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Review

Vibrational techniques applied to photosynthesis: Resonance Raman and fluorescence line-narrowing ☆

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ABSTRACT

Resonance Raman spectroscopy may yield precise information on the conformation of, and the interactions assumed by, the chromophores involved in the first steps of the photosynthetic process. Selectivity is achieved via resonance with the absorption transition of the chromophore of interest. Fluorescence line-narrowing spectroscopy is a complementary technique, in that it provides the same level of information (structure, conformation, interactions), but in this case for the emitting pigment(s) only (whether isolated or in an ensemble of interacting chromophores). The selectivity provided by these vibrational techniques allows for the analysis of pigment molecules not only when they are isolated in solvents, but also when embedded in soluble or membrane proteins and even, as shown recently, in vivo. They can be used, for instance, to relate the electronic properties of these pigment molecules to their structure and/or the physical properties of their environment. These techniques are even able to follow subtle changes in chromophore conformation associated with regulatory processes. After a short introduction to the physical principles that govern resonance Raman and fluorescence line-narrowing spectroscopies, the information content of the vibrational spectra of chlorophyll and carotenoid molecules is described in this article, together with the experiments which helped in determining which structural parameter(s) each vibrational band is sensitive to. A selection of applications is then presented, in order to illustrate how these techniques have been used in the field of photosynthesis, and what type of information has been obtained. This article is part of a Special Issue entitled: Vibrational spectroscopies and bioenergetic systems.

1. Introduction

In this article, we will review the application of two selective vibrational techniques, mainly resonance Raman spectroscopy but also fluorescence line-narrowing (FLN), to the study of the photosynthetic process. We will focus on the information provided by these methods on photosynthetic pigment structures and environments; we will not address their use for determining electron–phonon and electron-vibrational couplings.

From a practical point of view, resonance Raman and FLN yield similar information, i.e. they provide access to those vibrational modes coupled with the electronic transition of the studied molecule, and both exhibit very high selectivity in complex media. The application of resonance Raman has recently been reviewed by the authors of this current review [1,2]. In order to avoid redundancies in the current work, we have deliberately chosen to emphasize other, or more recent, achievements obtained by these techniques, which illustrate their varied applications in photosynthesis.

1.1. Resonance Raman and FLN: principles

The Raman effect is a change in light frequency when it is scattered by polyatomic molecules, this change occurring upon energy exchange between the incoming photon and the scattering molecule (i.e. non-elastic scattering). As the energy levels of the scattering molecule are discrete, if the frequency of the incident light is νo and that of the scattered light is νr, the energy difference $h * \Delta\nu = h * (\nu o - \nu r)$ must correspond to that of a transition between molecular energy levels (Fig. 1). Raman spectroscopy thus provides information on the energy of the vibrational levels of a given electronic state. The intensity of the Raman signal varies according to the fourth power of the incident frequency, νo. However, if this frequency matches that of an electronic transition of the irradiated molecule an enhancement of a subset of the Raman-active modes is observed, which may increase the signal by as much as six orders of magnitude. This is called the resonance effect (Fig. 1). In FLN, vibrational narrowing can be observed in fluorescence spectra at very low temperatures, when exciting a molecule into its lower-energy excited states. In these conditions, and provided that the energy of the photons chosen is low enough so that upper vibrational

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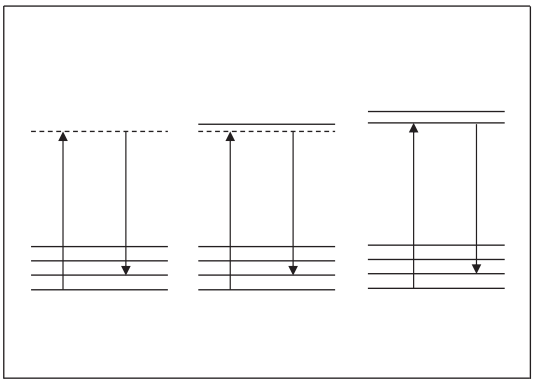

Fig. 1. (from left to right) Energy levels involved in Raman, resonance Raman and FLN spectroscopies.

levels of the excited states are not populated, the vibrational modes coupled with the electronic transition may be observed on top of the fluorescence envelope (Fig. 1). To summarize, resonance Raman yields highly selective vibrational information on individual scattering molecules in a complex medium, based on their absorption properties, while FLN gives similar information, but where the selectivity is based on the fluorescence properties of the molecule. Actually FLN may yield a full picture of the vibrational modes coupled with an electronic transition, particularly valuable for spectroscopic modeling (spectra and dynamics) of photosynthetic systems (see e.g. [3–5]).

The vibrational levels of a particular molecule depend on its structure, i.e. the nature of its constituent atoms, the chemical bonds between them, and its molecular symmetry. Resonance Raman and FLN spectroscopy can thus provide information for determining the chemical structure of molecules, as well as their conformation, and/or the intermolecular interactions they are involved in. It is worth noting that in resonance Raman and FLN spectra the unit of measure is usually wavenumbers (cm^{-1}), as this corresponds to the shift in energy between the exciting laser beam and the emitted signal — and therefore to the vibrational energy of the molecule under study.

1.2. Resonance Raman and FLN: selectivity and technical aspects

Resonance Raman and FLN spectroscopies yield selective information on a molecule in a complex medium, provided that it possesses an appropriate absorption transition or exhibits fluorescence. This makes it possible to study the interactions assumed by, or the conformation of, chromophores within proteins, including when these are still embedded in a biological membrane, only partially purified, or even in intact cells. Resonance Raman spectroscopy has been extensively used on biological chromophores, such as flavins, hemes, iron–sulfur clusters, bilins, chlorophylls and carotenoids. Fluorescence line-narrowing has found fewer applications in biology, mainly because the measurements require the sample to be maintained at very low temperatures (<15 K). However, since the first description of its application to chlorophyll molecules [6,7], it has found a niche in the field of photosynthesis.

In both methods, only a fraction of the vibrational modes of the scattering molecule are observed. More precisely, the signal arises from the vibrational modes which are coupled with the electronic transition that is probed during the measurement. In a simplified view these are the modes which involve nuclear motions that correspond to the distortions experienced by the molecule during its transition between the ground and excited states. This apparent limitation is actually an advantage, as the chemical groups which are not coupled with the transition are not expected to influence it. In a sense, these techniques yield specific information only on the parts of the molecule relevant for understanding the electronic transition used to produce selectivity. In turn, analysis of these active modes will yield valuable information about the nature of this transition, the nuclei involved, and the coupling between this transition and the different vibrational modes of the chromophore. As the function of pigmented cofactors usually involves their electronic transitions (such as for the absorption, transfer and conversion of light energy in photosynthesis), these techniques give precise molecular details on the functionally-active properties of these chromophores. The nature and position of the bands observed in resonance Raman and FLN yield information on the vibrational structure of the low-energy electronic state of the molecule, while their intensity yields information about the coupling of these modes with the electronic transition.

2. Applications to photosynthesis: carotenoids

2.1. Spectroscopic properties

Carotenoid molecules are among the most efficient resonance Raman scatterers in biology. Conversely, their fluorescence is intrinsically extremely low. Resonance Raman spectra of carotenoid molecules display four main groups of intense bands, termed ν_1 to ν_4, located at ca

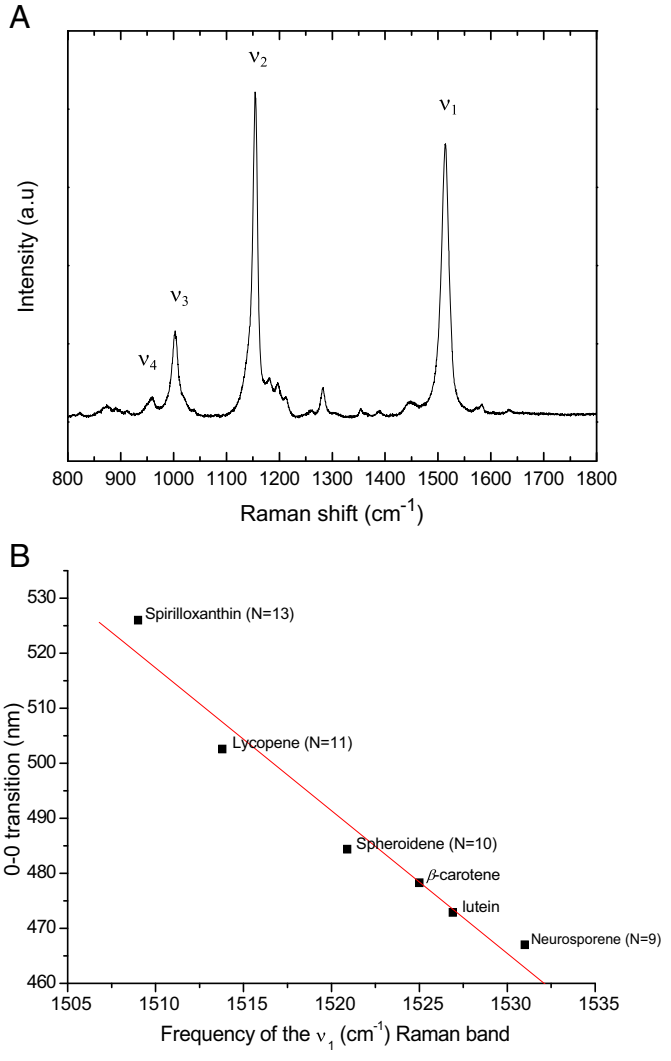

Fig. 2. A: Typical resonance Raman spectrum of a carotenoid molecule (lycopene). B: Correlation between the position of the $S_0 \rightarrow S_2$ electronic transition of carotenoid molecules and their observed ν_1 frequency, for carotenoids having different C═C conjugated chain lengths. As this frequency yields direct access to the C═C conjugated chain length, we conclude that the effective length of this chain is 9.3 and 9.6 for β-carotene and lutein, respectively. Accordingly, the end chain β-cycles contribute only 0.3 C═C to the total effective conjugation length of these molecules.

1520, 1150, 1000 and 950 cm^{-1}, respectively (Fig. 2A). The ν_1 mode arises from stretching vibrations of the carotenoid C═C bonds [8] — its position is actually a direct marker of the extent of conjugation (or conjugated chain length) of the scattering carotenoid [9] (see Fig. 2B). As such, its frequency is sensitive to the molecular configuration (*trans*/*cis*) [10–12]. The ν_2 mode arises from a more complex combination of vibrations, involving in particular stretching vibrations of C – C single bonds. Its structure, exhibiting a number of satellite bands, constitutes a sensitive fingerprint for carotenoid configuration [10–12]. The ν_3 mode at approximately 1010 cm^{-1} arises from in-plane rocking vibrations of the methyl groups attached to the conjugation chain, coupled with in-plane bending of the adjacent C – H. This band was recently proposed to be sensitive to the conjugated end-cycle conformation in cyclic carotenoid molecules [9]. ν_4 arises from C – H out-of-plane wagging motions coupled to C═C torsions. These modes are not formally coupled with the in-plane electronic transition of the carotenoid molecule for symmetry reasons, and these bands accordingly exhibit very low intensity. However, upon molecular distortions around C – C bonds they may gain intensity — in some cases the ν_4 intensity may even approach that of ν_1 [13,14]. Additionally, if the carotenoids under study are located in homogeneous binding sites which induce small but consistent distortions of the whole carotenoid population, fine structures will be observed in this spectral region, reflecting on the uniformity of the detailed structure of these molecules in the bulk sample (see e.g. [9,15]).

2.2. Carotenoid identification: from isolated proteins to in vivo situations

In the photosynthetic membrane, as well as in multi-chromophore photosynthetic complexes, the electronic transitions of the different carotenoids are degenerate, and given that they may also be tuned by their protein environment, it is often difficult to assess with any accuracy where each carotenoid population will contribute in the measured electronic absorption spectrum. Resonance Raman, which provides a chemical fingerprint of the scattering molecules, was first used to perform such attributions for LHCII, the major light-harvesting protein from higher plants. In this complex, the absorption positions of the two luteins and the 9-*cis* neoxanthin were determined by analyzing the resonance Raman spectra according to excitation wavelength [16–18]. In the last decade, the same approach has been used in media as complex as whole diatoms. Three carotenoids are generally present in the light-harvesting proteins of these organisms: fucoxanthin, diadinoxanthin and diatoxanthin [19]. The diadinoxanthin cycle in diatoms, the equivalent of the violaxanthin cycle in higher plants, consists of a one-step de-epoxidation of the mono-epoxy xanthophyll diadinoxanthin into diatoxanthin, which occurs in the chloroplast upon exposure to high light [20]. Moreover, in an added complication when compared to the situation in higher plants, the overall amount of these diadinoxanthin cycle carotenoids in the cell strongly depends on the light regime in which the diatoms are grown [21]. As the resonance Raman spectra of these three carotenoid molecules differ from each other significantly, it was possible, by scanning the wavelength used for inducing resonance, to selectively detect diadinoxanthin and diatoxanthin in intact *Cyclotella* cells in vivo, as well as to quantify the carotenoid levels relative to chlorophyll content [15]. Resonance Raman selectivity helped in discriminating between the different pools of diadinoxanthin on the basis of their conformation, and to follow the enzymatic cycle as a function of the illumination conditions. It was concluded that the additional diadinoxanthin molecules accumulated in cells under high-light growth conditions adopt a more twisted conformation than the constitutive population of diadinoxanthins present in low-light conditions, suggesting different protein binding sites in each case [15]. Similar measurements in the siphonous green alga, *Codium intricatum*, revealed the accumulation of *all-trans* neoxanthin when this organism was grown in high-light [22].

2.3. Tuning carotenoid absorption in photosynthetic proteins

As mentioned above, identifying the carotenoid(s) contributing to a given absorption transition is not always easy in photosynthetic complexes, partly because the protein environment may tune the absorption properties of different carotenoid sub-populations. In photosynthetic proteins, there are two well-known examples of such a phenomenon. The first example is the reaction center of photosystem II (PSII), where the (0, 0) absorption bands of the two bound β-carotenes peak at 489 and 507 nm [23]. The second example is of the two luteins bound to LHCII, exhibiting (0, 0) absorption transitions at 496 and 510 nm [16]. These absorption maxima are separated enough so that resonance Raman spectra of each population can be selectively obtained at low temperature, and even at room temperature in the case of the PSII reaction center. In both cases, it was observed that the frequency of the ν_1 Raman band downshifts for the red-absorbing carotenoid, reflecting an increase in effective conjugation length as compared to the blue-absorbing carotenoid (shown for PSII in Fig. 3). *Cis* isomerization is expected to promote the inverse phenomenon, i.e. a shortening of the effective length of the carotenoid conjugated chain. However, in solution β-carotene and lutein exhibit a shorter effective conjugation length than expected when counting the number of C═C double bonds in their isoprene chain, including the terminal β-cycles [9]. This shorter conjugation length is likely to be due to an s-*cis* configuration of the β-cycles due to steric hindrance, such that they are twisted slightly out of the conjugated plane [24]. It was thus proposed that, in both PSII reaction centers and LHCII, the pigment binding site of the red-absorbing carotenoid brings the conjugated cycle(s) back into the plane of the molecule through steric hindrance, red-shifting its absorption by increasing its effective conjugation length [9].

2.4. Carotenoid excited states

Resonance Raman spectra of excited states require that these states be populated prior to the measurement, usually by using a laser pulse. The structure of carotenoid and chlorophyll excited states are of primary interest in photosynthesis, as these are the active excited states during excitation energy transfer and charge separation. Due to their large resonance Raman cross-section, triplet and singlet states of carotenoids have already been reported, though usually in solvents and seldom in photosynthetic proteins. One-photon carotenoid excitation promotes an electron to the second excited state (termed S_2), as the transition from the ground state to the lower-energy excited state (S_1) is forbidden by symmetry [25]. The S_2 lifetime is extremely short (in the sub-picosecond range; [26]) and obtaining a clear resonance Raman spectrum from this ultra-short carotenoid S_2 state has been shown to be very demanding, even using stimulated Raman [27]. Resonance Raman spectra of the carotenoid S_1 state (ca 10 ps lifetime) have been reported since the 80's for many carotenoids in solvents [28,29]. Although there were, at that time, some attempts to perform similar experiments in vivo, very few spectra of the S_1 state of a carotenoid bound to a photosynthetic protein have been reported up to now [30–32]. Carotenoid molecules were actually one of the first examples where time-resolved resonance Raman spectroscopy was applied to a molecular excited state, when Dallinger et al. (1979) recorded a triplet state spectrum [33]. Triplet carotenoid spectra differ from those of ground state molecules because of the effect of the electron promotion on the C – C and C═C bond order. In solvents, the ν_1 band downshifts by more than 25 cm^{-1}, and the main component of the ν_2 band also downshifts from 1164 to 1125 cm^{-1} [28,34,35]. This very large shift probably reflects that this band arises from a rather complex mixture of C – C and C═C stretching with C – H bending modes. Recently, triplet spectra of carotenoids bound to the antenna of higher plants and purple bacteria were recorded [36]. The downshift of the ν_1 in LH2 from the purple bacterium *Rhodoblastus acidophilus* is nearly identical to that observed for the carotenoid in solvents. In contrast, in plant antenna

proteins, the downshift experienced by this band is about 25% less than that observed in solvents. In plant light-harvesting complexes, the triplet–triplet transfer from chlorophyll to carotenoid is ultrafast [36,37], while it occurs in the nanosecond timescale in bacteria [38,39]. It was proposed that the ultrafast triplet–triplet transfer observed in the plant light-harvesting complex is a consequence of a complex interaction between the carotenoid molecule and its neighboring chlorophylls, altering the structure of the carotenoid triplet state as observed by resonance Raman spectroscopy, and that in these complexes the triplet state is partially delocalized on these neighboring Chls. More recent experiments, conducted in artificial carotenoid-porphyrin dyads, have revealed that a similar signature of the carotenoid triplet was observed for dyads exhibiting ultrafast triplet–triplet transfer — and in those dyads only (Galzerano et al., in preparation).

3. Applications to photosynthesis: (bacterio)chlorophylls

3.1. Chlorophyll-like molecules

The resonance Raman spectrum of chlorophyll (Chl) has been extensively analyzed (see e.g. [40]) while its FLN complement has only been the subject of a few systematic studies [41,42]. The vibrational spectra contain about 60 bands (Fig. 4). The selection rules between the two methods being different (only those vibrational modes which induce a change in the molecule polarizability are active in Raman), thus making the content in bands as well as the observed intensities different (Fig. 5), they may be classed into about three principal regions: i) *the low frequency region* (200–500 cm^{-1}), where, in resonance Raman, modes directly involving the central Mg ion of these molecules contribute [40]; ii) *the mid-frequency region* (900–1600 cm^{-1}), where a series of bands contribute whose frequency is sensitive to the conformation of the conjugated Chl macrocycle [42–45] (note that the precise relationship between a set of frequencies observed for these bands and the precise geometric deformation of the macrocycle has yet to be modeled); and iii) *the high-frequency region* (1620–1710 cm^{-1}) where stretching modes of the conjugated vinyl and carbonyl groups of Chl molecules contribute [40,42,46]. The frequency of the carbonyl stretching modes tightly depends on the intermolecular interactions these groups are involved in. For instance, the BChl acetyl carbonyl stretching mode is observed at ca. 1660 cm^{-1} when free-from-interactions, while it shifts down to 1620 cm^{-1} upon H-bond formation [47]. Other factors, such as the dielectric properties of the environment as well as the conformation of the (B)Chl macrocycle, also influence these frequencies (though to a lesser extent; [48]). For instance, the stretching frequencies of (bacterio)pheophytin molecules are at a slightly higher frequency than those of (bacterio)chlorophylls, because of the difference in macrocycle conformation induced by the central magnesium ion (see e.g. [49] and for a review [50]).

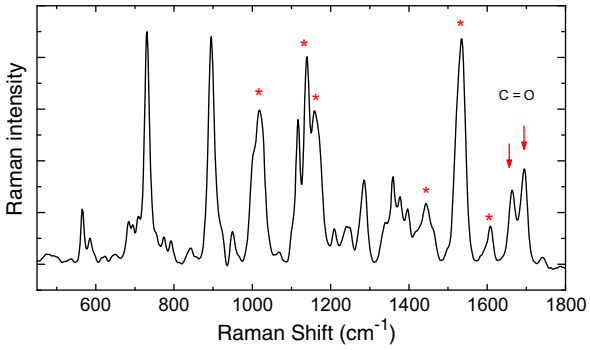

Fig. 4. Resonance Raman of bacteriochlorophyll *a* in THF, obtained with 1064 nm excitation. The modes marked with * are sensitive to the conjugated macrocycle conformation, termed R_1 to R_6 [44]; those marked with an arrow arise from stretching modes of the conjugated acetyl and keto carbonyl groups [40].

3.2. Characterizing the partner amino acids of (bacterio)chlorophylls: the primary electron donor in bacterial reaction centers

As described above, the frequency of the conjugated carbonyl groups of (bacterio)chlorophyll molecules is highly sensitive to the strength of the intermolecular interactions these groups are involved in. This property was extensively used to study the interactions of the BChl molecules in bacterial reaction centers (see e.g. [49,51,52]). Early experiments showed that the acetyl carbonyl of the two BChl molecules

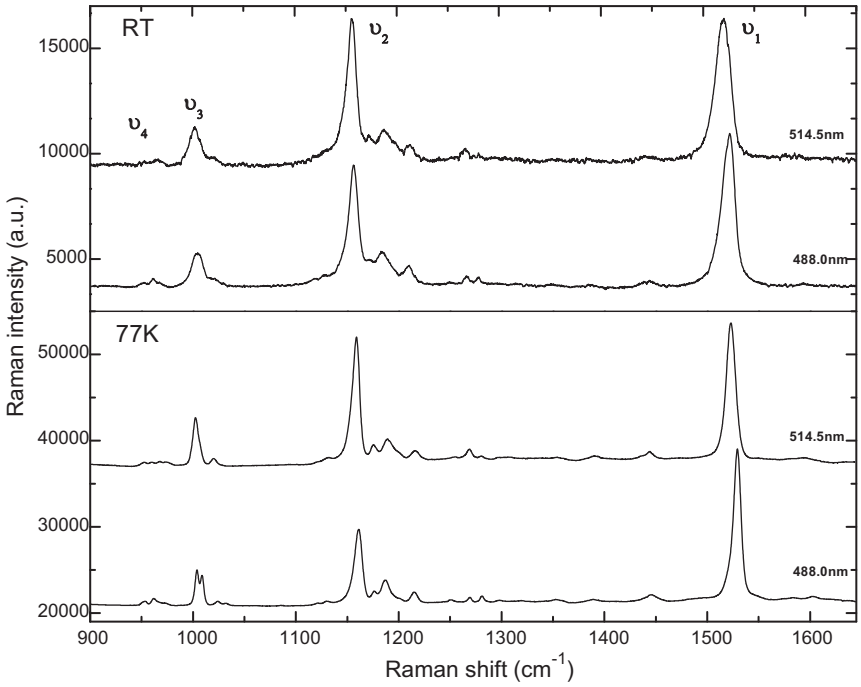

Fig. 3. Resonance Raman spectra of PSII-RC recorded with 488.0 and 514.5 nm excitation at room temperature (panel 1) and 77 K (panel 2). Note the ν_1 frequency shift between these two excitations, revealing a difference in the effective conjugation length of bound β-carotenes, and the difference in the ν_4 band structure, indicating different molecular conformations.

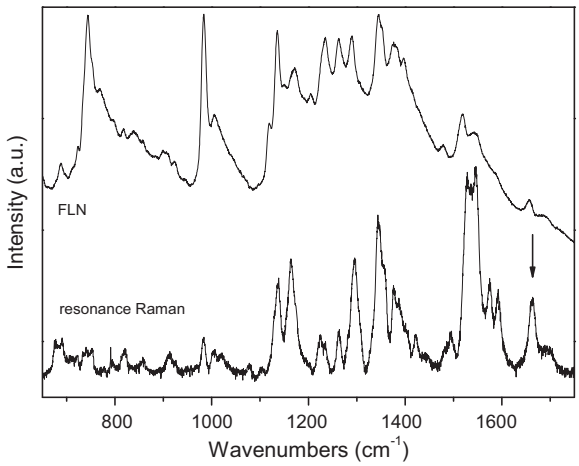

Fig. 5. Resonance Raman (77 K, 441.6 nm excitation) and FLN spectra of chlorophyll *d* in THF. Note the presence of a band at ca 1660 cm^{-1}, arising from the stretching mode of the conjugated acetyl group of this molecule (indicated by an arrow).

constituting the primary electron donor in this protein were asymmetrically H-bonded, in contrast with the first protein structure derived from X-ray crystallography [53]. Subsequent Raman studies, in combination with site-directed mutagenesis, led to the conclusion that the M210 tyrosine was not involved in an H-bond interaction with this BChl pair, but confirmed the presence of a very strong H-bond between the L168 histidine and the carbonyl acetyl of the special pair BChl located on the L side of the reaction center [54,55]. It is of note that the extreme selectivity of resonance Raman allowed such experiments to be performed on bacterial reaction centers that were still embedded in the photosynthetic membrane, provided that bacterial light-harvesting complexes were absent through gene deletion. Such experiments have in particular shown that the interactions between the apoprotein of the bacterial reaction center and its active sites were not perturbed upon membrane extraction [56]. Replacement of this histidine by an amino acid unable to H-bond the carbonyl induced a change in redox potential of the special pair of ca 100 mV, and a large number of bacterial reaction centers were designed as a result, in which H-bonding amino acids were introduced in front of each of the four carbonyls of the two BChl molecules of the special pair (in WT reaction centers, only one is H-bonded) [57]. Resonance Raman was used to control H-bond formation in each of these mutants, and to evaluate the strength of the newly-engineered bond. From this set of experiments, it was shown that the redox potential of the primary electron donor correlates with the number of H-bonds to its carbonyls, and more precisely to the total strength of these interactions [57,58]. It is of note that, from this set of experiments, a correlation could also be drawn between the redox potential of the primary electron donor and the total dipole born by the amino acids positioned in front of its carbonyl groups [59].

Near the two BChls constituting the primary electron donor two accessory BChls are located, which are on the path of electron transfer to the primary electron acceptor bacteriopheophytin. In 1988, resonance Raman experiments predicted that the oxidation of the primary electron donor would induce the formation of an H-bond between one of these BChl molecules (the one located on the electron-transfer-active side of the protein) and an unknown partner [51]. As the molecular structure of the bacterial reaction center could not at that time provide the identity of this partner, it was proposed that this unknown partner could be a water molecule not yet detectable in the X-ray-derived structure. Six years later, water molecules were indeed found in more refined structures of the reaction center at a position corresponding to the prediction deduced from these earlier resonance Raman experiments [60]. Indeed fifteen years after the initial Raman experiments, it was actually shown that one of these water molecules, located on the electron-active side of the protein, has a dramatic influence on both the rate of primary charge separation and the protein dynamics accompanying electron transfer [61,62].

3.3. Fluorescence line-narrowing of plant light-harvesting complexes

After the pioneering experiments of Avarmaa's group [7], vibrational studies based on FLN spectroscopy suddenly revived in the late 90's when Peterman et al. applied the technique to the major light-harvesting complex from higher plants, LHCII [4]. Since then, this method has been applied to a vast range of complexes. It is of note that vibrational narrowing has never been observed in BChl-based complexes from purple bacteria, except for the LH1 from *Rhodopseudomonas viridis* (Robert B., unpublished results). While it is reasonably easy to obtain vibrational narrowing from the BChl-containing Fenna–Matthews–Olson protein from green sulfur bacteria [3], such phenomena were never observed with LH1 or LH2 from purple bacteria. Similarly, while it is possible to get vibrational narrowing from the fully-dissociated form of LH1, namely B777, which is composed of monomeric LH1 polypeptides still retaining a bound BChl molecule, it was impossible to get similar results from the B820 form, which binds the basic spectroscopic dimer of BChl (Robert, B., unpublished results). Although it is difficult to formally discard experimental flaws, measurements performed on B820/B777 mixtures revealed that no vibrational narrowing of the B820 could be obtained at any excitation, in conditions where the vibrational narrowing of B777 was observed. This suggests the presence of very strong interactions in LH1, LH2 and B820 between the excited state and the surrounding medium, which may wipe out the narrowing by energy exchange. Of note is that these three complexes exhibit a strong absorption Stark effect [63,64], revealing the coupling of the excited state with a charge-transfer state, whose presence could very well explain the absence of vibrational narrowing in these complexes.

In contrast, vibrational narrowing could be observed for all the studied plant complexes, namely LHCII, CP29 and CP43 as well as for the Chl in cytochrome b_6f [4,65–69]. As stated above, from the mid-to-high frequency bands observed in these spectra, the coordination of the contributing Chl molecules can be deduced, as well as the interactions engaged by their conjugated carbonyl groups (in this case the keto carbonyl, as fluorescence generally arises from Chl *a* molecules which possess only one conjugated carbonyl). However, in FLN spectra, the contribution of the keto carbonyl stretching modes is extremely weak, and it is a challenge to access the structure of this band. On the other hand, as the frequency of this group is extremely sensitive to its protein environment, analyzing this spectral area may give direct access to the minimum number of molecules contributing to the fluorescence emission. The first experiments where such contributions were observed were performed on photosystem II reaction centers [66]. However, as the carbonyl of the different pigments bound to this protein shares quite similar environments, there was no dramatic differences between the carbonyl stretching regions of the FLN and resonance Raman spectra. When similar experiments were performed on the minor light-harvesting protein CP29, which bind a greater number of Chls *a* and *b*, a considerable reduction of the number of contributions is observed in the FLN spectra when compared to resonance Raman [65]. Nevertheless, no less than three unequivalent Chl molecules are still necessary to explain the complexity of the carbonyl stretching region in the FLN spectra — and indeed, this turns out to be in line with recent modeling of the excitation dynamics in plant light-harvesting complexes [5,70].

In recent years, single molecule experiments have shown that the main light-harvesting protein from higher plants, LHCII, explores a large conformational space at ambient temperatures, in which wells exist where the protein exhibits very red-shifted fluorescence properties [71]. As the bulk LHCII fluorescence spectrum is fitted very well by the addition of every observed single molecule spectrum, including the most red-shifted ones, the conclusion must be that these are not an artifact of the single molecule experiment [71]. Thus, FLN spectroscopy offers the possibility to highlight unexpected protein conformations,

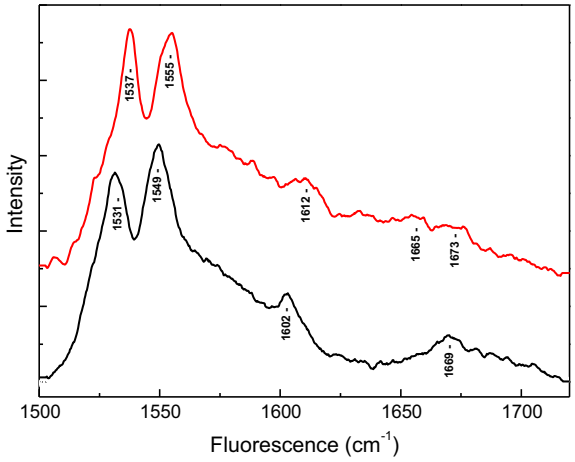

Fig. 6. Fluorescence line-narrowing spectra of LHCII trimers, excited at 685 nm (bottom) and 693 nm (top).

particularly if these exhibit red-shifted fluorescence emissions. In order to achieve this, FLN spectra are recorded with higher and higher excitation wavelengths, until the protein exhibiting the most red-shifted fluorescence properties is probed exclusively. This experiment was recently performed on LHCII (Robert, B. in preparation), and FLN spectra were obtained with excitation wavelengths as low as 695 nm, i.e. in a spectral range where there is virtually no LHCII absorption. Fig. 6 shows the rapid evolution of the FLN spectrum between 685 and 693 nm. Excitation at 685 nm results in a spectrum reasonably similar to that of a 5-coordinated Chl *a*, as indicated by the frequency of the main cycle bands (1536 and 1555 cm^{-1}), and the spectrum displays at least two, and probably three components in the carbonyl region (between 1655 and 1700 cm^{-1}). Excitation at 693 nm results in the presence of down-shifted cycle bands (1531 and 1550 cm^{-1}), and a carbonyl region exhibiting one principal frequency at 1671 cm^{-1}. These spectra suggest that, in this minor conformation of LHCII which fluoresces (and probably absorbs) to the red, the structure of the emitter is dramatically different — probably a single chromophore in a strained conformation.

4. Final word

Resonance Raman and FLN spectroscopies are extremely selective techniques which may unravel very fine structural details of the chromophores bound to photosynthetic complexes, and specifically those structural phenomena which rule the functional physico-chemical properties of these cofactors. Resonance Raman has been shown to be applicable to samples as complicated as cells and whole leaves, and FLN spectroscopy can not only pinpoint details of the bulk structure, it can also disclose unlikely conformations of these complexes. It seems likely that these techniques have a bright future in front of them. In addition, progress in DFT and time-dependent DFT, which now result in reasonable modeling of the vibrational spectra of molecules as complex as the photosynthetic pigments (see e.g. [24]), should in the next decade become an unavoidable support to formalize the data obtained by vibrational spectroscopy.

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