The contribution of the carotenoid to the visible circular dichroism of the light-harvesting antenna of *Rhodospirillum rubrum*

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The visible c.d. spectrum of wild-type *Rhodospirillum rubrum* shows positive bands [Dratz, Schultz & Sauer (1966) Brookhaven Symp. Biol. 19, 303–318] that are largely due to the B880 antenna pigments, bacteriochlorophyll *a* and carotenoids. The bacteriochlorophyll c.d. band was absent from the spectrum of *R. rubrum* G9, a mutant unable to synthesize coloured carotenoids, and could be partly restored by adding extracted carotenoids to freeze-dried membrane vesicles isolated from that mutant. Therefore it seems to arise from either bacteriochlorophyll–carotenoid interactions or bacteriochlorophyll–protein interactions that are induced by the carotenoid. The more complex carotenoid c.d. band had different shapes in native and reconstituted carotenoid-containing membranes. Such differences suggest that the optical activity of the carotenoid in the B880 antenna arises from both non-degenerate and degenerate interactions.

INTRODUCTION

Carotenoids are usual components of the photosynthetic pigment–protein complexes or holochromes, in which they perform light-harvesting and protective functions (for reviews, see Cogdell & Frank, 1987; Siefermann-Harms, 1987). The interactions of the carotenoid with other holochrome constituents induce changes in the properties of such constituents and of the carotenoid itself. Thus, in purple phototrophic bacteria, the carotenoid elicits red shifts of the near-i.r. (Q_y) bands of both antenna and reaction-centre bacteriochlorophyll (Boucher *et al.*, 1977; Thornber *et al.*, 1978), while simultaneously the visible absorption band of the carotenoid shifts also to the red and becomes optically active (Davidson & Cogdell, 1981). Additional changes take place in the resonance-Raman spectrum of the carotenoid (Iwata *et al.*, 1985) and in the sensitivity of some of the apoproteins to proteinases (Brunisholz *et al.*, 1986).

In the present study the influence of the carotenoid on the c.d. spectrum of the light-harvesting antenna of *Rhodospirillum rubrum* was investigated. The optical activity of the pigments in the photosynthetic complexes is largely the result of specific molecular interactions (Dratz *et al.*, 1966) and, consequently, the c.d. spectrum can be used to detect changes in such interactions, for example, during solubilization of the complexes or in dissociation–reconstitution experiments (Miller *et al.*, 1987; Ghosh *et al.*, 1988). We report here that, apart from lesser changes in the near-i.r. c.d. spectrum, the carotenoid induces optical activity in the visible (Q_x) transition of bacteriochlorophyll. The resulting single positive c.d. band appears to reflect either a bacteriochlorophyll–carotenoid interaction or a modification of bacteriochlorophyll–protein interactions. It is also reported that the c.d. of carotenoids that have been inserted *in vitro* in the antenna complex is different from the c.d. of native carotenoids. The differences suggest that two independent types of interactions give rise to the optical activity of the carotenoid in the *R. rubrum* antenna complex.

MATERIALS AND METHODS

Three strains of *R. rubrum* were used in this work: S1 (wild-type), G9 (carotenoidless mutant) (Sistrom, 1978) and T102

(reaction-centre-defective mutant) (Gómez *et al.*, 1982). *Rhodobacter capsulatus* MW4422 was also used. This strain is a double mutant that lacks the peripheral antenna and has a block in the carotenoid-biosynthetic pathway. It contains the nonaene carotenoids neurosporene, hydroxynurosporene (chloroxanthin) and methoxynurosporene at comparable levels (Scolnik *et al.*, 1980). The phototrophic strains were grown in the light and in the absence of O_2 , as described previously (Giménez-Gallego *et al.*, 1978). Non-phototrophic *R. rubrum* T102 was grown in the dark under limiting O_2 (Giménez-Gallego *et al.*, 1978), to allow formation of the photosynthetic system. Growth was allowed to enter the stationary phase to favour the formation of spirilloxanthin as the major carotenoid of the *R. rubrum* cultures (Lozano *et al.*, 1989).

Isolated membrane vesicles were obtained by differential centrifugation of cell-free extracts which had been prepared from cells suspended in 50 mM-potassium phosphate, pH 7, with a French press. The same buffer was used for washing and final suspension of the isolated vesicles. The method for the extraction and purification of the antenna complex of *R. rubrum* was described previously (Giménez-Gallego *et al.*, 1986). Nonaene carotenoids were extracted from freeze-dried cells of *Rb. capsulatus* MW4422 with acetone at 4 °C. The extract was filtered through paper and mixed with a similar volume of light petroleum (b.p. 50–70 °C). The solution was washed ten times with water (1:1, v/v) in order to remove acetone, and mixed with anhydrous Na_2SO_4 to remove traces of water. Then it was evaporated to dryness and the residue was dissolved in light petroleum. No detectable amounts of porphyrin were present in the resulting solution of carotenoids. For the reconstitution assay the extracted carotenoids were added to freeze-dried membrane vesicles obtained from *R. rubrum* G9 (2 mol of carotenoid/mol of bacteriochlorophyll) and, after dispersion of the solid phase and sonication, the mixture was kept at 27 °C for 2 h. Then it was dried in a rotary evaporator and the residue was suspended in 20 mM-Tris/HCl, pH 8, centrifuged, and washed twice and suspended in the same buffer. The suspensions were kept frozen at –20 °C. They were sonicated before recording spectra in order to minimize light-scattering.

Absorption spectra were obtained with a Shimadzu spectrophotometer (model UV-3000). The same instrument,

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supplemented with a fluorescence attachment and a S-1 type photomultiplier (Hamamatsu R-473) was used to obtain the excitation spectra of bacteriochlorophyll fluorescence. A solution of Rhodamine B ($3 \text{ mg} \cdot \text{cm}^{-3}$) in ethylene glycol was used as a photon counter. The spectrophotometer was interfaced to a Hewlett-Packard microcomputer (HP9826) for storage and processing of the data.

Near-i.r. c.d. spectra of samples in 1 mm-optical-pathlength cells were kindly scanned by Ms. A. M. Bardin (CEN, Saclay, France), using a Jobin Yvon IV dichrograph. Visible c.d. measurements were made with an older instrument (Roussel-Jouan II) in cells of 1 cm optical pathlength. All visible spectra were checked with either (or both) a Jobin Yvon III dichrograph at the Universidad Complutense (Madrid) or the Saclay instrument. The c.d. data were digitized and transferred to the Hewlett-Packard microcomputer with the aid of a Graphics Tablet (HP9111A) from photocopies of the original plots, since the high electrical conductivity of some recorder inks interfered with the digitalization process.

RESULTS

In the absorption spectrum of intact cells or isolated membranes of *R. rubrum*, the major visible and near-i.r. bands correspond to the most abundant type of pigment-protein complex, the core (or B880) light-harvesting antenna (Cogdell, 1986; Zuber, 1986a). This is a very convenient feature that allows one to study *in situ* those properties of the B880 chromophores that are directly related to their absorption spectrum. But, since the optical activity of all transitions is not the same, the signals in the c.d. spectrum of membranes cannot be unambiguously assigned to the B880 antenna when the absorption bands of this complex overlap with those of the less abundant photochemical reaction centre. Comparison with the spectra of membrane vesicles isolated from a reaction-centre-deficient mutant has been used throughout this work to circumvent such difficulties.

Fig. 1. shows the near-i.r. absorption (left) and c.d. (right) spectra of membranes isolated from a carotenoid-containing (a) and a carotenoidless (b) strain. In carotenoidless membranes, the changes detected in the double c.d. signal of the bacteriochlorophyll Q_y transition near 880 nm were a blue shift, which was the counterpart of the shift exhibited by the corresponding absorption band, and a broadening of the negative component of

Fig. 1. Near-i.r. absorption (left) and c.d. (right) spectra of *R. rubrum* membranes

Membrane vesicles were isolated from (a) wild-type strain S1, (b) carotenoidless strain G9 and (c) reaction-centre-less strain T102. In this Figure and Fig. 2, A_L and A_R are absorbances of left and right circularly polarized light.

Fig. 2. Visible absorption (left) and c.d. (right) spectra of bacterial membranes

Membrane vesicles were isolated from the following *R. rubrum* strains: (a) S1, wild-type, (b) G9, carotenoidless mutant, and (c) T102, reaction-centre-defective mutant. To the membranes of strain G9 shown in (d), exogenous carotenoids that had been extracted from *Rb. capsulatus* MW4422 were added *in vitro* (see the Materials and methods section). The spectra of membranes isolated from the yellow-green mutant of *Rb. capsulatus*, strain MW4422, are also shown (e). The absorbance of all samples at the main Q_y band of bacteriochlorophyll was 1.

the signal. No significant changes were detected in the intensity of the double c.d. band. This contrasts with the marked reduction observed in the red c.d. signal of chlorophyll in Photosystem-I particles obtained from a carotenoid-deficient mutant of maize (*Zea mays*) (Garay *et al.*, 1972). The spectra (absorption and c.d.) of carotenoidless membranes showed an additional alteration, the increased intensity of the band near 800 nm. This second Q_y transition corresponds to bacteriochlorophyll in the reaction centre and was accordingly absent from the spectra of a mutant that lacked that pigment-protein complex (Fig. 1c). It is not clear whether the higher relative intensity of the 800 nm band, which might reflect an increased reaction centre to antenna ratio in strain G9, is directly related to the absence of coloured carotenoids.

In the visible range, the prominent features of the absorption spectrum of wild-type *R. rubrum* membranes are the three-peaked band of the carotenoid (mainly spirilloxanthin in the cultures used here; see Lozano *et al.*, 1989) between 470 and 560 nm and the Q_x band of bacteriochlorophyll near 590 nm (Fig. 2a, left). Both absorption bands are largely due to antenna pigments and were consequently present in the spectrum of reaction-centre-less membranes (Fig. 2c, left). Both bands are optically active but, as the carotenoid of the *R. rubrum* antenna is only weakly dichroic, the bacteriochlorophyll band becomes the most prominent feature of the c.d. spectrum (Dratz *et al.*, 1966; Fig. 2a, right). The loss of the reaction centre in strain T102 had a noticeable effect on the intensity of the carotenoid c.d. signals (Fig. 2c, right), suggesting some influence of that complex that was not further investigated. In contrast, the c.d. of

the Q_x transition was little affected by the absence of the reaction centre, since only a small negative component above 600 nm was lost by the mutant (Fig. 2c, right).

As expected, the carotenoid bands were absent from both the absorption and c.d. spectra of membrane vesicles obtained from the carotenoidless strain (Fig. 2b). But, unexpectedly, also, the bacteriochlorophyll c.d. signal disappeared almost completely (Fig. 2b, right). In fact, the weak residual c.d. bands near 535 and 600 nm appear to be due to the reaction centre and not to the antenna porphyrins, since they are similar in location and shape to those reported for purified preparations of carotenoid-free reaction centres (Boucher *et al.*, 1977). Besides, the negative component above 600 nm (the only signal shown by carotenoidless membranes which could be directly observed in the spectrum of the wild-type strain; Fig. 2a, right), was not present in membranes of the reaction-centre-less mutant (Fig. 2c, right) or in reaction-centre-free preparations of solubilized antenna (results not shown). Therefore it seems that the loss of the carotenoid goes along with the disappearance of all antenna bands from the visible c.d. spectrum of *R. rubrum* membranes.

A trivial explanation for the absence of the 590 nm c.d. band from the spectrum of carotenoidless membranes is that it may correspond to the carotenoid and not to bacteriochlorophyll. The high extent of overlapping between the absorption bands of both pigments in the spectrum of *R. rubrum* (Fig. 2a, left) makes this possibility worth considering. But it can be readily discarded, because changes in the carotenoid composition of the membrane that have a marked influence on the carotenoid absorption and c.d. spectra had little effect on the 590 nm c.d. band. As an example, in *Rb. capsulatus* MW4422, a yellow-green strain that also has a B880 antenna as the major type of pigment-protein complex and contains only nonaene carotenoids (Scolnik *et al.*, 1980), the 590 nm band was similar to that of wild-type *R. rubrum* even though the absorption and c.d. bands of its carotenoids were located at shorter wavelengths and were much more intense than those of spirilloxanthin in the *R. rubrum* antenna complex (Fig. 2e). The band was also present at the same location in the c.d. spectrum of membranes isolated from exponentially growing *R. rubrum* (results not shown), which contains a mixture of spirilloxanthin and its metabolic precursors (Lozano *et al.*, 1989). It was also observed in a preparation of B880 complex purified from wild-type *Rb. sphaeroides* (Bolt *et al.*, 1981a), in which spheroidene and spheroidenone are the carotenoid constituents (Cogdell & Crofts, 1978).

It seems possible that the absence of visible optical activity in the antenna bacteriochlorophyll of strain G9 may be unrelated to the inability of this strain to synthesize coloured carotenoids, since it may have been caused by an independent defect of the mutant. In order to make this point clear, we used a reconstituted system obtained by adding carotenoids extracted from *Rb. capsulatus* MW4422 to freeze-dried membrane vesicles of *R. rubrum* G9. These carotenoids were preferred to the native *R. rubrum* pigments because, in our hands, they consistently yielded better reconstitution. As shown in the spectrum of Fig. 2(d), right, the added carotenoids partly restored the c.d. of the antenna Q_x transition in strain G9 vesicles. Although weak, the location and the intensity of the restored signal were consistent with the extent of reconstitution achieved, which was estimated to be close to 25% from the red shift elicited by the added carotenoids in the Q_y transition of antenna bacteriochlorophyll, and 35% from the degree of protection against aerobic photo-oxidation (as measured by the relative rate of irreversible bacteriochlorophyll photobleaching; results not shown). Other criteria indicated that a considerable fraction of the carotenoid that remained bound to the membrane had been successfully inserted into the antenna complexes: first, the carotenoid

Fig. 3. Absorption (A) and bacteriochlorophyll-fluorescence excitation (F) spectra of strain G9 membrane vesicles after reconstitution *in vitro* with nonaene carotenoids

absorption band shifted about 12 nm to the red upon binding. Consequently, its location (449 nm for the central peak) became close to that of the native carotenoids (451 nm) in *Rb. capsulatus*; and second, the efficiency of carotenoid-to-bacteriochlorophyll singlet energy transfer (estimated from the relative intensity of the carotenoid bands in the bacteriochlorophyll fluorescence-excitation and fractional-absorption spectra of the vesicles) ranged from 30 to 37% for wavelengths between 450 and 480 nm (Fig. 3), as compared with 23–26% for wild-type *R. rubrum* and 40–50% for *Rb. capsulatus* strain MW4422 (results not shown). However, in the fluorescence spectra of the reconstituted system and of *Rb. capsulatus* MW4422, the carotenoid bands were red-shifted 3–4 nm with respect to the corresponding absorption spectrum (Fig. 3). The shift, that was not observed in strains of *R. rubrum* and *Rb. capsulatus* which had the wild-type carotenoid phenotype (Lozano *et al.*, 1989), was probably due to the presence of significant levels of free pigments in the membrane vesicles that contained the mixture of nonaene carotenoids.

DISCUSSION

The near-i.r. (Q_y) transition of bacteriochlorophyll *a* in B880 complexes exhibits double c.d. that results from degenerate exciton interaction (Dratz *et al.*, 1966), but there is no evidence for a similar double c.d. band at the visible, Q_x , transition. This contrasts with the spectra of bacteriochlorophyll dimers in carbon tetrachloride (Dratz *et al.*, 1966) and of photochemical reaction centres (Philipson & Sauer, 1973), in which both transitions show double c.d. that crosses zero near the absorption maximum. Dratz *et al.* (1966) suggested that a negative component of the visible signal might be hidden within the overlapping carotenoid band, and proposed the investigation of a carotenoidless mutant to avoid the possible interference. Although the unexpected absence of the Q_x c.d. band in such a mutant prevents a definitive conclusion on this point, the observation that the absorption and

the c.d. bands are centred at approximately the same wavelength suggests that the major or sole component of the c.d. signal is a single positive band. Therefore it seems to correspond to the non-degenerate interaction of bacteriochlorophyll with another constituent of the antenna complex. The dependence of the bacteriochlorophyll c.d. signal on the carotenoid does not imply that it arises directly from carotenoid-bacteriochlorophyll interaction. It has been shown that the presence of the carotenoid in the B880 antenna complex influences the sensitivity of the apoprotein to proteinases (Brunisholz *et al.*, 1986), the ability of some detergents to dissociate the complex (Miller *et al.*, 1987) and the accessibility of bacteriochlorophyll to hydrophilic reagents (R. M. Lozano & J. M. Ramírez, unpublished work). Such results suggest that the pigment elicits conformational changes of the protein, and these changes might result in new bacteriochlorophyll-protein interactions that could be the direct cause of the modification of the porphyrin c.d. spectrum.

It appears that in other phototrophic bacteria the visible optical activity of B880-antenna bacteriochlorophyll may also depend on the carotenoid. Thus a 590 nm band similar to that of carotenoid-containing *R. rubrum* is exhibited by the c.d. spectra of the isolated B880 complex of wild-type *Rb. sphaeroides* (Bolt *et al.*, 1981a) and by the B880 antenna of membranes obtained from *Rb. capsulatus* MW4422 (Fig. 2e, right). Furthermore, the Ala⁺ strain of *Rb. capsulatus*, a double mutant that lacks both the peripheral antenna and coloured carotenoids, did not show the visible c.d. band of antenna bacteriochlorophyll (Bolt *et al.*, 1981b). Since the loss of the peripheral antenna did not cause the loss of the band in the *Rb. capsulatus* strain used here (Fig. 2e, right), it seems likely that, as in *R. rubrum* G9, the absence of the Q_x c.d. band in the Ala⁺ mutant was also associated with the inability to synthesize coloured carotenoids.

An observation that deserves some comment is the following: although the c.d. signal of the native carotenoid in *R. rubrum* and *Rb. capsulatus* membranes resembles the first derivative of the corresponding absorption band, both the c.d. and the absorption spectra of the carotenoid inserted *in vitro* in membranes obtained from *R. rubrum* G9 have the same shape (Fig. 2). The double (or first-derivative shaped) c.d. of some bacteriochlorophyll transitions is a reflection, as mentioned above, of degenerate exciton interaction, whereas non-degenerate protein-bacteriochlorophyll or carotenoid-bacteriochlorophyll interactions would lead only to single c.d. bands (Dratz *et al.*, 1966). The appearance of visible c.d., with splitting and change of sign across the band, upon addition of water to ethanolic solutions of xanthophylls has also been taken as indicative of molecular aggregation leading to exciton interaction (Lematre *et al.*, 1980). If a similar interpretation were given to the c.d. signal of the native carotenoid in the antenna of *R. rubrum* S1 and *Rb. capsulatus* MW4422, both non-degenerate and degenerate components would appear to contribute to the positive double band, and the absorption-like c.d. spectrum of the reconstituted system would indicate that only the non-degenerate interactions of carotenoids with other antenna constituents had been restored. In any case, the hypothetical degenerate interaction would not seem to be a requisite for the binding of the carotenoid at its specific site, because efficient singlet carotenoid-to-bacteriochlorophyll energy transfer is observed in the reconstituted system, and this process may occur only between specifically bound pigments (Cogdell & Frank, 1987; Siefermann-Harms, 1987). Since the *R. rubrum* B880 complex contains one carotenoid molecule per $\alpha\beta$ unit (Cogdell, 1986), the proposed degenerate interaction would have to take place between carotenoids of contiguous heterodimers.

This does not raise any problem, since the functional antenna unit is considered to be a multimer of the $\alpha\beta$ unit (Zuber, 1986b; Miller *et al.*, 1987; Ghosh *et al.*, 1988). In the reconstituted system, where only a small fraction of the $\alpha\beta$ units would contain a carotenoid molecule, carotenoid-carotenoid interaction would not be frequent. In the *Rb. capsulatus* type of B880 antenna, which contains two carotenoids per $\alpha\beta$ (Cogdell, 1986), additional carotenoid-carotenoid interactions within the heterodimer would be possible. Such extended interactions might explain the significantly stronger dichroism of the carotenoid in this subclass of B880 antenna (Fig. 2).

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