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Characterization of the core complex of *Rubrivivax gelatinosus* in a mutant devoid of the LH2 antenna

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Abstract

The core complex of purple bacteria is a supramolecular assembly consisting of an array of light-harvesting LH1 antenna organized around the reaction center. It has been isolated and characterized in this work using a *Rubrivivax gelatinosus* mutant lacking the peripheral LH2 antenna. The purification did not modify the organization of the complex as shown by comparison with the intact membranes of the mutant. The protein components consisted exclusively of the reaction center, the associated tetraheme cyt c and the LH1 αβ subunits; no other protein which could play the role of pufX could be detected. The complex migrated as a single band in a sucrose gradient, and as a monomer in a native Blue gel electrophoresis. Comparison of its absorbance spectrum with those of the isolated RC and of the LH1 antenna as well as measurements of the bacteriochlorophyll/tetraheme cyt c ratio indicated that the mean number of LH1 subunits per RC-cyt c is near 16. The polypeptides of the LH1 antenna were shown to present several modifications. The α one was formylated at its N-terminal residue and the N-terminal methionine of β was cleaved, as already observed for other *Rubrivivax gelatinosus* strains. Both modifications occurred possibly by post-translational processing. Furthermore the α polypeptides were heterogeneous, some of them having lost the 15 last residues of their C-terminus. This truncation of the hydrophobic C-terminal extension is similar to that observed previously for the α polypeptide of the *Rubrivivax gelatinosus* LH2 antenna and is probably due to proteolysis or to instability of this extension.

Keywords: Light-harvesting antenna; Reaction center-LH1 core complex; Purple bacteria; *Rubrivivax gelatinosus*

1. Introduction

In all photosynthetic organisms from bacteria to plants, specialized pigment–protein complexes called light-harvesting antenna (LH) harvest the light energy and transfer it efficiently to the reaction centers (RCs) where it is converted into charge separation, initiating electron transfer. In most purple photosynthetic bacteria two types of LH can be distinguished from their in vivo localisation and their spectral properties: LH1 which are tightly associated with the RC in the so-called (LH1–RC) core complex, and LH2 which are more loosely interacting with the core complex and which are present in variable amounts with the growth conditions. In the near infrared the LH1 bacteriochlorophyll (Bchl) absorption occurs at about 870 nm, and the LH2 one at about 850 nm. Our knowledge about the energy transfers between these complexes in the photosynthetic membranes has greatly progressed (see [1,2]), owing to extensive biophysical and spectroscopic studies, and to the description at atomic resolution of the structure of the RC [3–5] and of LH2 [6,7] from several bacterial species. Lower resolution models are also available for LH1s [8] as well as for LH1–RC core complexes [9–11]. LH1 and LH2 are oligomeric integral membrane proteins, built from two hydrophobic polypeptides α and β, each containing a single transmembrane α helix. Each αβ pair is associated with pigments (bacteriochlorophyll (Bchl) and carotenoids). When isolated in detergent solution, or reconstituted in lipid bilayers, the antenna adopt a ring-like structure formed by association of 8–9 αβ pairs for LH2, and 15–16 αβ pairs for LH1. The reversible dissociation of the isolated LH1 antenna into αβ

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subunits has been demonstrated for several bacteria [12–15] and used extensively for evaluating the forces stabilizing the LH1 structure [16]. In the LH2 structures the α polypeptides form the inner part of the ring and the β ones the outer part. In the core complex, the central cavity of the LH1 ring is occupied by the RC. Recent studies performed on native membranes reveal that these oligomeric structures are also present in vivo [17–23]. However there is still a debate about the core complex structure, as to whether the LH1 ring around the RC is complete or not in some bacteria. The arguments against the formation of a closed ring of LH1 rest on functional and structural observations. It has been stressed that a closed ring would hinder the diffusion of quinone molecules exchanging between the membrane quinone pool and the RC-bound secondary quinone Q_B. In *Rhodobacter* (*Rb.*) *sphaeroides* a small hydrophobic protein called PufX is associated with the core complex, and plays a role in facilitating this exchange [24,25]; it is encoded by a gene located in the *puf* operon coding for the LH1 subunits and for two of the subunits of the RC, L and M [26]. In strains expressing PufX the LH1 ring is incomplete and adopts a S-shape around two RCs [11,17,18]; whereas in $PufX^-$ strains the ring is closed and the LH1–RC complex is monomeric [18,27,28]. A S-shape dimeric assembly has also been observed for *Rb. blasticus* [21], another bacterium with a *pufX* gene located in the *puf* operon [29]. On the other hand, several reports concluded to the presence of a closed LH1 ring around one RC in various species such as *Blastochloris* (*Bl.*) *viridis* [19], *Rhodospirillum* (*Rs.*) *rubrum* [7,11,30] and *photometricum* [20]; in the two former species the *puf* operon has been sequenced and does not contain *pufX*. Based on these observations, it would be tempting to conclude that the absence of PufX correlates with a closed, monomeric core complex structure. But it is not consistent with the structure of the core complex isolated from *Rhodopseudomonas* (*Rps.*) *palustris*, another bacterium without PufX. In this structure [10] the LH1 ring around the RC is interrupted by a single trans-membrane helix replacing one of the LH1 αβ pairs. This helix could belong to a 15 kDa polypeptide detected in the core complex; this polypeptide would thus be functionally equivalent to PufX.

In this context we present a biochemical characterization of the core complex of *Rubrivivax* (*R.*) *gelatinosus*. This purple bacterium belongs to the β group of proteobacteria and the organisation of its *puf* operon is known to be different from those of the α group such as *Rb. sphaeroides* and *Rb. capsulatus* [31,32]. Notably it does not contain a gene analogue to *pufX*; the *pufC* gene coding for the tetraheme cyt c bound to the RC is found instead. Furthermore the genes *pufB* and *pufA* coding respectively for the β and α polypeptides of LH1 are preceded by *orf1* (coding for a putative soluble protein) and separated by *orf2* (coding for a putative transmembrane protein); the function of these two orfs is unknown. The photosynthetic apparatus of *R. gelatinosus* is located in the cytoplasmic membrane which is devoid of extensive invaginations and which presents at most a few short tubules [33]. Whereas the nonameric structure of *R. gelatinosus* LH2 has been recently demonstrated [34,35], the structure of its core complex has not been studied so far. Isolation of the core complex from wild type strains was already reported [36,37] but in our hands the yield was quite low. As a prerequisite for structural studies we have now isolated in good yield and in a native state the core complex of a *R. gelatinosus* mutant lacking the LH2 antenna. We paid particular attention to the composition of the complex, in order to determine if it contained another component in addition to the RC, to the tetraheme cyt c and to the LH1s. No such component was found. On the other hand we observed that the LH1 subunits of the isolated core complex presented several modifications. In particular, the α polypeptide was heterogeneous, consisting of a mixture of the full-length polypeptide and of a truncated one lacking the last 15 residues of its C-terminus. Only the truncated polypeptide was detected previously by direct sequencing of the protein [38], and it was proposed later to result from a post-translational processing [31]. In fact this truncation is similar to that observed for the α polypeptide of the isolated LH2 antenna [34] and probably occurs by the same mechanisms.

2. Materials and methods

2.1. Materials

The following detergents were used: *n*-nonyl β-D-glucopyranoside (C9G, Bachem), 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS, Fluka) and sodium cholate (Serva).

2.2. Bacterial growth conditions

Wild type *R. gelatinosus* (strain S1) and the PUCAΩ strain (containing a *pucA* gene interrupted by a Ω cartridge, therefore devoid of LH2) were grown at 30 °C anaerobically in the light in completely filled 2 L bottles in malate medium [39], with streptomycin (25 μg/ml) and spectinomycin (25 μg/ml) added to the growth medium for the mutant.

2.3. Purification of membranes

Cells were suspended in 50 mM Tris–HCl buffer, pH 8, containing 5 mM EDTA and 1 mM PMSF and broken in a French press. Crude membranes were isolated by differential centrifugation, washed in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8) and suspended in TE buffer containing 5% (w/w) sucrose. They were purified on a discontinuous sucrose gradient, formed by 4 layers containing respectively 15%, 30%, 40% and 50% (w:w) sucrose in TE buffer; after 2 h centrifugation at 200,000 g the coloured bands were recovered, diluted with TE buffer and spun down (200,000 g, 45 min). In a few experiments a continuous 15–50% sucrose gradient and overnight centrifugation (200,000 g) were used.

2.4. Isolation of photosynthetic complexes

R. gelatinosus strain S1 was used for isolating the RC and the LH1 antenna following published procedures [15,37]. The mutant PUCAΩ (lacking LH2) was used for the isolation of the core complex. Crude membranes were isolated as above and washed in TE buffer containing 1 M NaCl. The pellet was suspended in TE buffer to an OD_{877nm} of 40 and solubilised at 5 °C by the addition of C9G and CHAPS at final concentrations of 1% and 0.5% respectively. After 30 min incubation and centrifugation (1 hr, 200,000 g) the crude core complex was recovered in the supernatant. It was purified by a combination of ion exchange chromatography on DEAE-Sepharose Fast Flow (Amersham) with gradient elution (0 to 0.4 M NaCl in 10 mM Tris–HCl buffer, 1 mM EDTA, 0.2% C9G, 0.2% CHAPS, pH 8), followed by molecular sieve chromatography on Sepharose CL-6B (Amersham) in 50 mM Tris–HCl

buffer pH 8, containing 50 mM NaCl, 0.2% C9G and 0.2% Na cholate. In some experiments 1 mM benzamidine hydrochloride and 5 mM ε-amino caproic acid Na salt were added as protease inhibitors to all the above buffers. In several preparations, an additional chromatography was finally performed on a Superdex 200 (Amersham) FPLC column eluted with the same buffer as for the Sepharose. The purification was monitored by SDS-gel electrophoresis of selected fractions and by the absorption spectra measured on a Cary 500. The purity index of the core complex (I) was expressed as the (A_{875nm}/A_{280nm}) ratio.

2.5. Biochemical techniques

Native Blue-electrophoresis was performed as described [40] at 5 °C on 4–13% acrylamide gradient gels using the "slightly blue" cathode buffer containing 0.001% Coomassie Blue G-250 (Serva); the core complex samples contained 15% glycerol and 0.1% Na cholate. SDS-PAGE [41] was performed on minigels stained either with Coomassie blue R250 or with silver reagent (Biorad). Samples were prepared for SDS-PAGE either by direct dilution in the sample buffer or by acetone/methanol (7:2, v/v) precipitation at 5 °C followed by a low-speed centrifugation, and dissolution of the precipitate in a sample buffer containing 40 mM dithioerythritol. Heme c staining was performed by its (tetra-methyl-benzidine)-peroxidase activity [42]; for these assays dithioerythritol was omitted from the samples. Blots of SDS gels on polyvinylidene fluoride membranes (Amersham) were used for the determination of the N-terminal aminoacid sequences of selected bands by automated microscale Edman degradation.

Bchl concentration was measured after cold ethanol extraction using $\varepsilon = 62$ $mM^{-1}cm^{-1}$ at 770 nm [43]. Comparison with the absorption measured at 875 nm for the intact core complex in detergent solution indicated that in the latter case $\varepsilon = 127$ $mM^{-1}cm^{-1}$ for the total Bchl.

Protein concentration was measured with the bicinchoninic acid reagent (Pierce). In the purified complex, 1 mg/ml corresponded to an $OD_{875nm} = 13$.

The amount of tetrahemic cytochrome *c* in purified core complex was determined by the pyridine hemochrome method, using $\Delta\varepsilon = 23.97$ $mM^{-1}cm^{-1}$ for the differential absorption of 1 heme c measured at (550 nm minus 535 nm) [44].

The carotenoids present in the purified core complex were extracted and analysed by HPLC as already published [45].

2.6. Characterization of the LH1 polypeptides

The α and β polypeptides of the LH1 antenna were separated by reverse phase HPLC on a Kromasil C4 column as described [34], using core complex samples previously delipidated [46] and solubilized in 60% acetic acid. The column resolved the preparations in two well separated peaks, which were dried and submitted in parallel to SDS-PAGE (after dissolution in SDS buffer [41]) and to mass spectrometry (after dissolution in 60% acetic acid).

2.7. Spectroscopic techniques

Absorption spectra were recorded with a Cary 500 (Varian) spectrophotometer. A multiple regression analysis program provided by Origin.6 software (Microcal) was used to fit the core complex infrared spectrum by combining those of the isolated LH1 and of the RC. The following extinction coefficients were used for deriving from the best fit the molar LH1/RC ratio:for the RC $\varepsilon_{802\ nm} = 288$ $mM^{-1}cm^{-1}$; for LH1 $\varepsilon_{875\ nm} = 250$ $mM^{-1}cm^{-1}$ [15].

MALDI-TOF mass spectrometry experiments were performed by using a Voyager DE STR instrument (Applied Biosystems, Les Ulis, France) fitted with a UV pulse nitrogen laser (337 nm). The matrix used was the di-hydroxy-benzoic acid at 20 mg/ml in a acetonitrile/H_2O /trifluoroacetic acid (70/30/0.1%, v/v/%) solution. Three types of treatment were used before mixing with the matrix: dilution with water of core complexes in C9G solutions; precipitation of core complexes by acetone/methanol 7/2 (v/v) followed by dissolution of the protein precipitate in 60% acetic acid; and 60% acetic acid solutions of isolated antenna polypeptides (see above). Then the sample was deposited on the MALDI target plate according to the so called "dried droplet" method and analysed without further treatment. Instrument parameters were the following: linear acceleration voltage of 25 kV; grid value of 94%; delay extraction of 300 nsec. Mass spectrum calibration was realized by using a standard peptide mixture (pepmix 3) with masses ranging from 3495 to 6508 (LaserBiolabs, SophiaAntipolis, France).

2.8. Freeze-fracture and electron microscopy of purified membranes

A membrane suspension was placed between a thin copper holder and a thin copper plate and quenched in liquid propane, as described [47,48]. The frozen sample was fractured at −125 °C in vacuum of about $1 \cdot 33 \times 10^{-5}$ Pa by removing the upper plate with a liquid-nitrogen-cooled knife in a Balzers 301 freeze-etching unit. The fractured sample was etched at −105 °C for 3–5 min and a replica was produced with platinum-carbon or tungsten-tantalum (1.0–1.5 nm of metal deposited), backed with about 20 nm of carbon. The replica was cleaned by overnight incubation with chromic acid, washed with distilled water and observed in a Philips CM120 electron microscope.

3. Results

3.1. Fractionation of the cytoplasmic membranes of the wild type and PUCAΩ strains

The PUCAΩ strain is a LH2 deficient, photosynthetic mutant harboring a *pucA* gene (coding for the LH2 α polypeptide) disrupted by a Ω cartridge; it differs from the wild type strain by a lower growth rate under anaerobic illumination, and by its infrared absorption reflecting the absence of LH2 [39] (Fig. 1). Whereas the wild type infrared absorption is dominated by the LH2 contribution peaking at 860 and 800 nm, the spectrum of the mutant shows predominantly that of the LH1 antenna peaking at 877 nm, and minor bands at 800 and 756 nm ascribed respectively to the RC accessory Bchls and Bpheophytins. We observed also that the carotenoid absorption bands were blue-shifted and better resolved in the mutant as compared to the wild type (Fig. 1). It is known that *R. gelatinosus* contains a variety of carotenoids belonging to the spheroidene and spirilloxanthin biosynthetic pathways [32]. The blue-shift indicates that the mutant contains relatively more carotenoids of the spheroidene branch than the wild type (see below).

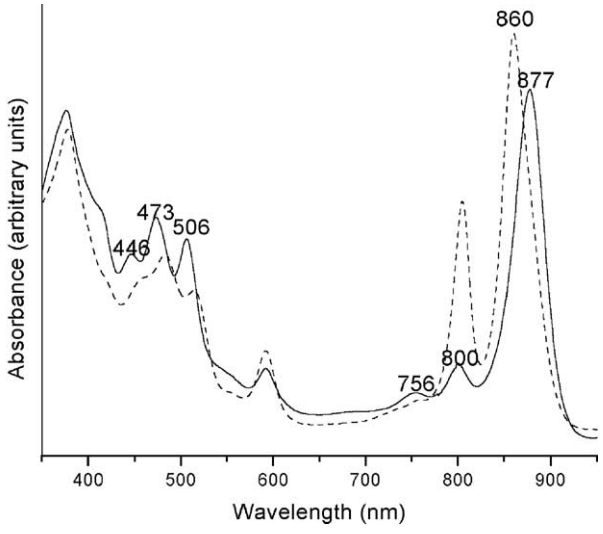

Fig. 1. Absorption spectra of *R. gelatinosus* membranes, suspended in 40% sucrose. Solid line : PucAΩ mutant. Dashes: wild type S1 strain. The spectra have been arbitrarily normalized to a similar Soret band absorbance.

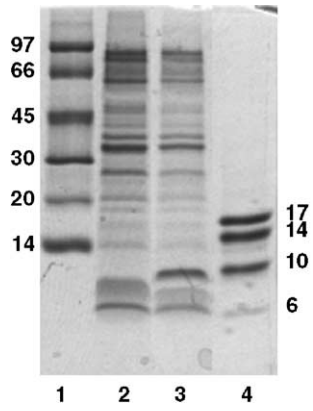

Fig. 2. Coomassie Blue-stained SDS-gel of membranes from the PucAΩ mutant (lane 2) and the wild type S1 strain (lane 3). Lanes 1 and 4 show the position of molecular weight standards with molecular weights in kDa.

The absence of LH2 in the mutant is clearly illustrated by SDS gel electrophoresis, where the only difference between the two strains concerned the low molecular weight range: two bands identified with the LH1 polypeptides were observed for the mutant, whereas three bands were observed for the wild type originating probably from the superposition of the LH1 and LH2 polypeptides (Fig. 2).

The lack of LH2 in the mutant could promote a modification of the membrane morphology as observed for *Rb. sphaeroides*, where this type of mutation resulted in the formation of tubular intracytoplasmic vesicles [17,18,49]. Therefore we compared the membrane particles isolated from photosynthetically grown cells of the wild type and of the mutant. After fractionation on a discontinuous sucrose gradient by rate zonal centrifugation, two pigmented bands located respectively at sucrose boundaries of 30%–40% and 40%–50% were observed for both strains; the faster migrating band was less abundant for the wild type than for the mutant. For each strain the two bands presented the same absorbance spectrum (apart of a higher turbidity of the faster migrating band); their SDS-PAGE polypeptide patterns showed only slight differences in the relative amounts of minor components (not shown). Furthermore when the gradient centrifugation was prolonged to equilibrium, only one pigmented band was observed (at 42% sucrose). Therefore the transient fractionation of the membrane particles came probably from a broad size distribution of broken membrane particles, rather than from the presence of two classes of vesicles differing by their proteic components.

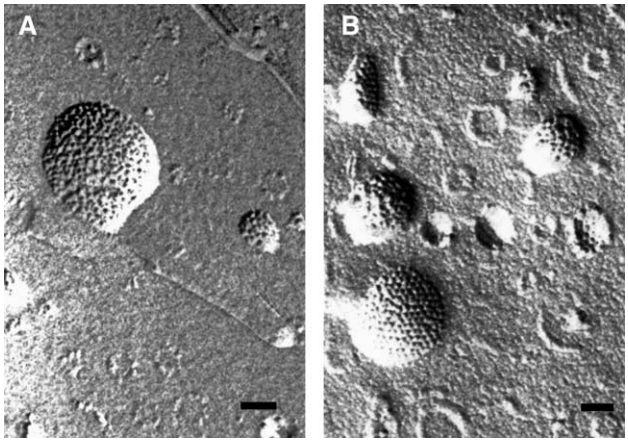

Fig. 3. Freeze-fracture electron microscopy images of purified membranes of *R. gelatinosus* wild type (A) and PUCAΩ mutant (B). The vesicles are full of densely packed proteins. In panel B the packing shows a more organized arrangement, indicating a possible two-dimensional lattice. Scale bar: 100 nm.

Freeze-fracture experiments were performed on purified membrane fractions of the wild type and mutant strains. In both cases we observed mainly small vesicles overcrowded with particles. With the mutant ordered arrays of particles were sometimes observed (Fig. 3). Otherwise no particular membrane structures, such as tubes or stacked membrane sheets, were apparent, as have been observed in *Rb. sphaeroides* [17,18,49] or *Bl. viridis* [50]. Hence the ultrastructure of the membranes was not modified by the absence of LH2.

3.2. Purification and spectroscopic characterization of the core complex

In a previous work the isolation of the core complex from wild type *R. gelatinosus* was described [51]. The complex was shown to consist of the L, M and H subunits of the RC (which still contained quinones), the tetraheme cytochrome *c* and the LH1 subunits. However the yield of this preparation was very low. Therefore in the present work we tried to improve it by starting from a mutant devoid of the LH2 antenna.

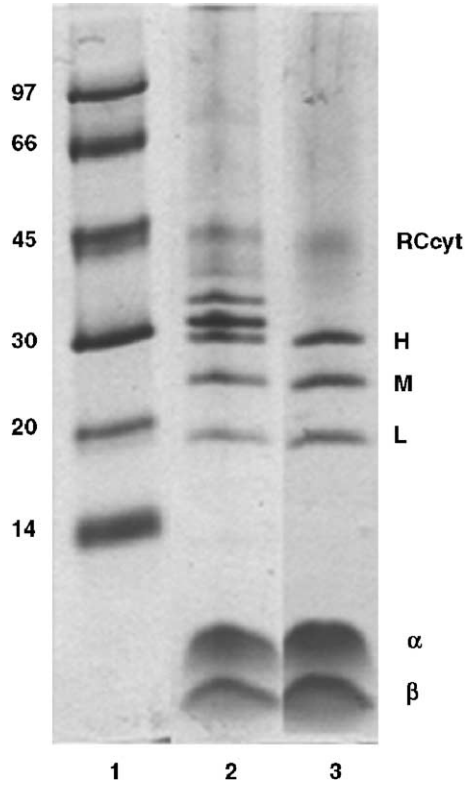

Fig. 4. Coomassie blue-stained SDS-gel of core complex samples at two stages of purification. Lane 1 shows the position of molecular weight standards with molecular weights in kDa. Lane 2: after DEAE-Sepharose chromatography. Lane 3: after final purification by molecular sieve chromatography; the polypeptides are labelled on the right.

The protocol used for the wild type strain, which consisted in a preferential solubilisation of the membranes by octylthioglucoside, did not work for the mutant. Instead the solubilisation was achieved by a mixture of C9G and CHAPS, resulting in a recovery of >80% of a crude core complex (purity index: I=0.99). When analysed on a sucrose gradient at equilibrium, this crude core complex fraction migrated as a single band (I=1.36). However analysis of this band by SDS-PAGE showed the presence of a few non-assigned polypeptides. A better purification was thus tried by directly subjecting the crude core complex to several chromatographic separations. After a first ion-exchange chromatography the purity index of the core complex was better (I=1.60) but SDS electrophoresis revealed two strong bands which ran just above the slowest RC subunit (Fig. 4). These bands did not belong to the core complex, as they could be separated from it by a 2D-gel electrophoresis, where the first dimension was a native Blue-gel electrophoresis [40] and the second one a Tris–Tricine SDS gel [41] (not shown). A mixture of C9G and Na cholate was used for the following gel filtration step(s). The proteins were eluted from a Sepharose and/or from a Superdex column in an asymmetrical peak; the leading edge contained the core complex (mean purity index 1.79±0.06, for 10 preparations), whereas the contaminants were more retarded and could be eliminated (Fig. 4). This protocol resulted in a high yield of the purified core complex, which was also quite stable (at least 2 months at 5 °C), a necessary condition for forthcoming structural studies.

The core complex ran as a broad band in native Blue-gel electrophoresis performed in presence of Na cholate (Fig. 5), confirming the monodispersity of the preparation otherwise indicated by sucrose gradient centrifugation (see above). The migration relative to native standard proteins is only indicative of the mass of the core complex/detergent particle, because it may depend on the presence and nature of the detergent [40]. It is however compatible with the absence of core complex dimers.

The infrared absorbance spectrum of the purified core complex in detergent solution (Fig. 6) showed a main band located at 875 nm, attributed to the LH1 Bchls (overlapping a

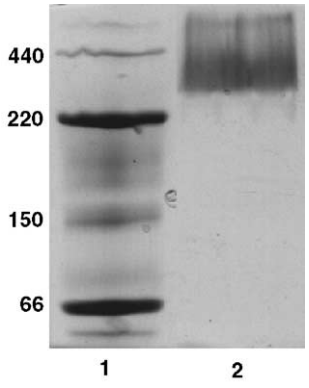

Fig. 5. Native Blue gel electrophoresis with Coomassie blue staining. Lane 1: protein standards (apoferritin, alcool dehydrogenase, catalase and bovine serum albumin), with their molecular weights indicated in kDa. Lane 2: purified core complexes.

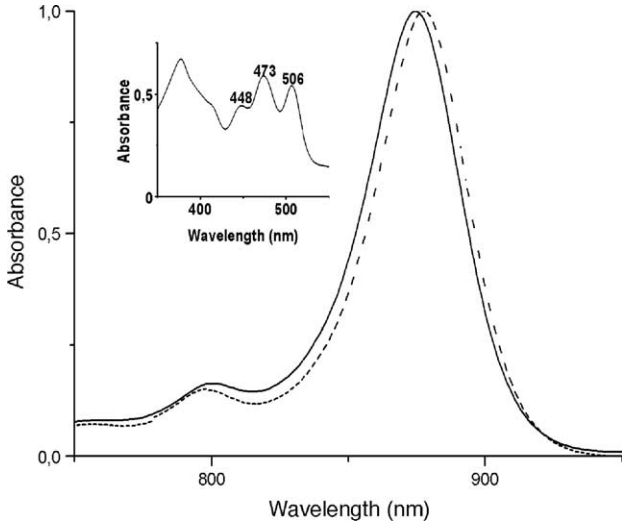

Fig. 6. Infra-red absorption spectrum of the core complex in 50 mM Tris–HCl buffer pH 8, containing 1 mM EDTA, 0.2% C9G, 0.2% Na cholate (solid line) compared to that of a membrane suspension in 50 mM Tris–HCl buffer pH 8 containing 50% sucrose (dashes). Inset: core complex absorption bands in the near UV-visible range.

weak absorption due to the RC Bchl dimer), and two weak bands at 802 and 760 nm originating from the RC monomeric Bchls and Bpheos, respectively. In this wavelength range it showed negligible differences from the native membranes of the PUCAΩ mutant, apart from a slight blue shift from 877 nm to 875 nm due presumably to detergent solubilisation (Fig. 6). Thus the RC:LH1 stoichiometry, which is indicated by the ratio of absorbances at 875–877 nm versus 802 nm, was not modified during the purification.

In the visible range, carotenoids are responsible for the bands observed at 448, 473 and 506 nm (Fig. 6) as in the PucAΩ membranes (Fig. 1). Qualitative HPLC analysis of pigment extracts indicated that hydroxyspheroidene and hydroxyspheroidenone were the major species, with lower amounts of spirilloxanthin and spheroidene (not shown). Hydroxyspheroidene and to a less extent other carotenoids of the spheroidene family were already shown to be bound preferentially to *R. gelatinosus* LH1 whereas spirilloxanthin was bound exclusively by the RC [15,52,53].

The Soret band peaking at 377 nm is mainly due to the Bchls, but it presents a shoulder located near 410 nm, which was not seen in the purified LH1 antenna [15]. This shoulder can be attributed to the tetraheme cyt c bound to the reaction center. Presence of this cyt c could be detected by a difference redox spectrum (ascorbate reduced *minus* ferricyanide oxidized) showing maxima at 552 and 524 nm originating from the cyt c α and β bands [54]. Another indication was given by the pyridine hemochrome spectrum and by heme staining on SDS gels (see below).

3.3. Polypeptide composition of the core complex and modifications of the antenna polypeptides

SDS-PAGE analysis of the core complex showed the presence of only 6 polypeptides (Fig. 4). The largest one,

migrating with a apparent M_w of 45 kDa, was broad and weak when stained with Coomassie Blue or Ag; it was however clearly revealed by heme c staining (not shown) and thus identified with the tetraheme cyt c. Three bands in the 38–20 kDa range were assigned to the subunits of the RC. As their apparent molecular weights were quite different from the values derived from the corresponding gene sequences these bands were isolated after Western blotting and the N-terminal sequence was determined for each of them (Table 1). The H, M and L subunits of the RC could thus be identified. As already noticed for *Rb. sphaeroides* RC, the migration of L and M subunits is faster than expected, probably because of their abnormal SDS binding due to high hydrophobicity [55]. We observed also that the L subunit was susceptible to heat: it was very weak or even absent when the samples were heated with SDS at 60 or 100 °C prior to electrophoresis and in the same time aggregated protein did not enter the gel.

In the 8–6 kDa range, two bands were attributed to the LH1 polypeptides. After Western blotting their N-terminal sequences were probed, identifying the smallest one with the β polypeptide and showing that the other one (presumably α) had a blocked N-terminus (Table 1). Their more precise identification was performed by MALDI-TOF mass spectroscopy combined with sequence data (Tables 1 and 2). When the whole core complex in C9G solution was examined by this technique after simple dilution in water before deposition on the matrix, 2 prominent peaks and a minor one were observed in the 2000–10,000 *M/z* range. The minor peak corresponded to the formylated α polypeptide, α(formyl)(Met1–Lys67), as its mass was higher by 29 Da than the theoretical one (Table 1). As its N-terminus was blocked (see above) this provided evidence for the formylation of the N-terminal methionine. In a preliminary experiment, a peptidic cartography was recorded from the α polypeptide excised from 1 D SDS gel, using a standard proteomic protocol. This cartography confirmed that no other polypeptide co-migrated in this SDS gel band.

The major MALDI-TOF peaks corresponded respectively to the β polypeptide having lost its N-terminal methionine, β(Ala2–Leu48), and to a truncated form of α having lost its C-terminal last 15 residues, α(formylMet1–Ala52) (Table 1). We should note that the relative peak intensities are not directly related to the abundance of these polypeptides, so a quantitation is not possible. In the 10,000–20,000 *m/z* range all the peaks observed could be explained by αβ oligomers (not shown). In a higher *M/z* range we were not able to detect the RC components which were probably not ionised with our experimental conditions.

The same MALDI-TOF spectra were obtained with a different treatment of the samples, i.e. using organic solvents to extract lipids, pigments and detergent molecules before solubilisation of the precipitated core complex in acetic acid.

For confirming these results, we isolated the LH1 polypeptides from the core complex by a reverse phase chromatography. On the column used (which is of low porosity) only two fractions were separated during gradient elution; they were identified by SDS-gel electrophoresis as pure α and β polypeptides, respectively. The α polypeptide was eluted earlier in the gradient than the β one. When subjected to MALDI-TOF spectroscopy, the β polypeptide showed the β(Ala2–Leu48) component, whereas the α one showed only the α(formylMet1–Ala52) one.

These results demonstrated that in the core complex, the C-terminal extension of the α polypeptide was cleaved in part and that the LH1 contained a mixture of α(formylMet1–Lys67) and of α(formylMet1–Ala52). The cleavage site (see Table 2) is located within a C-terminal hydrophobic sequence (see discussion), similarly to the proteolysis of the *R. gelatinosus* LH2 antenna observed in vitro [34,35]. However in comparison with the LH2 α polypeptide no intermediates corresponding to sequential cleavages of α between Lys67 and Ala52 were observed.

Altogether these experiments indicated that no other polypeptide co-migrated with the α or β ones in the SDS gel. Additionally a phosphorylation of the LH1 polypeptides was not detected, a modification reported for *Rs. rubrum* LH1 [56] but still disputed [57]. It should be stressed also that these experiments never indicated the presence of other polypeptides which could correspond to the product of *orf 2* (putative M_w=8.7 kDa) or to a PufX homologue.

Table 1
Characterization of the polypeptides of the *R. gelatinosus* core complex

Subunit	M_{app} (Da)$^{+}$	N-terminus$^{\#}$	M* (Da)	MALDI-TOF	
				M_{exp}(Da)	Identification
RC:cyt c	45,000	(blocked cf [54])	36944[a]	n.d.	–
RC:H	32,000	G–T–T–A–I–T–S–	27560	n.d.	–
RC:M	26,500	A–E–Y–Q–N–I–	36266	n.d.	–
RC:L	21,500	A–M–L–S–F–	31327	n.d.	–
LH1:α	7000	blocked	7347	7376	α(formylM1–K67)
				5913	α(formylM1–A52)
LH1:β	6300	A–E–R–K–G	5550	5419	β(A2-L48)

M_{app}^{+}: measured on SDS-PAGE gels.
N-terminus$^{\#}$: measured by Edman degradation; 1 letter code for the residues.
*M**: calculated from the gene sequence (wild type strain S1, accession number AY234384).
[a] Value calculated from the sequence assuming that the signal sequence (M1-G21) was cleaved (as for *Rps.viridis* cyt c), the mature cyt c N-terminus being C22.

Table 2
Sequences of the *R. gelatinosus* LH1 polypeptides (PufB and PufA) (accession number AY234384) and of the LH2 ones (PucB and PucA) (accession number AF312921), aligned with the conserved histidines which are the putative Bchl dimer ligands

```
PucB (LH2 β)   MADDANKVWPSGLTTAEAEELQKGLVDGTRIFGVIAVLAHILAYAYTPWLH 51
PufB (LH1 β)   MAERKGSI--SGLTDDEAQEFHKFWVQGFVGFTAVAVVAHFLVWVWRPWL- 48
               **:  ...:  ****  **:*::*  *:*   * .:**:**:*.:.:  ***

                                                                  ⇨
PufA (LH1 α)   --MWRIWRLFDPMRAMVAQAVFLLGLAVLIHLMLLGTNKYN---WLDGAKKAPAATAVAPVPAEVTSLAQAK 67
PucA (LH2 α)   MNQGKVWRVVKPTVGVPVYLGAVAVTALILHGGLLAKTDWFGAYWNGGKKAAAAAAAAPAPVAAPQAPAQ- 71
                  ::**:..*  .: .  :  *:::*  **.....:    * .* * *.**:*.**.*. ⇦ ... .
                                                                  ⇦
```

The black arrow denotes the cleavage site of PufA observed in this work. The proteolyses of PucA in vitro reported previously [33] occur at Ala 64 and at residues Ala58 to Ala53, located between the two grey arrows.

3.4. Mean size of the photosynthetic unit

For determining the mean number (n) of LH1 αβ subunits per RC in the core complex we used two approaches. First, we tried to reconstruct the absorption spectrum of the core complex in the infrared range (650 to 950 nm), $A_{core}(\lambda)$, by combining linearly those measured respectively for the purified RC, $A_{RC}(\lambda)$ [37] and for the isolated LH1, $A_{LH1}(\lambda)$ [15]. The infra-red absorption of the isolated LH1 was previously shown to depend weakly on its concentration [15]; for the fit we adopted the low-concentration spectrum peaking at 875 nm. The function

$$A_{core}(\lambda) = aA_{LH1}(\lambda) + bA_{RC}(\lambda)$$

was fitted using a multiple regression analysis provided by the Origin software. From the best global fit obtained (Fig. 7) it was possible to determine the peak absorbance values of the spectra $aA_{LH1}(\lambda)$ and $bA_{RC}(\lambda)$ respectively. The stoichiometric ratio, n, could then be calculated from these values and from the molar extinction coefficients of LH1 at 875 nm and of RC at 802 nm. As the extinction coefficient of *R. gelatinosus* RC is unknown, we adopted that of *Rb. sphaeroides* RC in LDAO solution. Likewise we used for LH1 an extinction coefficient measured previously for the antenna isolated in presence of the detergent decyl-tetraethyleneglycol. With these hypotheses the value of n was found to be 17. The error affecting this value is difficult to estimate, as it depends mostly on the assumed extinction coefficients. These coefficients might depend on the detergent and also on the state of association of LH1 with RC. This last point is quite critical. Indeed the differential extinction coefficient (oxidised minus reduced) of *Rb. sphaeroides* RC was found to be lower by 20% in the membranes (where it is associated with LH1) than in the detergent solubilised state [58]; the absolute extinction coefficient is probably also modified. Therefore an accuracy not better than ±10% is to be presumed for n.

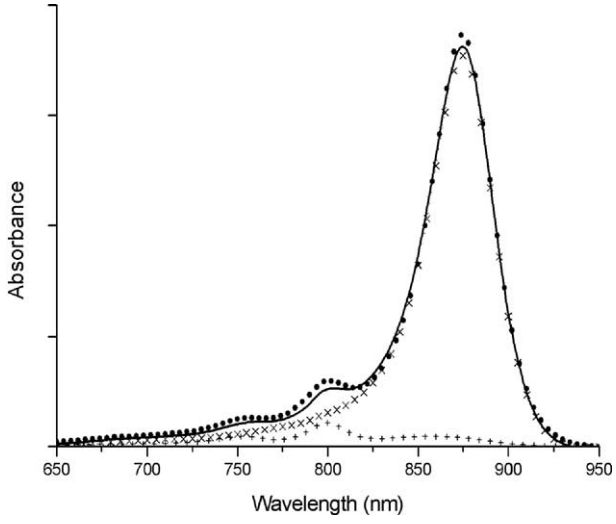

Fig. 7. Experimental (solid line) and reconstructed (●) infrared spectrum of the core complex, given along with the absorption spectra of the isolated LH1 (×) and RC (+) used for the fit.

In the second method, the tetraheme cyt c was quantitated by several measures of the pyridine hemochrome spectrum on samples which were also analysed repeatedly for their Bchl content. Assuming that one cyt c was bound to each RC, the molar ratio R=(Bchl)/(RC) could be obtained. It is known that the RC contains 4 Bchls and that 2 Bchls are bound per LH1 subunit. Therefore, assuming further that the samples contained only core complexes (i.e., no empty LH1 rings and no RCs without LH1), n can be calculated:

$$n = (R - 4)/2$$

For 3 independent preparations values of $n = 15.8 \pm 1.1$, 16.5 ± 0.6 and 17.6 ± 0.8 were found with this method.

The results obtained by the two methods are compatible. As the second method requires less assumptions it should be more reliable, and a value of 16.6 ± 1.2 is obtained.

4. Discussion

In this work we prepared and characterized a stable core complex using a *R. gelatinosus* mutant devoid of LH2. As compared to a previous preparation from the wild type strain [59], the main advantage of this new procedure resides in its much higher yield. Indeed the recovery of the core complex from the mutant is about 80% in the first solubilisation step, whereas in the wild type a selective but inefficient solubilisation was achieved as to avoid contamination by LH2. The mixed detergents used here (a glycosidic type plus a steroid derivative one) seemed furthermore to stabilise the complex as judged from its spectroscopic properties. The core complex was isolated as a monodisperse particle, as shown by its migration as a single band in a sucrose gradient at equilibrium, and as a main band in native electrophoresis compatible with a monomeric state.

In this core complex only the expected components were detected, i.e. the RC and tetraheme cyt c polypeptides, and the LH1 ones. The absence of a pufX component was anticipated, as a gene coding for a pufX analogue has not been detected in the photosynthetic gene cluster of *R. gelatinosus* (strains S1 [60] and IL234 [31]). It has been recently noticed that putative *PufX* genes have only been found up to now in *Rhodobacter* species not containing the tetraheme cyt c [29]. Recently the genome of a non-photosynthetic *R. gelatinosus* strain PM1 has been sequenced (accession number: NZ AAEM00000000); a Blast search did not identify a protein presenting a significant analogy with *Rb. sphaeroides* or *capsulatus* PufX. This negative result is however not conclusive, as the known pufX sequences are of low homology. For *Rb. sphaeroides*, PufX is essential for photosynthetic growth; it is required for the exchange of quinones between the RC and the bc_1 complex via the quinone pool [24]. Several studies indicate that its key role in vivo is to promote the dimerisation of the core complex [18,27] concomitant with a break in the LH1 ring around the RC; however its localization within the core complex assembly is still a matter of debate (see [11,61]). In bacteria lacking PufX, it could eventually be replaced by an other small membrane protein, such as the W protein detected in the core complex of *Rps. palustris* [10]. In the case of *R. gelatinosus*, a possible candidate could be the product of *orf2*, a protein of M=8174 Da with one putative transmembrane helix. But it has been observed that a constructed mutant with a disrupted *orf2* was still capable of photosynthetic growth (albeit at a lower rate) [62], suggesting that orf74 did not have the same function as PufX. Furthermore we did not observe a polypeptide in the 7–15 kDa range in addition to the LH1 ones, either in silver-stained gels or in MALDI-TOF spectra of the core complex. These negative results indicate that orf74 is probably not associated with the core complex, or that it is weakly bound and released during the purification.

In this work we have used MALDI-TOF spectroscopy for assessing the presence of the LH1 polypeptides and in the same time for characterizing several modifications. The β polypeptide was only modified by the loss of its N-terminal methionine, as already reported for two other *R. gelatinosus* strains [38]. Methylation at the amino group of β(Ala1) has been observed in three bacterial species where the sequence of the N-terminal end was (Ala)–(Glu/Asp)–(X)–(Lys)...with X uncharged; it was proposed that the methylation was related to this particular charge distribution [63]. In *R. gelatinosus*, the β N-terminus bears an extra positive charge, X being a Arg (Table 2). Accordingly this methylation does not occur. We did not detect either an appreciable oxidation of the α N-terminal methionine, which has been observed in vitro in *Rs. rubrum* LH1 [63]. More surprisingly, we observed that two α polypeptides coexisted in the core complex: the full-length α polypeptide and a shorter one having lost its 15 last C-terminal residues. Both were formylated presumably at their N-terminal methionine, as their N-terminus was blocked and their observed mass were 29 Da greater than the expected ones. As regard the formylation, earlier determinations of the α amino-acid sequence in two other *R. gelatinosus* strains [38] and in *Rs. rubrum* [64] also showed that the N-terminal methionine was blocked, presumably by a formyl group. The absolute molecular mass of the *Rs. rubrum* α polypeptide [63] provided further evidence for the presence of a formyl group, which was supposed to modify the N-terminal methionine.

The truncated α polypeptide detected here by mass spectrometry corresponds exactly to the amino acid sequence published previously [38], which by comparison with the gene sequence led to the hypothesis that the C-terminus was processed by cleavage after α(Ala52) [31]. Our results show however that the full-length polypeptide was also present, indicating a partial cleavage occurring after the assembly of full-length polypeptide in the core complex. This truncation is quite similar to that observed previously for the LH2 α polypeptide during aging of the isolated antenna [34]. In both cases the cleaved sequence is highly enriched in aliphatic residues (specially alanines) and in prolines (see Table 2), and it forms probably a disordered extension on the membrane periplasmic surface. It is interesting to note that these cleavages have been observed by us and other authors [38] after solubilisation of LH2 or of LH1 or of the core complex, in various *R. gelatinosus* strains and in presence of different detergents (LDAO, or C9G/CHAPS), as well as after direct extraction of the antenna polypeptides from

membranes with organic solvent mixtures. The action of proteases could be considered, as this bacterium was named after its capacity of liquefying gelatine, and another strain was shown to excrete a serine protease [65]. In our hands however the presence of protease inhibitors (see Materials and methods) during disruption of the cells and throughout the isolation did not have any effect. Therefore one should rather consider that these C-terminal extensions are inherently unstable as soon as the membranes are disrupted. In any case the C-terminus cleavages of the α polypeptides did not affect the stability of the antenna nor their functional properties, as judged from their spectroscopic characteristics.

It should be stressed that the cleavages of the α subunit that we observed in vitro in *R. gelatinosus* LH1 and LH2 are located in the hydrophobic C-terminal extension which is not present in the antenna of most other purple bacteria. For LH1, the cleavage occurs at His+23, using the convention of taking the conserved His30 as numbering reference (Table 2). Truncation in vivo of the α C-terminus of *Rb. sphaeroides* has been shown to affect the biogenesis of LH1 and the organization of the core complex [66]; however these truncations were more extensive, the critical residues being located from His+17 to His+10. For *R. gelatinosus* a minimal size of the C-terminal extension is required for the biogenesis of the LH2 antenna [39]; generalization of this observation to LH1 biogenesis is under way.

The isolated core complex showed the same spectral characteristics as the native membranes of the mutant, indicating that the relative number of LH1 subunits per RC was not modified during the isolation. This number is still questioned, at least for *R. gelatinosus*. Akiyama et al. concluded to a quite high ratio of 21.5±2.8 LH1 subunits per RC in intact cells of the IL144RL2 strain, based on the relative amounts of Bchl (bound to LH1 and RC) and Bpheo (bound only to RC) [67]. However this ratio might be overestimated, because this strain has a very depressed, but not totally absent LH2 antenna which contributed to the Bchl content. In a previous work [59] we found 14±1 LH1 subunits per RC for the core complex isolated from a wild type strain, by measuring the RC by its photooxidation (using the differential extinction coefficient of *Rb. sphaeroides* RC) and the Bchl by solvent extraction. Our present estimation of this ratio in the LH2-minus mutant was obtained by two different methods. The first was based on a deconvolution of the infra-red absorption spectrum of the core complex and required also assumptions about the absolute extinction coefficients of LH1 Bchl and of the RC in the complex. These parameters are critical but somewhat arbitrary, thus it is difficult to estimate the accuracy of the results. The second method was based on the determinations of Bchl and tetraheme cyt c contents using well established extinction coefficients. However a slight loss of tetraheme cyt c could occur during the purification as it is not strongly bound to the RC [37]; this would result in overestimating the ratio. With these remarks in mind, we concluded that the mean number of LH1 subunits per RC should be near 16 or sligthly lower. From this point of view the *R. gelatinosus* core complex seems similar to that of *Bl. viridis*, which contains 16 LH1 subunits encircling a RC-tetraheme cyt c assembly [19]. However the tetrahemic cytochromes of these two species are not structurally equivalent as they seem to be differently oriented towards the RC [68], a feature which may play a role in the core complex assembly. A more detailed structural model would be provided by forthcoming X-ray crystallography and electron microscopy studies; 2D-crystals of the core complex have already been obtained in preliminary experiments.

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