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Bacteriochlorophyll–protein interaction in the light-harvesting complex B800-850 from *Rhodobacter sulfidophilus*: A Fourier-transform Raman spectroscopic investigation

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Near-infrared excited Fourier-transform Raman spectra have been obtained from different spectral forms of *Rhodobacter sulfidophilus* light-harvesting II complexes. This complex, when isolated in lauryldimethylamine oxide, exists in a 805–828 nm form, which can be reversibly converted to the native 805–851 nm form upon addition of salt. The FT-Raman spectra predominantly show contributions of the carotenoid in the light-harvesting complex, with small but significant contributions of the bacteriochlorophylls excited in preresonance in the Q_y transition. One strongly and one weakly interacting 2a acetyl C=O group as well as one moderately strong interacting and one non-interacting 9-keto C=O carbonyl modes of the bacteriochlorophylls can be discerned for the 805–828 nm form. Changes of relative band intensities caused by different resonance conditions for the different spectral forms lead to an assignment of the strongly interacting 2a acetyl C=O and the moderately strong interacting 9 keto C=O to bacteriochlorophylls organized in the 828 pigment moiety. Shifts of these bands to higher frequencies upon the salt-induced transition indicate a perturbation of the pigment–protein interaction, probably caused by a local protein conformational change.

Introduction

Bacteriochlorophyll-protein complexes show red-shifted absorption maxima with respect to free bacteriochlorophyll (BChl). The mechanisms of this in vivo regulation of the lowest singlet transition of BChl are not well understood, but depend on the BChl association state and on environmental interactions in the protein matrix. The major light-harvesting complex from purple photosynthetic bacteria, named B800–850 according to its near-infrared absorbance maxima, has been isolated and well characterized [1–3], and crystallization of B800–850 complexes from several organisms has been reported [4–6]. Nevertheless, high-resolution X-ray structures are not yet available, and present knowledge of the organization of the pigment molecules thus relies on spectroscopic techniques.

The B800–850 complex isolated in mild detergents seems to consist of trimers, tetramers or hexamers of units consisting of bacteriochlorophylls and carotenoids embedded in two (in some cases three) polypeptides [7,8]. Kramer et al. [9] have proposed a model for the pigment organization in a minimum complex of two such units, with two monomeric BChl molecules forming B800 and four BChl molecules in two pairs, each of them strongly exciton-coupled, forming B850. Spectroscopic analysis of crystallized B800–850 complex has led to a somewhat different model [10].

Since growth conditions, but also environmental conditions after isolation alter the relative absorption strength of the 800 and 850 nm BChls, a number of subgroups of the B800–850 complex have been derived. Among those are the so-called 'high 800 nm-absorbing' complexes obtained from *Rps. acidophila* or *Rps. palustris* cells grown at low illumination levels [11,12]. Further subgroups were complexes obtained by proteolytic treatment of *Rb. capsulatus* chromatophores [13],

Abbreviations: BChl, bacteriochlorophyll; FT, Fourier transform; LH, light-harvesting; *Rb., Rhodobacter.*

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complexes obtained by treatment with lithium dodecyl sulfate (LDS) [14,15], and complexes treated with borohydride [16], all of which showed a decrease, differently strong for the individual treatment, of the 800 nm absorption. Some of these modified complexes, for example the LDS-treated low-800 nm form, could be almost completely reconverted to the original form upon removing of the reagent by dialysis.

The molecular origin of these shifts of the relative absorption strength presumably relies on a modified interaction of the pigments with their protein environment. In the case of strong excitonic coupling of the pigments, a change of tertiary structure or of local protein conformation could also modify the excitonic interactions between the pigments. In any case, the investigation of the modified B800–850 complexes, by comparison to the native forms, can help to elucidate the mechanisms which modulate the spectroscopic properties of the complexes.

Recently, isolation of a B800–850 complex from the semihalophilic purple nonsulfur bacterium *Rb. sulfidophilus* was reported [17]. This detergent-solubilized complex exists in different spectral forms, affected by many factors such as detergent concentration, salt and pH [17,18]. In contrast to the above-mentioned alterations of the relative absorption of the 800 and 850 nm BChls, the spectral modifications of the *Rb. sulfidophilus* light-harvesting complex affect exclusively the 850 nm absorption and appear to be perfectly reversible [17]. Solubilized in LDAO, this complex exists in a B800–830 form, which is converted to the native B800–850 form upon addition of salt. Upon removing the salt by dialysis, the B800–830 form is fully restored. More interestingly, the influence of salt drastically depends on the valence, the polarity and the ionic radii [18]. Very subtle perturbations such as the addition of trivalent cations in a 1:1 stoichiometry are sufficient to achieve the absorbance shift [18].

In the light of these observations, the *Rb. sulfidophilus* light-harvesting complex appears to be an ideal system to study the interaction of pigments in BChl-protein complexes. Previous investigations of BChl *a* in antenna complexes from purple bacteria have used resonance Raman spectroscopy (for reviews, see Refs. 19,20) to study the binding sites and the local environment of the pigment molecules. These studies exclusively implied resonant Raman excitation either in the Soret band or the Q_x transition of the BChl molecules. No resonance Raman spectra of light-harvesting complexes excited in either of the Q_y transitions have been reported to date due to unwanted luminescence from these lowest singlet transitions.

While these problems are inherent to 'classical' resonance Raman spectroscopy, the relatively new technique of near-infrared-excited Fourier-transform (NIR-FT) Raman spectroscopy allows one to obtain Raman spectra with excitation far from resonance. In this technique, the strongly reduced Raman scattering cross section is partly compensated by the advantages of Fourier-transform spectroscopy. Earlier applications of FT-Raman spectroscopy [21–23] for organic dyes have demonstrated that the problem of fluorescence can be overcome using excitation in the near-IR (for example, using the 1064 nm line of an Nd:YAG laser), a FT-spectrophotometer and a combination of a sensitive NIR detector with filters to reduce unwanted scattering. The potential of NIR-FT Raman spectroscopy to study biological systems has been recognized [23–26]. The lack of fluorescence and the absence of photoreactions initiated by the exciting laser appear to be ideal for the study of photobiological systems. Recently, NIR-excited FT-Raman spectra of rhodopsin, bacteriorhodopsin and phycocyanin were reported [27], demonstrating preresonant Raman scattering, with dominating vibrational contributions of the chromophores in the spectra.

The first NIR-FT Raman study of BChl *a* in vitro was reported recently by Mattioli et al. [28]. The authors were able to demonstrate that vibrational spectra of chlorophyllic systems can be obtained unperturbed by fluorescence, at room temperature, without the risk of photodegradation, and yielding a wealth of Raman lines. The possibility of obtaining NIR-FT Raman spectra of the photosynthetic bacterial reaction center was recently demonstrated by Johnson and Rubinovitz [29]. In the study presented here, we have used NIR FT-Raman spectroscopy to obtain vibrational spectra of the light-harvesting II complex from *Rb. sulfidophilus*. The carbonyl modes of the BChl a associated in the different spectral forms are used as markers for the interaction of the pigments in situ.

Materials and Methods

Cells of *Rb. sulfidophilus* (strain W4) were grown semiaerobically in the dark under conditions described earlier [17]. Intracytoplasmic membranes and isolated light-harvesting complexes were prepared following the procedure described in [17]. Briefly, the harvested cells were disrupted in a French pressure cell, unbroken cells were removed by centrifugation, and a sucrose gradient was used to obtain introcytoplasmic membranes. The light-harvesting II (LH II) complex was solubilized in 0.5% (w/v) lauryldimethylamine *N*-oxide (LDAO) and purified by a sucrose gradient and a DEAE column. Purified LH II complexes were eluted at a concentration of approx. 200 mM to 230 mM NaCl.

For spectroscopic measurements, the purified LH II complex was transferred into Tris buffer (10 mM, pH 7.5) with 0.1% LDAO and concentrated to a final protein concentration of approx 10 mg/ml in centricon-10 microconcentrators at $5000 \times g$. Spectra in the visi-

ble/near-infrared region of the light-harvesting complex diluted to approx 20. μg protein/ml were recorded with a spectrophotometer built in our laboratory.

FT-Raman spectra were recorded at 4 cm^{-1} resolution on a Bruker IFS 66 Fourier transform spectrophotometer with a Bruker Raman FRA 106 accessory. A 5 mm pathlength quartz cell in backscattering geometry was used for all measurements. FT-Raman spectra were obtained from protein solutions at a concentration of 1 mg/ml at room temperature. A diode-laser pumped Nd:YAG laser at approx. 220 mW cw laser power at 1064 nm was used for excitation. Typically 200 interferometer scans were collected and transformed using a Blackman-Harris apodization. Spectra of the buffer solutions were subtracted for correction.

Results and Discussion

Fig. 1 shows the near-infrared excited FT-Raman spectrum of the B805–828 form of light-harvesting complex from *Rb. sulfidophilus*. The corresponding absorbance spectrum in the visible-NIR taken in a thin-layer cell is shown in the right inset. The low background intensity of the spectrum indicates that very few fluorescence contributions are recorded, and Raman lines can be detected down to 200 cm^{-1} shift with respect to the laser line. The Raman spectrum exhibits prominent lines at 1507 cm^{-1}, 1282 cm^{-1}, 1150 cm^{-1}, 1017/1003 cm^{-1} and at 895 cm^{-1}, strongly indicative for carotenoid bands [30,31]. Although there are differences in the positions of individual peaks, the spectrum shows considerable similarity with the resonance Raman spectra of *C. vinosum* light-harvesting complex [31], deficient in its intrinsic carotenoids but incubated with spheroidene.

The wavelength of excitation in the FT-Raman experiments is far from carotenoid absorption. Nevertheless, the strong contribution of the carotenoid ν_1 and ν_2 vibrations indicates that preresonant Raman scattering is prevalent.

In the spectral region above 1600 cm^{-1}, four small Raman bands are detected between 1625 and about 1710 cm^{-1} (see inset of Fig. 1), about a factor of 20 smaller than the carotenoid ν_1 vibration at 1507 cm^{-1} (the broad peak at 1850 cm^{-1} is an artifact due to the characteristics of the filter module used to get rid of the Raleigh line). These four peaks are highly reproducible in position and intensity from sample to sample, provided that the same spectral forms of the light-harvesting complexes are compared. Four to five peaks in this spectral region were also observed in FT-Raman spectra from intracytoplasmic membranes of *Rhodobacter capsulatus* (Mäntele, W. and Sawatzki, J., unpublished observations).

It is reasonable to assume that these four peaks represent the carbonyl-stretching vibrations of the bacteriochlorophylls associated in the 800 and the 828 nm form, respectively. The scattering cross section of these vibrations appears to be considerably smaller than that of the carotenoids. In addition, only very weak scattering is observed at 1605–1615 cm^{-1}, where the C–C modes of bacteriochlorophylls should appear [20]. The almost complete absence of this mode in near-infrared excited FT-Raman spectra of BChl *a* in vitro (in THF

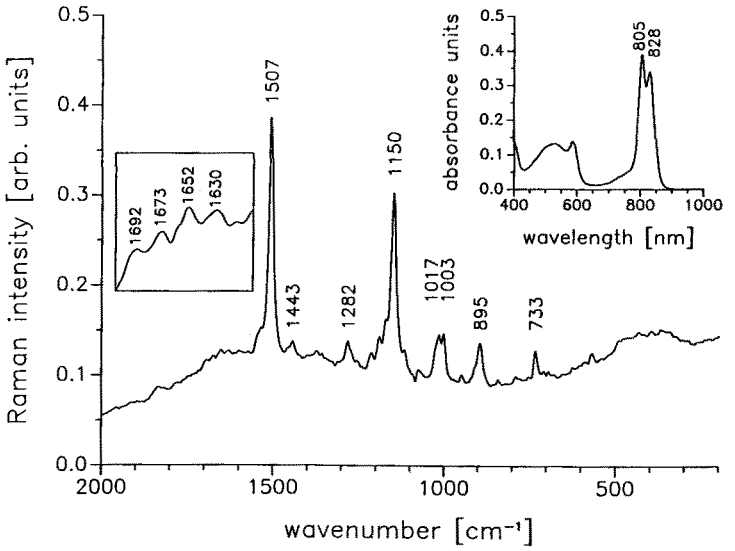

Fig. 1. Near-infrared excited FT-Raman spectrum of the B805–828 nm LH II complex from dark-semiaerobic grown *Rb. sulfidophilus*. Experimental conditions: resolution 4 cm^{-1}, 200 interferometer scans, 1064 nm laser power 220 mW. The light-harvesting complex was solubilized in Tris buffer (10 mM, pH 7.5) with 0.1% LDAO at a protein concentration of approx. 1 mg /ml. Spectra were taken in a 0.5 cm Infrasil quartz cell. Left inset: 1600 cm^{-1} to 1725 cm^{-1} region (enlarged); right inset: Absorption spectrum of the same complex at 0.01 cm pathlength.

solution) was also noted by Mattioli et al. [28]. Instead, a weak band at 1595 cm^{-1} in their spectra was assigned to the C–C mode.

Additions of small amounts of salt drastically modify the near-infrared absorbance spectrum (Fig. 2). Starting from the salt-free LH II complex with maxima at 805 nm and at 828 nm, addition of NaCl results in a decrease and red-shift of the 828 nm band. At 40 mM NaCl, the absorbance of this band is lowest, and the band appears to be broader. Further addition of NaCl leads to a maximum red shift of this peak to 851 nm and to a considerable increase of absorbance. The titration is complete at approx 100 mM NaCl, half of the shift being attained at approx 50 mM. This half-point depends drastically on the ionic radii and the valence of the salt, and varies between micromolar concentrations for trivalent cations, and 300–500 mM for some monovalent ions. A detailed analysis of this salt shift, which, at the limit, is attainable with a molar ratio of one ion per LH II, will be reported in a further paper (Mäntele et al., unpublished data).

At first sight, one might be tempted to describe the salt-induced shift (Fig. 2) in terms of a two-state model, with only one transition of the 828 nm band to 851 nm. However, the lack of a clear isosbestic point and the increased half-width of the band in the course of the transition rather suggests an additional change of extinction coefficients or one intermediate form with low absorbance at 840 nm. This intermediate form is less clearly seen in titrations with trivalent cations, for example yttrium chloride (data not shown).

Since the FT-Raman spectra are taken at room temperature in a 5 mm cuvette, additions of salt to perform

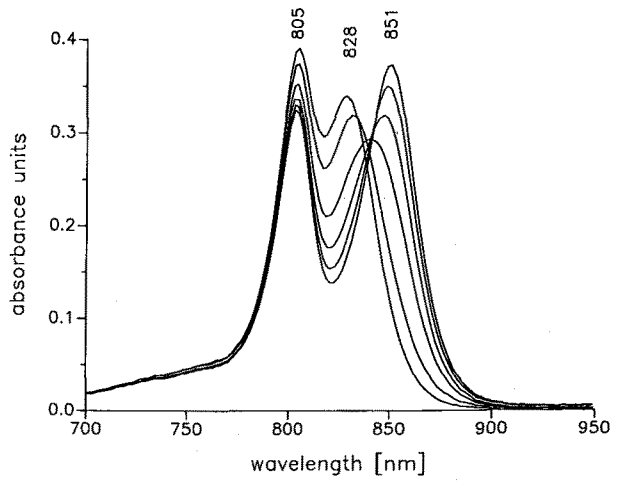

Fig. 2. Salt titration of the spectral forms of the LH II complex from dark-aerobic grown *Rb. sulfidophilus*, starting with the 805–828 nm form (0 mM NaCl) and finishing with the 805–851 nm form (100 mM NaCl). Experimental conditions: LH II protein concentration approx. 20 μg/ml; Tris-buffer (10 mM, pH 7.5) with 0.1% LDAO. The NaCl concentration was adjusted in steps of 20 mM by addition of small aliquots of a 4 M NaCl solution.

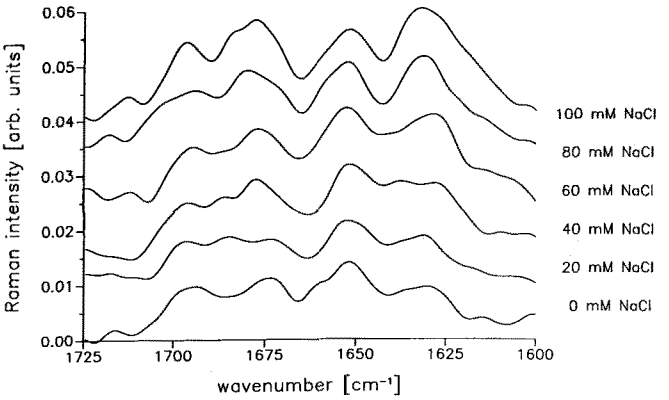

Fig. 3. Near-infrared excited FT-Raman spectra of the different spectral forms of the LH II complex from dark-aerobic grown *Rb. sulfidophilus* taken in a 0.5 cm quartz cell. A straight baseline fitted at 1750 cm^{-1} and at 1600 cm^{-1} was subtracted and the spectra were displaced vertically for easier discussion: Experimental conditions were as in Fig 1. The NaCl concentration was adjusted by addition of small aliquots of a 4 M NaCl solution.

the titration from the 805–828 to the 805–851 nm form are equally possible. FT-Raman spectra of these forms were obtained by addition of small aliquots of a 4 M NaCl solution to the Raman cuvette, resulting in a negligible dilution of the sample. Spectra at full wavenumber scale (200–2000 cm^{-1}, data not shown) closely resemble that in Fig. 1, confirming that the carotenoid vibrational modes are only weakly influenced. In fact, only a small carotenoid band shift is observed upon salt titration for LH II from dark-aerobic grown cells (Doi et al., unpublished results).

Fig. 3 shows the carbonyl frequency region of these Raman spectra at expanded scale. In order to compensate the weak fluorescence background leading to an inclined baseline in the 1800–1600 cm^{-1} spectral region, a straight baseline, fitted at 1750 cm^{-1} and at 1600 cm^{-1}, was subtracted from the Raman spectra and the spectra were displaced vertically for easier discussion.

The 805–828 nm form obtained at 0 mM NaCl displays at least four Raman bands at approx 1630 cm^{-1}, 1652 cm^{-1}, 1673 cm^{-1} and approx 1690 cm^{-1}, attributed above to the carbonyl modes of the bacteriochlorophylls associated in the two pigment moieties. Carbonyl modes of chlorophylls and bacteriochlorophylls present unique probes for the association state of the pigments and their interaction with the proteic environment (for reviews, see Refs. 20,32). As deduced from in vitro studies, the 2a-acetyl mode should appear between 1620 and about 1660 cm^{-1} and the 9-keto mode between 1655 cm^{-1} and approx. 1700 cm^{-1}. Within this coarse frequency range, increasing polarity of the environment and bonding of the carbonyl finetunes their stretching modes, in that it lowers the

frequency. The 10a and 7c ester modes, vibrating between 1710 cm^{-1} and 1750 cm^{-1} [20], should not contribute in these preresonant FT Raman spectra.

The four bands observed in the FT-Raman spectrum of the 805–828 nm form (0 mM NaCl) can thus be tentatively attributed to strongly interacting (1630 cm^{-1}) and weakly interacting (1652 cm^{-1}) 2a-acetyl C=O stretching modes and to moderately strong interacting (1673 cm^{-1}) and non-interacting (1690 cm^{-1}) 9-keto C=O stretching modes.

There is no evidence for a contribution of the amide I vibrational mode in these preresonant FT-Raman spectra. This agrees well with near-IR-excited FT-Raman spectra from other photobiological systems such as bacteriorhodopsin, where only very weak contributions from the amide I band were observed [27]. In contrast, the same authors were able to assign an amide I band in FT-Raman spectra of the visual pigment rhodopsin.

When the 805–828 nm form is gradually shifted to the 805–851 form by additions of small aliquots of NaCl, the band pattern in the 1700 cm^{-1} to 1620 cm^{-1} spectral region exhibits characteristic changes. The lowest-frequency peak (approx. 1630 cm^{-1} at 0 mM NaCl) appears to gain a satelite peak at its higher-wavenumber side upon increasing the salt concentration. From this doublet structure seen at intermediate NaCl concentration, a single peak at 1635 cm^{-1}, characteristic for the 805–851 nm form, develops towards higher salt concentration. The shift of this band, although small (from approx. 1630 cm^{-1} to 1635 cm^{-1}) to higher wavenumbers suggests a slightly weaker interaction in the 805–851 form than in the 805–828 form. In addition, the doublet structure seen at 40–60 mM NaCl suggests an intermediate form, with one acetyl C=O (at approx. 1640 cm^{-1}) weakly and one acetyl C=O (at approx. 1630 cm^{-1}) strongly interacting.

In contrast to this, the second peak at approx 1652 cm^{-1} remains almost unchanged in position. We attribute this peak to an acetyl C=O group free from interaction, and unperturbed upon the 805–828 to 805–858 transition.

The third peak at 1673 cm^{-1} can be attributed to a moderately strong interacting 9-keto C=O group. This group shifts to approx. 1680 cm^{-1} at intermediate and high salt concentrations, indicating a weaker interaction. Again, the band feature is not homogeneous, but with shoulders at its low- and high-frequency side.

Finally, the fourth peak at 1690 cm^{-1} (0 mM NaCl), typical for a non-interacting 9 keto C=O group in a polar environment [32], appears at the same frequency at the 805–828 and the 805–851 form. However, the high-frequency shoulders of this band, clearly evident at intermediate salt concentrations, reveal a heterogeneous structure, too. Due to the preresonance conditions, there are only weak or no contributions from the 10a or 7c ester C=O groups in the FT-Raman spectra. This is in agreement with FT-Raman spectra of BChl *a* in vitro [28].

It is interesting to note that the integral intensity of the two peaks at 1673 cm^{-1} and at 1630 cm^{-1} (0 mM) gradually increases with the red-shift of the 828 nm peak and reaches about 5-fold intensity in the high-salt form. In contrast to this, the intensity of the peaks at 1652 cm^{-1} and at 1690 cm^{-1}, the frequencies of which are only little influenced upon the salt-induced transition, remains constant. We attribute this remarkable change in relative peak intensity to different preresonance conditions of the two pigment moieties. Modes of a BChl a molecule associated with the 828 nm form, upon transition to the 851 form, should increase in intensity due to intensified resonance conditions.

This allows us to assign the 1652 cm^{-1} band, unchanged in position and intensity, to a free 2a-acetyl C=O group of a 805 nm BChl. The 1690 cm^{-1} band can then be attributed to a non-interacting 9-keto C=O group of the same pigment moiety. The bands at 1630 cm^{-1} and at 1673 cm^{-1}, both shifting and with increasing intensity, can be assigned to strongly bound 2a-acetyl and moderately interacting 9-keto C=O groups of the BChls associated in the 828 to 851 nm absorbing form.

Robert et al. [33] have characterized four modes in the C=O frequency region of Soret-excited resonance Raman spectra from *Rhodobacter sphaeroides* 2.4.1 B800–850 complexes. In a comparison of B800–850 complex from three different species (*Rb. sphaeroides, Rb. capsulatus* and *Rps. palustris*), they found three of these bands around 1632 cm^{-1}, 1642 cm^{-1} and 1667 cm^{-1} common to all three species, whereas only *Rb. sphaeroides* and *Rb. capsulatus* B800–850 exhibited a 1700 cm^{-1} band, indicative for a non-interacting keto C=O group [34]. The band pattern of the B805–851 form (100 mM NaCl, Fig. 3) of the *Rb. sulfidophilus* light-harvesting complex in general corresponds to the one observed for *Rb. sphaeroides* and *Rb. capsulatus* B800–850, with minor differences in band positions which may indicate variations in local polarity or the strength of bonding. No differences in carbonyl frequencies were detected by Robert et al. between 'normal' and 'high 800' (by growth conditions) light-harvesting complexes [35]. Since this result could be explained by the insertion of an additional BChl molecule with similar interaction properties, it is difficult to assign from these spectra the C=O vibrators to the 800 or 850 pigment moieties. In contrast, the salt-shift induced enhancement of preresonance for the 828/851 pigments allows us to attribute these carbonyl groups.

Conclusions

The salt-induced transition of the light-harvesting II complex from *Rb. sulfidophilus* appears to be related to a change of interaction of the 9-keto and 2a-acetyl C=O

bond(s) of the BChl(s) associated in the 828 to 851 nm absorbing form. The different bonding can account for at least part of this spectral shift. However, a perturbation of excitonic coupling through a modified geometry has to be taken into account as well.

It is not yet clear whether this modified bonding and/or change in exciton splitting are caused by a direct influence of the ion charge(s) on the conjugated system of the pigment(s), or if an influence of the ion charge on the protein conformation, for example by charge screening of the protein surface charges, is responsible. In preliminary Fourier-transform infrared (FTIR) spectroscopic investigations of the amide I absorbance (Mäntele, W. and Beck, M., unpublished data), only very minor protein conformational changes have been detected.

A further exploration of these Q_y excited preresonance Raman spectra has to include the low-frequency modes as well, and will need an analysis of relative band intensities and preresonant scattering cross sections in order to understand the scattering mechanism. Since part of the selectivity of resonance Raman spectroscopy is retained, it appears that the new technique of NIR-excited FT-Raman spectroscopy provides a non-invasive probe for the bonding of pigments in photosynthetic structures, and thus can help to elucidate mechanisms of regulation of pigment properties. This regulation clearly involves bonding of the pigment carbonyl groups to protein residues.

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