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Covalent Modification of Lysines of the B880 Light-Harvesting Protein of *Rhodospirillum rubrum*†

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ABSTRACT: Dansyl chloride [5-(dimethylamino)-1-napthalenesulfonyl chloride] was used to modify amino acids of proteins covalently in intact, active chromatophores of *Rhodospirillum rubrum* S1. A significant degree of amino acid modification (detected by dansyl fluorescence from proteins) was achieved without significant disruption of the far-red shift of the bacteriochlorophyll absorption spectrum. However, electron transport activity in the chromatophores was severely reduced, to 5–10% of original activity. The α subunit, the organic solvent soluble subunit, of the B880 light-harvesting bacteriochlorophyll protein (which has the composition $\alpha_2\beta_2$) was isolated. It was determined that the single lysine (residue 47 of 52) near the carboxy terminus of the subunit was the only covalently modified residue in that protein chain. A large percentage (up to 70%) of this light-harvesting protein subunit could be modified without disrupting the bacteriochlorophyll binding sites in the $\alpha_2\beta_2$ complex.

The light reactions of bacterial photosynthesis originate in bacteriochlorophyll–protein complexes that are embedded in the cell membrane (Okamura et al., 1982; Feher & Okamura, 1978). These Bchl–protein[1] complexes can be divided into two classes: photoreaction centers and light-harvesting (or antenna) complexes. The main function of the light-harvesting complexes, which bind the bulk of the bacteriochlorophyll, is to capture light energy and to transfer the energy to the reaction centers where photochemistry can occur (Drews, 1985).

The sequences of many Bchl binding proteins from photosynthetic bacteria have been determined over the past several years, and the first crystal structure of an integral Bchl–protein complex, the *Rhodopseudomonas viridis* reaction center, has recently been determined (Deisenhofer et al., 1984). The acquisition of the detailed crystal structure for the *Rps. viridis* reaction center complex will allow for the analysis of the role of particular portions of the polypeptide chains in the various binding sites of the protein complex (Deisenhofer et al., 1984). No crystal structure for a membrane-bound, light-harvesting protein is yet available, although the crystal structure of a water-soluble light-harvesting protein from the green bacterium *Chloropseudomonas ethylica* has been reported (Fenna & Matthews, 1977). Therefore, until crystal structures are available for other Bchl binding membrane proteins, the best evidence for the structure of these proteins in the membrane is still provided by calculating the hydropathy profile for the protein with the amino acid sequence and by probing the surfaces of the proteins with chemical labels and proteases.

The bacterium *Rhodospirillum rubrum* contains a single core light-harvesting protein complex (B880) with an absorption peak about 880 nm in wild-type S1 and about 870 nm in the carotenoidless mutant G-9 (Picorel et al., 1983). This complex from *Rs. rubrum* has been characterized by Picorel et al. (1983) and shown to contain two different polypeptides, α and β, of apparent M_r 7600 and 6400 on SDS–polyacrylamide gels. The minimal oligomer for the B880 complex was found to be $\alpha_2\beta_2$. Bchl and the carotenoid spirilloxanthin were present in a 2:1 mole ratio in the complex from the wild-type strain. Both the α and β polypeptides of the complex have been sequenced (Brunisholz et al., 1981; Gogel et al., 1983; Brunisholz et al., 1984a).

With sequences of the light-harvesting proteins available, it is now possible to conduct structure–function studies to determine the position and roles of specific amino acid side chains in the Bchl binding sites of the proteins. In both the α and β polypeptides of the *Rs. rubrum* B880 complex, a conserved histidine, which has been suggested to serve as a ligand for Bchl (Brunisholz et al., 1981; Theiler & Zuber, 1984), is found near the carboxy-terminal end of a stretch of hydrophobic amino acids in the sequence. Other amino acids in the protein may serve as hydrogen bond donors to Bchl or may form part of salt bridges in the complex. In the α subunit, there is a single lysine residue in the carboxy-terminal portion of the sequence. In order to determine if the ϵ-amino group of this lysine serves some function in Bchl binding, we used dansyl chloride to react with the proteins while the proteins were still membrane bound in chromatophores. Dansyl chloride was chosen because it is a moderately specific reagent that reacts readily with amino groups and because the fluorescent modification products can be identified by chromatography (Woods & Wang, 1967; Hartley, 1970). After covalent modification of the chromatophore membranes with dansyl chloride, the integrity of the binding sites for Bchl in the proteins was monitored with visible absorption spectros-

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[1] Abbreviations: Bchl, bacteriochlorophyll; CM, carboxymethyl; dansyl chloride, 5-(dimethylamino)-1-naphthalenesulfonyl chloride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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copy. The α subunit of the B880 light-harvesting complex was then isolated in order to investigate the extent of its modification. Of all the amino acids, only lysine was modified in the α subunit. In addition, the single lysine in the α subunit of the light-harvesting protein could be modified extensively by dansyl chloride (up to approximately 0.7 mol of bound dansyl per mol of lysine) without significant disruption of bacteriochlorophyll binding by the protein.

Materials and Methods

Materials. Chromatophores were prepared from *Rs. rubrum* strain S1 as described previously (Tonn et al., 1977). Dansyl chloride was obtained from Sigma Chemical Co. (St. Louis, MO). Dansyl amino acid standards and polyamide sheets were obtained from Pierce Chemical Co. (Rockford IL). All other reagents were of analytical grade.

Reaction with Dansyl Chloride. Various reaction conditions were tested in order to vary the degree of modification of the membrane proteins. In a typical mild reaction, chromatophores were suspended in 100 mM $NaHCO_3$, pH 9.5, at a concentration such that A_{880} was 15. Dansyl chloride was dissolved in freshly distilled, dry dimethylformamide and added to the chromatophore suspension in several aliquots down the center of a vortex. The reactions were carried out at room temperature (about 25 °C) in a darkened room. The final dansyl chloride concentration was 5 mM. The final concentration of dimethylformamide in the samples was 1–2%. Samples were stirred for 1 h at room temperature in dim light.

The reaction was stopped by chilling the samples on ice and then spinning the samples at low speed in a table top centrifuge to remove precipitated dansyl chloride. Following this, the solution was passed through a Sephadex G-50 column (water as solvent), and the chromatophores were pelleted by ultracentrifugation. The chromatophores were resuspended in water and repelleted as a wash step. The wash step was repeated several times. Finally, chromatophores were resuspended and dialyzed extensively against distilled water at 4 °C in the dark. Control chromatophores were treated identically; however, only dimethylformamide was added instead of the solution of dansyl chloride.

In other experiments the chromatophores were treated identically; however, the A_{880} of the chromatophore suspension, or the final concentration of dansyl chloride in the reaction, was varied.

Absorption Spectroscopy. The absorption spectra of chromatophores and purified proteins were measured on a Cary 14 spectrophotometer, a Beckman Acta IV spectrophotometer, and/or a Cary 219 spectrophotometer.

Fluorescence Spectroscopy. The fluorescence spectra of chromatophores and purified proteins were taken with a Perkin-Elmer MPF 44-A fluorometer. The excitation wavelength for fluorescence spectroscopy of dansylated samples was 340 nm.

Assays. Electron transport activity in the chromatophores was assayed by the method of Hochman et al. (1977). This assay measured the light-induced, noncyclic electron transport from external lipophilic donors to O_2.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970) on slab gels 1.5 mm thick. The separation gel contained a gradient of 7–17% polyacrylamide. For some separations, 4 M urea was included in the gels, and samples were delipidated with 80% acetone (cold) before incubation with SDS. The samples were incubated in sample preparation buffer containing 0.068 M Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol for 3 min in a boiling water bath. Electrophoresis was carried out in a cooled tank (4 °C) for 3–4 h at 40–50 mA. Staining with Coomassie Brilliant Blue G-250 and destaining were performed as described by Fairbanks et al. (1971). Molecular weight markers were purchased from Sigma Chemical Co.

Isolation of the α Subunit of the B880 Light-Harvesting Complex. (*A*) *Gel Filtration Chromatography.* Chromatophores that had been extensively dialyzed against distilled water were freeze-dried and extracted extensively with 1:1 chloroform/methanol (v/v) (eight extractions, 2 mL of solvent/50 mg of membrane material). The extracts were pooled and concentrated by evaporation with N_2. Gel filtration chromatography was performed on Sephadex LH-60 in 1:1 chloroform/methanol (v/v) with 0.1 M ammonium acetate as previously described (Gogel et al., 1983). Column fractions were monitored with A_{280}; pooled fractions were used for SDS–polyacrylamide gel electrophoresis and for further chromatography.

(*B*) *Ion-Exchange Chromatography.* A second chromatographic step of ion-exchange chromatography (Theiler et al., 1983) was used after the gel filtration chromatography in order to ensure a complete separation of the α and β subunits of the B880 light-harvesting complex. (Carboxymethyl)cellulose was prepared as described by Theiler et al. (1983). Pooled samples from Sephadex LH-60 gel filtration chromatography were applied to a (carboxymethyl)cellulose column in 5:1 chloroform/methanol (v/v) with 33 mM ammonium acetate, and the column was washed with the same solvent. Proteins were eluted from the column in two steps: one of 1:1 chloroform/methanol, 0.1 M ammonium acetate, and 5% acetic acid; the second of 1:1 chloroform/methanol, 0.1 M ammonium acetate, and 20% acetic acid. Column fractions were monitored for protein with A_{280}. Amino acid analysis of the column fractions was used to confirm the identity of the α subunit of the light-harvesting protein.

Identification of Dansyl Amino Acids. Purified protein was dialyzed extensively against water to remove salts, and then it was freeze-dried. Dried protein was hydrolyzed with constant boiling HCl (Pierce Chemical Co.) in sealed vials under a N_2 atmosphere at 110 °C for 24 h. Subsequently, the hydrolyzed material was dried in vacuo. Dansyl amino acids were separated by two-dimensional chromatography on polyamide sheets according to the procedures described by Woods and Wang (1967) as modified by Hartley (1970). Dansyl amino acids were identified by comparison to dansyl amino acid standards.

Identification of Degree of Labeling. The isolated dansylated protein was used for absorbance measurements, and the A_{280}, A_{290}, and A_{335} were used to estimate the degree of labeling of the proteins, essentially as described by Kinoshita et al. (1974). The A_{280} and A_{290} were corrected for scattering by subtracting A_{450} and for the absorbance of the dansyl group at 280 nm and 290 nm as described by Kinoshita et al. (1974). A molar absorptivity of 3370 at 335 nm was used to calculate the concentration of the dansyl group. Solutions composed of weighed amounts of freeze-dried purified protein were used to estimate the molar absorptivity of the α subunit at 280 nm, assuming a molecular mass of 6100 for the polypeptide. An extinction coefficient of 13 mM^{-1} cm^{-1} at 280 nm was found. The extinction coefficient of 10.2 mM^{-1} cm^{-1} at 290 nm (Loach et al., 1985) was also used to estimate the molar concentration of the protein.

Results

Absorbance Spectra of Bchl in the Reacted Chromatophores. The effect of dansyl chloride modification of the

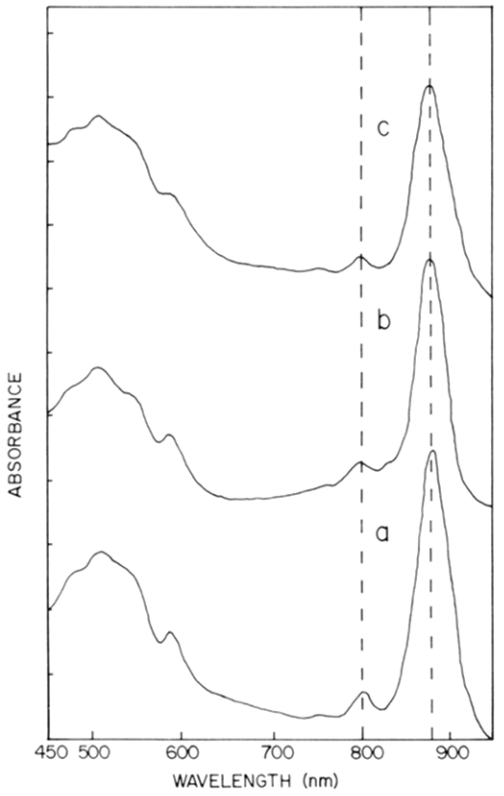

FIGURE 1: Absorbance spectra of *Rs. rubrum* chromatophores: curve a, control chromatophores (dimethylformamide, 2% of volume, when A_{880} = 15); curve b, chromatophores reacted with 5 mM dansyl chloride when A_{880} = 15 (dimethylformamide, 2% of volume); curve c, chromatophores reacted with 5 mM dansyl chloride when A_{880} = 7.5 (dimethylformamide, 2% of volume). The spectra have been corrected for scattering and have been height normalized at 800 nm in order to compare the relative heights of the 880-nm peaks. Vertical lines indicate the wavelengths of 880 and 800 nm.

membranes on the visible absorbance spectrum is illustrated by Figure 1. The addition of small quantities (1–2% by volume) of a membrane-disrupting organic solvent, such as dimethylformamide, to the control chromatophores did not have any discernible effect upon the red-shifted absorbance peaks of the bound Bchl (Figure 1a). Nor did chromatophores reacted with dansyl chloride under mild conditions (A_{880} = 15, 5 mM dansyl chloride, 1–2% dimethylformamide) show a change in the position of the absorbance peak (880 nm) for the bound Bchl (Figure 1b). Thus, the Bchl binding sites in the proteins were still intact. However, treatment with dansyl chloride at a lower concentration of chromatophores (A_{880} = 7.5), under the same conditions, led to increased destruction of the B880 complex (Figure 1c) as indicated by a decrease in absorbance in the far-red.

Many different reaction conditions were tested in order to determine whether conditions existed in which the membrane proteins would be modified extensively while the Bchl binding sites in those proteins would remain intact. Increasing the percentage of organic solvent in the reaction mixture increased the intensity of dansyl fluorescence seen on SDS–PAGE of a given quantity of chromatophores but also resulted in increased disruption of the Bchl binding site in proteins in the control chromatophores, as evidenced by a decrease seen in the absorbance at 880 nm and the appearance of a peak for free Bchl seen at 770 nm. The Bchl peak at 800 nm was much less affected by the increase in organic solvent concentration.

Electron Transport Abilities. Because dansyl chloride modification of the chromatophore membranes results in covalent modification of any available reactive groups on proteins, it was expected the dansyl modification would result in a significant decrease in electron transport activity, for many electron transport components in the membrane have been modified. Prolonged treatment of the control chromatophores through the various stages in the reaction process resulted in a loss of activity. Control chromatophores [activity 150 μmol of O_2 h^{-1} (mg of Bchl)$^{-1}$] evidenced approximately 50% of the activity of freshly prepared chromatophores [activity 320 μmol of O_2 h^{-1} (mg of Bchl)$^{-1}$]. The electron transport activity of the chromatophores was reduced even further, to 5–10% of the original activity, upon dansylation of the membranes. This reduction in activity occurred even when the absorption spectrum of the bound Bchl in the membranes remained unchanged (as in Figure 1b).

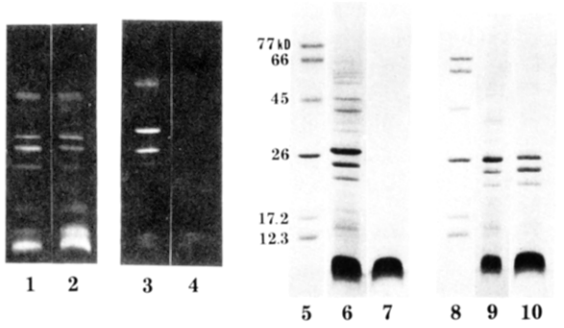

FIGURE 2: SDS gel electrophoresis of *Rs. rubrum* S1 chromatophores and chloroform/methanol, 1:1 (v/v), extracts from chromatophores on 7–17% polyacrylamide slab gels according to Laemmli (1970): lane 1, fluorescence from heavily dansylated chromatophores (unstained gel); lane 2, fluorescence from organic solvent extract of heavily dansylated chromatophores (unstained gel); lane 3, fluorescence from chromatophores dansylated to a lower degree than those in lane 1 (unstained gel); lane 4, fluorescence from an organic solvent extract of chromatophores dansylated to a lower degree than those in lane 1 (unstained gel); lane 5, protein standards, molecular masses indicated in kilodaltons (stained gel); lane 6, control chromatophores (stained gel); lane 7, organic solvent extract from control chromatophores (stained gel); lane 8, protein standards (stained gel); lane 9, dansylated chromatophores (stained gel); lane 10, organic solvent extract from dansylated chromatophores (stained gel).

Number of Proteins Modified. Modified chromatophores were subjected to SDS–PAGE in order to separate the proteins and identify which proteins had been modified. When electrophoresis was complete, several lanes of the slab gel were sliced from the whole gel and merely fixed, while duplicate lanes were fixed and stained. Fluorescent bands from covalently bound dansyl groups on proteins were detected in the gel lanes that were merely fixed (Figure 2). Gel lanes of control chromatophores showed no detectable fluorescence if treated in the same manner. A comparison of the fluorescent bands in unstained gel lanes with the stained bands in duplicate lanes (Figure 2) shows that most, if not all, of the proteins present were at least partially labeled with the dansyl group. Both subunits of the light-harvesting protein and all three subunits of the reaction center reacted with dansyl chloride. The chromatophores used in these gels had absorption spectra like that in Figure 1b.

Gel lanes (both unstained and stained) from a sample of the organic solvent [chloroform/methanol, 1:1 (v/v)] extract from dansylated chromatophores are also shown in Figure 2. A comparison of these lanes with the stained gel lane from a sample of the organic solvent extract from control chromatophores (Figure 2) shows that the β subunit of the light-harvesting protein and proteins of higher molecular weight, including the reaction center subunits, can be extracted from the dansylated membranes. The only protein extracted from the control membranes is the α subunit of the light-

harvesting protein (less than 10% contamination with β subunit). The alteration in the extraction properties of the labeled membranes indicates that the addition of a dansyl group(s) to the proteins has increased their solubility in organic solvents. This fact complicated the purification of the α subunit (normally the only protein extracted); however, two chromatographic steps were sufficient to achieve its purification.

Purification of the α Subunit of the B880 Light-Harvesting Complex. Two steps of chromatography—gel filtration and ion exchange—were used to purify the α subunit of the light-harvesting protein from both the control membranes and membranes that had been modified with dansyl chloride. SDS–PAGE of the purified protein indicated that this protein was the α subunit of the light-harvesting protein. Amino acid analysis was used to confirm the identity of the protein since the α and β subunits have very different ratios of several amino acids and can easily be distinguished by the ratios of the amino acids. The results of amino acid analysis indicated that the purified protein was over 90% pure α subunit.

Identification of Dansyl Amino Acids. The only dansyl amino acid found in purified α subunit was dansyl-ϵ-aminolysine (by comparison to Pierce Chemical Co. standards). In samples of lesser purity (used prior to ion-exchange chromatography), slight traces of dansyl-*O*-tyrosine from the β subunit (the α subunit contains no tyrosine) were also present.

Absorbance and Fluorescence Spectra of Purified Protein. Since the purified protein contains the covalently bound dansyl group, the visible absorbance spectrum for the protein (Figure 3A) shows the peak at 335 nm indicative of a dansyl group. This absorbance peak is missing in the visible absorbance spectrum of the control protein.

The purified protein also exhibited the fluorescence characteristics expected for a dansyl-labeled protein (Figure 3B). When the protein was excited at the A_{max} of the dansyl group, the emission spectrum characteristic of the dansyl group was obtained.

Determination of Degree of Protein Labeling. By use of the Loach et al. (1985) extinction coefficient and the corrections described by Kinoshita et al. (1974), the absorbance spectra of various purified samples of dansylated α subunit (in organic solvent) were used to estimate the degree of labeling of the proteins. As expected, the degree of labeling found varied with the reaction conditions and ranged from the maximum degree of labeling possible (1.0 mol of dansyl group/mol of α subunit) to a minimal degree of labeling (as little as 0.2 mol of dansyl group/mol of protein). A maximum degree of labeling found in samples with an absorbance spectrum like that in Figure 1b was approximately 0.7 mol of dansyl group/mol of protein. Using our extinction coefficient in the same calculations led to a slightly higher estimate (0.7–0.8 mol of dansyl group/mol of lysine). These ratios indicate that the majority of the lysines in the α subunits can be covalently labeled without disruption of the red-shifted Bchl binding site in the light-harvesting protein.

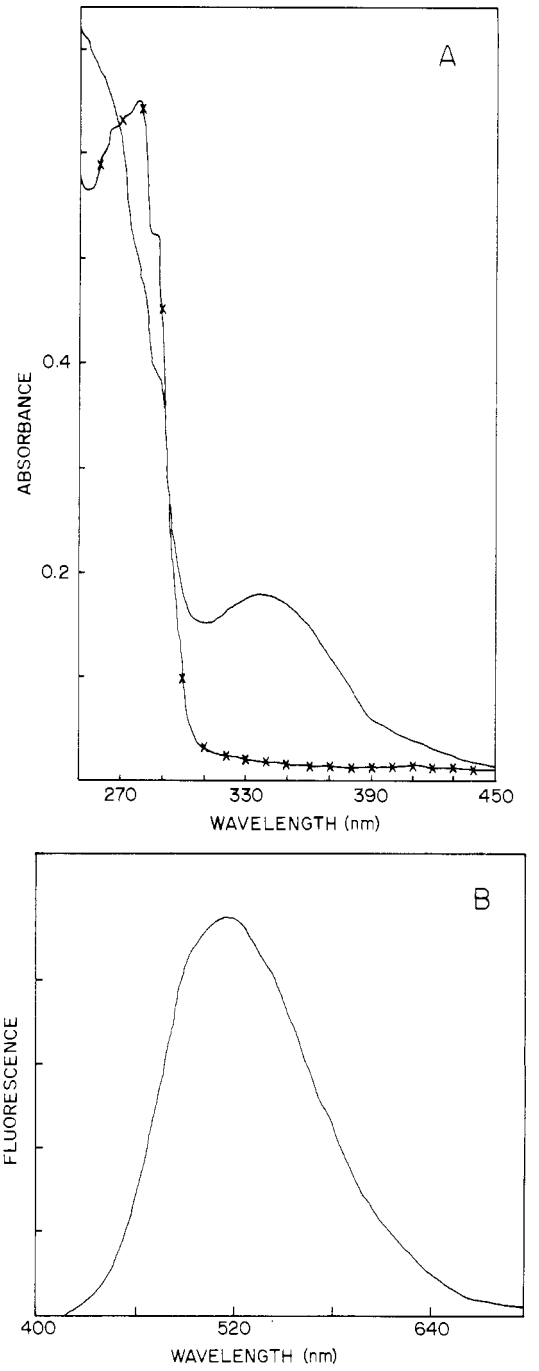

FIGURE 3: (A) Absorbance spectrum of isolated, dansylated α subunit of B880 light-harvesting protein of *Rs. rubrum* S1 (—) and of isolated control α subunit (–×–). (B) Fluorescence emission spectrum of isolated, dansylated α subunit of B880 light-harvesting protein of *Rs. rubrum* S1. The excitation wavelength was 340 nm.

Discussion

Dansyl chloride is a hydrophobic reagent that can partition into the membrane. It reacts primarily with amino, imino, phenolic hydroxyl, thiol, and imidazole groups (Gray, 1972). In the case of the α subunit of the B880 light-harvesting protein, the reagent reacts primarily with the ϵ-amino group of lysine for several reasons: the amino terminus of the protein is blocked; there are no thiol or phenolic hydroxyl groups in the subunit; the imino groups are involved in peptide bonds; the only imidazole group present may be involved as a ligand to the Mg in light-harvesting Bchl (Brunisholz et al., 1981; Theiler & Zuber, 1984). Dansyl chloride was chosen as the modification reagent because we could modify a single amino acid in the target protein and could subsequently identify the modified amino acid with known chromatographic procedures (Woods & Wang, 1967; Hartley, 1970). The α subunit was chosen as the target because of the position of the lysine in the carboxy-terminal portion of the protein. The reactivity of amino acids on other proteins was not investigated (although the lysines and the tyrosine of the β subunit are probably also affected). Interestingly, our results indicate that the single lysine in the α subunit could be modified at the ϵ-amino group without major disruption of the protein.

The modified lysine residue in the carboxy-terminal portion of the α subunit is 18 amino acid residues away from the

histidine residue implicated as a ligand for Bchl in the protein. Histidine residues have been implicated as the ligands to Bchl in the protein on the basis of evidence that histidine residues are the ligands to Bchl in a water-soluble Bchl *a* containing protein (Matthews et al., 1979) and resonance Raman spectral evidence (Robert & Lutz, 1985), and the fact that a histidine near the carboxy-terminal end of the hydrophobic stretch of the sequence seems to be conserved in the sequences of many light-harvesting proteins (Brunisholz et al., 1984a, 1985; Theiler et al., 1984). It has also been suggested that aromatic amino acids (phenylalanine, tyrosine, tryptophan) may play a role in part of the red shift (Brunisholz et al., 1984a). Charged and polar residues have been suggested in Bchl binding because model studies have indicated that some of the red shift in the absorbance of the Bchl in the light-harvesting protein binding site could be caused by strong hydrogen bonding (Katz, 1973) or that a positive charge near the Bchl could account for a portion of the red shift (Eccles & Honig, 1983). Lysine could potentially serve a function either as a hydrogen-bonding amino acid side chain or a charged center. The lysine in the α subunit is one of only three positively charged residues (one lysine and two arginines) at the carboxy-terminal side of the $\alpha\beta$ dimer, the portion of the dimer at the same side of the membrane as the histidines that may be Bchl ligands. Our results indicate, however, that the lysine in the α subunit carboxy terminus is clearly not part of the Bchl binding site in the protein.

At the present time, it is not clear if Bchl is bound near both the periplasmic side and the cytoplasmic side of the $\alpha\beta$ dimer or near only one side. A histidine is very close to the end of the membrane-spanning helix at the periplasmic side of the membrane in both subunits. The carboxy-terminal portion of the β subunit at the periplasmic side after the membrane-spanning region is very short (only 11 residues). Since the red-shifted Bchl binding site is a very tight binding site (the Bchl is very resistant to aqueous acetone extraction, unpublished observation), it might be suggested that both the α and β carboxy termini fold to form α "cap" for a tight and specific binding "pocket" if both Bchl subunits are bound at the periplasmic side of the $\alpha\beta$ dimer.

Loach et al. (1985) have suggested a model for the B880 complex in which both the amino-terminal and carboxy-terminal portions of the α subunits are involved in forming the binding sites for Bchl. In this model, the β subunits are involved in the aggregation of the subunits. In the β subunit, there is also a conserved histidine residue of unknown function at the cytoplasmic side (N-terminal side) of the membrane-spanning helix. However, protease treatment, which clipped off the amino-terminal portions of both the α and β subunits, has been reported to have little effect on the absorbance spectrum of Bchl (Brunisholz et al., 1984b). Further chemical and biochemical experiments will be needed to determine precisely which amino acids of the α and β subunits are involved as ligands to the Bchl and which amino acids form other parts of the red-shifted Bchl binding site.

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