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STRUCTURE AND FUNCTION OF CAROTENOIDS IN THE PHOTOREACTION CENTER FROM *RHODOSPIRILLUM RUBRUM*

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SUMMARY

The bacteriochlorophyll (*P*-800 and *P*-870) of the carotenoidless photoreaction center isolated from *Rhodospirillum rubrum* (strain G9) is bleached irreversibly when the preparations are exposed to intense near infrared light in the presence of oxygen. This effect is much smaller in preparations, extracted from the wild type, which contain, as shown earlier, 1 mol of spirilloxanthin per mol of *P*-870. This photodynamic effec is shown to be due to singlet O_2. The oxidation of adrenaline in the presence of superoxide dismutase and the oxidation of 1,3-diphenylisobenzofuran are used as reporter reactions. Singlet oxygen is presumably generated by the triplet-triplet energy transfer ^{3}bacteriochlorophyll $\rightarrow O_2$ ($^3\Sigma$).

Four purified bacterial carotenoids, spirilloxanthin, sphaeroidene, sphaeroidenone and chloroxanthin were attached onto the carotenoidless photoreaction center from strain G9 in nearly 1 : 1 mol ratios with respect to *P*-870. Once fixed, these carotenoids confer protection against the photodynamic bleaching of bacteriochlorophyll. The relative photoprotection efficiency was 1.0 for spirilloxanthin and sphaeroidene, 0.4 for chloroxanthin and 0.2 for sphaeroidenone. The fixed carotenoids display optical activity and their molar ellipticity appears to be correlated with their relative photoprotection efficiency. The efficiency of energy transfer to *P*-870 is 0.90 for sphaeroidene, 0.35 for sphaeroidenone, 0.30 for chloroxanthin and 0.20 for spirilloxanthin. The energy transfer efficiency from the carotenoids to bacteriochlorophyll is suggested to be governed by the rate of the internal conversion processes of the excited singlet state of the carotenoids.

A study of the difference absorption and CD spectra of the reconstituted minus carotenoidless preparations leads to the interpretation that the fixed carotenoids are in a central *monocis* conformation.

INTRODUCTION

In the photosynthetic bacteria, carotenoids are known to be antenna pigments and therefore, to transfer light energy ultimately to the photoreaction center [1].

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However, their most universal function appears to be protection against the photodynamic effect [2]. This role has been demonstrated in photosynthetic bacteria by the elegant studies of Stanier and coworkers [3–5].

In the course of our characterization of the photoreaction center from *Rhodospirillum rubrum*, we found that the preparations obtained from the wild type reproducibly contained close to 1 mol of spirilloxanthin per equivalent of the primary electron donor, *P*-870 [6]. Preparations from the G9 strain (carotenoidless) not only were devoid of this pigment but were also found to be clearly more photosensitive [7]. It seemed reasonable, therefore, to assume that spirilloxanthin plays a role not only in the photosynthetic membrane as a whole but also at the level of the photoreaction center. Further support in favor of this hypothesis came from the ubiquitous presence of carotenoid pigments in the photoreaction center preparations obtained from wild type bacteria [6, 8–12]. In spite of this circumstantial evidence one could not exclude, however, that the carotenoids are adventitious contaminants of such preparations.

It was later confirmed by Cogdell et al. [13] that photoreaction center prepared from *Rhs. rubrum* and *Rhodopseudomonas sphaeroides* (*Rhp. sphaeroides*) contains 1 mol of a carotenoid per mol of *P*-870. Moreover, in the latter organism which contains several types of carotenoids, only sphaeroidene or chloroxanthin, according to the strain studied, were copurified with the photoreaction center. Cogdell et al. [14] in another work, provided evidence for the quenching by carotenoids of state P^R in isolated photoreaction center.

The present work provides evidence for the photoprotective role of certain carotenoids against the photodynamic bleaching of bacteriochlorophyll. In order to play this role, the carotenoid must be bound onto a site of attachment of the photoreaction center. Binding necessitates certain structural features and appears to entail a central *monocis* conformation of the carotenoid.

MATERIALS AND METHODS

Chemicals. Dodecyldimethylamine *N*-oxide (LDAO) was obtained from Onyx Chemicals, Jersey City, N. J., Triton X-100 from J. T. Baker, Chemical Co. Philipsburg, N. J., adrenaline from Sigma Co., Saint Louis, Mo., bovine erythrocyte superoxide dismutase from Truett Laboratories, Dallas, Texas, 1,3 diphenylisobenzofuran from Aldrich Chemical Co., Montréal, Qué., methylene blue from British Drug House, Montréal, Qué. and *N,N'*-diethyl aminoethyl cellulose from Eastman Kodak Co. The other chemicals (reagent grade) were obtained from Fisher Scientific Co. Silica gel G (E. Merk A. G., Darmstadt) was used for thin layer plate chromatography.

Organisms. Wild type strain (ATCC no 11170) and strain G9 of *Rhs. rubrum* were grown as described previously [6]. Wild type *Rhp. sphaeroides* (ATCC no 17023) and strain Ga were grown semi-anaerobically in Hutner's medium [15] in a manner similar to wild type *Rhs. rubrum*. Strain Ga was a gift from Dr. W. W. Parson, University of Washington, Seattle, Wash. Spinach and carrots were purchased on the local market.

Preparation of photoreaction center. Photoreaction center from wild type *Rhs. rubrum* was extracted by the procedure of Noël et al. [8]. Ammonium sulfate fractionation was followed by chromatography on DEAE cellulose in the presence of 0.03 % LDAO as described by Noël [16]. Photoreaction center from strain G9 was isolated by the method of Vadeboncoeur and Gingras (unpublished).

Preparation and purification of the carotenoids. From 10 to 15 g of frozen bacteria were extracted twice with 150 ml portions of acetone at 4 °C. This extract was filtered on Schleicher and Schuell No. 595 filter paper, diluted with 150 ml of light petroleum (b.p. 38–48 °C) and washed four times with 1.5 l portions of distilled water to remove acetone. The light petroleum phase was then washed with a methanol/water (95/5, v/v) mixture in order to extract the major part of bacteriochlorophyll and bacteriopheophytin. The crude carotenoid extract in light petroleum was either used as such or purified by thin-layer plate chromatography. The plates were coated with silica gel and developed in a light petroleum (b.p. 38–48 °C)/acetone (9/1, v/v) mixture. The carotenoid bands were scraped, collected and eluted with acetone. After evaporation in vacuo, the pigment was dissolved in light petroleum (b.p. 38–48 °C). β-Carotene and lutein were prepared in a similar manner from carrots and from spinach leaves.

Reconstitution of the carotenoid-photoreaction center complex. About 1–2 mg carotenoid, either chromatographically pure or in the form of a crude extract freed of most of its porphyrins, was deposited on the walls of a test tube (15 mm × 150 mm). This was done by evaporation of a light petroleum solution under a stream of nitrogen gas. After complete removal of the solvent, 2 ml ($A_{865\,nm} = 1$) of photoreaction center from strain G9 in 10 mM Tris · HCl (pH 8.0)/0.1 % Triton X-100 was pipetted into this test tube. After 30 s of vigorous shaking, the preparation was incubated at 4 °C for 3 h. The preparation was then adsorbed on top of a DEAE-cellulose column (9 mm inside diameter, 50 mm high) and washed with 70 ml of 10 mM Tris · HCl (pH 8.0)/0.1 % Triton X-100/25 mM NaCl. The loosely bound carotenoids were eluted with this washing. The photoreaction center was then eluted with 10 mM Tris · HCl (pH 8.0)/0.1 % Triton X-100/200 mM NaCl and dialysed against 10 mM Tris · HCl (pH 8.0).

Carotenoid assay. The assays were performed on known amounts of quantitatively lyophilized photoreaction center extracted with acetone/methanol (7/2, v/v) according to the method of van der Rest and Gingras [6]. The extinction coefficient of spirilloxanthin in this solvent is $\varepsilon_{475} = 94\ mM^{-1} \cdot cm^{-1}$ [6]. The extinction coefficients of sphaeroidene, sphaeroidenone and chloroxanthin were determined by quantitative transfer from ether where their extinction coefficients are known [17–19], to acetone/methanol. The extinction coefficients ($mM^{-1} \cdot cm^{-1}$) thus obtained were as follows: for sphaeroidene $\varepsilon_{475} = 91$, for sphaeroidenone, $\varepsilon_{475} = 120$ and for chloroxanthin $\varepsilon_{475} = 93$.

Spectroscopic techniques. Circular dichroism was measured in 1-cm pathlength cuvettes with a Jasco circular dichroism recorder and spectropolarimeter (Model S-20).

Absorption spectra were obtained with a Cary 14R recording spectrophotometer. The bleaching of *P*-870 was measured with the Cary 14R spectrophotometer modified so as to permit cross-illumination of the sample cuvette. The actinic beam in this case was provided by a 75 W Xenon lamp through a Baush and Lomb High Intensity Monochromator. The wavelength of this beam was varied from 450 to 600 nm so as to obtain action spectra for the bleaching of *P*-870. The phototube was protected from scattered actinic light by an 870 nm Baird Atomic interference filter (15 nm half band width). The $\Delta A_{870\,nm}$ provoked by the actinic light was monitored under steady-state conditions. The light intensity incident on the surface of the sample

cuvette was monitored by a Yellow Springs Instruments model 65 radiometer. Only relative intensity measurements were sought. The intensities varied from $0.4 \cdot 10^3$ to $1 \cdot 10^3$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} between 480 and 600 nm. The highest intensities caused less than 10 % bleaching at 870 nm under steady-state conditions. Within this range of 0–10 %, the light intensity curve can be safely approximated to a straight line. Screening effect was minimized by using a cuvette with a 3×10 mm cross section, 3 mm being the pathlength of the actinic beam. Absorbance of the samples was < 0.3 (10 mm) in the 480–600 nm region.

Adrenochrome formation. For the measurement of photoinduced adrenochrome formation, adrenaline (final concentration $5 \cdot 10^{-4}$ M) was added to the photoreaction center preparation (final concentration 1 μM) in the spectrophotometric cuvette just before the experiment. The preparation was dispersed in 50 mM K_2CO_3 (pH 10.0)/0.1 % Triton X-100. The reaction was followed by means of a Cary 14R spectrophotometer set at 480 nm. An actinic beam from a Sylvania 650 W halogen-tungsten lamp was filtered by 2 cm of water and a colored glass Schott RG9 filter (cutoff wavelength, 690 nm). The photomultiplier tube was protected by a combination of Schott BG18 and BG38 filters. The preparation was illuminated for 15 s with this set up and the slope of absorbance versus time of illumination was used to measure the rate of the reaction. An extinction coefficient of $\varepsilon_{480} = 4.020$ mM$^{-1} \cdot$ cm^{-1} for adrenochrome [20] was employed. Experiments under N_2 gas were carried out in an anaerobic cuvette supplied by Hellma Canada Ltd.

RESULTS

Photodynamic effect

When aerobic photoreaction center preparations are submitted to high enough light intensities, *P*-800 and *P*-870 may be bleached irreversibly. This is illustrated by Fig. 1a which shows the $\Delta A_{800\text{ nm}}/A_{800\text{ nm}}$ of such preparations as a function of time under the bright white light (IR 2 mode) of the Cary 14R spectrophotometer. This effect is nearly abolished if the preparations are made anaerobic by the bubbling of N_2 gas for 10 min prior to illumination.

Fig. 1b shows light intensity curves for this photodynamic effect on *P*-800. In this experiment, the aerobic preparations were exposed to far red light for 30 s before recording their spectra with the weak dispersed beam (IR 1 mode) of the spectrophotometer. These preparations were characterized by a rapid rate of charge recombination due to the absence of secondary acceptors, thereby insuring complete reduction of the primary electron donor before the spectra were recorded. Moreover 800 nm is an isosbestic wavelength for reversible photochemistry of the photoreaction center. In a separate experiment, but with the same preparation and experimental set up, the reversible bleaching at 600 nm was followed by means of the weak dispersed measuring beam of the instrument. These light intensity curves show again the photoprotective role of spirilloxanthin. Furthermore, they indicate that photodynamic damage is proportional to light intensity even after the primary electron donor has been completely converted into its oxidized form. Under these conditions *P*-870 is completely bleached and therefore cannot participate in destructive photochemistry. Only *P*-755, *P*-800 and conceivably P^+-870 are left as potential photosensitizers.

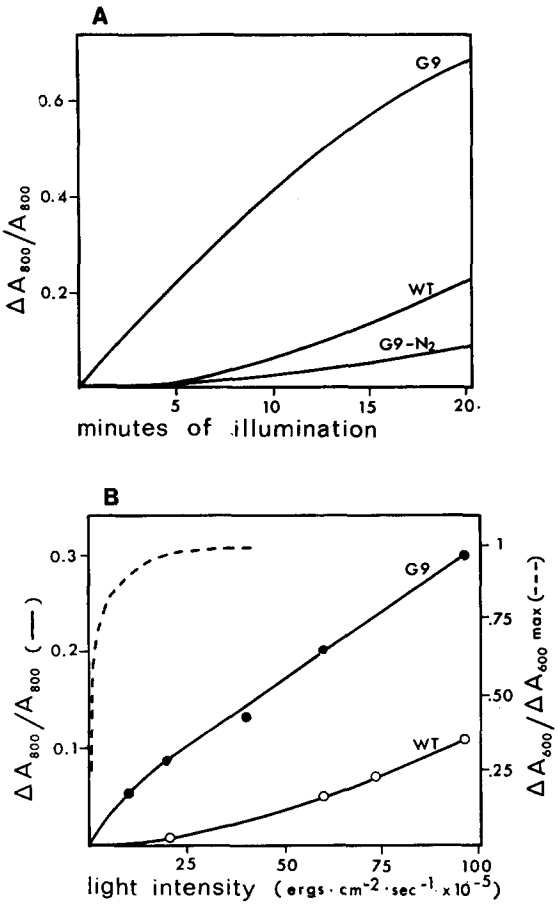

Fig. 1. Photodestruction of *P*-800 in photoreaction center isolated from two strains of *Rhs. rubrum*: a wild type strain (WT) and a carotenoidless strain G9. (A) Time course of the photodestruction of *P*-800 under bright white light provided by the IR 2 mode of illumination of the Cary 14R spectrophotometer. Curve G9-N_2 was obtained after bubbling nitrogen gas in the preparation from strain G9 for 10 min prior to illumination. 2.5 μM photoreaction center in 50 mM carbonate (pH 10.0)/0.1 % Triton X-100. (B) Initial rate of photodestruction of *P*-800 as a function of light intensity (—). Photoreaction center (1.4 μM) in 50 mM carbonate (pH 10.0)/0.1 % Triton X-100 was illuminated for 30 s through a Schott RG9 filter (cutoff at 690 nm) and 2 cm of water before its absorption spectrum was measured. A new sample was used for each point. The reversible oxidation of the primary electron donor was monitored at 600 nm under the same actinic light (– – –). In that case, the photodetector was protected by Corning BG18 and BG38 filters.

Adrenochrome formation

These experiments indicated that *P*-800 is the target of a photodynamic effect involving $^1O_2$ since the presence of O_2 is required and since spirilloxanthin has an inhibitory action. Further support for this interpretation was provided by following the oxidation of adrenaline to adrenochrome, a chain reaction characteristically initiated by O_2^- [21] but which occurs also in the presence of $^1O_2$ and excess superoxide dismutase.

TABLE I

METHYLENE BLUE-SENSITIZED ADRENOCHROME FORMATION

Adrenochrome formation was monitored by the absorbance change at 480 nm as described under Materials and Methods. The actinic beam was filtered by an Optics Technology 650 nm interference filter. Detection of this light was prevented by placing BG 18 and BG 38 Corning filters in front of the photomultiplier tube. Reaction mixture, in 1.0 ml of 50 mM carbonate buffer (pH 10): methylene blue ($A_{660\,nm} = 0.18$), adrenaline 1 mM, superoxide dismutase 0.66 mg (2000 units).

Additions	Initial rate of adrenochrome formation (nmol/min)
Methylene blue+adrenaline+superoxide dismutase: dark (aerobic)	0.0
Methylene blue+adrenaline+superoxide dismutase: light (aerobic)	18.6
Methylene blue+adrenaline: light (N_2 bubbling for 10 min)	0.0

As shown in Table I, the absorbance change at 480 nm by which this reaction is followed can also be photosensitized by methylene blue, a classical photogenerator of $^1O_2$. When adrenaline is incubated with methylene blue and superoxide dismutase in the dark no $\Delta A_{480\,nm}$ is observed. In red light, however, a $\Delta A_{480\,nm}$ is observed, provided oxygen is also present in the reaction mixture. This clearly indicates that, under these experimental conditions, $^1O_2$ is responsible for the reaction which can therefore serve as a probe for the presence of $^1O_2$ in other systems. Although the nature of the reaction product has not been established, we will call it adrenochrome for convenience.

When an aerobic mixture of photoreaction center and adrenaline is illuminated by far red light ($\lambda > 690$ nm), the formation of adrenochrome can be observed. A plot of the initial rate of adrenochrome formation against light intensity by a preparation from wild type *Rhs. rubrum* is shown in Fig. 2. The curve is multiphasic, leveling off at about $30 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} and rising steeply again (Fig. 2 upper part, "WT, no additions"). Deoxygenation of the reaction mixture by the bubbling of N_2 gas prior to the addition of adrenaline abolishes adrenochrome formation at all light intensities (not shown). Azide, a well known quencher of $^1O_2$ [22] almost completely eliminates the second portion of this curve (Fig. 2 upper part, "WT+NaN_3"). The lower part of Fig. 2 shows the effect of superoxide dismutase which leaves only the second portion of the curve (Fig. 2, "W.T.+S.O.D."). It is also seen that, in the absence of spirilloxanthin on the photoreaction center, the quantum yield of a adrenochrome formation observed in the presence of superoxide dismutase is considerably increased (Fig. 2, "G9+S.O.D."). As expected, the effects of superoxide dismutase and of azide are cumulative: together they nearly abolish the photo-oxidation of adrenaline (W.T.+S.O.D.+NaN_3, upper Fig. 2).

To sum up, adrenochrome is photogenerated by photoreaction center via two distinct pathways. One of the pathways is predominant at low light intensities and is inhibited by superoxide dismutase. In this reaction, the chain initiator for the oxidation of adrenaline is $O_2^{\cdot -}$ generated by illuminated photoreaction center as shown previously [23]. The other pathway predominates at higher light intensities and is not inhibited by superoxide dismutase but is inhibited by azide and by spirilloxanthin. It may be inferred to involve the presence of singlet oxygen.

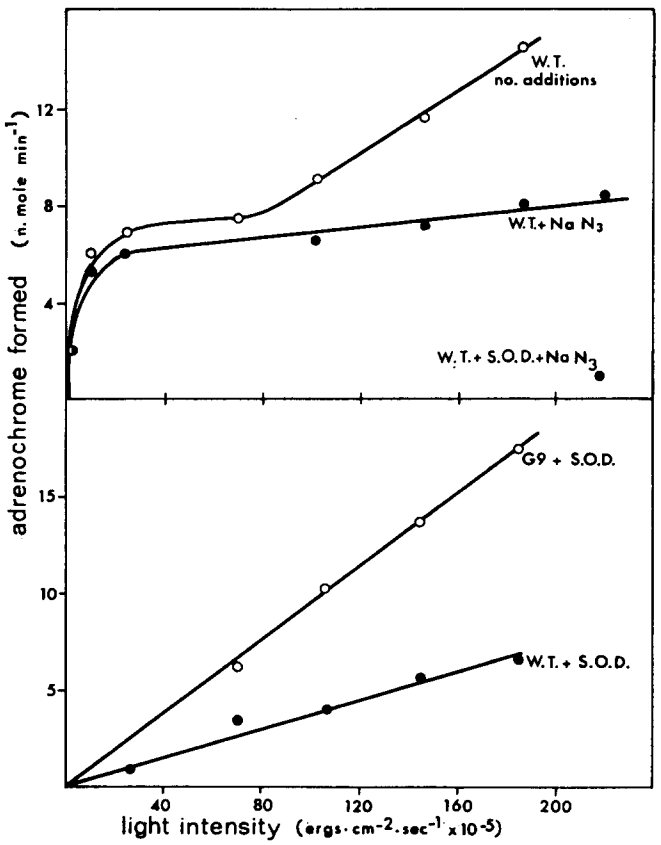

Fig. 2. The formation of adrenochrome by photoreaction center preparations from *Rhs. rubrum* as a function of light intensity. W.T. and G9 indicate preparations from wild type and from carotenoidless strain G9, respectively. Reaction mixture contained 1 μM photoreaction center and $5 \cdot 10^{-4}$ M adrenaline in 50 mM carbonate (pH 10.0)/0.1 % Triton X-100. Actinic light was as in Fig. 1b. Adrenochrome formation was monitored by the $\Delta A_{480\,nm}$ observed after 15 s of illumination. Upper: the effect of azide on the formation of adrenochrome by a preparation from the wild type strain. NaN_3 concentration was 10^{-2} M. Lower: the effect of spirilloxanthin on adrenochrome formation as a function of light intensity. This experiment as performed in the presence of 2000 units of bovine erythrocyte superoxide dismutase (S.O.D.).

The latter conclusion is corroborated by the oxidation of 1,3-diphenylisobenzofuran, a well known indicator for the presence of $^1O_2$ [24, 25]. In a typical experiment, a 1.4 μM solution of photoreaction center was illuminated ($\lambda > 690$ nm, $1.8 \cdot 10^7$ ergs $\cdot$ $cm^{-2} \cdot s^{-1}$) in the presence of 1,3-diphenylisobenzofuran: the rate of bleaching measured at 415 nm corresponded to the consumption of 42.5 nmol/min of 1,3-diphenylisobenzofuran. This reaction is blocked by the presence of azide or of spirilloxanthin attached onto the photoreaction center.

Reconstitution studies

In principle, comparison between the photoreaction center isolated from the wild type and from strain G9 should reveal specific effects of spirilloxanthin. However,

to our knowledge, strain G9 has not been shown to differ from the wild type by a single point mutation. Moreover, the two photoreaction center preparations are obtained through different procedures and are solubilized in different detergents. Hence, it is conceivable that they might differ in points other than their carotenoid content. The simplest answer to these objections appeared to study preparations reconstituted from carotenoidless photoreaction center and purified carotenoids.

Reconstitution was carried out as described in Materials and Methods. No reconstitution from purified β-carotene or lutein could be observed. Reconstitution starting with crude extracts from *Rhs. rubrum* and from *Rhp. sphaeroides* left 1.2 mol of spirilloxanthin or of sphaeroidene per mol of *P*-870 in the first and second case, respectively. With crude extracts from *Rhp. sphaeroides*, about 5 % sphaeroidenone was also bound along with 95 % sphaeroidene. Purified carotenoids yielded similar results except that not only spirilloxanthin and sphaeroidene but also sphaeroidenone were bound in nearly 1 : 1 molar ratios. Reconstitution was also effected with chloroxanthin but with molar ratios of carotenoid to *P*-870 varying between 0.4 and 1.5 depending on the preparations. Binding of chloroxanthin to photoreaction center appears to be looser than that of other carotenoids since it can be removed beyond the 1 : 1 ratio by column chromatography. These results are shown in Table II.

TABLE II

CERTAINS PROPERTIES OF CAROTENOIDS FIXED ON THE PHOTOREACTION CENTER FROM *RHODOSPIRILLUM RUBRUM*

Carotenoid	Carotenoid/*P*-870	Energy transfer efficiency	Photoprotection efficiency	$[\theta]_{\lambda\ max}^{car}$ (degree · cm² · dmol⁻¹)
β-Carotene	0	—	—	—
Lutein	0	—	—	—
Spirilloxanthin (wild type)	1.2	0.20	1.0	$8 \cdot 10^4$ (506 nm)
Spirilloxanthin (reconstituted)	1.2	0.20	1.0	$7.7 \cdot 10^4$ (506 nm)
Sphaeroidene	1.2	0.90	1.0	$6 \cdot 10^4$ (478 nm)
Sphaeroidenone	1.0	0.35	0.2	$< 5 \cdot 10^3$
Chloroxanthin	0.4–1.5	0.30	0.4	$2 \cdot 10^4$ (480 nm)

Energy transfer efficiency

Energy transfer and photoprotection efficiencies are certainly more stringent tests for the success of reconstitution than the final molar ratio of carotenoid/*P*-870. The efficiency of energy transfer from carotenoids to *P*-870 was measured from the reversible photobleaching of this species, as described in Materials and Methods. The efficiencies were measured relatively to that of 600 nm light which is known to be close to 1.0 [26]. Action and absorption due to the reinserted carotenoid molecule were obtained using a carotenoidless preparation from strain G9 as a baseline. Similar efficiencies of 0.20 at 490 nm were obtained for spirilloxanthin in preparations from the wild type or in reconstituted preparations. The energy transfer efficiencies of sphaeroidene, sphaeroidenone and chloroxanthin were, respectively, of 0.90, 0.35 and 0.30 in reconstituted preparations (Table II).

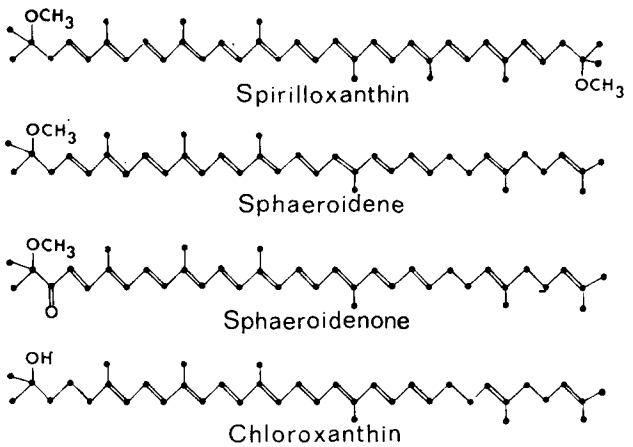

Fig. 3. The chemical structures of four carotenoids of bacterial origin.

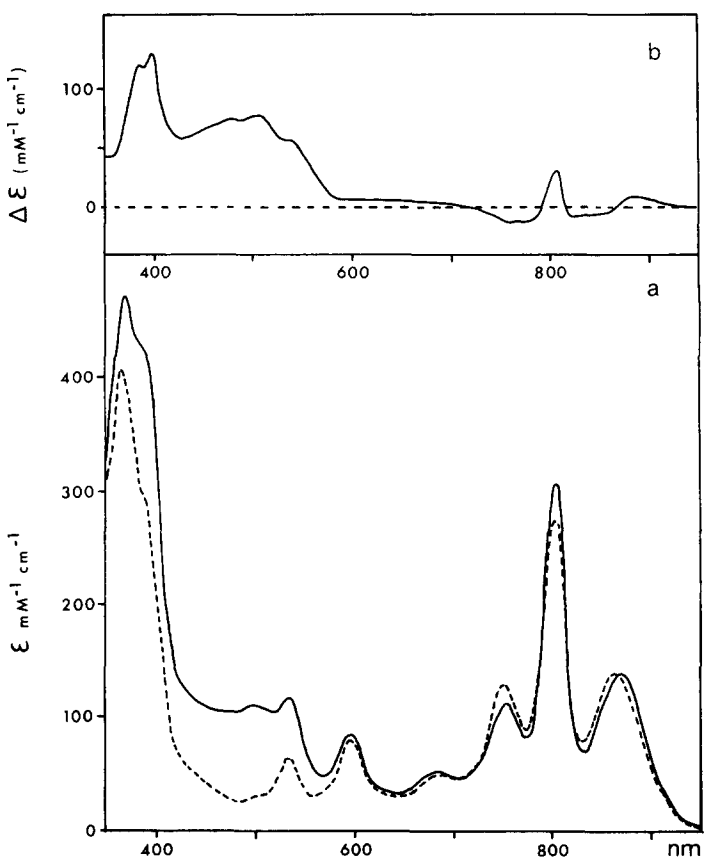

Fig. 4. Absorption spectra of photoreaction center prepared from wild type (—) or from carotenoidless strain G9 (– – –) of *Rhs. rubrum*. The preparations were dispersed in 10 mM Tris · HCl (pH 8.0) containing either 0.03 % LDAO (wild type) or 0.1 % Triton X-100 (strain G9). They were adjusted to an equal $A_{870\,nm}$ before recording their spectra. (a) Absorption spectra. (b) Measured difference spectrum of carotenoid containing minus carotenoidless preparation.

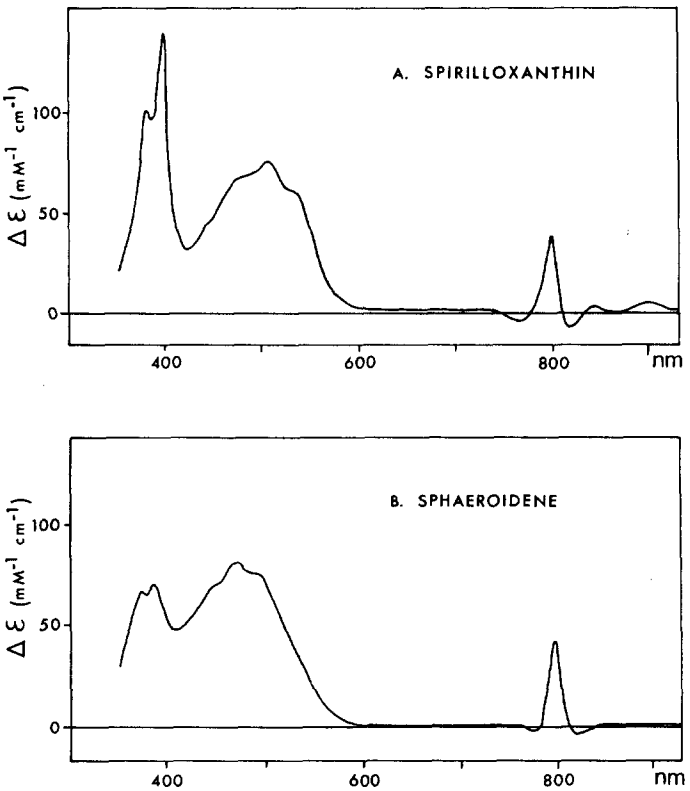

Fig. 5. See opposite page for legend.

Photoprotection efficiency

Photoprotection was measured by monitoring the irreversible absorbance change of the preparation at 800 nm caused by the bright white light beam (1R 2 mode) of the Cary 14R spectrophotometer. Photoprotection efficiency (P.E.) is defined as:

$$\text{P.E.} = \frac{\Delta A^{r}_{800\,nm} - \Delta A^{G9}_{800\,nm}}{\Delta A^{w}_{800\,nm} - \Delta A^{G9}_{800\,nm}}$$

where $\Delta A_{800\,nm}$ is the initial rate of the irreversible absorbance change at 800 nm. The superscripts indicate whether the preparation was reconstituted (r) or, came from the wild type (w) or from strain G9 (G9).

As shown in Table II, spirilloxanthin and sphaeroidene have a photoprotection efficiency of 1.0. Hence, reconstitution with these carotenoids confers a photoprotection equivalent to that supplied by the "native" spirilloxanthin in the preparation from the wild type strain. Sphaeroidenone and chloroxanthin which are also fixed in a nearly 1 : 1 fashion are poor photoprotectors in comparison. The photoprotection efficiency of chloroxanthin was found to be rather insensitive to the amount of carotenoid fixed on the protein. It remained in the neighbourhood of 0.4 for molar ratios varying between 0.4 and 1.5. It is noteworthy that mere suspensions of spirilloxanthin or of sphaeroidene in Triton X-100 exhibited no photoprotective effect.

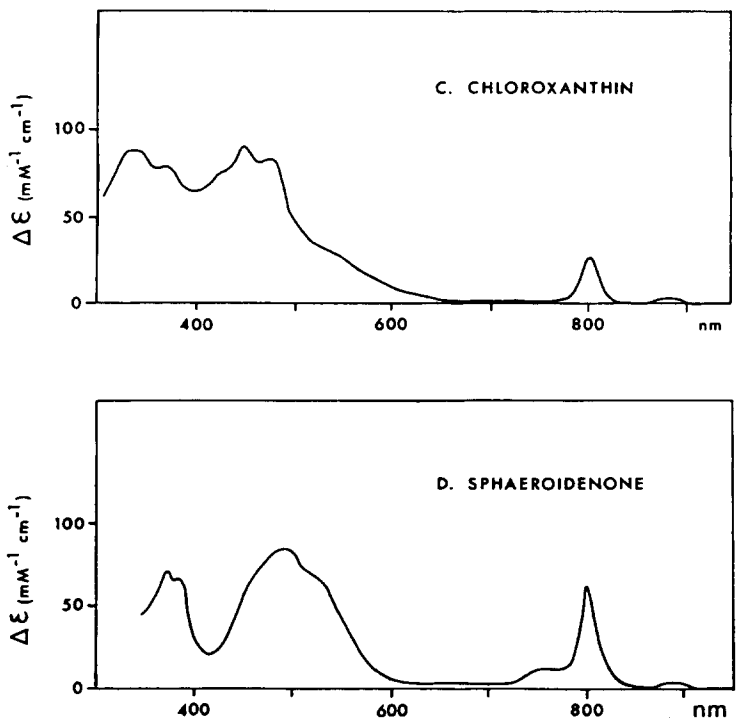

Fig. 5. The measured difference spectra of reconstituted minus carotenoidless photoreaction center from strain G9 of *Rhs. rubrum*. The carotenoids used for reconstitution are indicated in the figure. Buffer was 10 mM Tris · HCl (pH 8.0)/0.1 % Triton X-100.

Absorption spectroscopy

The absorption spectra of the photoreaction center from the wild type and from strain G9 are superimposed on Fig. 4a. For comparison purposes, the preparations were adjusted to an equal $A_{865\ nm}$. Also shown on this figure (Fig. 4b) is the experimentally measured difference spectrum of these preparations (wild type minus G9). Besides an increase in absorbance in the 400–600 nm region clearly due to spirilloxanthin itself, the presence of this pigment is at the origin of some interesting effects: a small but significant bathochromic shift (865 → 868 nm) of the far red band, hyperchromicity of the 800 nm band and of the 350–400 nm region, with peaks, in the latter case, at 382 nm and at 398 nm; one observes also a hypochromic effect at 760 nm.

Some of these differences might possibly arise from the use of different preparation procedures rather than from the presence of spirilloxanthin. This objection was answered by comparing the carotenoidless preparations from strain G9 before and after reconstitution with spirilloxanthin. The corresponding difference spectrum of Fig. 5a is very similar to that of Fig. 4b. The analogous difference spectra taken with preparations reconstituted with sphaeroidene, sphaeroidenone and chloroxanthin are shown on Figs. 5b, 5c and 5d. All four carotenoids induce a hyperchromic effect of *P*-800 and a bathochromic shift of *P*-870 (*P*-865 → *P*-868). They all induce the appearance of an intense band in the 350–400 nm region. This band is particularly intense in preparations which contain spirilloxanthin. The positions of its components

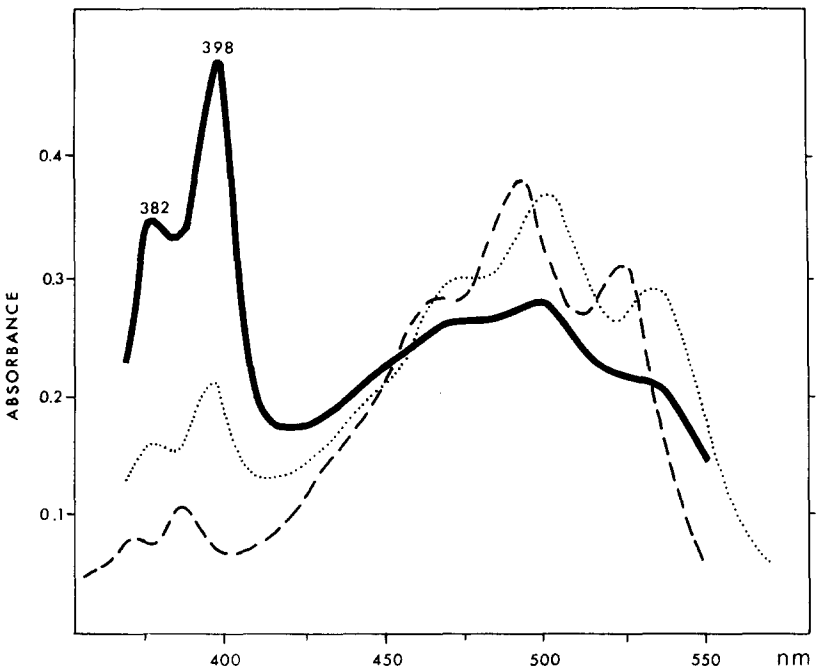

Fig. 6. Absorption spectra of spirilloxanthin in different environments. Difference spectrum of reconstituted minus carotenoidless preparations of photoreaction center from *Rhs. rubrum* (—), spirilloxanthin in diethyl ether (– – –) and spirilloxanthin dispersed in 10 mM Tris · HCl (pH 8.0)/0.1 % Triton X-100 (· · ·).

vary from one carotenoid to the next: 382 and 398 nm with spirilloxanthin, 387 and 393 nm with sphaeroidene, 386 and 393 nm with sphaeroidenone and 368 and 386 nm with chloroxanthin. All the difference spectra except that of Fig. 5c (chloroxanthin) appear to be free of marked light scattering artifacts.

Fig. 6 compares the absorption spectra of spirilloxanthin in different environments: in ether, in micelles of Triton X-100 or fixed in a 1 : 1 fashion on the photoreaction center. There is a bathochromic shift of all the absorption bands when going from ether to photoreaction center or to Triton X-100. Fixation of spirilloxanthin on the photoreaction center is also accompanied by a general loss of structure and of intensity of its absorption bands in the visible ($^1B \leftarrow {}^1A$ transition). At the same time, there is a large increase in the intensity of its "cis bands" ($^1C \leftarrow {}^1A$ transition).

Circular dichroism

Fig. 7 shows CD spectra of photoreaction center preparations both from the wild type and from the G9 strain. The preparations were adjusted to an equal $A_{870\ nm}$ of 0.77 and their spectra were recorded in the presence of 10 mM ascorbate in order to keep the primary electron donor in the reduced state.

The CD spectrum of the carotenoidless preparation is very similar to the spectra published by Sauer et al. [27] and by Reed and Ke [28] for analogous preparations obtained from *Rhp. sphaeroides* (strain R26). In the infrared, *P*-800 is split into two

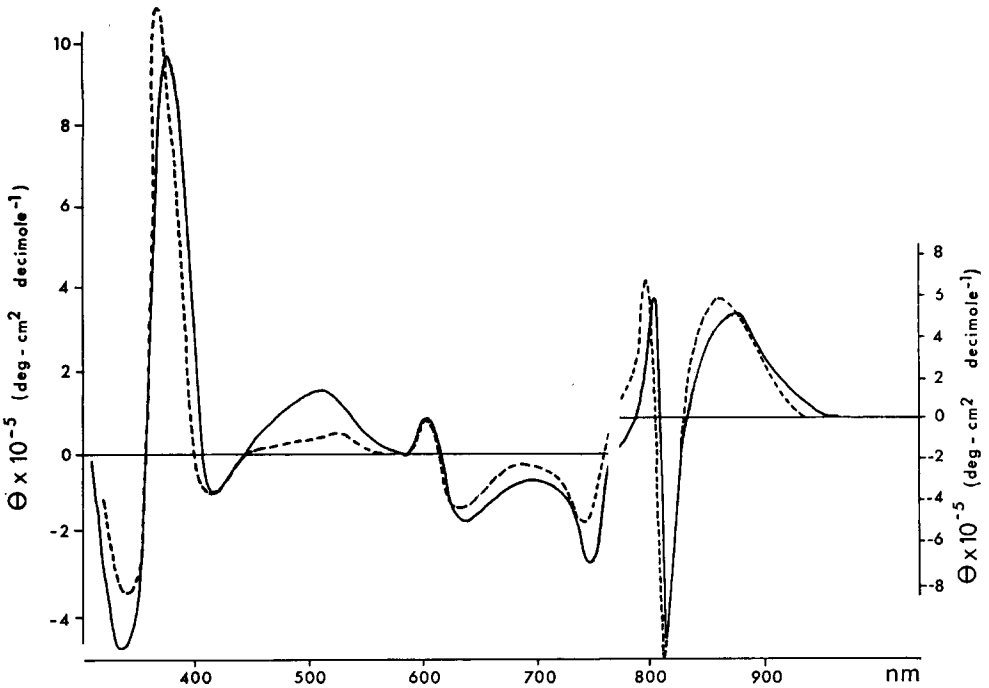

Fig. 7. Circular dichroism spectra of photoreaction center preparations from wild type (—) or from carotenoidless strain G9 (– – –) of *Rhs. rubrum*. The preparations in 10 mM Tris · HCl (pH 8.0)/0.03 % LDAO (wild type) or /0.1 % Triton X-100 (strain G9) were adjusted to an equal $A_{870\,nm}$ before recording their CD spectra.

components, one of positive ellipticity with a peak at 796 nm and the other of negative ellipticity with a peak at 807 nm; *P*-870 shows a positive CD peak at 862 nm. The presence of spirilloxanthin induces a decrease in the absolute values of the molar ellipticity of these three components as well as bathochromic shifts of the 796 nm and of the 862 nm peaks. The left portion of Fig. 7 also shows some interesting effects, namely a decrease in the molar ellipticity at 750 nm and increase in the 500 nm region coinciding with the $^1B \leftarrow {}^1A$ transition of spirilloxanthin. There is also a bathochromic shift and a decrease in the molar ellipticity in the 350–400 nm region.

Reconstituted preparations have the same spectroscopic behavior. Fig. 8 shows the calculated CD difference spectra (solid lines) of reconstituted minus carotenoidless preparations in their reduced form. The dotted lines are reminders of the absorption difference spectra. Preparations reconstituted with spirilloxanthin and sphaeroidene exhibit positive ellipticity in the 500 nm region corresponding to the $^1B \leftarrow {}^1A$ transition in these pigments. Optical activity is also present in the 350–420 nm region.

Table II gives the maximal molar ellipticities, $[\theta]^{car}_{\lambda max}$ in the visible for the carotenoids after their association with the photoreaction center isolated from strain G9. These values were obtained after subtracting the molar ellipticity of the carotenoidless preparation at the corresponding wavelength. No optical activity could be detected for sphaeroidenone and we estimate its $[\theta]^{car}_{\lambda max}$ to be less than $5 \cdot 10^3$ degree ·

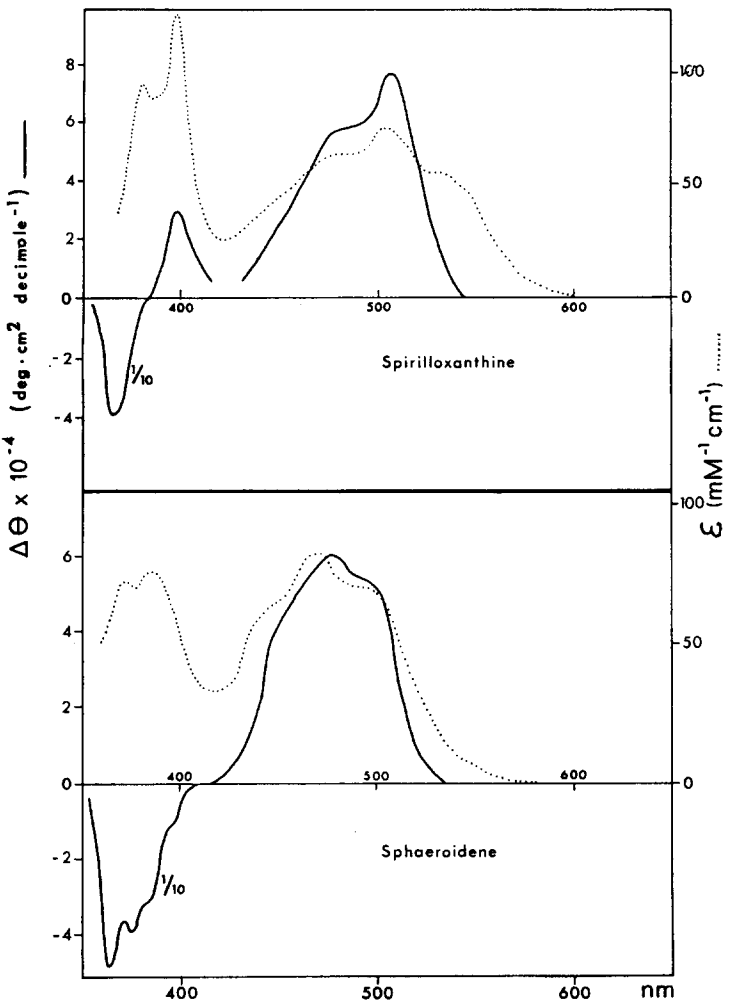

Fig. 8. Calculated difference CD spectra of reconstituted minus carotenoidless preparations of photoreaction center from *Rhs. rubrum*. Difference CD spectra (—) and difference absorption spectra (· · ·). The preparations in 10 mM Tris · HCl (pH 8.0)/0.1 % Triton X-100 were adjusted to an equal $A_{870\,nm}$ before recording their spectra. Upper figure: reconstitution with spirilloxanthin. Lower figure: reconstitution with sphaeroidene.

$cm^2 \cdot dmol^{-1}$. None of the carotenoids studied in this work showed any optical activity either in solution or dispersed in Triton X-100.

DISCUSSION

The first part of this work clearly indicates that singlet oxygen is involved in the photodynamic action observed on the photoreaction center. The evidence is as follows.

(1) Some of the pigments, and notably *P*-800 and *P*-870 are bleached irreversi-

bly under high light intensity, provided oxygen is present. This photodynamic effect is counteracted by certain carotenoids provided they can be fixed either naturally or artificially on the preparations. (2) The oxidation of adrenaline is not only triggered by $O_2^{\cdot-}$ as shown by Fridovich and Misra [21], but also occurs in the presence of $^1O_2$ since it can be photosensitized by methylene blue, a classical photogenerator of $^1O_2$, in the presence of high concentrations of bovine superoxide dismutase. The photooxidation of adrenaline is used here as a probe for the photogeneration of both $O_2^{\cdot-}$ and $^1O_2$ by the photoreaction center. Since the photogeneration of $O_2^{\cdot-}$ has already partially been discussed elsewhere [23], we will not dwell on it here. The rate of production of $^1O_2$ is directly proportional to light intensity (Fig. 2) and is not inhibited by superoxide dismutase. It is inhibited, however, by 10^{-2} M azide or by fixed spirilloxanthin. Azide is known to be a quencher of $^1O_2$ [22] and carotenoids can quench both ^{3}chlorophyll [29] and $^1O_2$ [30] in solution. (3) Further evidence is provided by the oxidation of 1,3-diphenylisobenzofuran, a classical probe for $^1O_2$ [24, 25].

Since adrenaline is insoluble in polar and in non-polar organic solvents it may safely be assumed to be dissolved in the aqueous phase of the medium rather than in its hydrophobic micellar phase. Hence, it may be considered to probe for $^1O_2$ that diffuses out of the detergent-photoreaction center micellar complex. The reverse situation where $^1O_2$, generated in the aqueous phase, diffuses into detergent micelles has been studied by Gorman et al. [31]. The smaller $^1O_2$ flux from the micellar into the aqueous phase observed when carotenoids are present may receive three different explanations. Carotenoids may quench ^{3}bacteriochlorophyll, they may quench $^1O_2$ or they may be chemically oxidized. Evidence for the first mechanism stems from the work of Cogdell et al. [14]: the rate of decay of state P^F was the same in photoreaction center preparations whether they contained carotenoids or not. However, the presence of carotenoids precluded the detection of state P^R; instead, a new signal was observed which was attributed to ^{3}carotenoid. Since P^R is assumed to be triplet in character, it would appear as a likely donor of triplet energy to O_2 ($^3\Sigma$) thus generating $^1O_2$. However, the light intensity curves of Fig. 1b indicate that other triplet donors may be present as well. For if state P^R was the sole photosensitizer present, the light intensity curve for photodynamic damage to *P*-800 should level off at light intensities giving a maximal steady-state concentration of P^+-870. It seems, therefore, that not only *P*-870 but also other pigments, namely *P*-800 or *P*-755, can act as photosensitizers.

We have so far no evidence for the quenching of $^1O_2$ by carotenoids although it may be pointed out that this reaction could also lead to the formation of ^{3}carotenoid. Although oxidation of the carotenoids has not been investigated, we have observed bleaching of the carotenoids when the preparations were submitted to extended periods of illumination under bright light.

Singlet energy transfer to the porphyrins is certainly negligible in vivo in terms of the amount of light energy collected, since these carotenoids represent but a small fraction (approx. 0.01) of the total antenna carotenoids. An interesting point, however, is the efficiency of energy transfer with respect to carotenoid structure. It is about 20 % for spirilloxanthin in *Rhs. rubrum* and about 90 % for sphaeroidene in *Rhp. sphaeroides* whether one measures transfer from antenna carotenoids to antenna bacteriochlorophyll in vivo [32, 33] or from the carotenoids to *P*-870 in the corresponding photoreaction center preparations [13, 34]. Moreover, in the present work the same efficiency values were found for carotenoids reinserted in a photoreaction center

prepared from a single source, namely strain G9. These data show that the transfer efficiency is governed by the molecular structure of the carotenoid itself rather than by the nature of its environment.

Assuming a resonance mechanism the rate constant of energy transfer, k_t, is described by Förster's equation [35].

$$k_t = \frac{9000\ (\ln 10)\ K^2 \phi_f}{128\pi^6 \eta^4 N \tau R^6} \int_0^\infty F(\nu)\varepsilon(\nu)\ \frac{d\nu}{\nu^4}$$

where K^2 is an orientation factor, ϕ_f is the fluorescence yield of the donor dipole, η is the solvent refractive index, N is Avogadro's number, τ is the actual mean lifetime of the emission, R is the intermolecular distance between donor and acceptor, $F(\nu)$ is the spectral distribution of the fluorescence of the donor (normalized to unity) and $\varepsilon(\nu)$ is the molar extinction coefficient of the acceptor as a function of ν, the frequency.

The relative values of k_t for the various carotenoids that can be attached on the photoreaction center can be estimated by making use of this equation. Since the carotenoids are very probably attached on the same specific site of the protein, factors η, K^2 and R may be assumed to be constant from one carotenoid to the next.

The factors which are expected to significantly affect k_t are therefore the value of the overlap integrals and $\phi_f/\tau = 1/\tau_r$ where τ_r is the radiative lifetime of the excited state. $1/\tau_r$ was calculated from the oscillator strength of the first absorption band of the fixed carotenoids. It is about twice greater for sphaeroidene than for spirilloxanthin and for sphaeroidenone. On the other hand, the overlap integrals of the theoretical emission spectra of the attached carotenoids with the Q_x band of bacteriochlorophyll in the photoreaction center were found to vary by a maximum of 10 % between the various carotenoids. Hence k_t is estimated to be twice as great for fixed sphaeroidene as for the other carotenoids.

The efficiency of energy transfer (E) is given by

$$E = \frac{k_t}{k_t + k_d}$$

where k_d is the deactivation rate constant for the excited singlet state of the donor by radiative and non-radiative processes. From this equation, k_d/k_t must increase by a factor of 40 when E varies from 90 % (as in sphaeroidene) to 20 % (as in spirilloxanthin). In other words k_d for spirilloxanthin is 20 times greater than k_d for sphaeroidene.

Carotenoids in solution do not fluoresce, presumably because the rate of deactivation by internal conversion is much faster than deactivation by radiative processes. If this applies also to carotenoids in chromatophores or on the photoreaction center, it would seem that the efficiency of energy transfer from carotenoids is governed primarily be their internal conversion rate constant.

There is clear evidence that the photoreaction center from several species of photosynthetic bacteria bears a site of attachment for carotenoids. The preparations so far obtained from normally grown wild type organisms contain carotenoids without any exception. In *Rhs. rubrum* and in *Rhp. sphaeroides*, the molar ratio of carotenoid/*P*-870 has been shown to be 1 : 1 [6, 13]. In these preparations the carotenoids are tightly held and cannot be removed by mild treatments such as DEAE-cellulose

chromatography in the presence of 0.03 % LDAO. In our opinion, the reconstitution experiments are the best evidence for the existence of a site of attachment for carotenoids. Even with high concentration ratios of carotenoids/*P*-870 in the reconstitution medium, only one molecule of carotenoid in tightly bound onto the preparation. Moreover, only certain carotenoids can be fixed in this manner. Comparison of the structure of these molecules (Fig. 3) shows that their carbon skeletons are identical from carbon 1 to carbon 10′ except for chloroxanthin which has a single bond instead of a double bond between carbons 3 and 4. Carbon 1 is substituted with a methoxyl or with a hydroxyl group. The affinity for the site of fixation as well as the photoprotection efficiency is definitely smaller for sphaeroidenone and for chloroxanthin than for spirilloxanthin or sphaeroidene. This points out to the importance of the structure of the "head" of these molecules which may be supposed to be involved in the fixation process.

The fixation of carotenoids on the photoreaction center is accompanied by modifications of the spectroscopic properties of the porphyrins and of the carotenoids themselves. As shown by difference spectroscopy (Figs. 4 and 5), *P*-800 undergoes a clearcut hyperchromic effect upon the fixation of carotenoids. Noteworthy also is the small but reproducible bathochromic shift of *P*-870. Analogous effects on *P*-800 and on *P*-870 may also be observed by CD spectroscopy (Fig. 7). These effects do not necessarily indicate direct carotenoid-bacteriochlorophyll interaction. It is quite possible that this interaction is mediated through a conformational change of the protein induced by the carotenoid molecule.

The absorption and CD spectra of the carotenoids in situ show significant differences with respect to the same carotenoids in solution. The difference spectra of Fig. 6 show a large hypochromic effect affecting the $^1B \leftarrow {}^1A$ transition (400–500 nm) and an equally large hyperchromic effect affecting the $^1C \leftarrow {}^1A$ transition ("cis band"). This effect is especially marked in the case of spirilloxanthin. In all-*trans*-carotenoids the $^1C \leftarrow {}^1A$ transition is forbidden but is allowed for symmetry reasons in *cis* forms. Since the $^1C \leftarrow {}^1A$ transition is very intense in fixed carotenoids we conclude that they are not present in an all-*trans* linear conformation but most likely in their central *monocis* conformation. In this line, the higher intensity of the $^1C \leftarrow {}^1A$ transition in fixed spirilloxanthin may be due to the fact that this molecule has a vertical plane of symmetry which gives rise to two identical dipoles per molecule.

As indicated in Table II, there is a correlation between the molar ellipticity of the fixed carotenoids and their efficiency as photoprotectors. Sphaeroidenone and chloroxanthin which have undetectable or low optical activity are relatively poor photoprotectors as compared to spirilloxanthin and sphaeroidene which have higher molar ellipticity values. Since carotenoids are optically inactive in solution, their optical activity induced by fixation on the photoreaction center may be attributed to an asymmetry of their environment or to an asymmetry of their conformation.

In the second case, the carotenoids which confer photoprotection may be imagined to be not only bent about their central double bond in a *monocis* conformation but to be also twisted in a protohelical shape. Whereas the $^1B \leftarrow {}^1A$ transition dipole is parallel to the long axis of the molecule, the $^1C \leftarrow {}^1A$ transition dipole is orthogonal to it. Their corresponding CD bands would therefore be expected to be of opposite signs. This appears to be the case for sphaeroidene but fixed spirilloxanthin shows a positive (398 nm) and a negative (366 nm) peak about a null point (382 nm)

corresponding to the "cis band" (Fig. 9). Since spirilloxanthin has a vertical plane of symmetry this CD difference spectrum may possibly arise from an exciton coupling of the two identical $^1C \leftarrow {}^1A$ transition dipoles. However, great caution is required in the interpretation of these difference spectra in the 350–400 nm region since they may also result from perturbations of the Soret band of bacteriochlorophyll by the carotenoids. The strongest statement that can be made is that these CD difference spectra are consistent with a *monocis* conformation of the carotenoid twisted in a protohelical shape. This interpretation is reminiscent of the model previously proposed for a caroteno-protein complex obtained from chromatophores of *Rhs. rubrum* [36].

All the available data indicate that the main function of the carotenoid of the photoreaction center is protection against $^1O_2$. Quenching of ^{3}bacteriochlorophyll, presumably in state P^R [14], is one mechanism by which this function comes into play. And this function is of sufficient importance to have conferred a selective advantage on the possession of a site of attachment for carotenoids on the photoreaction center. This site of attachment imposes a proximity relationship between the carotenoid and the bacteriochlorophyll; it imposes also a certain chemical structure and conformation of the carotenoid which must determine the efficiency of triplet-triplet energy transfer and therefore of photoprotection.

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