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Reconstitution of Carotenoids into the Light-Harvesting Complex B800–850 of *Chromatium minutissimum*

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Abstract—Chromatophores and peripheral light-harvesting complexes B800–850 with a trace of carotenoids were isolated from *Chromatium minutissimum* cells in which carotenoid biosynthesis was inhibited by diphenylamine. Three methods previously used for the reconstitution of carotenoids into either the light-harvesting (LH1) type complexes or reaction centers (RC) of carotenoidless mutants were examined for the possibility of carotenoid reconstitution into the carotenoid depleted chromatophores. All these methods were found to be unsuitable because carotenoid depleted complex B800–850 from *Chr. minutissimum* is characterized by high lability. We have developed a novel method maintaining the native structure of the complexes and allowing reconstitution of up to 80% of the carotenoids as compared to the control. The reconstituted complex has a similar CD spectrum in the carotenoid region as the control, and its structure restores its stability. These data give direct proof for the structural role of carotenoids in bacterial photosynthesis.

Key words: photosynthetic bacteria, carotenoid depleted complexes B800–850, carotenoids, reconstitution, structure, stability, structural role, spectroscopy, HPLC, electrophoresis

Among photosynthetic organisms, photosynthetic bacteria have one of the simplest and most stable systems for harvesting and effective transformation of sunlight energy. As a rule, such a system (bacterial photosynthetic apparatus) is composed of two light-harvesting complexes and a reaction center (RC). Light-harvesting complexes absorb the solar energy quanta and convert this energy into electronic excitation energy followed by its rapid and efficient transfer to reaction centers, where primary charge separation takes place [1, 2]. There are two distinct groups of light-harvesting complexes differing in their localization about the RC: peripheral antennas (LH2 or B800–850) and near-central antenna (LH1 or B880). The RC is localized inside a ring structure of the B880 complex to form an assembly B880–RC (the core complex) surrounded by the B800–850 complexes [3, 4].

Like in all photosynthetic organisms, in the bacterial cell the final products of three biosynthesis types, namely polypeptides, bacteriochlorophyll, and carotenoids, are precisely combined to form the pigment–protein complexes *in vivo*. However, unlike other protein–pigment complexes, low molecular weight (<10 kD) polypeptides are involved in the assembly of bacterial antennas; these polypeptides span the membrane only once, and their hydrophilic termini are exposed on the opposite sides of the membrane. These polypeptides (α and β) form two rings—the inner and the outer ones (the complex B800–850 consists of 8-9 polypeptide pairs, and the complex B880 consists of 16 polypeptide pairs)—between which (and closer to the periplasmic plane of the membrane) lie the bacteriochlorophyll dimers. An additional molecule of bacteriochlorophyll monomer is present in the peripheral antenna complex; this molecule is localized on the inner polypeptide ring closer to the cytoplasmic plane. Both the complex types also contain carotenoid molecules, which lie between the hydrophobic helixes of α and β polypeptides so that one end of the molecule is in contact with the bacteriochlorophyll dimer and the other end is exposed on the cytoplasmic plane of the membrane. Furthermore, the carotenoid molecule interacts with a series of amino acid residues of both the polypeptides and, in the complex B800–850, with the bacteriochlorophyll monomer. The complex LH1 contains 16 and the complex LH2 contains 13-14 carotenoid molecules (5-6 of them can lie on the outer side of the complex) [4-8].

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Thus, the bacterial antenna complexes are complex assemblies in which pigments and polypeptides are in harmonic interaction. Clearly bacteriochlorophyll and polypeptides compared with carotenoids are more significant, for the complexes cannot exist *in vivo* without them. So, the methods of gene engineering and/or chemical modification of these components are used in studies on the role of these components [9-11]. As for carotenoids, their biosynthesis can be suppressed by the methods of "classic" mutagenesis or using mutations in corresponding genes [12-14]. As a result, carotenoidless mutants are produced that differ from the initial strains in one antenna complex (LH1 or pseudo-LH1) instead of the two complexes described above. As a rule, in carotenoidless mutant cells the antenna complex LH1 is retained due to mutations in polypeptides, whereas the complex LH2 (pseudo-LH1) loses the site of binding with the bacteriochlorophyll monomer and becomes to resemble the complex LH1. These mutations stabilize the carotenoidless complex structure, and such a complex is a convenient object for study. Admittedly, the spatial structure of these complexes is modified so that the pigment-binding sites as well as general properties of pigment clusters could be significantly changed [15, 16]. Clearly, carotenoidless complexes of unchanged structure would be more convenient objects for studies. Until recent times from the studies on carotenoidless mutants, it seemed to be impossible that carotenoidless B800–850 type complexes could exist [1, 14-17]. We first found a series of bacterial strains in which the biosynthesis of carotenoids was almost completely suppressed with no loss of any light-harvesting complex. In carotenoid depleted membranes, a complete set of complexes was maintained, and the spectral characteristics of all complex types became unchanged [18-20]. Earlier we had developed a simple method for preparing such complexes from *Chromatium minutissimum* cells [19, 21]. The present study was undertaken to find conditions for the reconstitution of carotenoids into the carotenoid depleted B800–850 complexes and to examine some features of the complexes thus obtained.

MATERIALS AND METHODS

Chromatium minutissimum cells were grown under white light of intensity 2000 lx at 25°C as described previously [18, 21]. To inhibit carotenoid biosynthesis, the culture medium was supplemented with 12 mg/liter diphenylamine recrystallized from ethanol. The cells for experiments with the inhibitor were grown at strictly controlled temperature under white rather than red illumination since our experiments demonstrated that the inhibitor is not destroyed by white light, unlike thought earlier [18, 21], and the inhibitor activity remains unaffected. We obtained cells with carotenoid contents about 1-3% of the control level. Chromatophores were separated by differential centrifugation following cell destruction by ultrasonication as described elsewhere [3, 21]. The method of PAGE for the isolation of the B800–850 complex or its purification after carotenoid reconstitution is described in detail in [3]. The nonionic detergent Triton X-100 used previously for the treatment of membranes and isolation of the complexes causes partial destruction of carotenoid depleted complexes [18, 19]. So, it was replaced by the milder detergent dodecyl maltoside allowing isolation of complexes with native spectral characteristics and with no traces of degradation [20, 21]. The absorption spectra of the complexes were recorded directly in gels using UV-160 spectrophotometer (Shimadzu, Japan).

To extract carotenoids, 1 ml of *Chr. minutissimum* chromatophores with 40-50 optical density units at 850 nm was added to 10 ml of acetone–methanol mixture (7 : 2) under continuous stirring. Petroleum ether, 2-4 ml, and water, 20-25 ml, were subsequently added to the extract and stirred. The extracted pigments localized in petroleum ether, in the upper layer of the mixture. The pigments were collected using a pipette, transferred into a vial, and dried under nitrogen gas flow. The resulting film of pigments was rapidly washed 2-3 times with 10 ml of methanol for selective bacteriochlorophyll removal. This procedure is short enough for retaining carotenoids and allows removing the bacteriochlorophyll virtually completely (Fig. 1). The resulting fraction consists of carotenoids exclusively. Note that the presence of bacteriochlorophyll as a contamination in the carotenoid fraction would be an obstacle for reconstitution. The final carotenoid fraction was dried under nitrogen gas flow and either used immediately or stored under nitrogen at −20°C. As a result, a carotenoid mixture was obtained in which, from HPLC data, rhodopin, spirilloxanthin, and lycopene dominated.

Carotenoids were analyzed by HPLC as described previously [22, 23]. The equipment consisted of the LC 10AD*vp* pump with FCV 10A1*vp* module, which enabled solvent gradient formation under low pressure (both units from Shimadzu), two detectors—the first for monitoring at 280 nm (Gilson 111B, Gilson, France) and the second a UV160 spectrophotometer with HPLC cell for 380-600 nm registration, a 2000 double-channel integrator (Merck, Germany), and a 201 fraction collector (Gilson). Satisfactory separation of the pigments was achieved using a Separon C18 column (15 × 0.3 cm) (Tessek, Czech Republic). The solvent flow rate was 0.6 ml/min. The column was equilibrated with the mixture composed of 77% acetonitrile–water (9 : 1) and 23% ethyl acetate for 5 min. Then the mixture was linearly substituted by ethyl acetate. The total separation time varied from 25 to 30 min depending on the carotenoid composition. Pigment fractions were collected automatically. Their absorption spectra were recorded on the UV-160 spectrophotometer.

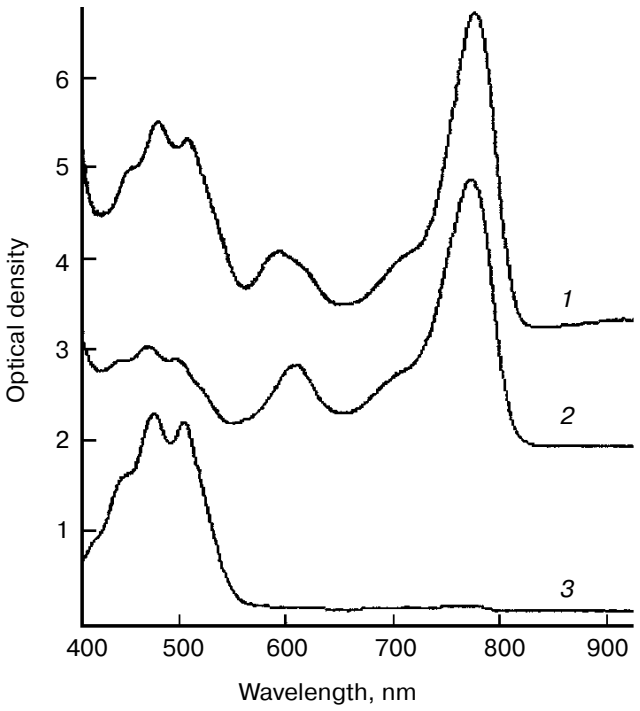

Fig. 1. Absorption spectra of acetone–methanol extract from *Chr. minutissimum* membranes (*1*), methanol extract of the dried film prepared from the same sample (*2*), and carotenoid fraction not dissolved in methanol (*3*). See details in the text.

Known techniques were tested in the present study for reconstitution of carotenoids or pheophytin into LH1 and RC complexes from carotenoidless mutants [24-29]. These techniques were changed to adapt for our sample because of high lability of *Chr. minutissimum* carotenoid depleted B800–850 complexes. The samples were homogenized in 50 mM Tris-HCl buffer, pH 8.0, in all experiments.

Incorporation in accordance with a method described in [24]. The carotenoid film was dissolved in 50-100 μl petroleum ether or benzene and the resulting solution was carefully layered on the sample surface. The chromatophores (A_{850} = 0.4) and the complex B800–850 (A_{850} = 0.4) were used as the samples. To stabilize the complex conformation, 0.1% SDS was added to the buffer. After the solvent evaporation, the carotenoids had to be transferred into buffer and, owing to their hydrophobic properties, reconstituted into the complex or form water insoluble aggregates. The method enables repeated addition of carotenoids to the sample thus increasing their concentration without destroying the structure of the complex.

The carotenoid film was dissolved in 50-100 μl of either acetone or acetone–methanol mixture (7 : 2) and added in portions of 10-15 μl to the sample—to chromatophores (A_{850} = 0.4) or to the complex B800–850 (A_{850} = 1-20), as described elsewhere [28, 29]. In this case, the solvent concentration did not exceed 20-25%, because higher solvent concentrations destabilize the complex structure disturbing the pigment–protein interaction. Since acetone causes a remarkable blue shift of the long-wave absorption band of the complex B800–850 (to 818 nm [19]), we added 0.1% SDS to stabilize its native conformation.

Incorporation by the method described in [25-27]. Dried carotenoids were diluted in 1 ml of buffer with 1% Triton X-100. After vigorous stirring, 0.5 ml of B800–850 complex (A_{850} = 18) was added to the carotenoids. The mixture was sonicated for 30 min and then an additional portion of carotenoid solution in petroleum ether (30 μl) was added to the mixture followed by repeated sonication for 30 min. The sample was incubated for 48 h at 4°C in the dark. Triton X-100 solubilized carotenoids well enough; however, in spite of substantial elevation of the sample concentration, it easily destroyed the B800–850 complex. Therefore, Triton X-100 was substituted by 1% *n*-octyl glucoside, which is commonly used for solubilization of pigments and polypeptides on reconstitution of LH1 complexes [29-31]. However, carotenoids were poorly dissolved in the buffer at low concentrations of the detergent; therefore, in further experiments the concentration of *n*-octyl glucoside was increased up to 20%. Thus, the dried carotenoids were dissolved in 50 μl 20% *n*-octyl glucoside and sonicated 2-3 times for 15 min in an ultrasonic bath. Then 150 μl of either B800–850 complex or chromatophores were added and repeatedly sonicated for 15 min. To prevent possible destruction of antennas, concentrated samples were used (A_{850} = 20-25). After incubation for 2 h, the sample was diluted with Tris-HCl buffer to the final *n*-octyl glucoside concentration of 0.8% and repeatedly sonicated for 15 min.

The following chemicals were used in the study: dodecyl maltoside (Anatrace, USA); Triton X-100, *n*-octyl glucoside, Tris, and SDS (Sigma, USA); the chemicals for electrophoresis, HPLC, and growing of the bacteria were produced in Russia.

RESULTS

Since the B800–850 complex had not yet been used for reconstitution with carotenoids, we needed to characterize it. We added diphenylamine, an inhibitor of carotenoid biosynthesis, into the culture medium. As a result, the biosynthesis of carotenoids in *Chr. minutissimum* cells was suppressed by 97-99%. The absorption spectrum of membranes isolated from the cells with highest suppression of carotenoid biosynthesis, in which spectrophotometry failed to detect carotenoids, is given in Fig. 2 (spectrum *1*). Nevertheless, these membranes con-

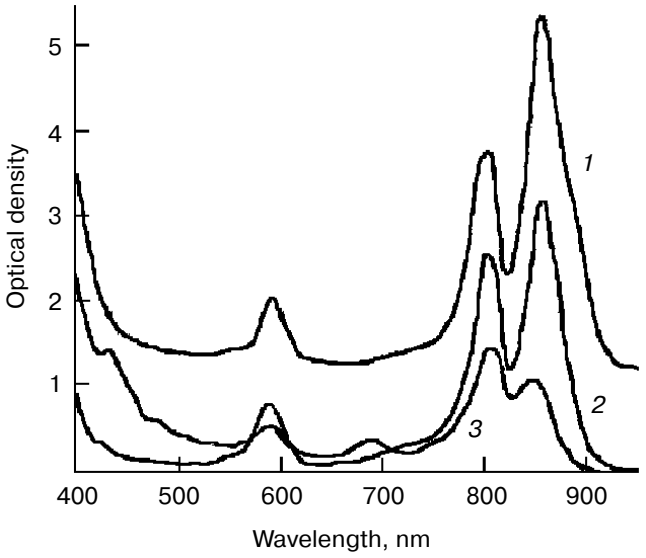

Fig. 2. Absorption spectra of membranes (*1*) isolated from *Chr. minutissimum* cells grown with diphenylamine and B800–850 complex isolated from the membranes with dodecyl maltoside (*2*) or Triton X-100 (*3*).

tained traces of carotenoids. HPLC analysis of concentrated pigment extracts of these membranes showed small amounts of rhodopin, neurosporene and its derivatives, ξ-carotene and its derivatives, and phytoene (Fig. 3). It is known that *Chr. minutissimum* membranes contain three B800–850 complexes per B880 complex (this is the photosynthetic unit) [1, 2, 19], so the total number of carotenoid molecules in this unit is 55 (100%). Hence, in *Chr. minutissimum* membranes in which the biosynthesis of carotenoids is inhibited by 98%, one carotenoid molecule is present per four mentioned complexes. So, in *Chr. minutissimum* membranes we prepared a population of B800–850 complexes containing no more than one carotenoid molecule per complex. The carotenoid precursor phytoene did not accumulate in significant amount in the samples (the carotenoid-to-phytoene ratio was 1 : 1). However, one can ignore the presence of this precursor in membranes because in carotenoidless mutants phytoene is abundantly synthesized [12], but not incorporated into the complexes. So, all studies on carotenoidless LH1 complexes from such mutants deal with the reconstitution of carotenoids rather than the substitution of phytoene by carotenoids [24-26].

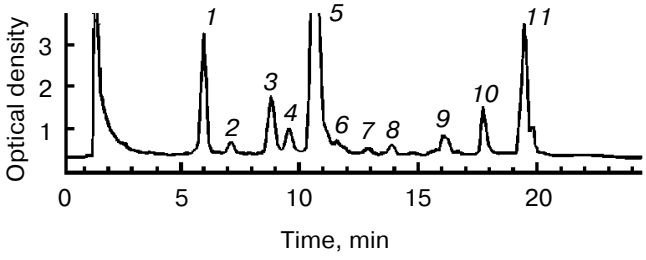

Fig. 3. HPLC of pigments in membranes isolated from the *Chr. minutissimum* cells grown with diphenylamine. Peaks: *1-5*) bacteriochlorophyll and its derivatives; *6*) rhodopin; *7*) methoxyneurosporene; *8*, *10*) ξ-carotene and its derivative; *9*) bacteriopheophytin; *11*) phytoene. Absorption monitoring at 280 nm.

We isolated the B800–850 complex from membranes by PAGE using various detergents (Fig. 2, spectra *2* and *3*). Using Triton X-100 in combination with 0.1% SDS, we did not succeed in preparing the B800–850 complex in its native conformation (the long-wave absorption band at 839 nm); moreover, some amount of bacteriochlorophyll in the complex was oxidized in the process of isolation. The complex isolated using dodecyl maltoside completely lacked these faults. It is worth noting that, unlike carotenoidless LH1 complexes from mutants, the complexes we isolated possessed highly labile structure and so were easily destroyed. Thus, all of the three described samples either are practically carotenoid depleted (the complexes B800–850 isolated by two methods) or contain carotenoid depleted complexes (*Chr. minutissimum* membranes) and, hence, are very convenient for carotenoid reconstitution studies.

The methods developed to date for the reconstitution of carotenoids are based on different ways of solubilization of a pigment film and can be divided into three groups. The first, carotenoids in a water-insoluble solvent are layered on a sample. The second, carotenoids in a water-soluble solvent are added directly to the sample. The third, a carotenoid film is solubilized in a detergent solution and added to the sample.

Considering the high lability of the carotenoid depleted B800–850 complex from *Chr. minutissimum*, the use of solvents not miscible with water seemed to be the mildest method for the reconstitution of carotenoids. In this case, there is no direct contact between the complex and the solvent, since the latter is above the sample. While the solvent evaporates, carotenoids should enter the buffer and, due to their hydrophobic nature, either incorporate into the complex or form aggregates. In this study we used petroleum ether, benzene, or their mixture. Both the solvents easily dissolve carotenoids from *Chr. minutissimum* and only differ in their rates of evaporation. After several portions of carotenoids in petroleum ether were added followed by electrophoresis, two colored bands were visible; the first inside and the second on the top of the gel (data not shown). The absorption spectra of these bands are displayed on the Fig. 4. It is easily seen that the band entering the gel (it should represent the LH2 complex because electrophoretically homogeneous complex

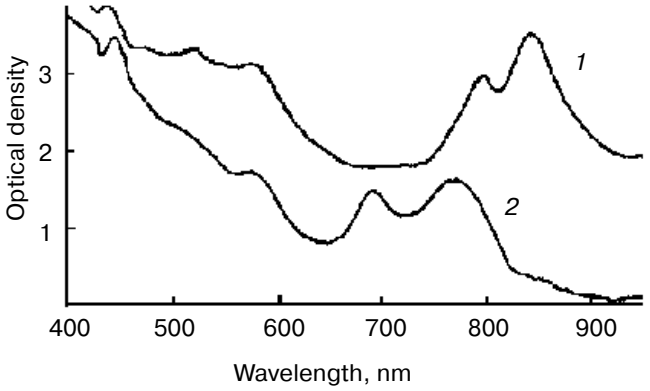

Fig. 4. Absorption spectra of electrophoretic bands of the reconstituted B800–850 complex after carotenoid incorporation with petroleum ether: *1*) the band on the top of the gel; *2*) the band penetrating into the gel.

was applied) is a destroyed complex with monomeric and oxidized bacteriochlorophyll (absorption maxima located between 650 and 800 nm). The band localized on the top of the gel according to near infrared absorption spectrum resembles the B800–850 complex (Fig. 4, spectrum *1*). However, in the range of the carotenoid absorption region and Q_x band of bacteriochlorophyll (400-600 nm) a small increase in absorption is only visible indicating a possible nonspecific (since, unlike the control complex, the Q_x band was not drawn up as a distinct peak) interaction of some portion of carotenoid into the complex. Similar results were obtained when benzene was used. Obviously, carotenoids cannot enter the water phase and incorporate into the complex in the absence of strong detergents used in the original protocols (Triton X-100 or lauryldimethylaminooxide) [25-27]. In this case, there appears to be the aggregation of carotenoids directly after the solvent was evaporated.

The experiments with solvents miscible with water were more successful (Fig. 5). Here, the membranes were used as a sample to improve stabilization of the B800–850 complex. A small blue shift of the long-wavelength band from 856 to 851 nm in the absorption spectrum of B800–850 complex and appearance of a small peak of oxidized bacteriochlorophyll (with maximum at 697 nm) were detected after carotenoids dissolved in acetone–methanol were added to the membranes. Simultaneously, the light absorption decrease detected in the NIR region at 880 nm was assessed for the B880 complex, and significant absorption increase in the carotenoid region was observed. Based on these results, one suppose that the reconstitution of carotenoids occurs simultaneously with destruction of the B880 complex. The data of electrophoresis confirm this supposition: band corresponding to the B880 complex was not detectable in the gel. Absorption spectra of the control B800–850 complex and of analogous complex with reconstituted carotenoids are given in the Fig. 5b. The comparison of these spectra suggests that the carotenoids are reconstituted into the complex; however, we failed to reconstitute the carotenoid absorption bands and their intensity in this way. It was impossible to carry out an additional reconstitution using this technique because the method results in the elevation of the solvent concentration and destruction of the complex.

Our experiments show that the reconstitution of carotenoids into the complex requires their preliminary solubilization in the solution added to the sample. This solution is to contain a detergent. The detergent should solubilize the carotenoid film and then prevent aggregation of carotenoids when added to the water phase con-

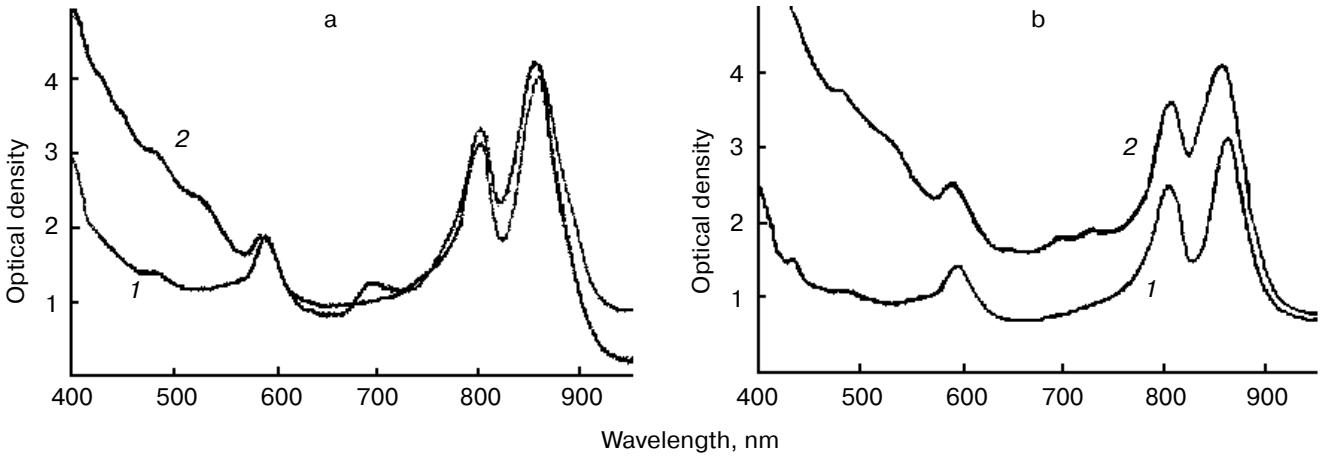

Fig. 5. a) Absorption spectra of membranes isolated from *Chr. minutissimum* cells (grown with diphenylamine) before (*1*) and after (*2*) the addition of carotenoid solution in acetone–methanol (final concentration 10%); b) absorption spectra of the control B800–850 complex (*1*) or the complex with incorporated carotenoids (*2*) isolated by PAGE with dodecyl maltoside. The spectra were registered directly in the gel.

taining the sample. It should possess high micelle-forming concentration to avoid carotenoid retention in the detergent micelles, thus allowing carotenoids to enter the buffer and reconstitute into the complex. *n*-Octyl glucoside with its micelle-forming concentration of 0.8% meets these requirements and is widely used for the reconstitution of LH1 type complexes and subcomplex B820 of purple bacteria from polypeptides and pigments [30-33]. This approach also allows elevation of the concentration of membranes (complexes) to increase the stability of B800–850 complex during the reconstitution. Based on the data presented in the studies considered above, we modified the earlier proposed technique [26] enabling the reconstitution of carotenoids into the B800–850 complex from *Chr. minutissimum* (see "Materials and Methods"). Solubilization of carotenoids in detergent solution is an important step of the technique. The source of the detergent is apparently of little importance because the detergents from four different sources were used with virtually the same results. The carotenoid film interacting with a detergent solution does not form a true solution, because the peaks characteristic of carotenoids are missing in its absorption spectrum, but solubilization of carotenoids in detergent micelles takes place. When this solution is added to the carotenoid depleted membranes, the following three processes occur simultaneously: 1) carotenoids are reconstituting into the B800–850 complex; 2) the B800–850 complex changes its conformation; 3) the B880–RC assembly was destroyed (Fig. 6a, spectrum *2*). One can judge the reconstitution of carotenoids from the appearance of absorption bands with the main peak at 483-484 nm characteristic of carotenoids. In this stage, the NIR absorption spectrum of membranes with reconstituted carotenoids resembles that of control light-harvesting complex B800–850 treated with either nonionic detergent Triton X-100 or *n*-octyl glucoside. These detergents cause a reversible blue shift of the long-wavelength absorption band of the complex to 830 nm [19, 34]. This band shift of light-harvesting complex should unmask the band of the B880–RC assembly, which looks like a shoulder in membrane spectra (Figs. 2 and 6a, spectrum *1*). However, this band is missing after the carotenoid reconstitution suggesting that in this process the B880–RC assembly is destroyed, which confirms the data of electrophoresis: the B800–850 complex is only isolated from membranes containing reconstituted carotenoids (Fig. 6b). Along with it, a small fraction of free pigments is detected, resulting apparently from the B880–RC assembly destruction. The absorption spectrum of the B800–850 complex containing reconstituted carotenoids (Fig. 6b) after the PAGE suggests that the complex practically returned to its native conformation (peaks at 847 and 798 nm; B850/B800 ratio > 1), and that carotenoids reconstituted into the complex. This fact is confirmed by the location of carotenoid absorption bands with main maximum at 485 nm corresponding to the control sample, and the intensity of these bands are also evidence for this fact. A minor peak with maximum at 431 nm arises apparently from the residual carotenoids remaining in the complex after the growing of *Chr. minutissimum* cells with the inhibitor. The comparison of absorption spectra of the control B800–850 complex and that with reconstituted carotenoids (Fig. 7) indicates that the spectra are virtually identical in location of absorption bands of bacteriochlorophyll and carotenoids. Preliminary calculations suggest that the efficacy of the carotenoid reconstitution in this case is about 80%. The analysis of the complexes by crystallography suggests that they consist of eight to nine carotenoid molecules localized inside the ring struc-

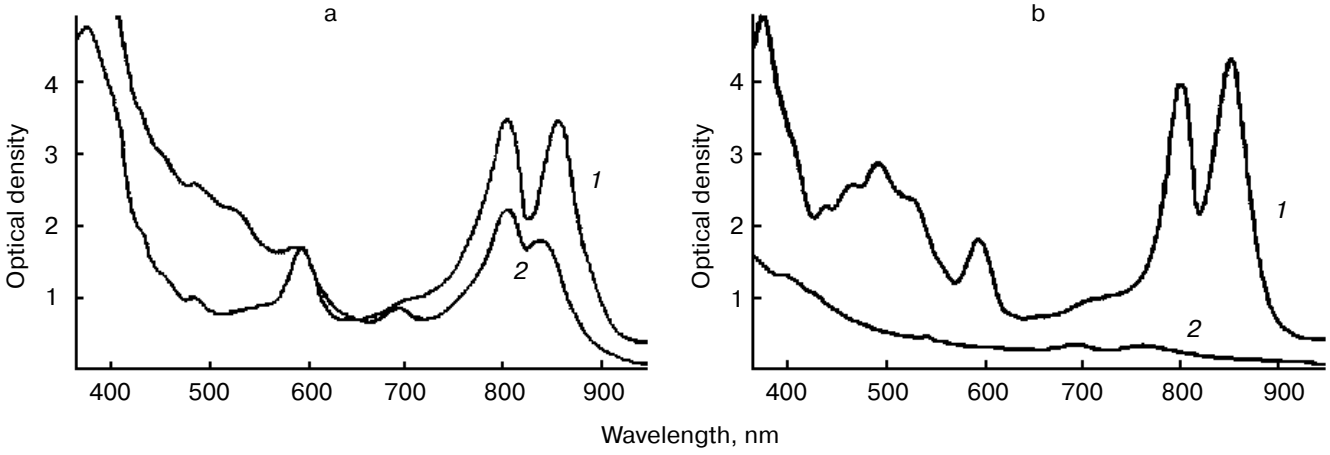

Fig. 6. a) Absorption spectra of membranes isolated from *Chr. minutissimum* cells (grown with diphenylamine) before (*1*) and after (*2*) the reconstitution of carotenoids in the presence of *n*-octyl glucoside; b) absorption spectra of the B800–850 complex with reconstituted carotenoids (*1*) and of the fraction of free pigments (*2*) isolated by PAGE from sample *2* (Fig. 6a).

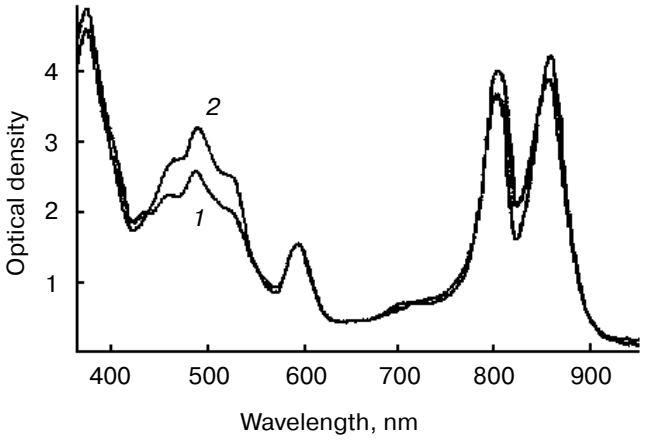

Fig. 7. Absorption spectra of the B800–850 complex with reconstituted carotenoids (*1*) and the control complex (*2*). The spectra are normalized at the Q_x band at 590 nm.

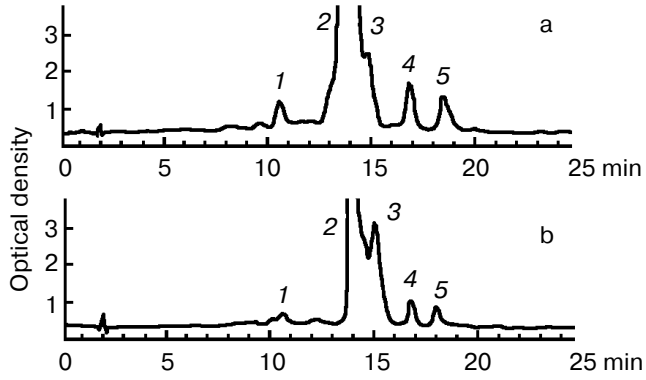

Fig. 8. HPLC of carotenoids in *Chr. minutissimum* membranes (a) and in the B800–850 complex with reconstituted carotenoids (b). Peaks: *1*) bacteriochlorophyll; *2*) rhodopin; *3*) spirilloxanthin; *4*) anhydrorhodovibrin; *5*) lycopene. Absorption monitoring at 485 nm.

ture of the light-harvesting complex, and four or five carotenoid molecules localized outside the ring [5, 6]. Thus, the B800–850 type complex contains fourteen carotenoid molecules, and we succeeded in reconstituted of eleven carotenoid molecules into the complex. The composition of carotenoids reconstituted into the complex, as judge from HPLC analysis, is virtually the same as those in membranes (Fig. 8). Only slight elevation of spirilloxanthin level is detected.

An important criterion for the evaluation of the accuracy of reconstitution of carotenoid into the pigment–protein complexes is the circular dichroism (CD) spectrum. On binding of carotenoids with pigment–protein complexes *in vivo*, the particular CD-spectra for the given complex are detected in the carotenoid absorption region. Three positive bands are present in the carotenoid region of the CD spectrum for LH2 complexes from *Rb. sphaeroides* [24], and the non-conservative CD spectrum for the *Chr. minutissimum* complex under study is detected with positive bands in the 480-580 nm region and negative bands in the 400-480 nm region (Fig. 9, spectrum *1*). Destabilization of carotenoid–protein interaction results in disappearance of the CD spectrum. The spectrum is absent in carotenoids in organic solvents or in detergent solution, as well as in carotenoids non-specifically bound to albumin [24]. Therefore, the virtually completely restored CD spectrum that we obtained after reconstitution of carotenoids into *Chr. minutissimum* B800–850 complex supports our assumption mentioned above of the correct reconstitution of carotenoids into the complex (Fig. 9, spectrum *2*).

Another important criterion confirming the correct reconstitution of carotenoids is an evaluation of the structural stability of the complex. As we demonstrated earlier, the standard B800–850 complex has very stable structure. It can only be destroyed by heating above 75°C. The complex responds to prolonged (several days) treatment with high Triton X-100 concentrations (>5%) with conforma-

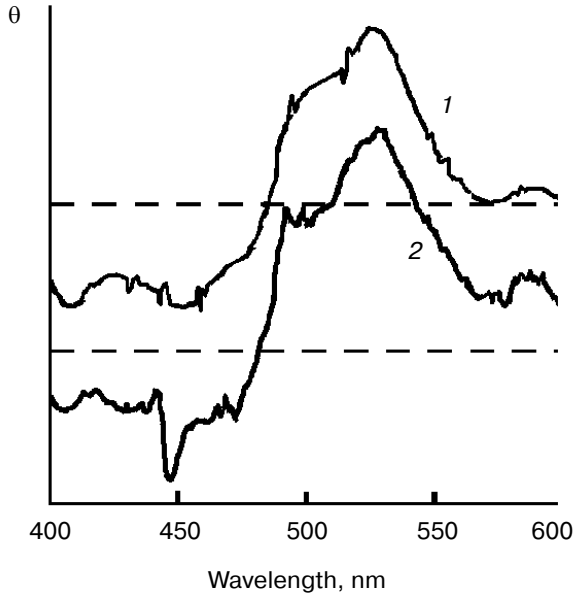

Fig. 9. Circular dichroism spectra of the control complex B800–850 (*1*) and the complex with reconstituted carotenoids (*2*).

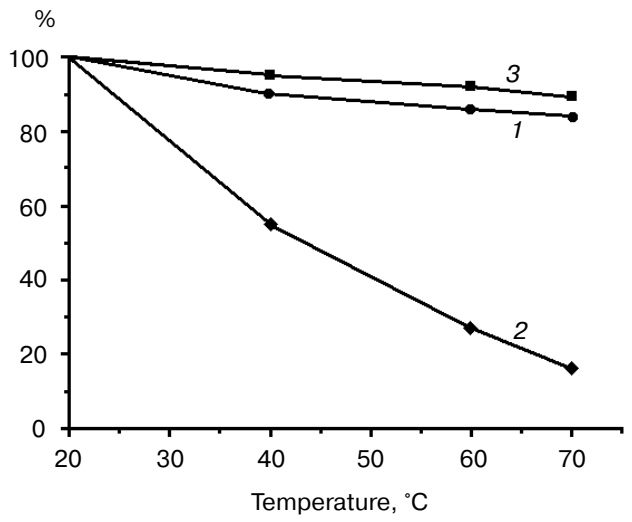

Fig. 10. Decrease in the long wavelength absorption band in the control (*1*), carotenoid depleted (*2*), and reconstituted (*3*) complex B800–850.

tional change (B850 → B830 transition), but any sign of the complex destruction (monomeric bacteriochlorophyll) is absent [19]. Carotenoid depleted complex B800–850 is very labile and undergoes complete destruction under heating at 70-75°C. The treatment of the complex with low concentration of Triton X-100 also resulted in its rapid destruction and appearance of monomeric bacteriochlorophyll [19]. From the heat denaturation curves presented in Fig. 10, it is obvious that the B800–850 complex being reconstituted with carotenoids becomes as highly thermostable as the control complex is. Similar features were observed in the presence of Triton X-100: the complex regained its structural stability after the reconstitution of carotenoids (data not shown). These results are direct evidence for the hypothesis stated above on the structural role of carotenoids in bacterial photosynthesis.

DISCUSSION

To determine the role of a distinct component (in this case, carotenoids) in the pigment–protein complex, one should compare the features proper to the control object with those of the complex without carotenoids. However, when carotenoids are removed by an inhibitor, the risk is that either additional factors (such as phytoene accumulated in membranes) can affect the structural stability of the complex or other essential components are removed together with carotenoids. The former is most significant in the opinion of leading scientists, Cogdell, Frank, Scheer, et al. (personal communication), so let us consider this aspect in greater detail.

So far, carotenoid depleted complexes are commonly prepared using mutants obtained via approaches of either classical genetics or gene engineering. These mutants are characterized by defects in the gene *crtI* encoding phytoene desaturase, the enzyme reducing a double bond in position 11-12 or 12′-11′ [35]. So, phytoene accumulates abundantly in the mutant cells. In all studied carotenoidless mutants, the B800–850 complex is not assembled. So it has been deduced that this complex can be only assembled in the presence of carotenoids [17]. It is also worth emphasizing that in carotenoidless mutants bacteriochlorophyll clusters are modified in surviving complexes. The LH1-type complex is the only one surviving, which assembles *in vivo* from modified polypeptides and are characterized by a blue-shifted long-wavelength absorption band [16]. The assembly of the complex *in vivo* is poorly understood, but it is beyond question that some factors providing the assembly of the native complex are changed.

The method we have applied in this study for preparing carotenoid depleted complexes was widely used in studies on carotenoid biosynthesis pathways in bacterial cells [36-38]. It implies the use of carotenoid biosynthesis inhibitors, and it was not actually applied for isolation of carotenoid depleted complexes. The possible reason is that according to the commonly accepted view these inhibitors, such as diphenylamine, also affect phytoene desaturase [39, 40]. So, a mutation in the gene encoding this enzyme is more convenient than the inhibitor. The data of the present study (Figs. 2 and 3), as well as the data of our previous experiments [18-22], show that the effect of the inhibitor differ from the result of mutation in the gene *crtI*. First, unlike mutants, in membranes isolated from the cells grown in the presence of diphenylamine the level of phytoene is not equal to the level of carotenoids in the control. As a rule, the phytoene to carotenoids ratio is about one in inhibited samples, whereas it ought to be 99 if the levels of phytoene synthesis and carotenoid synthesis in control were equal. Second, the traces of carotenoid (rhodopin) corresponding to one of the last stages of biosyntheses are found in the studied membranes. This fact is apparently indicative of the inhibition of phytoene synthetase rather than phytoene desaturase. And, third, unlike mutagenesis, the use of the inhibitor allows the preparation of membranes with a complete set of carotenoid depleted complexes from the *Chr. minutissimum* cells. Carotenoid depleted complexes isolated from these membranes are identical to the control complexes in their absorption spectra in the NIR region, indicating that the assembly principles remain unchanged with inhibition of carotenoid biosynthesis. Thus, the use of the inhibitor enables the preparation of not only carotenoid depleted light-harvesting B800–850 complexes (Fig. 2), which so far have not been success-

fully prepared with mutagenesis of the carotenoid biosynthesis genes, but also the B880 complexes with unchanged spectral characteristics. Surely, the inhibitor approach as the method for preparation of carotenoid depleted complexes has certain limitations. In some bacteria the carotenoid biosynthesis is slightly inhibited, because the inhibitor is metabolized, in others the inhibitor affects cell growth. Nonetheless, we have found several bacteria (*Th. sibirica*, *Chr. vinosum*, and *Ect. haloalkalliphila*) that respond to the inhibitor added into the culture medium in the same way as *Chr. minutissimum* [19-22]. The assembly of B800–850 complex in absence of carotenoids is apparently possible in some other bacteria, and the bacterium *Chr. minutissimum* is not unique in this regard.

The second question, regarding the supposition that some components that are important for the complex structure may have been removed together with carotenoids, can be solved only by the effective reconstitution of carotenoids into the complex and by the demonstration that the properties of the complex are restored after the reconstitution. The complexes from carotenoidless mutants that have been used so far for carotenoid reconstitution differ substantially from the complexes used in the present study on a number of counts. First, carotenoidless complexes from the mutants of LH1 type are noteworthy for the high stability of their structure in comparison with the complexes under the study. Hence, the complexes from these mutants can be treated with "heavy" detergents like Triton X-100 or lauryldimethyl-aminooxide that easily destroy carotenoid depleted complexes from *Chr. minutissimum*. Second, the B800–850 complex under investigation transfers irreversibly to the short-wave conformation with the long-wavelength band position at 815-818 nm after drying the sample (either membranes or complex) [19]. We therefore could not use an approach assuming carotenoid reconstitution into dried complex. Obviously, the indicated differences did not allow us to use efficiently the known methods for carotenoid reconstitution (Figs. 4 and 5).

The technique elaborated by us for carotenoid reconstitution based on the use of high octyl glucoside concentrations for the carotenoid solubilization in combination with sonication has enabled rather effective and correct reconstitution of the carotenoids into B800–850 complex from *Chr. minutissimum* (Figs. 6-10). The results demonstrate that the approach using an inhibitor enables removal of carotenoids only, whereas other components important for the structure of the complexes are not affected. Unfortunately, the technique was denaturing for the B800–RC assembly, which was subject to destruction during the reconstitution (Fig. 6). Development of a milder technique for the carotenoid reconstitution into this assembly is necessary, sending us in search of new approaches.

Based on the earlier data on the partial inhibition of carotenoid biosynthesis in *Chr. minutissimum* cells [22], one can anticipate that the sites of carotenoid binding in B800–850 complex are nonspecific and can be filled with other carotenoids. However, in *Chr. minutissimum* cells spirilloxanthin is accumulated preferentially in B880–RC assembly, whereas rhodopin is a major carotenoid of B800–850 complex [18]. The question arises, why the complexes from *Chr. minutissimum* membranes are enriched in different carotenoids when the sites of the carotenoid binding are unspecific. The simpler answer may be as follows: in spite of some non-specificity of the carotenoid binding sites at early stages of the biosynthesis (from ξ-carotene to hydroneurosporene) they are more specific for rhodopin and spirilloxanthin. These carotenoids differ in their hydrophobicity and side substitutive chains, and therefore *in vivo* they incorporate differently in different complexes. Hence, we purposely used for the reconstitution a carotenoid mixture including both rhodopin and spirilloxanthin, but not a single carotenoid, as in works with mutant carotenoidless complex [24-27]. The results obtained have demonstrated (Fig. 8) that all types of carotenoid present in membranes do reconstitute into B800–850 complex, and we failed to find any selectivity for rhodopin in carotenoid binding sites of the complex. Obviously, further more detailed experiments are necessary to answer the question of heterogeneity of carotenoid composition of complexes from *Chr. minutissimum*. One can assume that the indicated heterogeneity is due to the particular features of the assembly of the complexes *in vivo* and the strictly specified carotenoid composition corresponds to each complex.

A hypothesis on the structural role of the carotenoids in biosynthesis has been put forward in our works [41-43] on the basis of high lability of the complexes isolated from cells with inhibited carotenoid biosynthesis. However, the hypothesis was not accepted previously due to the false assumption of high level of phytoene synthesis in the presence of inhibitor [44], which is contrary to the facts as indicated above. Later, Zurdo, et al. [45] made a conclusion about the structural role of carotenoids. Using the selective extraction of carotenoids from dried *Rb. sphaeroides* membranes, they demonstrated that the removal of 40-45% carotenoids correlates with the decreasing of the absorption band for the Q_y transition of the bacteriochlorophyll in LH2 complex at 800 nm. Thus, about 50% of carotenoids have been retained in the LH2 type complex, and only the 800 nm band has been modified. In these complexes, the bacteriochlorophyll with absorption band at 800 nm is known to be very labile, and the absorption at 800 nm can be reduced by 80-100% by a number of treatments that cause only slight shift in the carotenoid absorption bands [46]. Therefore, these results cannot be considered as a convincing evidence for the structural role of carotenoids. Nevertheless, the

hypothesis about the structural role of carotenoids has been recognized after this work [47]. Later, when the data of X-ray diffraction analysis on the spatial structure of LH2 complexes was obtained, the structural role of carotenoids has been confirmed based on the position of the carotenoid molecules in the complexes. The authors anticipate that the central position of the carotenoid molecule between two polypeptides and their contacts with bacteriochlorophyll dimer and monomer provide the stability of the structure of the complex [48]. Nevertheless, we do not know any other direct demonstrations of the fact that carotenoid removal substantially weakens the structure of LH2 complexes, whereas carotenoid reconstitution restores their stability. Therefore, we anticipate that the results presented here on the carotenoid incorporation into *Chr. minutissimum* B800–850 complex that enable enhancing the structural stability up to the control level, are presently the unique and direct evidence for the structural role of carotenoid in bacterial photosynthesis.

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