Evaluation of Structure−Function Relationships in the Core Light-Harvesting Complex of Photosynthetic Bacteria by Reconstitution with Mutant Polypeptides†

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ABSTRACT: Seven mutant LH1 polypeptides of *Rhodobactor sphaeroides* have been isolated, and their behaviors in *in vitro* reconstitution of LH1 and its subunit complex have been characterized. Two mutants were selected to address the increased stability of the subunit complex of *Rb. sphaeroides* compared with that of *Rhodobacter capsulatus*. We found that this difference can be largely ascribed to the existence of Tyr at position +4 in the β-polypeptide (the numbering system used assigns position 0 to the His which provides the coordinating ligand to bacteriochlorophyll) of the former bacterium compared to Met in that position in the latter. The amount of energy involved in the increased interaction was 1.6 kcal/mol, which would be consistent with a hydrogen bond involving Tyr. Mutation of the His at position 0 to Asn allows an estimate of the binding energy for subunit formation contributed by coordination of the imidazole group of His to the Mg atom of bacteriochlorophyll of >4.5 kcal/mol per BChl. Finally, an evaluation of the role of amino acids in the C-terminal region of the α-polypeptide was begun. Reconstitution of a mutant α-polypeptide in which Trp at position +11 was changed to Phe resulted in optimal formation of an LH1-type complex whose λ_{max} was blue-shifted to 853 nm, the same as observed in the intact bacterium harboring this mutation. These results provide further confirmation that the environment of BChl in reconstituted LH1 complexes is the same as *in vivo* and support the assignment of this residue to a role in hydrogen bonding with the C3[1] carbonyl group of BChl. Two other mutants of the α-polypeptide in which 5 and 14 amino acids in the C-terminus were deleted were also examined. These were of interest because the latter mutant, unlike the former, resulted in a low level of expression of LH1 in intact cells. However, with both of these mutant polypeptides, reconstitution appeared identical to that of the native system. In the case of the mutant shortened by 14 amino acids, a small blue-shift in λ_{max} to 861 nm was observed, again reproducing the blue-shift exhibited by the intact cells. Thus, these results suggest that the lowered levels of *in vivo* expression observed in these two mutants are due to reduced incorporation of the α-polypeptide into the membrane or its increased degradation, rather than to decreased stabilization of the LH1 complex.

The light-harvesting apparatus of photosynthetic bacteria such as *Rhodobacter sphaeroides* consists of a peripheral complex, termed LH2, which donates excitation energy to the core light-harvesting complex, LH1.[1] The reaction center (RC), where photochemistry occurs, receives energy from LH1. All three of these complexes are composed of integral membrane proteins which bind bacteriochlorophyll (BChl) and carotenoid (Car). The structures of RC and LH2 complexes have been determined to atomic resolution (Deisenhofer et al., 1995; Lancaster et al., 1995; McDermott et al., 1995; Koepke et al., 1996). LH1 complexes are the exception in that there is no high-resolution structure yet determined. From a biological standpoint, LH1 complexes are interesting in that they are invariably present in the bacterial photosynthetic unit, unlike LH2, and also in that they must interact with both the RC and LH2 when it is present.

Core light-harvesting complexes (LH1) of photosynthetic bacteria are formed by the association of two small polypeptides, α and β, together with BChl and Car molecules with an approximate stoichiometry of $\alpha_{12}\beta_{12}$·24BChl·12Car per reaction center (RC) (Loach & Sekura, 1968; Loach & Parkes-Loach, 1995; Francke & Amesz, 1995). A somewhat greater stoichiometry has been proposed (16 α-polypeptides, 16 β-polypeptides, and 32 Bchl per RC) on the basis of cryoelectron microscopic results with isolated LH1 complexes of *Rhodospirillum rubrum* (Karrasch et al., 1995). A stable subunit complex has been isolated from the LH1 complex of many photosynthetic bacteria (Miller et al., 1987; Chang et al., 1990a; Heller & Loach, 1990; Meckenstock et al., 1992; Jirsakova & Reiss-Husson, 1993; Parkes-Loach et al., 1994; Kerfeld et al., 1994) and is believed to have the composition $\alpha_1\beta_1$·2BChl (Loach & Parkes-Loach, 1995),

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although the composition $\alpha_2\beta_2$·4BChl has also been suggested (Ghosh et al., 1988; Sturgis & Robert, 1994). Both the subunit complex and LH1 have been reconstituted from the separately-isolated components (Parkes-Loach et al., 1988; Loach et al., 1994; Davis et al., 1995).

On the basis of reconstitution of subunit and LH1 complexes with BChl analogs, important interactions between BChl and the protein have been illuminated (Parkes-Loach et al., 1990; Davis et al., 1996). These studies showed: (1) the Mg atom is required, presumably for coordinating a ligand provided by the protein; (2) the acetyl group at position C3 is important, presumably for participating in hydrogen bonds with groups of the protein; (3) the carbomethoxy group at position C13[2] is important either for hydrogen bonding or by influencing the binding of the C13[1] carbonyl group; and (4) the bacteriochlorin oxidation state of the macrocycle is required, suggesting a highly restrictive overall geometry for fitting the BChl binding site.

The recent determinations of the structure of the LH2 complexes of *Rhodopseudomonas acidophila* (McDermott et al., 1995) and *Rhodospirillum molischianum* (Koepke et al., 1996) by X-ray crystallography provide important insights into the structural features of LH1. In the LH2 structure of *Rps. acidophila*, there are two concentric cylinders of helical protein subunits which enclose the pigment molecules. At one end of the cylindrical structure, nine BChl molecules are positioned between the outer helices constituted by the β-polypeptides with the bacteriochlorin rings positioned perpendicular to the transmembrane helix axis. At the other end of the cylindrical structure, an additional 18 BChl molecules are sandwiched between the 2 rings of helices and form a continuous overlapping ring. The BChl molecules in this latter ring are responsible for the 850-nm absorption band of LH2 and exist in a structural environment which is believed to have some similarity to that of the BChl in LH1. These BChl molecules responsible for the 850-nm absorption band are coordinated to His_0 (see Figure 1 for the LH polypeptide amino acid numbering scheme) of the α- and β-polypeptides of LH2, as predicted (Zuber & Brunisholz, 1991; Robert & Lutz, 1985). The only hydrogen bonding to BChl observed in the 850 nm component of LH2 was between the C3 acetyl oxygens and Tyr_{+13} and Trp_{+14} of the α-polypeptides (McDermott et al., 1995). Many of these structural features were also observed in the crystal structure of *Rs. molischianum* LH2 except the oligomeric ring size contained eight repeating heterodimer units instead of nine (Koepke et al., 1996).

Mutagenesis and resonance Raman experiments on LH2 from *Rb. sphaeroides* have also demonstrated that there are hydrogen bonds from αTyr_{+13} and αTyr_{+14} to the C3 acetyl oxygens (Fowler et al., 1992, 1994); using the same approach, a hydrogen bond was also identified from LH1 αTrp_{+11} to the C3 acetyl oxygen (Olsen et al., 1994). Results from resonance Raman spectroscopy (Sturgis & Robert, 1994) and reconstitution experiments with BChl analogs (Parkes-Loach et al., 1990; Davis et al., 1996) and selectively modified polypeptides (Meadows et al., 1995; Loach & Parkes-Loach, 1995) support the expectation that additional hydrogen bonding exists in LH1 and its subunit complex compared to LH2. The fact that it has so far not been possible to isolate a stable subunit complex from LH2 also suggests that additional binding elements exist in LH1 to stabilize its subunit structure.

The combination of site-directed mutagenesis and reconstitution methodologies provides a powerful approach for evaluating structure−function relationships. The information obtained is complementary to the characterization of *in vivo* mutant LH1 complexes and can provide insights not available by any other method. In the results presented here, amino acids in the α- and β-polypeptides of *Rb. sphaeroides* were changed by site-directed mutagenesis, the modified polypeptides were isolated, and their ability to support reconstitution of subunit- and LH1-type complexes was examined. The particular mutants prepared were chosen to address these four questions: (1) Which amino acid(s) in the core region of the β-polypeptides of *Rb. sphaeroides* and *Rb. capsulatus* is (are) responsible for the 15-fold difference in K_{assoc} for formation of the subunit-type complex (Loach et al., 1994; Loach & Parkes-Loach, 1995)? (2) What is the magnitude of stabilization provided by ligation of the imidazole side chain of histidine to BChl? (3) What is the role of the C-terminal portion of the α-polypeptide in stabilization of the subunit and LH1 complexes? (4) How does the elimination of one of the hydrogen bonds to LH1 BChl influence the absorbance of both the subunit and LH1 complex *in vitro*?

MATERIALS AND METHODS

Site-directed mutagenesis was carried out according to the procedures of McGlynn et al. (1996) using the pAlter-1 vector (Promega). Mutant genes were mobilized into the recipient *Rb. sphaeroides* strain DD13 ($LH2^-LH1^-RC^-$) by conjugative transfer. This strain contains genomic deletions of *puf* and *puc* genes and insertions of antibiotic resistance genes (Jones et al., 1992), and is therefore deficient in RC, LH2, and LH1 complexes.

Mutagenized *Rb. sphaeroides* strains were propagated on M22+ medium (Hunter & Turner, 1988). Chromatophores either were prepared from mutant cells using a French pressure cell, following treatment with lysozyme and DNase I, or were prepared by sonication without lysozyme and

[1] Abbreviations: BChl, bacteriochlorophyll *a*; LH1, the core light-harvesting complex (also called B873); subunit complex (also referred to as B820), the subunit form of LH1 either isolated from LH1 or prepared by reconstitution using native α- and β-polypeptides and BChl; subunit-type complex, a reconstituted complex exhibiting absorption and CD spectra highly similar to the native subunit complex but containing a mutant polypeptide or only native β-polypeptide (without the α-polypeptide) and BChl; LH1-type complex, a reconstituted complex containing BChl and a mutant α- or β-polypeptide with a native β- or α-polypeptide, respectively, and displaying absorption and CD spectra highly similar to those of native LH1; LH2, accessory light-harvesting complex also referred to as B800−850; RC, reaction center; OG, *n*-octyl β-D-glucopyranoside; HFA, hexafluoroacetone trihydrate; near-IR, near-infrared; CD, circular dichroism; $\beta Ser_{-7}\rightarrow$Ile, mutant β-polypeptide of *Rb. sphaeroides* in which Ser at position −7 (see Figure 1 for numbering of amino acid positions) was changed to Ile; $\beta Tyr_{+4}\rightarrow$Met, mutant β-polypeptide of *Rb. sphaeroides* in which Tyr at position +4 was changed to Met; $\beta His_0\rightarrow$Asn, mutant β-polypeptide of *Rb. sphaeroides* in which His at position 0 was changed to Asn; $\alpha His_0\rightarrow$Asn, mutant α-polypeptide of *Rb. sphaeroides* in which His at position 0 was changed to Asn; $\alpha Trp_{+11}\rightarrow$Phe, mutant α-polypeptide of *Rb. sphaeroides* in which Trp at position +11 was changed to Phe; αArg_{+21t}, mutant α-polypeptide of *Rb. sphaeroides* in which the C-terminal amino acids from Val +22 to the C-terminus were deleted; αLeu_{+12t}, mutant α-polypeptide of *Rb. sphaeroides* in which the C-terminal amino acids from Glu +13 to the C-terminus were deleted. The term $\Delta\Delta G$ is used to indicate the difference in ΔG values determined for formation of a subunit-type complex using a mutant polypeptide compared to that determined using native polypeptides.

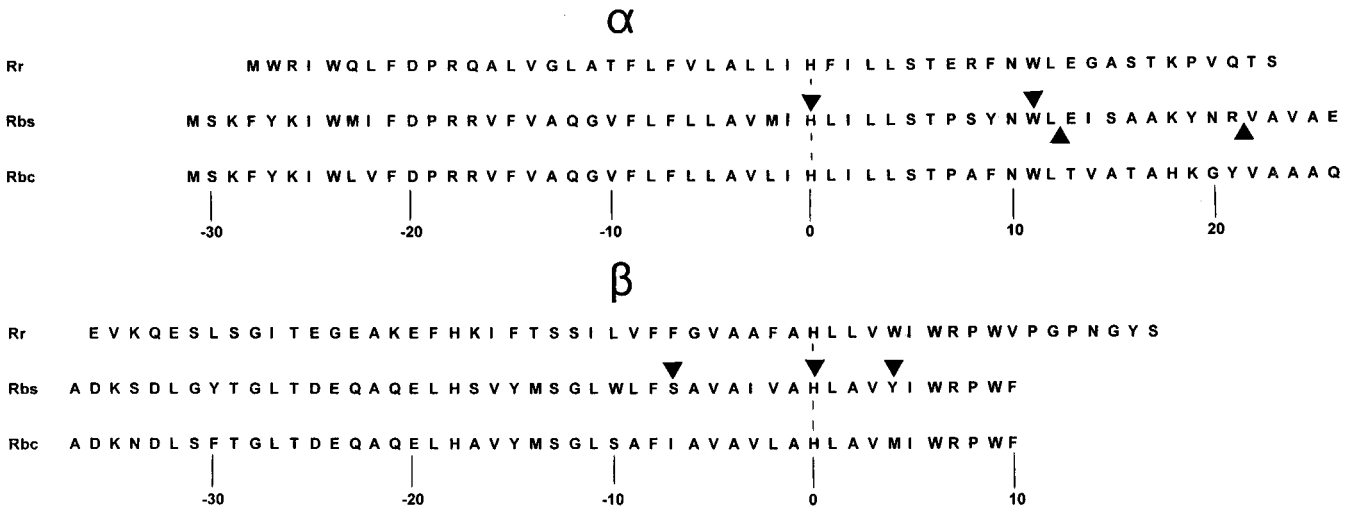

FIGURE 1: Amino acid sequences of the α- and *β*-polypeptides of *Rs. rubrum* (Rr), *Rb. sphaeroides* (Rbs), and *Rb. capsulatus* (Rbc). The conserved His which most likely provides the ligand occupying the fifth coordination site to Mg in BChl is assigned position zero. Amino acids toward the N-terminus from this position are assigned negative numbers, and those toward the C-terminus are assigned positive numbers. The ▼ above an amino acid indicates a site of mutation while the ▲ below two adjacent amino acids indicates where the C-terminus was deleted. See Abbreviations for naming of these mutant polypeptides.

DNase I treatment. Subsequently, the membranes were spun in a Beckman L8-70M ultracentrifuge using a Ti 50.2 rotor at 20 000 rpm for 30 min at 4 °C to remove cellular debris. Samples were then freeze-dried in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Some cultures were propagated in Sistrom's media containing 1 *μ*g/mL tetracycline and 20 *μ*g/mL kanamycin. These liquid cultures also contained 2 g/L casamino acids, and cultures were grown semi-aerobically in the dark in Fernbach flasks (filled to 75% capacity) on an orbital shaker at 30 °C. Following harvesting of the cells, chromatophores were prepared as described in Davis et al. (1995).

Isolation of LHI polypeptides and reconstitution procedures were described previously (Chang et al., 1990a; Loach et al., 1994; Davis et al., 1995). For $\beta Ser_{-7} \rightarrow Ile$, $\beta Tyr_{+4} \rightarrow Met$ and $\alpha Trp_{+11} \rightarrow Phe$, the nucleotide sequence of the mutant gene was verified in the cells used for preparation of the mutant polypeptides. Amino acid sequence analyses of some mutant polypeptides were performed by Dr. Joseph Leykam of the Biochemistry Department Macromolecular Structure Facility at Michigan State University. Electrospray mass spectral analyses and determination of amino acid compositions were conducted by Harvard Microchemistry Laboratories in Cambridge, MA.

Association constants were calculated for the equilibria between the fully dissociated components (BChl, α- and *β*-polypeptides) and the subunit complex. On the basis of determinations of stoichiometry (Loach et al., 1989), gel filtration experiments, and various spectroscopic analyses (Visschers et al., 1991; van Mourik et al., 1991; Loach & Parkes-Loach, 1995), it is assumed that the native subunit complex has the composition $\alpha_1\beta_1 \cdot 2BChl$ and the subunit-type complex formed with only BChl and the *β*-polypeptide has the composition $\beta_2 \cdot 2BChl$. Thus,

$$K_{assoc} = \frac{[\text{subunit complex}]}{[\alpha][\beta][\text{BChl}]^2}$$

and for formation of a similar complex with BChl and only the *β*-polypeptide

$$K_{assoc} = \frac{[\text{subunit-type complex}]}{[\beta]^2[\text{BChl}]^2}$$

The concentrations of α- and *β*-polypeptides were determined by measuring the absorbance at 287 nm of a solution of the selected polypeptides in HFA and using a molar absorptivity at 287 nm of 3400 M^{-1} cm^{-1} per Trp contained in the polypeptides (Miller et al., 1987; Loach et al., 1994). The concentrations of subunit complex and uncomplexed BChl were determined by deconvolution of the absorption spectrum in the Q_Y region (Loach et al., 1989). The data were fitted to two components using a molar absorptivity for BChl in OG of 55 mM^{-1} cm^{-1} at 777 nm and a molar absorptivity for the subunit complex of 172 mM^{-1} cm^{-1} at 823 nm based on 2 BChl per complex (Chang et al., 1990b).

Because the presence of low percentages of impurities in the BChl and polypeptides can have a major effect on determinations of K_{assoc}, and because BChl is especially difficult to maintain at greater than 98% purity, control reconstitution assays (native polypeptides and BChl) were always conducted in parallel to reconstitutions utilizing mutant polypeptides. In these control assays, all components were identical to those of the experimental reconstitution except for the polypeptide being tested. Furthermore, if any component in the control experiments showed evidence of being less than 90% pure, new materials were isolated. In this way, the comparative values for K_{assoc} reported here are probably accurate to within 15%, and calculated $\Delta\Delta G$ values are accurate to within 0.15 kcal mol^{-1}.

Absorption spectra were recorded with a Shimadzu UV-160 spectrophotometer. To reduce the effects of scattered light, opal glass was placed between the sample and the detector. CD spectra were recorded using a Jasco J500C spectropolarimeter.

RESULTS

The sites selected for mutation for this study are indicated in Figure 1. The reason for selecting two of these particular sites was to determine which amino acid(s) in the core region of the *β*-polypeptides of *Rb. sphaeroides* and *Rb. capsulatus*

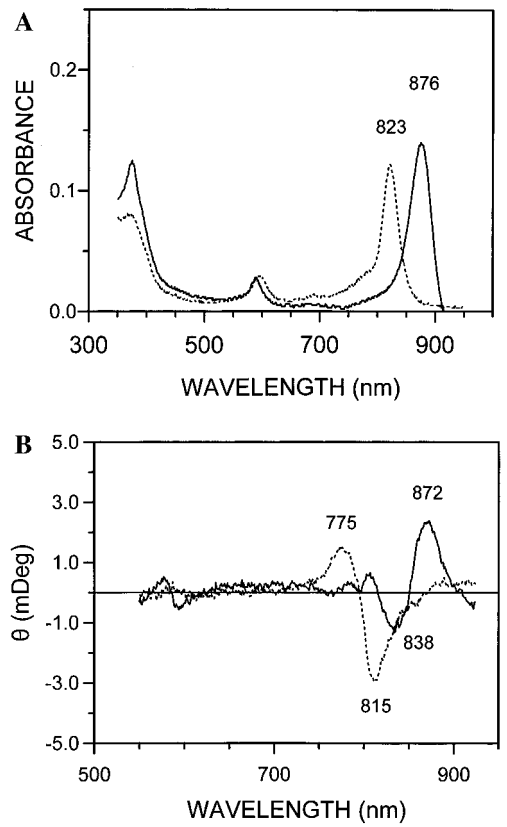

FIGURE 2: (A) Absorption spectra of the reconstituted subunit-type complex (dashed curve) using only the mutant βSer$_{-7}$→Ile polypeptide of *Rb. sphaeroides* and of the reconstituted LH1-type complex (solid curve) using the native α-polypeptide and mutant βSer$_{-7}$→Ile polypeptide of *Rb. sphaeroides*. For formation of the subunit complex, the polypeptide concentration was 2.2 μM and [BChl] = 1.4 μM in 0.60% OG. The concentrations of polypeptides for LH1 formation were 1.8 μM and 2.2 μM for the α- and β-polypeptides, respectively, and [BChl] = 1.4 μM at 0.60% OG. The LH1-type spectra were recorded after storage of the reconstituted sample at 5 °C overnight. Spectra were recorded in 1 cm cuvettes. (B) CD spectra of the reconstituted subunit- and LH1-type complexes with line designations as above. Samples were in 1 cm cuvettes.

might be responsible for the 15-fold difference in K_{assoc} for formation of the β-only subunit-type complex (Loach et al., 1994; Loach & Parkes-Loach, 1995). The mutants βSer$_{-7}$→Ile and βTyr$_{+4}$→Met were prepared for this purpose. A second goal in selecting the mutants studied here was to evaluate the contribution to BChl binding of His$_0$ in both the α- and β-polypeptides. The mutants βHis$_0$→Asn and αHis$_0$→Asn (Olsen, 1994) were used for this purpose. Finally, modifications of the C-terminal region of the α-polypeptide were studied to evaluate structural elements required for LH1 formation. The previously reported mutant αTrp$_{+11}$→Phe (Olsen et al., 1994) and two recently reported truncation mutants, αArg$_{+21t}$ and αLeu$_{+12t}$ (McGlynn et al., 1996), were utilized for these studies.

βSer$_{-7}$→Ile. Whole cells and chromatophores of this mutant showed normal LH1 spectral properties. The β-polypeptide isolated from this strain exhibited a slight increase in HPLC retention time in comparison to the native β-polypeptide isolated from *Rb. sphaeroides* PUC705-BA chromatophores (18.26 min vs 17.52 min). A shift to a longer retention time might be expected from the substitution of a more hydrophobic residue at position −7. Reconstitution of the subunit and LHI complexes using the mutant

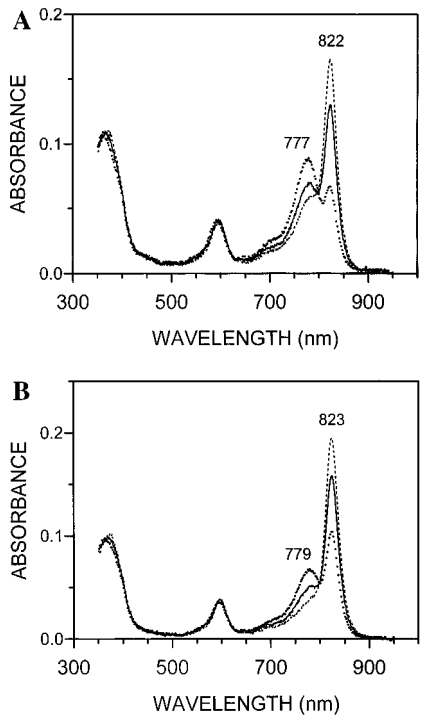

FIGURE 3: Comparison of reconstitutions using the mutant βTyr$_{+4}$→Met polypeptide only (A) or the native β-polypeptide only (B). In both cases, the polypeptide concentrations were 6.1 μM and [BChl] = 2.2 μM at 0.75% OG. The percent OG was 0.90%, 0.75%, and 0.66% for the samples represented by the dotted, solid, and dashed curves, respectively. Spectra were recorded in 1 cm cuvettes. Because the samples at 0.75% and 0.66% OG were prepared by dilution of the sample at 0.90% OG, the resulting spectra were multiplied by an appropriate dilution factor to normalize the spectra to a constant BChl concentration.

β-polypeptide and native α-polypeptide (Figure 2A) paralleled reconstitution results observed with the native α- and β-polypeptides (Figure 7B). Subunits prepared with the mutant β-polypeptide exhibited absorption maxima at 823 nm while LHI complexes absorbed at 874−876 nm. CD spectra of the reconstituted mutant subunit- and LH1-type complexes were also measured (Figure 2B) and found to be very similar to those obtained with native polypeptides (Loach et al., 1994). Determination of K_{assoc} for subunit formation using the mutant β-polypeptide also revealed values within 15% of the controls using the native β-polypeptide.

βTyr$_{+4}$→Met. The HPLC retention time of this mutant β-polypeptide was identical to that of the native β-polypeptide when an HFA extract of the mutant chromatophores was analyzed. Upon isolation of the polypeptide by organic solvent extraction and LH-60 gel filtration chromatography, the HPLC profile was altered somewhat. A doublet appeared in the HPLC chromatograms of the different mutant β-polypeptide preparations where only a single peak was observed previously. Individual collection of these two peaks and subsequent reconstitution with BChl *a* resulted in equivalent behavior for subunit formation. In both cases, subunit formation was noticeably decreased relative to control reconstitutions with the native β-polypeptide (Figure 3). Decreased subunit formation is apparent at all three concentrations of OG, giving a value for K_{assoc} of 5.4-fold, 5.5-fold, and 3.3-fold smaller than with the native β-polypeptide at 0.90%, 0.75%, and 0.66% OG, respectively. The values determined at 0.90% and 0.75% OG are more accurate

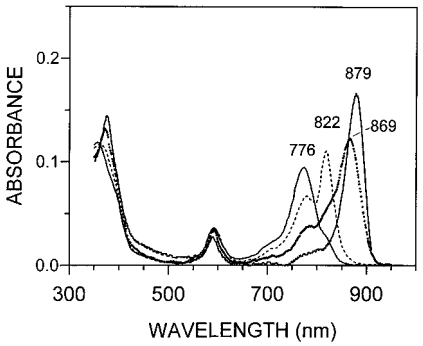

FIGURE 4: Absorption spectra of the reconstituted subunit- and LH1-type complexes with the mutant βTyr$_{+4}$→Met polypeptide and the native α-polypeptide of *Rb. sphaeroides*. The concentration of the polypeptides was 7.3 μM, and [BChl] = 2.2 μM in 0.90% OG. The spectra were recorded at 0.90% OG (solid curve with λ_{max} = 776 nm), 0.70% OG (dashed curve), 0.60% OG (dotted curve), and 0.60% OG after being chilled overnight at 4 °C (solid curve with the λ_{max} = 879 nm). Spectra were recorded in 1 cm cuvettes. Because the samples at 0.70% and 0.60% OG were prepared by dilution of the sample at 0.90% OG, the resulting spectra were multiplied by an appropriate dilution factor to normalize the spectra to a constant BChl concentration.

because sufficient amounts of both free BChl and the subunit-type complex were present, allowing accurate determination of their concentrations. The CD spectrum of the subunit-type complex formed with only the mutant β-polypeptide and BChl revealed a typical subunit-type CD signal (data not shown).

The combination of the mutant β-polypeptide with the native α-polypeptide also exhibited decreased subunit formation when compared with reconstitutions when both polypeptides were native. At 0.90% OG, only a very small shoulder was observed on the long-wavelength side of a 776 nm absorption band (Figure 4), indicating little subunit formation. In comparison, the native control reconstitution exhibited substantial subunit absorption at this OG concentration (cf. Figure 7B). At 0.70% OG, subunit absorption in the mutant reconstitution became more prominent (Figure 4), but much less subunit was formed than in control reconstitutions with native polypeptides (Figure 7B). A comparison of K_{assoc} gave a 5-fold lower value for the mutant β-polypeptide-containing system as compared with the native system. However, at 0.60% OG, the Q_Y absorption band for the mutant system shifted to 869 nm, whereas the corresponding Q_Y band for control reconstitutions remained at 825 nm. It thus appears that the equilibrium favors LHI formation during reconstitution with the mutant β-polypeptide compared to that with the native β-polypeptide. Upon chilling overnight, an LH1 species at 879 nm formed to the same extent as did LH1 in the native system. The mutant LH1 CD spectrum displayed a peak and trough at 891 and 853 nm, respectively (data not shown), which is similar to that observed for LH1 formed with native polypeptides.

βHis_0→Asn. HPLC analysis of this mutant β-polypeptide revealed a single peak significantly shifted to longer retention times than the native β-polypeptide (22.37 min vs 17.52 min). The amount of mutant β-polypeptide obtained from the starting material—8 L of cell culture—was quite small, slightly less than 0.2 mg (1−2 mg of native polypeptides is typically obtained from 1 L of PUC705BA cells ($RC^+LH1^+LH2^-$).

Initial reconstitutions using the mutant β-polypeptide provided no evidence for formation of a subunit complex.

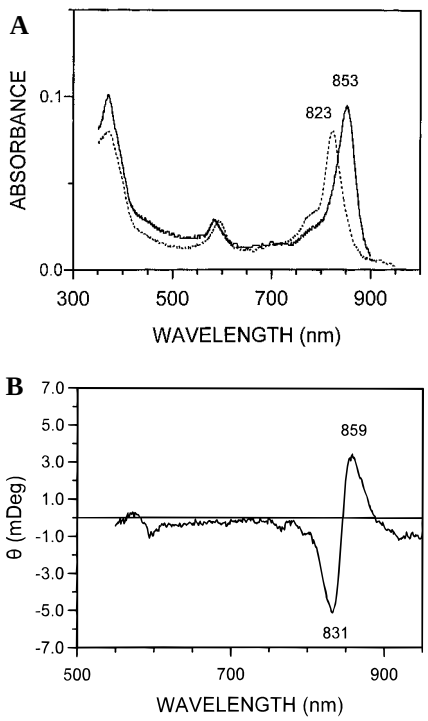

FIGURE 5: (A) Absorption spectra of the reconstituted subunit- and LH1-type complexes with the mutant αTrp$_{+11}$→Phe polypeptide and the native β-polypeptide of *Rb. sphaeroides*. The concentration of the mutant αTrp$_{+11}$→Phe polypeptide was 1.9 μM, that of the native β-polypeptide was 2.4 μM, and that of BChl *a* = 1.1 μM at 0.60% OG. The subunit-type complex was formed at 0.60% OG (dashed curve) at room temperature, and the LH1-type complex (solid curve) was formed upon cooling this sample overnight at 5 °C. Absorption spectra were recorded in 1 cm cuvettes. (B) CD spectrum of the reconstituted LH1-type complex formed with the mutant αTrp$_{+11}$→Phe polypeptide (1.9 μM) and the native β-polypeptide (2.4 μM) of *Rb. sphaeroides* with 1.7 μM BChl at 0.60% OG and 5 °C. CD spectra were recorded in cuvettes with a 2 cm path length.

Neither the β-only reconstitution (3.6 μM at 0.90% OG) nor the α−β reconstitution (containing 2.9 μM mutant β-polypeptide and 7.2 μM native α-polypeptide at 0.90% OG) formed a subunit-type complex. Additional reconstitution experiments with a higher concentration of the mutant β-polypeptide in the absence of the α-polypeptide also failed to demonstrate subunit formation.

αHis$_0$→Asn. HPLC analysis of this mutant α-polypeptide revealed a major peak shifted to longer retention times (18.95 min vs 17.95 min), but two smaller peaks at longer retention times were also present. The amount of mutant α-polypeptide obtained from 10 L of the starting cell culture was again quite small, approximately 0.4 mg. Reconstitution experiments with this polypeptide in the presence of the native β-polypeptide failed to form a LH1-type complex (data not shown). A subunit complex was formed attributable to the native β-polypeptide present.

αTrp$_{+11}$→Phe. The HPLC retention time of the mutagenized α-polypeptide was slightly increased relative to the native *Rb. sphaeroides* α-polypeptide (16.07 min vs 15.85 min). Initial purification of the mutant α-polypeptide resulted in incomplete separation from the native β-polypeptide and necessitated further purification by HPLC. Reconstitution of this mutant α-polypeptide with native β-polypeptide yielded both a subunit- and an LHI-type complex (Figure 5A). The absorption maximum of the quantitatively-formed subunit was at 823 nm, and the CD

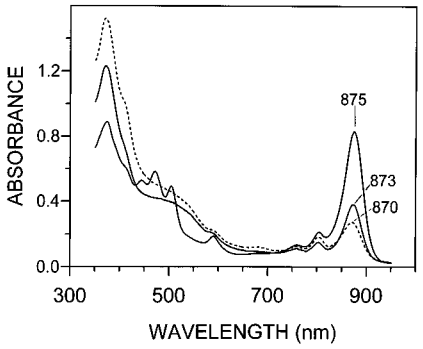

FIGURE 6: Absorption spectra of chromatophores prepared from PUC705-BA (native)(solid curve with λ_{max} = 875 nm), mutant αArg_{+21t}-containing (solid curve with λ_{max} = 873 nm), and mutant αLeu_{+12t}-containing (dashed curve) cells.

spectrum (data not shown) paralleled the subunit prepared with native polypeptides. The LHI complex exhibited an absorption maximum at 853 nm, and the CD spectrum mirrored the blue-shift of the Q_Y band, exhibiting a peak and trough at 859 and 831 nm, respectively (Figure 5B). The blue-shifted LHI absorption maximum obtained by reconstitution agrees well with that of the mutant LH1 *in vivo*, which is also at 853 nm (Olsen et al., 1994).

C-Terminal Truncated α*-Polypeptides.* Two α-polypeptides were examined in which amino acids in the C-terminus beyond residue +21 (αArg_{+21t}) or +12 (αLeu_{+12t}) were deleted. These mutants were part of a set of five mutants previously constructed to evaluate the role of the C-terminus of the α-polypeptide in the *in vivo* assembly and structure of LH1 (McGlynn et al., 1996). Whole cells and intracytoplasmic membranes prepared from these mutants exhibited reduced LH1/RC ratios (McGlynn et al., 1996, and Figure 6). In addition, the LH1/RC core size was reduced, and a progressive blue-shift in the LH1 absorption maximum was observed as the C-terminus became shorter. Such a reduction in LH1 content *in vivo* could result from a variety of causes such as inappropriate insertion of the α-polypeptide into the membrane, rapid degradation, or destabilization of LH1 complex formation. Some information on the latter possibility could be evaluated *in vitro* by determining whether the shortened polypeptides decrease LH1 formation in reconstitution experiments.

Bacterial cultures synthesizing mutant polypeptides were harvested and the truncated α-polypeptides isolated by organic solvent extraction, gel filtration on Sephadex LH60, and further purification by reverse phase HPLC (Davis et al., 1995). The isolated polypeptides were characterized by their retention times relative to the native α-polypeptide, by their amino acid composition, and by mass spectral analyses.

αArg_{+21t} exhibited a retention time only slightly increased relative to the native α-polypeptide (17.43 min vs 17.35 min). Its molecular weight according to mass spectral analysis was 6369.0, in exact agreement with the calculated molecular weight. Upon reconstitution, both the subunit and LH1 complexes were formed in a manner completely analogous to those found with native polypeptides (data not shown). In addition, the K_{assoc} for subunit formation was identical to that obtained with native polypeptides.

αLeu_{+12t} exhibited a retention time substantially greater than that of the native α-polypeptide (19.75 min vs 17.35 min). However, it was quite close to that of the native β-polypeptide. The molecular weight of the αLeu_{+12t} mutant

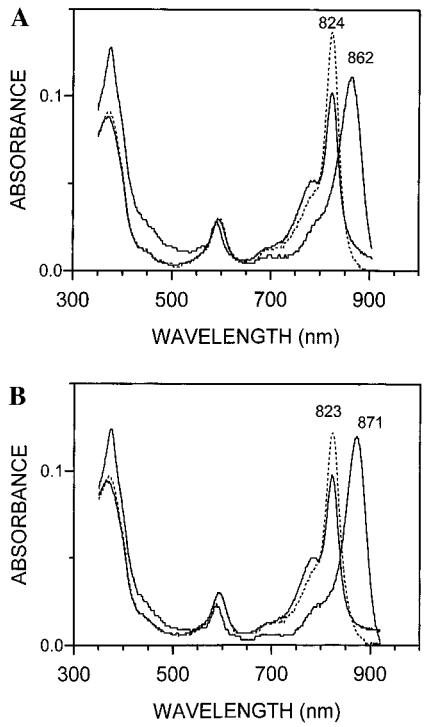

FIGURE 7: Comparative absorption spectra for systems reconstituted with the mutant αLeu_{+12t} and native β-polypeptide (A) or with native α- and β-polypeptides (B) of *Rb. sphaeroides*. Concentrations of the mutant αLeu_{+12} and native α-polypeptides were 3.4 μM, the native β-polypetides were 2.2 μM, and BChl *a* was 1.4 μM at 0.67% OG. Spectra were recorded at room temperature at 0.90% OG (solid curve with λ_{max} = 824 nm), 0.67% OG (dashed curve), and after chilling the 0.67% OG sample overnight at 5 °C (solid curve with λ_{max} = 862 nm). Spectra were recorded using 1 cm cuvettes.

(5335) is quite similar to that of the β-polypeptide (5441). In addition, the hydropathy profiles of these two polypeptides are quite similar. By adjustment of HPLC gradients, small quantitites of αLeu_{+12t} could be separated from the β-polypeptide for determination of its amino acid composition and mass spectral analyses. A mass of 5335 Da was obtained, in agreement with that expected for αLeu_{+12t}. Upon reconstitution, both the subunit- and LH1-type complexes were formed in a manner completely analogous to those found with native polypeptides (see Figure 7A,B). Again, the K_{assoc} for subunit formation was identical to that with native polypeptides. It should be noted that the λ_{max} of the LH1-type complex formed with αLeu_{+12t} and a native β-polypeptide is at 862 nm, approximately 9 nm blue-shifted from that of the native complex. This is in good agreement with the λ_{max} observed in the chromatophores isolated from these mutant bacteria (Figure 6).

DISCUSSION

As shown, the combination of site-directed mutagenesis and reconstitution methodologies provides a powerful approach for evaluating structure−function relationships in these intrinsic membrane proteins. It is able to provide a highly selective way of perturbing the factors that control the oligomerization of LH polypeptides. The information obtained is complementary to the results of characterizing mutated LH complexes *in vivo* and can provide insights not available by any other method. For example, changes in amino acids that appear to have no effect on LH1 formation

in vivo (e.g., $\beta Tyr_{+4} \rightarrow Met$) can be examined with much greater sensitivity by reconstitution methodology, and the collective role of many such amino acids can be evaluated. As part of this definition, binding energies for stabilizing the subunit complex can be quantitatively determined, and the mode of BChl binding can eventually be fully described. The results reported here provide the first step in this direction.

Determination of the Source of the Difference between the β-Polypeptides of Rb. sphaeroides and Rb. capsulatus in the Stabilization of the Subunit Complex. Out of 48 amino acids in the β-polypeptide of *Rb. sphaeroides* and *Rb. capsulatus*, only 10 are different (Figure 1). Of these 10 differences, only 4 occur in the region most likely to be involved with BChl binding. These are positions −7, −3, −2, and +4. In spite of these few differences, the K_{assoc} for formation of a subunit-type complex differs by 15-fold or by about 1.6 kcal mol^{-1}. One of the goals of the experiments reported here was to determine the basis for this difference. Because the differences at positions −3 (Ile vs Val) and −2 (Val vs Leu) might be considered conservative, we selected the Ser at position −7 and the Tyr at position +4 of the β-polypeptide of *Rb. sphaeroides* to change separately to Ile and Met, respectively, which are the amino acids at these locations in the β-polypeptide of *Rb. capsulatus*.

By comparing the data for reconstitution with the mutants $\beta Ser_{-7} \rightarrow Ile$ and $\beta Tyr_{+4} \rightarrow Met$ (Figures 2, 3, and 4), it is clear that the difference in stability of the subunit-type complex can largely be accounted for by the different amino acids at position +4. This implies that in addition to position −7, the eight other differences in amino acids between the two polypeptides have little effect on formation of the subunit complex, unless their possible enhancement and weakening effects should happen to cancel. These eight other differences occur at locations in the amino acid sequence either that are outside the core region or that are at positions which contain a highly variable amino acid in other comparable polypeptides from other photosynthetic bacteria. At these eight positions, the differences in amino acids found in the β-polypeptide of *Rb. sphaeroides* compared to those of *Rb. capsulatus* are highly conservative, and one would not expect them to cause a significant change in stabilization of the subunit complex. The data in Figure 4 show that the $\beta Tyr_{+4} \rightarrow Met$ mutation affects the formation of heterologous (αβ) subunit-type complexes in a manner similar to the homologous (β-only) subunit-type complex (Figure 3). It is interesting to note that in the case of the heterologous reconstitution, the decreased stability of the subunit-type complex is accompanied by an increased assembly of LH1 complexes; evidence for such an effect is seen at 0.6% OG when the 869-nm complex is seen with the mutant $\beta Tyr_{+4} \rightarrow Met$, but only the band at 825 nm is observed with the native β-polypeptide.

The results imply that Tyr at position +4 is involved in stabilizing the subunit complex and therefore LH1 as a whole. Except for *Rb. capsulatus*, the amino acid at this location in the β-polypeptide of LH1 of other bacteria is either Tyr or Trp, both of which are capable of hydrogen bonding. The involvement of Tyr_{+4} in stabilizing the subunit complex has been suggested to be due to hydrogen bonding between the side chain of this amino acid and an oxygen atom of BChl coordinated to His_0 of the α-polypeptide (Loach & Parkes-Loach, 1995). However, its contribution to stabilization of the subunit-type complex could equally well be explained by its participation in a hydrogen bond to another amino acid side chain or main chain oxygen atom. This latter explanation is particularly attractive if the C-terminal portions of the α- and β-polypeptides of LH1 are arranged similarly to those of LH2 (McDermott et al., 1995; Koepke et al., 1996). Then βTyr_{+4} is in an ideal location to form a hydrogen bond to the C-terminal end of an α-polypeptide. The existence of such a polypeptide interaction would be consistent with the truncation experiments of McGlynn et al. (1996) where decreased LH/RC ratios were observed when residues between αLeu_{+12} and αArg_{+21} were deleted. Yet another possible role in stabilization might be due to a "rudder" effect similar to that observed for porin (Kreusch et al., 1994; Schulz, 1995) and RC structures (Deisenhofer et al., 1985; Michel et al., 1995) where the flat amphipathic side chain polar groups are solvated by water and their nonpolar parts are embedded in the nonpolar boundary region of the membrane, detergent or protein.

Role of His in Binding BChl. On the basis of amino acid sequence data (Zuber & Brunisholz, 1991), resonance Raman information (Robert & Lutz, 1985), and site-directed mutagenesis experiments (Bylina et al., 1988; Olsen, 1994; unpublished results), His has been implicated as providing the coordinated ligand to BChl in LH1. Proof of such a role in the 850-nm component of *Rps. acidophila* (McDermott et al., 1995) and *Rs. molischianum* (Koepke et al., 1996) has come from their recently-solved crystal structures. In the RC, His coordination to BChl can be replaced by Gln (Coleman & Youvan, 1990) and by Gly (Goldsmith et al., 1996), apparently without seriously affecting properties of the complex. Also, Asn and Gln are among the ligands to Chl in the chlorophyll *a*/*b*−protein complex (Kuhlbrandt et al., 1994).

Mutants in which His_0 was changed to Asn, Tyr, or Leu (Olsen, 1994) were prepared from both the α- and β-polypeptides of *Rb. sphaeroides*. By spectral examination of the whole cells, only the mutant cells containing $\beta His_0 \rightarrow Asn$ exhibited a slight LH1 complex absorbance. A polypeptide was isolated in very low yield from the $\beta His_0 \rightarrow Asn$ mutant which appeared in place of the native β-polypeptide. When tested in the reconstitution assay, formation of a subunit-type complex could not be observed under our assay conditions. The inability to demonstrate formation of a subunit-type complex means that the stabilization of this complex by His coordination by the β-polypeptide is at least 4.5 kcal mol^{-1}. This is based on our ability to accurately measure as little as 50 nM subunit-type complex which, under conditions of the assay, would indicate a $K_{assoc} = 2 \times 10^3$ times smaller than that with the native β-polypeptide. From model studies, imidazole coordination to BChl to form a 5-coordinate complex is expected to occur with 4.5−5.5 kcal mol^{-1} binding energy (Cotton, 1976). Our results are consistent with this assumption.

In the case of the α-polypeptide isolated from the mutant $\alpha His_0 \rightarrow Asn$, an LH1-type complex could not be formed. Because the conditions used to reconstitute an LH1-type complex employ lowering the OG concentration to below the critical protein-OG micelle concentration, it is surprising that even this driving force is insufficient to overcome the absence of the His ligand.

αTrp_{+11} Affects the λ_{max} of the LH1 Complex, but Not That of the Subunit. Previous measurements of the whole cell

spectral properties of the mutants αTrp_{+11}→Phe and αTrp_{+11}→Tyr indicated that interactions involving this amino acid affected the λ_{max} of LH1 (Olsen et al., 1994). Evidence was provided in these studies that hydrogen bonding occurs between the carbonyl group on the side chain at position C3 of BChl and the αTrp_{+11} residue. In the experiments reported here, the use of the α-polypeptide of αTrp_{+11}→Phe is shown to cause a blue-shift of λ_{max} of reconstituted LH1 to 853 nm (Figure 5A), identical to that observed in chromatophores and whole cells of this mutant. This is an important result in that it further strengthens the likelihood that our reconstituted systems are reproducing the BChl environments of the native LH1 complexes. It also indicates that the λ_{max} *in vivo* most likely reflects the environment provided by the α- and β-polypeptides and does not depend on interactions with other components.

In contrast to the effect on the λ_{max} of the LH1 complex, no effect was observed on formation of the subunit-type complex (Figure 5A). In the case of the subunit complex formed with native polypeptides, both homodimer and heterodimer populations presumably coexist since their K_{assoc} values are similar. However, changing αTrp_{+11} to Phe may decrease the ability of the mutant α-polypeptide to support subunit formation relative to the β-only subunit-type complex. Thus, we cannot tell whether the subunit complex observed is that involving only the β-polypeptide, or whether it is a homodimer/heterodimer mixture as is assumed for the native polypeptides.

It might be proposed that the role of the side chain of αTrp_{+11} is to form a hydrogen bond to the $C3^1$ carbonyl oxygen of the BChl coordinated to His_0 of the same polypeptide. Support for such an assignment comes from the crystal structure of LH2 of *Rs. molischianum* in which the side chain of αTrp_{+11} participates in a hydrogen bond to the $C3^1$ carbonyl oxygen of the BChl coordinated to His_0 of the same polypeptide (Koepke et al., 1996). Since there is significant amino acid sequence identity at the C-terminal end of the α- and β-polypeptides of *Rb. sphaeroides* LH1 compared with that of the α- and β-polypeptides of *Rs. molischianum* LH2, respectively, similarity in roles of common amino acid side chains might be expected. Furthermore, the side chain of αTyr_{+13} in LH2 of *Rps. acidophila* plays a similar role in hydrogen bonding to the $C3^1$ carbonyl oxygen of the BChl coordinated to His_0 of the same α-polypeptide (McDermott et al., 1995). Association/dissociation studies involving more mutants are underway to allow us to examine this point further. The formation of LH1 must involve stabilizing interactions other than (or in addition to) those of αTrp_{+11} since LH1-type complexes are readily formed both *in vivo* and in the *in vitro* reconstitution with the αTrp_{+11}→Phe mutant.

It is interesting that a Trp side chain in a similar location on the β-polypeptide seems to play a similar role. Several lines of evidence indicate that βTrp_{+9} is also a key amino acid in stabilizing the formation of both the subunit- and LH1-type complexes by hydrogen bonding to BChl (Olsen, 1994; Koepke et al., 1996; Sturgis et al., 1997). Because this amino acid is involved in stabilizing the subunit complex (unpublished results), it might be suggested that it interacts with the $C3^1$ carbonyl oxygen of BChl coordinated to His_0 within the subunit complex.

C-Terminal Truncated α*-Polypeptides.* Removing amino acids from the C-terminus of the α-polypeptide of *Rb. sphaeroides* markedly reduced the ratio of LH1/RC from 26 BChl/RC in the control strain to between 6 and 10 BChl/RC in a series of mutants (McGlynn et al., 1996). Detergent-solubilized complexes obtained from these strains contained from 10 to 20 BChl/RC, as well as some RC unattached to LH1. This reduction in LH1 content could result from a variety of sources such as inappropriate processing of the polypeptide, defective insertion into the membrane, its rapid degradation, or decreased binding interactions in forming the LH1 complex. An *in vitro* approach was used to evaluate this last possibility in the current study by determining whether the shortened polypeptides decrease LH1 formation in reconstitution experiments.

Two important results are reported here. First, deleting 5 (αArg_{+21t}) and 14 (αLeu_{+12t}) amino acids had no effect on formation of a subunit-type complex. Measured K_{assoc} values were indistinguishable from those obtained with native polypeptides. Second, formation of LH1-type complexes was readily demonstrated with each mutant system, both after chilling the subunit-type complex for 1 h and after an overnight chill. The relative stability at room temperature of the LH1-type complex formed in each case was the same as that formed with native polypeptides. These results indicate that there is no apparent reduction in the ability of the subunit complexes to associate to form LH1-type complexes. These observations are consistent with the proteolytic truncation experiments of Meadows et al. (1995) in which the α-polypeptide of *Rs. rubrum* was shortened by 5 and 10 amino acids at the C-terminus without effect on reconstitution of subunit- and LH1-type complexes.

Interestingly, the reconstituted LH1-type complex using the αLeu_{+12t} polypeptide exhibits a blue-shift in the λ_{max} of BChl, just as observed in the absorption spectrum of this mutant LH1 examined *in vivo*. This suggests that the α-polypeptide amino acids between +12 and +21 have a small role in directly interacting with BChl or in helping to stabilize the LH1 complex in some other way. For example, a smaller LH1 complex (fewer associated subunits) might affect the delocalization of the excited state and therefore limit the extent of the red-shift of this assembly of BChl (Westerhuis et al., 1992). From the studies of McGlynn et al. (1996), the region most affecting this blue-shift in λ_{max} seems to be between residues +17 and +21, which encompasses hydrophilic residues capable of forming hydrogen bonds.

CONCLUSIONS

The fact that it is possible to isolate and reconstitute a stable subunit complex from LH1 but not from LH2 of photosynthetic bacteria suggests that stabilizing interactions exist in LH1 beyond those inferred from the crystal structure of LH2 (McDermott et al., 1995; Koepke et al., 1996). Furthermore, the ability to form a subunit-type complex with only the β-polypeptide of LH1 (Parkes-Loach et al., 1988; Loach et al., 1994) provides clues to additional stabilizing interactions (Loach & Parkes-Loach, 1995). Thus, examination of the mutants βSer_{-7}→Ile and βTyr_{+4}→Met allowed the conclusion that the amino acid at position +4 was primarily responsible for the difference in stability of the subunit complex of *Rb. sphaeroides* and *Rb. capsulatus*.

The power of applying a combination of reconstitution and site-directed mutagenesis methods to structure−function

studies is underscored by these experiments. Through this approach, the relative contributions of each binding element can be accurately determined. Thus, the contribution of ligation of BChl by His_0 of each polypeptide can be estimated to be at least 4.5 kcal/mol. Future extension of these studies to those employing higher concentrations of polypeptides and BChl should allow a more accurate determination of this important interaction.

From the studies of mutant α-polypeptides containing truncated C-termini, the reduced levels of LH1 expression observed by McGlynn et al. (1996) are most likely due to either inappropriate polypeptide processing, decreased incorporation of the α-polypeptide into the membrane, or its increased rate of degradation.

In addition, it has been possible to examine further the contribution made by the hydrogen bond from αTrp_{+11} to the C3 acetyl carbonyl of one of the LH1 BChl. Because the λ_{max} of the reconstituted LH1-type complex was blue-shifted to 853 nm, the same as observed in the intact bacterium harboring this mutation, these results provide further confirmation that the environment of BChl in reconstituted LH1 complexes is the same as *in vivo* and support the assignment of this residue to a role in hydrogen bonding with the $C3^1$ carbonyl group of BChl.

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