Effects on the Formation of Antenna Complex B870 of *Rhodobacter capsulatus* by Exchange of Charged Amino Acids in the N-Terminal Domain of the α and β Pigment-Binding Proteins†

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ABSTRACT: The N-terminal domains of the α and β polypeptides of the B870 antenna complex of *Rhodobacter capsulatus* are oppositely charged. In both polypeptides two charged amino acids are located close to the N-terminus, and two of them are close to the hydrophobic central domain. To test the hypothesis that charged amino acids in the N-terminus have a function for insertion and assembly of pigment-binding polypeptides, charged amino acids were replaced by amino acids of opposite charge. The results show that an exchange of amino acid positions 3 and 6 in α (Lys → Glu) or 2 and 5 in β (Asp → Lys, Arg) has little effect under semiaerobic conditions on the formation of B870 but the additional exchange of positions 14 and 15 in α (Arg → Glu, Asp) and/or 13 and 14 in β (Asp, Glu → Arg) inhibits strongly under semiaerobic dark and anaerobic light conditions the stable incorporation of the polypeptides into the membrane and the formation of the B870 complex. The mutant U43(pTXAB5) is able to grow without any antenna.

The photosynthetic apparatus of *Rhodobacter capsulatus* contains three pigment–protein complexes, the photochemical reaction center (RC) and the two light-harvesting antenna complexes, named according to their main absorption peaks in the near-infrared, B870 (LHI) and B800–850 (LHII). The LHI complex forms together with the RC the core complex and is the direct donor of excitation energy to the RC. The core complex has a relatively constant size (Aagaard & Sistrom, 1972; Drews, 1985). The LHII complex is the bulk antenna in cells grown at low light intensities and determines the variable size of the photosynthetic unit, i.e., the molar ratio of total bacteriochlorophyll (Bchl) per RC. The LHII complex interconnects at least at low light intensities the RC–LHI core complexes (Hunter et al., 1989).

The pigments of the light-harvesting complexes of non-sulfur purple bacteria are combined with two different low molecular weight polypeptides named α and β. Each polypeptide has one central domain of hydrophobic amino acids, which was predicted to span the membrane once as an α helix (Cogdell & Scheer, 1985; Drews, 1985). Two molecules of Bchl and one molecule of carotenoid are proposed to be bound noncovalently to the hydrophobic portion of the two subunits α and β (Drews, 1985). The amino acid sequences of LHI α (M_r 6588) and β (M_r 5341) of *Rb. capsulatus* have been determined previously (Tadros et al., 1984, 1985). The polypeptides are oriented in the membrane in the same direction: the N-terminal domains are exposed on the cytoplasmic surface, and the C-terminal domains are exposed or point to the periplasmic surface of the membrane (Tadros et al., 1986, 1987). The N-terminal regions of LH polypeptides from non-sulfur purple bacteria contain several charged amino acids (Zuber, 1986). In the α polypeptides dominate positively charged and in the β polypeptides dominate negatively charged amino acid residues [Figure 1 and Zuber (1986)]. These charged amino acids are supposed to be involved in the process of assembly of the pigment complexes in various species of photosynthetic bacteria (Drews et al., 1983).

The N-termini of numerous membrane proteins have a positively charged amphiphilic domain which is believed to direct a protein to the membrane (Saier et al., 1989). Such a domain is present in all α polypeptides and probably causes stable insertion of α polypeptides into membranes (Dierstein et al., 1984).

In order to learn more about the role of charged N-terminal amino acids in the insertion of α and β polypeptides and the formation of the LHI complex, charged amino acids in positions close to the N-terminus (positions 2 and 5 in β and positions 3 and 6 in α) and close to the hydrophobic central domain (13 and 14 in β and 14 and 15 in α) were replaced by amino acids in opposite charge. The effect of these mutations on the formation of the B870 complex is reported here.

Materials and Methods

Strains and Culture Conditions. The strains used during this work are listed in Table I. The phototrophic bacteria were cultivated on a malate yeast extract–mineral medium (RÄH; Drews, 1983) aerobically or semiaerobically in the dark on a rotary shaker or anaerobically in the light at 30 °C in screw-cap bottles. To investigate the influence of light intensity on formation of the photosynthetic apparatus, the bacteria were grown in a light turbidostate at a constant cell mass density of about $A = 0.3$ (660 nm, 0.5 cm; corresponding to about 240 μg of cell protein/mL) similar to that described in Reidl et al. (1983). The cells were adapted to high- or low-light conditions after about three generations (Reidl et al., 1983). The culture vessel was gassed with a low stream of purified nitrogen gas (99.99%).

Plasmid Construction. Synthetic oligonucleotides (5′ to 3′ antisense) were hybridized to template DNA derived from

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Table I: Strains of *R. capsulatus* and Plasmids Used

	genotype	phenotype	ref or source
strain 37b4	wild type	wild type	DSM 938
strain B10	wild type	wild type	ATCC 33303
strain NK3	Tn5 mutation downstream of *puc*BA	LHII negative	Kaufmann et al., 1984; Tichy et al., 1989
strain U43[a]	*puf*$^-$, *puc*$^-$	RC$^-$, LHI$^-$, LHII$^-$	Youvan et al., 1985
plasmid pTJS133		Tc^R	Schmidhauser & Helinski, 1985
plasmid pRK2013		Km^R	Figurski & Helinski, 1979
plasmid pTX35[b]		Tc^R	Klug & Cohen, 1988
	Strains of Mutant U43 Containing Plasmid pTX35 or Derivatives of That[c]		
U43(pTX35)	LHII$^-$, RC$^+$, LHI; wild type		
U43(pTXB26)	LHII$^-$, RC$^+$, LHI; β D(2) → K, D(5) → R		
U43(pTXA12-1)	LHII$^-$, RC$^+$, LHI; α K(3+6) → E		
U43(pTXB87)	LHII$^-$, RC$^+$, LHI; β D(2) → K, D(5) → R, D(13) → R, E(14) → R		
U43(pTXB0325)	LHII$^-$, RC$^+$, LHI; β D(13) → R, E(14) → R		
U43(pTXA6-10)	LHII$^-$, RC$^+$, LHI; α K(3,6) → E, R(14) → E, R(15) → D		
U43(pTXAB5)	LHII$^-$, RC$^+$, LHI; α K(3,6) → E, R(14) → E, R(15) → D; β D(2) → K, D(5) → R, D(13) → R, E(14) → R		

[a] The *puf* operon of this strain was deleted and expression of B800-850 inhibited by a point mutation. [b] pTJS133 derivate containing wild-type *puf* operon. [c] The symbols behind LHI indicate which amino acid (one-letter code + position from the N-terminus) has been exchanged by an amino acid of opposite charge (behind arrow).

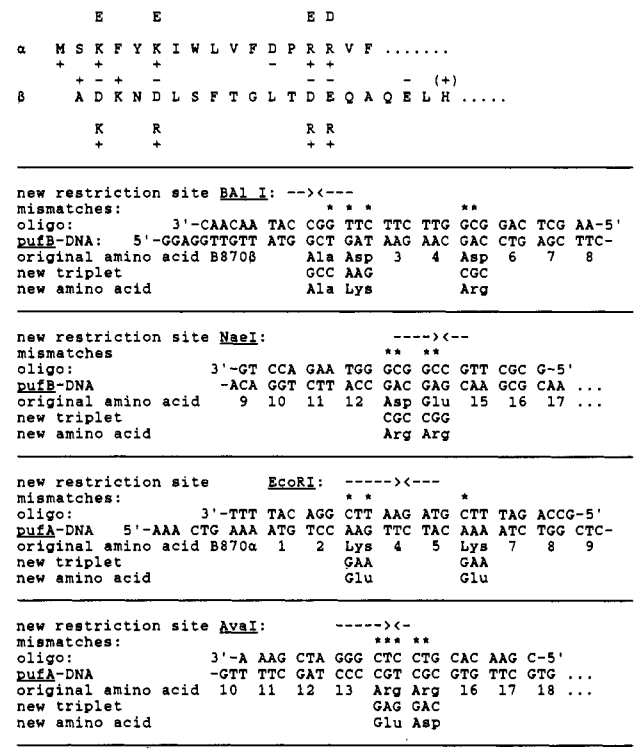

FIGURE 1: N-Terminal amino acid sequences of the α and β polypeptides of the B870 (LHI) light-harvesting complex of *Rb. capsulatus* and the exchange of amino acids by amino acids of opposite charge.

M13RC1 (Belasco et al., 1985), which was amplified and isolated from an *Escherichia coli dut*$^-$ *unc*$^-$ strain as described (Kunkel, 1985), and to M13mp8 DNA cut with *Nde*I following the gap duplex approach of Kramer et al. (1984). No forbidden codons were used in mutagenesis. The mutated DNA sequences contained new restriction sites, which were used for the screening of the desired clones (Figure 1). The preparation of oligonucleotides and the exchange of amino acids are described in Figure 1.

*Eco*RI–*Stu*I or *Bsm*I–*Stu*I fragments of a *pUC*19 derivative containing the *Rb. capsulatus* wild-type *puf* sequence were replaced by the corresponding DNA fragments originating from the mutated M13RC1 sequences. From these constructs the *Rhodobacter*-specific DNA was excised and inserted into a derivative of pTJS133 plasmid as described previously (Klug & Cohen, 1988). The pTJS133-derived plasmids were conjugatively transferred from *E. coli* to *Rb. capsulatus* with helper plasmid pRK2013 (Figurski & Helinski, 1979).

Analytical Methods. The cells were broken and the membrane fractions isolated as described in Tadros et al. (1987).

Membrane proteins were solubilized in SDS sample buffer for 30 min at 70 °C and separated on polyacrylamide gels (11.5–16.5% gradient of acrylamide) by electrophoresis following the method of Laemmli (1970).

The bacteriochlorophyll content was calculated from the 770-nm absorption of the acetone/methanol (2:7 v/v) extract with an extinction coefficient of 76 mM^{-1} cm^{-1} (Clayton, 1966). Absorption spectra were measured with a UVIKON 860 Kontron spectral photometer or a Beckman DU70 spectrophotometer. The reaction center content was determined by reversible bleaching in a CARY 14 spectral photometer (Aminco) as described by Reidl et al. (1983) with an extinction coefficient of 112 mM^{-1} cm^{-1} (referred to the molarity of reaction centers; Straley et al., 1973).

Fluorescence emission spectra were registered on a homemade spectral photometer.

RNA isolation, blotting, and hybridization were performed as described earlier (Klug et al., 1987). The values reported in Tables II and III are from representative single experiments. Each experiment with a specific strain was repeated at least once. Deviations of Bchl values of one preparation were maximally 10% and between different experiments with the same strain and the same culture conditions maximally 20%. The values for high-light and low-light cells were always from the same experiment.

RESULTS

Effects of Replacement of Charged Amino Acids in LHI Proteins on Bacterial Growth and Absorbance of Pigment–Protein Complexes. The pTJS133-derived plasmids were transferred into strain U43 of *R. capsulatus*, which has the *puf* operon deleted from the chromosome and lacks the LHII complex due to a point mutation (Table I). The plasmids were stably established in the presence of tetracycline. The transfer of plasmid pTX35 carrying the wild-type *puf* operon resulted in complementation of the RC–LHI$^-$ defect of U43 in trans [Table I, U43(pTX35), Table II). Growth rate and formation of the RC–LHI complex in strain U43(pTX35) were similar to those in strain NK3 (Table II), which has the same phenotype as U43(pTX35) but has the *puf* operon present in the chromosome (Table I). Because both strains are lacking LHII, the cell mass doubling time increased under low-light conditions (Table II).

The genes for *puf*BA were modified as described under Materials and Methods and in Figure 1 in order to replace

Table II: Cell Mass Doubling Time and Pigment Content of *Rb. capsulatus* Strains Grown in a Turbidostate at High and Low Light Intensities[a]

strains	cell mass doubling times (min)		nmol of Bchl/mg of cell protein	
	low irradiation	high irradiation	low irradiation	high irradiation
37b4	180	180[b]	18.3	2.2[b]
NK3	270	190[b]	5.4	1.6[b]
B10	200	160	9.4	0.8
U43(pTX35)	250	180	6.6	1.2
U43(pTXB26)	270	180	7.4	1.9
U43(pTXA12-1)	280	170	8.1	2.3
U43(pTXAB5)	nd[c]	190	nd	0.6
U43(pTXB87)	330	180	2.1	1.0

[a] See Materials and Methods and Table I. The results for each strain were from one representative experiment. [b] Values cited from Reidl et al. (1983, 1985). [c] nd, not determined.

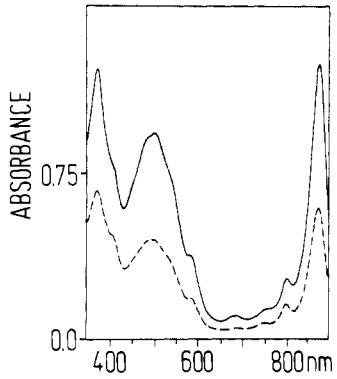

FIGURE 2: Absorption spectra of membranes isolated from cells of strains U43(pTX35) (full line) and U43(pTXB26) (broken line) grown semiaerobically in the dark. Both membrane suspensions were adjusted to 0.8 mg of protein/mL. The Bchl content was 24.4 nmol of Bchl/mg of membrane protein [U43(pTX35)] and 14.9 nmol of Bchl/mg of membrane protein [U43(pTXB26)].

charged amino acids of the LHI polypeptide by amino acids of opposite charge. An exchange of charged amino acids in positions 2 and 5 of the β polypeptide [strain U43(pTXB26), Figure 1, Table I] has an effect on the formation of LHI under semiaerobic growth conditions but not under anaerobic light conditions when compared with U43(pTX35). This was evident from absorption spectra taken from whole cells equilibrated to the same optical density (not shown) and from absorption spectra of purified membranes (Figure 2). The Bchl content of cells was higher in U43(pTX35) (2.8 nmol of Bchl/mg of cell protein) than in U43(pTXB26) (1.12 nmol of Bchl/mg of cell protein). A similar effect was observed when the lysine residues in positions 3 and 6 of the α polypeptide were replaced by glutamic acid residues [U43-(pTXA12-1), Figure 1, Tables I and II).

A strong effect on the formation of pigment–protein complex LHI, however, was observed when, in addition to the peripheral amino acid residues, two residues close to the central hydrophobic domain were replaced, U43(pTXB87). The cellular Bchl content (Table II) and the height of the 870-nm absorption peak were strongly lowered, and the peak around 800 nm became the dominating peak in the near-infrared region of the absorption spectrum (Figure 3). The cell doubling time of the mutant U43(pTXB87) was similar to those of wild-type strains when grown under high-light conditions (Table II). At low light intensities the cell doubling time increased, because the light intensity became the growth-limiting factor (Table II). The absorption spectra of membranes from strain U43-(pTXB87) were very similar whether the cells were grown at high or low light intensity (not shown).

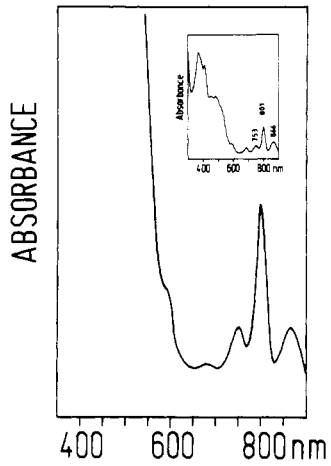

FIGURE 3: Absorption spectrum of membrane fraction II isolated from mutant U43(pTXB87) grown at high light intensity. Insert: near-infrared absorption spectrum of membrane fraction III from cells of U43(pTXAB5) grown at high light intensity.

In order to see whether the observed changes in the phenotypical properties of strain U43(pTXB87) were correlated to the number and sign of charges that were changed rather than the position of the converted amino acids, the codons corresponding to positions 13 and 14 of the β polypeptide were mutated. The absorption spectra of the cells and membranes from strain U43(pTXB0325) (amino acids 13 and 14 of β replaced) were found to be very similar to that of strain U43(pTXB87) (not shown). The results show that replacement of amino acid residues located near the N-terminus has only a very small effect while replacement of the amino acid residues closer to the central hydrophobic region effects the formation of the B870 complex strongly.

An exchange of four charged amino acid residues in both α and β polypeptides [U43(pTXAB5)] resulted in complete inhibition of LHI formation (Figure 3 insert).

Influence of Mutation on RC/LHI Content and Polypeptide Patterns in Membrane Fractions. In order to study the effect of amino acid replacement on the formation of the LHI complex more precisely, different membrane fractions were isolated from some representative strains, and the protein pattern was investigated.

Three membrane fractions were obtained by sucrose equilibrium density centrifugation of cell-free extracts from high-light-grown cells. A light, minor fraction I, the major middle fraction II, and a heavier fraction III were obtained (Figure 4). The fractions differed not only in density but also in concentrations of Bchl, RC content, and absorption spectra (Figure 4). However, in all membrane fractions under various growth conditions the photosynthetic unit in cells of U43-(pTXB87) was smaller than in cells of U43(pTX35) (Table III). Careful measurements of reversible bleaching at different light intensities have revealed that U43(pTXB87) contains a LHI antenna of reduced size, while in U43-(pTXAB5) no antenna was detectable (W. Mäntele, unpublished results).

It is known that Bchl synthesis and the synthesis of pigment-binding proteins are coordinated and the formation of complexes is dependent on the presence of all constituents (Klug et al., 1986).

To specify the steps in the formation of the LHI complex that were impeded by the mutations, the protein pattern of membranes was studied by SDS–polyacrylamide gel electrophoresis (Figure 5). Strains with the wild-type *puf* operon [U43(pTX35)] showed major bands of the α and β polypeptides especially when grown under low light intensity

Table III: Composition of Major Membrane Fraction II

strain	nmol of Bchl/mg of membrane protein from cells grown at		pmol of RC/mg of membrane protein from cells grown at		PSU[a] from cells grown at	
	low light intensity	high light intensity	low light intensity	high light intensity	low light intensity	high light intensity
U43(pTX35)	11.4	8.3	410	330	28	25
U43(pTXB87)	6.8	4.4	620	730	11	6

[a] PSU = size of the photosynthetic unit (mol of Bchl/mol of RC). The determination of the size of the photosynthetic unit was impeded by variable amounts of unknown tetrapyrrol compounds and LHI complexes in the preparations from mutant cells.

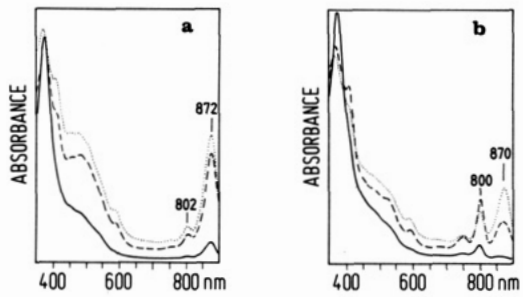

FIGURE 4: (a) Absorption spectra of membrane fractions isolated by sucrose gradient centrifugation from strain U43(pTX35); wild-type *puf* operon present, defect in LHII, grown at high light intensity. Full line: top (I) minor fraction; 3.5 nmol of Bchl/mg of protein; 0.6 mg of protein/mL. Broken line: major (II) middle fraction; 8.1 nmol of Bchl/mg of protein; 1.55 mg of protein/mL. Dotted line: lower (III) fraction; 8.1 nmol of Bchl/mg of protein; 1.0 mg of protein/mL. (b) Absorption spectra of membrane fractions isolated by sucrose gradient centrifugation from mutant strain U43(pTXB87), grown under high light intensity. Full line: top (I) minor fraction in the sucrose gradient; 1.9 nmol of Bchl/mg of protein; 0.9 mg of protein/mL. Broken line: middle (II) major fraction in the sucrose gradient; 5.3 nmol of Bchl/mg of protein; 1.1 mg of protein/mL. Dotted line: lower (III) fraction; 7.8 nmol of Bchl/mg of protein; 0.6 mg of protein/mL.

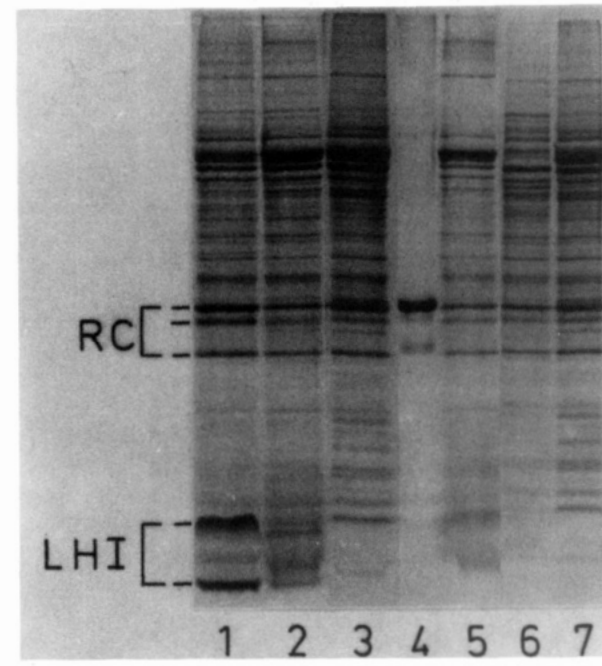

FIGURE 5: Protein patterns of membranes isolated by sucrose density gradient centrifugation from wild-type and mutant strains of *Rb. capsulatus* and separated by SDS–polyacrylamide gel electrophoresis. Polyacrylamide gradient 12.5–16.5%. To each lane 40 μg of protein was applied. Membranes and RC preparations were isolated from the following strains: Lane 1: U43(pTX35); from low-light-grown cells; 36.5 nmol of Bchl/mg of protein. Lane 2: U43(pTX35); high-light-grown cells; 7.2 nmol of Bchl/mg of protein. Lane 3: U43(pTXB87); from low-light-grown cells; fraction II; 6.8 nmol of Bchl/mg of protein. Lane 4: RC from the carotenoidless strain Ala^+. Lane 5: U43(pTXB87) from high-light-grown cells; fraction II; 6.5 nmol of Bchl/mg of protein. Lane 6: strain U43(pTXAB5); from high-light-grown cells; 2.2 nmol of Bchl/mg of protein. Lane 7: membrane fraction II; U43(pTXB87); high-light-grown cells; 6.5 nmol of Bchl/mg of protein.

(Figure 5, lane 1). Small amounts of the LHI α and β proteins were present in membranes of strain U43(pTXB87) (Figure 5, lanes 3 and 5). In the mutant U43(pTXAB5), which has four amino acid exchanges in each α and β polypeptide, two very faint polypeptide bands were visible at positions corresponding to relative molecular masses of about 12000 and 7000 (Figure 5, lane 6). From comparable studies with the mutant U43 we know that these are not the LHI α and β polypeptides (H. Stiehle, N. Cortez, and G. Drews, unpublished results). In those mutants where two amino acid residues near the N-terminus have been replaced and the 870-nm peak was fully expressed (Figure 2), the protein pattern was very similar to that of strain U43(pTX35) (not shown).

Fluorescence Emission Spectra. Fluorescence emission spectra of different mutant strains were recorded in order to see whether energy transfer from antenna to RC is visible. Whole cells and membrane preparations were excited at different wavelengths. In strains U43(pTX35) and U43(pTXB26) a strong emission band at 910/911 nm of the LHI complex and a small shoulder at 783 nm could be seen, indicating the presence of the B870 complex. In the mutant strain U43(pTXB87), where the B870 content is small, the major emission peak was shifted to 904 nm, and an additional maximum at about 780 nm became a strong component. Excitation at 572 nm induced emission bands at 714, 790, and 880 nm. At low temperature (4 K) and excitation at 400 nm, emission bands at 702, 768, 820, 892, and 907 nm were detected. the band at 768 nm was presumably emitted from pheophytin. It is not clear where the double emission band around 900 nm was coming from. As mentioned earlier, in the mutant strains different unidentified pigments were enriched. The various emission bands indicate that precursors or degradation products of Bchl or aggregates of Bchl increase when the amount of regular pigment–proteins decrease.

Discussion

The N-termini of the α and β polypeptides of the LHI complex are exposed on the cytoplasmic side of the intracytoplasmic membrane (Tadros et al., 1986, 1987). The fact that large pieces of these N-terminal domains are protected from proteolytic degradation (Tadros et al., 1987) led to the suggestion that the N-terminal domains of α and β polypeptides interact by ionic and/or hydrophobic bonds. The observed distribution of amino acids of opposite charge in the α and β subunits supports this model. An alignment of the amino acid sequences of many LHI polypeptides indicates that the positive net charge of the α and the negative net charge of β N-terminal are conserved (Zuber, 1986).

Mutant strains were constructed by replacing charged amino acids in the N-terminal regions by amino acids of opposite charge, thus preventing ionic bonds between the α and β N-terminal domains. The special changes were strongly dependent on the position of the charged amino acid residues. Replacement of the two charged amino acids close to the

N-terminus in β [U43(pTXB26)] or in α [U43(pTXA12-1)] had no or a very small effect on the LHI specific absorbance. But a replacement of the amino acids close to the hydrophobic central domain in β [U43(pTXB0325)] effects a strong reduction of the LHI formation, similar to the replacement of four amino acids in the β polypeptide [U43(pTXB87)]. An exchange of the four charged amino acids in α and β [(U43(pTXAB5)] inhibited completely the formation of the LHI complex (insert of Figure 3). Very recently it was shown that an exchange of four amino acids only in the α polypeptide results also in a complete inhibition of LHI formation (H. Stiehle, N. Cortez, and G. Drews, unpublished results). Thus, the two amino acid positions close to the central hydrophobic domain, especially those of the α polypeptide, are important for the assemply process.

The mean size of the photosynthetic unit in strain U43-(pTXB87) was smaller than in strain U43(pTX35) with a wild-type LHI complex (Table III). Since the mutant U43-(pTXB87) can adapt to low light intensities (Table II), it could be supposed that some RC's have a complete LHI while others have no antenna. In support of this hypothesis, variable ratios of LHI/RC were observed in different membrane fractions (Figure 4b). We cannot exclude the possibility of unequal distribution of LHI units to the RC population. But bacteria grow also without any antenna as shown with strain U43-(pTXAB5). The minimal light intensity for growth of wild-type and mutant strains has not been determined.

Gel electrophoretic analysis of the membrane proteins of these mutants supported the spectroscopical data: The replacement of the two charged amino acid residues of β near the N-terminus had a much smaller effect than the replacement of four charged amino acids in β (Figure 5). The same effect was observed when in α and β four amino acid residues were replaced [U43(pTXAB5)], although in this case opposite charges were present at corresponding positions of the α and β polypeptide. Since in the mutant strain U43(pTXAB5) α and β polypeptides were not detectable in the membrane, we assume that the major effect of the mutation in these mutants is the inhibition of a stable protein import into the membrane rather than the missing interaction between the α and β subunits (Drews et al., 1987).

In other reports of site-directed mutagenesis the Bchl-binding domain was mutated. The LHI complex was lost from the membrane when alanine 28 in the α polypeptide close to the conservative histidine is replaced by a residue larger than valine or if histidine 32^{α} is replaced by arginine or other amino acids (Bylina et al., 1988). This effect, however, is presumably caused by the disturbed binding of Bchl to the α polypeptide. Inhibition of Bchl synthesis (Klug et al., 1986) or Bchl binding (Bylina et al., 1988) inhibit the assembly of the complex.

It was reported that a loss of the LHI complex is correlated with a partial loss of functional RC complexes in the membrane of *Rb. capsulatus* (Jackson et al., 1986). We observed, however, that high-light-grown cells of strain U43(pTXAB5), which contains active RC's but no traces of LH complexes, grew with a cell doubling time similar to that of the wild-type strain (Table II). Thus, sufficient light energy was absorbed and an electric potential difference generated across the membrane. Kinetic measurements are planned to investigate energetic communication and energy dissipation in mutants like U43(pTXB87) and U43(pTXAB5).

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Registry No. L-Arg, 74-79-3; L-Glu, 56-86-0; L-Asp, 56-84-8; L-Lys, 56-87-1.

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