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¹³C Magic Angle Spinning NMR Evidence for a 15,15'-cis Configuration of the Spheroidene in the *Rhodobacter sphaeroides* Photosynthetic Reaction Center[†]

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ABSTRACT: The photosynthetic reaction center of *Rhodobacter sphaeroides* 2.4.1 contains one carotenoid that protects the protein complex against photodestruction. The structure around the central (15,15') double bond of the bound spheroidene carotenoid was investigated with low-temperature magic angle spinning ¹³C NMR, which allows an in situ characterization of the configuration of the central double bond in the carotenoid. Carotenoidless reaction centers of *R. sphaeroides* R26 were reconstituted with spheroidene specifically labeled at the C-14' or C-15' position, and the signals from the labels were separated from the natural abundance background using ¹³C MAS NMR difference spectroscopy. The resonances shift 5.2 and 3.8 ppm upfield upon incorporation in the protein complex, similar to the 5.6 and 4.4 ppm upfield shift occurring in the model compound β -carotene upon trans to 15,15'-cis isomerization. Hence the MAS NMR favors a cis configuration, as opposed to the trans configuration deduced from X-ray data.

Spheroidene is the carotenoid in the RC¹ of the photosynthetic bacterium *Rhodobacter sphaeroides* 2.4.1. The RC is a transmembrane protein complex containing three protein subunits supporting the spheroidene, a bacteriochlorophyll dimer, two bacteriochlorophyll monomers, two bacteriopheophytins, two ubiquinones, and an Fe²⁺ ion. Probably the most important role of the spheroidene is to act as a protective device against irreversible photodestruction of the RC by quenching chlorophyll triplet states and preventing the chlorophyll-sensitized formation of singlet-state oxygen, a major oxidizing agent (Foote, 1968; Krinsky 1968; Cogdell & Frank, 1988). Following the pioneering work of Deisenhofer and Michel (1989), the complex of the *R. sphaeroides* wild-type strain 2.4.1 has been crystallized, and its structure is now known in considerable detail (Frank et al., 1987; Yeates et al., 1988; Komiya et al., 1988; Allen et al., 1988; Feher et al., 1989). The spheroidene is located near the monomeric accessory chlorophyll of the photosynthetically inactive branch and is the only chromophore that does not adhere to the approximate 2-fold rotation symmetry of the RC (Allen et al., 1988; Feher et al., 1989). Unfortunately, the carotenoid structure deduced from the X-ray diffraction studies suffers from many ambiguities and uncertainties. The electron density map generated by the X-ray diffraction pattern contains only part of the carotenoid structure. A curved structure with a

15,15'-trans double bond fits the electron density map best,² although the β factors are extremely high (~ 50), indicating a large amount of uncertainty in the atomic coordinates for the carotenoid. The ground-state energy calculated for the X-ray structure is unreasonably high, and the transition energies and oscillator strengths do not match the experimental data for spheroidene in the RC (Zhang et al., 1989).

On the basis of a comparison of the Raman spectra of model compounds with the Raman spectrum of the RC, Koyama et al. (1982, 1983) proposed a central cis double bond for the spheroidene in the RC. The same conclusion was reached by Lutz et al. (1987), who extracted the spheroidene from the RC under low-light conditions followed by ¹H NMR analysis of its structure. However, for an unambiguous assignment of the Raman lines, an analysis with isotope-substituted derivatives is necessary. Moreover, the extraction experiments yielded an all-trans and 15,15'-cis isomer mixture, making an unambiguous assignment difficult. Clearly, an independent in situ analysis of the carotenoid in the reaction center is needed.

¹³C CP/MAS NMR of selectively enriched samples is a sensitive spectroscopic method and is the method of choice to obtain NMR access with atomic selectivity for membrane proteins as large as the RC (de Groot et al., 1990; Gebhard et al., 1991). The MAS NMR signal constitutes a sharp center band at the isotropic chemical shift, together with a set of rotational side bands at multiple integrals of the spinning speed, relative to the center band [for a review, see, e.g., Smith and Griffin (1988)]. From the intensities of these side bands, the principal components of the chemical shift anisotropy tensor may be deduced (Herzfeld & Berger, 1980; de Groot et al., 1991). The isotropic shift and the chemical shift tensor are sensitive to the configuration of the polyene system (Harbison et al., 1984, 1985).

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¹ Abbreviations: CD, circular dichroism; CP, cross-polarization; LDAO, *N,N*-dimethyldodecylamine-*N*-oxide; MAS, magic angle spinning; NMR, nuclear magnetic resonance; RC, reaction center; TMS, tetramethylsilane.

² Arnoux et al. (1989) fitted their X-ray data for *Y. R. sphaeroides* to a 15,15'-cis isomer, but the result was not unique.

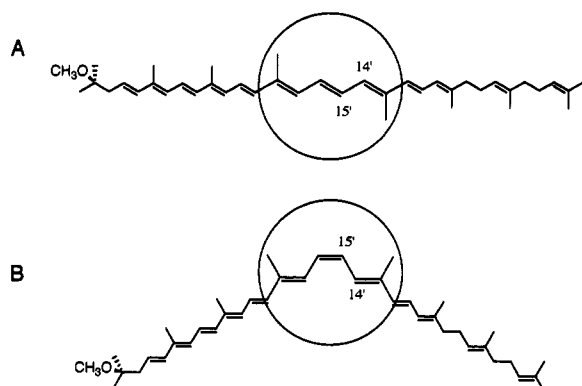


FIGURE 1: Structures of (A) *all-trans*- and (B) 15,15'-*cis*-spheroidene (IUPAC name: 1-methoxy-3,4-didehydro-1,2,7',8'-tetrahydro- ψ,ψ -carotene). The central parts are expanded, and the labeled positions are indicated.

In this work, we present ^{13}C CP/MAS NMR spectra of R26 RCs reconstituted with spheroidene specifically enriched at the C-14' or C-15' position. Previous CD studies provided compelling evidence that spheroidene bound to R26 complexes has the same structure as the spheroidene in the wild-type 2.4.1 (Frank & Violette, 1989). Comparison of the chemical shifts of the labels with the chemical shifts of the corresponding positions in *all-trans*-spheroidene and the model compounds *all-trans*- and 15,15'-*cis*- β -carotene allows us to examine the configuration of the RC-bound spheroidene. The chemical shift differences between RC-bound spheroidene and *all-trans*-spheroidene closely resemble those between 15,15'-*cis* and *all-trans*- β -carotene. The data therefore support a 15,15'-*cis* configuration for the spheroidene in the *R. sphaeroides* RC.

MATERIALS AND METHODS

The synthesis of the [$14'\text{-}^{13}\text{C}$] and [$15'\text{-}^{13}\text{C}$]spheroidene is described by Gebhard et al. (1990). The position of the labels is indicated in Figure 1. The labeled molecules were incorporated in RCs of the carotenoid-deficient strain *R. sphaeroides* R26, originally provided to us by Dr. G. Feher. Cells of this strain were grown in large quantities and purified with the procedure of Feher and Okamura (1978).

The reconstitution was carried out in subdued light. The enriched spheroidene was dissolved in 1 mg/mL petroleum ether. A 1.8-mL aliquot of the solution was pipetted into a 50-mL vial. The solvent was evaporated with a stream of nitrogen gas, and a thin film of spheroidene was deposited on the walls of the vial. Two milliliters of a solution of 15 mM Tris buffer, 1 mM EDTA, and 1% LDAO (v/v), pH 8.0, was added to the vial which was then vortexed for 10 s. The resulting solution was decanted into a 100-mL volumetric flask containing a small magnetic stirring bar. A suspension of R26 reaction centers (~ 15 mg of protein) in 100 mL of 15 mM Tris buffer, 1 mM EDTA, 1% LDAO (v/v), and 400 mM NaCl was added to the flask, which was secured above a stirring plate and insulated to prevent heating. The suspension was allowed to stir for 6 h. A DEAE Sephacel column (Sigma I-6505) was packed and equilibrated with 15 mM Tris buffer and 1% LDAO, pH 8.0. The reconstituted reaction center suspension was loaded onto the column and washed with 15 mM Tris buffer and 0.5% LDAO, pH 8.0, until there was no longer any unbound carotenoid in the eluent, which was assayed by absorption spectroscopy. The reaction centers were then removed from the column using 400 mM NaCl in the eluting buffer. The ratio of the spheroidene peak at 501 nm to the primary donor peak at 865 nm was used to

determine the amount of spheroidene bound to the reaction centers relative to that found in wild-type 2.4.1 reaction centers. It was determined that $\sim 70\%$ of the reaction centers were reconstituted with the labeled spheroidene.

Low-temperature 100-MHz ^{13}C CP/MAS NMR experiments were performed with a MSL-400 NMR spectrometer using a 7-mm MAS probe (Bruker, Karlsruhe, Germany). In these experiments, the sample is kept in a closed container and is shielded from the light. The spinning rate around the magic angle was kept at $\omega_r/2\pi = 6.00 \pm 0.01$ kHz with a spinning speed controller constructed in our laboratory (de Groot et al., 1988). The spectra were accumulated in 1024 channels with proton decoupling during acquisition. The 90° pulse lengths for the ^1H and ^{13}C were 4–5 μs , the cross-polarization time was 1 ms, the recycle delay was 2 s, and the sweep width was 50 kHz.

Since CP requires strong dipolar interactions between the abundant protons and the less-abundant ^{13}C nuclei, the protein complexes were immobilized by freezing using liquid nitrogen cooled bearing gas. The equipment for cooling the gas is described in some detail by Allen et al. (1991). The temperature of the bearing gas T_B was measured with a thermocouple just before the gas flows into the spinner assembly. The actual sample temperatures are higher and depend also on the amount of room temperature drive gas used for the spinning. The temperatures quoted are calculated according to $T \approx 0.86T_B + 50$, which is accurate to about 5 K.

Chemical shifts are referenced to TMS using the narrow line at 14.4 ppm from the ^{13}C -12 methyl of the LDAO contribution to the natural abundance background as an internal calibration. All spectra were recorded with the same dead time of 10 μs , and the first-order phase correction after Fourier transformation was always kept at the same small value of 25° . With this procedure, the absolute error in the chemical shift is ~ 0.2 ppm.

all-trans- β -Carotene was purchased from Aldrich (Brussels, Belgium) while its 15,15'-*cis* isomer was provided to us by F. Hoffman-La Roche & Co.

RESULTS AND DISCUSSION

The analysis of the configuration around the 15,15' double bond requires a characterization of the shifts of the ^{13}C resonances in spheroidene upon isomerization. However, the 15,15'-*cis*-spheroidene is highly unstable and isomerizes to the *all-trans* form. It is not available in sufficient quantities for a detailed MAS NMR analysis. Therefore *all-trans*- and 15,15'-*cis*- β -carotene were used as models. Both compounds are stable, and the symmetry of the molecule reduces the number of components in the MAS NMR spectra, permitting a detailed characterization of the isotropic shifts, the principal components of the shift tensors, and almost a complete assignment of the resonances from the olefinic carbons without the necessity for isotope labeling of individual positions.

In Table I the chemical shifts of the polyene carbons in *all-trans*- and 15,15'-*cis*- β -carotene are listed. The solid-state data were obtained from CP/MAS experiments at various spinning speeds. Our assignments of the resonances are slightly different from those of Harbison et al. (1985) and were obtained using nonprotonated carbon-selective experiments (Opella & Frey, 1979), by comparing the solid-state data with the solution data, and through a full analysis of the data, yielding the principal components of the chemical shift anisotropy for the individual positions (de Groot et al., 1991),

Table I: Chemical Shifts (ppm) of the Conjugated Carbons in *all-trans*-Spheroidene in Solution Compared with the Two Isomers of the Model Compound, *all-trans*- and 15,15'-*cis*- β -Carotene, in Solution and in the Solid State^a

	spheroidene			β -carotene					
	solution		solution ^b			solid			
	trans	$\Delta\sigma_{1,sp}$	trans	15- <i>cis</i>	$\Delta\sigma_{1,liq}$	trans	15- <i>cis</i>	$\Delta\sigma_{1,sol}$	
C-5	135.2	5.9	129.3	129.3	0.0	129.1	128.6	-0.5	
C-6	130.6	-7.4	138.0	138.1	0.1	139.1	138.6	-0.5	
C-7	124.6	-2.1	126.7	126.8	0.1	126.0 ^c	125.6 ^c	-0.4	
C-8	137.5	-0.3	137.8	137.8	0.0	139.5	140.2	0.7	
C-9	135.9	-0.1	136.0	136.1	0.1	138.5	138.0	-0.5	
C-10	132.6	1.8	130.8	130.8	0.0	131.3	130.6	-0.7	
C-11	124.9	-0.2	125.1	125.4	0.3	126.0 ^c	125.6 ^c	-0.4	
C-12	138.0	0.7	137.3	137.5	0.2	137.5	137.5	0.0	
C-13	136.1	-1.0	136.4	137.1	0.7	137.3	136.4	-0.9	
C-14	133.0	0.6	132.4	126.8	-5.6	132.7	127.8	-4.9	
C-15	129.4	0.6	130.0	125.6	-4.4	130.9	124.6	-6.3	
C-15'	130.3	0.3	130.0	125.6	-4.4	130.9	124.6	-6.3	
C-14'	131.4	-1.0	132.4	126.8	-5.6	132.7	127.8	-4.9	
C-13'	136.6	0.2	136.4	137.1	0.7	137.3	136.4	-0.9	
C-12'	135.2	-2.1	137.3	137.5	0.2	137.5	137.5	0.0	
C-11'	125.1	0.0	125.1	125.4	0.3	126.0	125.6	-0.4	
C-10'	125.8	-5.0	130.8	130.8	0.0	131.3	130.6	-0.7	
C-9'	139.7	3.7	136.0	136.1	0.1	138.5	138.0	-0.5	

^a $\Delta\sigma_{1,sp}$ are the chemical shift differences between spheroidene and *trans*- β -carotene, $\Delta\sigma_{1,liq}$ are the shifts for β -carotene in solution upon *cis*/*trans* isomerization, and $\Delta\sigma_{1,sol}$ are the isomerization shifts for the model in the solid state. ^b Data from Gebhard et al. (1990). ^c The difference between C-7 and C-11 could not be resolved in the spectra for β -carotene in the solid state.

Table II: Principal Components σ_{ii} (ppm) of the Chemical Shift Anisotropy Tensors in the Solid State for the 14' and 15' Polyene Carbons of *all-trans*-Spheroidene and the Two Isomers of β -Carotene

	C-14'			C-15'		
	σ_{33}	σ_{22}	σ_{11}	σ_{33}	σ_{22}	σ_{11}
<i>all-trans</i> -spheroidene	209	132	59	215	138	37
<i>all-trans</i> - β -carotene	211	130	57	213	144	35
15,15'- <i>cis</i> - β -carotene	214	127	42	215	141	17

which could then be related to anisotropy data for retinal (Harbison et al., 1985).

For the β -carotene, the variation of the isotropic shifts upon 15-*trans*/*cis* isomerization for C-14 and C-15 is significantly larger than for the remaining olefinic carbons, even in the solid state, where it is known from the X-ray structure that the molecule is subject to pronounced conformational distortions (Sterling, 1964). It appears that, both in solution and in the solid state, the shifts upon *trans* $\rightarrow$ *cis* isomerization are less than 1 ppm, except for the two carbons situated in the 15,15' double bond and the C-14 and C-14' on either side of it, which shift by 4–6 ppm. This phenomenon may be used to analyze the configuration of the 15,15' double bond of spheroidene in situ by using specific ^{13}C labeling at C-14' and C-15' in conjunction with CP/MAS NMR.

The isotropic shifts in the central part, from C-11 to C-13', of the *all-trans*-spheroidene and β -carotene molecules are also very similar. This is carried one step further in Table II, which lists the principal components of the chemical shift tensor for the labeled spheroidenes and the corresponding olefinic carbon resonances of the two β -carotene models. The chemical shift anisotropy is extremely sensitive to variations of the electronic structure of widely different origin (Veeman et al., 1984; Orendt et al., 1988). Since it is the same within experimental errors for the spheroidene and the *all-trans*- β -carotene, this suggests an almost identical electronic structure around the central double bond in these two compounds.

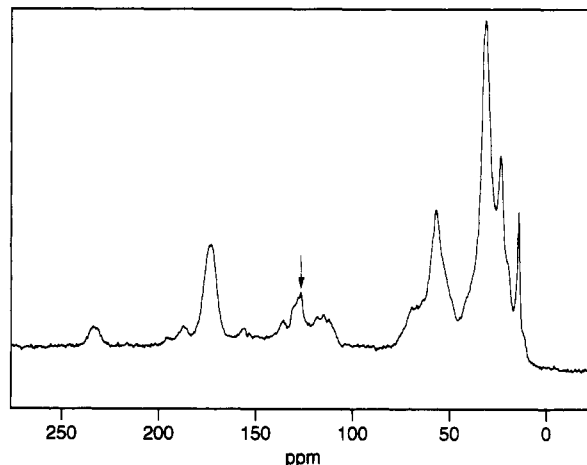


FIGURE 2: Proton-decoupled ^{13}C CP/MAS spectra for $[15'\text{-}^{13}\text{C}]$ -spheroidene incorporated into *R. sphaeroides* R26 RC. The data were collected with a spinning speed $\omega_r/2\pi = 6.00$ kHz at $T = 220$ K. The resonance from the label is indicated with an arrow.

The most upfield component, σ_{11} , which is directed perpendicular to the plane of the conjugated system (Veeman 1984), is the only one affected by the isomerization of β -carotene. Such localized shifts have been observed more often. It is known that ^{13}C nuclei one bond away undergo a upfield shift upon *trans* to *cis* isomerization, the so-called γ effect (Rowan & Sykes, 1974; Becker et al., 1974). It arises from the steric hindrance across the *cis* linkage between the 14 and 14' hydrogens, which are in close contact with each other. This gives rise to mutual repulsion of the electrons of the C–H bonds, causing an increased diamagnetic shielding and an upfield ^{13}C shift (Cheney & Grant, 1967; Englert, 1975). In addition, it has been argued that the shift is localized in the σ_{11} element of the chemical shift tensor (Harbison et al., 1985). The 14 and 14' shifts are therefore most likely associated with a γ effect. Concerning the 15 and 15' position, the origin of the upfield shift localized in σ_{11} upon isomerization is not clear, but the same effect can be observed for *trans* $\rightarrow$ *cis* isomerization of other olefinic molecules with some degree of symmetry with respect to both sides of the double bond, for instance butene and cyclooctene (Veeman, 1984).

In Figure 2 the spectrum of $[15'\text{-}^{13}\text{C}]$ spheroidene R26 RCs in frozen solution at $T = 220$ K and $\omega_r/2\pi = 6$ kHz is shown. The broad feature in the region from 0 to 70 ppm represents the natural abundance background of the aliphatic resonances of the protein and of the detergent. At ~ 175 ppm the center band from the natural abundance carbonyl resonances is found, with its side bands at ~ 115 and ~ 235 ppm. The peak at ~ 130 ppm is mainly due to the background signal from aromatic and olefinic carbons. In this sample, the signal of the C-15' is increased by a factor of 90 because of the 99% ^{13}C enrichment, and close inspection reveals a sharp resonance at 126.5 ppm indicated by an arrow.

The signal from the label can be separated from the natural abundance background using difference spectroscopy (de Groot et al., 1988). This is demonstrated in Figure 3 for the C-14'- and in Figure 4 for the C-15'-labeled complex. In both figures, the aromatic region of the spectrum is shown in more detail in trace A, while trace B yields the result after subtraction of a signal in the same region collected from a natural abundance R26 sample at the same spinning speed. In each figure, the single narrow resonance represents the contribution from a singly labeled carbon in the 125 000 Da RC complex! Another way of separating the label signal from the background is shown in Figure 5. The spheroidene is

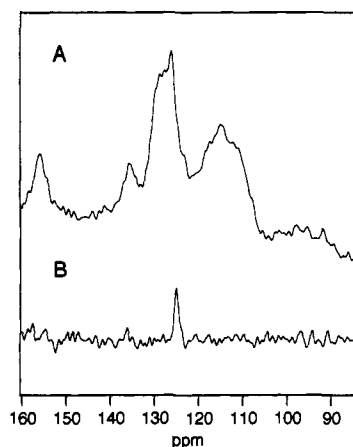


FIGURE 3: Olefinic and aromatic region of the proton-decoupled ^{13}C CP/MAS NMR data for *R. sphaeroides* RC reconstituted with $[14'\text{-}^{13}\text{C}]$ spheroidene. (A) Spectrum. (B) Difference spectrum. The data were collected with a spinning speed $\omega_r/2\pi = 6.00$ kHz at $T = 205$ K.

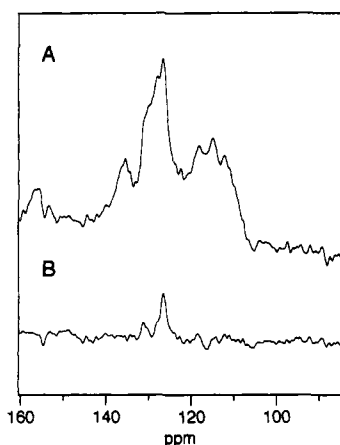


FIGURE 4: Olefinic and aromatic region of the proton-decoupled ^{13}C CP/MAS NMR data of Figure 2 for *R. sphaeroides* RC reconstituted with $[15'\text{-}^{13}\text{C}]$ spheroidene. (A) Spectrum. (B) Difference spectrum.

located in the rigid transmembrane region of the complex (Allen et al., 1988) and therefore should give rise to narrow NMR lines (Fischer et al., 1992). The background, however, consists of broad lines that are built up from a large number of small natural abundance contributions. Since the contribution from the label is the only narrow signal in the aromatic region, it can be separated from the background simply by taking the second derivative of the spectrum. This is shown in Figure 5, with trace A the second derivative of the spectrum in Figure 3A, and trace B the second derivative of the spectrum in Figure 4A. In both cases, one sharp signal is left, at the same chemical shift as the resonance in the corresponding difference spectrum.

The results of the experiments are summarized in Table III, where the protein data are compared with the data for spheroidene and the two β -carotene isomers, in solution. It appears that the signals from the labels of the spheroidene incorporated in the RC are both shifted upfield with respect to the all-trans molecule in solution, as is to be expected for the 15,15'-cis form. In addition, the magnitude of the shifts compares well with the values observed for the trans $\rightarrow$ cis isomerization of β -carotene.

It is unlikely that other mechanisms than isomerization could be responsible for the upfield shifts upon incorporation of the molecule in the RC. Raman studies by Lutz et al. (1987) have provided evidence for a planar center of the RC-bound spheroidene, and the chemical shift analysis presented

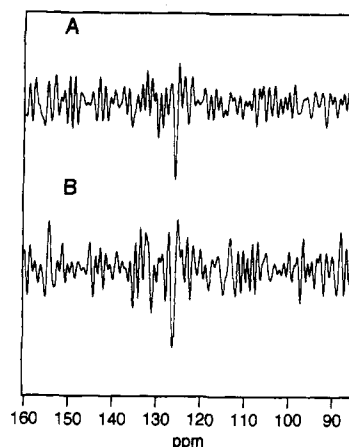


FIGURE 5: Noise-filtered second derivatives of the regions in Figure 3A for the $14'\text{-}^{13}\text{C}$ label (A) and Figure 4A for the $15'\text{-}^{13}\text{C}$ label (B).

Table III: Differences $\Delta\sigma$ (ppm) between the Isotropic Chemical Shifts (σ_I) for the $14'$ - and $15'\text{-}^{13}\text{C}$ -Labeled Spheroidene Incorporated into R26 RC and in Solution Compared with the Differences Observed for all-trans- and 15,15'-cis- β -Carotene in Solution

	σ_I ($14'\text{-}^{13}\text{C}$)	σ_I ($15'\text{-}^{13}\text{C}$)
reconstituted RC's	126.2	126.5
all-trans-spheroidene	131.4	130.3
$\Delta\sigma$	-5.2	-3.8
15,15'-cis- β -carotene	126.8	125.6
all-trans- β -carotene	132.4	130.0
$\Delta\sigma$	-5.6	-4.4

in Table I argues against an explanation in terms of small conformational distortions. According to the X-ray data, the labels are probably more than 6 Å away from the plane of the accessory B chlorophyll monomer. This is the nearest macroaromatic cycle, and any ring current shift should be less than 0.5 ppm (Giessner-Pretre & Pullman, 1971; Allen et al., 1988). Although shifts in the range of 4–6 ppm may in principle be caused by the presence of a polar group in the protein, such an explanation would be in contradiction with the X-ray structure, which reveals an apolar binding site (Komiya et al., 1988). In addition, polarization of a polyene chain by a polar group is expected to give rise to shifts that occur predominantly at either the even-numbered or the odd-numbered sites, as opposed to shifts that are comparable in magnitude for two adjacent sites (Harbison et al., 1985). Hence the NMR data of the specifically ^{13}C -enriched, reconstituted RC complexes provide convincing evidence for a 15,15'-cis conformation of the carotenoid.

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