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Characterization of LHI$^-$ and LHI$^+$ *Rhodobacter capsulatus pufA* Mutants

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The NH_2 termini of light-harvesting complex I (LHI) polypeptides α and β of *Rhodobacter capsulatus* are thought to be involved in the assembly of the LHI complex. For a more detailed study of the role of the NH_2-terminal segment of the LHI α protein in insertion into the intracytoplasmic membrane (ICM) of *R. capsulatus*, amino acids 6 to 8, 9 to 11, 12 and 13, or 14 and 15 of the LHI α protein were deleted. Additionally, the hydrophobic stretch of the amino acids 7 to 11 was lengthened by insertion of hydrophobic or hydrophilic amino acids. All mutations abolished the ability of the mutant strains to form a functional LHI antenna complex. All changes introduced into the LHI α protein strongly reduced the stability of its LHI β partner protein in the ICM. The effects on the mutated protein itself, however, were different. Deletion of amino acids 6 to 8, 9 to 11, or 14 and 15 drastically reduced the amount of the LHI α protein inserted into the membrane or prevented its insertion. Deletion of amino acids 12 and 13 and lengthening of the stretch of amino acids 7 to 11 reduced the half-life of the mutated LHI α protein in the ICM in comparison with the wild-type LHI α protein. Under the selective pressure of low light, revertants which regained a functional LHI antenna complex were identified only for the mutant strain deleted of amino acids 9 to 11 of the LHI α polypeptide [U43 (pTPR15)]. The restoration of the LHI$^+$ phenotype was due to an in-frame duplication of 9 bp in the *pufA* gene directly upstream of the site of deletion present in strain U43(pTPR15). The duplicated nucleotides code for the amino acids Lys, Ile, and Trp. Membranes purified from the revertants were different from that of the reaction center-positive LHI$^+$ LHII$^-$ control strain U43(pTX35) in doubling of the carotenoid content and increase of the size of the photosynthetic unit. By separating the reaction center and LHI complexes of the revertants by native preparative gel electrophoresis, we confirmed that the higher amount of carotenoids was associated with the LHI proteins.

The purple nonsulfur bacterium *Rhodobacter capsulatus* is able to produce ATP alternatively by photophosphorylation under anaerobic conditions or by oxidative respiratory phosphorylation in the dark (for a review, see reference 22). Changes of oxygen partial pressure or of light intensity govern biosynthesis of the intracytoplasmic membrane, of photosynthetic complexes, and of the pigments bacteriochlorophyll (Bchl) and carotenoids (reviewed in references 20, 23, 28, and 29). Pigments and pigment-binding polypeptides are coordinately assembled into the two light-harvesting antenna complexes I and II (LHI and LHII) and into the reaction center (RC) complex (for reviews, see references 19, 25, 29, and 40). The complexes are located in distinct membrane areas (9, 15) and contain two different pigment-binding polypeptides (LHI α and β, LHII α and β, and RC-M and RC-L). Additionally, one non-pigment-binding polypeptide is present in the LHII and RC complexes (19, 23, 40).

The LHI complex is directly associated with the RC complex. Both complexes form the core of the photosynthetic apparatus (19). LHI α and β and RC-M and RC-L are encoded by the *puf* operon of *R. capsulatus* (2, 57). The third subunit of the RC (RC-H), however, is encoded by the *puhA* gene at another site of the photosynthetic gene cluster (16, 47).

The LHI α and β polypeptides are present in a 1:1 ratio. The LHI apoproteins are integral proteins of the intracytoplasmic membrane (ICM) and span the membrane once with a central hydrophobic domain (51–53). The NH_2 terminus of the LHI α polypeptide is located in the cytoplasm and has a short hydrophobic central part of amino acid residues flanked by positively charged residues which are next to a conserved proline (51, 52). The LHI α protein is inserted into the ICM in wild-type amounts only in the presence of the LHI β protein (43). The LHI β protein, however, has a negatively charged NH_2 terminus and is able to integrate temporarily into the membrane in the absence of the LHI α protein (43). The LHI β protein was observed to be inserted earlier into the ICM than was the LHI α protein (43).

The described structures and features of the LHI α and β polypeptides are conserved among several species of the genus *Rhodospirillaceae* (50). Two highly conserved amino acid residues (Trp-8 and Pro-13) are present in the NH_2 terminus of the LHI α polypeptide (50). The aromatic structure of the Trp at position 8 seems to be of importance for the insertion of the LHI α polypeptide into the ICM (42). The conservative amino acid residue Pro-13, however, seems to be necessary for keeping the LHI β partner polypeptide in the membrane (42).

Deletions or insertions have been introduced into the NH_2 terminus of the LHI α protein to obtain more information on the structural requirements for the membrane insertion of the LHI α protein and the assembly of both LHI proteins into a functional complex. The construction and characterization of *R. capsulatus* LHI$^-$ strains containing a mutated LHI α polypeptide are described in this report. Additionally, LHI$^+$ revertant strains of one LHI$^-$ mutant strain are characterized.

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TABLE 1. *R. capsulatus* strains used

Strain (plasmid)	Phenotype	Reference	Genotype; changes in the LHI α protein[a]
U43	RC⁻ LHI⁻ LHII⁻	58	*puf*
U43(pTX35)	RC⁺ LHI⁺ LHII⁻	17	*puf*⁺
U43(pTPR9)	RC⁺ LHI⁻ LHII⁻	43	(*puf*⁺); deletion of the *pufB* gene
U43(pTPR13)	RC⁺ LHI⁻ LHII⁻	This study	*puf*⁺; αI ΔAsp-12–Pro-13
U43(pTPR14)	RC⁺ LHI⁻ LHII⁻	This study	*puf*⁺; αI ΔLys-6–Ile-7–Trp-8
U43(pTPR15)	RC⁺ LHI⁻ LHII⁻	This study	*puf*⁺; αI ΔLeu-9–Val-10–Phe-11
U43(pTPR16)	RC⁺ LHI⁻ LHII⁻	This study	*puf*⁺; αI ΔArg-14–Arg-15
U43(pTPR17)	RC⁺ LHI⁻ LHII⁻	This study	*puf*⁺; αI ↓ Ala-Ile-Leu-Met between Leu-9 and Val-10
U43(pTPR18)	RC⁺ LHI⁻ LHII⁻	This study	*puf*⁺; αI ↓ Ser-Lys-Arg-Thr between Leu-9 and Val-10
U43(pTPR15-R)[b]	RC⁺ LHI⁺ LHII⁻	This study	*puf*⁺; αI Lys-9–Ile-10–Trp-11
U43(pTPR15-R2n)[c]	RC⁺ LHI⁺ LHII⁻	This study	*puf*⁺; αI Lys-9–Ile-10–Trp-11

[a] Δ, deletion of amino acid residues; ↓, insertion of amino acid residues.
[b] One of the LHI⁺ revertant strains of U43(pTPR15), isolated directly after a low-light experiment. All revertant strains of U43(pTPR15) were characterized as being phenotypically and genotypically identical with U43(pTPR15-R); therefore, the other revertants are not listed here.
[c] Phenotypically and genotypically identical with strain U43(pTPR15-R); constructed as described in Materials and Methods and Results for excluding chromosomal mutations to be present in U43(pTPR15-R).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. All bacterial strains and plasmids used in this study are listed in Table 1. Growth conditions for *Escherichia coli* strains are described elsewhere (39, 43). Mutant strains of *R. capsulatus* were grown in malate mineral medium (18) semiaerobically in the dark in Erlenmeyer flasks (32°C; rotary shaker, 120 rpm). Low-light batch cultures of *R. capsulatus* strains were cultivated in malate mineral medium in airtight screw-cap bottles at 28°C without shaking (2,500 lx; distance to the lamps, 10 cm). If strains contained plasmids conferring tetracycline resistance, tetracycline was added to the medium in a concentration of 20 μg (*E. coli*) or 2 μg (*R. capsulatus*) per ml.

Analytical methods. ICMs of *R. capsulatus* strains were purified by discontinuous sucrose gradient centrifugation as described elsewhere (28, 51). Total amounts of the pigments Bchl and carotenoids were determined according to Clayton (10). Protein concentrations of purified membranes were determined as described by Lowry et al. (38). Determination of the RC has been described elsewhere (48, 49). The amounts of Bchl and RC per single cell were calculated from the values per milligram cell protein, the cell number per milliliter (A_{660} [1 cm] = 1.2 corresponds to 5×10^8 cells per ml), and the milligrams of protein per milliliter of cells. Absorption spectra were measured with a Kontron spectrometer (UVICON 860) and plotted by using the computer program Diagramme (Atari).

Plasmid constructions. Plasmid derivatives of pTJS133:: 2fd (31, 45) containing a 3.8-kb *Xho*II DNA fragment of *R. capsulatus* including the *puf* operon were constructed. Synthetic oligonucleotides (Fig. 1) synthesized at the University of Bielefeld were used for introduction of mutations in the *pufA* gene coding for the LHI α polypeptide. The mutations were carried out in a manner analogous to that described for deletion of the *pufA* and *pufB* genes (43). The mutations were confirmed by sequencing with appropriate primers (42–44). The plasmid constructions containing mutated *puf* operons were designated serially as pTPR13 to pTPR18 (Table 1). These plasmids were used for transfer (26, 32) from *E. coli* to *R. capsulatus* U43 (58).

In vivo pulse-labeling and pulse-chase experiments. Conditions for in vivo labeling of membrane proteins from *R. capsulatus* and chase of radioactivity were described recently in detail (43). Proteins from crude membranes and soluble fractions (43) were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gradient gels (12.5 to 17.5%) according to Laemmli (36). Protein patterns of radiolabeled proteins were analyzed after exposure of dried gels to X-ray film. Each labeling experiment was repeated at least three times for reproducibility. In this report, we present results of only one representative experiment for each mutant strain.

Determination of insertion kinetics and of the $t_{1/2}$ of the LHI proteins. For each mutant strain, two to three autoradiographs showing the protein pattern after pulse or chase experiments were scanned with an LKB 2202 UltroScan laser densitometer. Only the lower part of the autoradiographs, which included the RC and LHI proteins, (as shown, for example, in Fig. 3 and 4) was scanned. The values scanned were integrated with the 3390^A integrator (Hewlett-Packard Co.). From these values, we determined the insertion kinetics and the half-life ($t_{1/2}$) of the LHI proteins on each X-ray film, taking into account a variation of 10 to 15% according to the variation that we have determined for the control strain U43(pTX35) in different experiments. Each lane on an autoradiograph was measured three times at slightly different positions (left, middle, and right), and the arithmetic mean was determined.

Because of high mean variations determined for absolute amounts of the LHI proteins in the control strain U43 (pTX35), we will not present quantitative values for the mutant strains U43(pTPR13) to U43(pTPR18) but only indicate whether amounts are similar to, higher than, or lower than amounts in the control strain.

Isolation of plasmids from *R. capsulatus* mutant strains; subcloning strategy for sequencing reactions. Cells of *R. capsulatus* carrying plasmid derivatives of pTJS133::2fd (31, 45) were grown semiaerobically in the dark in the presence of tetracycline up to A_{660} (1 cm) = 1.2 to 1.4; 50 ml of these cultures was used for isolation of plasmid DNA as described by Birnboim and Doly (3). Only small amounts of plasmid DNA can be obtained from *R. capsulatus* cells. Therefore, plasmid DNA was transfered to *E. coli* HB101 (4) directly after isolation from *R. capsulatus* cells.

A 903-bp/912-bp *Eco*RI-*Pst*I DNA fragment (57) of the *puf* operon (2) containing plasmid pTPR15 or of its mutated derivatives (Table 1) was inserted into the replicative form of phage M13mp18 (55) and amplified in *E. coli* TG1 (purchased

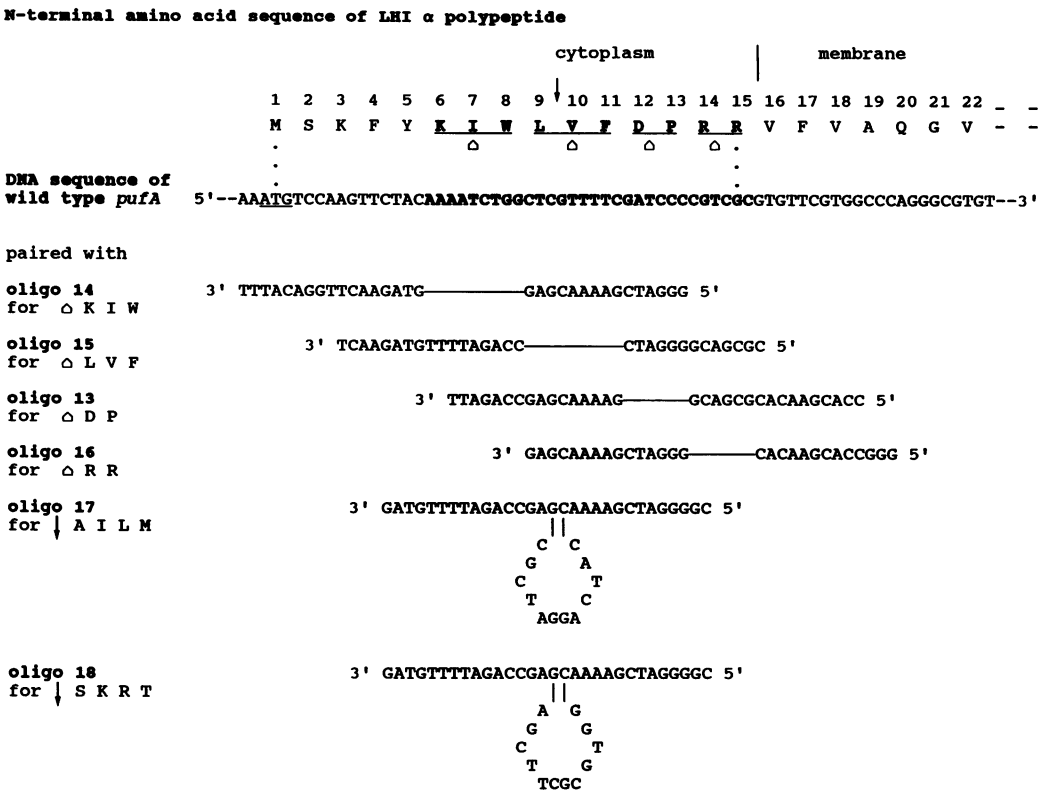

FIG. 1. Sequences of the synthetic oligonucleotides used for deletions and insertions of nucleotides in the *pufA* gene of *R. capsulatus* and pairing with the wild-type *pufA* gene. The first 22 amino acid residues of the LHI α protein of *R. capsulatus* (52) are shown at the top. The region facing the cytoplasm and that located in the ICM (51) are indicated. The site of insertion of additional amino acids between Leu-9 and Val-10 is marked by an arrow; sites of deletions are underlined and marked by open arrowheads. The wild-type *pufA* DNA sequence of *R. capsulatus* strains (57) is written in 5′→3′ direction below the amino acid sequence of the LHI α protein. Each codon is written directly below the amino acid residue for which it codes. The synthetic oligonucleotides used in the construction of mutated *pufA* genes are paired in 3′→5′ direction with the wild-type *pufA* DNA sequence. Regions of nucleotide deletions are indicated by solid lines; those of insertions are represented as loops.

from Amersham International plc). The *Eco*RI-*Pst*I DNA fragment contains the *pufB* (coding for LHI β) and *pufA* (coding for LHI α) genes and part of the *pufL* gene (coding for RC-L) of *R. capsulatus* U43(pTPR15) or of its LHI$^+$ revertants (Table 1). The DNA sequence of the coding strand of the *Eco*RI-*Pst*I DNA fragment was determined by single-strand sequencing (44) with appropriate primers (primers described in references 42 and 43 and the universal primer for M13 sequencing from Pharmacia LKB, Germany). The DNA sequence of the coding strand was determined in the *Eco*RI-to-*Pst*I direction by reading it from the back side of the autoradiograph.

Retransfer of pTPR15 plasmid derivatives from *E. coli* to *R. capsulatus*. The 912-bp *Eco*RI-*Pst*I DNA fragment containing the *pufB* and *pufA* genes and part of the *pufL* gene (2, 57) were isolated from the revertant *puf* operons and reintroduced into the wild-type *puf* operon in vector pTJS133::2fd (31, 45). These plasmid derivatives were transferred from *E. coli* HB101 to *R. capsulatus* U43 by triparental mating as described previously, using pRK2013 as conjugative helper plasmid (26, 32).

Separation of RC and LHI complexes by preparative gel electrophoresis. RC and LHI complexes of purified membranes were separated on preparative polyacrylamide gradient gels (7.5 to 15%) as described by Broglie et al. (5). The gels contained 0.4% dodecyl sulfate Li salt and 1 mM EDTA in the upper buffer reservoir.

After separation of the complexes, bands containing the RC or LHI complex were cut out of one half of the gels, and spectra were taken in the presence of 10 mM Tris HCl (pH 7.8). The complex-containing bands of the other half of the gels were directly transferred to SDS sample buffer (62 mM Tris HCl [pH 6.8], 3% SDS, 10% glycerol, 5% mercaptoethanol, 1% bromphenol blue) to remove the proteins from the gel matrix. After incubation of the gel pieces in SDS sample buffer overnight at room temperature, the proteins were separated by SDS-polyacrylamide gradient gels (36, 43).

Search for LHI$^+$ revertants of the LHI$^-$ mutant strains of *R. capsulatus* under the selective pressure of low light. Cells were grown under low light in special screw-cap bottles which contained rubber seals in the middle of the caps. Samples were taken with syringes by perforating the rubber seals of the caps.

A single colony was used to inoculate precultures, which were grown semiaerobically up to A_{660} (1 cm) = 1.0 to 1.2. Equal amounts of the precultures were used for dilution to A_{660} (1 cm) = 0.02 to 0.04 in 50 ml of medium with tetracycline and for dilution in medium without the antibiotic in airtight screw-cap bottles. The cultures were incubated in the dark for 12 h at 32°C (consumption of oxygen) and then exposed to light; 500-μl samples were taken each 3, 6, 9, or 12 h, and the bottles were refilled with medium also standing in the light. The optical density (A_{660}) was determined for each sample, and adequate dilutions were plated on agar

plates with or without tetracycline for determination of growth rate, mutation rates, and eventually loss of the plasmids.

Strains were cultivated under low light for up to 14 to 28 days. RC^+ LHI^+ $LHII^-$ cells grew about two times faster under low-light conditions than did RC^+ LHI^- $LHII^-$ cells (see Results). Therefore, rare mutations leading to LHI^+ revertants should be selected and accumulated by growth under low light. RC^+ LHI^- $LHII^-$ strains of *R. capsulatus* had a pale pink-orange color. Strains which regained an RC^+ LHI^+ $LHII^-$ phenotype were dark red.

To look for cells which regained the ability to form an LHI antenna complex, cells of the mutant strains were plated out on agar plates (malate mineral medium with or without tetracycline) in dilutions ranging from 10^5 to 10^8 cells per plate. The plates were incubated under anaerobic light conditions (1,200 lx; distance to the lamps, 20 cm; 30°C) in transparent acrylic glass containers. The light intensity could not be increased to more than 1,200 lx; otherwise, the temperature in the containers reached 34 to 36°C, temperatures known to prevent the growth of *R. capsulatus* cells.

RESULTS

Characterization of mutant strains with deletions or insertions in the NH_2-terminal segment of the LHI α protein of *R. capsulatus*. **(i) Spectroscopic analysis.** *R. capsulatus* U43, used as the recipient for the plasmids constructed in this study (Table 1), is unable to synthesize pigment-protein complexes and therefore is photosynthetically inactive (RC^- LHI^- $LHII^-$ phenotype). The major part of the *puf* operon has been deleted in strain U43, and a point mutation in the *pucC* gene blocks the expression of the LHII complex (54a). Because of its phenotype, U43 is a suitable strain for expression of *puf* genes in *trans* and analysis of effects caused by mutated *puf* genes.

Transfer of plasmids pTPR13 to pTPR18 (Table 1), coding for the RC-M, RC-L, LHI β, and mutated LHI α polypeptides, to strain U43 restored the RC^+ but not the LHI^+ phenotype. Absorption spectra taken from purified membranes of all mutant strains did not show the absorption peak at 872 nm typical for the LHI antenna complex. Representative for the absorption spectra of all mutant strains characterized in this study, the typical RC absorption spectrum of membranes from mutant strain U43(pTPR15) is shown in Fig. 2C. The lack of any light-harvesting complex was confirmed by reversible bleaching of the RC (not shown). Photochemical activity of the RC and growth of all strains in special screw-cap bottles under low light (2,500 lx; distance to the lamps, 10 cm) confirmed that the RC was active in all strains. The growth rate of all mutant strains was determined to be about two times lower (data not shown) than that of the RC^+ LHI^+ $LHII^-$ control strain U43 (pTX35) (17, 31). Growth under high-light conditions was not tested.

Cells were grown under low-light conditions for at least 28 days to look for revertants which regained an LHI antenna complex. In only one case [mutant U43(pTPR15)] did we identify revertants which restored the LHI^+ phenotype (see below).

(ii) In vivo pulse-labeling. All deletion and insertion mutations introduced into the NH_2-terminal segment of the LHI α protein inhibited completely the formation of the LHI antenna complex. To analyze why a functional LHI complex could not be formed, the insertion of membrane proteins was examined by in vivo pulse-labeling experiments.

The LHI β protein of all mutant strains except one was integrated into the ICM with wild-type kinetics and in amounts similar to that of the wild type (Fig. 3). In the mutant strain U43(pTPR17), however, the LHI β polypeptide was inserted into the membrane with wild-type kinetics but in smaller amounts (Fig. 3D) than in the control strain (Fig. 3F).

In contrast to the LHI β polypeptide, the mutated LHI α polypeptide of U43(pTPR17) was integrated into the ICM in amounts similar to those for U43(pTX35) (Fig. 3D and F), taking into account that the mutated protein of U43(pTPR17) has an additional methionine which was also labeled by L-[^{35}S]methionine in pulse experiments. The mutated LHI α proteins present in U43(pTPR13) (Fig. 3A) and in U43 (pTPR18) (Fig. 3E) were inserted into the membrane in amounts and with kinetics similar to those for U43(pTX35) (Fig. 3F). In membranes of strain U43(pTPR15), the mutated LHI α protein was integrated only in small amounts, as shown for the LHI α protein in the absence of the LHI β protein in U43(pTPR9) (Fig. 3C; Table 1). In membranes of mutant strains U43(pTPR14) and U43(pTPR16) (Fig. 3B), no protein could be detected at the position of the LHI α protein. Because three and two, respectively, amino acyl residues have been deleted from the LHI α polypeptide in these strains, the mutated LHI α protein may have run at the position of the LHII α protein on gels. In this case, the mutated protein could have been present in only small amounts, if at all, because the intensity of the LHII α protein band on the autoradiograph was not increased significantly compared with that of the LHII α protein of U43(pTX35) (Fig. 3F).

Proteins from the soluble fractions of all mutant strains of this study were also separated on SDS-polyacrylamide gradient gels. The LHI β and LHII α and β proteins were present in the soluble fractions of all strains (unpublished results), as shown for control strains (43). In some mutants, the mutated LHI α protein could also have been present in the soluble fraction. However, this could not be proven because antibodies against LHI polypeptides were not available.

(iii) In vivo pulse-chase experiments. Pulse-chase experiments were carried out to examine the stability of the LHI α and LHI β proteins after their insertion into the membrane. In all strains tested, the LHI β protein was integrated temporarily into the ICM (Fig. 4). The $t_{1/2}$ of the LHI β protein was determined to be 2 to 3 min in all mutant strains.

In U43(pTPR13), U43(pTPR17), and U43(pTPR18), the mutated LHI α polypeptide was detectable in the membrane fraction for at least 60 min [Fig. 4F for U43(pTPR18); $t_{1/2}$ = 30 ± 3 min] or 120 min [Fig. 4B for U43(pTPR13); $t_{1/2}$ = 50 ± 5 min; Fig. 4E for U43(pTPR17); $t_{1/2}$ = 55 ± 6 min]. The mutated LHI α polypeptide which had been inserted into the ICM of U43(pTPR15) in small amounts (Fig. 3C) disappeared as fast from the membrane fraction as did its LHI β partner polypeptide (Fig. 4C; $t_{1/2}$ for LHI α = 2.5 ± 0.5 min).

Isolation of LHI^+ revertants of the LHI^- strain U43 (pTPR15). Screening light cultures of the mutant strains described in this study (Table 1) for the presence of revertant strains which regained the ability to form a LHI complex, we obtained LHI^+ revertants only for strain U43(pTPR15). Inadvertently, tetracycline had been added to the medium in that experiment. Normally, tetracycline was omitted from light cultures because of the known autooxidation of this antibiotic. Therefore, we repeated the experiment with medium that did not contain tetracycline. Surprisingly, we were not able to find revertants of U43(pTPR15) in that control

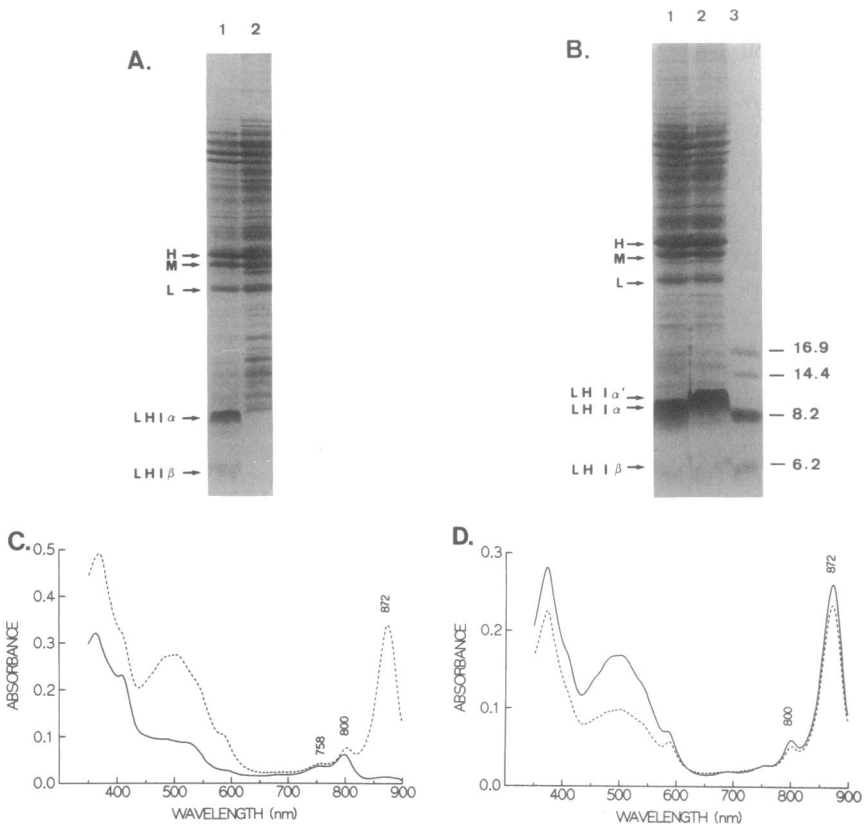

FIG. 2. Protein patterns and absorption spectra of purified membranes. *R. capsulatus* U43(pTX35), U43(pTPR15), and U43(pTPR15-R) were grown semiaerobically in the dark to A_{660} (1 cm) = 1.2. Cells were collected, and membranes were isolated from French press-broken cells by sucrose discontinuous gradient centrifugation. Proteins of the main pigmented membrane fraction were separated by SDS-polyacrylamide gradient gels (12.5 to 17.5%) (20 μg of protein per lane). Absorption spectra of purified membranes (C and D) were taken after adjusting protein solutions to 100 (D) or 150 (C) μg of protein per ml. The protein patterns of isolated membranes are shown in panels A and B; the RC subunits H, M, and L and the LHI polypeptides α and β are marked by arrows. (A) Protein pattern of purified membranes of the RC^+ LHI^- $LHII^-$ strain U43(pTPR15) (lane 2) compared with that of membranes isolated from the RC^+ LHI^+ $LHII^-$ control strain U43(pTX35) (lane 1). The absorption spectra of membranes purified from U43(pTPR15) (solid line) and U43(pTX35) (dashed line) are also shown (C). (B) Protein pattern of membranes isolated from U43(pTPR15-R) (lane 2) compared with that of purified membranes of U43(pTX35) (lane 1). The LHI α protein of the revertant strain U43(pTPR15-R) ran slower in gels than did that of the control strain; therefore, it was named LHI α'. A myoglobin polypeptide kit (Pharmacia) was used as a marker (lane 3). Numerals at the right side indicate the M_r in thousands. The absorption spectra taken from ICM of mutant and control strains (D) were almost identical beside the height of the absorption peaks for the LHI antenna (872 nm), for the carotenoids (504 nm), and for the tetrapyrroles (365 nm). Symbols: solid line, spectra taken from membranes of U43(pTPR15-R); dashed line, spectra from membranes of the control strain U43(pTX35).

experiment. It was concluded that tetracycline could stimulate or select for mutations in cultures of *R. capsulatus* U43(pTPR15) grown under low light. We have done several experiments to examine the role of tetracycline in the appearance of LHI^+ revertants of strain U43(pTPR15). These results, however, are not discussed in this report.

Single colonies of LHI^+ revertants of U43(pTPR15) from six experiments were grown semiaerobically, stock cultures were made, and these strains were analyzed. All of the LHI^+ revertant strains were then characterized as being genotypically and phenotypically identical. Therefore, only the revertant strain U43(pTPR15-R) is discussed in detail as an example (Table 1).

(i) Characterization of membranes purified from LHI^+ revertants of U43(pTPR15). The LHI α protein present in membranes of all revertants (called LHI α') ran slower in SDS-polyacrylamide gradient gels than did the LHI α protein of strain U43(pTX35) (Fig. 2B). The behavior of all other proteins was the same as that of the control.

In contrast to the absorption spectrum of membranes from strain U43(pTPR15) (Fig. 2C), the absorption spectra taken from membranes of the LHI^+ revertants were similar to that taken from membranes of U43(pTX35). However, the peak for the carotenoids at 504 nm was higher than in the control membranes (Fig. 2D). The pigment concentrations per milligram of protein and the sizes of the photosynthetic units were higher in membranes of the revertants than in those from U43(pTX35) (Table 2). However, the total moles Bchl and RC per cell were similar in the revertant strains and U43(pTX35) (Table 2).

(ii) Isolation of plasmids containing the *puf* operon of revertants of U43(pTPR15); determination of the DNA sequence of a 912-bp *Eco*RI-*Pst*I DNA fragment of the *puf* operon. Derivatives of plasmid pTPR15 were isolated from the six revertants (see above and Materials and Methods; Table 1), and the DNA sequences of the LHI structural genes *pufB* (coding for LHI β) and *pufA* (coding for LHI α) were determined from appropriate DNA fragments (*Eco*RI-*Pst*I) cloned in M13mp18.

The sequences of the whole 912-bp *Eco*RI-*Pst*I DNA

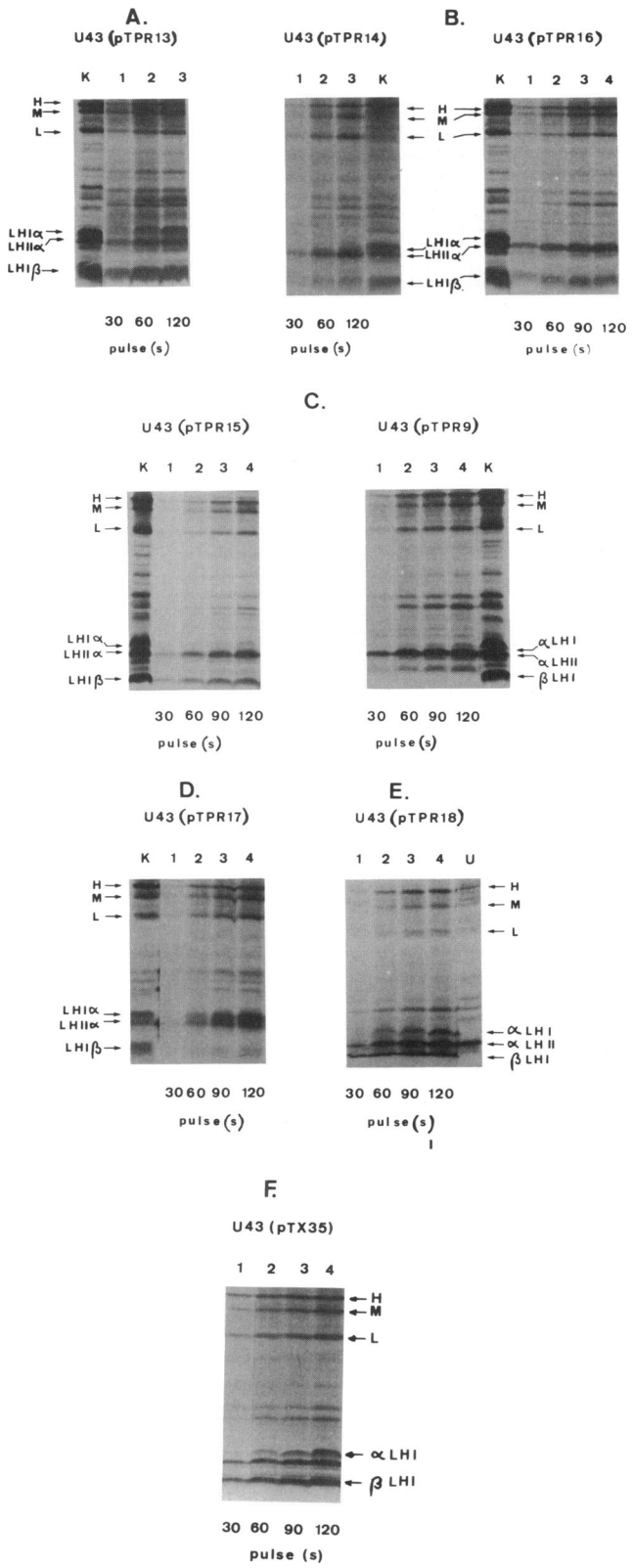

FIG. 3. In vivo pulse-labeling experiments. In vivo pulse-labeling of cells was carried out after semiaerobic induction with L-[^{35}S] methionine as described previously (43). Samples were taken after the indicated times of pulse. Crude membranes were isolated (43), and proteins were separated by SDS-polyacrylamide gradient gels (36). Gels were dried, and the protein pattern of radiolabeled proteins were determined by exposing gels to X-ray films. Only the lower parts of the autoradiographs are shown. The RC polypeptides H, M, and L, the LHI polypeptides α and β, and the LHII polypeptide α are marked. Lane K shows membrane proteins from U43(pTX35); lane U shows those of U43 for a control (Fig. 3E). In the numbered lanes, membrane proteins from mutant strains are shown. (A) U43 (pTPR13), deletion of Asp-12 and Pro-13 of the LHI α protein; (B) U43(pTPR14), deletion of Lys-6, Ile-7, and Trp-8, and U43 (pTPR16), deletion of Arg-14 and Arg-15; (C) U43(pTPR15), deletion of Leu-9, Val-10, and Phe-11 of the LHI α protein, and U43 (pTPR9), deletion of the *pufB* gene; (D) U43(pTPR17), insertion of Ala-Ile-Leu-Met between amino acids Leu-9 and Val-10 of the LHI α polypeptide; (E) U43(pTPR18), insertion of amino acids Ser-Lys-Arg-Thr between Leu-9 and Val-10 of the LHI α NH_2 terminus; (F) U43(pTX35), wild-type LHI proteins (control). Although formation of the LHII complex is inhibited completely in the parental strain U43 (58), the LHII α protein was present in all membrane preparations (43; this work) and impeded the identification of the mutated LHI α proteins. The LHII β protein was not present in the membrane fraction (43).

fragment upstream and downstream of the *pufA* gene of all revertant strains were identical to the wild-type sequence (57). The sequences determined for the *pufA* gene were identical in all revertants of U43(pTPR15) (Fig. 5B) but different from the sequences of wild-type *pufA* (57) and the *pufA* of plasmid pTPR15 (Fig. 5A). Nine base pairs have been duplicated in the *pufA* gene of the revertant strains compared with the *pufA* gene of U43(pTPR15). This duplication was in frame with the code for the LHI α protein and caused a duplication of the sequence Lys-Ile-Trp in the NH_2-terminal segment of the LHI α protein (Fig. 5C). This Lys-Ile-Trp stretch is present only once in the LHI α polypeptide of U43(pTPR15) and in the wild-type LHI α polypeptide (52) (Fig. 5C).

The finding of an additional positive charge is consistent with the different behavior of the LHI α′ protein present in the revertants compared with the LHI α protein of U43(pTX35) (Fig. 2B) on gels.

(iii) Cloning of the mutated *pufA* gene into the wild-type *puf* operon and retransfer to *R. capsulatus* U43. Mutations in the carotenoid biosynthetic pathways could result in altered colony pigmentation similar to that found in the LHI$^+$ revertants of U43(pTPR15). Therefore, the observed increase in the carotenoid content of membranes from revertant strains could have been due to changes in the carotenoid gene cluster which is located on the chromosome of *R. capsulatus* U43 (59).

The *pufA*-containing 912-bp *Eco*RI-*Pst*I DNA fragment of the revertant plasmid pTPR15-R, whose DNA sequence has been determined, was cloned again into the background of the wild-type *puf* operon of *R. capsulatus* (vector pTJS133:: 2fd) and retransferred to strain U43. Membranes isolated from the strain constructed by this in *trans* complementation [U43(pTPR15-R2n); Table 1] showed an absorption spectrum identical to that of membranes from U43(pTPR15-R) (Fig. 2D). This finding excluded the possibility that chromosomal mutations caused the restoration of the LHI$^+$ phenotype and doubling of the carotenoid content in the ICM of the revertants (Table 2; Fig. 2D).

(iv) Preparation of native RC and LHI complexes from revertant strains of U43(pTPR15). DNA sequencing of the *pufA*-containing DNA fragment of revertant strain U43 (pTPR15-R) and retransfer of this fragment to U43 in the background of the wild-type *puf* operon showed that doubling of the carotenoid content must result in some way from

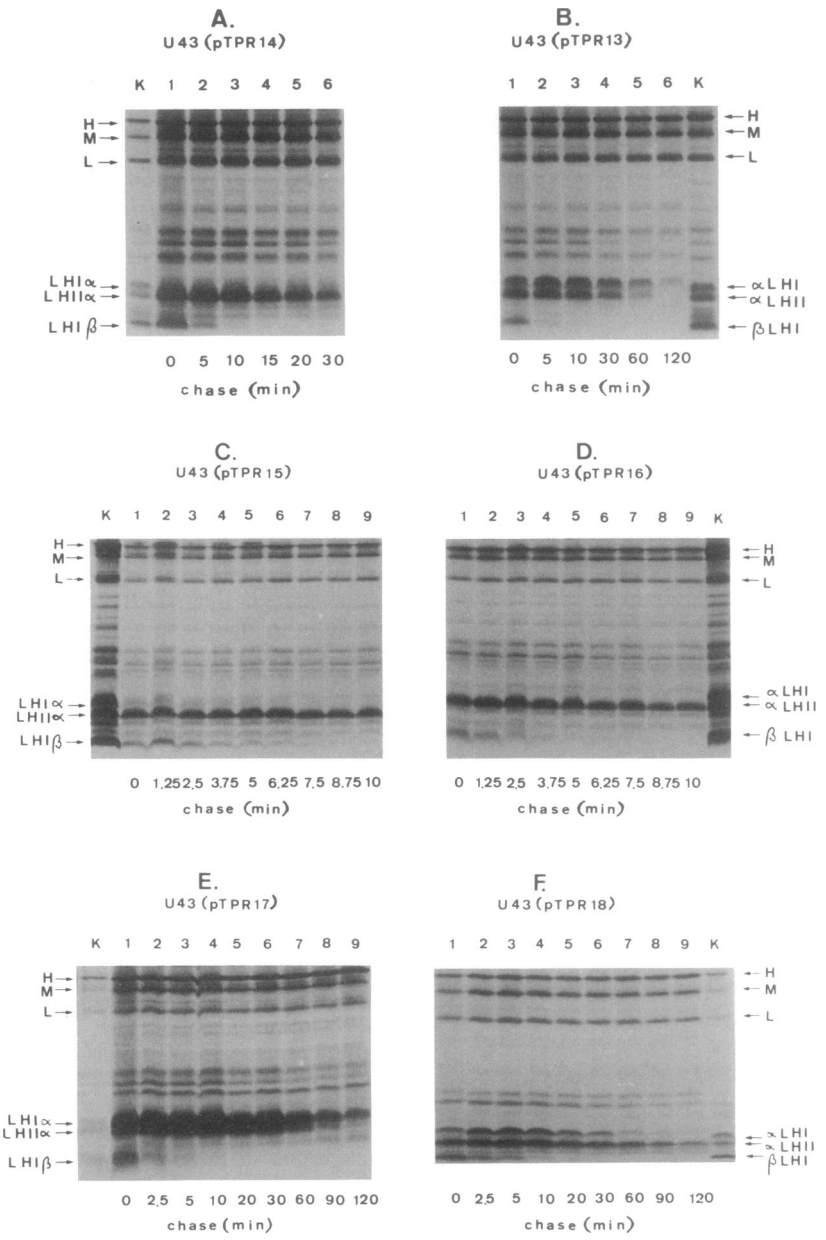

FIG. 4. In vivo pulse-chase experiments. Cells were pulse-labeled for 2 min, and radioactivity was chased as described by Richter and Drews (43). Samples were taken at the indicated times after chase. Membrane proteins were isolated and subjected to gel electrophoresis (36), and the protein pattern of radiolabeled proteins was analyzed (43). Only the lower parts of the autoradiographs are shown. The RC-H, -M, and -L proteins and the LHI α and β and LHII α proteins are marked by arrows. In lane K, membrane proteins of the control strain U43(pTX35) were separated; numbered lanes show the protein patterns of membrane proteins isolated from mutant strains (see Table 1 and legend to Fig. 3).

the duplication of Lys-Ile-Trp in the LHI α′ protein. To determine whether additional amounts of carotenoids are indeed bound to the LHI antenna complex of U43(pTPR15-R), the RC and LHI complexes of the revertant strain were isolated and compared with those of the control, U43 (pTX35) (Fig. 6).

The LHI-containing band of U43(pTPR15-R) was colored more deeply orange on native gels than was the band containing the LHI complex of U43(pTX35) (Fig. 6A). The absorption spectra taken from the RC complex-containing bands of the revertant and control strains were nearly

TABLE 2. Pigment content of the major membrane fraction isolated from U43(PTPR15-R) and from the control strain U43(pTX35)

Strain	Amt/mg of protein			Photosynthetic units (mol of Bchl/mol of RC)	Amt (10^{-7})/cell	
	nmol of Bchl	nmol of carotenoids	pmol of RC		nmol of Bchl	pmol of RC
U43(pTX35)	15.5	14.8	510	29	0.99	1.75
U43(pTPR15-R)	19.8	31.0	540	36	1.12	1.73

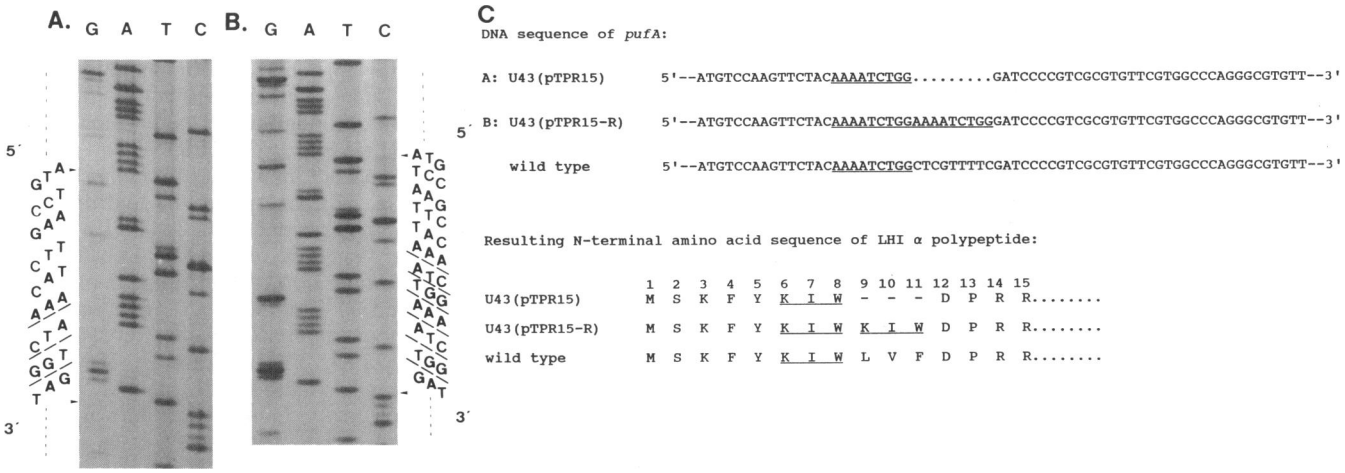

FIG. 5. Comparison of the DNA sequences determined for the 5′ ends of the *pufA* genes of mutant strain U43(pPR15) (A) and revertant strain U43(pTPR15-R) (B). The DNA sequences of the *pufA* genes of the mutant and revertant strains were determined with appropriate primers as described in Materials and Methods. The sequences derived from the autoradiographs are shown at the left (A) and right (B) in 5′→3′ direction. Aside from a duplication of the 9-bp sequence 5′-AAAATCTGG-3′, the sequences of *pufA* are identical in both strains and identical with the wild-type *pufA* gene sequence (57). (C) Comparison of the *pufA* DNA sequences and derived amino acid sequences of the LHI α proteins of mutant strain U43(pTPR15), revertant strain U43(pTPR15-R), and the wild-type strain of *R. capsulatus* (57).

identical (Fig. 6B). The absorption spectra taken from the LHI-containing bands of revertant and control strains, however, were different in carotenoid content (Fig. 6C). In contrast to other species, the native LHI complex of *R. capsulatus* is very unstable after isolation from cells. The Bchl dissociates from its original position and appears in its monomeric form. This was true not only for mutant but also for wild-type strains which have been studied so far. Therefore, in all native LHI complex preparations, only the absorption spectrum typical for Bchl at 775 nm could be demonstrated, not the absorption spectrum at 875 nm typical for the LHI complex.

However, the LHI α and β polypetides were present in the LHI complex-containing band of the control strain, as shown by SDS-polyacrylamide gel electrophoresis (Fig. 6D, lane 4). They were also present in that band of the revertant strains (Fig. 6E, lane 4) which contained the higher amount of carotenoids (Fig. 6C).

(v) Growth of the revertants in low-light cultures. The growth rates of the LHI^+ revertants of U43(pTPR15) determined in low-light cultures were almost identical to those determined for U43(pTX35) (not shown). Even in light-grown cultures with tetracycline, the revertants were as stable as was the control strain U43(pTX35), and no pale colonies which have again lost the LHI^+ phenotype have been detected. The experiments were repeated twice in the presence or absence of tetracycline with the same result.

DISCUSSION

Characterization of LHI^- mutant strains of *R. capsulatus* with deletions or insertions in the NH_2-terminal segment of the LHI α protein. Our knowledge of the assembly process of the photosynthetic complexes is restricted, but it has become evident that this process is very complex (9, 30, 33, 54, 59). Translocation of the polypeptides of the photosynthetic complexes across the lipid bilayer of the ICM depends on the proton motive force (14). Pigments are also necessary for proper and stable assembly of the photosynthetic complexes (33, 41) as well as protein-protein interactions (56).

It has been reported that separately isolated LHI β polypeptides but not LHI α polypeptides are able to form in vitro with Bchl a molecules the structural subunit (B820-like complex) of the B875 (LHI) complex (37). A functional B875-type complex, however, could be reconstituted only by addition of the LHI α protein (37). In vivo, no B875 complex was formed when the LHI α polypeptide was not synthesized (43). However, there is some evidence for a definite order in the LHI assembly; by in vivo pulse-labeling experiments, it was shown that the LHI β protein inserts earlier into the membrane than does the LHI α protein (43).

The oppositely charged amino acids of the LHI polypeptides α and β NH_2 termini are likely to be involved in the assembly of functional LHI complexes (17, 48). The results of this study support the finding that the charges near the membrane-spanning segment of both LHI proteins are of importance in LHI complex assembly. After the deletion of the two positively charged Arg residues present at positions 14 and 15 of the LHI α polypeptide, the mutated protein was not detectable in the ICM [Fig. 3B, U43(pTPR16)].

After deletion of amino acids 6 to 8 (Lys-Ile-Trp) of the LHI α polypeptide, the mutated LHI α protein was also not detectable by the methods used in this study [Fig. 3B, U43(pTPR14)]. It has been shown before that the exchange of positive charges near the NH_2 terminus of the LHI α protein has only a slight effect on the formation of the LHI antenna complex (17). Therefore, the absence of the mutated LHI α protein or its presence in very small amounts at the position of the LHII α protein in U43(pTPR14) is believed to be caused by the deletion of Trp-8 and not by the loss of the positively charged Lys-6. Aromatic residues are known to be involved in the integration of other membrane proteins into the lipid bilayer (12), and they are conserved in the NH_2-terminal segments of light-harvesting α polypeptides (50). It has been shown that the aromatic substructure of Trp-8 is of importance for the insertion of the LHI α protein into the ICM (42). Additionally, Trp-8 of the LHI α polypeptide of *R. capsulatus* possibly contributes in binding of carotenoids to the LHI antenna complex (1; our unpublished results).

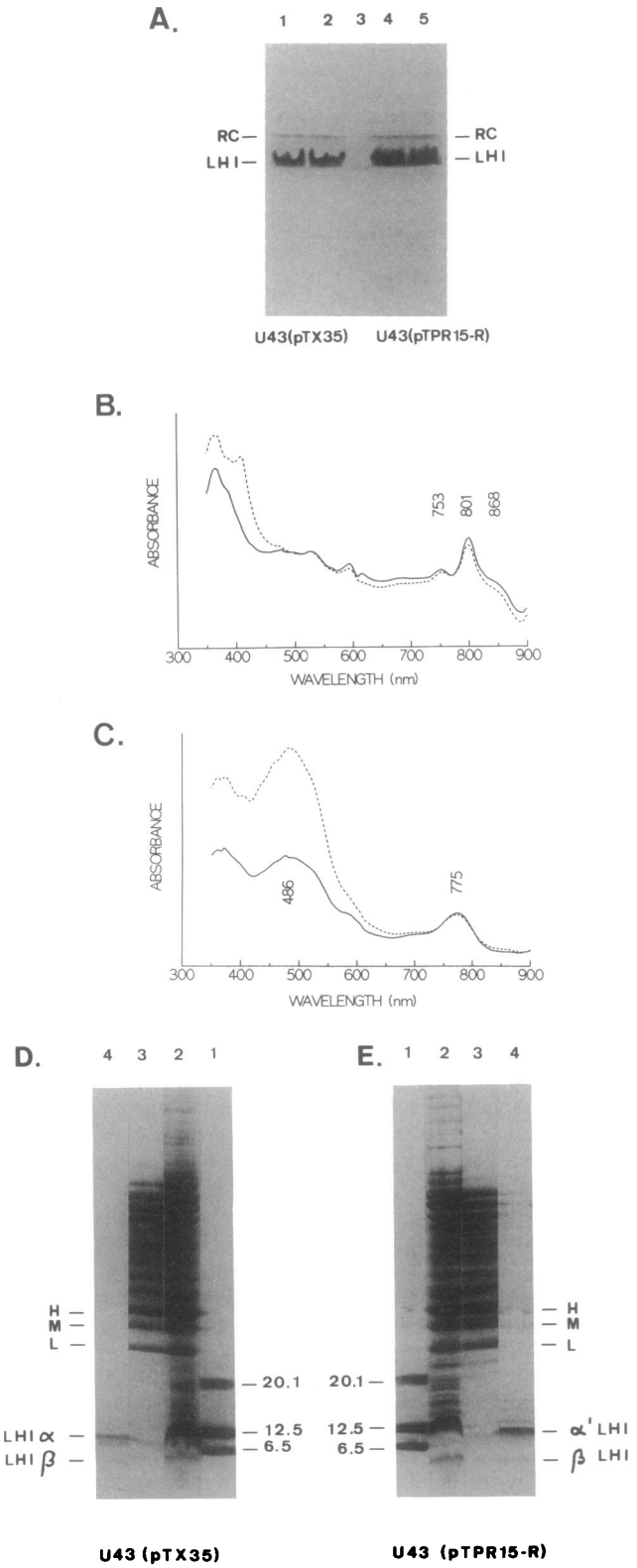

FIG. 6. Isolation of RC and LHI complexes by native preparative gel electrophoresis. Gel electrophoresis was carried out as described in Materials and Methods. (A) Middle part of a preparative gel. Each lane was loaded with 100 μg of proteins of purified membranes. The photosynthetic complexes of control strain U43(pTX35) (lanes 1 and 2) and of U43(pTPR15-R) (lanes 4 and 5) were separated. Lane 3 was empty. The RC and the LHI complex bands are indicated at the left [U43(pTX35)] and right [U43(pTPR15-R)]. (B and C) Absorption spectra. The pigment-containing bands present on the preparative gels were cut out, and absorption spectra were measured in 10 mM Tris (pH 7.8). (B) Overlay of absorption spectra taken from gel pieces which contained the RC complex of U43(pTX35) (solid line) and U43(pTPR15-R) (dotted line). (C) Overlay of absorption spectra taken from LHI complex-containing gel pieces of U43(pTX35) (solid line) and U43(pTPR15-R) (dotted line). (D and E) SDS-polyacrylamide gels. Proteins of the gel pieces cut out of preparative gels were solubilized in SDS sample buffer and separated on SDS-polyacrylamide gels. (A) U43(pTX35); (B) U43(pTPR15-R). Lanes: 1, molecular weight marker purchased from Bethesda Research Laboratories (Neu-Isenburg, Germany) (6.5, 12.5, and 20.1 indicate the M_r in thousands); 2, whole proteins from purified ICM (80 μg per slot); 3, whole proteins resolved from RC complex-containing gel pieces (the RC-L, -M, and -H proteins are indicated); 4, 1/10 of the proteins resolved from LHI complex-containing gel pieces. It was apparent that in addition to the RC polypeptides, polypeptides of higher molecular weight were present in the RC complex-containing fraction (lanes 3). However, mainly the LHI polypeptides were present in the LHI complex-containing bands (lanes 4). The background present in lanes 1 and 4 of panel D was due to overflow caused during loading of the lanes.

The hydrophobic stretch of amino acids 7 to 11 of the LHI α protein (Ile-Trp-Leu-Val-Phe) seems to be necessary for a efficient integration of this protein into the membrane. If parts of it were deleted [U43(pTPR15), ΔLeu-Val-Phe], the mutated LHI α protein was integrated into the membrane only in small amounts [Fig. 3C, U43(pTPR15)] and disappeared from the membrane fraction with a $t_{1/2}$ similar to that of the LHI β protein (Fig. 4C). Extending the hydrophobic stretch either by hydrophobic residues [U43(pTPR17)] or by hydrophilic and positively charged residues [U43(pTPR18)] did not inhibit efficient integration of the mutated proteins into the ICM (Fig. 3D and E). The mutated LHI α polypeptide was inserted temporarily into the membranes of U43(pTPR17) and U43(pTPR18) (Fig. 4E and F). However, the $t_{1/2}$s of about 30 min in U43(pTPR18) and 55 min in U43(pTPR17) were much lower than in the control strain U43(pTX35), in which the LHI proteins are known to be stable in the ICM for hours (48).

Therefore, the region of amino acids 7 to 11 of the LHI α polypeptide seems to be essential for this polypeptide in targeting to and insertion into the membrane. The exact number of five residues at positions 7 to 11 of the NH_2 terminus of the LHI α protein is also important. Shortening or lengthening of this region obviously prevented the formation of α-β pairs and resulted in a rapid degradation of the LHI β protein (Fig. 4C, E, and F), similar to results obtained in the absence of the LHI α partner protein (43). Lengthening of the hydrophobic region of the LHI α protein still enabled the contact of both LHI proteins upon integration (Fig. 4E and F) but not the assembly to functional complexes.

After deletion of Asp-12 and Pro-13 of the LHI α polypeptide [mutant strain U43(pTPR13)], both LHI proteins were inserted into the ICM (Fig. 3A) with kinetics and in amounts similar to those found in the control strain U43(pTX35) (Fig. 3F). The LHI α polypeptide was inserted into the ICM for at least 120 min of chase and with a $t_{1/2}$ of about 50 min (Fig. 4B). However, the LHI β protein was soon lost from the membrane fraction. Additionally to findings published earlier (42), this was another indication that specifically the region around the Pro-13 of the LHI α protein is needed for keeping the LHI β partner protein in the membrane. This

result may also explain that the LHI β protein of mutants U43(pTPR17) and U43(pTPR18) was lost from the membrane fraction (Fig. 4E and F) although the LHI α protein was present temporarily in the ICM. The Pro was no longer at position 13 in the mutated LHI α proteins of these mutant strains. The amino acid Pro is known as a helix-breaking residue. Therefore, one possible explanation of the results of our work and those of former studies (42) could be that the NH_2 termini of the LHI α and β polypeptides are wound around each other and that the LHI α Pro-13 is needed for fixation of the LHI β partner polypeptide in the ICM. Deletion of Pro-13 or change of its relative position will change the normal tertiary structure of the LHI α NH_2 terminus and therefore the interactions between the two LHI polypeptides.

With one exception [U43(pTPR17); Fig. 3D], the LHI β polypeptide of all mutant strains studied in this work was integrated in the membrane in wild-type amounts but was soon lost from the membrane fraction, similar to results for a strain in which the LHI α protein was not synthesized (43). In mutants U43(pTPR14), U43(pTPR15), and U43(pTPR16), this may be due to the lack of wild-type amounts of the LHI α protein in the membrane. Though both LHI proteins were present in nearly wild-type amounts in membranes of U43(pTPR13) and U43(pTPR18), the LHI proteins seem not to interact correctly. The mutated LHI α protein was much more stable than the nonmutated LHI β partner protein. This finding was surprising because the LHI α protein, though it seems to be dependent on the LHI β protein in the membrane insertion process (43), was able to remain in the membrane fraction without its partner protein. The LHI β protein, however, was able to integrate into the ICM in the absence of the LHI α protein (43), but its stability in the membrane seems to be dependent on a definite contact with the LHI α protein.

Characterization of LHI^+ revertants of *R. capsulatus* U43 (pTPR15). In *R. capsulatus* U43(pTPR15), nine nucleotides of the *pufA* gene, coding for the amino acid sequence Leu-Val-Phe, have been deleted, resulting in a RC^+ LHI^- $LHII^-$ phenotype. Growing this strain under low-light conditions, we were able to isolate revertants which regained the ability to form an LHI complex.

In all revertants of U43(pTPR15) tested so far, the DNA sequence 5′-AAAATCTGG-3′ of the *pufA* gene was duplicated directly upstream of the site of the deletion present in U43(pTPR15) (Fig. 5C). The duplicated nucleotides were in frame with the code for the LHI α polypeptide of wild-type cells and code for the amino acid sequence Lys-Ile-Trp. Therefore, the LHI α′ polypeptide present in the revertant strains has the same number of amino acids as does the wild-type LHI α protein (Fig. 5). In the wild-type LHI α protein, there are five hydrophobic residues at positions 7 to 11 (Ile-Trp-Leu-Val-Phe) which are likely to play a role in the membrane insertion process of the LHI α protein (see above). In the mutant LHI α′ of the LHI^+ revertants of U43(pTPR15), an additional positive charge has been introduced into the hydrophobic region in comparison with the wild-type LHI α protein (Fig. 5C). This, however, did not change the ability of the mutated LHI α polypeptide to integrate stably into the ICM and to assembly with its LHI β partner protein into a functional LHI antenna complex (Fig. 2D). Therefore, the additional positive charge seems not to affect the interaction between the NH_2 termini of the LHI α and β polypeptides. As shown by alignment of the primary structures of light-harvesting α polypeptides (50), the number of charged amino acyl residues is not conserved. However, a positive net charge in the NH_2 terminus of the LHI α protein is conserved (50). Because the net charge is still positive in the revertant LHI α′ protein, this may be the reason why LHI formation is not disturbed.

The functional LHI antenna complex present in the revertants like U43(pTPR15-R) was different from that of the control strain U43(pTX35) by a 25% larger photosynthetic unit value and by a 100% higher content of carotenoids (Fig. 2D; Fig. 6C; Table 2). Since the size of the photosynthetic unit of U43(pTX35) can vary between 25 and 35 (unpublished data), the observed differences between mutant and control strain membranes may be within the normal range for *R. capsulatus*. However, the increase of the carotenoid content was significant in all membrane preparations of the revertant strains.

Carotenoids are bound noncovalently to the photosynthetic polypeptides. They have a dual function in photosynthetic reactions: accessory light harvesting in photosynthetic complexes and photoprotection against the destructive effects of light or oxygen (reviewed in references 11, 13, 34, and 46). Carotenoids have been reported to be involved in light harvesting of RC and LHII complexes but not of LHI complexes (27). It has been reported that the long-living singlet state O_2* species rapidly reacts with unsaturated fatty acids, purines, and aromatic amino acids (24) and initiate processes leading to cellular damage and cell death (35). Polar functional groups probably were involved in noncovalent binding of carotenoids of the xanthophyll type at special positions in pigment-binding proteins (8). Both one aromatic amino acid (Trp-8) and one polar, charged amino acid (Lys-6) are present near each other in the NH_2-terminal segment of the wild-type LHI α protein of *R. capsulatus* (52). Therefore, it was supposed that this region could be one of the contact sites between the carotenoid sphaeroidenone (xanthophyll type) and the LHI antenna proteins (6).

This idea is supported by our results. The duplication of the Lys-Ile-Trp stretch in the LHI α protein of a mutant of *R. capulatus* caused a 100% increase of the amount of carotenoids bound to the LHI complex (Fig. 6). We have demonstrated that the observed effect was not caused by chromosomal mutations or additional mutations in the *puf* operon present in *trans*.

The conserved amino acid Trp-8 probably is one of the sites necessary for insertion of the LHI α protein into the ICM (42). Carotenoids bound to Lys-Ile-Trp could function to prevent Trp-8 from oxidative degradation, thus establishing the role of Trp-8 in the membrane integration process of the LHI α protein. Carotenoids could also fulfill another important role in the assembly of the LHI complex. It was reported that the NH_2 terminus of the B890 α protein of *Rhodospirillum rubrum* is protected against proteinase K digestion only if carotenoids were present (7). The B890 β protein, however, was always accessible to proteinase K whether carotenoids were bound or not. Brunisholz et al. (7) concluded that the B890 α protein undergoes a conformational change in the presence of carotenoids. The B890 α polypeptide of *R. rubrum* has an Arg-Ile-Trp stretch at about the same position as the Lys-Ile-Trp stretch of the LHI α protein of *R. capsulatus* (50). Arg-Ile-Trp and Lys-Ile-Trp stretches were conserved among numerous LH proteins from photosynthetic bacteria (6, 50). It has been shown that the aromatic substructure of Trp is necessary for the integration of the LHI α protein into the ICM (42). Exchange of Trp-8 of the LHI α protein of *R. capsulatus* to Ala reduced drastically the amount of the LHI α protein integrated into the membrane (42). If carotenoids are associated with the

aromatic structure of Trp and are involved in a conformational change of the LHI α protein (as postulated in reference 7), and if this change is necessary for insertion of the protein into the ICM, this would explain why the Ala-8 LHI α protein could not efficiently contact the membrane (42).

The results presented in this work and previously (17, 42, 43, 48) support the idea that the two LHI proteins interact with each other in the membrane insertion and LHI assembly processes. The detailed molecular mechanism of the LHI assembly remains to be determined.

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