Reconstitution of Core Light-Harvesting Complexes of Photosynthetic Bacteria Using Chemically Synthesized Polypeptides. 2. Determination of Structural Features That Stabilize Complex Formation and Their Implications for the Structure of the Subunit Complex†

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ABSTRACT: Chemically synthesized polypeptides have been utilized with a reconstitution assay to determine the role of specific amino acid side chains in stabilizing the core light-harvesting complex (LH1) of photosynthetic bacteria and its subunit complex. In the preceding paper [Meadows, K. A., Parkes-Loach, P. S., Kehoe, J. W., and Loach, P. A. (1998) *Biochemistry 37,* 3411−3417], it was demonstrated that 31-residue polypeptides (compared to 48 and 54 amino acids in the native polypeptides) having the same sequence as the core region of the β-polypeptide of *Rhodobacter sphaeroides* (sphβ31) or *Rhodospirillum rubrum* (rrβ31) could form subunit-type complexes. However, neither polypeptide interacted with the native α-polypeptides to form a native LH1 complex. In this paper, it is demonstrated that larger segments of the native *Rb. sphaeroides* β-polypeptide possess native behavior in LH1 formation. Polypeptides were synthesized that were six (sphβ37) and ten amino acids (sphβ41) longer than sphβ31. Although sphβ37 exhibited behavior nearly identical to that of sphβ31, sphβ41 behaved more like the native polypeptide. In the case of rrβ31, a polypeptide with four additional amino acids toward the C terminus was synthesized (rrβ35). Because LH1-forming behavior was not recovered with this longer polypeptide, one or more of the three remaining amino acids at the C-terminal end of the native β-polypeptide seem to play an important role in LH1 stabilization in *Rs. rubrum*. Three analogues of the core region of the *Rb. sphaeroides* β-polypeptide were synthesized, in each of which one highly conserved amino acid was changed. Evidence was obtained that the penultimate amino acid, a Trp residue, is especially important for subunit formation. When it was changed to Phe, the λ_{Max} of the subunit shifted from 823 to 811 nm and the association constant decreased about 500-fold. Changing each of two other amino acids had smaller effects on subunit formation. Changing Trp to Phe at the location six amino acid residues toward the C terminus from the His coordinated to Bchl resulted in an approximately 10-fold decrease in the association constant for subunit formation but did not affect the formation of a LH1-type complex compared to sphβ31. Finally, changing Arg to Leu at the location seven amino acid residues toward the C terminus from the His coordinated to Bchl decreased the association constant for subunit formation by about 30-fold. In this case, no LH1-type complex could be formed. On the basis of these results, in comparison with the crystal structure of the LH2 β-polypeptide of *Rhodospirillum molischianum*, two possible structures for the subunit complex are suggested.

The identity and relative contributions of interactions that stabilize the core light-harvesting complex (LH1)[1] of photosynthetic bacteria can be determined using structural analogues of the cofactors and the proteins, together with reconstitution methodology. In the preceding paper (*1*), the chemical synthesis, isolation, and characterization of polypeptides whose amino acid sequences reproduce portions of the amino acid sequence of the β-polypeptides of LH1 of *Rhodobacter sphaeroides* or *Rhodospirillum rubrum* were described. Polypeptides containing either the 31 amino acids at the C terminus of the *Rb. sphaeroides* β-polypeptide (sphβ31) or the equivalent 31 amino acids of the *Rs. rubrum* β-polypeptide (rrβ31) were fully competent in forming a subunit-type complex and exhibited association constants for complex formation comparable to or exceeding those of the native β-polypeptides. Importantly, these results demonstrated that all structural features required to make the subunit complex are present in the well-defined, chemically synthesized polypeptides. However, neither polypeptide appeared to interact with the native α-polypeptides to form a LH1-type complex.

In the experimental results reported here, the chemical synthesis methodology has been used to synthesize three longer polypeptides with more of the N- or C-terminal portions of the native β-polypeptide of *Rb. sphaeroides* or *Rs. rubrum*, respectively. These syntheses were undertaken

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to evaluate interactions between the α- and *β*-polypeptides near the N and C termini that appear to be necessary in forming a native LH1 complex. In addition, three synthetic polypeptides were prepared in which one amino acid at a time was changed relative to the sequence of sph*β*31 in order to determine its contribution to the interaction energy for complex formation. The amino acids chosen are highly conserved residues whose side chains are near the Bchl binding site and are capable of hydrogen bonding interactions. Finally, carboxypeptidase Y treatment of the native *Rb. sphaeroides β*-polypeptide was employed to prepare an analogue with two fewer amino acids at the C-terminal end which could be compared with one of the synthesized analogues. The results of these experiments, together with earlier reconstitution results (*2*−*6*), site-directed mutagenesis results (*7*−*13*), and a consideration of the recent crystallographic information on the structure of LH2 (*14*, *15*), are used to suggest two possible structures for the subunit complex.

METHODS AND MATERIALS

Reconstitution assays were conducted as previously described (*1*, *4*, *5*). Descriptions of methods used for chemical syntheses and purification of synthetic polypeptides have been given previously (*1*). Fmoc-Asn(trt)−PEG−PS resin was used in addition to those previously reported (*1*). The identity of the synthesized polypeptides was confirmed by electrospray mass spectral analysis [conducted by either the Biochemistry Department Structure Facility (Michigan State University, East Lansing, MI) or the Harvard Microchemistry Facility (Cambridge, MA)]. The method used for determination of association constants has been summarized (*1*).

Enzymatic Truncation of the Native β-Polypeptide of Rb. sphaeroides LH1. Carboxypeptidase Y (CY) was obtained from both Sigma and Boehringer Mannheim. The native *Rb. sphaeroides β*-polypeptide was isolated as previously described (*4*). The protocol used for proteolysis was similar to the one previously published by Klemm (*16*). *β*-Polypeptide (0.3 mg) was dried out of HFA into a teardrop flask and then taken up with 0.1 mL of 5% SDS buffered with 0.05 M pyridine acetate (pH 5.6). After 5 min, the solution was diluted with 0.4 mL of 0.1 M pyridine acetate (pH 5.6) for a final protein concentration of 110 *μ*M. CY in 0.1 M pyridine acetate (pH 5.6) was then added for a polypeptide:CY ratio of 50:1 (w/w). The mixture was shaken at room temperature, and the reaction was monitored by HPLC. As the reaction progressed, the native *β*-polypeptide peak decreased and a separate peak increased which was later shown by mass spectral analysis to be the modified *β*-polypeptide which had lost its C-terminal Phe. With longer times, this second peak then decreased and a third peak formed which has been demonstrated by mass spectral analysis to be the *β*-polypeptide minus two amino acids (Phe and Trp) at the C-terminal end (sph*β*CP2). When most of the polypeptide was converted to this product, usually requiring about 3 h, the reaction was quenched by addition of 100 *μ*L of glacial acetic acid and the sample lyophilized to dryness. The product was then purified by reverse phase HPLC using gradient G2 of Meadows et al. (*5*) with a C-18 column.

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

RESULTS

sph*β*31 formed a subunit-type complex with properties nearly identical to those found with the native *β*-polypeptide (*1*). However, its behavior under LH1-forming conditions was not like that of the native *β*-polypeptide but identical to that of the protease-truncated polypeptide from LHSV to the C terminus (see Figure 1 for amino acid sequences of the peptides synthesized and the numbering system used). sph*β*31 readily formed a LH1-type complex absorbing at 849 nm without the α-polypeptide and seemed to show no real interaction with the α-polypeptide when the α-polypeptide was present, as it still formed a complex absorbing at 849 nm (*1*). Thus, amino acids in the sequence toward the N terminus from residue −20 appear to play an important role in allowing interaction with the α-polypeptide to stabilize the heterologous α*β* subunit complex, which is necessary for formation of LH1 absorbing at 872 nm. To determine how many of these residues are required, longer polypeptides were synthesized and isolated and their properties in reconstitution evaluated.

Chemical Synthesis of sph β37. The synthesis of sph*β*37 proceeded smoothly as did its purification. The behavior of sph*β*37 was very similar to that of sph*β*31. It readily

[1] Abbreviations: Bchl, bacteriochlorophyll *a*; $Bchl_{\alpha}$ and $Bchl_{\beta}$, Bchl molecules whose fifth coordination sites are occupied by the imidazole group of His at position zero of the α- and *β*-polypeptides, respectively (see Figure 1 for the numbering system used for the amino acid sequence); LH1, core light-harvesting complex (also called B873, named after the long-wavelength absorption maximum); subunit complex (also referred to as B820), subunit form of LH1 either isolated from membranes containing LH1 or prepared by reconstitution using native α- and *β*-polypeptides and Bchl; subunit-type complex, reconstituted complex exhibiting absorption and CD spectra highly similar to those of the native subunit complex but containing an analogue polypeptide or only native *β*-polypeptide (without the α-polypeptide) and Bchl; LH1-type complex, reconstituted complex containing Bchl and an analogue 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 [reconstituted systems in which a subunit-type complex or LH1-type complex was formed with only a *β*-polypeptide are sometimes referred to as a *ββ* subunit-type complex or a *ββ* LH1-type complex to distinguish them from the heterologous α*β* complexes]; LH2, accessory light-harvesting complex (also known as B800−850); OG, *n*-octyl *β*-D-glucopyranoside; HFA, hexafluoroacetone trihydrate; near-IR, near-infrared; CD, circular dichroism; CY, carboxypeptidase Y. The term ΔΔ*G* is used to indicate the difference in Δ*G* values determined for formation of a subunit-type complex using an analogue polypeptide compared to that determined using native polypeptides. The following nomenclature was used to refer to the chemically synthesized polypeptides. sph*β*16, sph*β*31, sph*β*37, and sph*β*41 represent the chemically synthesized polypeptides that have amino acid sequences identical to the last 16, 31, 37, and 41 amino acids of the native *β*-polypeptide of *Rb. sphaeroides*, respectively; rr*β*31 and rr*β*35 represent the chemically synthesized polypeptides that have amino acid sequences identical to that of the native *β*-polypeptide of *Rs. rubrum* from residues −20 to +10 or −20 to +14, respectively (see Figure 1 for the numbering of amino acids in the sequences); $sph\beta31_{W+6\rightarrow F}$, $sph\beta31_{R+7\rightarrow L}$, and $sph\beta31_{W+9\rightarrow F}$ represent chemically synthesized polypeptides that have amino acid sequences identical to the last 31 amino acids of the native *β*-polypeptide of *Rb. sphaeroides* except that Trp at position +6, Arg at position +7, or Trp at position +9 was changed to Phe, Leu, or Phe, respectively; sph*β*CP2 represents the native *β*-polypeptide of *Rb. sphaeroides* after removal of two amino acids at the C-terminal end by carboxypeptidase Y treatment.

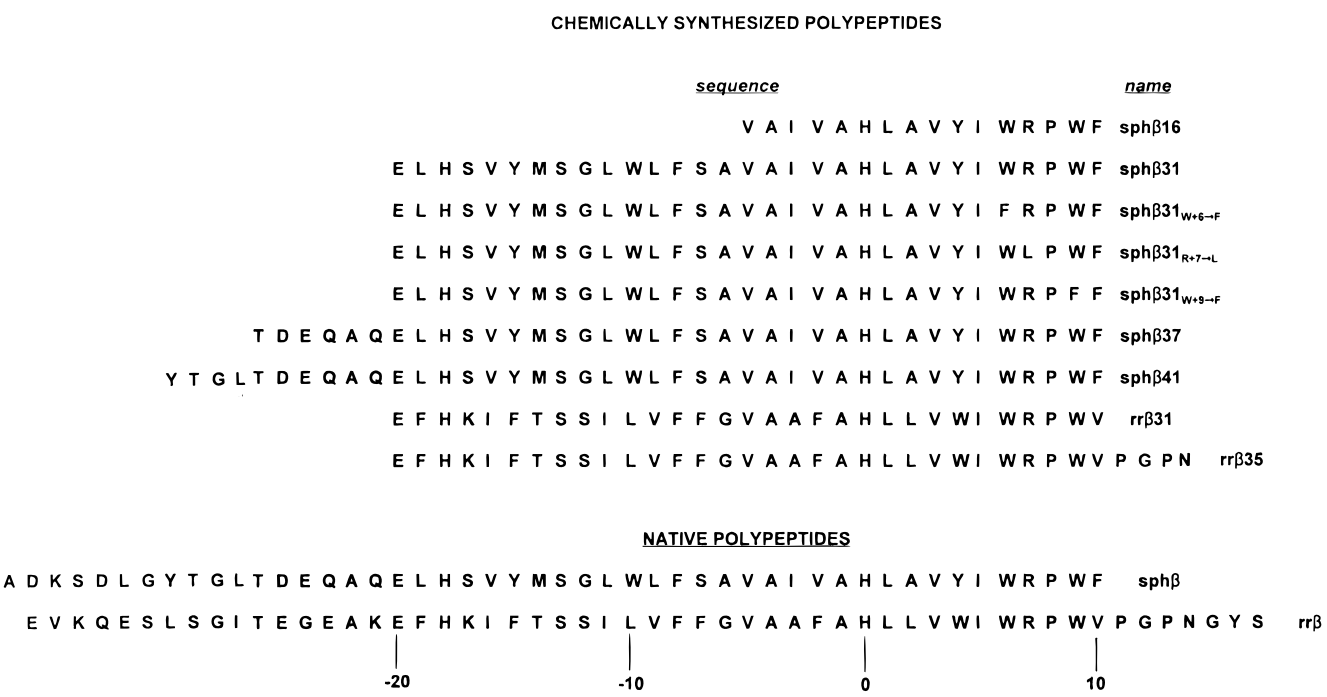

FIGURE 1: Amino acid sequences of chemically synthesized analogues and native β-polypeptides of LH1 of *Rs. rubrum* (rrβ) (*17*, *18*) and *Rb. sphaeroides* (sphβ) (*19*, *20*). The His to which Bchl is coordinated was assigned position 0, and each polypeptide was aligned accordingly. The abbreviation used for each polypeptide in the text is indicated on the right side.

formed a subunit complex with a K_{Assoc} similar to that found with sphβ31 or the native β-polypeptide (Figure 2A and Table 1). Also like sphβ31, a LH1-type complex absorbing at 847 nm was formed with or without an added α-polypeptide. The LH1-type complex was formed more slowly, however, and upon the sample being warmed to room temperature, it reverted to the subunit-type complex more rapidly than was the case with the sphβ31 system, indicating that the additional amino acids destabilize the homologous $\beta\beta$ LH1-type complex slightly. The CD spectrum of the subunit-type complex (Figure 2B, dotted curve) was very similar to that of the native subunit complex (*21*) and that exhibited by sphβ31 (*1*). The CD spectrum of the LH1-type complex was similar to that obtained using sphβ31 (*1*) and again exhibited much greater molar ellipticity than that of the native LH1 complex (*21*) and exhibited a more asymmetric shape (Figure 2B, solid curve).

Chemical Synthesis of sphβ41. sphβ41 was synthesized from a portion of the completed sphβ37 which had not been removed from the resin. Isolation of sphβ41was carried out by reverse phase HPLC using a C-18 column and a gradient similar to G3 of Meadows et al. (*5*). Mass spectral analysis of the purified material gave the value of 4771.0 Da, in excellent agreement with the calculated value (4770.6 Da). Reconstitution experiments with sphβ41 proved to be quite interesting. While a subunit-type complex formed in the usual way (similar to that with sphβ31, sphβ37, and native β-polypeptide; see Figure 3 and Table 1), a LH1-type complex did not readily form under our standard assay conditions unless the α-polypeptide was also present, thus showing more of the characteristic behavior of the native β-polypeptide. At higher concentrations of Bchl, a LH1-type complex could be partially formed in the absence of an α-polypeptide (λ_{Max} at approximately 844 nm), but it rapidly converted to the subunit complex upon warming to room temperature. Importantly, a stable LH1-type complex with a λ_{Max} at 870 nm formed when the *Rb. sphaeroides* α-polypeptide was present (Figure 3A). Clearly, the additional N-terminal length plays an important role in preventing association of $\beta\beta$ subunit-type complexes in forming red-shifted species and in supporting formation of a heterologous $\alpha\beta$ subunit-type complex which can then oligomerize to form a more native LH1-type complex. The CD spectra of both the subunit- and LH1-type complexes (Figure 3B) were very similar to those of the native subunit and LH1 complexes (*21*).

Chemical Synthesis of rrβ35. rrβ31 also formed a subunit-type complex but did not form LH1 alone or with the *Rs. rubrum* α-polypeptide (*1*). In contrast, the enzymatically truncated β-polypeptide (from EFHK to the C terminus) did form LH1 (*5*). The only difference between these two polypeptides is that rrβ31 does not have the last seven amino acids at the C terminus of the native β-polypeptide. To identify the portion of the C terminus which was not present in rrβ31 but must be involved in stabilizing interaction with the α-polypeptide to form the LH1 complex, a polypeptide containing four more amino acids toward the C terminus than that of rrβ31 was synthesized. This synthesis of rrβ35 proceeded smoothly, and the polypeptide was purified by reverse phase HPLC using a C-18 column and the G3 gradient of Meadows et al. (*5*). The activity exhibited by this polypeptide was quite similar to that of rrβ31 (Figure 4), but a somewhat lower K_{Assoc} was obtained (Table 1). No LH1-type complex could be formed either in the presence or in the absence of an α-polypeptide (data not shown). Thus, a specific interaction between some portion of the last three C-terminal amino acids of the native *Rs. rubrum* β-polypeptide and the native *Rs. rubrum* α-polypeptide seems to be required to stabilize formation of a heterologous $\alpha\beta$ LH1-type complex.

Analogues of sphβ31−sph$\beta31_{W+6\rightarrow F}$. Since the properties of sphβ31 were exactly like those of the protease-truncated

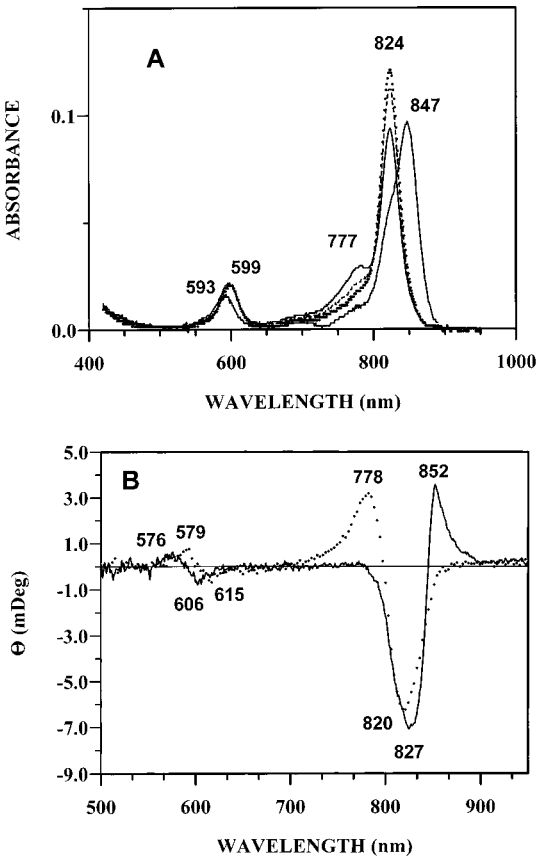

FIGURE 2: (A) Absorption spectra of a reconstitution experiment using sphβ37 and Bchl. Spectra were recorded at 0.90% (solid curve with absorption peaks at 777 and 824 nm), 0.75% (dashed curve), 0.66% (dotted curve), and 0.66% OG stored at 4 °C overnight (solid curve with a λ_{Max} of 847 nm). Concentrations were as follows: sphβ37, 4.8 μM; and Bchl, 1.4 μM at 0.66% OG. Spectra were recorded in 1 cm cuvettes and multiplied by an appropriate dilution factor to normalize the spectra to a constant BChl concentration. (B) CD spectra of the subunit-type complex (dotted curve) at 0.75% OG at room temperature, formed using sphβ37 and Bchl, and the corresponding LH1-type complex (solid curve) formed after diluting to 0.66% OG and storage overnight at 4 °C. Concentrations were as follows: sphβ37, 10.8 μM; and Bchl, 3.1 μM at 0.75% OG. Spectra were recorded at room temperature (subunit-type complex) and at 10 °C (LH1-type complex) in 2 cm cuvettes. Four spectra were averaged to improve the signal-to-noise ratio.

native polypeptide (from LHSV to the C terminus), and since this sequence was all that was required to form a subunit-type complex, structural requirements for complex formation could be systematically probed by chemical synthesis of selected analogues. A highly conserved amino acid residue near the Bchl coordination site, Trp+6 (see Figure 1 for amino acid numbering), was changed to Phe and the chemically synthesized polypeptide isolated in the same manner as with sphβ31. The polypeptide was purified by HPLC and its identity confirmed by mass spectral analysis (peaks at 3624.4 and 3640 Da compared with a calculated value of 3624.3 Da; the additional peak at 3640 Da is attributed to an oxidized Met in some of the polypeptide). In reconstitution experiments with this polypeptide, formation of the subunit-type complex was decreased somewhat, exhibiting a K_{Assoc} about 10-fold smaller than that for the sphβ31 (Table 1). Thus, Trp+6 of the native polypeptide does have some effect in stabilizing the subunit-type complex, possibly by a weak hydrogen bonding interaction.

Table 1: Association Constants of Reconstituted Subunit Complexes

system	polypeptide (μM at 0.75% OG)	K_{Assoc} ($M^3 \times 10^{-16}$) 0.90% OG	0.75% OG
Rs. rubrum	native β (2.0) + native α (1.7)	126	≥300
	native β (3.7)	2	24
chemical synthesis *Rs. rubrum*	rrβ31 (4.9)	12	120
	rrβ35 (6.9)	0.7	3
Rb. sphaeroides	native β (1.8) + native α (1.2)	38	≥300
	native β (6.1)	23	200
enzyme-truncated *Rb. sphaeroides*	sphβCP2 (4.9) + native α (2.4)	≤0.1	0.7
chemical synthesis *Rb. sphaeroides*	sphβ31 (5.5)	40	≥300
	sphβ37 (5.4)	26	≥300
	sphβ41 (5.6) + native α (6.8)	19	≥300
	sphβ41 (5.6)	8.3	79
chemical synthesis *Rb. sphaeroides* analogues	sph$\beta31_{W+6\rightarrow F}$ (5.5)	1.5	22
	sph$\beta31_{R+7\rightarrow L}$ (6.0)	0.5	6
	sph$\beta31_{W+9\rightarrow F}$ (14.2)	≤0.1	0.5

No change was observed in the λ_{Max} of the subunit complex, its CD spectrum, or in formation of a LH1-type complex relative to sphβ31 (data not shown).

Analogues of sphβ31−sph$\beta31_{R+7\rightarrow L}$. In this synthesis, Leu was substituted for the strictly conserved Arg+7. The results of reconstitution experiments were quite interesting (Figure 5). A subunit-type complex was formed with a λ_{Max} of 820 nm, indicating a structure similar to that formed with the native β-polypeptide, although with a substantially decreased K_{Assoc} relative to that obtained with sphβ31 (Table 1). Because this polypeptide was especially difficult to solubilize, it is possible that the K_{Assoc} measured was decreased because the concentration of sph$\beta31_{R+7\rightarrow L}$ in solution may have been lower than assumed. Interestingly, a LH1-type complex was not formed, either with or without an α-polypeptide. Because a subunit-type complex was formed, the lack of LH1 formation cannot be attributed to a lack of solubility of sph$\beta31_{R+7\rightarrow L}$. That a LH1-type complex would not form with the native α-polypeptide was expected because that is the behavior exhibited by sphβ31. However, the fact that sph$\beta31_{R+7\rightarrow L}$ would not form a LH1-type complex at all strongly implicates Arg+7 in the assembly of a LH1-type complex from the homologous $\beta\beta$ subunit and implies that it may also be important in forming LH1 from the native heterologous αβ subunit, as well.

Analogues of sphβ31−sph$\beta31_{W+9\rightarrow F}$. Trp+9 is strictly conserved in all known β-polypeptides of both LH1 and LH2. An analogue was synthesized in which Phe was substituted for this Trp. Isolation of the polypeptide proceeded smoothly in a fashion parallel to that for sphβ31. Mass spectral analysis gave a mass of 3624.0 and 3641.0 Da compared with the calculated value of 3624.3 Da. The peak with the higher mass is attributed to an oxidized Met in some of the polypeptide. From reconstitution assays using this analogue, three interesting results were obtained (Figure 6). First, the subunit complex did not readily form, as K_{Assoc} had decreased nearly 500-fold compared with that for sphβ31 (Table 1). Second, the subunit λ_{Max} was blue-shifted by 13−14 nm (to 811 nm). The CD spectrum also exhibits this blue shift but

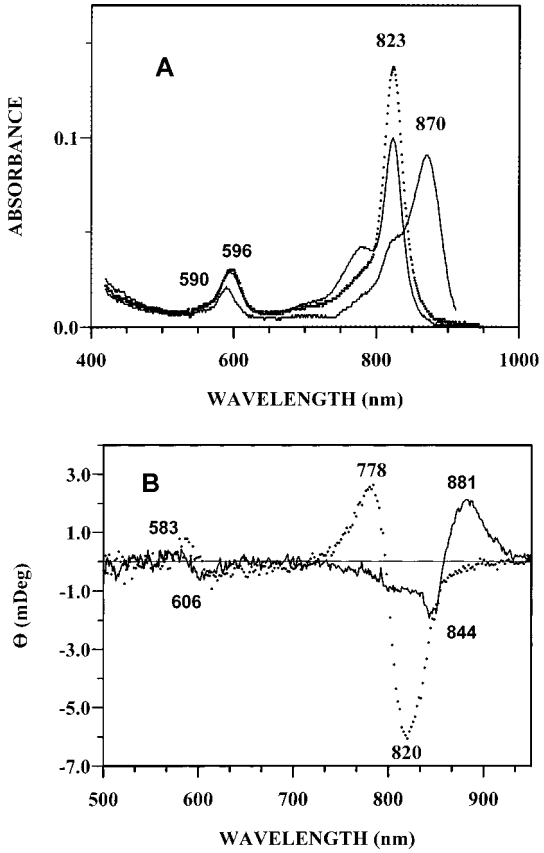

FIGURE 3: Reconstitution using sphβ41, the native α-polypeptide of *Rb. sphaeroides*, and Bchl. (A) Absorption spectra at 0.90% (solid curve with absorption peaks at 777 and 823 nm), 0.66% (dotted curve), and 0.66% OG stored at 4 °C overnight (solid curve with a λ_{Max} of 870 nm). Concentrations were as follows: α, 3.0 μM; sphβ41, 2.4 μM; and Bchl, 1.7 μM at 0.66% OG. Spectra were recorded in 1 cm cuvettes and multiplied by an appropriate dilution factor to normalize the spectra to a constant BChl concentration. (B) CD spectra of the subunit-type complex (dotted curve) at 0.75% OG at room temperature formed using sphβ41, the native α-polypeptide of *Rb. sphaeroides* and Bchl, and the corresponding LH1-type complex (solid curve) formed after diluting the sample to 0.66% OG and storage overnight at 4 °C. Four spectra were averaged to improve the signal-to-noise ratio. Concentrations at 0.75% OG were as follows: α, 6.8 μM; sphβ41, 5.5 μM; and Bchl, 2.9 μM. Spectra were recorded in 2 cm cuvettes at room temperature (subunit-type complex) and at 10 °C (LH1-type complex). The data for the LH1-type complex were corrected for the presence of the subunit complex due to incomplete formation of a LH1-type complex at this temperature.

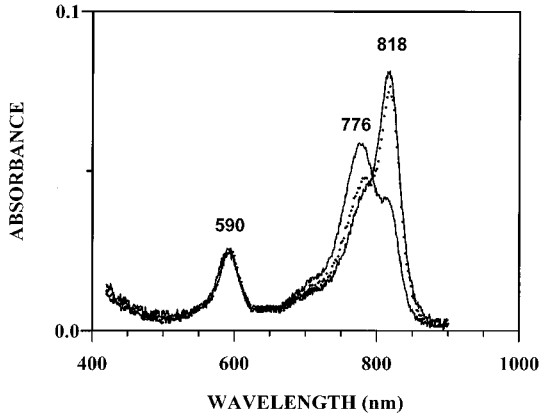

FIGURE 4: Absorption spectra of a reconstitution experiment using rrβ35 and Bchl. Spectra were recorded at 0.90% (solid curve with an absorption peak at 776 nm), 0.75% (dotted curve), and 0.66% (solid curve with a λ_{Max} of 818 nm). Concentrations were as follows: rrβ35, 6.9 μM; and Bchl, 1.1 μM at 0.66% OG. Spectra were recorded in 1 cm cuvettes and multiplied by an appropriate dilution factor to normalize the spectra to a constant BChl concentration.

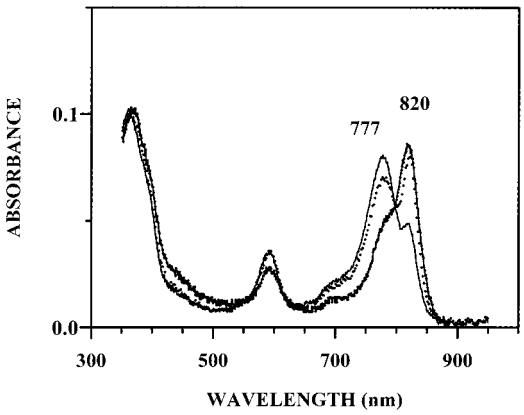

FIGURE 5: Reconstitution using sph$\beta 31_{R+7\rightarrow L}$, the native α-polypeptide, and Bchl. Absorption spectra at 0.90% (solid curve with an absorption peak at 777 nm), 0.75% (dotted curve), and 0.59% OG stored at 4 °C overnight (solid curve with a λ_{Max} of 820 nm). Concentrations were as follows: α, 2.4 μM; sph$\beta 31_{R+7\rightarrow L}$, 3.4 μM; and Bchl, 1.9 μM at 0.75% OG. Spectra were recorded in 1 cm cuvettes and multiplied by an appropriate dilution factor to normalize the spectra to a constant BChl concentration.

otherwise has a shape which is very similar to those of subunit-type complexes (Figure 6B). Although we have previously observed such a blue shift with the α- and β-polypeptides of *Rs. rubrum* and a Bchl analogue in which a vinyl group replaced the acetyl group at the C3 side chain of Bchl (see Figure 8C for the structure and numbering of Bchl) (*6*), this is the first amino acid we have observed to significantly effect the λ_{Max} of the subunit complex. And third, a LH1-type complex was not formed under our standard assay conditions. The results obtained with this polypeptide analogue are especially insightful when taken together with those obtained with the native β-polypeptide after removal of two amino acids from the C terminus (see below).

Carboxypeptidase Removal of the Two C-Terminal Amino Acids from sphβ. Trp+9 and Phe+10 were removed from the C terminus of the native *Rb. sphaeroides* β-polypeptide by protease treatment with carboxypeptidase Y. The polypeptide was isolated by HPLC. Mass spectral analysis gave a mass of 5124.5 Da compared with the calculated value of 5124.1 Da. Whereas removal of one amino acid (Phe+10) had little effect on reconstitution behavior (*5*), removal of two amino acids (Trp+9 as well as Phe+10) resulted in major differences. Figure 7 shows the results of a reconstitution assay using a native α-polypeptide and a *Rb. sphaeroides* β-polypeptide from which the two C-terminal amino acids had been removed (sphβCP2). This polypeptide poorly formed a subunit-type complex, as was observed with sph$\beta 31_{W+9\rightarrow F}$ (Table 1), and again exhibited a blue-shifted λ_{Max} at 811 nm. The similar inhibitory effect on subunit formation displayed by sph$\beta 31_{W+9\rightarrow F}$ and sphβCP2 indicates that the lack of the N terminus in the former does not significantly impact the effect of changing Trp+9 to Phe. However, unlike the results with sph$\beta 31_{W+9\rightarrow F}$, in the presence of the native α-polypeptide, a native-like LH1-type

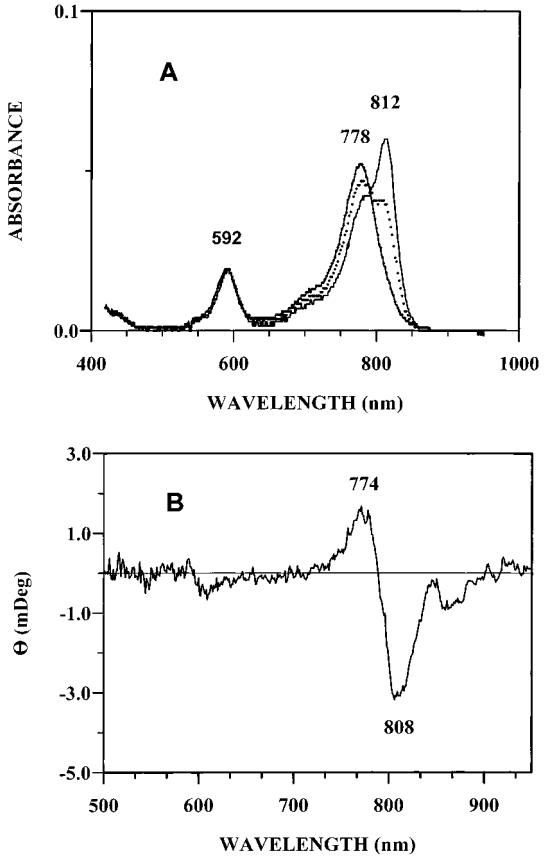

FIGURE 6: (A) Absorption spectra of a reconstitution using sphβ31$_{W+9\rightarrow F}$, the native α-polypeptide of *Rb. sphaeroides*, and Bchl taken at 0.90% (solid curve with an absorption peak at 778 nm), 0.75% (dotted curve), and 0.66% OG stored at 4 °C overnight (solid curve with a λ_{Max} of 812 nm). Concentrations were as follows: α, 1.9 μM; sphβ31$_{W+9\rightarrow F}$, 3.2 μM; and Bchl, 1.2 μM at 0.66% OG. Spectra were recorded in 1 cm cuvettes and multiplied by an appropriate dilution factor to normalize the spectra to a constant BChl concentration. (B) CD spectrum of the subunit-type complex at 0.75% OG at room temperature formed in a reconstitution using sphβ31$_{W+9\rightarrow F}$ and Bchl. Concentrations were as follows: sphβ31$_{W+9\rightarrow F}$, 14 μM; and Bchl, 3.0 μM. Spectra were recorded in 2 cm cuvettes. Four spectra were averaged to improve the signal-to-noise ratio.

complex was formed with a blue-shifted λ_{Max} at 860 nm (Figure 7), again underscoring the importance of the N-terminal region for stabilizing LH1.

DISCUSSION

The chemical syntheses of selected polypeptides, along with the reconstitution methodology, allow a comprehensive evaluation of structure−function relationships. The knowledge obtained by reconstitution is complementary to that obtained from the characterization of in vivo mutant LH1 complexes and can provide information, such as binding energies, not available by any other method. Although other factors, such as post-translational modification, incorporation of polypeptides into the membrane, or rates of degradation, may effect LH1 formation in vivo, the fact that native-like LH1 complexes can be formed by reconstitution using chemically well-defined components allows one to address fundamental structure−function relationships unencumbered by other events occurring in vivo.

Minimal Requirements for LH1 Formation in Rb. sphaeroides. All the interactions required for subunit formation are

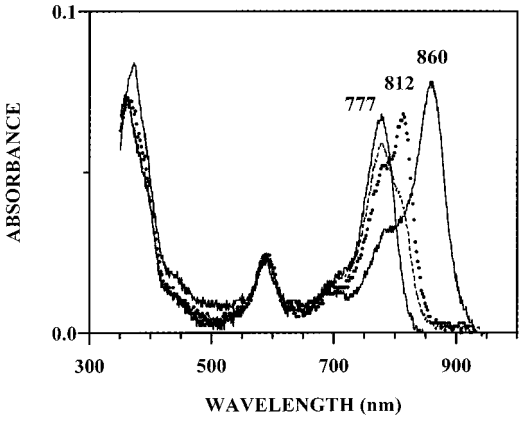

FIGURE 7: Absorption spectra of a reconstitution using sphβCP2, the native α-polypeptide, and Bchl. Spectra were taken at 0.90% (solid curve with an absorption peak at 777 nm), 0.75% (dashed curve), 0.66% (dotted curve), and 0.66% OG stored at 4 °C overnight (solid curve with a λ_{Max} of 860 nm). Concentrations were as follows: α, 1.7 μM; sphβCP2, 2.2 μM; and Bchl, 1.5 μM at 0.66% OG. Spectra were recorded in 1 cm cuvettes and multiplied by an appropriate dilution factor to normalize the spectra to a constant BChl concentration.

also important for the formation of LH1 since this latter complex appears to arise from a simple association of the subunit complex (*2*, *4*, *6*, *22*−*24*). However, there are additional interactions in the N-terminal region of the α- and β-polypeptides that have been implicated as being important for LH1 formation (*4*, *5*, *25*, *26*). Experiments reported here underscore the importance of the N-terminal regions.

In the case of the *Rb. sphaeroides* polypeptides, it is interesting that removal of 11 amino acids from the N terminus of the native β-polypeptide results in a polypeptide (sphβ37) that not only readily forms a subunit-type complex with Bchl in the absence of an α-polypeptide but also oligomerizes to form a LH1-type complex in the absence of an α-polypeptide. In fact, the presence of an α-polypeptide seems to make little difference in the K_{Assoc} of the subunit-type complex or the location of the blue-shifted λ_{Max} (847 nm) of the LH1-type complex. This indicates that self-oligomerization of $\beta\beta$ subunit-type complexes is stronger than oligomerization of $\alpha\beta$ subunit-type complexes. However, in the case of the polypeptide analogue containing four additional amino acids on the N terminus (sphβ41), the behavior changes markedly, approaching that of the native β-polypeptide. Thus, although formation of the $\beta\beta$ subunit-type complex appears essentially unchanged according to its λ_{Max} and the K_{Assoc} for subunit formation, this species does not oligomerize to form a stable LH1-type complex. Moreover, in the presence of a native α-polypeptide, a stable LH1-type complex is formed which appears to be due to oligomerization of $\alpha\beta$ subunit-type complexes as in native LH1. This conclusion is based on the observations that the λ_{Max} is shifted to longer wavelengths [closer to that of the native system (Figure 3A)] and the CD spectrum is similar to that of native LH1 (Figure 3B). These results could be explained by considering that the additional length of sphβ41 simply gets in the way of oligomerization of $\beta\beta$ subunit-type complexes but does not inhibit oligomerization of $\alpha\beta$ subunit-type complexes. This explanation will be further discussed below after introducing two possible structures for the subunit complex.

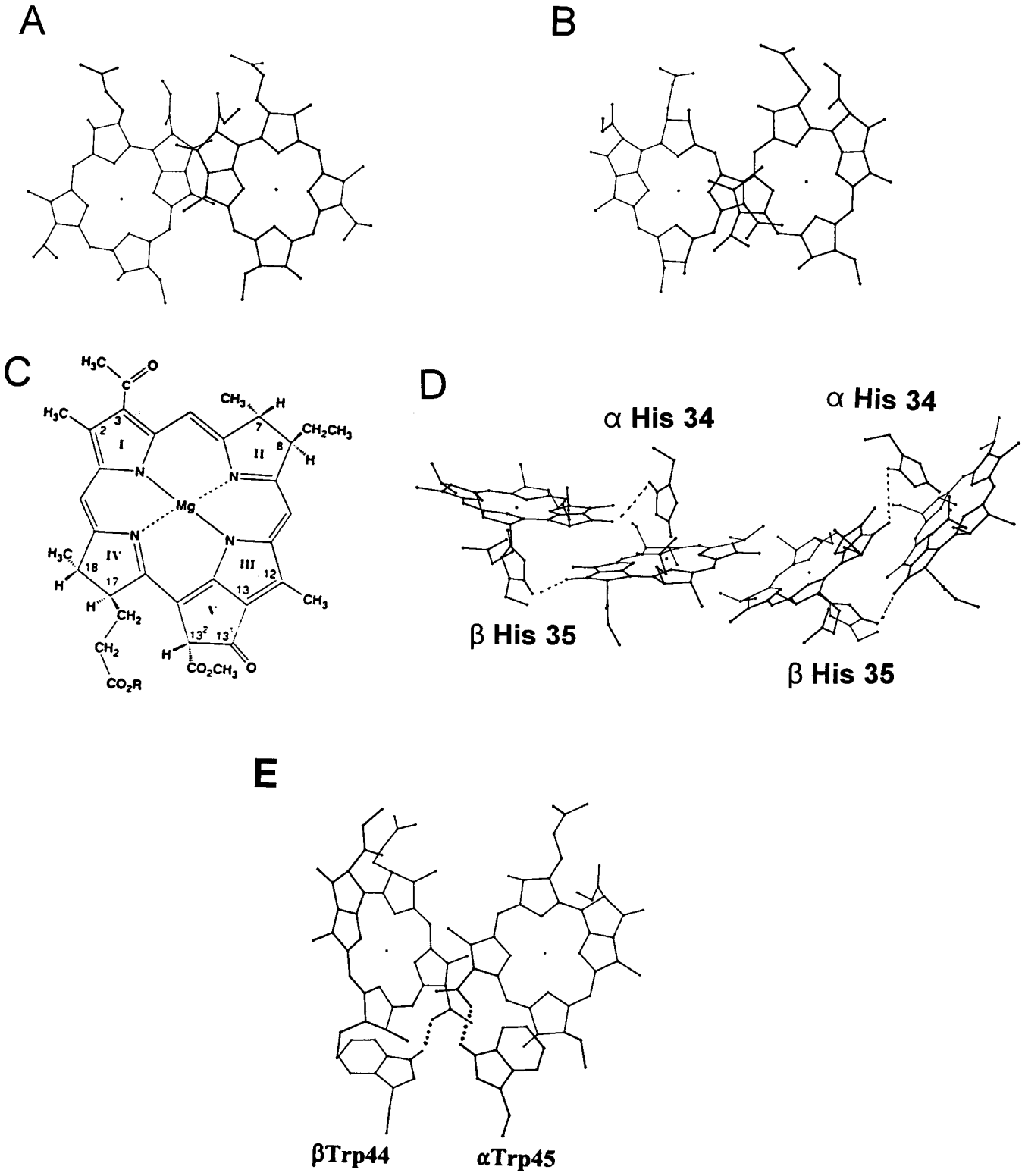

FIGURE 8: Pair of Bchl overlapping at rings III/V (A) or rings I (B) using the coordinates for the LH2 crystal structure of *Rs. molischianum* reported by Koepke et al. (*15*). (C) Structure of Bchl. (D) Four Bchl of *Rs. molischianum* B850 ring showing coordinated imidazole groups from His0 of each polypeptide. (E) Pair of Bchl overlapping at rings I showing the hydrogen bonding interaction from the $C3^1$ carbonyl group of $Bchl_\beta$ to βTrp+9 (βTrp44 when numbered from the N terminus) and from the $C3^1$ carbonyl group of $Bchl_\alpha$ to αTrp+11 (αTrp45 when numbered from the N terminus).

In addition to causing interference with oligomerization of the $\beta\beta$ subunit-type complexes, the four additional amino acids and the adjacent N-terminal region of the α-polypeptide may also be involved in some specific attractive interactions. Numerous protein−protein interactions are found between the N-terminal region of the α- and β-polypeptides of LH2 of *Rhodospirillum molischianum* (*15*). Since there is an additional Bchl molecule in LH2 (B800) that is not present in LH1, there should be an even greater interaction of these regions of the protein in LH1.

Minimal Requirements for LH1 Formation in Rs. rubrum. As with *Rb. sphaeroides*, the N-terminal regions of the *Rs.*

rubrum α- and *β*-polypeptides contribute to the stabilization of LH1 (*5*). However, there appears to be an additional interaction in the C-terminal region of the α- and *β*-polypeptides of *Rs. rubrum* since rr*β*31 does not seem to interact with the native α-polypeptide of *Rs. rubrum* to form a LH1-type complex, whereas the protease-truncated EFHK to C terminus polypeptide does (*1*, *5*). In this paper, it was shown that synthesis of a polypeptide, rr*β*35, containing four additional amino acids on the C terminus compared with rr*β*31 was not sufficient to recover the ability to form LH1-type complexes (Figure 4). This focuses attention on the three remaining amino acids at the C terminus of the native *Rs. rubrum β*-polypeptide.

Although the structures of LH2 (*14*, *15*) are very useful for anticipating the site of Bchl binding in LH1, the *β*-polypeptide of *Rs. rubrum* LH1 is seven amino acids longer than that of the LH2 *β*-polypeptides of *Rhodopseudomonas acidophila* or *Rs. molischianum*. Therefore, one can only use the LH2 crystal structures to tentatively predict the *β*-polypeptide conformation up to Val+10. The additional sequence of the *Rs. rubrum β*-polypeptide of LH1 is PGPNGYS. This sequence strongly suggests a turn motif. It might therefore be proposed that because the *β*-polypeptide in the *Rs. molischianum* LH2 structure approaches the C-terminal α-helical region of the α-polypeptide at about residue +10 of the *β*-polypeptide (*15*), the additional length of the *β*-polypeptide of *Rs. rubrum* LH1 may allow an interaction between the last three amino acids of this polypeptide and the C-terminal region of the *Rs. rubrum* α-polypeptide. The examination of native *Rs. rubrum β*-polypeptides shortened by one and two amino acids at the C-terminal end should help further pinpoint the proposed interaction.

Side Chain Interactions Stabilizing Subunit Formation. On the basis of reconstitution experiments with heterologous polypeptides (*4*) and site specific mutants (*12*) and resonance Raman measurements on membrane preparations of native (*27*) and mutant systems (*8*), a number of amino acids have been implicated in stabilizing the subunit complex. (1) His0 of the α- and *β*-polypeptides provides the coordinated ligands to Bchl (*7*, *10*, *27*). This is consistent with the crystal structure of LH2 where the corresponding histidines provide ligands to the B850 Bchl (*14*, *15*). The energy of stabilization contributed by this interaction (determined by measurements of K_{Assoc} and calculation of $\Delta\Delta G$) is placed at about 6 kcal/mol for each Bchl in the subunit complex (*12*). (2) sph*β*Tyr$_{+4}$ provides a little over 1 kcal/mol of stabilization to subunit formation (*12*). (3) αTrp+11 is involved in hydrogen bonding to the C3 acetyl carbonyl of Bchl (see Figure 8C for the structure and numbering of Bchl) (*8*). This latter interaction is also observed in the crystal structure of LH2 prepared from *Rs. molischianum* (*15*).

From the experimental results reported here, three amino acids can be added to this list; sph*β*Trp$_{+6}$ and sph*β*Arg$_{+7}$ provide about 1.4 and 2.0 kcal/mol, respectively, to the stabilization energy for subunit formation, and sph*β*Trp$_{+9}$ is of greater significance in contributing about 3.7 kcal/mol to subunit stabilization. This latter result is again consistent with observations from the crystal structure of LH2 of *Rs. molischianum* which shows that in LH2 *β*Trp+9 is involved in hydrogen bonding to the C3 acetyl carbonyl group of Bchl (*15*) and the recent resonance Raman study of LH1 in membranes of *Rb. sphaeroides* and of a mutant lacking *β*Trp+9 (*13*).

Structure of the Subunit Complex of LH1. Although it is not yet possible to fully define the structure of the subunit complex of LH1, it is useful to place the experimental results in the framework of a structural model. There are many compelling reasons to begin modeling the Bchl-binding region of LH1 by assuming that the conformations of the backbones of the α- and *β*-polypeptides in LH1 of *Rb. sphaeroides* and *Rs. rubrum* will be the same as those in LH2 of *Rs. molischianum* in the C-terminal region. (1) There are significant amino acid sequence identities between the α- and *β*-polypeptides of LH2 of *Rs. molischianum* and the α- and *β*-polypeptides of LH1 of *Rs. rubrum* (24 and 47%, respectively). When highly conserved differences are considered, the percent homology approaches 40% for the α-polypeptides and 60% for the *β*-polypeptides. Of even greater significance is the Bchl binding region of the *β*-polypeptide of *Rs. rubrum* whose amino acid sequence from residue −4 to +10 has 10 out of 15 amino acids that are identical with that region of the *β*-polypeptide of *Rs. molischianum*, and the other five could be considered highly conserved. (2) The only two hydrogen bonds from the protein to the B850 Bchl in LH2 of *Rs. molischianum* are also found in LH1 (*8*, *13*). These are provided by Trp+11 of the α-polypeptide and Trp+9 of the *β*-polypeptide, and both hydrogen bonds involve the carbonyl group of the acetyl side chain of Bchl at position C3. (3) As in LH2, His0 of the α- and *β*-polypeptides provides the ligands to the Mg atom of Bchl (*5*, *7*, *10*, *27*). (4) The spectral properties of the exciton pair of Bchl in the subunit complex predict a structure very similar to that observed for the two B850 Bchl in LH2 (*28*−*31*).

Some significant differences exist in the structure of the LH1 complex compared with that of the LH2 complex. One difference is that the absence of the B800 Bchl in LH1 should result in structural differences in the N-terminal region. Another difference is that a stable subunit complex can be isolated from LH1, but so far has not been isolated from LH2 (*31*, *32*). This difference in behavior indicates that there are some special stabilizing interactions between Bchl and the protein in LH1 complexes which are not exhibited by the polypeptides of the LH2 complex. One further point is that the LH1 complex can readily be reconstituted without carotenoid, and many carotenoid-less mutants exist that contain LH1 complexes. On the other hand, the only reported success in reconstituting LH2 complexes required carotenoid (*32*), and there are as yet no clear examples of carotenoid-less mutants where LH2 is expressed. Therefore, in our current working model of the subunit complex of LH1, special consideration is given to additional forces that may be involved in holding the subunit complex together.

As summarized above, strong evidence exists for two major interactions between the protein and Bchl in the subunit complex, as well as in LH1. These are coordination of αHis0 and *β*His0 to Bchl (to identify each Bchl, they are referred to as Bchl$_{\alpha}$ and Bchl$_{\beta}$, respectively) and the hydrogen bonding interaction of *β*Trp+9 and αTrp+11 to the C3^1 carbonyl of Bchl. A further possible interaction which is suggested from the LH2 crystal structure (*15*) would be that involving hydrogen bonding between N1 of the imidazole ring of αHis0 and of *β*His0 and the carbonyl group at C13^1

of Bchl$_\beta$ and Bchl$_\alpha$, respectively. However, the distance between the hydrogen of His and the C13^1 oxygen of Bchl is between 3.3 and 3.8 Å, and the N−H−O bond angle approaches 90° (see Figure 8D) so that the interaction is, at best, very weak. In the two structures discussed below, these potential interactions (αHis0 and βHis0 coordination to Bchl, hydrogen bonding from βTrp+9 and αTrp+11 to Bchl, and hydrogen bonding involving αHis0 and βHis0) will be considered as the major stabilizing interactions holding the subunit complex together.

In the crystal structures of LH2, two different pairs of $\alpha_1\beta_1$·2Bchl units of B850 can be chosen as a reference point (*14*, *15*). In one, the two Bchl overlap at the adjoining rings (III/V, Figure 8A), and in the other, they overlap near the C3 side chains of ring I (Figure 8B; see Figure 8C for the numbering system used for Bchl). The first will be referred to as a C13−C13 pair and the second as a C3−C3 pair. Two possible structures for the subunit complex are considered below.

Structure Suggestion 1. In this structure, the subunit complex of LH1 would contain one α-polypeptide, one β-polypeptide, and two Bchl arranged as a C3−C3 pair. There are two major reasons why this pair is considered to be the most likely orientation for the subunit complex. First, this pair is chosen instead of a C13−C13 pair because in the latter case, if both polypeptides were a β-polypeptide where one of them played a role equivalent to that of an α-polypeptide, their N-terminal regions would collide. This is because the N terminus runs directly toward the other polypeptide in a C13−C13 pair (this may be seen by viewing the N terminus of the two polypeptides of *Rps. acidophila* LH2 shown in panels A and B of Figure 9 which have the relationship of a C13−C13 pair). On the other hand, if the $\beta\beta$ subunit-type structure were that of a C3−C3 pair based on *Rps. acidophila* LH2 (panels C and D of Figure 9), these N-terminal regions would not experience such steric restrictions and thus they would have little effect on subunit formation, as we observe. Such C3−C3 subunit complexes would then not be able to undergo further oligomerization to form $\beta\beta$ LH1-type structures because this would again involve formation of C13−C13 pairs. This is again consistent with experimental results (*4*). On the other hand, $\alpha\beta$ subunit-type complexes would not have these constraints in oligomerization because the α-polypeptide N terminus and the β-polypeptide N terminus have presumably evolved to be juxtaposed so that one can lay on top of the other (Figure 9A) and thus provide additional stabilizing interactions without steric interference. The results obtained with sphβ31, sphβ37, and sphβ41 are thus readily explained by this suggested subunit structure. sphβ31 and sphβ37 would have an α-helical secondary structure for their entire length from Trp+6 to the N terminus, and therefore, the amino acid side chains in this region would not be sufficiently close to interact with each other. On the other hand, for sphβ41, if the additional polypeptide at the N terminus has turned to run parallel to the membrane, as it does in the LH2 β-polypeptide, the four additional amino acids would form an extended chain with Tyr−30 approaching the other polypeptide to within about 4.8 Å (Cα−Cα; Asn-20 in the α-polypeptide of LH2 of *Rps. acidophila* and *Rs. molischianum*). For the case when only sphβ41 polypeptides are present, the N termini would experience serious steric conflict as a C13−C13 pair in the LH2 conformation. However, these N-terminal regions would be well-separated in a C3−C3 pair in the LH2 conformation. If these proposed C3−C3 subunit complexes were to attempt to oligomerize to form a LH1-type complex, the N-terminal regions of adjacent $\beta\beta$ subunits would conflict with each other, thus preventing the formation of higher states of association.

The second reason for choosing a C3−C3 pair is that, by a slight structural shift of LH2 coordinates, the two well-established hydrogen bonding interactions involving Bchl with βTrp+9 and αTrp+11 can be utilized to stabilize the subunit complex. It should first be noted that, for a C3−C3 pair in the LH2 structure of *Rs. molischianum*, βTrp+9 is hydrogen bonded to the Bchl$_\beta$ at its C3 side chain carbonyl and αTrp+11 is hydrogen bonded to Bchl$_\alpha$ at its C3 side chain carbonyl (Figure 8E). In the proposed model of the subunit complex, we suggest switching these two hydrogen bonds so that αTrp+11 hydrogen bonds to Bchl$_\beta$ (this is actually the same interaction as seen for αTyr+13 in the LH2 structure of *Rps. acidophila* if a C3−C3 pair is selected in that crystal structure) and βTrp+9 is proposed to be hydrogen bonding to Bchl$_\alpha$. Because of the symmetry in the structure, only a small adjustment in the rotation of the side chains of βTrp+9 and αTrp+11, together with a small movement in the α-helical segment to which they are attached, may be required to allow formation of these hydrogen bonds. Whereas such small adjustments may be energetically unfavorable in LH2 or LH1 because of restrictions imposed by the neighboring subunits, the isolated LH1 subunit is micellated by small flexible detergent molecules which would permit small conformational changes. These two interactions could then result in substantial "cross-linking" of the two polypeptides and their Bchl molecules, thus stabilizing the subunit complex. Two important experimental results reported here are explained by this model. (1) When βTrp+9 is removed, or changed to Phe, a blue shift in the λ_{Max} of the subunit-type complex occurs (Figures 6 and 7), and (2) a substantial decrease in K_{Assoc} is measured (Table 1) due to the loss of this stabilizing interaction. The formation of a $\beta\beta$ subunit-type complex is also explained since βTrp+9 of each of two β-polypeptides could cross hydrogen bond in the same manner as discussed for the $\alpha\beta$ subunit-type complex, resulting in a symmetrical structure for the subunit complex and one highly similar to the native $\alpha\beta$ subunit complex.

Suggested Structure 2. A second type of cross-hydrogen bonding interaction could be considered for stabilization of the subunit complex according to the results with the LH2 crystal structures. This interaction involves N1 of the imidazole ring of αHis0 and βHis0 and the C13^1 carbonyl group of Bchl$_\beta$ and Bchl$_\alpha$, respectively. Although these interactions are presumably weak in the LH2 crystal where the "bond" distances are long (3.5−3.8 Å) and the bond angles are considerably less than ideal (see Figure 8D), the isolated subunit in detergent solution might be sufficiently free of external restrictions to support more ideal distances and angles. A structural requirement for invoking these stabilizing interactions in the subunit structure is that they would require a C13−C13 pair because, in the C3−C3 pair, the imidazole side chains are on the wrong side of the Bchl macrocycle to allow a hydrogen bond to the adjacent Bchl (Figure 8D) (*14*, *15*). For this suggested structure, βTrp+9

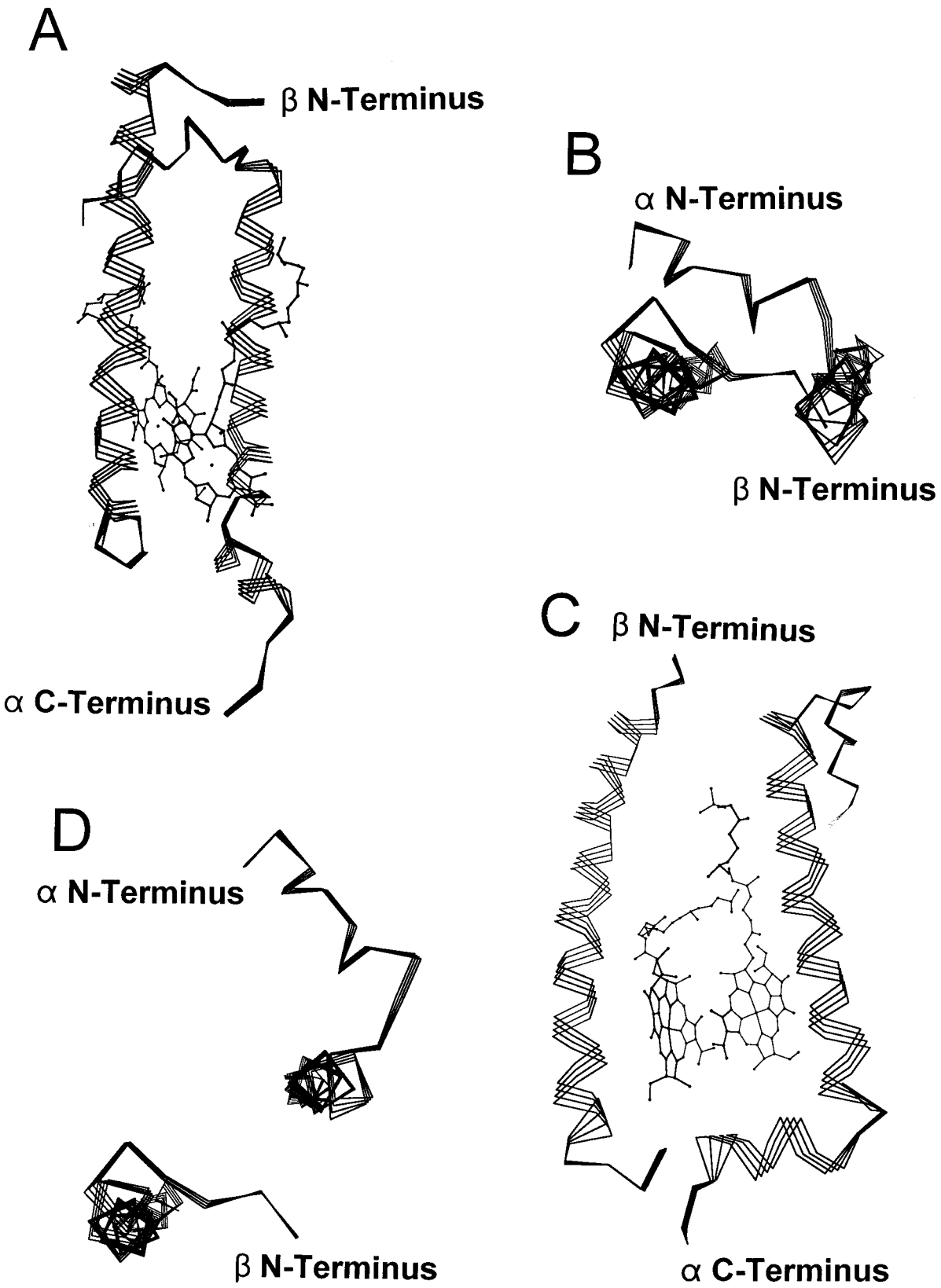

FIGURE 9: (A) Side view of an $\alpha_1\beta_1$·2Bchl unit of the LH2 structure of *Rps. acidophila* with Bchl overlapping at rings III/V (a C13−C13 pair) with the B800 Bchl, carotenoid, and detergent molecules omitted for clarity. (B) View of the structure in panel A from the N terminus with the C-terminal portion of the polypeptides and Bchl omitted for clarity. (C) Side view of an $\alpha_1\beta_1$·2Bchl unit of the LH2 structure of *Rps. acidophila* with Bchl overlapping at rings I (a C3−C3 pair) with the B800 Bchl, carotenoid, and detergent molecules omitted for clarity. (D) View of the structure in panel C from the N terminus with the C-terminal portion of the polypeptides and Bchl omitted for clarity. The program MOLW was used to project these structures.

and αTrp+11 would be involved in self-hydrogen bonding as in the LH2 structure of *Rs. molischianum* (Figure 8E). To accommodate this subunit structure, a different backbone conformation would be required for the N-terminal region

of the β-polypeptide than is observed for LH2 because, as discussed above, steric conflict at the N termini would not allow the $\beta\beta$ subunit-type complex to form. Furthermore, an explanation of why LH1-type complexes cannot be formed with only the β-polypeptides of either *Rs. rubrum*, *Rb. sphaeroides*, or *Rhodobacter capsulatus* is not apparent.

Relationship of the Subunit Complex and LH1. Finally, if one assumes that the subunit complex consists of a C3−C3 pair as per suggested structure 1 or a C13−C13 pair as per suggested structure 2, what additional interactions, or changes in interactions, might occur upon oligomerization to form LH1? It is possible that the cross-hydrogen bonding suggested for βTrp+9 and αTrp+11 in suggested structure 1 may change upon oligomerizing to form a structure that mimics the hydrogen bonding pattern of *Rs. molischianum* LH2. Evidence exists that an initial rapid dimerization of the subunit occurs as the first associative step, followed by a slower "annealing" period (*2*, *4*, *22*). The small changes in structure that would be required for shifting from βTrp+9 and αTrp+11 "cross-hydrogen bonding" to "self-hydrogen bonding" might explain the slower annealing phase for LH1 formation. Alternatively, if the subunit structure is that of suggested structure 2, then oligomerization may involve a weakening of each of the hydrogen bonds involving His0 and a change in the conformation of the N terminus.

As discussed earlier, important attractive interactions also occur between the N-terminal region of the α- and β-polypeptides of adjacent subunits. The importance of such interactions was first indicated by the study of site-selected mutants of LH1 in *Rb. capsulatus* (*25*, *26*). In the case of *Rs. rubrum* LH1, an important interaction also seems to exist involving the C-terminal end of the β-polypeptide.

Conclusion. The combination of reconstitution methodology and chemical synthesis of specific polypeptide analogues has facilitated better definition of interactions that stabilize LH1 and its subunit complex. By extension of these studies, it should be possible to determine which of the two suggested structures, if either, correctly describes the subunit complex and whether any significant structural changes occur upon oligomerization. In addition to the identification of all interactions important for complex formation, it is also possible to determine the magnitude of their contribution to the overall energy of stabilization. Such measurements are important not only for understanding Bchl binding in photosynthesis but also in evaluating forces that stabilize membrane proteins and their cofactors in general.

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