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The stereoisomerism of bacterial, reaction-center-bound carotenoids revisited: an electronic absorption, resonance Raman and ^{1}H-NMR study

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In order to solve discrepancies between earlier assignments we have reinvestigated the stereoisomerism of the spheroidene molecule bound to reaction centers (RC) of *Rhodobacter sphaeroides*. A stable *cis* isomer could be extracted and purified from the reaction centres by working at very low ambient light. Resonance Raman spectroscopy showed that this *cis* isomer assumed the same configuration as that of the RC-bound molecule. Proton-NMR spectroscopy of the extracted isomer permitted to assign it the 15–15′ mono *cis* configuration. Comparisons between resonance Raman spectra of the native form and of the 15 *cis* extract showed that, in the reaction center, 15 *cis* spheroidene is in addition twisted into a non-planar conformation. Comparisons of extraction-induced changes in relative intensities of Raman bands of the 760–1060 cm^{-1} regions, which largely correspond to out-of-plane modes, further indicated that the out-of-plane twist of RC-bound spheroidene should predominantly affect C_8–C_{12} and/or $C_{8'}$–$C_{12'}$ regions of the molecule rather than the central region. Comparisons between difference electronic absorption spectra of RC-bound spheroidene and of RC-bound methoxyneurosporene showed that the out-of-plane twisting of both these native forms results in a drastic weakening of their $^1C \leftarrow {}^1A$ electronic transitions, compared with those of the planar, 15 *cis* forms. Finally, it is proposed, on the basis of their resonance Raman spectra, that spirilloxanthin bound to RCs of *Rhodospirillum rubrum* as well as dihydroneurosporene or dihydrolycopene bound to RCs of *Rhodopseudomonas viridis* shares 15 *cis* configurations and out-of-plane twisting with carotenoids bound to RCs of various strains of *Rb. sphaeroides*.

Introduction

Reaction centers of wild-type strains of purple photosynthetic bacteria appear to generally contain a carotenoid molecule [1], the function of which is largely photoprotective [2]. Early structural studies based on resonance Raman spectrometry [3] and on electronic absorption and circular dichroism [2] indicated that these carotenoids should assume chain configurations and/or conformations differing from those of the bulk carotenoids of bacterial intracytoplasmic membranes. Although these stereoisomers appeared to be identical in several species and strains of bacteria [4], no general agreement has yet been reached about their precise nature [2,4–6].

On the basis of their CD and electronic absorption data, Gingras and his coworkers proposed that, in carotenoid-reconstituted RCs of *Rhodo-*

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Abbreviations: COSY, correlated spectroscopy; RC, reaction center; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; RR, resonance Raman; DC, circular dichroism; BChl, bacteriochlorophyll, PED, potential-energy distribution.

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spirillum rubrum, strain G9, the carotenoids adopted a 15–15′ *cis* configuration twisted into a protohelical shape [2]. Lutz et al. [4] applied resonance Raman (RR) spectroscopy to RCs from three strains of *Rhodobacter sphaeroides* and from wild-type *R. rubrum* and *Rhodopseudomonas viridis*, confirming their previous proposal of a *cis* configuration [3]. They tentatively proposed that the native configuration might be di-*cis*, and sterically hindered. More recently, Koyama et al. [5,7] compared RR spectra of several *cis* isomers of β-carotene to those of spheroidene and methoxyneurosporene in reaction centers of *Rb. sphaeroides*. They concluded that the RC-bound carotenoids merely assumed the 15-15′ *cis* configuration, and questioned the previous interpretation by Lutz et al. [4] of the 300–400 nm regions of differential absorption spectra of these carotenoids. Still more recently, Chadwick and Frank [6] suggested from EPR data on triplet states of various carotenoids reconstituted in *Rb. sphaeroides*, strain R26-1, that the native conformation might involve twists at the 6–7 and 6′–7′ positions.

None of the studies quoted actually was free of uncertainty concerning the assignment of the conformations of RC-bound carotenoids. For instance, the tentative assignment of Boucher et al. [2] as a 15 *cis* form was solely based on the very high intensity of the band attributed to the $^1C \leftarrow {}^1A$ electronic transition of spirilloxanthin bound to *R. rubrum* G9. On the contrary, Lutz et al. found a very small oscillator strength for the $^1C \leftarrow {}^1A$ band of spheroidene in wild type [4] or reconstituted R26 [8] RCs from *Rb. sphaeroides*. Similarly, their tentative di-*cis* assignment was largely based on the high apparent instability observed for the *cis* configuration when extracted (together with the bacteriochlorin pigments) in dim light from the reaction center [4]. A more recent study of *cis* → *trans* photoizomerisation of 15 *cis* β carotene sensitized by BChl *a*, however, pointed at the very high quantum yield of this process [9]. Finally, the study of Koyama et al. [5,7] presented the difficulty of comparing RR spectra of open-chain carotenoids in a proteic environment to RR spectra of β-carotene in *n*-hexane, and did not propose a clue to the weakness of the $^1C \leftarrow {}^1A$ absorption of RC-bound spheroidene.

On the other hand, the detailed conformation of RC-bound carotenoids, which may be of importance in understanding their exact function in the RC, is not yet available from X-ray crystallographic data on RCs from *Rps. viridis*, which do not yet give a clear picture of the dihydrolycopene molecule (Deisenhofer, J., personal communication).

We thus attempted to clarify this question further, by reconsidering the possibility of extracting carotenoids from the RC without altering their native configuration, hence making possible a direct assignment of this configuration, using ^{1}H-NMR spectroscopy. Recent progress in assignments of Raman-active modes [11] also was likely to permit comparisons of conformational differences between RC-bound and extracted carotenoids. We also reconsidered the problem of the $^1C \leftarrow {}^1A$ transition which is made difficult by the fact that, in the RC, this band lies under the strongly allowed Soret transition of the bacteriochlorin pigments, which in addition is sensitive to the presence or absence of the carotenoid [3,8], and may be overlapped by other contributions, e.g., from cytochromes.

Partial reports of this study have been made previously [12,13].

Material and Methods

Rhodobacter sphaeroides 2.4.1 (wild-type) cells were grown anaerobically on a modified Hutner's medium. Reaction centers were extracted and purified using HPLC, following the procedure of Berger et al. [14].

Extraction of the reaction center-bound carotenoid

In order to avoid photoisomerization of the native *cis* configuration which is sensitized by bacteriochlorin pigments, the extraction of RC-bound pigments was conducted as far as possible in complete darkness. For visually controlled steps, indirect illumination of the working area was achieved by using a 635 nm band emitted by a Bausch and Lomb 338602 monochromator coupled to a 50 W halogen lamp, and diffused into 2π steradians by a white surface. The resulting irradiance at the sample was less than 3 $\mathrm{nW \cdot cm^{-2}}$.

After several attempts, two distinct procedures were selected for preparing spheroidene in its native *cis* configuration. In the first method, used when studying RR spectra of the whole pigment extract, an acetone-isooctane mixture (3 : 1, v/v) was added (1.5 : 1, v/v) to a concentrated (absorbance 20 at 800 nm) suspension of 2.4.1. RCs, at 0 °C. After rapid mixing with a syringe, an organic phase demixed at the upper part of the mixture. This organic phase essentially consisted of pure isooctane and contained most of the RC content in pigments. A droplet of this solution was frozen down to 77 K and transferred into the Raman cryostat. At these low temperatures, no isomerization of the carotenoid occurred even under high irradiance conditions.

The second method was designed for preparing the higher amounts of purified carotenoid necessary to the NMR experiments. The reaction center pigments were extracted by adding ethanol (10 : 1, v/v) to a concentrated (absorbance 20 at 800 nm) suspension of reaction centers. After 5 min incubation at room temperature, water was added up to a final, total concentration of 20% and the mixture was injected through a SEP-PAK C18 cartridge (Waters). In these conditions, carotenoid and bacteriopheophytin were adsorbed by the nonpolar phase of the cartridge, while most of the bacteriochlorophyll and protein were eluted. The cartridge was then rinsed with 5 ml of a 1 : 5 (v/v) ethanol-water mixture. The adsorbed pigments were eluted using 2 ml of acetone. This solution was chromatographed through a 0.9 × 50 cm 10 ODS2 Partisil HPLC column (Whatman). Elution was achieved by a 6 ml/min flow of ethanol containing 3% water (vol.). A typical elution profile, shown on Fig. 1, presented a weak peak from bacteriochlorophyll traces, a bacteriopheophytin peak, and successively two carotenoid peaks. The first peak grew at the expense of the second one when the starting mixture was exposed to dim light; it is hence ascribed to the *all trans* form, and the second one to the native *cis* isomer. The fractions corresponding to this latter elution peak were rapidly dried under secondary vacuum. The dry extracts corresponding to about 100 absorbance · ml of reaction centers were deuterated by dissolution in methanol-d$_4$ (99.8%, CEA-SMM). After rapid evaporation of the solution, this step was repeated. The sample was finally dissolved in 0.3 ml of $CDCl_3$ (99.8%, CEA-SMM) to a final concentration of about $5 \cdot 10^{-4}$ M.

Spectroscopic methods

Electronic absorption spectra were recorded on a Shimadzu UV-160 spectrometer. Difference electronic absorption spectra were recorded on a Cary 17 spectrometer coupled to a Tracor Northern 1710 multichannel analyzer.

Resonance Raman spectra were recorded at 20 K using a 496.5 nm excitation wavelength. This wavelength insured selective observations of the carotenoid over potential contributions from the bacteriochlorin pigments. The equipment and methods were the same as in Ref. 15, except that a Tracor Northern 1710 multichannel analyzer was used in storing and manipulating the spectra. ^{1}H-NMR spectra were recorded at 1 °C on a 500 MHz Bruker WM-500 spectrometer and were referenced relative to internal tetramethylsilane. Two-dimensional J-correlated experiments (COSY and relayed coherence transfer COSY) were performed by using the pulse sequences ($90°-t_1-45°-t_2$) and ($90°-t_1-90°-t-180°-t-90°-t_2$), respectively, with $t = 3$ ms [16,17]. A total of 256 free induction decays consisting of 2048 data points each were recorded yielding 1K × 1K matrices of real points after zero-filling and Fourier transformation.

Results and Discussion

A stable cis isomer of spheroidene can be extracted from 2.4.1. reaction centers

Two carotenoid bands are present in HPLC chromatograms of the pigment mixture extracted according to procedure II (cf. Material and Methods) from reaction centers of *Rb. sphaeroides* 2.4.1 (Fig. 1). The first eluted one (band I) must correspond to a phototransformed species of the native spheroidene stereoisomer. Indeed, brief illumination of the pigment mixture prior to HPLC results in an increase of this band I, at the expense of the second eluted one (band II). Electronic absorption spectra of both eluted forms (when transferred in light petroleum ether) clearly show that form I is, as expected, *all-trans*, while form II is *cis* (Fig. 2). Indeed, the electronic spectrum of form I exhibits

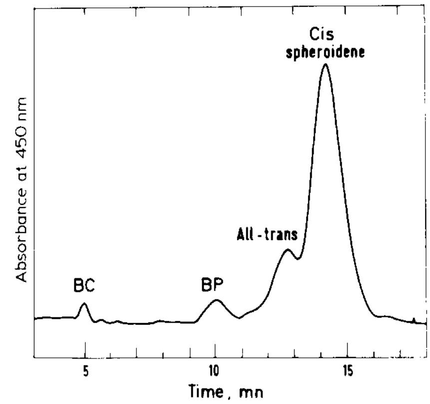

Fig. 1. Typical preparative HPLC elution profile of a pigment mixture extracted in the dark from reaction centers of *Rb. sphaeroides* 2.4.1. (see text). BC, bacteriochlorophyll *a*; BP, bacteriopheophytin *a*.

a very weak $^1C \leftarrow {}^1A$ band only, in the 340 nm region, and its visible maxima, located at 486, 452 and 430 nm, correspond to those observed for the *all-trans* isomer [18]. Form II, on the opposite, yields strong '*cis*', $^1C \leftarrow {}^1A$ transitions at 347 and 333 nm, as well as blue-shifted visible bands at 481, 451 and 425 nm.

Resonance Raman spectra of form II are characteristic of a *cis* stereoisomer as well (Fig. 3).

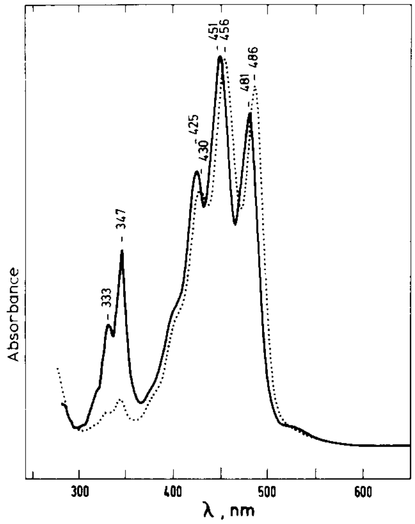

Fig. 2. Electronic absorption spectra of *all-trans* spheroidene and of the *cis* isomer extracted from reaction centers of *Rb. sphaeroides* 2.4.1. This latter isomer is shown to assume the 15–15′ *cis* configuration by ^{1}H-NMR (see text).

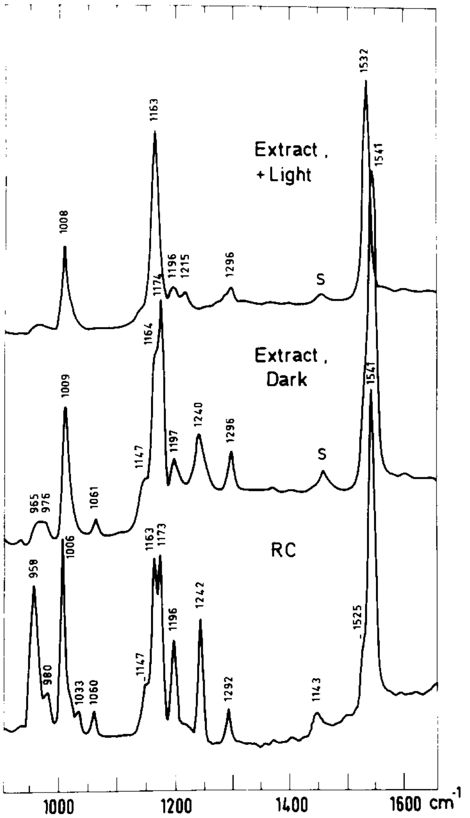

Fig. 3. Resonance Raman spectra (900–1650 cm^{-1} regions) of spheroidene, 30 K, 496.5 nm excitation. From bottom to top; RC, spheroidene bound to reaction centers of *Rb. sphaeroides* 2.4.1; Extract, Dark, spheroidene in a total pigment extract from the above RCs, kept in the dark until frozen at 30 K; Extract, Light, same sample, kept for 1 min in room light before freezing; S, isooctane lines.

Indeed, the main C=C stretching mode is observed at 1541 cm^{-1}, a wavenumber 9 cm^{-1} higher than observed for the *all-trans* isomer and identical to the wavenumber of the native, RC-bound *cis* form [4]. In addition, form II yields a RR band at 1240 cm^{-1}, which is absent from the *all-trans* spectra, but present in RR spectra of the native *cis* form [4].

The *cis* isomer of spheroidene extracted from 2.4.1 RCs is very likely to be the same as the *cis* form which has been characterized in native RCs using electronic absorption and resonance Raman [4]. As a matter of fact, although RR spectra of the native and extracted forms are not identical in terms of relative band intensities (see lower), they share the following common features: (i) the wavenumber of the C=C stretching band, which has been shown, in series of β carotene isomers, to depend on the nature of the *cis* isomer [5,7]; (ii) a sizeable band at 1240 cm^{-1}, shown by Koyama et al. [5] to be active in RR spectra of only a few *cis* isomers of β carotene; (iii) a common structure of the fingerprint 1130–1200 cm^{-1} region, including the presence of a 1173 cm^{-1} band [19]; and (iv) a 1060 cm^{-1} band. This set of common features constitutes an excellent indication for the *cis* isomers of spheroidene present in the RC and in the extract being identical. Moreover, HPLC chromatograms (Fig. 1) show that the limited photoisomerisation process which takes place during the extraction process yields two stereoisomers in sizeable amounts only, one of which is the *all-trans* isomer. When the illumination level of the sample is increased, no additional peak is still observed, but a mere increase of band I. It thus appears that, in the present experimental conditions, the native *cis* isomer is essentially photoconverted into the *all-trans* isomer, with no sizeable buildup of any other *cis* intermediate.

In earlier extraction experiments [4] very small amounts only of a *cis* isomer of spheroidene were recovered. It is now clear from the high quantum yield of BChl *a*-sensitized photoisomerization of 15 *cis* β carotene [9] and from the present results that the origin for these observations was not in some intrinsic unstability of the native *cis* configuration (which might then have been sterically hindered), but rather in an insufficient shading of the extract from any wavelength significantly absorbed by the bacteriochlorin pigments.

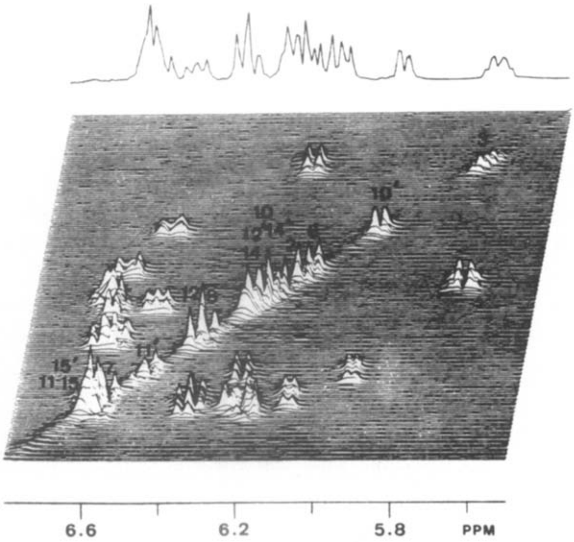

Fig. 4. Olefinic part of *all-trans* spheroidene. ^{1}H-NMR COSY spectrum recorded at 1° C in $CDCl_3$.

Determination of the cis isomer of spheroidene extracted from 2.4.1 reaction centers

The nature of the *cis* isomer of spheroidene recovered from RCs of *Rb. sphaeroides* 2.4.1 was determined using ^{1}H-NMR spectroscopy.

Assignments of all-trans spheroidene resonances. The olefinic part of the spheroidene spectrum contains five doublets with a coupling constant of 11 Hz (H6, H10, H14, H14′, H10′), four doublets with a coupling constant of 15 Hz (H2, H8, H12, H12′), seven quadruplets (H7, H11, H15, H15′, H11′, H4′, H6′) and one octuplet (H3). All these resonances as well as the CH_2 and CH_3 signals were assigned by recording two-dimensional COSY (Fig. 4) and relayed coherence transfer COSY

TABLE I

PROTON-CHEMICAL SHIFTS OF *ALL-TRANS* SPHEROIDENE 1 mM IN $CDCl_3$, 274 K, TMS AS INTERNAL REFERENCE

	Chemical shift (ppm)	proton numbering
CH	6.62 ± 0.04	11, 15, 15′
	6.595	7
	6.505	11′
	6.385	12
	6.355	8
	6.265	14
	6.250	12′
	6.230	10
	6.210	14′
	6.165	2
	6.11	6
	5.960	10′
	5.716	3
	5.111	4′, 6′
CH_2	2.325	4
	2.13	8′
	2.04 ± 0.15	2′, 3′, 7′
CH_3	1.97 ± 0.03	9, 13, 13′
	1.930	1
	1.820	5, 9′
	1.62	1′, 5′

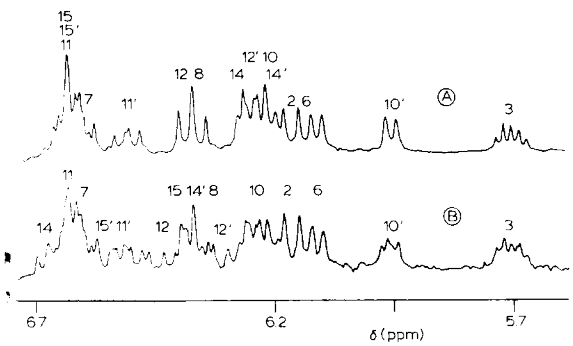

Fig. 5. Olefinic part of one dimensional ^{1}H-NMR spectrum (1 ° C, $CDCl_3$) of (A) *all-trans* spheroidene; (B) *all-trans* + *cis* (65 : 35) spheroidene (reaction center extract). The labeled signals correspond to the 15–15′ *cis*-isomer protons.

spectra, which were compared to previously published proton spectra of various retinoids [20–22]. The chemical shifts of the *all-trans* spheroidene protons are listed in Table I.

Spectral analysis of the cis / all-trans isomeric mixture. The deuteration process of the *cis* isomer recovered from HPLC, as well as its stay in solution during the ^{1}H-NMR experiments (although at 0 ° C and in the dark) resulted in partial isomerization towards the *all-trans* form. As estimated from the ratio of the absorbances of the $1C \leftarrow {}^1A$ and ${}^1B \leftarrow {}^1A$ electronic transitions, 21% of the sample remained in the *cis* form after 12 h in these conditions, and an average 35% during the NMR experiments. Fig. 5 shows the proton spectrum of the *all-trans* molecule and that of the isomeric mixture in the range 7.0–5.6 ppm. Comparison of the two spectra leads to the following observations.

The signals of H2, H6, H3 and H10′ protons are practically identical in both spectra. The set of resonances located at 6.23 ± 0.03 ppm which contains the H12′, H14′, H14 and H10 signals of the *all-trans* molecule is significantly decreased in the isomeric mixture spectrum. Taking into account a 65 : 35 concentration ratio between the *all-trans* and *cis* isomers, signal integration shows the presence of only one resonance of the *cis* isomer in this region. Moreover, one doublet with a coupling constant of 11 Hz is found at 6.68 ppm. This resonance, which does not exist in the *all-trans* isomer spectrum, can only be assigned to either H14, or H14′ or H10 protons of the *cis* isomer. Since these resonances are located at 6.23 ± 0.03 ppm in the *all-trans* isomer spectrum, the observed isomeric shift $\Delta\delta = \delta_{cis} - \delta_{trans}$ is about 0.45 ppm. This value is characteristic of a *cis* isomerization [20,21]. Two *cis* isomers, 15–15′ and 11–12 are consistent with the above results. The doublet located at 6.68 ppm should be assigned to H14 in the first case and to H10 in the second case. A decoupling experiment indicates that this doublet is scalar coupled to a signal located at 6.37 ppm which can be assigned to either H15 or H11. The corresponding isomeric shift $\Delta\delta$ is about -0.25 ppm in both cases, in agreement with the expected value [20,21].

However, the presence of an 11–12 isomer is unlikely; indeed, such an isomerization should induce a high-field shift of the H14 signal [20,21] which is undetected in the observed spectrum. Moreover, the H14′ and H12′ resonances should be unaffected (with respect to the *all-trans* molecule spectrum) while only one resonance of the *cis*-isomer is found in the region 6.23 ± 0.03 ppm. By contrast, the 15–15′ isomerization which should induce a low-field shift of the H12 and H12′ resonances is consistent with the data. Two doublets with a coupling constant of 15 Hz are found at 6.45 and 6.31 ppm and do not exist in the *all-trans* isomer spectrum. These resonances can be assigned to the H12 and H12′ resonances of the *cis*-isomer. The corresponding isomeric shift ($\Delta\delta \approx 0.065$ ppm for both signals) is consistent with the expected value [20,21]. Finally, one quadruplet is located at 6.54 ppm but is absent from the *all-trans* isomer spectrum. This quadruplet can be assigned to the H15′ resonance of the *cis* isomer. The corresponding isomeric shift (-0.08 ppm) is also in agreement with a 15–15′ *cis* isomerization.

Hence, the *cis* isomer extracted from RCs of *Rb. sphaeroides* assumes the 15–15′ mono *cis* configuration. According to the above comparisons of resonance Raman spectra of the native and extracted forms, this must also be the native configuration of reaction center-bound spheroidene, as recently proposed by Gingras and coworkers [2] and by Koyama and coworkers [5,7] on more indirect bases.

The stereoisomer of spheroidene present in 2.4.1 reaction centers is non planar

Although spheroidene is clearly assuming a 15–15′ *cis* configuration both in the raction center and in the extract, RR spectra recorded from this species in each of these two environments differ by several features (Fig. 3). These essentially consist of differences in relative band intensities, the most conspicuous of which concerns a complex band located near to 960 cm^{-1}. This band is indeed four times weaker in the extract spectra recorded at 496.5 nm than in those from the reaction center. As far as the 960 cm^{-1} band is also weak in the *all-trans* photoisomerized extract (Fig. 3), the question arises whether this difference might be due to partial isomerization of the dark extract. This hypothesis can actually be ruled out by noting that (i) the C=C stretching mode occurs exactly at the same frequency, 1541 cm^{-1}, in RR spectra of the native form and of the dark extract; and (ii) the 1173 cm^{-1} band, which occurs in spectra of the native form but lacks in spectra of the *all-trans* form, is stronger in RR spectra of the dark extract than in those of the native form (Fig. 3).

It has long been known [23,24] that RR-active modes around 960 cm^{-1} arise from out-of-plane motions of the chain hydrogens. These modes are not totally symmetric in the C_{2h} point group, and hence give rise to very weak Raman bands only when resonance is with the strongly allowed $^1B \leftarrow {}^1A$ transition. They are known to gain some intensity when the carotenoids are *cis*, hence assuming lower molecular symmetry [4,19].

In the present situation, however, spheroidene clearly assumes the same 15 *cis* configuration in both the RC and the dark extract. The still higher intensity of the 960 cm^{-1} band in RR spectra of 15 *cis*, RC-bound spheroidene thus most probably originates from a still lower molecular symmetry, i.e., out of plane distortion from the basic 15 *cis* confugration [12]. This distortion may occur either in the ground or excited electronic states, or in both. The latter possibility appears the more likely, considering that the RC protein constrains the carotenoid into this otherwise unstable conformation [8], which in addition is likely retained in the lowest excited triplet state [19].

QCFF-π calculations [25] by Mathies and coworkers [26] have shown that small twists in the conjugated chain of 3,7-dimethyl-2,4,6,8,10-dodecapentaenal, in the ground state, can result in large increases of the Raman intensities scattered by out-of-plane wagging modes of chain hydrogens. Moreover, this enhancement effect was predicted to be largely localised to modes of those hydrogens adjacent to the twisted bonds.

Recent normal mode calculations by Tasumi and coworkers [11] indicated that several Raman active bands of β carotene should predominantly involve out-of-plane motions. We attempted to make use of these bands, in addition to the 960 cm^{-1} ones, in order to localize the out-of-plane

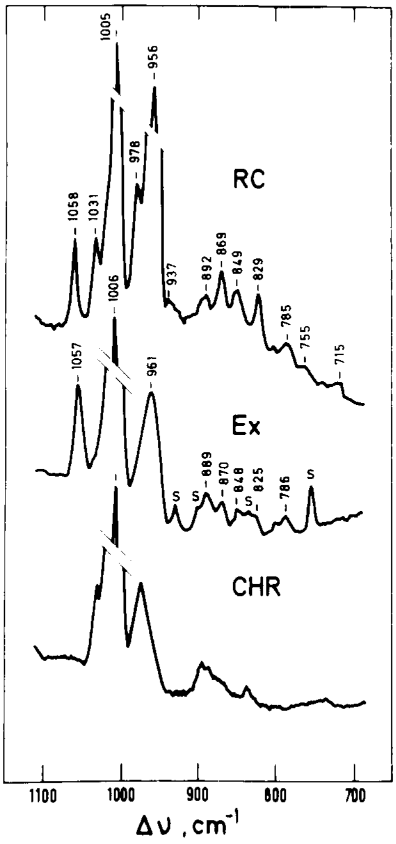

Fig. 6. Resonance Raman spectra (700–1100 cm^{-1} regions) of spheroidene, 30 K, 496.5 nm excitation. RC, spheroidene bound to reaction centers of *Rb. sphaeroides* 2.4.1.; Ex, spheroidene in a total pigment extract from the above reaction centers, experiment conducted in the dark; CHR, spheroidene in chromatophores from *Rb. sphaeroides* (*all-trans* isomer). Peak intensities of the 1006 cm^{-1} bands are identical. S, isooctane lines.

TABLE II

CONFORMATION-SENSITIVE RAMAN BANDS OF 15 *cis* SPHEROIDENE IN THE 760–1060 cm^{-1} RANGE

All experimental data are from spectra excited at 496.5 nm and 30 K. The classifications are: A, relative intensities divided by at least 2 upon extraction of spheroidene from the reaction center, in darkness; B, other bands; Ref.: intensity reference. The calculated frequencies and assignments are from ref. 11; participations in the PED from symmetry-related coordinates have not been distinguished; only participations at least equal to 20% have been indicated. R, rocking; S, stretching; T, torsion; W, wagging; ip, in plane; op, out of plane.

15 *cis* Spheroidene					15 *cis-β* Carotene			
RC-bound		Dark extract		Classification	Solution		Assignment	
obs (cm^{-1}) [a]	I_{rel}	obs (cm^{-1})	I_{rel}		obs (cm^{-1}) [a]	calcd. (cm^{-1})		
1058	13	1057	12	B	1056	1058	13Me R,	op
1031	11	1031 sh	1	A	1026	1013	9Me R,	ip
1005	100	1005	100	Ref.	1006	1006	13Me R,	ip
978	18	972	18	B	970	968	11=12T,	op
956	70	962	18	A	957	952 [c]	7=8 T, 7H W,	op
937	2.9	–	–	A	–	899	10H W,	op
892	5.7	889	5.7	B	889 [b]	890	14H W,	op
869	10.3	870	4.5	A	868 [b]	864	9Me S,	ip
849	10.3	848	4	A	849	848	8H W,	op
829	9.7	825	2.3	A	831	822	12H W,	op
785	4	786	2.5	B	784	764	15H W,	op

[a] From Ref. 4.

[b] Frequencies misindicated in Ref. 4 as 939 and 918 cm^{-1}, respectively.

[c] See text and Note added in proof.

deformation(s) assumed by RC-bound spheroidene.

Fig. 6 and Table II show that, in the 700–1100 cm^{-1} region and taking the 1006 cm^{-1} band intensity (13 and 13′-methyl rock, Ref. 11) as a reference, bands at 1030, 956, 937, 869, 849 and 829 cm^{-1} (group A) are much weaker in the extract spectrum than in the RC spectrum. On the other hand, bands at 1058, 978, 892 and 785 cm^{-1} (group B) have similar relative intensities in the two spectra.

A very close analogy, both in band frequencies and relative intensities, is observed between RR spectra of 15-*cis* spheroidene (dark extract, Fig. 3) and of 15-*cis-β*-carotene [4] obtained in the same conditions (Table II). This indicates that the normal mode calculation that Saito and Tasumi [11] performed for the C_4–$C_{4'}$ moiety of 15-*cis-β*-carotene is largely transposable to 15-*cis* spheroidene. This calculation furthermore indicates that the only conspicuous intensity difference between the two spectra, at 1174 cm^{-1}, concerns a mode of the C_5–C_7 region, which has to be structurally different in the two molecules. Fig. 7 presents a tentative localization of the frequencies observed for 15-*cis* spheroidene in the 1100–700 cm^{-1} region, according to the major PED components calculated by Saito and Tasumi for the C_4–$C_{4'}$ portion of a 15 *cis* carotenoid [11]. Except for the 1030, 1005 and 869 cm^{-1} ones, all of these modes are out-of-plane in the 15 *cis* isomer. It is clearly seen from Fig. 7 that group A bands correspond

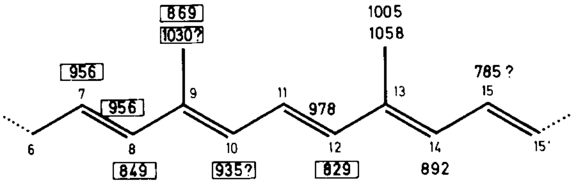

Fig. 7. Molecular model of the 6–15′ section of spheroidene, displaying the wavenumbers (cm^{-1}) of Raman-active modes of the 750–1060 cm^{-1} region which can be localized on the molecule on the basis of the major contributions in their PED, according to calculations by Saito and Tasumi [11]. Squared numbers represent group A (see text and Table II) modes, which are weakened by at least a factor 2 upon extraction of spheroidene, in the dark, from 2.4.1. RCs. Other numbers represent group B modes (see text, Table II and Note added in proof).

to modes which all are localized in the C_7–C_{12} and/or $C_{7'}$–$C_{12'}$ regions of the chain, while group B bands correspond to modes of the C_{11}–$C_{11'}$ region. This spatial segregation of the two groups of modes appears marked enough to allow to conclude safely, despite some uncertainties * in band assignemnts, that the out-of-plane twisting of RC-bound 15-*cis* spheroidene concerns at least one of the C_7–C_{11} and $C_{7'}$–$C_{11'}$ sections of the chain, and not the C_{13}–$C_{13'}$ central section. The fact that the RR intensities of several modes of the C_7–C_{12} region of spheroidene appear to be similarly affected by its extraction from the RC suggests that the out-of-plane deformation of the native form might concern more than one C=C bond.

The conclusion that spheroidene, in its native site of the RC, assumes both a 15-*cis* configuration and an out-of-plane conformation agrees qualitatively with the earlier proposal of Gingras and co-workers [2] based on CD measurements of RCs of *R. rubrum* G9 reconstituted with various carotenoids, including spheroidene. It similarly agrees qualitatively with the recent proposal of Chadwick and Frank [6] that twists occurring at the 6–7 and 6′–7′ bonds should account for their EPR data on triplet states of RC-bound carotenoids.

The oscillator strength of transition $^1C \leftarrow {}^1A$ of reaction-center-bound spheroidene is small

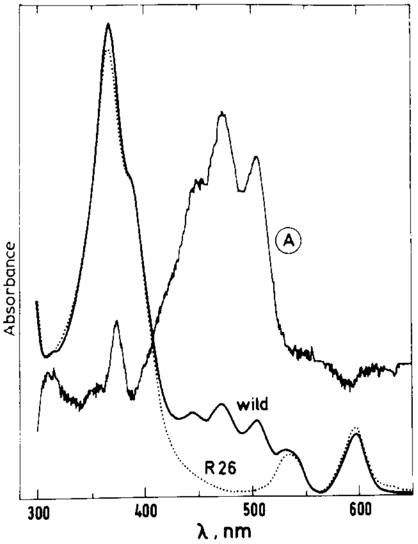

Fig. 8. Difference electronic absorption spectrum (A) obtained by subtracting a spectrum of reaction centers of the carotenoidless R26 mutant of *Rb. sphaeroides* (dotted line) from a spectrum of wild-type reaction centers (heavy line). The 600 nm Q_x band of BChl *a* of the latter spectrum was overcompensated by 10% (see text and references 4 and 8).

Previous diagnoses of the conformations of RC-bound carotenoids were in part based on evaluations of the oscillator strength of their next-to-lower singlet electronic transition around 340 nm [2,4,5]. It is indeed well known that in linear polyenes with a symmetry center this transition is $g \rightarrow g$ and hence symmetry-forbidden. It becomes allowed when molecular symmetry is lowered, e.g., by *trans* → *cis* isomerisation. Among *cis* isomers of C_{40} carotenoids, the highest oscillator strength for this band is actually observed for the 15–15′ *cis* isomer, which departs most from C_{2h} symmetry. Peak absorption of the $^1C \leftarrow {}^1A$ band is then approx. 50% that of the visible $^1B \leftarrow {}^1A$ transition [10]. This is in particular observed for 15–15′ *cis* spheroidene extracted from 2.4.1. RCs (Fig. 2), for which the $^1C \leftarrow {}^1A/{}^1B \leftarrow {}^1A$ peak absorbance ratio is 0.53 in petroleum ether solution. Evaluating the absorbances of the $^1C \leftarrow {}^1A$ bands of RC-bound carotenoids is difficult, because of their near coincidence with the strongly allowed Soret transitions of the bacteriochlorin pigments [4]. Differences between absorption spectra of RCs from the carotenoidless G9 strain of *R. rubrum*, and of the same RCs reconstituted with spheroidene, chloroxanthin or spirilloxanthin yielded very strong near ultraviolet bands (up to twice the

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* In particular, our assignment of the 956–962 cm^{-1} band of 15 *cis* spheroidene and of 15 *cis* *β* carotene to the C_7=C_8 torsion mode calculated at 952 cm^{-1} by Saito and Tasumi [11] is at variance with their own assignment of a 941–953 cm^{-1} band they observed for 15 *cis* *β* carotene to a C_{15}=$C_{15'}$ torsion mode, calculated by them at 955 cm^{-1} (Table II). Our choice was based on the fact that all RR spectra that we have recorded, at 30 K, from *all-trans* and 15 *cis* (i.e., RC-bound) carotenoids each displayed two bands only in the 940–980 cm^{-1} region where five modes (two pairs of them being nearly degenerate) are predicted [11]. These two bands are located around 960 and 970 cm^{-1} for *all-trans* and 15 *cis* *β* carotene and spheroidene. Hence, we assign them the same vibrational origins in these four derivatives. From selection rules for the *all-trans* derivatives, the 960 cm^{-1} mode should be $T(7{=}8)$ (A_g) of the 15 *cis* forms rather than $T(15{=}15')$ (A_u) (see Note added in proof).

peak-visible absorption) to Gingras and coworkers [2]. These bands were identified with the ${}^1C \leftarrow {}^1A$ bands of the carotenoids and were thus taken as indicative of 15 *cis* configuration for these molecules. Lutz et al. [4] and Agalidis et al. [8], similarly working on RCs from *Rb. sphaeroides*, wild-type and R26 strain, obtained difference spectra which contained much weaker bands near to 370 and 348 nm (Fig. 8), in addition to occasional, artifactual bands in the 390–410 nm range (cf. also the 397 nm component in Ga spectrum of Fig. 9). They further showed that the presence of the carotenoid in the RC induced a redshift of about 1.5 nm and a 10% hyperchromic effect on the Soret transitions of bacteriochlorin pigments. Lutz et al. [4] thus ascribed the relatively stronger difference band near to 370 nm to this phenomenon, and suggested that the very weak 348 nm band might constitute one component of the ${}^1C \leftarrow {}^1A$ transition of spheroidene. Koyama et al. [5,7] later questioned this interpretation, in the context of their planar 15 *cis* assignment of the native conformation of RC-bound carotenoids.

In order to solve this difficulty, we compared difference spectra of spheroidene bound to 2.4.1. RCs and of methoxyneurosporene bound to RCs of the Ga strain of *Rb. sphaeroides.* Methoxyneurosporene has one conjugated double bond less than spheroidene. Hence, its ${}^1B \leftarrow {}^1A$ transition is to the blue of that of spheroidene, by about 20 nm (Fig. 9). Extensive comparisons of electronic spectra of carotenoids with 9–13 conjugated double bonds, and assuming various configurations, enabled Zechmeister [10] to formulate the empirical rule that, in a given solvent, the ${}^1C \leftarrow {}^1A$ transition was located at a relatively constant spectral distance from the O–O component of the ${}^1B \leftarrow {}^1A$ transition. In hexane, this wavelength difference was found to be 142 ± 2 nm.

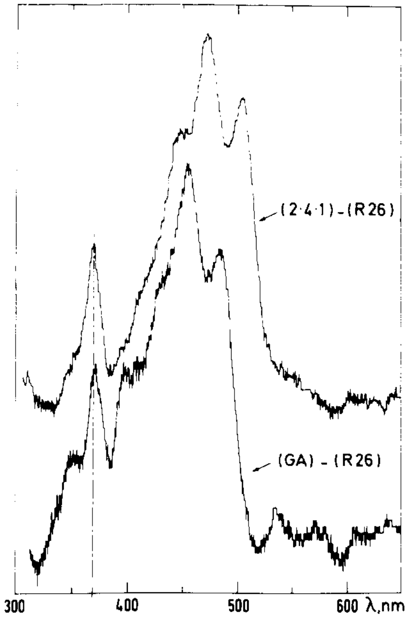

Fig. 9. Difference electronic spectra (see Fig. 10) of reaction centers from the spheroidene-containing, wild-type strain of *Rb. sphaeroides* (2.4.1.R26) and of the methoxyneurosporene-containing, GA mutant of the same species (Ga-R26)

Spheroidene and methoxyneurosporene are very likely to share identical environments in RCs of *Rb. sphaeroides*. It is thus expected, from these bases, that the ${}^1C \leftarrow {}^1A$ bands of these two carotenoids should be located about 20 nm apart. Fig. 9 shows that, in the 370–330 nm region, both (2.4.1) – (R26) and (Ga) – (R26) difference spectra contain positive bands at 367–368 nm of quite similar absorbances and widths, which hence cannot primarily arise from the ${}^1C \leftarrow {}^1A$ transitions of spheroidene and methoxyneurosporene, respectively. This fully confirms our previous assignment of this band to carotenoid-induced hyperchromic and bathochromic effects on the Soret bands of bacteriochlorin pigments. The two components of the actual ${}^1C \leftarrow {}^1A$ transitions of spheroidene and methoxyneurosporene must be weaker than the 367 nm band and may lay under it, or at a lower wavelength (Fig. 9). In any case, as stated earlier [4] these bands must be much weaker than those of planar 15-15′-*cis* isomers.

This conclusion is quite consistent with the fact that, in the reaction center of *Rb. sphaeroides*, the carotenoid assumes an out-of-plane twisting superimposed over the basic 15-*cis* configuration. Oddly enough, the very similar proposal made by Gingras and coworkers [2] for RC-bound spirilloxanthin [2] was essentially based on the opposite conclusion that the ${}^1C \leftarrow {}^1A$ band of this carotenoid in RCs from *R. rubrum* was unusually strong. This rises the question whether the conformational assignments made in the present paper for RC-bound spheroidene may be valid for other carotenoids and other bacterial species, including *R. rubrum*.

A twisted 15-cis conformation is common to reaction center-bound carotenoids of different Rhodospirillaceae.

Resonance Raman spectra of spheroidenone, neurosporene and methoxyneurosporene bound to reaction centers of *Rb. sphaeroides* [4,8,19] leave no doubt that they assume the same 15 *cis* configuration as spheroidene [4]. In addition, these RR spectra all share strong bands at approx. 960 cm^{-1} with those of RC-bound spheroidene. These higher intensity bands are indicative that these three carotenoids also assume out-of-plane deformations when in their native sites. These properties are also clearly shared by reaction center carotenoids of two other genera of the Rhodospirillaceae family. Indeed, dihydroneurosphorene or dihydrolycopene bound to RCs of *Rps. viridis* as well as spirilloxanthin bound to RCs of *R. rubrum* yielded RR spectra presenting characteristic features for both 15 *cis* configurations and out-of-plane twisting [4]. Hence, the above-noted discrepancies in interpretations of the 300–400 nm regions of electronic spectra of RCs from *Rb. sphaeroides* and from *R. rubrum* cannot arise from differences in these basic conformational properties of their bound carotenoids. Further resonance Raman spectroscopy of extraction-induced changes in activities of the 750–1100 cm^{-1} region modes of RC-bound spirilloxanthin might indicate whether the precise nature of the out-of-plane twisting of the conjugated part of this molecule is equivalent to that of spheroidene bound to *Rb. sphaeroides* RCs.

Note added in proof

After this paper was accepted, we were informed by Prof. M. Tasumi that the assignments of the approx. 960 and 970 cm^{-1} modes of *all trans β* carotene made in Ref. 11 had to be inverted, on the basis of RR spectra recently obtained by his group on selectively deuteriated samples. Hence, the 956 and 978 cm^{-1} modes of RC-bound spheroidene are most likely to arise actually from $T(7=8)$ and $T(11=12)$, respectively. Table II and Fig. 7 should be accordingly modified; the out-of-plane distortion of RC-bound spheroidene thus should essentially concern the C_8-C_{12} and/or $C_{8'}$-$C_{12'}$ regions. We thank Prof. Tasumi for this unpublished information.

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References

1 Cogdell, R.J., Parson, W.W. and Kerr, M.A. (1976) Biochim. Biophys. Acta 430, 83–93
2 Boucher, F., Van der Rest, M. and Gingras, G. (1977) Biochim. Biophys. Acta 461, 339–357
3 Lutz, M., Kléo, J. and Reiss-Husson (1976) Biochem. Biophys. Res. Commun, 69, 711–717
4 Lutz, M., Agalidis, I., Hervo, G., Cogdell, J. and Reiss-Husson, F. (1978) Biochim. Biophys. Acta 503, 287–303
5 Koyama, Y., Takii, T., Saiki, K. and Tsukida, K. (1983) Photobiochem. Photobiophys. 5, 139–150
6 Chadwick, B.W. and Frank, H.A. (1986) Biochim. Biophys. Acta 851, 257–266
7 Koyama, Y., Kito, M., Takii, T., Tsukida, K. and Yamashita, J. (1982) Biochim. Biophys. Acta 680, 109–118
8 Agalidis, I., Reiss-Husson, F. and Lutz, M. (1980) Biochim. Biophys. Acta 589, 264–274
9 Jensen, N.H., Nielsen, A.B. and Wilbrandt, R. (1982) J. Am. Chem. Soc. 104, 6117–6119
10 Zechmeister, L. (1962) *Cis-trans* Isomeric Carotenoids, Vitamins A and Arylpolyenes, Springer-Verlag, Wien
11 Saito, S. and Tasumi, M. (1983) J. Raman Spectrosc. 14, 310–321
12 Szponarski, W., Robert, B., Szalontai, B. and Lutz, M. (1984) Abstract of the 7th International Symposium on carotenoids, München, 27–31 August, p. 37
13 Lutz, M. (1987) in Spectroscopic and Structural Studies of Biomedical Materials, (Twardowski, J., ed.), Sigma Press, Wilmslow, in the press
14 Berger, C., Tiede, D.M. and Breton, J. (1984) Biochem. Biophys. Res. Commun. 121, 47–54
15 Lutz, M., Hoff, A.J. and Bréhamet, L. (1982) Biochim. Biophys. Acta 679, 331–241
16 Aue, W.P., Bartholdi, E. and Ernst, R.R. (1986) J. Chem. Phys. 64, 2229–2246
17 Wagner, G. (1983) J. Magn. Reson. 55, 151
18 Davies, B.H. (1965) in Chemistry and Biochemistry of Plant Pigments (Goodwin, T.W., ed.), 1st Edn., Ch. 18, Academic Press, London
19 Lutz, M., Chinsky, L. and Turpin, P.Y. (1982) Photochem. Photobiol. 36, 503–515
20 Englert, G. and Vecchi, M. (1980) Helvetica Chimica Acta 63, 1711–1718
21 Tsukida, K., Saiki, K. and Sugiura, M. (1981) J. Nutr. Sci. Vituminol. 27, 551–561
22 Tsukida, K. and Saiki, K. (1982) J. Nutr. Sci. Vitaminol. 28, 311–313
23 Rimai, L., Gill, D. and Parsons, J.L. (1971) J. Am. Chem. Soc. 93, 1353–1357
24 Warshel, A. and Karplus, M. (1974) J. Am. Chem. Soc. 96, 5677–5689
25 Warshel, A. and Dauber, P. (1971) J. Chem. Phys. 66, 5477–5488
26 Eyring, G., Curry, B., Mathies, R., Franzen, R., Paling, I. and Lugtenburg, J. (1980) Biochemistry 19, 2410–2418