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Reconstitution of Okenone into Light Harvesting Complexes from *Allochromatium minutissimum*

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Abstract—Okenone was reconstituted into light harvesting (LH) complexes of the purple photosynthetic bacterium *Allochromatium minutissimum* possessing the spirilloxanthin pathway for carotenoid biosynthesis. Suppression of this pathway by diphenylamine, an inhibitor of carotenogenesis, yielded nearly carotenoidless complexes preserving their native spectral properties. Using a previously developed technique, okenone was readily reconstituted into LH1 complex (>90%) whereas its reconstitution into LH2 complex was of low efficacy (10-20%). The absorption band of the reconstituted okenone was shifted to shorter wavelength compared with its position *in vivo*. This is typical for other reconstituted carotenoids. The reconstitution of okenone was confirmed by Li-DS electrophoresis (in contrast to free okenone the reconstituted okenone migrated with complexes), circular dichroism spectra (reconstituted okenone exhibited optical activity), and fluorescence excitation spectrum (energy transfer from okenone to bacteriochlorophyll was at the control level).

Key words: photosynthetic bacterium, light harvesting complexes, okenone, reconstitution, electrophoresis

Light harvesting (LH) complexes are an important component of the photosynthetic machinery; they are needed for more effective accumulation of light energy. Photosynthetic bacteria possessing the simplest photosynthetic machinery usually contain two light harvesting complexes, peripheral (LH2) and pericentral (LH1). The latter surrounds a reaction center and forms LH1–RC assembly also known as the "core" complex [1]. The light harvesting complexes consist of low molecular weight peptides (α and β), bacteriochlorophyll, and carotenoids. The medial part of each polypeptide contains an α-helical transmembrane region, whereas the C- and N-ends are exposed to the two sides of the membrane. The LH2 and LH1 complexes consist of 8-12 and 15-16 polypeptide pairs, respectively. The polypeptides form two rings (external and internal), whereas carotenoids and bacteriochlorophyll clusters (dimers) are located between these rings. In the LH2 and LH1 complexes, bacteriochlorophyll molecules absorb at 820-855 and 870-890 nm, respectively. They are located closer to the periplasm. At the outer surface of the ring closer to the cytoplasm the LH2 complex contains two molecules of monomeric bacteriochlorophyll absorbing at 795-805 nm and some carotenoids [2-5]. All carotenoids present in bacterial cells usually absorb at 400-560 nm.

Five pathways of carotenoid biosynthesis are known in photosynthetic bacteria, the spirilloxanthin and okenone pathways being the best studied ones. Besides "normal" end product spirilloxanthin, spirilloxanthin biosynthesis yields the unusual spirilloxanthin spheroidene and also rhodopinal glucoside. Carotenoid molecules formed in this biosynthetic pathway contain methoxy-, hydroxy-, keto-groups, etc. as side group substituents [6]. In bacteria with okenone biosynthesis, okenone and hydroxythiotece-474 are the end products. These differ from other bacterial carotenoids by the presence (besides keto- and methoxy-groups) of an aromatic ring in χ position [6]. The presence of okenone in bacterial membranes is suggested to influence spectral characteristics of LH2 complex [7]. In contrast to other complexes characterized by two spectral bands, the absorption spectrum in the near infrared (IR) region of such LH2 complex has one major band at 825-835 nm and a shoulder at 800 nm [1, 8].

Insertion of carotenoids containing various side group substituents into the light harvesting complexes still requires detailed investigation. The main approach employed for solution of this problem consists in the use of carotenoidless mutants [9-12]. However, this approach

Abbreviations: *Alc. minutissimum*) *Allochromatium minutissimum*; *Mac. purpuratum*) *Marichromatium purpuratum*; *Rba. sphaeroides*) *Rhodobacter sphaeroides*; *Rsp. rubrum*) *Rhodospirillum rubrum*; Li-DS) lithium dodecyl sulfate.

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has some shortcomings. For example, the carotenoidless mutants differ from corresponding wild strains by the presence of only one antenna complex. In such cells only the LH1 complex usually remains due to mutations in genes encoding polypeptides, and LH2 (pseudo-LH1) loses monomeric bacteriochlorophyll binding sites; it is present in membranes in small quantities, and its absorption spectrum resembles that of LH1 complex [13-16]. Such mutations result in stabilization of carotenoidless complexes and changes in their spectral characteristics (blue shift of the main band in the near IR region, loss of bacteriochlorophyll absorbing at 800 nm) and spatial structure; these changes may significantly influence pigment binding sites [17-21]. Carotenoids of spirilloxanthin biosynthesis may be reconstituted to such modified LH1 complexes [9, 10]. The other approach for solution of this problem consists in employment of carotenoidless mutants constructed by gene engineering methods causing removal of the corresponding part of the bacterial genome. In the genome of such mutant genes encoding carotenogenesis components from other bacteria are then inserted. However, this approach has been developed for a limited number of nonsulfur photosynthetic bacteria [18, 19].

Earlier we demonstrated that using the carotenogenesis inhibitor diphenylamine, it is possible to obtain carotenoidless LH1 and LH2 complexes from *Alc. minutissimum* bacterial cells [20-23]. These complexes did not change their spectral characteristics and did not contain large amounts of phytoene. We also developed a method for reconstitution of crude carotenoid extract into the LH2 complex [24]. In the present study, we have investigated the possibility of okenone reconstitution to carotenoidless LH1 and LH2 complexes of the purple photosynthetic bacterium *Alc. minutissimum*.

MATERIALS AND METHODS

Cells of *Alc. minutissimum* and *Mac. purpuratum* were grown under white light (intensity of 2000 lx) and temperature of 25°C as described earlier [20, 25, 26]. For inhibition of carotenoid biosynthesis, *Alc. minutissimum* cultivation medium was supplemented with diphenylamine (12 mg/liter), which was re-crystallized from ethanol [20, 24]. Chromatophores were isolated from sonicated cells by differential centrifugation as described earlier [20, 24].

For carotenoid extraction, 1 ml of *Mac. purpuratum* chromatophores (OD_{850} = 40-50) was mixed with 10 ml of acetone–methanol (7 : 2) under constant stirring. The resultant extract was sequentially mixed with 2-4 ml of petroleum ether and then 20-25 ml of water under stirring. The extracted pigments were localized in the petroleum ether on the top of the resultant mixture. They were pipetted into a penicillin vial and dried under nitrogen. Crude carotenoid fractions were isolated by column chromatography using a column (2 × 1.1-1.3 cm) packed with silica gel (ICN Silica Active I, 100-200 μm) and washed with hexane. Pigments applied to the column in 1 ml of hexane and adsorbed on this column were eluted by hexane–acetone mixture (93 : 7). The pigments were dried under nitrogen and immediately used or stored under nitrogen at –20°C.

Carotenoid reconstitution was carried out by the method described in [24]. Carotenoids were added to a sample of 500 μl in sequential portions of 10 μl. The resultant complexes were separated by gel electrophoresis as described in [24, 27]. For better separation of free carotenoids from complexes, 0.05% Li-DS was added to the upper buffer. Gels were photographed using a QV4000 digital camera (Casio, Japan).

Absorption spectra were registered in gels using a UV-160 spectrophotometer (Shimadzu, Japan). For preparation of spectra for printing, they were digitized using Graph2Digit 0.52b (designed by V. Plisko: plsoft.narod.ru) in "negative" mode with step 2 (1 nm = 2 pixels) and also Photoshop, Excel, and Origin software as described [28]. Fluorescence excitation spectra were registered using a Hitachi-850 spectrofluorimeter (Hitachi, Japan); circle dichroism spectra were registered using a J600 spectropolarimeter (Jasco, Japan).

Carotenoids were analyzed by HPLC as described in [24, 28] at flow rate 1.0 ml/min using a Spherisorb ODS2 column (250 × 4.6 mm, 5 μm; Waters, England). The rate of solvent flow is 1.0 ml/min. The column was equilibrated with a solvent mixture (77% of acetonitrile–water (9 : 1) mix and 23% of ethyl acetate). The elution was carried out as follows: 3 min with the mixture, which was then linearly substituted for ethyl acetate for 20 min and finally 5 min with ethyl acetate. Carotenoid samples with reconstituted okenone were analyzed after elution of the corresponding complex from one gel.

The following reagents were used in this study: dodecyl maltoside (Anatrace, USA); Tris, octyl glucopyranoside, and Li-Ds (Sigma, USA); and silica gel (ICN, USA). Reagents for electrophoresis and cultivation of bacteria were purchased from Russian suppliers.

RESULTS AND DISCUSSION

Pigment containing membranes from *Alc. minutissimum* cells treated with the carotenogenesis inhibitor diphenylamine were used for carotenoid reconstitution. It was previously shown that such membranes contain small quantities of rhodopin, neurosporene and its derivatives, ζ-carotene and its derivatives, and also the carotene precursor phytoene [21, 24]. Under these conditions, the *Alc. minutissimum* membranes contained populations of LH1 and LH2 complexes lacking carotenoids, or the carotenoid content did not exceed one molecule per complex. However, these membranes contained the

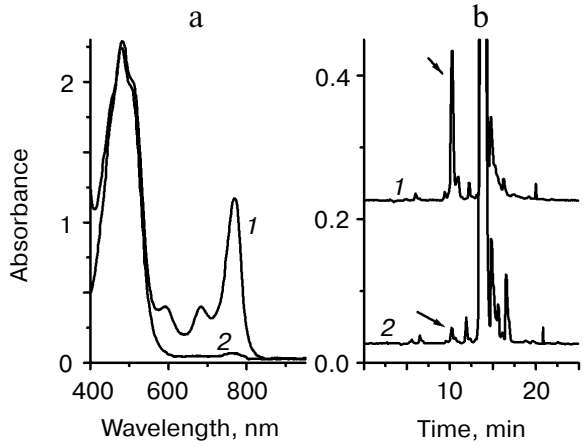

Fig. 1. Absorption spectrum of the pigment fraction from *Mac. purpuratum* before (*1*) and after (*2*) bacteriochlorophyll removal by column chromatography (a) and HPLC analysis of pigments in *Mac. purpuratum* membranes before (*1*) and after (*2*) bacteriochlorophyll removal by column chromatography (b). The analysis was carried out at 486 nm. The arrow indicates the hydroxythiotece-484 peak; other peaks are related to okenone and its derivatives.

whole set of light harvesting complexes with unchanged spectral characteristics in the near IR region. These features provide a unique opportunity for study of carotenoid insertion into both types of light harvesting complexes.

In this work, we have used carotenoids from the bacterium *Mac. purpuratum*, which belongs to a group of bacteria containing okenone as the main carotenoid [6-8]. Previously we found that the presence of bacteriochlorophyll in the carotenoid fraction prevents carotenoid reconstitution into LH2, and so reconstitution required removal of bacteriochlorophyll [24]. Washing of pigment film with methanol easily removed bacteriochlorophyll from pigment extracts from control *Alc. minutissimum* membranes. However, in the case of *Mac. purpuratum* membranes methanol solubilized both carotenoids and bacteriochlorophyll. So we used column chromatography. Figure 1a shows absorption spectra of *Mac. purpuratum* extracts before and after the column stage. This approach effectively removed bacteriochlorophyll without significant changes in the carotenoid composition. Data of HPLC analysis of carotenoids indicates that hydroxythiotece-484 content was reduced after the column chromatography stage (Fig. 1b).

Increasing the amount of carotenoids added to *Alc. minutissimum* membranes caused a gradual shift to shorter wavelength (by 10-14 nm) of the band at 852 nm and reduction of the 852/798 band intensity ratio from 1.3 to ⩾1 (Fig. 2). These bands are related to LH2 complex and such spectral changes reflect reversible conformational transitions observed in this complex during treatment with low concentrations of nonionic detergents (~0.1%) or solvents (20-40%) [20, 29]. Increase in absorbance in the carotenoid region occurred simultaneously with these changes. This was characterized by appearance of a wide band with the main maximum at ~500 nm related to okenone. The carotenoid band red-shifted by 18 nm compared with its spectrum in solvent (Fig. 1a). This suggests aggregation of carotenoids or their reconstitution into complexes.

Initially we used electrophoresis in the presence of dodecyl maltoside (data not shown). In the presence of the nonionic detergent, the main band corresponding to carotenoids was localized on the top of the gel and gave a colored background in bands of the complexes. For exclusion of co-migration of free pigments and complexes, we added the ionic detergent Li-DS (0.05%). During such type of electrophoresis, free pigments are inserted into detergent micelles and migrate with the electrophoretic front. Electrophoresis of a control sample revealed the presence of greenish-blue bands of LH1–RC assembly and LH2 complex (Fig. 3a). Addition of an okenone fraction to the sample followed by subsequent electrophoresis resulted in appearance of two new bands red in color on the top of the gel and in the electrophoretic front. They corresponded to the band of aggregated (not bound to complexes) and free carotenoids (Fig. 3b, zones *1* and *4*). Absorption spectra of these bands revealed the presence of only carotenoids, traces of monomeric bacteriochlorophyll, and products of its degradation (Fig. 4a). The latter may be attributed to partial decomposition of LH1 complex in the presence of Li-DS. Simultaneously, there was a change in color of the bands of LH2 complex and LH1–RC assembly. The latter band became red-brown.

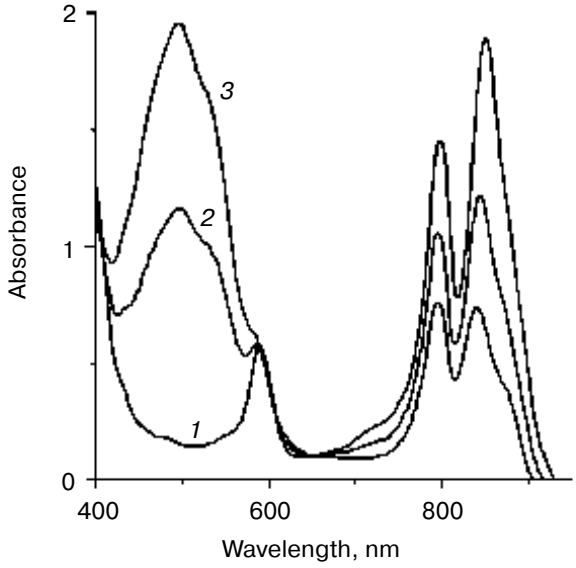

Fig. 2. Absorption spectrum of *Alc. minutissimum* membranes before (*1*) and after addition of 20 μl (*2*) and 40 μl (*3*) of *Mac. purpuratum* carotenoids.

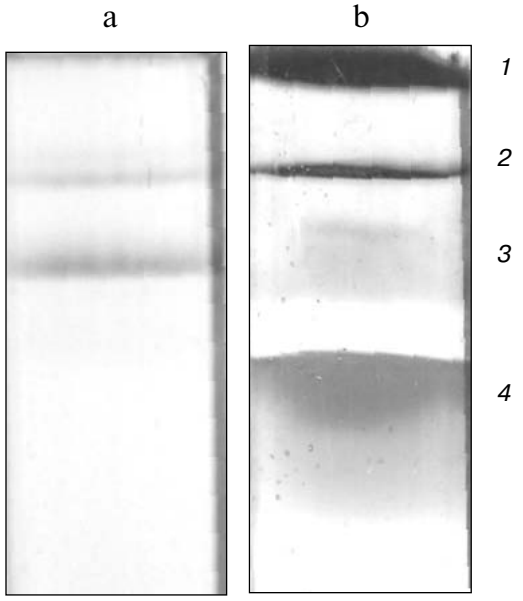

Fig. 3. Electrophoresis of *Alc. minutissimum* membrane preparations in the presence of dodecyl maltoside (a) and dodecyl maltoside with Li-DS after addition of 60 μl of carotenoids from *Mac. purpuratum* (b). *1*) Zone of free aggregated carotenoids; *2*) LH1–RC assembly; *3*) LH2 complex; *4*) zone of free carotenoids.

Figure 4b shows absorption spectra of LH2 complex. This complex is characterized by a small number of reconstituted carotenoids (Fig. 4b, spectrum *2*). According to our evaluation, it did not exceed 20% of the control (Fig. 4b, spectrum *3*). HPLC analysis revealed the presence of okenone and a small amount of its derivatives in this complex (Fig. 5a). Although carotenoids containing hydroxy- and methoxy-groups could be easily reconstituted [24], it should be noted that the process of okenone reconstitution into this complex was of low efficacy (>10%). Its reconstitution required additional sonication of the sample. Due to low content of okenone reconstituted into the LH2 complex, we failed to measure effectiveness of energy transfer from carotenoids to bacteriochlorophyll in this complex and to evaluate reconstitution effectiveness by measurement of circular dichroism spectra. In contrast to LH2 complex, gradual addition of carotenoids to the LH1–RC assembly resulted in the increase in intensity of the carotenoid absorption band compared with the corresponding control (Fig. 6). Comparison of carotenoid absorption band intensity in the control assembly LH1–RC (Fig. 6, spectrum *6*) and in the sample with maximal amount of reconstituted carotenoids (Fig. 6, spectrum *5*) revealed that the intensity of carotenoid band absorbance was reduced to 90%. This effect could not be attributed to equal electrophoretic mobility of LH1–RC assembly and carotenoids because the electrophoresis buffer additionally contained an ionic detergent forming a band of free pigments (Fig. 3). Consequently, increase in carotenoid band intensity in the LH1–RC assembly occurred due to carotenoid binding to this assembly. After reconstitution with LH1–RC assembly, HPLC analysis detected only okenone (Fig. 5b). Registration of circular dichroism spectra is one of the approaches used for evaluation of effectiveness of carotenoid reconstitution. It is known that free carotenoids or carotenoids adsorbed on albumin lack optical activity [9]. The circular dichroism spectrum of the LH1–RC assembly with reconstituted okenone is characterized by positive (maximum at 466 nm) and negative (maximum at 541 nm) absorption bands (Fig. 7a). The circular dichroism spectra of *Mac. purpuratum* membranes (which are characterized by predominance of LH2 complex with okenone) contain one negative band with maximum at 487 nm and a positive shoulder at 545 nm. Registration of fluorescence excitation spectra revealed that in the LH1–RC assembly effectiveness of energy transfer from okenone was comparable to energy transfer from spirilloxanthin to bacteriochlorophyll in the control assembly (Fig. 7b).

In this study, we have demonstrated okenone reconstitution to the light harvesting complexes of *Alc. minutissimum* cells. Carotenoid biosynthesis in these cells is characterized by preferential accumulation of spirilloxanthin (the end product) in LH1 complex and rhodopin (an intermediate product of biosynthesis) in LH2 complex [20]. (Figure 8 shows structural formulas of these carotenoids.) Maximal inhibition of carotenogenesis resulted in dramatic reduction in carotenoids or their precursors in these complexes to trace levels [20, 21]. This

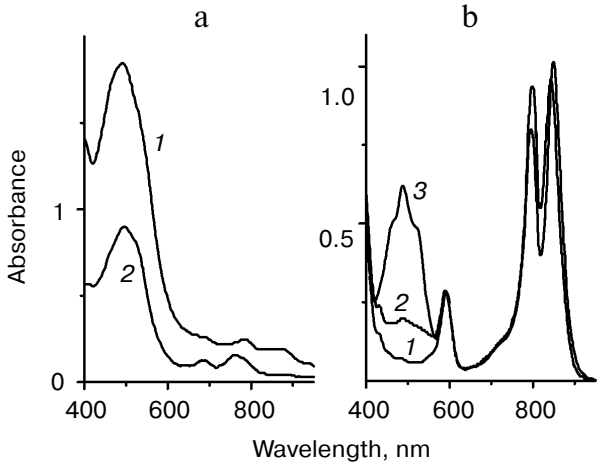

Fig. 4. Absorption spectra of free aggregated carotenoids (*1*) and free carotenoids (*2*) (a) and also absorption spectra of the LH2 complex isolated from cells with inhibited carotenoid biosynthesis (*1*), LH2 complex reconstituted with okenone (*2*) and LH2 complex from control cells (*3*) (only the part of the spectrum containing a region of carotenoid absorbance is shown) (b). The spectra were normalized in the Q_x band of the bacteriochlorophyll transition at 590 nm.

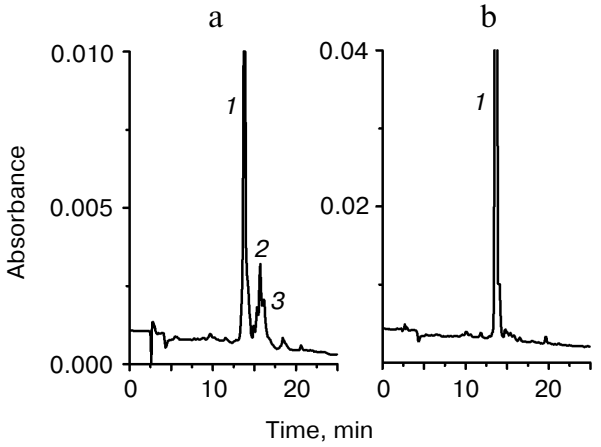

Fig. 5. HPLC analysis of carotenoids of LH2 complex reconstituted with okenone (spectrum *2* of Fig. 4) (a) and carotenoids from LH1–RC assembly after addition of carotenoids from *Mac. purpuratum* followed by subsequent isolation of the assembly by electrophoresis (Fig. 6) (b). The analysis was carried out at 486 nm. Peak identification: *1*) okenone; *2*, *3*) okenone derivatives.

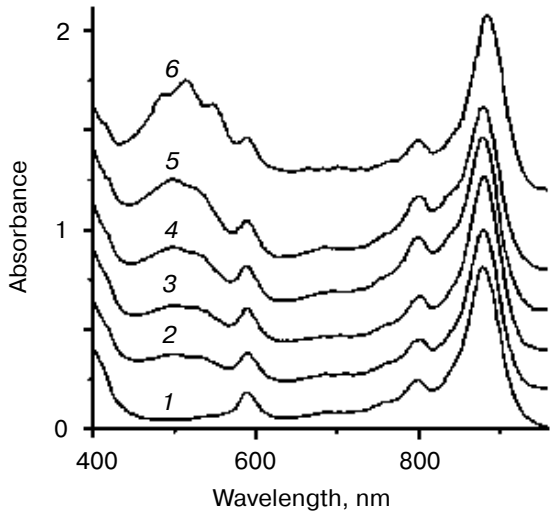

Fig. 6. Absorption spectra of LH1–RC from *Alc. minutissimum* cells. *1*) Samples from cells with inhibited carotenoid biosynthesis; *2-5*) samples after addition of 10, 20, 40, and 60 μl of carotenoids from *Mac. purpuratum*, respectively; *6*) a sample from *Alc. minutissimum* cells with normal carotenoid synthesis.

allowed successful reconstitution of spirilloxanthin carotenoids into these complexes [24]. Okenone (Fig. 8) contains one aromatic ring in χ position, one methoxy-group at C-1 (as in spirilloxanthin), and a keto group at C-4. It is possible that this keto group "extends" the conjugated double bond and induces transition of an ordinary bond into trans-conformation [6]. Thus, the okenone molecule differs from spirilloxanthin and rhodopin in length of the molecule and in side groups.

It is important to emphasize that we have used carotenoidless LH complexes with unmodified structure. These complexes preserved native spectral characteristics of bacteriochlorophyll clusters. Earlier studies of carotenoid binding sites used only carotenoidless mutants lacking normal light harvesting complexes. Neurosporene and spheroidene were reconstituted into modified LH1 complex from *Rba. sphaeroides* (strain R26) [9]. Spirilloxanthin, spheroidene, and various derivatives of spheroidene were reconstituted into the B850 complex (modified LH2 complex) from *Rba. sphaeroides* (strain R26.1) lacking binding sites for monomeric bacteriochlorophyll [30-32]. Attempts to reconstitute carotenoids containing aromatic rings (like β-carotene) with reaction centers were not successful [33]. Thus, for the first time we have demonstrated here reconstitution of the carotenoid containing one aromatic ring into light harvesting complexes from *Alc. minutissimum*. This reconstitution was of different efficacy: okenone was readily reconstituted into LH1 complex and exhibited low effectiveness of reconstitution into LH2 complex. Nevertheless, the level of okenone reconstitution into unmodified LH2 complex from *Alc. minutissimum* is consistent with literature data. For example, Papagiannakis et al. [32] reported reconstitution of only 5-10% of spirilloxanthin to the B850 complex of *Rba. sphaeroides* (strain R26.1). (We believe that reconstitution with such complex would be more effective.) This may be attributed to loss of monomeric bacteriochlorophyll which is one (although not the principal) component involved in carotenoid binding site formation [2, 3]. Consequently, loss of monomeric bacteriochlorophyll reduces specificity of the carotenoid binding site, which facilitates reconstitution of other carotenoids.

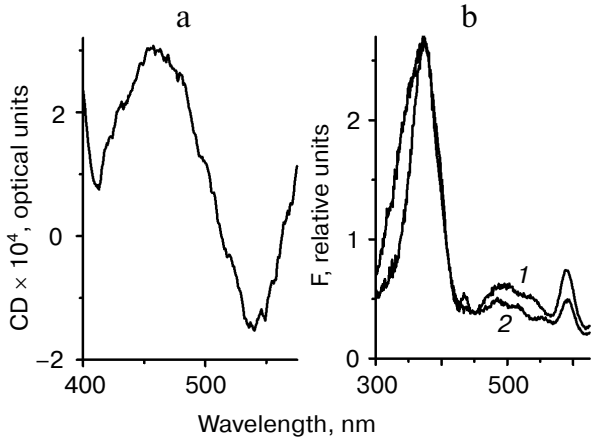

Fig. 7. Circular dichroism spectrum of LH1–RC complex with reconstituted okenone (a) and fluorescence excitation spectrum of LH1–RC reconstituted with okenone (*1*) and control LH1–RC assembly (*2*) (b).

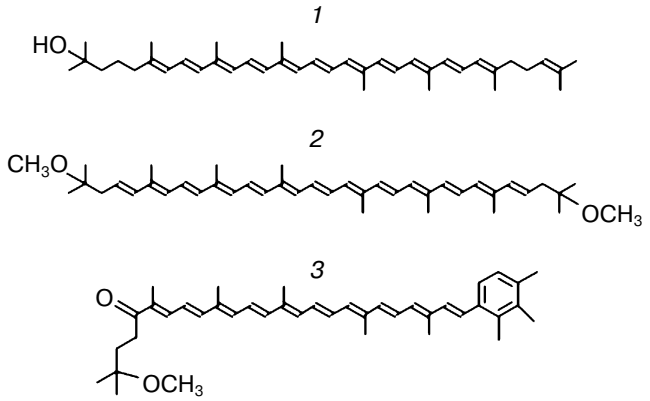

Fig. 8. Structural formulas of carotenoids: rhodopin (*1*), spirilloxanthin (*2*), and okenone (*3*).

Different efficacy of okenone reconstitution into *Alc. minutissimum* LH1 and LH2 complexes may be explained by different specificity of carotenoid binding sites in these complexes. Earlier we found the existence of a second minor spirilloxanthin pathway of carotenoid biosynthesis in some okenone containing bacteria. The whole spirilloxanthin pool was localized in LH1 complex, and its content represented ~30% of the total carotenoid content in this complex; during assembly of LH1 complex *in vivo* about 4-6 spirilloxanthin molecules and 10-12 okenone molecules and its derivatives were inserted into this complex [8, 25, 26]. Thus, taking into consideration universal principles of assembly of light harvesting complexes we suggest that carotenoid binding sites in LH1 complexes are less specific than those in LH2 complexes.

It remains unclear how accurate okenone reconstitution into light harvesting complexes from *Alc. minutissimum* was, i.e., whether okenone occupied hydrophobic pockets normally occupied by rhodopin and spirilloxanthin *in vivo*. Since the amount of reconstituted okenone never exceeded carotenoid content observed in the complex *in vivo*, this fact would indirectly indicate accuracy of reconstitution. Taking into consideration the tendency of okenone to aggregate (Figs. 3 and 4a), we would expect significantly larger amounts of carotenoids bound to the complex in the case of simple okenone sorption on LH1 or LH2 complexes. Since LH2 complex has external and internal carotenoid binding sites, it is clear that the external sites are more susceptible to okenone.

One criterion of accurate okenone binding into the complex is the position of its absorption bands. In both light harvesting complexes from *Alc. minutissimum*, this band has a maximum at 500 nm, whereas in solvent it has maximum at 482 nm and in absorption spectra of membranes from okenone containing bacteria its maximum is positioned at 512 nm [8, 25, 26]. Similar shorter wavelength position of absorption spectra of the reconstituted carotenoid was found during reconstitution of spirilloxanthin into B850 complex from *Rba. sphaeroides* (strain R26.1) compared with the control samples from *Rsp. rubrum* [32]. In the first case, the spirilloxanthin molecule had planar configuration, whereas in the second case it had twisted configuration. Spectral properties of reconstituted carotenoids (and geometry of their molecules) are significantly influenced by the polypeptides accommodating a carotenoid molecule. This is confirmed by circular dichroism spectra of LH1 complex with reconstituted okenone and okenone containing membranes; these have different orientation of absorption bands. It is possible that reduction of the absorption spectrum and position of absorption bands is possible only during reconstitution with carotenoids from the same bacterial species [24] or during the use of carotenoids possessing similar structure with carotenoids of the investigated object (Moskalenko et al., unpublished data).

The other criterion of carotenoid reconstitution into the light harvesting complex is energy interaction with bacteriochlorophyll [30-32]. Registration of fluorescence excitation spectra revealed that effectiveness of energy transfer from okenone to bacteriochlorophyll is at the level of the control sample. In okenone containing membranes, effectiveness of energy transfer from okenone to bacteriochlorophyll is 75-85% [1, 34]. Consequently, the carotenoid environment (i.e., complex accommodating the reconstituted carotenoid) plays a decisive role in this process. Such conclusion is consistent with experimental results [32], demonstrating the formation of carotenoid triplets; the latter depended on the type of complex used for reconstitution rather than the type of carotenoid molecule.

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