pressure on resonance enhancement.

The behavior of the frequencies of the various BChl vibrational modes active in FT preresonance Raman with macrocycle distortion has been recently systematically studied (*25*). These authors identified 6 modes sensitive to the macrocycle conformation, the sensitivity of which was sufficient to ascribe conformational changes of the macrocycle resulting from atomic displacements as small as 1 pm (*25*). For pentacoordinated BChl molecules in diethyl ether, these modes are observed at 1609, 1536, 1445, 1159, 1140, and 1017 cm^{-1}. Of these modes, five modes with slightly changed frequencies (1607, 1539, 1175, 1141, and 1028 cm^{-1}) are clearly visible in the protein spectra presented in Figure 2. A weak band near 1445 cm^{-1}, though visible, is at the lower limit of intensity for its position to be accurately measured. Unfortunately the presence of reaction centers in our membrane preparation precludes the use of the methine bridge mode near 1607 cm^{-1} for diagnostic purposes, since this mode is significantly affected by the reaction center contributions (*15*) and shows complex changes in shape and position with pressure. It is probable that the changes we observe here result from alterations in the relative contributions of reaction centers and antenna complexes as the resonance conditions change due to the various pressure-induced red shifts of different absorption transitions.

The frequencies of the four remaining modes all show pressure-induced displacements; however none of them exhibits a sensitivity to pressure higher than that measured on average for the other bands present in the FT Raman spectra.

As has previously been discussed, the hydrogen-bonding environment of the BChl C_2 acetyl groups has a definite influence on the absorption spectrum of light-harvesting complexes from purple bacteria in general and, in particular, on the absorption spectrum of LH1. In the different systems studied, that is, the 800 nm absorbing transition of BChl in LH2 (*16*), the 850 nm transition of LH2 (*10*, *13*, *14*), and the 875 nm transition of LH1 (*11*, *15*), there appears to be a linear relationship between the hydrogen (H)-bond strength in which the acetyl carbonyl is involved and the red shift experienced by the absorption transition (*15*, *31*).

The frequencies of the Raman bands at 1643 and 1664 cm^{-1} (Figure 2 and Table 1) indicate the strength of the H-bonds in which both the C_2 acetyl and the C_9 keto groups of BChl are acting as acceptors (*26*). The H-bonds under discussion are those between the C_2 acetyl groups and the δNH of the tryptophan residues α_{+11} and β_{+9} (*11*, *15*) and between the C_9 keto group and the δNH of the liganding histidine residues (*12*, *32*). The frequencies of these two carbonyl bands are both almost insensitive to pressure up to 0.625 GPa.

DISCUSSION

To assess the possible effects of pressure on pigment macrocycle distortion and H-bonding interactions involving the C_2 carbonyl group of the Bchl molecules, we have measured the effects of pressure on the preresonance Raman spectrum. Of the four Raman bands that we highlighted above as being sensitive to the BChl macrocycle conformation, two show large displacements when their positions are compared in diethyl ether and LH1 protein (Näveke, A., unpublished results, ref *25*). These two bands are those at 1028 and 1175 cm^{-1} in Figure 2, with displacements of +11 and +15 cm^{-1} relative to their positions in the solvent. Such large displacements of the bands sensitive to conformation probably reflect a distortion of the BChl molecule induced by insertion into the binding site. However, as discussed by Näveke (*33*), the physical nature of a distortion able to cause large shifts in the frequencies of some modes and relatively minor shifts to other conformation-sensitive modes must await further studies or robust normal mode calculations on these large asymmetric conjugated systems.

The pressure-induced displacements observed in the Raman bands sensitive to the macrocycle conformation are small and nonspecific. The frequencies of these modes do not exhibit larger pressure sensitivity than the other modes shown in Table 1 and Figure 2. If some pressure-induced distortion of the BChl macrocycle at 0.625 GPa cannot be completely ruled out, it should in any case be much smaller than that experienced by the BChl molecule when it is inserted into the binding site. As discussed in Näveke et al. (*25*), such a conformational change should result in a displacement of no more than 0.5 pm for the atoms of the bacteriochlorin macrocycle, to be at the limit of the accuracy of Raman studies. We come to the conclusion that the pressure-induced distortions of the BChl macrocycle are most probably absent under the experimental conditions used.

The two carbonyl stretching frequencies at 1643 and 1664 cm^{-1} remain practically unaffected by pressure. Indeed these modes seem less sensitive to pressure than the bulk of the other modes included in Table 1; it should, however, be remembered that, unlike the majority of vibrational modes of BChl, the carbonyl stretching modes are nearly entirely localized on these peripheral substituents and so a different sensitivity is probably to be expected. The frequencies of these carbonyl modes have been shown to be highly sensitive to the strength of the H-bonds that the C_9-keto and C_2-acetyl groups are involved in downshifting by more than 35 cm^{-1} in some cases (the stronger the H-bond, the lower the corresponding mode frequency). The C_9-keto group stretching frequency is also sensitive to the dielectric properties of its environment (*16*). The observed pressure insensitivity of the frequency of the carbonyl modes implies that both of the H-bonds remain intact and virtually unaltered, in terms of both distance and geometry. Furthermore, the invariance of the C_9-keto group stretching frequency suggests that the dielectric properties in the vicinity of this group are relatively insensitive to pressure. The observed invariance of these parameters suggests that the structural changes in the vicinity of the carbonyl groups of the chromophores induced by pressure are limited. The conclusion may be reached that the H-bond interactions are not at the origin of the pressure-induced red shift of the absorption of antenna proteins and indeed do not participate in this red shift.

Various effects have been proposed as being partially responsible for the red shift of the Q_y absorption band in the antenna complexes, namely, interactions with carotenoids (*34*); distortions of the bacteriochlorophyll macrocycle (*35*); specific protein−BChl interactions; changes in local polarizability (*16*); and interactions with the neighboring BChl molecules (*17*−*19*, *36*).

Of the various factors mentioned, carotenoid−BChl interactions cannot play a role in the *Rsp. rubrum* G9$^+$ strain we have studied, since it is a carotenoid-less strain. The formation of H-bonds between the C_2 acetyl groups of BChl molecules and the surrounding protein can tune the red-most absorption transition by as much as 430 cm^{-1}, both in LH1 and LH2 complexes (*10*, *11*, *13*−*16*). Further a red shift of no more than 130 cm^{-1} in the Q_y transition of the monomeric, B800, BChl of LH2 was attributed to the local dielectric properties of its protein binding site (*16*). The evidence from the spectra presented above suggests that although pigment distortion, hydrogen bonding, and dielectric properties near the C_9-keto group binding sites may be factors involved in the red-shift, they are all relatively insensitive to pressure. Taken together these measurements seem to indicate a considerable resistance of the pigment−protein interactions to applied pressure since all of the interactions are expected to respond to compression of the binding site. Therefore the changes in the absorption spectrum induced by pressure must thus largely be attributed to alterations in pigment−pigment interactions. Although resonance Raman spectroscopy does not directly yield information on nonspecific (dispersion-type) pigment−protein interactions and we thus cannot formally discard the involvement of such interactions in the observed pressure-induced red shift, it seems to us likely that the role of such interactions is limited. We believe this since their modulation should affect the specific interactions we observe with Raman spectroscopy and also as nonspecific they should be of the same order of magnitude for all the BChl binding sites. As previously noted, the extent of the red shift is much larger for strongly coupled BChl molecules than for weakly coupled (as in the FMO protein) or uncoupled BChl molecules (as for the 800 nm absorbing BChl of LH2 complexes from purple bacteria). We thus favor the interpretation that changes in the pigment−pigment interactions are mostly responsible for the large pressure-dependent red shift observed, this through a combination of increases in excitonic coupling and increases in the nonspecific dispersion interactions. In the closely packed BChl structure of LH1, by analogy with the reaction center structure, one cannot totally rule out the influence of charge-transfer interactions.

The red shift in the Q_y band can thus be attributed to various types of interactions and how these interactions appear to vary as a function of pressure described. The red shift attributable to the H-bonding of the C_2 acetyl group has been calculated from the Raman spectrum as described previously (*15*, *31*). The estimated shift at atmospheric pressure is −250 cm^{-1}, and according to the present measurements, it is almost independent of pressure. The red shift caused by other local factors, distortion of pigments, dielectric constant, and nonspecific pigment−protein interactions, is harder to determine. However, on the basis of the Raman data the sum of these contributions has been estimated to be less than −200 cm^{-1} (ref *16*, Sturgis et al., manuscript in preparation). On the basis of the measurements in this work the contributions to this from pigment distortion and dielectric constant effects near the carbonyl groups must be nearly independent of pressure. These considerations imply that the pressure-sensitive red shift is attributable to nonspecific pigment−protein interactions on one hand and pigment−pitment interactions, through classical solvatochromic (dispersion) effects and exciton coupling effects.

It is worth noting how these different factors might be expected to behave under compression. Assuming an isotropic compression, the dispersion shift is inversely proportional to the intermolecular separation, R, raised to the sixth power. In the first-order theory of dipolar interaction and ignoring any changes in dielectric constant, the exciton shift increases with R^{-3} (*37*), while the charge-transfer shift would be proportional to R^{-1} (*38*). The different distance dependence of the various mechanisms also lead to different expected relationships between the shift and the pressure, with the dispersion shift following a quadratic dependence, the dipolar interaction showing a linear dependence, and the charge-transfer shift a cube-root dependence.

To estimate the distance changes that can be expected in a membrane protein at pressures up to 1 GPa is difficult, depending as it does on a value for the isothermal compressibility, the behavior of this parameter with pressure, and assumptions about the isotropy and homogeneity of the compression. The bulk isothermal compressibility of globular water-soluble proteins is relatively low, usually in the range of 0.05−0.15 GPa^{-1}, while that of hexane is about 0.9 GPa^{-1} (see ref *39*); assuming that the compressibility is constant, homogeneous, and isotropic leads to estimates for distance changes in a protein at 1 GPa of −1.7% to −5.3%. Changes of this order are consistent with the observed changes in the vibrational spectrum and our interpretation of these changes as indicating a relatively rigid and invariant binding pocket; however, such distance changes appear barely reconcilable with the observed quasi-linear −916 cm^{-1} GPa^{-1} pressure dependence of the 1532 cm^{-1} red shift, especially if about 400 cm^{-1} of this is considered to be insensitive to the pressure. Such a sensitivity implies a larger compressibility, in the range of 0.25−0.45 GPa^{-1} (depending on the relative contributions of dispersion and dipole−dipole type interactions), at least in the regions and along the axes of interaction. A relaxation of the assumptions of homogeneous and isotropic compression seems entirely reasonable in a proteic environment, and indeed is observed for soluble proteins. Thus the observed pressure sensitivities probably result from a moderately compressible but somewhat anisotropic and inhomogeneous protein.

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