Optically probing structure and organization : single-molecule spectroscopy on polyethylene films and a resonance Raman study of a carotenoid

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Citation

Wirtz, A. C. (2006, October 26). *Optically probing structure and organization : single-molecule spectroscopy on polyethylene films and a resonance Raman study of a carotenoid*. *Casimir PhD Series*. Retrieved from https://hdl.handle.net/1887/4928

5 Two Stereoisomers of Spheroidene in the *Rhodobacter sphaeroides* R26 Reaction Center. A DFT Analysis of Resonance Raman Spectra.

abstract – In order to determine the structure of spheroidene incorporated in the photosynthetic reaction center (RC) of *Rhodobacter sphaeroides*, we have performed a theoretical analysis of the resonance Raman spectra of 19 isotopomers of spheroidene reconstituted into the R26 *Rh. sph.* RC. The normal mode underlying the transition characteristic for spheroidene in the RC at 1240 cm^{-1} has been identified and found to uniquely refer to the cis nature of the 15,15' carbon double bond. Detailed analysis of the isotope-induced shifts of transitions in the carbon-carbon double bond stretch region, proves that besides 15,15'-cis spheroidene another configuration occurs.

5.1 Introduction

Carotenoids in the membranes of purple photosynthetic bacteria are found both in the light harvesting complexes (LHC) and in the photosynthetic reaction centers (RC) [124,125]. For anaerobically grown *Rhodobacter sphaeroides* wild-type strain 2.4.1, the carotenoid bound to the RC is spheroidene [126]. It takes part in light harvesting and protects the bacteriochlorophyll pigments from photo-destruction by preventing the formation of singlet oxygen [124,127].

Spheroidene in the LHC is known to occur in the all-trans form [128,129]. As early as 1976, Lutz et al. suggested on the basis of resonance Raman scattering that in the RC, spheroidene adopts a cis conformation [128], probably 15,15'-cis [130]. They studied spheroidene reconstituted in the carotenoidless R26 *Rh. sphaeroides* RC. The latter was shown to have the same characteristics and structure after reconstitution as the wild type RC, which naturally contains spheroidene [57,131–133]. Lutz et al. later combined resonance Raman and ^{1}H-NMR spectroscopy on spheroidene extracted from the RC to conclude that the RC contained 15,15'-cis spheroidene [134]. In order to determine the precise location of the cis bond in the conjugated system, Koyama et al. compared resonance Raman data for the RC and various cis-isomers of β-carotenes [135, 136]. De Groot et al. relied on the same principle for the interpretation of ^{13}C magic angle spinning NMR data of the RC [137]. Such investigations have indicated with increasing confidence that spheroidene adopts a 15,15'-cis conformation. Bautista et al. demonstrated that the natural selection of a cis-isomer of spheroidene for incorporation into RCs is mainly determined by the actual structure of the RCs [138]. The crystallization and X-ray diffraction of the *Rh. sphaeroides* RC allowed for ever more accurate determination of the structure of the RC [57, 139–142]. The resolution of the derived electron density maps around the spheroidene molecule, however, does not suffice to unequivocally determine the structure of the carotenoid. As late as 2000, McAuley et al. considered the 13,14-cis conformer as a possible (though less likely) fit of their data [142].

In order to understand the function of spheroidene in the photophysical cycle of the photosynthetic complex, one needs to know its structure. Despite the impressive indirect evidence of the 15,15'-cis conformer occurring in the RC, this configuration still remains to be observed directly. Some time ago, we embarked on a project that involves (1) the synthesis of specific ^{13}C and ^{2}H labeled spheroidenes, (2) the reconstitution of these spheroidenes into the R26 RC, and (3) recording resonance Raman spectra of the spheroidene isotopomers both in solution and bound to the RC. We have reported the spectra for a range of ^{13}C and ^{2}H labeled spheroidenes in earlier articles [143,144]. Since then we have measured the spectra of many more isotopomers of spheroidene in the RC. Recently, we have also demonstrated that a complete description of the resonance Raman spectra of isotope labeled *all-trans*-spheroidene in solution could be obtained, using DFT geometry optimization and frequency calculations [145]. This success has inspired confidence that we can use the same theoretical approach to analyze the resonance Raman spectra of the spheroidene isotopomers in the R26 RC, in order to learn more about the structure of the carotenoid.

In this chapter we report on the progress we have made using DFT analysis for determining the structure of spheroidene in the RC. In our attempts to reproduce the experimental spectra, we have calculated spectra for a variety of structures. Both planar and a range of non-planar 15,15'-cis, 13,14-cis, 13,14-15,15'-13',14'-triple-cis and 15,15'-cis-10,11-12,13-double-s-cis spheroidene structures have been subjected to examination. None of these calculations reproduce the changes observed in the resonance Raman spectra upon isotope substitution entirely, particularly in the $1500 - 1550\,\mathrm{cm}^{-1}$ region. Our analysis will demonstrate that the RC must contain 15,15'-cis spheroidene. We cannot explain all isotope-induced shifts, however, if we suppose that all spheroidene in the RC exists in the 15,15'-cis configuration. Another conformer, probably 13,14-cis, also occurs in significant proportion.

Before commencing in section 5.4 with the DFT analysis of the resonance Raman spectra of spheroidene in the RC, we first present certain relevant experimental details and will briefly discuss several representative spectra in section 5.2. The computational methods used to optimize the molecular geometries and calculate the normal modes and frequencies are described in section 5.3.

5.2 Resonance Raman Spectra

The synthesis of isotope-labeled spheroidenes and their reconstitution into the *Rhodobacter sphaeroides* R26 photosynthetic reaction center have been described in previous publications [146–149]. Resonance Raman spectra of spheroidene in the reaction centers (RC) were obtained in a bath cryostat at 1.4 K from a glass of $30 - 50\,\%$ glycerol and the RC/Tris-buffer solution. In addition to the natural abundance (NA) spectrum we have determined the spectra for a total of 18 isotopomers of reconstituted spheroidene, 11 labeled with ^{2}H and 7 labeled with ^{13}C. These isotopomers are: 10-^{2}H, 11-^{2}H, 12-^{2}H, 14-^{2}H, 15-^{2}H, 15'-^{2}H, 14'-^{2}H, 11'-^{2}H, 10,12-^{2}H$_2$, 12,14-^{2}H$_2$, 15,15'-^{2}H$_2$, 8-^{13}C, 10-^{13}C, 11-^{13}C, 13-^{13}C, 15'-^{13}C, 14'-^{13}C and 13,14-$^{13}C_2$.

For the resonance Raman measurements we excited at 496.5 nm, in resonance with the first allowed $\pi^* \leftarrow \pi$ ($S_2 \leftarrow S_0$) transition. Only transitions corresponding to vibrational modes that contain conjugated C–C or C=C stretch character are resonance enhanced in the spectra. Hence for the normal-mode analysis we consider a truncated structure, comprising the C3 to C9' part of the molecule (see Figure 5.1) terminated by carbon atoms that were assigned the masses of the corresponding terminal groups (87 and 151 for C_5H_9O and $C_{11}H_{17}$ respectively). Figure 5.1 contains a schematic representation of spheroidene and explains our labeling. For simplicity spheroidene is shown in the *all-trans* form.

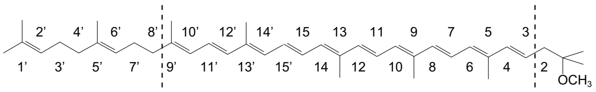

Figure 5.1: Schematic representation of the spheroidene molecule and our labeling system. The molecule is shown in the *all-trans* form. The conjugated part of spheroidene is indicated between dashed lines.

In Figure 5.2 the resonance Raman spectra in the range $400 - 1600\,\text{cm}^{-1}$ are displayed for NA spheroidene in petroleum ether, and NA and 15,15'-$^2H_2$ labeled spheroidene in the R26 reaction center. Three regions can be recognized in the spectrum of *all-trans* spheroidene in Figure 5.2(a). Between 1500 and $1600\,\text{cm}^{-1}$ transitions are seen that correspond to C=C stretch vibrations. The so-called fingerprint region between 1100 and $1300\,\text{cm}^{-1}$ is where normal modes comprising stretch vibrations of C–C bonds and in-plane H-bend vibrations are found. In the region around $1000\,\text{cm}^{-1}$, there is a band at $1002\,\text{cm}^{-1}$ which belongs to a normal mode composed of methyl-rock vibrations. The absence of out-of-plane H-bend transitions, normally found around $950\,\text{cm}^{-1}$, indicates that this structure is planar. For planar spheroidene, symmetry rules forbid the mixing of out-of-plane modes with the C–C and C=C stretch modes, and they cannot gain intensity in the resonance Raman spectrum.

The spectra of the spheroidenes in the RC in Figure 5.2 do clearly show out-of-plane H-bend transitions around $950\,\text{cm}^{-1}$, and hence these spheroidene structures cannot be planar. In general, these two spectra are much richer in transitions in all three aforementioned regions than the spectrum in Figure 5.2(a). In the fingerprint region, among the signals that stand out in comparison to the *all-trans* spectrum, we see one or several bands at or slightly below $1240\,\text{cm}^{-1}$. In the C=C stretch region we see two or more transitions appear.

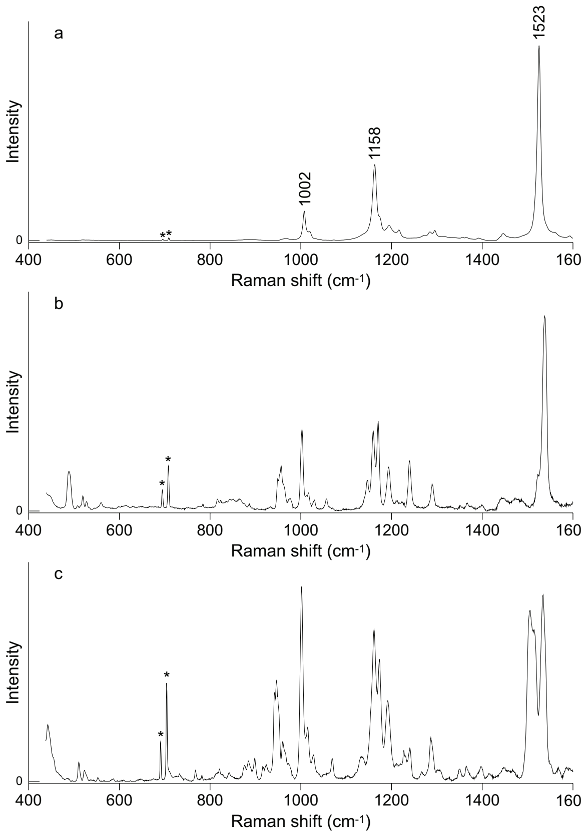

Figure 5.2: Resonance Raman spectra of (a) NA spheroidene in petroleum ether, and (b) NA spheroidene and (c) 15,15'-$^2H_2$ spheroidene in the photosynthetic reaction center of *Rhodobacter sphaeroides.* Peaks denoted with * are argon plasma lines.

The spectrum for NA spheroidene in the RC displays an intense C=C stretch band at $1538\,\mathrm{cm}^{-1}$ with a clear shoulder at $1523\,\mathrm{cm}^{-1}$. The peak at $1538\,\mathrm{cm}^{-1}$ has a width of $11\,\mathrm{cm}^{-1}$, which could indicate the presence of two overlapping transitions. The shift of the most intense transition to a higher frequency for spheroidene in the RC with respect to *all-trans*-spheroidene ($1523\,\mathrm{cm}^{-1}$) is larger than expected upon introducing a cis double bond in the conjugated part of the molecule. Such upward shifts are related to a reduced conjugation length and are normally found to be about $10\,\mathrm{cm}^{-1}$ for β-carotenes [135, 136, 150]. The fact that the most intense peak shifts upward by $16\,\mathrm{cm}^{-1}$ is most probably due to the non-planarity of the structure of RC spheroidene. In Figure 5.2(c) more than the aforementioned two bands are apparent in the region between

1500 and 1540 cm^{-1}. This is also the case for many other isotopomers of spheroidene in the RC, most notably all those labeled at or somewhere between chain positions 11 and 14'. We have checked the possibility that C=C stretch signals arise from residual isotope-labeled *all-trans* spheroidene present in our samples. Careful comparison of the spectra of spheroidene isotopomers in the RC and in petroleum ether has shown that this is not the case. The positions of transitions and distinct shoulders in the C=C stretch regions of the resonance Raman spectra displayed in Figure 5.2, as well as for all other isotopomers, are listed in Table 5.1. For some isotopomers, denoted with a ‡ symbol in Table 5.1, the spectra display broad peaks in this region as a result of the close proximity of various transitions. The overlap of signals therefore prohibits the determination of the precise frequencies associated with these transitions. Note that this is not a consequence of limited resolution of our monochromator, but of the intrinsic width of the signals associated with molecular transitions. For the marked isotopomers the peak width (FWHM) is given in the caption of Table 5.1. The width of a single transition is approximately 8 cm^{-1}, so any significantly broader band in principle consists of more than one transition. Some of the indicated spectra will be treated in more detail in the discussion of the normal-mode analysis.

5.3 Computational Methods

In a previous article we described our method for the calculation and subsequent analysis of the resonance Raman spectra of spheroidene in solution [145]. The success of our method in quantitatively describing the resonance Raman spectra of a large conjugated molecule like *all-trans*-spheroidene makes us confident that this method can also be applied to analyze cis-spheroidene bound to the RC. Furthermore, we have performed test calculations on 9- and 11-cis-retinal and found that the calculated frequencies agree well with experimental resonance Raman frequencies [150]. The spectra are calculated in four steps:

(1) Geometry optimization. The GAUSSIAN 98 package, Rev. A.5 [151] on an IBM SP2 computer and later the GAUSSIAN 03 package, Rev. B.05 [152] were used for performing the DFT calculations on an 18 dual processor node Beowulf cluster (1.6 GHz Xeon CPUs). We used a 6-31G* basis set combined with the hybrid B3LYP functional. Geometry optimization was done in Cartesian coordinates and carried out with the Berny algorithm and an ultra-fine integration grid for numerical calculation of the two-electron integrals.

Our starting structure was taken from the X-ray structure of the reaction center as determined by McAuley et al. [142] for a mutant bacterium at a

Isotopomer	Experimental C=C stretch frequencies (% max int.)				Calc. C=C str. freq.			
					15,15’-cis		13,14-cis	
NA	1523 (19)		1538 (100)		1525	1537	1526	1539
8-^{13}C	1524 (100)	1533 (55)	1538 (29)	1550(19)	1523	1535	1525	1532
10-^{2}H‡	1534 (100)				1525	1531	1523	1539
10-^{13}C‡	1518 (100)				1525	1528	1522	1539
11-^{2}H	1516 (54)	1529 (100)	1540 (46)		1516	1534	1524	1528
11-^{13}C	1519 (76)	1528 (100)			1517	1536	1525	1529
12-^{2}H‡	1524 (100)	1530 (62)			1519	1534	1519	1531
13-^{13}C	1524 (93)	1528 (100)	1537 (81)		1522	1529	1512	1539
14-^{2}H	1522 (26)	1532 (100)			1523	1531	1516	1539
15-^{2}H	1514 (26)	1529 (100)	1535 (87)		1517	1535	1524	1529
15’-^{2}H	1514 (22)	1529 (100)	1537 (82)		1517	1537	1525	1531
15’-^{13}C	1508 (11)	1522 (32)	1539 (100)		1511	1537	1522	1533
14’-^{2}H‡	1520 (25)	1531 (100)	1535 (86)	1541 (34)	1523	1535	1523	1538
14’-^{13}C‡	1529 (100)		1534 (85)	1555 (10)	1525	1533	1521	1539
11’-^{2}H	1528 (51)		1538 (100)		1523	1537	1525	1537
10,12-^{2}H$_2$‡	1518 (100)		1537 (8)		1517	1530	1516	1531
12,14-^{2}H$_2$‡	1519 (100)				1514	1530	1509	1530
15,15’-^{2}H$_2$	1506 (92)	1515 (81)	1534 (100)	1551 (9)	1506	1535	1514	1524
13,14-^{13}C$_2$	1505 (40)	1511 (90)	1526 (78)	1539 (100)	1514	1526	1499	1539

Table 5.1: This table lists the positions and intensities for peaks and visible shoulders in the C=C stretch regions of both experimental and calculated resonance Raman spectra. The intensity value between brackets is proportional to the most intense peak in the spectrum.

‡ Due to the proximity of transitions, a single experimental peak is found for which assignments of maxima and shoulders are tentative or even impossible. The widths (FWHM) are: 10-^{2}H 16 cm^{-1}, 10-^{13}C 18 cm^{-1}, 12-^{2}H 17 cm^{-1}, 14’-^{2}H 14 cm^{-1}, 14’-^{13}C 20 cm^{-1}, 10,12-^{2}H$_2$ 18 cm^{-1}, 12,14-^{2}H$_2$ 18 cm^{-1}

resolution of 2.1 Å. This structure is deposited in the RSCB Protein Data Bank as 1QOV. The *Rhodobacter sphaeroides* bacterium whose RC was studied by McAuley et al. was grown under semi-aerobic conditions in the dark and therefore the bound carotenoid is actually spheroidenone ($C_{41}H_{58}O_2$) instead of spheroidene ($C_{41}H_{60}O_1$). Spheroidenone contains an additional carbonyl group bound to C-atom 2 (see Figure 5.1). The carbonyl group does not appear to interact with the surrounding protein [142] so as to significantly affect the spheroidenone configuration compared to that of spheroidene. The conjugated part of the spheroidenone structure we have based our calculations on, can therefore be considered to be the same as that of spheroidene.

As was mentioned in section 5.2, the presence of out-of-plane H-bends in the resonance Raman spectra of incorporated spheroidenes indicates that the molecule cannot be planar. Geometry optimizations of isolated 15,15’-cis spheroidene all resulted in a planar structure, due to the absence of a protein scaffold. In order to prevent the DFT optimized structures from becoming planar we have fixed the Cartesian coordinates of C-atoms of methyl groups in our calculations. These coordinates were taken from the X-ray file.

(2) Numerical calculation of the Hessian in Cartesian coordinates and transformation to mass-weighted coordinates, using GAUSSIAN 98 or GAUSSIAN 03 with the Freq(ReadIsotopes) keyword and option.

(3) Calculation of the normal modes and corresponding frequencies for all isotopomers, using the Wilson GF formalism. In this chapter we refer to calculated spectra for spheroidene containing only the most abundant isotopes ^{1}H and ^{12}C as natural abundance (NA) spectra, despite the fact that in actuality naturally occurring carbon contains 1.1 % ^{13}C and hydrogen 0.015 % ^{2}H. The DFT calculated frequencies are scaled by a factor of 0.963 [153].

(4) Resonance Raman intensity. We estimated the intensity of a normal mode I_a according to Equation 5.1:

$$I_a \propto \nu_\alpha \left(\sum_i A_{\alpha i} \delta_i \right)^2 \tag{5.1}$$

In this equation, which is only an approximation [145,154], ν_α is the frequency of normal mode α, A represents the transformation matrix from internal into normal coordinates that was determined in step (3) and δ_i equals the change in internal coordinate i as a result of the (near-)resonant electronic $\pi^* \leftarrow \pi$ (HOMO to LUMO) transition.

Reproduction of the experimental frequencies for all isotopomers is in fact the most significant indicator that we have obtained a correct molecular structure. For this reason the primary goal of our work was to reproduce the frequencies of the experimental spectra in our calculations. The intensity estimation in step (4) mainly served to reveal which transitions display any resonance Raman intensity at all. We have used the same set of δ_i-values (for the C–C and C=C bonds) as for *all-trans*-spheroidene [145]. In that case the δ_is were determined from the best fit between experimental and calculated resonance-Raman intensities. Later on electronic calculations (semi-empirical ZINDO method) showed that these values could be rationalized on the basis of the increase/decrease of the double-bond character of each carbon-carbon bond from the HOMO to the LUMO. Similar calculations for cis-spheroidenes revealed negligible changes compared to *all-trans*-spheroidene.

5.4 DFT analysis

In this section we will discuss the results from our calculations. Presently, the consensus in literature is that spheroidene in the Rhodobacter sphaeroides photosynthetic reaction center exists in the 15,15'-cis configuration. We have therefore started our analysis from the same assumption. In our discussion we will show that, especially for the C=C stretch region, although the calculated frequencies nicely reproduce some resonance Raman bands and their shifts upon isotope substitution, other peaks remain that cannot be explained with a 15,15'-cis configuration.

5.4.1 C=C stretch region

As Figure 5.2 and Table 5.1 illustrate, reconstituted NA and isotopically labeled spheroidenes in the R26 RC show two or more C=C stretch modes in the region between 1500 and 1540 cm^{-1}. For several experimental spectra the bands in this region are quite broad ($> 10\,\mathrm{cm}^{-1}$) and probably contain more than one normal-mode peak. The relevant isotopomers have been denoted with a ‡ symbol in Table 5.1. In this section we focus on how our calculations reproduce the transitions associated with C=C stretch modes.

Although Figure 5.2 shows that spheroidene in the RC is not planar we have started our calculations with a planar 15,15'-cis spheroidene configuration. The calculations, as expected, result in two NA C=C stretch frequencies that are too low, giving 1520 and 1530 cm^{-1}. The earlier work on *all-trans*-spheroidene also revealed two C=C stretch normal modes, visible as distinct transitions only in the spectra of some isotopomers. The two C=C stretch modes were shown by Dokter et al. to consist of two in-phase combinations, one containing stretch vibrations of double bonds that are substituted with a methyl group, and one containing stretch vibrations of unsubstituted C=C bonds [145]. The calculations for cis-spheroidene yield similar mode compositions, except that already for NA they are calculated at more separated frequencies. For planar 15,15'-cis spheroidene, the mode at 1520 cm^{-1} corresponds to the normal mode that comprises unsubstituted local C=C stretch modes and the mode at 1530 cm^{-1} to the normal mode that consists of a linear combination of local methyl-substituted C=C stretch modes. For brevity we will in the following discussion refer to the former as the C=C stretch mode and the latter as the Me–C=C stretch mode.

In order to prevent optimized spheroidene structures from becoming planar, we have frozen the carbon positions of all five methyl groups at the coordinates found for the X-ray structure. The positions of the methyl groups show

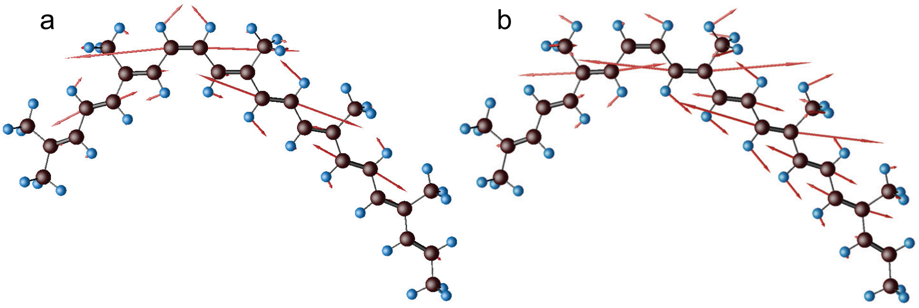

Figure 5.3: Mode compositions for the C=C stretch (a) and Me–C=C stretch modes (b) of non-planar 15,15'-cis spheroidene. Double bonds have been drawn as thicker sticks. Atom sizes and bond lengths have not been drawn to scale. Arrows indicate relative displacement, but are also not drawn to scale. Displacement for hydrogen atoms has been scaled down. The molecule is drawn in approximately the same orientation as the schematic representation in Figure 5.5(a).

up clearly in the electron density map as protrusions, making them probable points of fixture. The non-planar 15,15'-cis structure obtained this way yielded two NA frequencies, 1525 and 1537 cm^{-1}. The former is the C=C stretch mode and the latter the Me–C=C stretch mode. Figure 5.3 shows what the compositions are for these two modes (see Figure 5.5(a) for labeling). As predicted, non-planarity results in higher stretch frequencies and these correspond well with the experimental values of 1523 cm^{-1} and 1538 cm^{-1}. No normal modes are calculated to occur in this region other than these two modes. The calculated frequencies between 1500 and 1550 cm^{-1} for all isotopomers of this structure have been summarized in Table 5.1. The bond lengths, bond angles and dihedral angles of the optimized geometry are given in Table 5.2 in the supplementary material. Our calculations produce two normal modes in the C=C stretch region for each isotopomer. The experimental spectra of isotope-labeled spheroidenes in the RC, however, often show three or four bands in the C=C stretch region. We will discuss this phenomenon more in-depth later in this chapter.

In the following paragraphs we will examine in more detail the results of our DFT calculations for the non-planar 15,15'-cis spheroidene structure. Our calculations show that the C=C stretch mode compositions for the isotopomers remain roughly the same, while frequencies shift as expected from mass-effects upon isotope-substitution. The discussion will focus on the C=C stretch regions of the experimental spectra of NA spheroidene and 7 isotopomers in the RC, as displayed in Figure 5.4. Above each spectrum 8 cm^{-1} wide bars show the calculated frequencies from Table 5.1 for the respective isotopomer. Continuous bars denote C=C stretch modes and dashed bars Me–C=C stretch modes.

Consider the spectrum of 15'-^{2}H (Figure 5.4(b)) substituted spheroidene. It displays at least three clear transitions as listed in Table 5.1. At first glance it appears that the Me–C=C mode at 1538 cm^{-1} is unaffected, as may be expected when labeling an unsubstituted C=C bond. Instead of a signal at 1523 cm^{-1} we see one that has shifted down 9 cm^{-1} and one that has shifted up 6 cm^{-1} with respect to the NA spectrum (Figure 5.4(a)). This seems to indicate a splitting of the C=C stretch mode. The peak at 1514 cm^{-1} might correspond to a C=C stretch mode in which the labeled 15=15' bond participates more, and the one at 1529 cm^{-1} to a mode in which it does not participate much. The fact is that our calculations only produce two normal modes in this spectral region, whose frequencies correspond to the two *outermost* signals quite well, but no mode is calculated to occur around 1529 cm^{-1}. Our method produces no splitting for the lower frequency C=C mode upon 15'-^{2}H substitution.

The spectrum for doubly substituted 15,15'-$^2H_2$ spheroidene (Figure 5.4(c)) in the RC also shows three maxima. Moreover, the signal does not approach zero at 1524 cm^{-1}, despite the fact that the maxima to either side are 19 cm^{-1} apart. As was mentioned earlier, each transition is expected to give rise to a peak of approximately 8 cm^{-1} width (FWHM). This indicates the presence of a fourth transition at approximately 1524 cm^{-1}. The maximum at 1505 cm^{-1} is probably a C=C stretch mode related to the one found at 1514 cm^{-1} in the singly labeled 15'-^{2}H spectrum, as it has shifted roughly twice as far from the NA position. In this spectrum it has acquired significantly more intensity. A strong transition is also visible at 1515 cm^{-1}, which is harder to explain. It was verified that it does not arise from residual 15'-^{2}H spheroidene present in the sample. Our calculations still do not produce any C=C mode splitting, and once again reproduce the two *outermost* signals at 1505 cm^{-1} and 1534 cm^{-1} deriving from C=C and Me–C=C normal modes respectively.

When we label the carotenoid on a methyl-substituted double bond, like in 13-^{13}C spheroidene, we see two maxima and two shoulders appear in Figure 5.4(d). Curiously, a clear band at 1537 cm^{-1} is still visible, like it is in the NA spectrum. We had initially interpreted this transition as belonging to the Me–C=C stretch mode. Such a mode should have been affected by this labeling. In fact, our calculations for non-planar 15,15'-cis spheroidene show two frequencies: a C=C stretch mode at 1522 cm^{-1} and a Me–C=C vibration at 1529 cm^{-1}. These reproduce the two *innermost* features in the experimental spectrum, as opposed to when we label the unsubstituted dou-

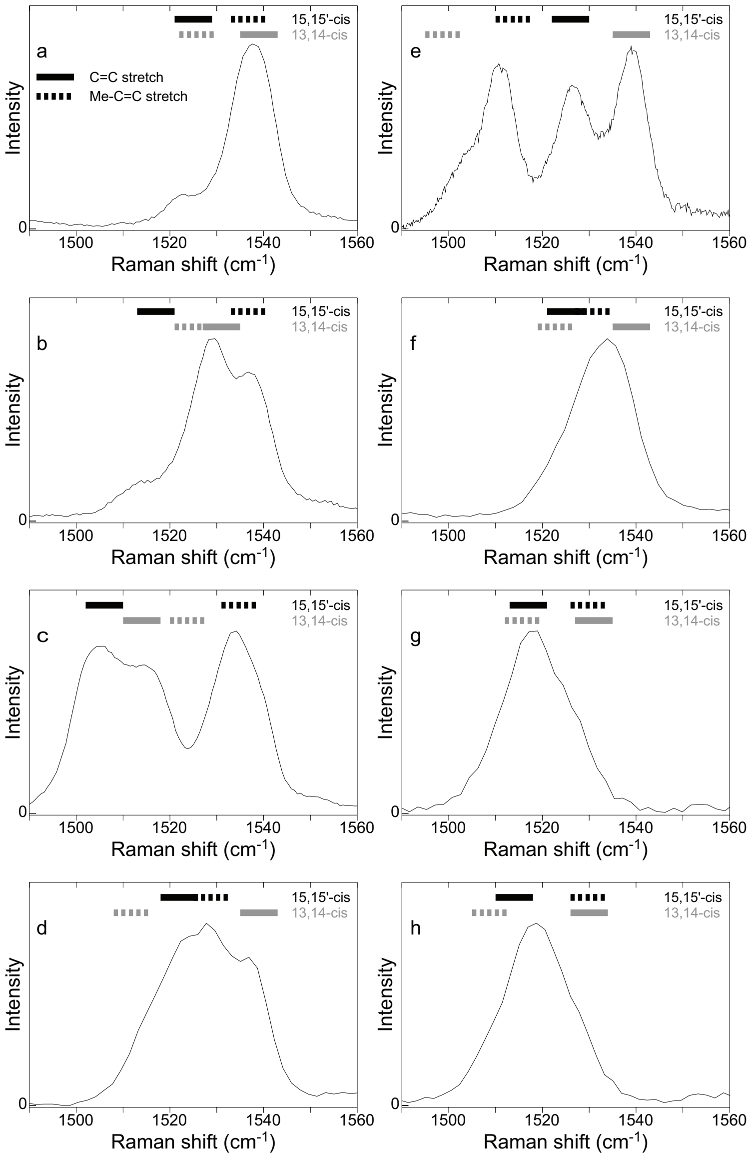

Figure 5.4: The C=C stretch regions of the resonance Raman spectra of reconstituted spheroidene in the *Rhodobacter sphaeroides* R26 photosynthetic reaction center. (a) NA, (b) 15'-^{2}H, (c) 15,15'-$^{2}H_2$, (d) 13-^{13}C, (e) 13,14-$^{13}C_2$, (f) 10-^{2}H, (g) 10,12-$^{2}H_2$, (h) 12,14-$^{2}H_2$. Calculated frequencies have been indicated above each spectrum by bars. Values calculated for the 15,15'-cis structure are displayed in black and values for the 13,14-cis structure in gray.

ble bonds. The C=C stretch mode is largely unaffected by labeling at the 13 position, whereas the Me–C=C mode shifts to a lower frequency. The signal at 1537 cm^{-1} cannot correspond to a Me–C=C normal mode containing the local 13=14 coordinate. Again transitions are visible in the spectrum that our method does not reproduce for this configuration. The experimental bands are all the more interesting, because of the presence of the shoulder at 1515 cm^{-1}. If this shoulder corresponded to the Me–C=C stretch mode shifting down due to a single ^{13}C substitution at position 13, it would have shifted down by no less than 23 cm^{-1}. This seems an unreasonably large shift.

The experimental spectrum for 13,14-^{13}C$_2$ spheroidene (Figure 5.4(e)) in the RC is even more surprising at first glance. We can make out four transitions. A shoulder is clearly visible at 1505 cm^{-1} and the three distinct peaks each display a width characteristic of a single transition. Once again we note a peak at 1539 cm^{-1}, which cannot be compatible with a Me–C=C mode that includes the 13=14 internal coordinate. Likewise, one would not expect a Me–C=C normal mode to shift by 33 cm^{-1} to 1505 cm^{-1}, even when doubly ^{13}C-labeling a methyl-substituted double bond. Our calculations suggest the signal at 1511 cm^{-1} corresponds to the Me–C=C stretch mode and the 1526 cm^{-1} peak to the C=C delocalized vibration, quite in line with the results for 13-^{13}C labeled spheroidene. No other modes are calculated to occur in this frequency range.

The entry for 10-^{2}H labeled spheroidene in Table 5.1 was denoted with a ‡. This symbol indicates an experimental spectrum that does not allow for an unambiguous determination of the frequencies corresponding to the overlapping transitions. Figure 5.4(f) shows the C=C stretch region of the experimental spectrum of this isotopomer. It displays a single broad peak with a maximum at 1534 cm^{-1}. The experimental peak's width (16 cm^{-1} FWHM) indicates, however, that it in fact encompasses more than a single resonance Raman transition. The calculated values of 1525 and 1531 cm^{-1} lie within the range corresponding to the broad experimental band.

The final two spectra in Figure 5.4 are (g) 10,12-^{2}H$_2$ and (h) 12,14-^{2}H$_2$. They illustrate another striking phenomenon in our experimental spectra. These doubly labeled spheroidenes yield significantly different spectra from the other two discussed previously (Figures 5.4(c) and (e)). Instead of showing three or more distinct C=C or Me–C=C stretch resonance Raman transitions, whose maxima cover a range from 1500 to 1540 cm^{-1}, the two spectra in Figures 5.4(g) and (h) both consist of single broad features. The two isotopomers are distinct in that they have been labeled on both a C=C and a Me–C=C bond, causing a downward shift in both stretch modes. Whereas labeling a single type of double bond, be it with one or two heavier isotopes, consistently left a high frequency (just below $1540\,\mathrm{cm}^{-1}$) feature visible in the spectrum, substituting with ^{2}H on both types of double bonds, causes all features to shift downwards. This is an indication that the high frequency features visible at approximately $1538\,\mathrm{cm}^{-1}$ belong both to a mode with C=C and a mode with Me–C=C character. The $11\,\mathrm{cm}^{-1}$ width of the $1538\,\mathrm{cm}^{-1}$ peak in the NA spectrum is not inconsistent with this interpretation. Before elaborating on this, however, we will discuss some attempts at varying the non-planar 15,15'-cis structure.

For 15,15'-cis spheroidene, the most crucial section of the molecule for our calculations lies around the 15=15' bond. In an attempt to characterize the effect on the calculated modes and frequencies of both the 15=15' bond length and the 14–15=15'–14' dihedral angle, we have systematically varied their values between 1.35 and 1.39 Å and 0 and 10 ° respectively. Whereas these small variations of the dihedral angle are not found to have any significant impact on calculated frequencies, the 15=15' bond length—not surprisingly—is quite critical for the value of the unsubstituted C=C stretch frequency. Neither parameter, however, strongly influences the mode compositions and neither therefore induces a splitting of normal modes. Even a dihedral angle as large as 60 ° does not effect the number of modes calculated in the C=C stretch region. The latter of course does lead to a lengthening of the 15=15' bond, which results in a strong drop of the frequency of the unsubstituted stretch mode.

We have established that major changes to the structure of 15,15'-cis spheroidene do influence the position of the calculated modes in the C=C stretch region, while they do not give rise to extra modes for isotope labeled spheroidenes. We have therefore turned our attention towards finding an alternative structure that might produce multiple bands in the C=C stretch region upon isotope substitution. A range of alternative configurations have been investigated. These are shown in Figure 5.5(b) 13,14-cis, (c) 13,14-15,15'-13',14'-triple-cis and (d) 15,15'-cis-10,11-12,13-double-s-cis. These configurations have been considered, because they might be compatible with the electron density map as determined by McAuley et al. and refined by Roszak et al. [57, 142]. Configurations (b), (c) and (d) have in common that for the NA calculation their lowest frequency C=C stretch mode is the methyl-substituted mode, in contrast to the 15,15'-cis structures. In the case of (b) this is because of the presence of a methyl-substituted cis double bond, instead of an unsubstituted C=C cis bond. Structure (c) has two methyl-substituted C=C cis bonds and one unsubstituted C=C cis bond and therefore the Me–C=C stretch mode has the lowest frequency. For (d) the Me–C=C mode is the lowest of the two, because of the diminished participation of the 11=12 bond in the

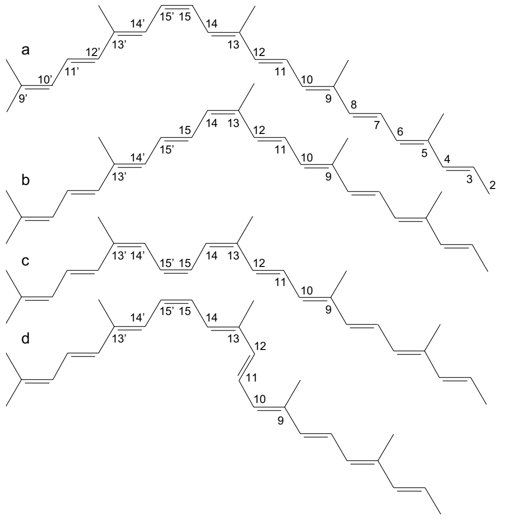

Figure 5.5: Schematic representations of spheroidene configurations (a) 15,15'-cis (b) 13,14-cis, (c) 13,14-15,15'-13',14'-triple-cis (d) 15,15'-cis-10,11-12,13-double-s-cis.

unsubstituted C=C normal mode, raising the frequency of the unsubstituted stretch mode above that of the methyl-substituted one. Only the 13,14-cis configuration produces NA frequencies close to the experimental values. The calculated values are listed in Table 5.1. What calculations on these three structures have in common with all other spheroidene structures studied, is that they—again—do not result in more than two bands in the C=C stretch region. We will nonetheless more closely examine the mode structure of the calculated spectra for the 13,14-cis, because of the close correspondence of the NA frequencies with the experiment.

The non-planar 13,14-cis spheroidene structure was obtained by restraining methyl groups in a similar way as for the 15,15'-cis structure discussed above. In this case we only fixed the four methyl groups at the 5, 9, 13 and 13' positions (see Figure 5.5(b)). As is made clear in Table 5.1, the calculated frequencies for NA 13,14-cis spheroidene come close to those for the 15,15'-cis structure and fit the experimental results as well. In this case the high frequency mode is the C=C stretch mode, a reversal with respect to the 15,15'-cis calculations. Undoubtedly this will have some consequences for the direction in which the bands shift with isotope labeling.

Regarding the spectra in Figures 5.4(b) and (c) (15'-^{2}H and 15,15'-$^{2}H_2$), we immediately see that also the 13,14-cis calculations fail to produce all features. Due to the reversed mode structure, the signal that shifts upon

^{2}H-substitution is now also the opposite of that in the 15,15'-cis calculations. The previously unexplained strong signal at 1529 cm^{-1} in Figure 5.4(b) is now reproduced in our calculations (1531 cm^{-1}). The calculated Me–C=C frequency of 1525 cm^{-1} is not visible, but might be drowned out by the signal at 1529 cm^{-1}. The Me–C=C mode calculated at 1524 cm^{-1} for the 15,15'-^{2}H$_2$ isotopomer could explain the fact that the signal does not approach zero in the experimental spectrum. Likewise do our calculations now explain the feature at 1515 cm^{-1} in Figure 5.4(c), but not that at 1505 cm^{-1}. The two outermost peaks in both experimental spectra are not produced in this case, exactly the opposite of what was lacking in our calculations for the 15,15'-cis structure.

The same conclusions hold for the spectra in Figures 5.4(d) and (e) (13-^{13}C and 13,14-^{13}C$_2$). The peaks reproduced by our calculations for a 13,14-cis configuration are precisely those that were not explained by our 15,15'-cis calculations. For the latter configuration we could only reproduce the innermost peaks, and for the 13,14-cis structure only the outermost come out of these two calculations. Although the Me–C=C stretch mode at 1499 cm^{-1} for 13,14-^{13}C$_2$ is found a bit too low, we can now at least explain the origin of the shoulder at 1505 cm^{-1} in Figure 5.4(e): It results from the Me–C=C mode, shifted down 18 cm^{-1} with respect to the NA value. Such a shift is more reasonable than the 33 cm^{-1} needed to explain it using our 15,15'-cis calculated results. It should be clear by now that the 13,14-cis calculations reproduce several experimental bands and correctly predict how they shift upon isotope substitution, while they are completely unable to explain those that the calculation on the 15,15'-cis configuration predicts. As such, the two calculations appear complementary.

A significant result is obtained for the 10,12-^{2}H$_2$ and 12,14-^{2}H$_2$ isotopomers. Our calculations produce nearly the same values and shifts for both the 13,14-cis and 15,15'-cis spheroidene configurations. Such results are to be expected, since the labeling takes place on both types of double bonds. This brings us back to the point made earlier about the appearance of the two experimental spectra for these doubly substituted isotopomers. Instead of showing several peaks spread over the range of $1500 - 1540$ cm^{-1}, like the 15,15'-^{2}H$_2$ and 13,14-^{13}C$_2$ spectra, they display a relatively narrow signal around 1518 cm^{-1}. This can only be explained if we assume the signals in the NA spectrum each represent both a Me–C=C and a C=C stretch mode. Such an assumption in turn can only be made by supposing that the photosynthetic reaction centers of *Rhodobacter sphaeroides* contain spheroidene in more than one configuration. Moreover, the configurations present must have inverted mode compositions in the C=C stretch region, like for example the 15,15'-cis structure and the 13,14-cis structure.

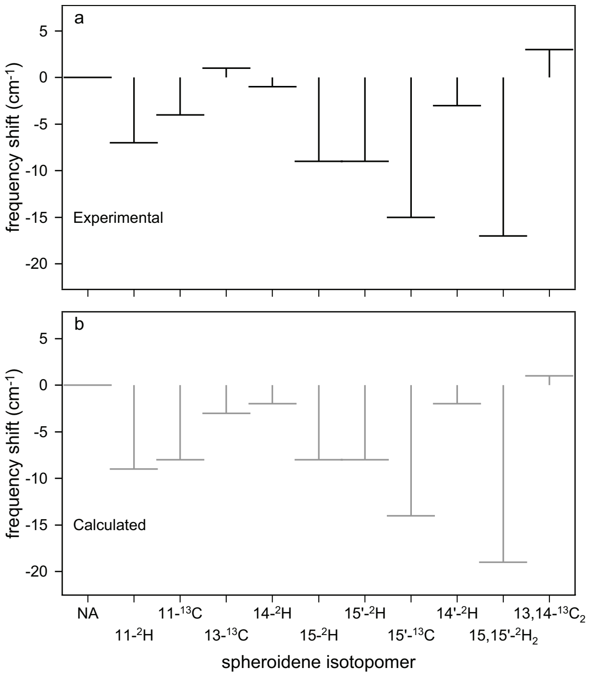

Figure 5.6: A comparison of experimental (a) and calculated (b) shifts for the C=C stretch mode of 15,15'-cis spheroidene with respect to the NA values. The experimental NA frequency is 1523 cm^{-1} and the calculated one is 1525 cm^{-1}. Only for the isotopomers shown it is possible to assign a transition, whereas in the other spectra the C=C stretch and Me–C=C stretch modes show too much overlap.

The interpretation of the experimental results as originating from two structures enables us to assign the transitions in the C=C stretch region of the spectra to either 15,15'-cis spheroidene or to the second stereoisomer, and to a C=C or Me–C=C stretch mode. Figure 5.6 shows a comparison of experimental shifts of the C=C stretch mode of 15,15'-cis spheroidene to the shifts that we calculated for this mode. We have included shifts of only 11 out of 19 isotopomers. The resonance Raman spectra of the displayed isotopomers show clearly resolved transitions for the assigned mode, whereas the other spectra do not (e.g. Figures 5.4(f)–(h)). The close correspondence of the experimental and the calculated values serves to illustrate that our 15,15'-cis calculations correctly reproduce the frequencies found in the C=C stretch region of the resonance Raman spectra, as long as we assume that another spheroidene configuration is present as well. The same holds true for the Me–C=C stretch mode of 15,15'-cis spheroidene (not shown here).

5.4.2 Fingerprint region: 1150–1240 cm^{-1}

The normal modes visible in the fingerprint regions of the resonance Raman spectra (see Figure 5.2 between 1150 and 1300 cm^{-1}) comprise in-plane C–C stretch and C–H bend vibrations. The spectra reveal intense signals lying close together between 1150 and 1180 cm^{-1} for all isotopomers. Our calculations for the 15,15'-cis structure reveal several strong transitions in this region, which are delocalized throughout the conjugated part of the chain. The transitions calculated in this region are found close together and consist of many local modes. As a result, the compositions and frequencies of the calculated vibrational modes are found to be quite sensitive to even minor changes in the spheroidene structure. For this part of the spectrum the correspondence of the calculated frequencies with the experimental spectra is less quantitative than for the C=C stretch region. This is to be expected as it was already found to be the case for *all-trans*-spheroidene [145].

There is one strong transition in the NA spectrum at 1193 cm^{-1}, which is observed at roughly the same frequency for all isotopomers. This transition can also be seen in the spectrum of *all-trans*-spheroidene (see Figure 5.2(a)). The corresponding normal mode is an in-phase combination of in-plane bend vibrations of the C–H bonds and stretch vibrations of the C–C bonds on the non-prime side of the conjugated chain (for 15,15'-cis spheroidene). Our calculations for 15,15'-cis spheroidene reproduce this transition quite well for NA (at 1189 cm^{-1}) as well as the isotope labeled spheroidenes.

A distinctive transition observed in the resonance Raman spectra of spheroidene in the *Rhodobacter sphaeroides* RC (see Figure 5.2) is found at 1239 cm^{-1} [130, 134, 136]. For the 15,15'-cis structure we calculate a mode at 1242 cm^{-1}. Just like in the experimental NA spectrum, it is the only transition in the calculated spectrum between 1200 and 1250 cm^{-1} with non-zero intensity. Figure 5.7 shows that the normal mode calculated at 1242 cm^{-1} consists of a linear combination of local modes in the 14–15=15'–14' section of the spheroidene molecule. This makes it the only intense mode in the entire calculated spectrum that is not considerably delocalized across the conjugated chain. Note that a similar mode composition could not occur for a different cis-spheroidene structure, like 13,14-cis spheroidene for example. Indeed, the occurrence of the transition at 1239 cm^{-1} in the NA resonance Raman spectrum seems to refer uniquely to the cis nature of the 15=15' bond.

The calculated frequency was found to depend only weakly on the 14–15=15'–14' dihedral angle for values between 0 and 10 °. Such changes to the dihedral angle have no effect on the composition of the mode. We do expect the normal mode to be strongly influenced by ^{2}H substitution at the 15

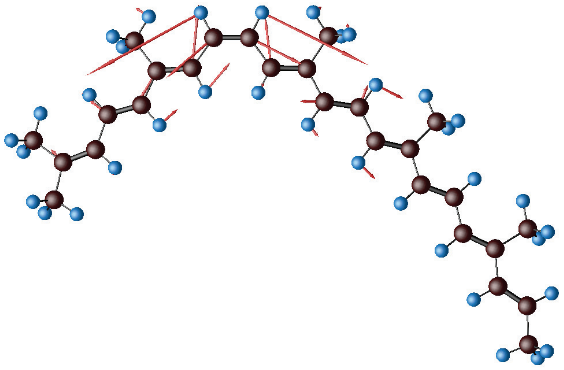

Figure 5.7: The composition of the normal mode corresponding to the transition calculated to occur at 1242 cm^{-1} for the 15,15'-cis structure. The mode is composed almost exclusively of the 14'–15' and 15–14 stretches and the C–H bend vibrations at the 15 and 15' positions.

or 15' positions, however, since this would significantly lower the frequencies of the local C–H bend vibrational modes. Indeed, the mode around 1240 cm^{-1} disappears altogether in our calculations for 15-^{2}H, 15'-^{2}H and 15,15'-$^2H_2$ labeled spheroidene. The only mode with non-zero intensity around 1240 cm^{-1} is calculated at 1223 cm^{-1} for 15,15'-$^2H_2$ spheroidene. A similar result is found for 15-^{2}H and 15'-^{2}H spheroidene. The mode at 1223 cm^{-1} is a rather delocalized mode consisting of many different local modes. The corresponding mode is not found to have any intensity in the calculation for NA spheroidene, nor is it seen in the experimental NA spectrum. It is observed in the spectra of 15-^{2}H, 15'-^{2}H and 15,15'-$^2H_2$ labeled spheroidene, as well as for several other isotopomers for which it is also calculated. Thus far the 15,15'-cis calculations agree well with the experimental observations. Our experimental spectra, however, still show remaining signals at roughly 1240 cm^{-1} for the 15-^{2}H, 15'-^{2}H and 15,15'-$^2H_2$ isotopomers, where our calculations do not calculate any vibrational mode. In the 15-^{2}H and 15,15'-$^2H_2$ spectra the transitions are considerably weaker than in all other spectra. The corresponding modes must be composed of different local modes than those constituting the mode shown in Figure 5.7 and are therefore likely to be more delocalized. Since no such mode is calculated for the 15,15'-cis structure, this could indicate that an additional cis-structure might be present in the experiments. As in the subsection on the C=C stretch region (5.4.1), we have considered the 13,14-cis structure of spheroidene as a possible candidate.

In the frequency range between 1200 and 1245 cm^{-1} the calculations for

NA 13,14-cis spheroidene only produce a transition with non-zero intensity at 1209 cm^{-1}. This transition does not appear in the experimental NA spectrum. It is observed, however, in the spectra of 8-^{13}C, 10-^{2}H, 11-^{2}H, 11-^{13}C, 13-^{13}C, 15-^{2}H, 14'-^{13}C, 11'-^{2}H, 13,14-^{13}C$_2$, and 15'-^{2}H labeled spheroidene, for which the transition is also calculated with considerable intensity. The fact that our calculations do not produce a transition around 1210 cm^{-1} for the 15,15'-cis structure suggests that the calculations for the two structures are complementary, as was the case for the C=C stretch region. The 13,14-cis structure, however, does not yield any transitions around 1240 cm^{-1}, and fails to explain the observed transition in the resonance Raman spectra of the 15-^{2}H, 15'-^{2}H and 15,15'-^{2}H$_2$ isotopomers.

The calculated results for the fingerprint region support the presence of a 15,15'-cis spheroidene structure in the reconstituted R26 RCs. The transitions found in this region are not as easily assigned as those in the C=C stretch region. Nonetheless, our analysis has revealed that the transition at 1239 cm^{-1}, which is reproduced at 1242 cm^{-1}, is not only indicative of spheroidene in the RC, but also a unique marker of the 15,15'-cis stereoisomer. Other expected transitions in the fingerprint region are also obtained, although they do not correspond as closely to experimental values as for the C=C stretch region. The signal at 1193 cm^{-1} is accurately reproduced at 1189 cm^{-1}, but between 1150 and 1180 cm^{-1} the agreement is less quantitative. Calculations for a 13,14-cis stereoisomer in the fingerprint region do not disagree with the possibility of the additional structure being 13,14-cis.

5.5 Conclusions

The theoretical analysis of the resonance Raman spectra of reconstituted spheroidene in the R26 photosynthetic reaction center has demonstrated conclusively that the RC contains 15,15'-cis spheroidene. The DFT optimized geometry, obtained by fixing the five methyl groups at the coordinates from the X-ray structure of Roszak et al. [57,142], accurately reproduces the trends of isotope-induced shifts for two transitions in the C=C stretch region. Furthermore, our calculations have revealed that the transition at 1239 cm^{-1}, which was hitherto already known to be distinctive of spheroidene in the RC, in fact corresponds to a normal mode unique to the 15,15'-cis stereoisomer. It is calculated at 1242 cm^{-1} for NA spheroidene.

The resonance Raman spectra of several isotopomers of spheroidene contain additional transitions that we do not observe in our calculations for 15,15'-cis spheroidene. Most notably the C=C stretch regions in our spectra display a pattern of isotope-induced shifts, which our analysis has shown cannot result from a 15,15'-cis structure. This fact has led us to conclude that some RCs contain an alternative stereoisomer of spheroidene. The explanation of the complete resonance Raman spectra is not possible by presuming spheroidene is bound in one form only.

There can be no doubt concerning the purity of the isotope-labeled spheroidenes used in reconstituting spheroidene in the R26 RC. Our earlier work on spheroidene in petroleum ether [145] has demonstrated the presence of only single isotopomers in the samples and the same compounds were used in the process of reconstitution. Moreover, we are confident that the discrepancies between our calculations and experimental results are not related to faults in the computational method used. We base this conclusion on our success in describing the spectra of 19 isotopomers of spheroidene in organic solvent, combined with positive test results from calculations of the resonance Raman spectra of 9- and 11-cis-retinal.

To the best of our knowledge, the possibility of spheroidene incorporated in the RC occurring in two different cis-configurations, has never been considered in earlier studies. As such, our conclusions are not incompatible with findings from previous publications. The presence of two stereoisomers in the RC crystals might be the cause of the lower resolution of the electron density maps at the carotenoid position compared to that for other cofactors in the RC [57, 142]. In 1989 Kolaczkowski noticed additional shoulders in the triplet EPR spectrum of the fully deuterated RC of *Rhodobacter sphaeroides* 2.4.1. He interpreted these shoulders as originating from a second conformer that had undergone a twist around a sigma bond near one of the ends of the conjugated part of the spheroidene molecule [155]. Such a structure is incompatible with our resonance Raman results.

As a possible candidate for the additional structure present in our samples, we have considered 13,14-cis spheroidene, which seems compatible with the X-ray density map determined by McAuley et al. and refined by Roszak et al [57, 142]. If we superimpose the results from the theoretical analysis of the 15,15'-cis and 13,14-cis structures, all transitions in the C=C stretch regions of the resonance Raman spectra and trends upon isotope substitution are well reproduced and understood. This is clearly illustrated by the calculated frequency bars plotted over the spectra in Figure 5.4 and the close correspondence between the experimental and calculated shifting patterns in Figure 5.6. The intensities of the transitions in the C=C stretch region that are not related to the 15,15'-cis structure, suggest that the additional structure makes up a significant proportion of the spheroidene bound to the RC. It should be noted that as far as the C=C stretch results are concerned, *any* additional configuration might do, provided the mode composition of the two C=C stretch modes is reversed with respect to that of a 15,15'-cis structure. The 13,14-cis stereoisomer is a possibility, but not a necessity. In order to ascertain that it is indeed present in our samples, further attention needs to be given to the geometry optimization of the 13,14-cis structure in relation to the calculated resonance Raman spectrum.

Acknowledgments

We would like to acknowledge the following people: I. van der Hoef and R. Gebhard synthesized the isotope labeled spheroidenes. C. A. Violette and R. Farhoosh performed the reconstitution of labeled spheroidenes into the R26 RCs. Resonance Raman spectra were recorded by J. Köhler, P. Kok, C. Th. J. van den Nouland, Y. Nagano and A. M. Dokter. J. W. F. Venderbos was responsible for the 13,14-cis DFT calculations and analysis. We are grateful to A. W. Roszak and R. J. Cogdell for making their electron density maps of the RCs available to us.

5.6 Supplementary Material

Bond	**Bond length (Å)**	**Bond angle (°)**	**Dihedral angle (°)**
2–3	1.500		
3=4	1.347	124.5	
4–5	1.458	126.8	-180.0
5=6	1.366	118.4	-180.0
6–7	1.437	128.2	-180.0
7=8	1.364	123.2	-179.4
8–9	1.445	126.5	179.6
9=10	1.372	118.7	-179.4
10–11	1.432	127.6	179.2
11=12	1.365	123.9	176.2
12–13	1.442	128.8	-178.2
13=14	1.370	119.1	173.3
14–15	1.432	126.8	-175.1
15=15’	1.370	125.9	174.6
15’–14’	1.430	125.1	0.0
14’=13’	1.368	128.3	-168.9
13’–12’	1.446	117.0	180.0
12’=11’	1.356	127.5	-177.0
11’–10’	1.445	122.4	179.4
10’=9’	1.352	127.4	-177.7
9’–8’	1.506	121.0	180.0
5–Me	1.509	118.0	0.0
9–Me	1.511	118.3	1.0
13–Me	1.505	118.6	-4.7
13’–Me	1.509	123.4	1.7
9’–Me	1.503	123.7	0.3

Table 5.2: This table lists bond lengths, bond angles and dihedral angles for the optimized non-planar 15,15’-cis spheroidene structure used for our frequency calculations reported in Table 5.1. The Cartesian coordinates in the X-ray structure [57, 142] of the five methyl groups were fixed. For this particular optimization the 14–15=15’–14’ dihedral angle was also fixed at 0.0 °. The value for this dihedral was not found to significantly influence the calculated frequencies or compositions. For the numbering system see Figure 5.5(a). The angles are defined from top to bottom in the table. The bond angle listed by the 3=4 bond therefore refers to the 2–3=4 bond angle. The dihedral angle listed by the 4–5 bond refers to the 2–3=4–5 dihedral angle, etc..