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A specific carotenoid is required for reconstitution of the *Rubrivivax gelatinosus* B875 light harvesting complex from its subunit form B820

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Abstract The core light-harvesting complex B875 from *Rubrivivax gelatinosus* has been reconstituted from its subunit form B820 with hydroxyspheroidene, the carotenoid which is bound to native B875 antenna. Other carotenoids which are chemically similar to hydroxyspheroidene (spheroidene, spheroidenone, neurosporene and spirilloxanthin) gave only low levels of partial reconstitution. Absorption and circular dichroism spectra of the hydroxyspheroidene-containing, reconstituted B875 were identical with those of original B875 antenna isolated directly from chromatophores, indicating that the two hydroxyspheroidene molecules bind to their native sites within the $(\alpha\beta)Bchl_2$ subunit during the reconstitution process. These observations point to a structural role for this carotenoid in determining the architecture of *Rv. gelatinosus* B875 antenna.

Key words: Photosynthesis; Purple bacteria; *Rubrivivax gelatinosus*; Carotenoid; Core light-harvesting complex; Antenna

1. Introduction

The core light-harvesting antenna absorbing at about 875 nm (known as B875) has been successfully purified from several species of purple non-sulfur bacteria [1–7]. The complex is a large oligomer built up from small heterodimers α/β, each α/β unit binding non-covalently 2 molecules of BChl *a* and 1 or 2 molecules of carotenoid, depending on the species. It was estimated that the size of the B875 oligomer in vivo is 12, that is, 12 heterodimers surround a reaction center [7,8]. This size is also indicated by analytical centrifugation experiments done on the isolated B875 complex from *Rubrivivax gelatinosus* (Jirsakova et al., unpublished results). The B875 complexes from *Rhodospirillum rubrum* or *Rhodobacter sphaeroides* have been intensively studied; it has been discovered that, by treating them with high concentration of detergent [3,7–9], they can be converted to a smaller oligomer (which is probably a heterotetramer $(\alpha/\beta)_2$ [8,10] or a heterodimer α/β [11]) absorbing at 820 nm. However, this cleaveage occurs only if the carotenoid molecules are removed from the B875 complex at some stage during B820 preparation, indicating the involvement of carotenoids in the structural stability of the B875 complex. *Rv. gelatinosus* B875 can also be transformed into a smaller B820 form, but by a different treatment where a high concentration of ammonium sulfate is used [12]. The formation of B820 occurs together with the loss of carotenoids which were irreversibly adsorbed on the gel matrix during hydrophobic chromatography.

Moreover, by decreasing the detergent concentration, the B820 complexes from *R. rubrum* [3,5] and *Rb. sphaeroides* [9] could be reassociated into an oligomer with an infrared absorption slightly blue-shifted from the original B875 one, i.e. at 873 nm. Other spectral properties of these reconstituted complexes are close to that of native B875. Conversely, various attempts at the reconstitution of *Rv. gelatinosus* B875, starting with B820, have been unsuccessful. Under different conditions supposed to favour reconstitution (high protein concentration, absence of ammonium sulfate and/or lowering detergent concentration) we have observed that the infrared absorption band was shifted to about 850 nm and became very broad [12]. We interpreted the 850 nm broad band as due to an incorrect reaggregation of B820 into abnormal oligomers. This heterogeneity was supported by low temperature absorption spectra (taken in presence of glycerol) where several spectral bands were clearly resolved (unpublished).

In this work, we describe a succesful reconstitution of *Rv. gelatinosus* B875 from its subunit form B820. The reconstitution occurred only if the carotenoid which is bound to this antenna in its native state, hydroxyspheroidene, was added to the B820 and if the ammonium sulfate was dialysed out. The absorption and CD spectra of reconstituted B875 and of purified, native, B875 were identical. Other carotenoids which are structurally very close to hydroxyspheroidene: spheroidene, hydroxyspheroidenone, or spirilloxanthin gave no reconstitution or a much less efficient one. These results demonstrate an important role of carotenoid molecules in mediating the structure of *Rv. gelatinosus* core antenna, which has not been observed for *R. rubrum* or *Rb. sphaeroides*.

2. Materials and methods

Core antenna, B875, and its subunit form, B820, were isolated and purified as described previously [12]. The B820 samples were prepared and stored in 0.1 M Tris-HCl, 1 mM EDTA, 3 mg/ml $C_{10}S$, 2 M $(NH_4)_2SO_4$, pH 8.0.

Carotenoids (hydroxyspheroidene, neurosporene, spheroidene, hydroxyspheroide-none and spirilloxanthin) were prepared from chromatophores as follows: 5 vols. of acetone/methanol (7:2, v/v) were added to 1 vol. of chromatophores (OD at 860 nm was about 100). After a 10 min extraction a low speed centrifugation (2000 × g) was performed. The supernatant was collected and the pellet re-extracted with acetone/methanol until it was colourless. The pooled supernatants were dried under vacuum and immediatly dissolved in diethylether. TLC on Silica gel 60 (Merck) was performed with acetone/petroleum ether (1:9, v/v) as developing solvent. The spots containing the required carotenoids were scraped off and put into 1 ml of acetone. Acetone extracts of carotenoids were stored at −20°C.

Reconstitution of B875 from B820 was performed at 20°C by two alternative methods. In method A, carotenoid in acetone solution was

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Abbreviations: BChl *a*, bacteriochlorophyll *a*; CD, circular dichroism; $C_{10}S$, decanoylsucrose; EDTA, ethylenediaminetetraacetate; Tris, Tris(hydroxymethyl)amino-methane; *Rv.*, *Rubrivivax*; *R.*, *Rhodospirillum*; *Rb.*, *Rhodobacter*.

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dried under nitrogen as a thin film in a glass tube and dispersed with extensive stirring in 100 mM Tris-HCl buffer, containing 1 mM EDTA and 3 mg/ml $C_{10}S$ (Calbiochem). As the carotenoids are susceptible to photooxidation, their absorption spectra were checked. An aliquot of this solution was mixed with B820 sample. In method B, a B820 sample was added directly to dried carotenoid, mixed on a Vortex and incubated 30 min. In both methods, an excess of carotenoid (about 2 times relative to BChl *a* molarity) was necessary because of its low solubility.

Microdialysis was performed to remove the ammonium sulfate from the mixture. Purification of B875 after reconstitution was performed by gel filtration on a small Ultrogel AcA44 (IBF Biotechnics) column (0.9 cm of diameter, 12 cm of length). The column was equilibrated with 0.1 M Tris-HCl buffer, 1 mM EDTA, 3 mg/ml $C_{10}S$, pH 8.0.

Absorbance spectra were recorded on a Cary-2300 spectrophotometer. CD spectra were measured on a Jobin-Yvon Mark 5 dichrograph coupled with a minicomputer; cylindrical cuvettes of 2 mm optical path were used.

3. Results and discussion

Two hydroxyspheroidene molecules are originally bound to the basic subunit (α/β)$Bchl_2$ of *Rv. gelatinosus* B875 when the antenna is isolated in a native state [12]. These carotenoid molecules are released when B875 is dissociated into the B820 subunit form. We tried therefore to reconstitute the B875 antenna from B820 and different carotenoids found in chromatophores of *Rv. gelatinosus*. Fig. 1 shows the structural formulae of these carotenoids, which differ in the number of conjugated double bonds and in nature of substituents at the extremities

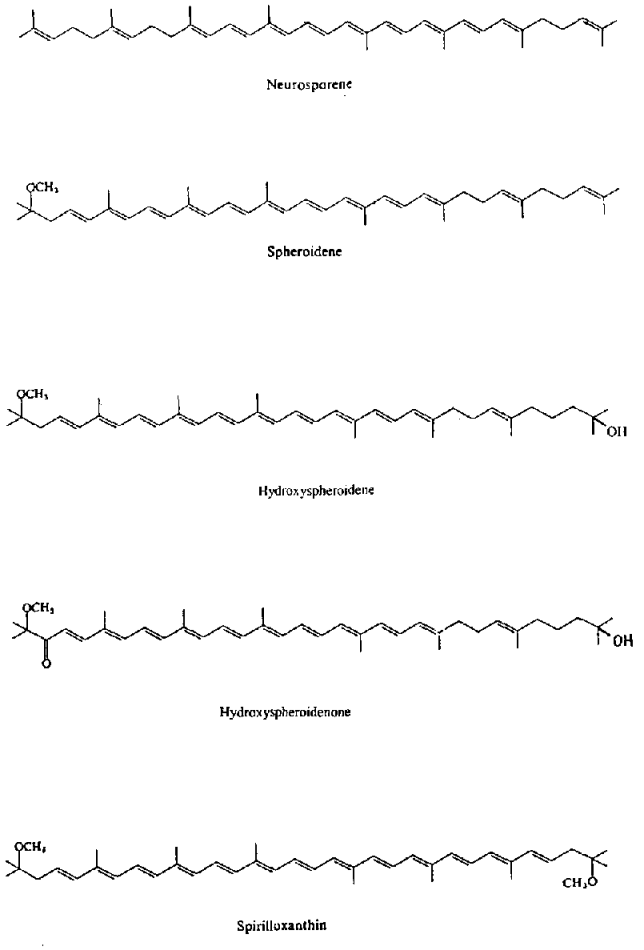

Fig. 1. Structural formula of the carotenoids used in this work.

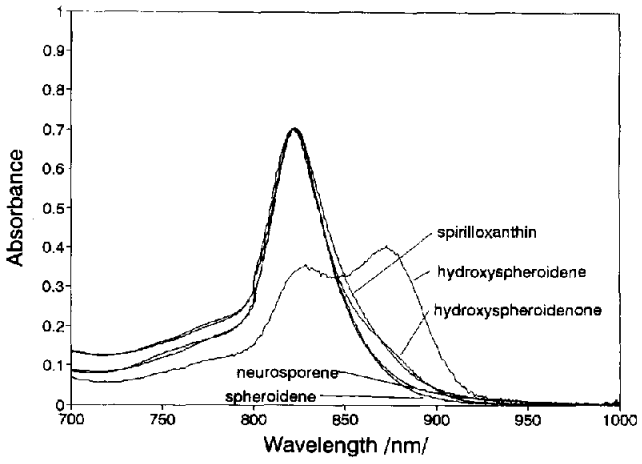

Fig. 2. Infrared absorption spectra of B820 with addition of different carotenoids, after 1 h incubation.

of the molecules. Fig. 2 demonstrates changes in the Q_y absorption band of B820/carotenoid mixtures, observed one hour after carotenoid addition. When hydroxyspheroidene was added, we observed an immediate shift of part of the 820 nm band to 875 nm although ammonium sulfate was still present; this salt was shown to be necessary for dissociation of B875 and for stability of the B820 form [12]. Much smaller shifts were observed with spirilloxanthin and hydroxyspheroidenone, as indicated by a slight broadening of the Q_y band on its redmost side. Almost no shift was induced by neurosporene and spheroidene.

After dialysis of these mixtures against buffer without ammonium sulfate the absorption spectra were again recorded (Fig. 3). Note that the absorbance spectrum of the sample containing hydroxyspheroidene has been scaled down two times for easier comparison with the other spectra. Taking into account the dilution during the dialysis, reconstitution of B875 was almost complete with hydroxyspheroidene; small amounts of 'free' BChl *a* absorbing at 777 nm and of residual B820 were observed, but the spectrum was dominated by the 875 nm band.

With all other carotenoids, the mixtures became quite turbid after dialysis to remove ammonium sulfate. Absolute infrared absorbance strongly decreased in the order spirilloxanthin > hydroxyspheroidenone > neurosporene > spheroidene; and the samples presented a band at 777 nm and another very broad one, with maxima at 820 and in the 850–870 nm range. Thus these carotenoids were unable to bring about a reversible B820–B875 transition; and furthermore they did not protect the B820 subunit form against degradation due to the absence of ammonium sulfate. This degradation was observed in control experiments performed in the absence of carotenoid (not shown).

The reconstituted B875 antenna was separated from excess hydroxyspheroidene by gel filtration of the dialyzed mixture and the absorption and CD spectra of the purified complex were checked. Fig. 4 shows an almost perfect identity in normalized absorption spectra of the original B875 antenna and the reconstituted complex; only very slight differences were observed in the Soret band intensity and the position of the main infrared maximum which was slightly concentration dependant. The ratio of bound carotenoid to Bchl, i.e. the ratio A_{875}/A_{480}, was the same in both preparations. The CD spectra of native B875 [12] and of reconstituted one (Fig. 5 and insert)

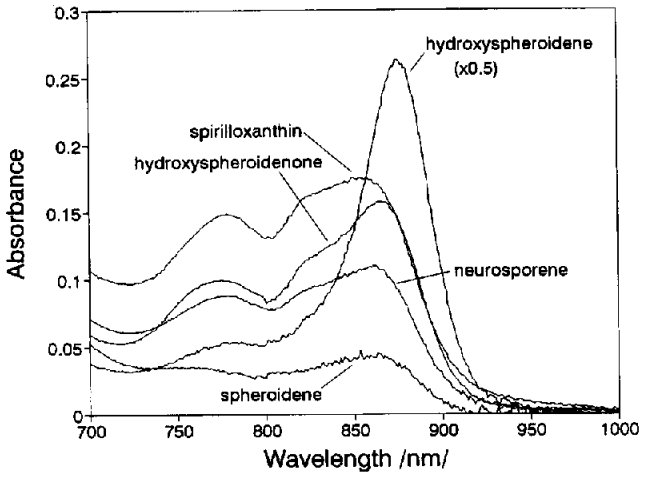

Fig. 3. Infrared absorption spectra of the same solutions as in Fig. 1, after removal of the ammonium sulfate by dialysis.

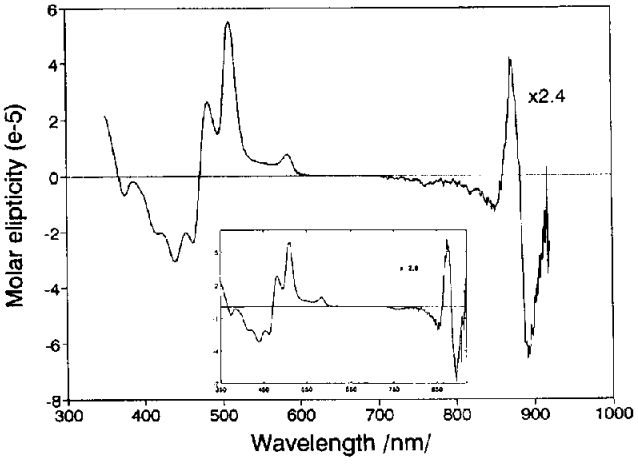

Fig. 5. CD spectum of reconstituted B875 and isolated B875 (insert, taken from [10]).

were identical, both in the BChl and in the carotenoid absorption bands. It should be noted that the infrared CD spectrum of the B820 form is blue-shifted comparing with the B875 one [12]. Dichroism of these pigments is a very sensitive probe of their localisation, that is their interactions with other pigments and with the protein matrix. It may be concluded that during reconstitution of the B875 antennae the hydroxyspheroidene molecules were correctly inserted at the same time as the interactions between the Bchl molecules within the pigment–protein assembly were restored.

These results indicate a structural role of carotenoid molecules in the reversible reconstitution of *Rv. gelatinosus* B875 from the B820 form. This reconstitution occurs specifically with hydroxyspheroidene; other carotenoids which are not bound to this antenna in vivo cannot be substituted for it. In other bacteria (*Rb. sphaeroides, R. rubrum*), B875 could be reconstituted from B820 without the addition of any carotenoids [3,8,9]. In *R. rubrum*, B875 has even been reconstituted from isolated α,β polypeptides and free BChl *a* [13]. But in these reconstituted

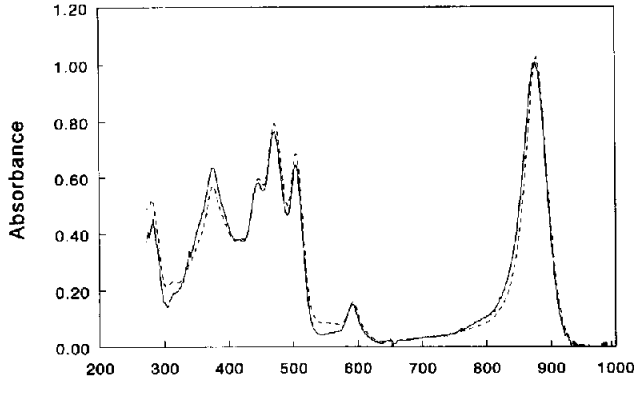

Fig. 4. Absorption spectra of isolated B875 (dashed line) and of B875 reconstituted from B820 and hydroxyspheroidene (full line), after purification by gel filtration. Both samples were solubilized in 0.1 M Tris-HCl, 1 mM EDTA, 3 mg/ml $C_{10}S$, pH 8.0. Spectra were normalized to the same infrared absorption.

antennae the CD spectra were not strictly identical with those of these antennae measured either in chromatophores, or in the isolated state (when its cleavage into B820 is avoided). The diversity of the biological and chemical properties amongst the photosynthetic complexes of different purple bacteria is demonstrated in recent work of Kerfeld et al. [14] who showed that B820 form can be prepared also from *Chromatium purpuratum* with the carotenoid remaining bound to the protein; however, no reconstitution of B875 was observed when the concentration of detergent was lowered.

The localisation and conformation of carotenoids have been recently described in the LHCII antenna from green plants [15]. Molecular models for the carotenoids bound to bacterial antenna are still lacking, despite promising crystallization of several of these complexes (see [16] for a recent review). Carotenoids are essential for peripheral antenna complex formation in both *Rb. capsulatus* [17] and *Rb. sphaeroides* [18]; none of these complexes being formed when carotenoid synthesis was genetically interrupted in *Rb. sphaeroides*. In the core antenna of *R. rubrum*, biochemical arguments indicate that the carotenoids interact with the N-termini of the α/β polypeptides probably near the cytosolic side of the membrane, and protect the N-terminus of the α polypeptide against proteinase K digestion [19]. A series of site directed mutations in *Rb. capsulatus* core antennahave revealed that one residue of the α polypeptide, Trp8 (in *Rv. gelatinosus*, the equivalent residue is in position 5) could be involved in interactions with carotenoid [20]. This Trp residue is highly conserved in all α subunits of core and peripheral antennae sequenced to date from about 30 purple sulfur and non-sulfur bacteria [21]. When this Trp was changed for Ala or Leu in *Rb. capsulatus*, the mutants failed to synthetise the core complex. When Trp was changed for Tyr, the bound carotenoid content was reduced by 60%. We can speculate that in *Rv. gelatinosus* the terminal substituents of hydroxyspheroidene molecules mediate the interactions between (α/β) subunits which determine the precise architecture of B875. A possible hypothesis for the specificity of this carotenoid would be that the hydroxyl group on the C1′ end is H bonded to the α Trp5 residue. Neurosporene does not posses any terminal polar group and spheroidene has only one methoxy group, and so cannot replace hydroxyspheroidene. Spirilloxanthin also contains a methoxy group instead of a hydroxyl group; besides

it is is more planar and rigid than hydroxyspheroidene because of 13 conjugated double bonds. Hydroxyspheroidenone differs from spheroidene by an additional keto group at the C1 end, which could prevent reconstitution because of steric or polar effects. It would be of interest to test these hypotheses by a detailed study of the interactions of carotenoids with the protein matrix, especially by Raman spectroscopy.

The reconstitution of B875 has been tested in this work using only absorption and CD spectral characteristics. Measurements of stationary and time-resolved fluorescence would be useful for further checking the functional integrity of the reconstituted antenna; such measurements are in progress.

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