Biochimica et Biophysica Acta, 635 (1981) 295—303

BBA 48024

RECONSTITUTION OF CAROTENOIDS INTO THE LIGHT-HARVESTING PIGMENT-PROTEIN COMPLEX FROM THE CAROTENOIDLESS MUTANT OF *RHODOPSEUDOMONAS SPHAEROIDES* R26

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(Received October 14th, 1980)

Key words: Carotenoid; Bacteriochlorophyll; Light-harvesting complex; Pigment-protein complex; Bacterial photosynthesis; (Rps. sphaeroides)

Summary

Two carotenoids, neurosporene and spheroidene, have been successfully added to chromatophores from the carotenoidless mutant of *Rhodopseudomonas sphaeroides* R26. Carotenoids reconstituted in this way into the B-850 light-harvesting pigment-protein complex both sensitise bacteriochlorophyll fluorescence and protect the complex from the photodynamic reaction.

Introduction

Carotenoids have a dual role in photosynthesis. They act as accessory light-harvesting pigments and as photoprotective agents, preventing the chlorophylls from sensitising the harmful photodynamic reaction [1,2]. In whole cells of photosynthetic bacteria the efficiency of energy transfer from the carotenoid to the bacteriochlorophyll has been found to vary from 30 to 90%, depending on the species [3]. However, it is not clear whether this variation is a function of the carotenoid type, or rather depends upon how the carotenoids and bacteriochlorophylls are arranged with respect to each other within the various pigment-protein complexes.

One approach to studying energy transfer between the carotenoid and the bacteriochlorophyll is to make use of a series of mutant strains of a single species of bacteria, which differ only in their carotenoid compositions (cf. Ref. 4). In this way the same pigment-protein complex could be obtained but with a variety of carotenoid types present within the complex. However, this

Abbreviations: BChl, bacteriochlorophyll; SDS, sodium dodecyl sulphate.

approach has the disadvantage that only a limited number of mutants are available and that most of these mutants contain more than one carotenoid type.

Another method of varying the carotenoid type present within a given pigment-protein complex is to reconstitute carotenoids with carotenoidless mutants. In principle this method should allow a wider range of carotenoid types to be studied.

Carotenoids have indeed been added successfully to reaction centres isolated from the carotenoidless strains of *Rhodospirillum rubrum* (G9) and *Rhodopseudomonas sphaeroides* (R26) [5,6]. Boucher et al. [5] showed that carotenoids reconstituted with *Rhs. rubrum* (G9) reaction centres will both transfer energy to the bacteriochlorophyll and protect the reaction centres from photodestruction. The efficiency of the carotenoid to bacteriochlorophyll energy transfer was found to vary between 20 and 90% depending very strongly upon the carotenoid type present. Evidence was presented to show that in each case the carotenoid was bound to the same single site upon the reaction centre. Boucher et al. [5] therefore suggested that the efficiency of energy transfer was mainly determined by the structure of the carotenoid rather than its binding site. In contrast, however, Cogdell et al. [4] found that the efficiency of energy transfer from the carotenoid to bacteriochlorophyll in the B-800—850 light-harvesting pigment-protein complex from *Rps. sphaeroides*, was rather insensitive to carotenoid composition. This suggested that for this light-harvesting complex the energy transfer efficiency was mainly determined by the binding site.

In this present study we describe a method for adding carotenoids to a light-harvesting pigment-protein complex, B-850, from *Rps. sphaeroides* R26. This complex has been isolated previously by Sauer and Austin [7]. It contains two bacteriochlorophyll molecules per pair of approx. 9000 molecular weight polypeptides [8] and this pair of bacteriochlorophylls are exciton coupled [7]. This complex is probably analogous to the B-870 light-harvesting pigment-protein complex recently isolated from wild-type *Rps. sphaeroides* [9]. When the carotenoids are bound to this complex they fulfil both their accessory light-harvesting role and their photoprotective role.

Materials and Methods

Cells of *Rps. sphaeroides* R26 were grown anaerobically in the light with succinate as the sole carbon source. The cells were harvested and then disrupted by passage through a French Pressure cell at 10 tons/inch^2. Chromatophores were then isolated from the broken cells by differential centrifugation [10], resuspended in 20 mM Tris-HCl, pH 8.0, freeze-dried and stored at −20°C until used. The concentration of bacteriochlorophyll in the chromatophores was determined by extraction into acetone/methanol (7 : 2) using an extinction coefficient of 76 $mM^{-1} \cdot cm^{-1}$ at 772 nm [11].

Preparation of the carotenoids. Two carotenoids, spheroidene and neurosporene, were extracted and purified from *Rps. sphaeroides* 2.4.1 and *Rps. sphaeroides* G1C, respectively. In each case the required carotenoid was the major carotenoid type present in the strain of bacteria used [12,13]. The carotenoids were initially extracted and purified as described previously [14]. However, at

the stage of the bacteriochlorophyll-free, dry petroleum spirit extract, the bulk purification required was achieved by chromatography on alumina [15]. The concentrations of the carotenoids were determined from their $E^{1\%}_{1cm}$ values [16]. The carotenoids were stored in petroleum spirit 40—60°C b.p. in the dark at −20°C until use. Their purity was verified by chromatography on silica gel thin-layer plates [13], by recording their absorption spectra on a Unicam SP-8000 spectrophotometer and by mass spectrometry [17]. The mass spectra were recorded on an AEI M530 Mass Spectrometer. The samples were inserted directly into the probe at an ionising voltage of 70 eV with a probe temperature of 100°C.

Reconstitution of the carotenoids with the chromatophores and preparation of the B-850 light-harvesting pigment-protein complex. The carotenoids, in petroleum spirit, were added to 0.25 g of freeze-dried chromatophores in a ratio of 5 mol of carotenoid/1 mol of bacteriochlorophyll. The mixture was briefly sonicated (30 s—1 min) to disrupt any aggregated chromatophores, and then the petroleum spirit evaporated off in a rotary evaporator. The chromatophores were then resuspended in 20 mM Tris-HCl, pH 8.0. Centrifugation at 100 000 × *g* for 1 h pelleted the chromatophores leaving the excess carotenoid on top of the supernatant as an oily layer. The pelleted chromatophores were then either washed twice more for the further studies on the reconstituted chromatophores, or used directly for the preparation of the B-850 light-harvesting pigment-protein complex. For the carotenoid-binding studies the initial carotenoid: bacteriochlorophyll ratio was varied from 0.5 : 1 to 20 : 1.

For the preparation of the B-850 light-harvesting pigment-protein complex a method suggested to us by Drs. J. Bolt and K. Sauer, University of California at Berkeley, was used. The reconstituted chromatophores were resuspended in 20 mM Tris-HCl, pH 8.0, to give an A at 855 nm of 50 cm^{-1}. Sodium dodecyl sulphate (SDS) was added from a 10% (w/v) solution to give a final concentration of 1% (w/v). After stirring for 10 min at room temperature the solution was diluted to 0.2% (w/v) with 5 mM sodium phosphate, pH 7.0. This extract was loaded onto a hydroxyapatite column (50 ml onto a 10 × 1 cm column) which had been equilibrated with 5 mM sodium phosphate, pH 7.0. The column was washed with 5 mM sodium phosphate, pH 7.0, 100 mM NaCl, 0.1% SDS. The phosphate concentration was increased stepwise (while the concentration of NaCl and SDS were kept contant) from 5 to 10 mM, to 100 mM, to 150 mM and finally, to 250 mM where the B-850 light-harvesting pigment-protein complex was eluted. The eluted light-harvesting pigment-protein complex was immediately dialysed against 10 mM Tris-HCl, pH 8.0, since it was found to be unstable in high phosphate concentrations.

Photochemical studies. The absorption spectra were recorded on an Unicam SP8000 spectrophotometer (400—850 nm) and on a SP500 (800—950 nm). The fluorescence emission and excitation spectra were recorded on a simple homemade fluorimeter [4]. The fluorescence excitation spectra were recorded for exciting wavelengths between 400 and 630 nm monitoring the fluorescence intensity at 858 nm. The actinic light intensity was measured at each excitation wavelength and the fluorescence excitation spectra was corrected for variations in the actinic light intensity as previously described [4]. The efficiency of carotenoid to bacteriochlorophyll energy transfer was determined by normalising

the fluorescence excitation spectra and the fractional absorption spectra at 590 nm, in the bacteriochlorophyll absorption band.

The photodestruction of the B-850 light-harvesting pigment-protein complex, with and without carotenoids, was measured by the irreversible bleaching at 850 nm induced by illumination in the presence of oxygen with strong white light (900 $W \cdot m^{-2}$). The CD spectra were recorded on a Cary 60 spectrophotometer.

Results and Discussion

Binding of carotenoid to chromatophores

It was very obvious that carotenoid was being incorporated into the chromatophore membranes since the membranes changed their colour in the presence of the carotenoids (e.g. with neurosporene they were green and with spheroidene they were brown). Fig. 1 shows a typical carotenoid-binding curve. The amount of carotenoid bound per bacteriochlorophyll saturates at approx. a carotenoid : BChl ratio of 0.4. The saturation is reached by adding carotenoid to the chromatophores at ratios between 2 and 3 mol carotenoid/mol of bacteriochlorophyll. This ratio of carotenoid : BChl is well within the range found in chromatophores of carotenoid-containing strains of *Rps. sphaeroides* (0.38—0.59 [18]). However, it should be pointed out that a direct comparison between the two situations is difficult since the carotenoid-containing strains have both types of antenna complex (the B-800—850 and B-870 light-harvesting pigment-protein complexes), while R26 contains only the B-850 light-harvesting pigment-protein complex. When the carotenoid is added to the chromatophore membrane its absorption spectrum is shifted to the red.

The positions of the absorption maxima of the carotenoid depend very strongly upon its environment. This is illustrated in Table I for spheroidene. As the carotenoid is transferred from petroleum spirit to liposomes there is a large red shift of 11—15 nm. Addition of the carotenoid to bovine serum albumin causes a further red shift of 3—4 nm. While incorporation of the carotenoid into the B-850 light-harvesting pigment-protein complex results in an additional 3—4 nm red shift. It is interesting to note that the positions of the absorption

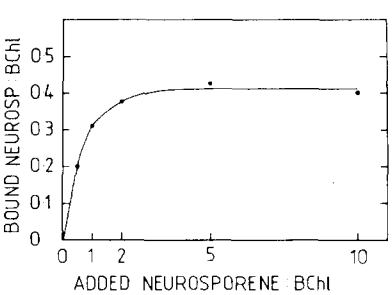


Fig. 1. Binding of neurosporene to chromatophores of *Rps. sphaeroides* R26 carotenoidless mutant. Neurosporene in petroleum spirit was added to freeze-dried chromatophores. After brief sonication the petroleum spirit was evaporated off under vacuum in a rotary evaporator and the chromatophores resuspended in 20 mM Tris-HCl, pH 8.0. Excess neurosporene was washed free by centrifuging at 100 000 × *g* for 1 h (three times) before determination of the neurosporene : BChl ratio.

TABLE I

ABSORPTION MAXIMA OF SPHEROIDENE IN DIFFERENT ENVIRONMENTS

Spheroidene environment	Absorption maxima (nm)		
Petroleum spirit (40—60° C)	427	452	482
Liposomes *	436	464	497
Bovine serum albumin	440	466	500
R26 B-850	443	469	504
NC1B 8253 SPH.B-870 **	439—446	466—472	500—505

* The lipid used to make these liposomes was phosphatidylcholine.
** Holmes et al. [19].

maxima of spheroidene in the isolated B-850 light-harvesting pigment-protein complex are the same as those of spheroidene in the analogous B-870 light-harvesting pigment-protein complex recently isolated from wild-type (carotenoid-containing) *Rps. sphaeroides* [19]. This suggests that the spheroidene is being incorporated into very similar environment to that which it occupies in the native B-870 light-harvesting pigment-protein complex. Although addition of spheroidene to the protein bovine serum albumin causes a rather similar red shift as reconstitution with the B-850 light-harvesting pigment-protein complex, with bovine serum albumin there is a large spectral distortion. In wild-type membranes, wild-type B-870 light-harvesting pigment-protein complex and the reconstituted B-850 light-harvesting pigment-protein complex the middle of the three carotenoid absorption peaks is the most intense. However, in the case of spheroidene bound to bovine serum albumin the lowest energy absorption peak is the most intense. This suggests a non specific binding in which the conformation of the spheroidene has been significantly altered.

Fluorescence excitation

Reconstituted chromatophores. When the chromatophores were excited with broad band blue light the fluorescence emission spectrum showed a single strong peak at approx. 860 nm. (Note this has not been corrected for the transmission characteristics of the analysing monochromator, cf. Ref. 4.) Light absorbed by the reconstituted carotenoids does sensitise bacteriochlorophyll fluorescence (monitored at 858 nm). However, the efficiency of this carotenoid to bacteriochlorophyll energy transfer is rather low, in each case approx. 20%. This figure is probably low for two reasons: (a) undoubtedly a proportion of the carotenoid incorporated into the chromatophores is nonspecifically bound at sites other than in the pigment-protein complex, and (b) the reconstituted samples are rather turbid and therefore it is difficult to obtain an accurate absorption spectrum for the normalisation.

In an attempt to overcome these problems, so that the effect of different carotenoids could be accurately assessed, it was decided to work only with the isolated B-850 light-harvesting pigment-protein complex.

Reconstituted B-850 light-harvesting pigment-protein complex. Fig. 2 shows the absorption spectrum of the isolated B-850 light-harvesting pigment-protein complex, with and without carotenoid (in this case neurosporene). The pres-

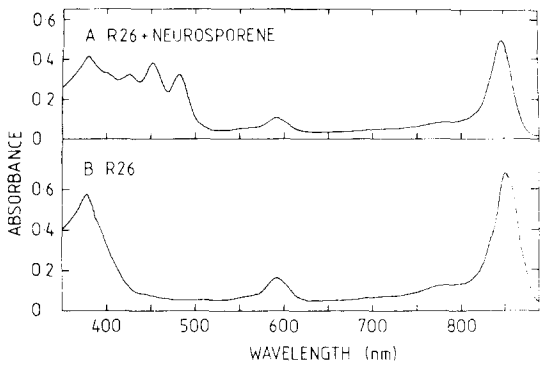


Fig. 2. Absorption spectra of B-850 light-harvesting pigment-protein complexes from *Rps. sphaeroides* R26 with and without neurosporene. Complexes were isolated from chromatophores (B) and from chromatophores reconstituted with neurosporene (A). The concentration of bacteriochlorophyll was 4.6 μM in (A) and 6.4 μM in (B).

ence of the reconstituted carotenoid is clearly shown by the presence of the characteristic three carotenoid absorption peaks between 400 and 500 nm.

It is important to try and assess whether the carotenoid is being bound specifically to the B-850 light-harvesting pigment-protein complex or whether it is binding randomly at several different sites. It is interesting to note therefore that the absorption spectrum of the bound spheroidene corresponds exactly with that of the carotenoid bound to the B-870 light-harvesting pigment-protein complex from carotenoid-containing strains of *Rps. sphaeroides* [19].

When the carotenoids are bound to either reaction centres [5,14] or the B-800—850 light-harvesting pigment-protein complex from *Rps. sphaeroides* [18] they show strong induced CD spectra. Denaturation of these complexes disrupts the carotenoid-protein interaction and the carotenoid CD spectrum disappears.

When the carotenoid is bound to the B-850 light-harvesting pigment-protein

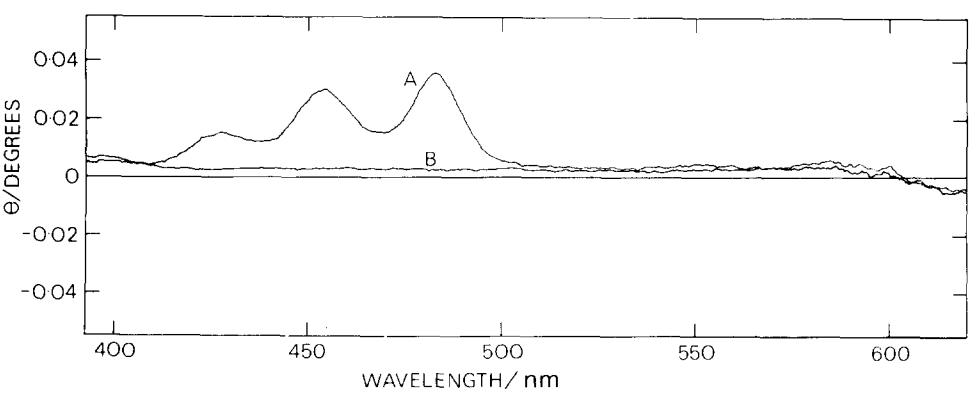
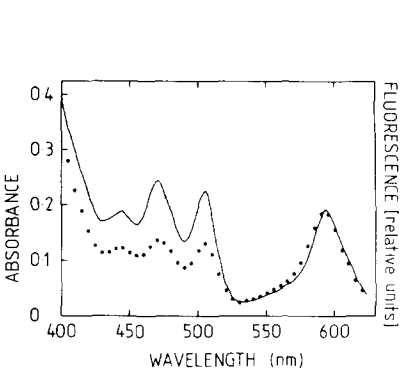


Fig. 3. Circular dichroism of B-850 light-harvesting pigment-protein complexes from *Rps. sphaeroides* R26 with and without neurosporene. (A) Complex isolated from chromatophores reconstituted with neurosporene, 6.5 μM bacteriochlorophyll. (B) Complex isolated from chromatophores alone, 6.5 μM bacteriochlorophyll.



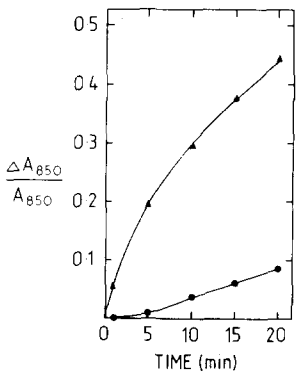


Fig. 4. Absorption and fluorescence excitation spectra from B-850 light-harvesting pigment-protein complex containing spheroidene. The excitation spectrum (●●●), measured at 858 nm using an RG715 and 858 interference filter over the photomultiplier, was normalized to the absorption spectrum (———) at the 590 nm BChl absorption band. For determination of the efficiency of carotenoid → BChl energy transfer the fluorescence excitation spectrum was normalized to the fractional absorption spectrum of the complex. The concentration of bacteriochlorophyll was 4 μM.

Fig. 5. Photodestruction of B-850 light-harvesting pigment-protein complex with and without neurosporene. The irreversible bleaching of the 850 nm absorption band in complexes with (●———●) and without (▲———▲) neurosporene. The complexes (in 10 mM Tris-HCl, pH 8.0) were illuminated in a cuvette by strong white light (900 $W \cdot m^{-2}$) previously passed through 5 cm of water acting as a heat filter. Air was bubbled through the cuvette during illumination. In each case the concentration of bacteriochlorophyll was 6 μM.

complex it shows a strong induced CD spectrum (Fig. 3). This induced CD spectrum is only apparent when the carotenoid is bound to the B-850 light-harvesting pigment-protein complex. No CD spectrum was detected for the carotenoid in organic solvent, in detergent solution, in liposomes or bound to bovine serum albumin. Because of this and the strength of the induced CD bands the data suggest that the carotenoid is indeed being bound specifically to the B-850 light-harvesting pigment-protein complex.

The isolated, solubilized B-850 light-harvesting pigment-protein complexes are optically clear. Fig. 4 shows a typical fluorescence excitation spectrum for a sample of B-850 light-harvesting pigment-protein complex reconstituted with spheroidene. With both neurosporene and spheroidene the efficiency of energy transfer from the carotenoid to the bacteriochlorophyll, 60—70%, is higher than that estimated for the reconstituted chromatophores. This compares very well with the efficiency of the carotenoid to bacteriochlorophyll energy transfer in the B-870 light-harvesting pigment-protein complex of *Rps. sphaeroides* isolated by lithium dodecyl sulphate polyacrylamide gel electrophoresis [20].

The fact that this energy transfer has been reconstituted with such a high efficiency again suggests that most of the carotenoid must be binding specifically to the B-850 light-harvesting pigment-protein complex. Carotenoids are generally assumed to be non-fluorescent [21] and this therefore implies that they have an extremely short-lived first excited singlet state. If singlet-singlet energy transfer from the carotenoid to the bacteriochlorophyll is going to occur with high efficiency then those molecules must be held very close to each other and have their respective transition moments favourably aligned.

Photodestruction of the B-850 light-harvesting pigment-protein complex

In the absence of added carotenoids the bacteriochlorophyll in the B-850 light-harvesting pigment-protein complex is rapidly and irreversibly photodestroyed by illumination with strong white light in the presence of oxygen (Fig. 5). The presence of carotenoid (in this case neurosporene) gives a large measure of protection against this photodestruction (Fig. 5). It is probable that the true degree of photoprotection is higher than that shown in Fig. 5 since there are undoubtedly some complexes present without carotenoid. Spheroidene also gives good photoprotection.

During the progress of the photodestruction of the bacteriochlorophyll the absorption maximum of the near-infrared absorption band shifted proressively to shorter wavelengths. For the experiment depicted in Fig. 5 with the carotenoidless B-850 complex, after 20 min the maximum had shifted from 850 nm to 845 nm. This agrees well with the recent finding of Rafferty et al. [22], who showed that this small shift was due to one of the two bacteriochlorophylls which make up this absorption band being bleached (i.e. the bacteriochlorophyll goes from a dimeric state to a monomeric state).

We are at present continuing this study with a wider range of carotenoids and also trying to determine accurately how many carotenoids bind to the B-850 complex per bacteriochlorophyll.

Acknowledgements

This study was supported by a grant from the S.R.C., E.D. is an S.R.C. predoctoral scholar. We wish to thank Professor G. Wood and Mr. G. Medlow, Strathclyde University, Glasgow, for running the CD spectra for us, Mrs. I. Durant for expert technical assistance and Dr. V.B. Math for determining the mass spectra of the carotenoids used in the reconstitution. We are also grateful to Dr. M.F. Hipkins for critically reading the manuscript.

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