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BINDING OF CAROTENOIDS ON REACTION CENTERS FROM *RHODOPSEUDOMONAS SPHAEROIDES* R 26

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Summary

The carotenoid-less reaction centers isolated from *Rhodopseudomonas sphaeroides* (strain R 26) bind pure all-*trans* spheroidene as well as spheroidenone in a nearly 1 : 1 molar ratio with respect to *P*-870. Neither β-carotene nor spirilloxanthin, both absent from wild-type *Rps. sphaeroides*, could be bound in appreciable amounts. Resonance Raman spectra of the carotenoid-reaction center complex indicate that the carotenoid is bound as a *cis* isomer, its conformation being very close, although probably not identical, to that assumed by the carotenoid in the wild-type reaction centers. The electronic absorption spectra of the carotenoid-reaction center complexes are in good agreement with such a interpretation. When bound to the R 26 reaction centers, spheroidene displays light-induced absorbance changes identical in peak wavelengths and comparable in amplitudes to those observed in the wild-type reaction centers. Thus the binding of the carotenoid to the R 26 reaction centers most likely occurs at the same proteic site as in the wild-type reaction centers. This site shows selectivity towards the nature of carotenoids, and has the same sterical requirement as in the wild type, leading to the observed all-*trans* to *cis* isomerisation.

Introduction

It seems now generally accepted that a carotenoid molecule is bound to the reaction centers of photosynthetic bacteria as an integral part of the complex.

Abbreviation: BChl, Bacteriochlorophyll.

Thus in a number of bacterial species about 1 mol of carotenoid is present per *P*-870 [1,2]. The conformation of this carotene molecule, as shown by circular dichroism [1] and Raman resonance spectroscopy [3,4] is strikingly different from that of the bulk carotene present in the chromatophore membrane; in the reaction center the carotenoid assumes a particular *cis* (presumably di-*cis*) conformation, whereas the bulk carotenoids are in the all-*trans* form [3,4]. The functional roles that have been ascribed to the carotenoid bound to the reaction center are those of increasing the light-collecting efficiency and protecting BChl against photooxidative damage. When the normal photochemistry is blocked by chemical reduction of the first electron acceptor the photoexcited state of *P*-870 is deactivated by energy transfer to the carotenoid and results in formation of the carotenoid triplet state [5]. In *Rhodospirillum rubrum* reaction centers, native spirilloxanthin, as well as various carotenes artificially fixed on the carotenoid-less reaction center isolated from the G9 mutant, are able to protect BChl against the photodynamic effect of $O_2{}^1$ [6]. Thus the question arises of the relationship between the particular conformation of the carotene in the reaction center and its functional role.

In this work, we have tried to bind carotenoids to reaction centers isolated from the carotenoid-less mutant *Rhodopseudomonas sphaeroides* R 26, in line with the experiments carried out by Boucher et al. on *R. rubrum* [6]. These authors easily bound various bacterial carotenoids on their G9 reaction center preparations. Their interpretation of the electronic absorption and circular dichroism spectra of the complexes, as well as those of spirilloxanthin in wild-type reaction centers, indicated a central mono-*cis* conformation of the bound carotenoid. However, we have questioned this interpretation [4] and in this work, we have tried to determine whether spheroidene may be bound in vitro to *Rps. sphaeroides* R 26 reaction centers; if so, what are the structural and functional properties as compared to those it assumes in the wild-type reaction center. We have also obtained some insights about the existence and the specificity of a carotenoid binding site in the *Rps. sphaeroides* reaction center.

Materials and Methods

Samples. Rps. sphaeroides bacteria strains Y (wild type) and R 26 were grown as previously described in the synthetic L medium [7] and in the semi-synthetic liquid medium of Cohen-Bazire et al. [8] supplemented with yeast extract (1 g/l), respectively. Reaction centers from *Rps. sphaeroides* R 26 were purified according to Okamura et al. [9] except that a linear NaCl gradient was used for DEAE-cellulose chromatography instead of a stepwise elution. Reaction centers from the strain Y were purified as already described [10] with an additional purification on DEAE-cellulose as above.

Spheroidene, and spheroidenone were extracted from *Rps. sphaeroides* cells and purified by column chromatography on aluminium oxide [11,12]; their purity was checked by chromatography on Schleicher and Schüll· No. 287 kieselguhr paper [11]. Spirilloxanthin was extracted [11] from aged *R. rubrum* cells (grown for 4—7 days) in which it has been shown to be the major carotenoid [13]. All-*trans* β-carotene was a gift from Mr J. Kleo.

Preparation of complexes of carotenoid and reaction center. Carotenoids are

insoluble in water and are not well solubilized when merely added to an aqueous detergent solution. Thus before incubation with reaction centers, it was necessary to disperse them in solution by the following steps: a 0.2—0.3 mg carotenoid solution in acetone or light petroleum ether was dried under N_2 in a sonication cell and 5—8 ml of 1% Triton X-100, 10 mM Tris-HCl buffer, pH 8.0, were added. After vigorous vortexing the mixture was sonicated during 20 min in a MSE 150 W disintegrator and centrifuged 10 min at 3000 × *g* to eliminate the titanium probe debris and any insolubilized fraction of the carotenoid. The solubilized carotenoid content of the final solution, measured spectroscopically after dilution of an aliquot of the supernatant in acetone, was usually 20—60 μM.

To this solution a reaction center aliquot was added (final concentration 1—3 μM). The mixture was stirred overnight at 20°C and applied to short Whatman DEAE 52-cellulose column (0.9 × 4.5 cm), equilibrated with 1% Triton X-100, 10 mM Tris-HCl buffer, pH 8.0. The free excess carotenoid was not retained and was washed off. The column was then washed with 0.1% lauryldimethylamine oxide, 10 mM Tris-HCl buffer, pH 8.0, and the carotenoid-reaction center complex was eluted with 0.4 M NaCl; when necessary the preparation was concentrated first on a Amicon PM 30 membrane, then by pelleting overnight at 200 000 × *g*.

Spectroscopic assays. Absolute absorption spectra and 'light minus dark' difference spectra were recorded on a Cary 14 R spectrophotometer, equipped with an home-made cross-illumination device. Infrared saturating continuous actinic light was provided by a Sylvania tungsten-iodide lamp and a Wratten 89 B filter; the photomultiplier was protected by a 10% $CuSO_4$ solution (1 cm optical path). Absorption differential spectra were computed using a Cary 17 spectrometer and a multichannel analyzer (Didac 4000, Intertechnique).

Resonance Raman spectra were recorded at 30 K as previously described [3].

The published extinction coefficients were used for measuring the concentration of reaction centers [14] and of carotenoids after acetone or light petroleum ether extraction [15]. The amount of spheroidene bound per reaction center after recombination was estimated from the absolute absorption spectrum of the complex, and from its light-induced absorption changes (see Results).

Results

Binding of carotenoids

Before describing our results, we would stress that an appreciable binding of carotenoid to *Rps. sphaeroides* R 26 reaction centers could only be observed when several conditions were fulfilled. The carotenoid must be solubilized first and this required sonication in 1% Triton. Lauryldimethylamine oxide at any concentration or 0.1% Triton were inefficient. The formation of mixed micelles between the detergent and the pigment [16], favoured by a comparable hydrocarbon chain length of the two compounds, may occur during this treatment. Omission of the sonication step led to a low (if any) amount of binding to reaction centers; a low degree of binding was also observed when aged reac-

tion center preparations (stored at 5°C longer than 2 weeks) or frozen and thawed ones were used.

Up to 1 mol spheroidene/mol *P*-870 could be bound to R 26 reaction centers in several experiments performed with four different reaction center preparations. The spheroidene content was estimated by comparison of absolute absorption spectra of R 26, of spheroidene-R 26 and of wild-type reaction centers (see below), all normalized with respect to the Q_x band of BChl. Similarly spheroidenone could also be bound to R 26 reaction centers. In this latter case we did not determine the binding stoichiometry; it should be not far from unity, as inferred from the intensities of the carotenoid absorption bands.

In spite of a number of experiments, we did not observe any binding of all-*trans* β-carotene or spirilloxanthin on R 26 reaction centers.

Some of our results are at variance with those reported for *R. rubrum* G9 reaction centers [6], where the solubilization of the carotenoid was apparently not required: the binding reportedly occurred by mild shaking of reaction centers in a very dense suspension of carotenoid (0.8—1.6 mM) in 0.1% Triton. Additionally, the G9 reaction centers were reported to easily bind four different bacterial carotenoids in 1 : 1 molar ratios; two of these carotenoids are not synthetized by *R. rubrum* cells.

Resonance Raman spectra of spheroidene and spheroidenone bound to R 26 reaction centers

When bound to R 26 reaction centers, these two carotenoids yielded characteristic resonance Raman spectra, indicative of a *cis* conformation. Resonance Raman spectra of bound spheroidene (Fig. 2) indeed displayed a number of features already observed in wild-type reaction centers [4] which distinguished them quite clearly from those observed in chromatophores. Among these features the most conspicuous were: (i) a +10 cm^{-1} shift and alteration of the structure of the band located at 1540 cm^{-1} (ν_1); (ii) a splitting into two components (1164, 1174 cm^{-1}) of the ν_2 band; (iii) a strong increase in relative

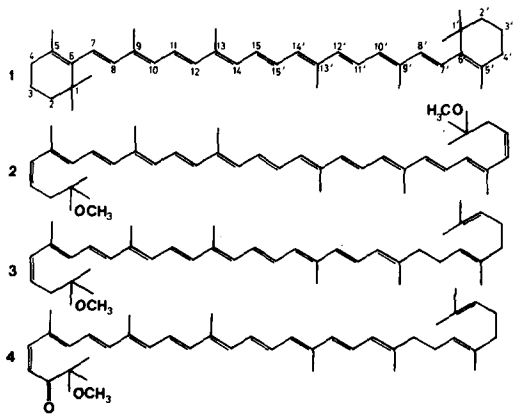

Fig. 1. Chemical formula of the carotenoids used in the binding experiments. 1, All-*trans* β-carotene; 2, spirilloxanthin, present in *R. rubrum*; 3, spheroidene, and 4, spheroidenone present in *Rps. sphaeroides*. 2—4, Chain-end conformations shown here are arbitrary.

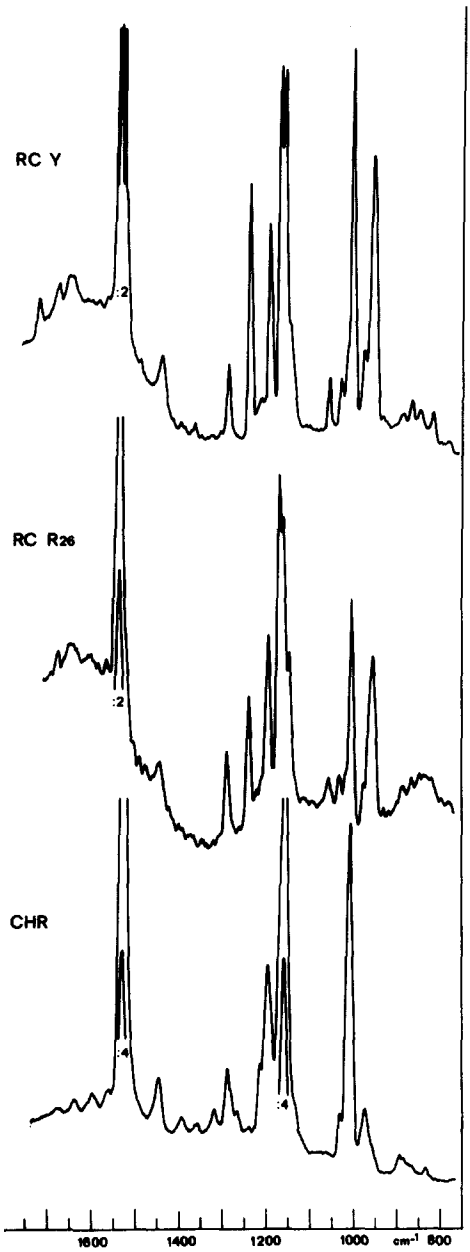

Fig. 2. Resonance Raman spectra of spheroidene in various preparations from *Rps. sphaeroides*, recorded at 30 K, 800—1700 cm^{-1} region. Excitation at 496.5 nm. RCY, wild-type reaction centers. RC R 26, spheroidene bound to R 26 reaction centers. CHR, chromatophores.

intensity of a band at 956 cm^{-1}, and (iv) additional or strongly enhanced bands at 1241, 1058, 490 and 258 cm^{-1}. However, there were some differences in relative intensities between resonance Raman spectra of the spheroidene-R 26 and the wild-type reaction center preparations. These differences were: (i) an enhancement of the ν_2 doublet, by 10—20% depending on the preparation;

the 1174 cm^{-1} component was always stronger than the 1161 cm^{-1} (these two components have the same intensity for wild-type reaction centers); (ii) a 2-fold enhancement of a ν_2 satellite at 1150 cm^{-1}; (iii) a weakening of the characteristic '*cis* band' at 1240 cm^{-1} by 15—30%; (iv) an enhancement of a weak band at 1294 cm^{-1} by a factor of 1.2—2 depending on the preparation, and (v) a 2-fold enhancement of a band at 285 cm^{-1} (not shown), also characteristic of spheroidene bound to wild-type reaction centers. These intensities were measured relatively to the peak intensity of the ν_3 band near 1005 cm^{-1}, which likely corresponds to a group frequency, possibly arising from the stretching mode of C_{chain}-CH_3 bonds [17,18].

Resonance Raman spectra of spheroidenone bound to R 26 reaction centers were quite similar to those observed for spheroidene, but they also displayed relative intensity differences with respect to those of spheroidenone in wild-type reaction centers extracted from cells grown aerobically.

The results clearly indicate that these two carotenoids adopt after binding to R 26 reaction center a *cis* conformation identical, or very similar, to the one we have previously observed in the wild-type reaction center [4]. In all three cases the same sets of vibrational modes were active, at the same frequencies and with nearly the same relative intensities. The differences noted in several band intensities for spheroidene are not originating from the presence of any detectable amount of all-*trans* molecules in the preparations, either bound to the reaction centers or not. This is demonstrated by the fact that the 1174 cm^{-1} component of the ν_2 band, which is characteristic of the *cis* form, increases in relative intensity relatively to the 1161 cm^{-1} component, present for both the *cis* and all-*trans* forms. The strain exerted on spheroidene when binding to the host polypeptide most probably results in minor conformation changes of the polyene chain, in addition to the main *cis* folding which yields the main characteristics of the resonance Raman spectra [4]. It appears likely that the intensity differences observed between resonance Raman spectra of spheroidene, either bound to wild-type or to R 26 reaction centers, arise from differences in these minor conformation changes. We have previously discussed the possible conformation of spheroidene in the wild-type reaction centers [4]. The Raman and electronic absorption spectra sugggest it assumes a di-*cis* conformation, possibly involving one methylated double bond and one unmethylated; this hypothesis should now be extended to spheroidene and spheroidenone recombined to the R 26 reaction center. However, a precise identification of these conformers must await the study of a series of well-characterized isomers of a C_{40} carotenoid.

Absorption spectrum of the spheroidene-reaction center complex

The binding of spheroidene to the R 26 reaction center was accompanied by a number of spectral changes best observable in the difference spectra. When compared to the original R 26 reaction center (Fig. 3), the three well-resolved vibrational bands of the $B_1 \leftarrow A_1$ electronic transition of the spheroidene molecule were apparent. Much less conspicuous was a very weak band at 348 nm, which we considered to constitute the so-called *cis* band (in the wild-type reaction center, a *cis* band has been already observed at 348 ± 3 nm [4]). A 369 nm-positive band likely resulted from an hyperchromic effect

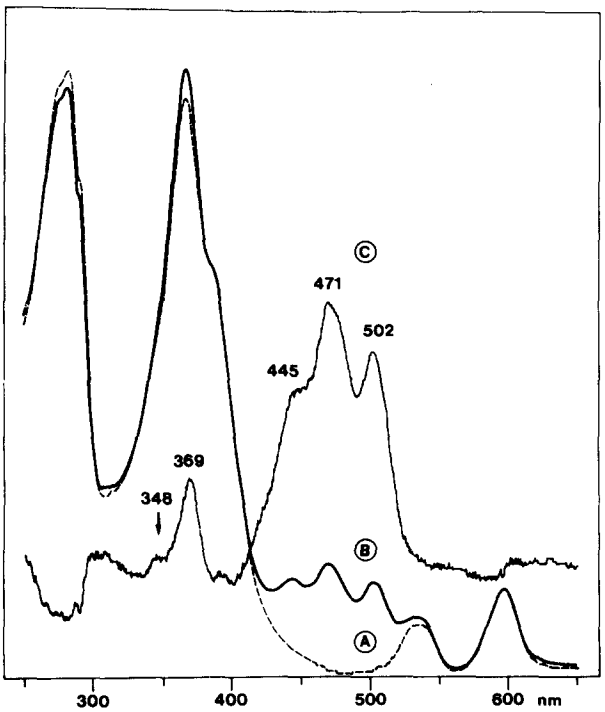

Fig. 3. Absorption spectra at 20°C of (A) R 26 reactions centers; (B) spheroidene-R 26 reaction center complex; (C) computed differential absorption spectra 2.5 × [(B) − (A)].

(approx. 15%) and a slight red shift of the Soret band of BChl: such a phenomenon, probably due to BChl-spheroidene interactions, was already observed when comparing R 26 and wild-type reaction center spectra [4].

The electronic absorption spectrum of spheroidene bound to R 26 reaction centers differed only very slightly from that of spheroidene present in wild-type reaction centers (Fig. 4). The 0-0 and lowest 0-1 components of the $B_1 \leftarrow A_1$ transition of the former were blue-shifted by 1.5 and 0.7 nm, respectively, as calculated from the difference spectrum of Fig. 4.

These absorption data strongly suggested, as did the Raman data, that the conformation of spheroidene in R 26 reaction centers is the same as, or very similar, to that observed in wild type.

Spheroidene band shifts in R 26 reaction centers

Continuous actinic illumination induced absorbance changes in the visible bands of spheroidene bound to R 26 reaction centers (Fig. 5): a series of peaks and troughs in the 450-530 nm region was observed, at the same wavelengths as those observed for wild-type (strain Y) reaction centers. The amplitude of these changes expressed relatively to the bleaching of the Q_x band of BChl as $\Delta A_{510\ nm} - A_{495\ nm}/\Delta A_{600\ nm}$, were nearly equal (0.21 in the wild type, 0.19 in the complex). Such spectral changes have been studied previously in reaction centers isolated from another wild-type [19—21] and mutant strains [21] of *Rps. spheroides* and they were interpreted as resulting from an electrochromic red shift of the carotenoid bands, due to the local field created by the primary

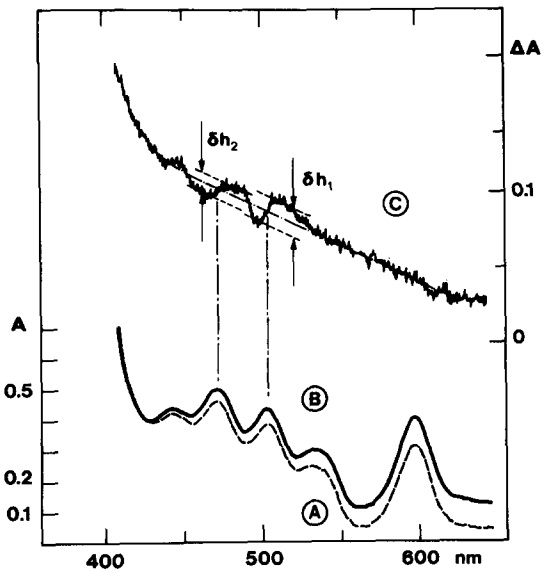

Fig. 4. Comparison of absorbances of the wild-type reaction center and of the spheroidene-R 26 reaction center complex at 20°C, in the visible absorption bands of spheroidene. (A) Wild-type reaction center; (B) spheroidene-R 26 reaction center complex; (C) computed difference spectrum: 5 X [(A) — (B)] (see Material and Methods). The wavelength differences between the 0-0 and between the lowest 0-1 ($B_1 \leftarrow A_1$) transitions of spheroidene in the two samples were calculated using δh_1 and δh_2, respectively (see text).

charge separation. More precisely, some authors considered that the carotenoid was only sensitive to the positive charge of the BChl dimer (in the state $P\text{-}870^+$, or ($P\text{-}870^+$, FeUQ)) [20]; others [21]; were able to observe another band shift, of opposite direction, when the reaction center bore a negative charge (state P-870, $(UQFe)^-$).

This latter point was examined by light minus dark spectra in the presence

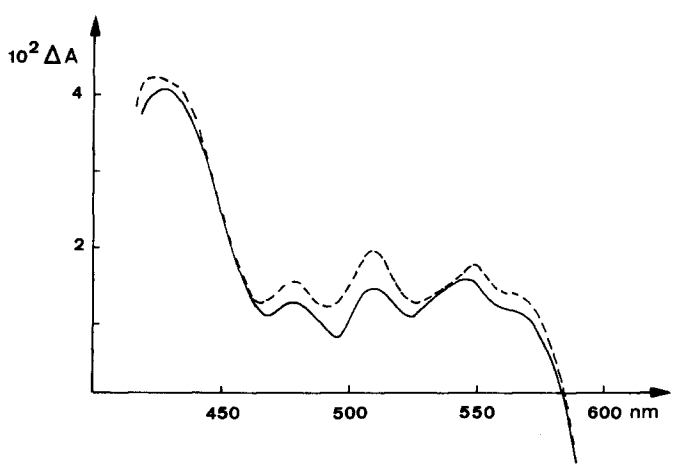

Fig. 5. Light minus dark difference spectra of wild-type reaction centers (- - - - -) and spheroidene-R 26 reaction center complex, both 2.1 μM in 10 mM Tris-HCl buffer, 0.1% lauryldimethylamine oxide, pH 8.0.

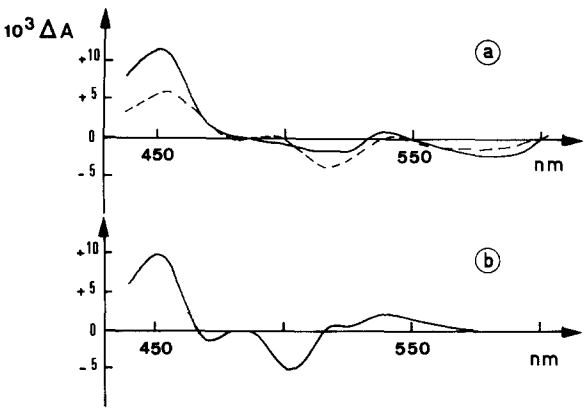

Fig. 6. A comparison of the light minus dark difference spectra in presence of the phenazonium methosulfate-ascorbate couple (a) 2 μM wild-type reaction center (------) and 1.5 μM R 26 reaction center (———). (b) 2.1 μM Spheroidene-R 26 reaction center complex. All spectra were run in presence of 66 μM phenazonium methosulfate, 1.2 mM ascorbate in 10 mM Tris-HCl buffer, 0.1% lauryldimethylamine oxide, pH 8.0.

of an excess of the phenazonium methosulfate-ascorbate redox couple, which is able to keep *P*-870 reduced, and upon illumination leads to the state (*P*-870, $(UQFe)^-$) (Fig. 6). In R 26 and in wild-type reaction center preparations, the absorbance changes in the 480—530 nm range were identical; the band shifts of spheroidene were abolished, in agreement with the former interpretation [20]. However, under the same conditions the complex of spheroidene on R 26 reaction centers behaves differently: one could still observe a weak negative peak at 500 nm, which likely originates from a residual carotenoid shift. This effect was not studied in further detail.

Discussion

The reported results indicate that the protein of *Rps. sphaeroides* R 26 reaction center possesses a carotenoid binding site sharing the same properties as that of the wild-type reaction center protein.

This site has the remarkable feature of imposing a unique *cis* conformation to the carotenoid, leading to an all-*trans* to *cis* isomerisation of spheroidene (or spheroidenone) during the binding process. The origin of the energy required for this steric rearrangement is unknown; it may arise from Van der Waals forces favouring the inclusion of the carotenoid in a hydrophobic region of defined and restricted size, or from specific forces acting between the carotenoid, the BChl molecules, and (or) some protein residues. Previously we have presented the experimental data, based on the resonance Raman spectra, which indicated the formation of a *cis* isomer; the conformation of the polyene backbone, possibly di-*cis*, appears to be grossly the same for spheroidene and spheroidenone bound to R 26 reaction centers, as for various carotenoids present in several wild-type reaction centers [4].

A central mono-cis conformation has been previously proposed for the carotenoids bound to *R. rubrum* reaction centers (either in the wild-type, or recom-

bined with the G9 preparation) [6], however, this proposal was based on the analysis of circular dichroism and absorption data, which did not yield precise information about the steric conformation of the carotenoid molecule. One should note that the conformations of spheroidene (and spheroidenone) may not be strictly identical when bound either to R 26 or to the wild-type reaction centers. The alterations we observed in the relative intensities of several Raman bands could reflect structural or environmental differences; structural factors are more plausible, as Raman spectra of carotenoids are very weakly sensitive to environment changes, and the electronic absorption spectra, which generally are very sensitive to the environment of carotenoids, are hardly modified.

Not only does the binding of the carotenoid impose a 'native' *cis* conformation to the molecule but it likely occurs with the same spatial arrangement with respect to the other chromophores as in the wild-type reaction centers. Indeed an electrochromic effect is observed identical to that exhibited by the carotenoid in the wild-type preparation; the Soret absorption band of BChl is also similarly modified. Thus the binding of the carotenoid to the R 26 reaction center most likely occurs at the same site as in the wild-type reaction center.

The binding site also appears to display some specificity. Among the four carotenoids tested, only two could be bound: spheroidene, and spheroidenone, which both occur in wild-type reaction centers; the major carotenoid present in these reaction centers is spheroidene when the bacteria are grown anaerobically [1] and spheroidenone when they are grown aerobically (Reiss-Husson, F., unpublished results). Neither β-carotene, nor spirilloxanthin, both with the same total chain length as spheroidene, could be bound in a detectable amount. Thus the conformation of the ends of the chain, and/or the nature of the substituents, play a role in the binding process. This is different to the behavior of the *R. rubrum* G9 reaction center. In addition to spirilloxanthin, this reaction center was reported to bind three bacterial carotenoids exogenous to *R. rubrum* in 1 : 1 molecular ratios [6]; at least two of them, spheroidene and spheroidenone, seemed to be bound at a site similar to that present in wild-type *Rps. sphaeroides*, as inferred from the circular dichroism data. Resonance Raman studies should resolve the conformation of these bound pigments. However, one may already conclude that the G9 reaction preparation has fewer binding restrictions with regard to the chemical nature of the carotenoids than *Rps. sphaeroides* R 26. The reason for this difference is unknown but perhaps is related to the reaction center proteins of these two bacterial species which are not identical, as shown by their immunological and electrophoretic properties [9].

Acknowledgements

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