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THE TYPE, AMOUNT, LOCATION, AND ENERGY TRANSFER PROPERTIES OF THE CAROTENOID IN REACTION CENTERS FROM *RHODOPSEUDOMONAS SPHAEROIDES*

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SUMMARY

Analysis of photosynthetic reaction centers from *Rhodopseudomonas sphaeroides* strains 2.4.1 and Ga shows that each contains approx. 1 mol of a specific carotenoid per mol of reaction center. In strain 2.4.1. the carotenoid is spheroidene (1-methoxy-3,4-didehydro-1,2,7′,8′,-tetrahydro-ψ,ψ-carotene); in strain Ga, it is chloroxanthin (1-hydroxy-1,2,7′,8′-tetrahydro-ψ,ψ-carotene). The carotenoid is bound to the same pair of proteins as are the bacteriochlorophylls and bacteriopheophytins of the reaction center. This binding induces strong circular dichroism in the absorption bands of the carotenoid. The carotenoid is close enough to the other pigments of the reaction center so that light energy transfers efficiently from the carotenoid to the bacteriochlorophyll, sensitizing bacteriochlorophyll fluorescence. The fluorescence polarization spectrum of the reaction centers shows that the transition vectors for the visible absorption bands of the carotenoid lie approximately parallel to the 600 nm (Q_x) transition of the bacteriochlorophyll complex.

INTRODUCTION

The isolation of photosynthetic reaction centers from chromatophores of *Rhodopseudomonas sphaeroides* was first described for the carotenoidless strain R26 [1, 2]. These reaction centers have been well characterized; they contain three protein subunits, four molecules of bacteriochlorophyll, two molecules of bacteriopheophytin, one atom of iron, and one or two molecules of ubiquinone [3, 4]. By suitable modifications of the preparative technique, it is possible to isolate similar reaction centers from carotenoid-containing strains of *Rps. sphaeroides* [5–7] and other carotenoid-containing species including *Rhodopseudomonas viridis* [8], *Chromatium vinosum* [9] and *Rhodospirillum rubrum* [10]. In all of these cases, the reaction center

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preparations also contain carotenoids [5–10]. It is pertinent to ask, therefore, whether carotenoids are an integral part of the reaction center, and if so, how they are bound, and what roles they play. This report addresses these questions in a study of the carotenoids in reaction centers from *Rps. sphaeroides* strains 2.4.1 and Ga. Strain 2.4.1 is the wild type and strain Ga is a mutant defective in carotenoid biosynthesis at a stage following the formation of neurosporene and chloroxanthin.

MATERIALS AND METHODS

Preparation of reaction centers

Cells of *Rps. sphaeroides* strains R26, 2.4.1, and Ga were grown photosynthetically in batch culture, with succinate as the sole carbon source. The cells were harvested, and chromatophores were prepared after passing the cells through a French pressure cell at 10 tons/inch^2 [11]. Reaction centers were isolated following lauryldimethylamine oxide treatment of the chromatophores, by the method of Clayton and Wang [1] for strain R26 and by a modification [6] of the method of Jolchine and Reiss-Husson [5] for strains 2.4.1 and Ga. All of the reaction centers were further purified by DEAE-cellulose chromatography [4]. Only preparations with ratios of $A_{280\,nm}/A_{800\,nm}$ of less than 1.5 were used. Reaction center concentrations were calculated from the absorbance at 802 nm, using the extinction coefficient 280 $mM^{-1} \cdot cm^{-1}$ [3].

Analysis of carotenoids

A measured amount of aqueous sample was extracted with 10 volumes of acetone and then with 10 volumes of methanol. This procedure was repeated twice more. The pooled extracts were diluted with light petroleum (b.p. 37–49 °C) and washed with warm water to remove the acetone and methanol. Emulsions which sometimes formed at this stage were broken by the addition of a small amount of NaCl. The petroleum extract was dried with Na_2CO_3 and then partitioned at least four times against 95 % methanol/5 % water. This procedure removed bacteriochlorophyll and bacteriopheophytin. At this stage, the visible absorption spectrum of the petroleum extract was recorded. The extract was then evaporated to dryness under N_2, taken up in a minimal volume of diethylether, and chromatographed on silica gel thin-layer plates with benzene/chloroform (1 : 1, v/v) as the solvent. The colored, carotenoid-containing bands were scraped off the plate, and the carotenoids were eluted with ether and analyzed spectrophotometrically, using the following extinction coefficients:spheroidene, $1.49 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ at 453 nm [12]; spheroidenone, $1.29 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ at 483 nm [13]; neurosporene, $1.69 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ at 437 nm [13]; and chloroxanthin, $1.37 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ at 437 nm [14]. (Values given for the extinction coefficients by different authors differ by approx. ± 10 %.) Knowing the composition of the mixture, it then was possible to calculate the total carotenoid concentration in the original petroleum extract.

Sodium dodecyl sulfate gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the reaction centers was performed by the method of Laemmli [15], except that the gels were more highly cross-linked (10 % acrylamide and 0.53 % methylene-bis-acrylamide). In addi-

tion, some gels containing 15 % acrylamide and 0.5 or 0.8 % methylene-bis-acrylamide were used. Typically the gels were electrophoresed at 3 mA per tube for 4 h in the dark. Following the electrophoresis, the gels were scanned at several different wavelengths, using a Gilford 240 spectrophotometer equipped with a linear transport device, and then stained for 4 h with Coomassie brilliant blue R250 (0.25 % (w/v) in 50 % methanol/5 % acetic acid). After soaking overnight in 5 % methanol/7.5 % acetic acid, the gels were destained electrophoretically. Apparent molecular weights were determined by comparison of the mobilities of the reaction center proteins with those of the following standards: bovine serum albumin (molecular weight, 68 000), catalase (60 000), ovalbumin (43 000), alcohol dehydrogenase (41 000), carboxypeptidase A (34 600), bovine trypsin (23 300), myoglobin (17 200), bovine lysozyme (14 400), and equine cytochrome *c* (11 700).

Spectroscopy and fluorescence

Circular dichroism spectra were measured with a Cary model 60 instrument; ordinary absorption spectra, with an Aminco-Chance spectrophotometer. For the measurement of fluorescence excitation spectra, exciting light from a 45 W tungsten-I_2 lamp passed through a monochromator with a band-pass of 10 nm, a Schott HA-11 (IR absorbing) filter, and a 2 Hz chopper. The sample was held in a 1 cm glass cuvette with four clear sides. Fluorescence emitted at 90 ° to the excitation beam was collected with a lens, passed through two Corning 2540 filters (transmitting beyond 900 nm) and detected with an RCA 7102 photomultiplier which was cooled with solid CO_2. The photomultiplier output was amplified, digitized, and stored in a computer of average transients. An auxiliary lamp and a second photomultiplier provided timing signals for synchronizing the sweep of the signal averager with the excitation light chopper. Typical measurements involved averaging 100 sweeps. At each excitation wavelength, the intensity of the beam incident on the sample was measured with a calibrated silicon solar cell (EG and G, Inc., SGD-100); the spectrum in Fig. 4 is corrected for the variation of this intensity with wavelength. For the measurement of fluorescence polarization spectra, a Polaroid filter was placed in the excitation path, and a Glan-Thomson prism in the detection path. The polarization, P, is

$$\left(\frac{F_{||}-F_{\perp}}{F_{||}+F_{\perp}}\right)_{V} - \left(\frac{F_{||}-F_{\perp}}{F_{||}+F_{\perp}}\right)_{H},$$

where $F_{||}$ and $F_{\perp}$ are the fluorescence intensities measured with the analyzing polarizer parallel and perpendicular to the excitation polarizer, and the subscripts V and H indicate measurements made with vertical and horizontal alignment of the excitation polarizer, respectively [16].

RESULTS AND DISCUSSION

The absorption spectrum of the light petroleum extract obtained from reaction centers of strain 2.4.1 has maxima at 427, 453, and 485 nm and corresponds closely to the spectrum of spheroidene [12]. We therefore confirm the identification [7] of the major carotenoid in these reaction centers as spheriodene. The extraction yielded 1.24 mol of spheroidene per mol of reaction centers. This is very similar to the content of spirilloxanthin that has been reported for reaction centers from *R. rubrum* strain

S-1 [17]. Thin-layer chromatography of the extract from *Rps. sphaeroides* 2.4.1 reaction centers showed that there were in fact two carotenoids present, spheroidene (R_F 0.6), which made up 98–99 % of the mixture, and a red carotenoid (R_F 0.45) which made up the remainder. The red component had absorption maxima at 461, 483, and 515 nm, as expected for spheriodenone [12]. The structures of spheroidene, spheroidenone, and other carotenoids are given in Fig. 1.

Analysis of the carotenoids of reaction centers from strain Ga is more difficult because the two major carotenoids that occur in chromatophores of this strain (neurosporene and chloroxanthin) have the same absorption spectrum [14]. However, previous studies have shown that chromatophores from strain Ga usually contain about twice as much neurosporene as chloroxanthin [14, 18]. We therefore initially analyzed the carotenoids in Ga chromatophores, in order to verify that we could distinguish between neurosporene and chloroxanthin. Thin-layer chromatography of a chromatophore extract gave three prominent yellow carotenoid bands, with R_F values of 0.94, 0.56, and 0.25. All three carotenoids had identical absorption spectra in light petroleum, with maxima at 414, 437, and 466 nm. The component with R_F 0.94 constituted 58 % of the total; the one with R_F 0.56 constituted 38 %; and the one with R_F 0.25, 4 %. The relative concentrations and R_F values identify the first component as the hydrocarbon carotenoid neurosporene, and the second one as the hydrated derivative of neurosporene, chloroxanthin (Fig. 1). The third component is possibly a doubly hydrated derivative of neurosporene, but we did not attempt to verify this supposition.

Extraction of reaction centers from strain Ga yielded 1.06 mol of carotenoid per mol of reaction centers. The absorption spectrum of the extract was essentially the same as that of the chromatophore extract. Thin-layer chromatography showed the presence of two carotenoids, with R_F values of 0.92 and 0.60. The slower com-

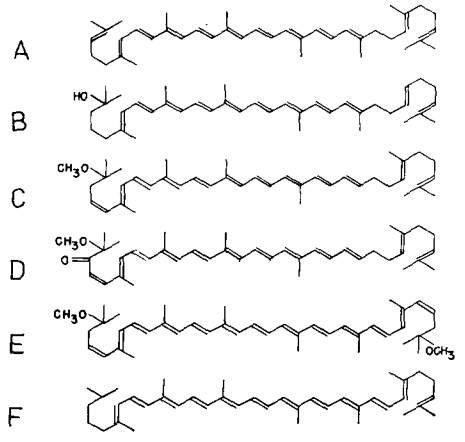

Fig. 1. Structures of the carotenoids mentioned in the text: A, neurosporene (7,8-dihydro-ψ,ψ-carotene); B, chloroxanthin (1-hydroxy-1,2,7′,8′-tetrahydro-ψ,ψ-carotene); C, spheroidene (1-methoxy-3,4-didehydro-1,2,7′,8′-tetrahydro-ψ,ψ-carotene); D, spheroidenone (1-methoxy-3,4-didehydro-1,2,7′,8′-tetrahydro-ψ,ψ-caroten-2-one); E, spirilloxanthin (1,1′-dimethoxy-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene); F, dihydrolycopene(1,2-dihydro-ψ,ψ-carotene). After ref. 20.

ponent co-migrated with chloroxanthin purified from the chromatophore extract when they were rechromatographed on the same plate. (This time the R_F was 0.58.) The chloroxanthin made up 94 % of the total carotenoid present; the remaining 6 % was neurosporene.

Jolchine and Reiss-Husson [7] found 0.7–0.8 mol of spheroidene per mol of reaction centers in their preparation from *Rps. sphaeroides* strain Y, which is similar to strain 2.4.1. Because the molar ratio was less than one, Jolchine and Reiss-Husson suggested that the carotenoid was a contaminant, rather than a true component of the reaction center. However, they estimated the amount of carotenoid present in the reaction center preparation after two chromatographic steps. Because losses could have occurred during the chromatography, their value of the molar ratio seems likely to be an underestimate. If the carotenoid were a contaminant, one might expect that the carotenoid composition of the reaction center preparation would reflect that of the parent chromatophores, but this is not the case. Chromatophores from strain 2.4.1 contain spheroidene and spheroidenone in a molar ratio of about 2.2 : 1 [19]. In reaction centers from this strain, the ratio of spheroidene to spheroidenone is $\geqslant$ 50 : 1. The situation in strain Ga is equally striking. The major carotenoid of the chromatophores, neurosporene, is a very minor component of the reaction centers. This reasoning, and the finding of nearly 1 : 1 stoichiometry of carotenoids : reaction centers, favor the idea that in these two strains a specific carotenoid is an integral component of the reaction center.

The specificity that reaction centers exhibit with regard to carotenoid is puzzling. Referring to the structures shown in Fig. 1, one might be tempted to generalize that reaction centers require a carotenoid with a hydroxyl or methoxyl group at carbon 1 and two hydrogens at carbon 2. Chloroxanthin and spheroidene would satisfy these requirements, as would spirilloxanthin, the carotenoid present in reaction centers of *R. rubrum* strain S-1. However, dihydrolycopene, which occurs in reaction centers from *Rps. viridis* [21], would not.

From *R. rubrum* chromatophores, Schwenker et al. [22] have isolated a protein with a molecular weight of 12 000 which binds approximately one equivalent of spirilloxanthin. The carotenoid that this protein carries is likely to belong to the light-harvesting antenna of the chromatophores, rather than to the reaction center. If a carotenoid also is an integral part of the reaction center, it is interesting to ask whether the reaction center contains a similar carotenoprotein, or if not, which of the reaction center subunits is most closely associated with the carotenoid. Fig. 2 shows sodium dodecyl sulfate-polyacrylamide gel electrophoretograms of reactions center from *Rps. sphaeroides* strain 2.4.1, and from the carotenoidless strain R26. If the reaction centers are denatured prior to electrophoresis (by boiling for 1 min in 1 % sodium dodecyl sulfate), both types of reaction center yield only three protein bands (Figs 2A and 2B). Comparison with the mobilities of standard proteins gives molecular weights of 21 000, 24 000, and 29 000 for the three components. These values agree well with previous analyses of the R26 reaction centers [2, 4, 11]. (The molecular weights reported for the reaction center proteins must be considered only as approximations. In 15 % polyacrylamide gels, the mobility of the largest of the three proteins is greater than expected, and in the doubly cross-linked 15 % gels the two largest proteins electrophorese together as a single band.) No extra protein with a lower

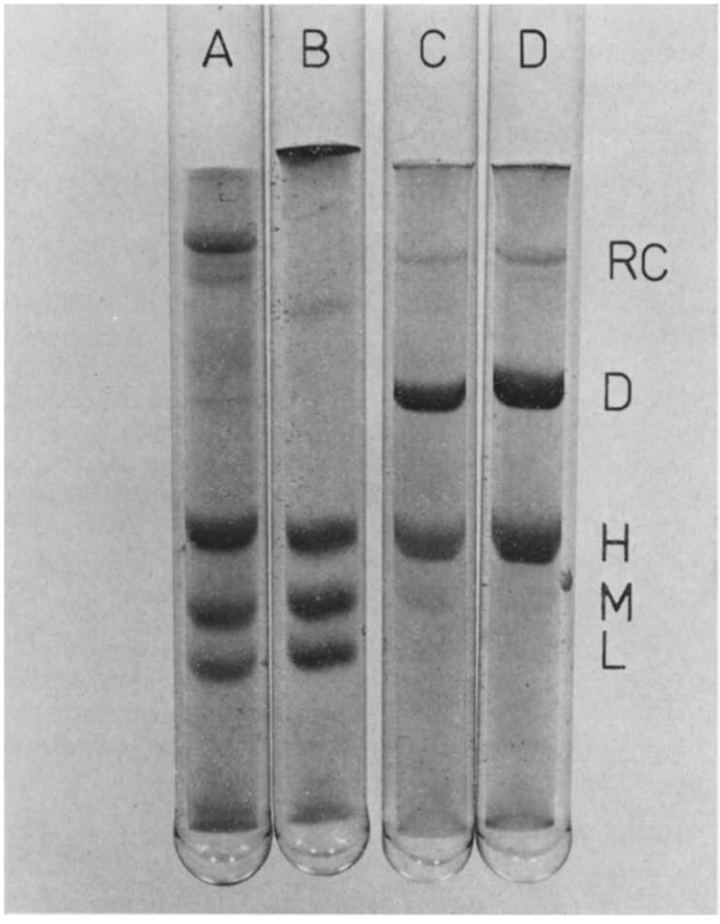

Fig. 2. Polyacrylamide gel electrophoretograms of reaction centers from *Rps. sphaeroides* strains R26 (gels A and C) and 2.4.1 (gels B and D). For A and B, the reaction centers were denatured by boiling in 1 % sodium dodeyl sulfate prior to the electrophoresis, and the electrophoresis buffer contained 1 % sodium dodecyl sulfate. For C and D, the reaction centers were not boiled, and the buffer contained 0.01 % lauryldimethylamine oxide and 0.1 % sodium dodecyl sulfate. For details, see the text. RC, intact reaction centers; D, dimer of M and L subunits; H, M, and L, heaviest, middle and lightest subunits. The gels were cut off at the ion front.

molecular weight was detected in the 2.4.1 reaction centers, even when the polyacrylamide concentration was increased to 15 %.

After denaturation by boiling in 1 % sodium dodecyl sulfate, all of the reaction center pigments, including the carotenoids, are released so that they electrophorese with the ion front. Okamura et al. [4] have described a milder procedure which splits the reaction center trimer into a monomer and a dimer of the two lightest subunits. In their procedure, the reaction centers are not boiled, and 0.01 % lauryldimethylamine oxide is included with 0.1 % sodium dodecyl sulfate for the electrophoresis. The dimer retains the bacteriochlorophyll and the bacteriopheophytin and is photochemically active. Figs 2C and 2D compare the effects of treating R26 and 2.4.1 reaction centers in this way. Both types of reaction centers split predominantly into

monomers and dimers, with a small fraction of the reaction centers remaining intact and some dissociating completely into the three subunits. The dimer has an apparent molecular weight of 43 000, in agreement with the value obtained by Okamura et al. [4], and the intact reaction center has an apparent mocleular weight of 78 000.

Prior to fixation and staining, the gels were scanned at various wavelengths. Nearly all of the bacteriochlorophyll and bacteriopheophytin and (in the case of the 2.4.1 preparation) the carotenoid were retained on the dimer, so that the dimer from the R26 reaction center was blue and that from the 2.4.1 reaction center was brown. The absorption spectrum of the dimer from strain 2.4.1, as measured from scans of the gel, agreed with the spectrum of the intact preparation, measured before the electrophoresis. Identical results were obtained with reaction centers from strain Ga. It seems clear that the carotenoid does not reside on a separate protein in the reaction center,

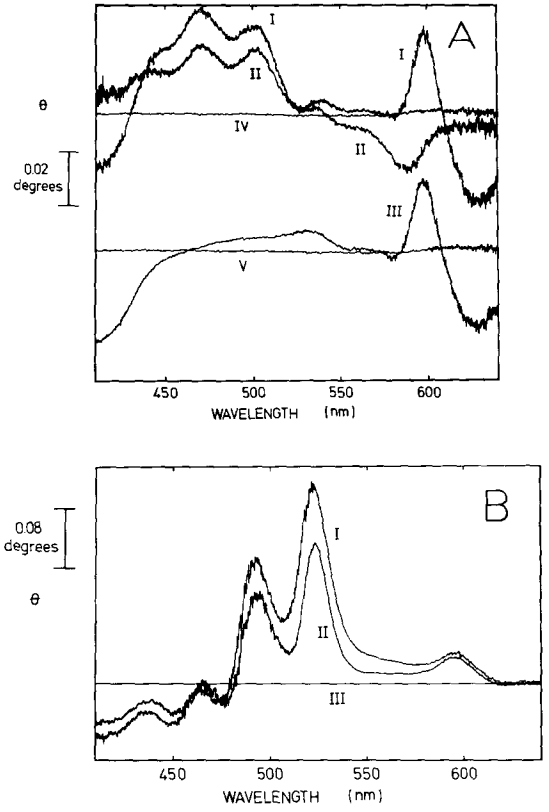

Fig. 3. (A) Circular dichroism spectra of reaction centers from *Rps. sphaeroides*. Curve I, 17.0 μM reaction centers from strain 2.4.1 in 20 mM Tris · HCl, pH 7.5, 0.05 % Triton X-100. Curve II, same as I, but the reaction center bacteriochlorophyll complex was oxidized by titrating with KIO_4. Curve III, 16.5 μM reaction centers from strain R26 in 20 mM Tris · HCl, pH 7.5, 0.05 % Triton X-100. Curves IV and V, baselines. (B) Circular dichroism spectra of chromatophores from *Rps. sphaeroides* strain 2.4.1. Curve I, untreated chromatophores (70 μM bacteriochlorophyll in 20 mM Tris · HCl, pH 7.5). Curve II, the same as I but with 2 % lauryldimethylamine oxide added. Curve III is a baseline. The total carotenoid concentration was approx. 44 μM, or about 2.6 times that in the reaction center samples of A.

but rather joins the bacteriochlorophyll and bacteriopheophytin on the dimer of the two lightest subunits.

Additional information on the carotenoid binding site can be obtained from the CD spectrum of the reaction centers. The CD spectrum of 2.4.1 reaction centers exhibits three strong, positive bands with peaks at 503, 472, and 446 nm (Fig. 3A, curve I). These bands can be attributed to carotenoids, because they coincide with the carotenoid bands of the absorption spectrum, and because they are absent in the CD spectrum of the carotenoidless R26 reaction centers (Fig. 3A, curve III). But circular dichroism is not an intrinsic property of the carotenoids. When the carotenoids are extracted into organic solvents, they are optically inactive and a CD spectrum of the solution is absolutely flat. The strong CD of the reaction centers must result from asymmetry in the site at which the carotenoid binds. Oxidation of the reaction center bacteriochlorophyll complex causes a decrease of about 30 % in the strength of the carotenoid CD bands, presumably reflecting a change in the environment of the carotenoid (Fig. 3A, curve II).

For comparison, Fig. 3B shows the CD spectrum of intact chromatophores of strain 2.4.1. The carotenoids of the light-harvesting antenna also exhibit circular dichroism [23], but their CD spectrum is quite different from that of the reaction centers in that the long wavelength band is very intense relative to the two bands at shorter wavelengths. Although the origin of this effect is obscure, it would appear that the environment of the bulk carotenoids differs substantially from that of the reaction center carotenoid. The addition of the detergent lauryldimethylamine oxide in amounts comparable to or greater than those used in the preparation of reaction centers causes a decrease in the strength of the carotenoid CD bands, but it has little effect on the shape of the spectrum (Fig. 3B, curve II).

Although the carotenoid of the reaction center must be located close to the bacteriochlorophyll complex, it does not appear to exert a major influence on the interactions among the bacteriochlorophyll molecules themselves. The CD spectrum of reaction centers includes a double (positive and negative) band near 600 nm which has been attributed to exciton interaction among the bacteriochlorophyll molecules [24, 25], and the spectra of the 2.4.1 and R26 reaction centers are indistinguishable in this region (Fig. 3A, curves I and III). In addition, the near infrared absorption spectra of the 2.4.1 and Ga reaction centers are essentially identical to those of the R26 preparation. These observations suggest that the carotenoid lies on the periphery of the bacteriochlorophyll complex, rather than fitting in among the bacteriochlorophylls. Circular dichroism studies have suggested that the carotenoid lutein can intercalate between the two chlorophyll molecules of chlorophyll *a* dimers in solution [26], but this appears not to occur in the reaction center.

One consequence of the close proximity between the carotenoid and the bacteriochlorophyll complex of the reaction center is the possibility of energy transfer between the two types of pigments. Cogdell et al. [6] have shown recently that the transfer of energy from the bacteriochlorophyll complex can excite the carotenoid to a triplet state. Fig. 4A shows that the carotenoid in its excited singlet state can transfer energy in the opposite direction, generating an excited singlet state of the bacteriochlorophyll. Judging from the yield of fluorescence from the bacteriochlorophyll when one excites the carotenoid at 470 or 510 nm, relative to that when one excites the

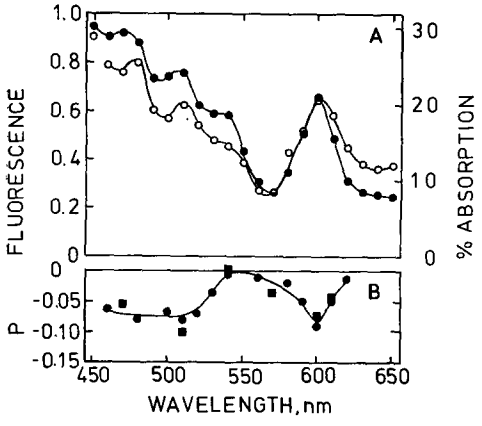

Fig. 4. (A) Excitation spectrum for fluorescence from the bacteriochlorophyll complex of *Rps. sphaeroides* strain 2.4.1 reaction centers (○–○), and absorption spectrum of the same preparation (●–●). The sample contained 1.5 μM reaction centers in 50 mM Tris · HCl, pH 7.6, 0.05 % Triton X-100. To increase the fluorescence yield, the photochemical electron transfer reaction in the reaction centers was blocked by reduction of the electron acceptor with $Na_2S_2O_2$. See the text for experimental details. (B) Fluorescence polarization spectrum of reaction centers from *Rps. sphaeroides* 2.4.1. The sample was as in A, except that the reaction center concentration was twice as high. See the text for definition of the polarization, *P*, and for experimental details. The squares and circles represent measurements with two different samples.

bacteriochlorophyll directly at 600 nm, the efficiency of energy transfer is approx. 80 %. This agrees with results reported by Slooten. [27].

Information on the relative orientations of the carotenoid and bacteriochlorophyll molecules in the reaction center can be obtained from an examination of the fluorescence polarization spectrum. Fig. 4B shows that when one excites the carotenoid the polarization of the bacteriochlorophyll fluorescence is essentially the same as it is when one excites the bacteriochlorophyll directly. Excitation into the 540 nm absorption band of the bacteriopheophytin of the reaction center gives significantly different results; in this case, as Ebrey and Clayton [16] found for R26 reaction centers, the polarization of the bacteriochlorophyll fluorescence is virtually zero.

The transition vectors for the visible absorption bands of an all-*trans* carotenoid are aligned parallel to the long axis of the molecule [28]. In the reaction center, the long axis of the carotenoid would appear to lie approximately parallel to the direction of the 600 nm (Q_x) transition vector of the bacteriochlorophyll complex. Because the reaction center contains four bacteriochlorophyll molecules which are not all aligned in parallel [16, 24, 25], the 600 nm absorption band includes more than one electronic transition, and we are not able to describe its resultant orientation in terms of the positions of the individual molecules. However, if one considers the 600 nm absorption band to be a property of the complex as a whole, the orientation of the carotenoid molecule would appear to be optimal for energy transfer into this band. Measurements of linear dichroism have suggested that the bulk carotenoids of intact chromatophores also are aligned approximately parallel to the Q_x transition moment of the antenna bacteriochlorophyll [29].

In conclusion, a carotenoid appears to be an integral component of the photo-

synthetic reaction center in both strain 2.4.1 and strain Ga. In both cases, the reaction center contains approximately one equivalent of a specific carotenoid which differs from the predominant carotenoid of the light-harvesting antenna of chromatophores. The carotenoid is bound in sufficient intimacy with the bacteriochlorophyll complex of the reaction center, so that the transfer of energy from the carotenoid to the bacteriochlorophyll occurs efficiently, and the orientation of the carotenoid appears to be optimal for such transfer. However, it seems unlikely that the transfer of energy in this direction is the major role that the carotenoid of the reaction center plays in vivo. Chromatophores of *Rps. sphaeroides* strain 2.4.1 contain bacteriochlorophyll and carotenoids in a ratio of approx. 1.6 : 1. If the photosynthetic unit size is approximately 100 bacteriochlorophylls per reaction center, the carotenoid of the reaction center would comprise only 1–2 % of the total carotenoids of the chromatophores. The amount of light that is absorbed by the reaction center carotenoid would therefore be negligible. Energy absorbed by the bulk carotenoids finds its way to the reaction center, but this presumably occurs by way of the antenna bacteriochlorophyll, rather than by transfer through the carotenoid of the reaction center. A more likely role for the carotenoid in vivo would be to provide a mechanism for the removal of energy from the reaction center, under conditions which prevent the photosynthetic electron transfer reaction from occurring. Under these conditions, the transfer of energy to the carotenoid prevents the formation of a triplet state of the bacteriochlorophyll complex of the reaction center [6]. This may be important for the avoidance of side reactions that could lead to degradation of the reaction center bacteriochlorophyll.

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