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Isolation and Characterization of an Organic Solvent Soluble Polypeptide Component from Photoreceptor Complexes of *Rhodospirillum rubrum*†

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ABSTRACT: An organic solvent soluble polypeptide has been isolated from photoreceptor complexes and chromatophores of *Rhodospirillum rubrum*. After extraction of the protein from lyophilized samples with 1:1 chloroform–methanol, it was purified by column chromatography. Its isoelectric point determined by isoelectric focusing was 7.10. When analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the purified polypeptide ran as a single band of an apparent molecular weight of 12 000. However, according to amino acid analysis, the minimal molecular weight based on one histidine residue per polypeptide is 19 000. The polypeptide contains no cysteine and no tyrosine. Amino acid analyses indicated that three methionines were present per histidine residue and cyanogen bromide cleavage gave four smaller peptides which were isolated by two-dimensional electrophoresis and chromatography. Spectroscopic analysis indicated the presence of three tryptophan residues per histidine and *N*-bromosuccinamide cleavage also gave four smaller peptides which could be isolated by two-dimensional electrophoresis and chromatography. The C-terminal amino acid was shown to be glycine by two methods, while the N-terminal amino acid appears to be blocked. The organic solvent soluble polypeptide accounts for approximately 50% of the chromatophore protein and seems to bind the antenna bacteriochlorophyll and carotenoid molecules. Using this procedure, organic solvent soluble polypeptides were isolated from several photosynthetic bacteria and were found to have substantially different amino acid contents.

The light harvesting unit in photosynthetic bacteria such as *Rhodospirillum rubrum* is contained in, or on, the membrane structure proliferating from the cell membrane of the bacterium. Cell disruptive procedures such as sonication have been used to convert the membrane into small vesicles called chromatophores, and these vesicles can be converted into even smaller particles by various detergent treatments. Some of these preparations contain the antenna pigments or "bulk bacteriochlorophyll" (Loach et al., 1963, 1970a,b; Hall et al., 1973), while others contain only the phototrap pigments (Clayton and Wang, 1971; Feher, 1971; Clayton and Haselkorn, 1972; Noel et al., 1972). The former are called photoreceptor complexes, while the latter are called reaction centers.

Photoreceptor complexes (Hall et al., 1973) appear to contain the polypeptides found in reaction centers, plus one or more smaller polypeptides, depending on the species of bacterium. The antenna bacteriochlorophyll and the carotenoids also remain in photoreceptor complexes. We have found that extraction of lyophilized photoreceptor complexes of *R. rubrum* with organic solvents will totally dissolve the low-molecular-weight polypeptide, phospholipids, carotenoids, and bacteriochlorophyll, while the remaining polypeptides are insoluble. This same low-molecular-weight polypeptide has been isolated from chromatophores by a similar procedure, and relatively large amounts of it have been purified. The work described here is concerned with the isolation of this organic solvent soluble polypeptide (OSSP[1]) from *Rhodospirillum rubrum*, as well as initial chemical and physical characterization. A preliminary report of these results has been given (Tonn et al., 1974).

Materials and Methods

Bacterial Growth. Rhodospirillum rubrum, strain 1.1.1, was propagated anaerobically in modified Hutner's growth medium (Cohen-Bazire et al., 1957) at 25–30 °C. The bacteria were grown in a light box illuminated by 500 ft-c (1×10^4 erg cm^{-2} s^{-1}) fluorescent lights.

^{14}C Bacterial Cultures. Protein contents of samples were determined by radiolabeling the bacteria with [^{14}C]phenylalanine (UL, spec act. 350 to 500 μCi/mM, International Chemical and Nuclear Corp.). [^{14}C]Phenylalanine was added to modified Hunter's growth medium at concentrations varying

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[1] Abbreviations used are: OSSP, organic solvent soluble polypeptide; $NaDodSO_4$, sodium dodecyl sulfate; $NaDodSO_4$–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

from 25 to 50 μCi/L. About 70% of the labeled amino acid was incorporated into the cells.

Analysis of Radioactivity. The amount of radioactivity in ^{14}C-labeled samples was measured with a TriCarb scintillation spectrophotometer (Model 544, Packard Instrument Co.), in scintillation fluid containing 10 mL of toluene (Baker), 5 mL of Triton X-100 (Rohm and Haas), 49 mg of 2,5-diphenoloxazole, and 1 mg of 1,4-bis(2,5-diphenyloxazolyl)benzene (Packard).

Preparation of Biological Materials. Chromatophores were prepared from whole cells by modification of the method of Loach et al. (1963), using sonication and differential ultracentrifugation. Cells were suspended in 0.1 M glycylglycine buffer, pH 7.5 (Sigma) and then sonicated at maximum intensity for 3 min with a Biosonik III (Bronwill Scientific). Cellular debris was removed by centrifugation at 47 000*g* for 30 min, and the chromatophore fraction was pelleted from the first supernatant by centrifugation at 218 000*g* for 50 min. The pelleted chromatophores were resuspended in glycylglycine buffer and again centrifuged at 218 000*g* for 45 min. Chromatophores used for protein isolation were washed by suspending them in water and collected by centrifugation at 218 000*g* for 45 min. Photoreceptor complexes were prepared from chromatophores by alkaline-urea-Triton X-100 treatment (Loach et al., 1970a,b) followed by electrophoretic purification (Hall et al., 1973). The photoreceptor complexes were extensively dialyzed (4 to 5 days with ten changes of water) to remove Triton X-100. They were pelleted by centrifugation at 218 000*g* for 30 min. The chromatophore and photoreceptor complex preparations were suspended in minimum volumes of water and were lyophilized to dryness.

Phospholipid Determination. The total lipid phosphorus of the chloroform–methanol extract and the Sephadex LH-60 column fractions was determined using the method of Smith et al. (1959).

Carbohydrate Determination. The phenol-sulfuric acid test for determining glucose equivalents (Dubois et al. (1951)) was used to determine the carbohydrate content of the polypeptide. The samples of OSSP used for analysis were dissolved in 0.5% $NaDodSO_4$. Controls on $NaDodSO_4$ were conducted for both glucose as a standard curve and also on ovalbumin as a well-characterized glycoprotein. Their was no significant color contribution by the $NaDodSO_4$.

Amino Acid Analysis. Protein samples of about 0.4 mg were added to 1 mL of constant boiling hydrochloric acid (Sequanal Grade, Pierce) and were hydrolyzed under vacuum at 108 °C for 20, 24, 36, 48, and 72 h. The samples were dried by rotary evaporation at 40 °C. To remove any remaining hydrochloric acid, water was added and the sample was again dried. This was repeated three times. The samples were then dissolved in 100 μL of 0.2 N sodium citrate buffer, pH 2.2 (Pierce). Analyses were carried out on a Durrum Model D-500 amino acid analyser.

Tryptophan was determined spectrophotometrically by the method of Edelhoch (1967) in 8 M urea (Pierce) and in 1:1 chloroform–methanol, and by titration with *N*-bromosuccinimide (Spande and Witkop, 1967). The *N*-bromosuccinimide (Eastman) was recrystallized from water, yielding white crystals which were stored at −20 °C in the dark.

The amide content of the protein was determined by modification of the Conway microdiffusion technique (Conway, 1947; Wilcox, 1967). Ammonia was released from the protein by hydrolysis in concentrated hydrochloric acid for 10 days at 37 °C. The hydrochloric acid was removed by drying over sodium hydroxide pellets, and the ammonia was isolated by microdiffusion in Conway vessels. The free ammonia was determined colorimetrically by a phenol–hypochlorite–manganese assay (Russell, 1946; Exley, 1956). Horse heart cytochrome *c* (Sigma) was used as a standard.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis ($NaDodSO_4$–PAGE). $NaDodSO_4$–PAGE was carried out using the method of Shapiro et al. (1967), modified by Okamura et al. (1974). The apparatus used was a Canalco Model 66 electrophoresis unit. The 5 × 65 mm gels were run at 3 mA per gel until the tracking dye reached the bottom of the gel. The gels were fixed overnight in 50% trichloroacetic acid and then stained with Coomassie blue R-250. They were scanned with a Quick Scan spectrometer (Helena Laboratories) which employed a color filter (maximum transmission at 570 nm) for selecting the detecting wavelengths. Standard proteins used were cytochrome *c* (Sigma), β-lactoglobulin (Nutritional Biochemicals), ovalbumin (Pharmacia), and bovine serum albumin (Mann).

Determination of Protein-Bound Sodium Dodecyl Sulfate. The concentration of $NaDodSO_4$ bound to protein was determined colorimetrically by the method of Reynolds and Tanford (1970). The $NaDodSO_4$ was recrystallized twice from ethanol. The protein was dissolved in 8 M urea (Pierce) which had been deionized with Bio-Rad AG501-X8 (D) mixed bed resin. The urea was removed and simultaneously replaced by $NaDodSO_4$ by dialysis against 0.05 M Tris buffer, pH 7.5, containing 3.5 mM $NaDodSO_4$. The concentrations used were the same as those used for $NaDodSO_4$–PAGE.

Gel Electrofocusing in 1% Triton X-100. Electrofocusing was carried out using a Canalco Model 66 electrophoresis unit. The procedure was developed from the method of Wrigley (1971). Each 5 × 65 mm gel was cast from the following solution: 0.8 mL of protein solution containing 1.5% Triton X-100, 0.3 mL of acrylamide stock solution, 1 μL of *N,N,N′,N′*-tetramethylethylenediamine, 30 μL of carrier ampholyte (LKB) in the pH range desired, and 70 μL of ammonium persulfate (10 mg/mL). The acrylamide stock solution contained 7.5 g of acrylamide (Gold Label, Aldrich), and 0.25 g of *N,N′*-methylenebisacrylamide (Bio-Rad) per 25-mL solution. Horse heart myoglobin (Sigma), with an isoelectric point of 7.0, was chosen as a standard. The anode vessel contained 0.2% sulfuric acid and the cathode vessel contained 0.4% ethylenediamine. Electrofocusing was started at 2 mA per gel and allowed to run until myoglobin had formed a sharp band, in about 3 h. One of each type of gel was immediately sliced into 1-mm slices and each slice placed in 1 mL of water for measurement of pH. Other gels were placed in 5% trichloroacetic acid overnight for protein fixing and ampholyte removal. The gels were stained in 0.1% bromphenol blue–10% mercuric chloride in 50% ethanol for 2 h and then destained in 30% ethanol–5% acetic acid. This stain was preferred because it could be used before all ampholytes were removed while Coomassie blue could not.

Cyanogen Bromide Cleavage. Lyophilized protein was dissolved in 70% formic acid (Pierce) at a concentration of 10 mg/mL. Cyanogen bromide (Pierce) was added at a 40-fold excess over the number of methionine groups, and the reaction was allowed to proceed for 24 h at 23 °C (Corradin and Harbury, 1970). The sample was diluted with 5 volumes of water and lyophilized to dryness. The digest was characterized by peptide mapping.

N-Bromosuccinimide Cleavage. Lyophilized protein was dissolved in 70% acetic acid (Pierce) at a concentration of 2 mg/mL and 0.15 mL per mL of 20 mM *N*-bromosuccinimide (recrystallized, Eastman) was added (Ramachandran and

Witkop, 1967). The solution was allowed to stand 60 min at 23 °C, and 0.15 mL of *N*-bromosuccinimide was added a second time. After 60 min more, the solution was diluted fivefold with water and lyophilized. The digest was characterized by peptide mapping.

Trypsin Cleavage. Two milligrams of protein was dissolved in 2 mL of 0.01 M ammonium carbonate, pH 7.9, containing 1% $NaDodSO_4$. This solution solubilized the protein, but the high detergent concentration was shown to cause smearing of the peptide maps run on the digest. Consequently, after the protein dissolved, the solution was dialyzed against two changes of ammonium carbonate (no $NaDodSO_4$). The resulting solution was cloudy but did not precipitate, and later smearing of the peptide maps was reduced. Trypsin was dissolved in 0.001 M hydrochloric acid at a concentration of 2 mg/mL and 25 μL was added to the protein solution. After 10 h an additional 25 μL of the trypsin solution was added. After 15 h the solution was heated in a boiling water bath for 5 min, and the precipitated trypsin was removed by centrifugation. The digest was examined by peptide mapping.

Amino-Terminal Determination. Methods used for determination of the amino terminal residue of the polypeptide were: dansylation (Gray, 1972), Edman degradation with a Beckman sequencer (Fast Quadrol Program), and leucine aminopeptidase hydrolysis (Light, 1972). The peptide and protein procedures for dansylation were taken directly from Gray (1972) with no modifications. In the automated Edman degradation using the Beckman 890C sequencer, 5 mg of protein in 0.2 mL of glacial acetic acid (PHIX, Pierce) was added to the spinning cup. A Beckman gas–liquid chromatograph (Model 65) was used to monitor phenylthiohydantoins. Enzymatic cleavage with leucine aminopeptidase (Light, 1972) was performed with the following modifications. Three different buffers were used: 0.1 M Tris, 0.14 M triethylamine, and 0.2 M *N*-ethylmorpholine acetate containing 0.5% $NaDodSO_4$. All were at pH 8.5 and contained 0.0025 M Mg^{2+} for enzyme activation. After 5 h the reaction was stopped by adding Dowex 50 (Bio-Rad) until the pH dropped to a value between 2.5 and 3. The samples were then handled in the same way as the carboxypeptidase A and B digests (described below).

Carboxyl-Terminal Determination. The carboxyl-terminal amino acid was determined by hydrazinolysis using the method of Fraenkel-Conrat and Tsung (1967). Anhydrous hydrazine (Pierce) (0.2 mL) was added to 2 mg of lyophilized protein, and the sample was sealed under vacuum. After 24 h at 80 °C, the excess hydrazine was removed by lyophilization, and the free amino acids were separated from the hydrazides by chromatography on Amberlite IRC-50 (H^+ form). The free amino acids were determined by amino acid analyses.

The carboxy-terminal amino acids of the polypeptide were also determined enzymatically by carboxypeptidases A and B treatment (Ambler, 1972). The polypeptide was dissolved in 0.2 M *N*-ethylmorpholine acetate, pH 8.5, containing 0.5% $NaDodSO_4$, at a final protein concentration of 1 mg/mL. Carboxypeptidase A (diisopropyl fluorophosphate treated, Sigma) was added to one set of samples to give a final enzyme concentration of 2.5 nmol/mL, and two samples of 1 mL each were incubated at various time intervals from 1 min to 12 h. Carboxypeptidase B (diisopropyl fluorophosphate treated, Sigma) was added at a final enzyme concentration of 0.2 unit/mL to a second set of protein samples. Duplicate samples were incubated for 5 h. Both carboxypeptidases A and B were added to a third set of samples in the same concentrations as above, and duplicate samples were incubated 5 h. Blanks containing enzyme but no polypeptide were run at each time period for each enzyme. All incubations were at 37 °C. The reactions were stopped by adding Dowex 50 (International Chemical and Nuclear Corp.) until the pH of the supernatant was 2.5 to 3 (indicator paper). The mixtures were shaken 20 min, the supernatants removed, and the resin beads washed with 2 resin volumes of water. The adsorbed amino acids were then eluted with 5 M ammonium hydroxide and were taken to dryness by lyophilization. One sample at each time interval was dissolved in 100 μL of 0.2 M citrate buffer, pH 2.2, and was submitted for amino acid analysis. The other sample was dissolved in 1 mL of constant boiling hydrochloric acid (Pierce) and hydrolyzed under vacuum at 108 °C for 24 h and then was prepared for amino acid analysis in the usual fashion. This allowed the determination of glutamine and asparagine by difference.

TABLE I: Protein Extracted from *R. rubrum* Chromatophores.

Solvent	% Total ^{14}C Counts
Methanol	6
1:2 chloroform–methanol	46
1:1 chloroform–methanol	50
2:1 chloroform–methanol	23
9:9:2 chloroform–methanol–water	49
2:2:1 chloroform–methanol–water	
Chloroform phase	25
Water phase	13
Interface	62
Chloroform	0.5
1:2 dimethyl sulfoxide–methanol	16
1:1 dimethyl sulfoxide–methanol	25
Dimethyl sulfoxide	9
Ethanol	0.8
Carbon tetrachloride	0.3
Benzene	0.2
Acetone	0.3
1:1 acetone–methanol	25
7:2 acetone–methanol	12
Dimethylformamide	2
1:1 dioxane–water	8
Dioxane	0.4
1:2 petroleum ether–methanol	16
1:1 petroleum ether–methanol	25
Petroleum ether	0.2

Peptide Mapping. Various peptide fractions were mapped on Polygram Cel 300 thin-layer plates (Brinkmann) by electrophoresis in one dimension followed by chromatography in the second dimension (Borden and Margoliash, 1976). Electrophoresis was performed with a DeSaga electrophoresis chamber (Brinkmann) and a Gelman Delux regulated power supply at 400 V and 10 to 12 mA. The standard electrophoresis time was 1 h, and the electrophoresis buffer was pyridine–acetic acid–water (200:7:1800), which was pH 6.5. The chromatography solvent was either butanol–pyridine–acetic acid–water (10:15:3:12) or butanol–acetic acid–water (3:1:1). The fingerprints were developed after chromatography with ninhydrin (Nin-Sol, Pierce). Butanol and acetic acid were purchased from Pierce and were Sequanal and PHIX grades, respectively. Reagent grade pyridine (Aldrich) was distilled from tosyl chloride, or Sequanal grade was used directly.

Results

Isolation of an Organic Solvent Soluble Polypeptide. Early experiments in this laboratory involving lipid extraction of chromatophores and photoreceptor complexes showed that protein was also dissolved in chloroform–methanol. This

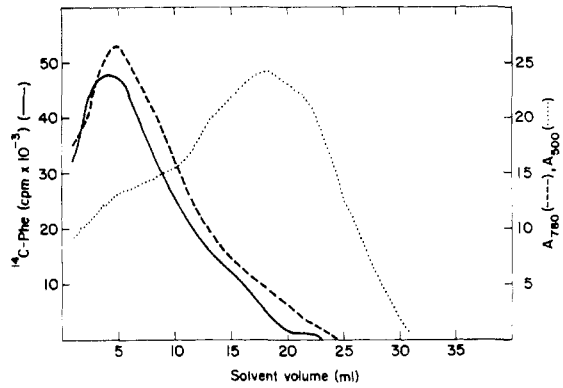

FIGURE 1: Repeated extraction of *R. rubrum* chromatophores with small volumes of 1:1 chloroform–methanol. [^{14}C]Phenylalanine-labeled lyophilized chromatophores (100 mg) were repeatedly extracted with 1-mL volumes of chloroform–methanol, and the absorbance spectrum and amount of radioactivity extracted were measured for each sample.

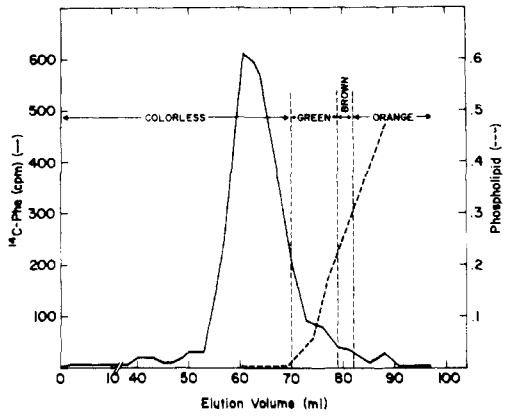

FIGURE 2: LH-60 preparative column chromatography of a chloroform–methanol extract of [^{14}C]phenylalanine-labeled *R. rubrum* chromatophores. After chromatophores were extracted, the protein–pigment solution was applied to a Sephadex LH-60 column, and eluted with 1:1 chloroform–methanol. The colorless fractions containing protein were pooled and concentrated by rotary evaporation, and then lyophilized to dryness (see Results).

suggested that extraction with organic solvents could be used for isolation of a membrane protein or proteins from photosynthetic organisms.

Lyophilized chromatophores isolated from cells supplied with [^{14}C]phenylalanine in their growth media were exhaustively extracted with various solvents. Solvents were added to the chromatophores, mixed briefly on a Vortex mixer, and allowed to stand 20 min. The residue after extraction was removed by centrifugation in a table top centrifuge. The residues were repeatedly extracted until the supernatant solution was colorless and the radioactivity in it reached the background level. As shown in Table I, the most protein was solubilized with 1:1 (v:v) chloroform–methanol, so this solvent system was chosen for preparative purposes. Figure 1 shows the results of repeated extraction of 100 mg of radiolabeled chromatophores with 1-mL volumes of 1:1 chloroform–methanol at room temperature. After exhaustive extraction, the residue was buff colored. For preparative purposes, 15 mL of 1:1 chloroform–methanol was added to 100 mg of lyophilized chromatophores and the suspension mixed briefly on a Vortex mixer and allowed to stand 15 min. It was then centrifuged and the clear dark green supernatant solution was decanted.

The extracted protein was separated from the pigments and phospholipids by gel permeation chromatography on Sephadex LH-60[2] or Sephadex LH-20 (Pharmacia Fine Chemicals). Figure 2 shows a typical preparative column profile of an LH-60 column. About 80% of the fractions containing protein were colorless. These fractions were pooled and dried by rotary evaporation under vacuum to yield a white solid. The solid obtained in this way adhered tightly to glass, so it was dissolved in 1:1 pyridine–water, diluted tenfold with water, and lyophilized. Combined electrophoresis and chromatography, as used in peptide mapping, showed that a single spot moved from the origin. In the chromatography step it had an R_f 0.2 (with the solvent pyridine–butanol–acetic acid–water).

Analyses of other components separated by gel permeation chromatography showed that the radiolabel was not incorporated into bacteriochlorophyll, the carotenoids, or the phospholipids to a significant extent. While the radioactive protein often trailed into the green band on preparative chromatography, with carefully run analytical columns only about 3% of the radioactivity was found in the fractions containing bacteriochlorophyll, and less than 0.05% was found in fractions containing phospholipids and carotenoids.

NaDodSO$_4$-PAGE. NaDodSO$_4$-PAGE utilizing a Tris buffer system (Shapiro et al., 1967) was used for determining the polypeptide compositions of various fractions. Figure 3 compares sodium dodecyl sulfate–polyacrylamide gels of chromatophores, the chloroform–methanol soluble polypeptide, and the residue left after extraction of chromatophores with chloroform–methanol. The chloroform–methanol-soluble extract showed a single protein band which ran slightly faster than horse heart cytochrome *c* and had an estimated apparent molecular weight of 10 000. No pigments were associated with this band but were visible in the vicinity of the tracking dye. The significance of the 10K band remaining in the residue will be discussed after the amino acid analyses data are presented.

Lyophilized [^{14}C]phenylalanine-labeled photoreceptor complexes were also extracted with 1:1 chloroform–methanol and up to 80% of the radioactivity was dissolved. Pigments and the soluble protein were separated by gel permeation chromatography on Sephadex LH-20. The results of extraction of photoreceptor complexes with 1:1 chloroform–methanol are shown in Figure 4. In this case, the major band at 10 K was completely extracted. This latter experimental result has been reproduced in several such experiments so far attempted. The chloroform–methanol insoluble residue resulted in three protein bands which resembled the *R. rubrum* reaction center preparation reported by Noel et al. (1972).

Protein Bound NaDodSO$_4$. Because this polypeptide is an integral membrane protein with unique solubility properties, the amount of NaDodSO$_4$ bound under the conditions used in NaDodSO$_4$–PAGE (Shapiro et al., 1967) was measured. While many water soluble polypeptides bind 1.4 g of NaDodSO$_4$/g of protein (Reynolds and Tanford, 1970), membrane polypeptides have been reported to bind widely varying amounts of NaDodSO$_4$ (Nelson, 1971; Simons and Kääriänen, 1970) and to give anomalous results on NaDodSO$_4$–PAGE. The average of five determinations of NaDodSO$_4$ binding to the organic solvent soluble polypeptide was 1.00 g of NaDodSO$_4$/g of protein, with all values within ±8%.

Isoelectric Focusing. The isoelectric point of the organic solvent soluble polypeptide was determined by gel electrofo-

[2] Sephadex LH-60 was a gift from Pharmacia and was initially used because it gave better separation of protein from bacteriochlorophyll than did Sephadex LH-20, particularly at 4 °C. It was not put into commercial production, so Sephadex LH-20 was substituted.

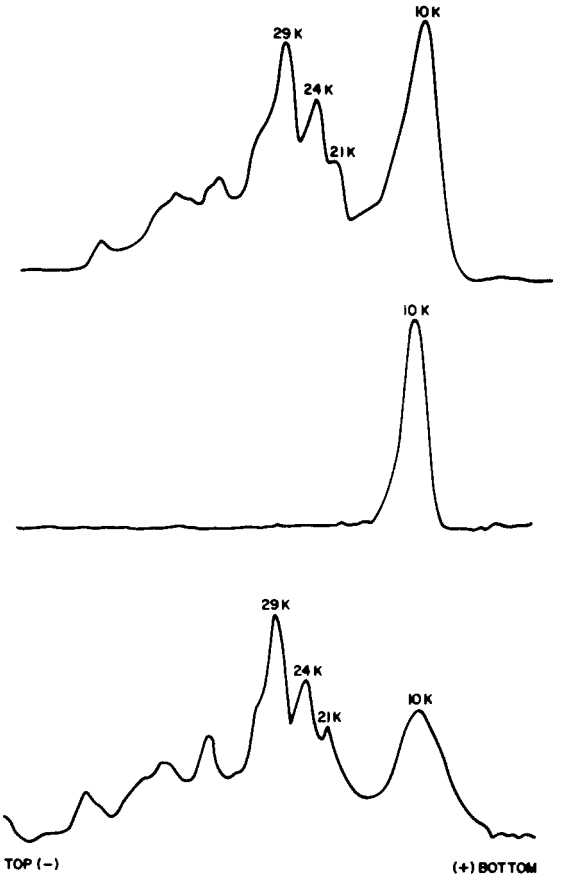

FIGURE 3: $NaDodSO_4$-PAGE scans. (Top) Unextracted chromatophores isolated from *R. rubrum*. (Middle) The chloroform–methanol soluble polypeptide. (Bottom) The residue from *R. rubrum* chromatophores left after extraction.

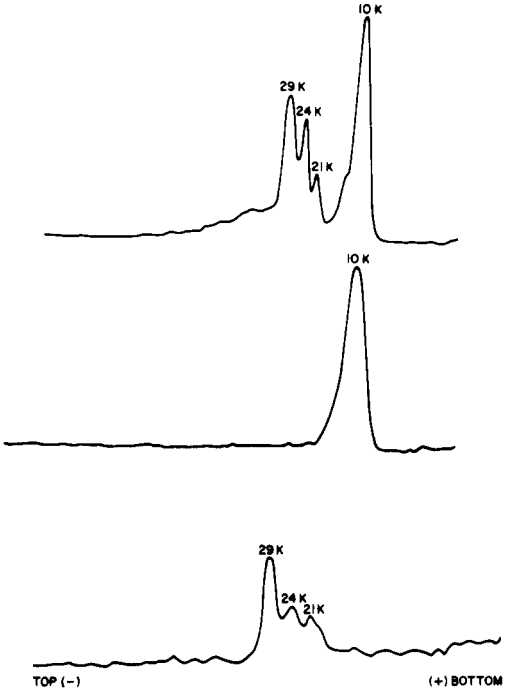

FIGURE 4: $NaDodSO_4$-PAGE scans. (Top) Unextracted photoreceptor complexes isolated from *R. rubrum*. (Middle) The chloroform–methanol soluble polypeptide extracted from photoreceptor complexes. (Bottom) The residue from *R. rubrum* photoreceptor complexes after extraction.

cusing in a pH gradient formed by ampholytes of a pH range from 6 to 9. The polypeptide focused at a pH of 7.10 ± 0.10. This value is consistent with our expectations because of the solubility of the polypeptide in organic solvents, but it is considerably higher than the value of p*I* = 5.0 calculated for the isoelectric point assuming no ion binding. The discrepancy suggests that either OSSP binds cations or, because of its unique pigment binding and membrane location role, it may have several protonated carboxyl groups in an apolar environment.

Solubility of the Organic Solvent Soluble Polypeptide. A known weight of [^{14}C]phenylalanine-labeled polypeptide isolated from chromatophores was dissolved in 1:1 chloroform–methanol or 1:1 pyridine–water, and 0.15-mL aliquots were transferred to glass stoppered conical centrifuge tubes. The solvent was removed by evaporation under prepurified nitrogen (chloroform–methanol) or by lyophilization (pyridine–water). An equal volume of test solvent was added, and the tube was stoppered and carefully mixed on a Vortex mixer. After 15 min the solution was centrifuged and 0.1 mL was removed and placed in a scintillation vial. Standards were run with each solvent to determine quenching by the solvent, and all samples were appropriately corrected. Protein solubility in mg/mL was calculated from the amount of radioactivity in each solvent. The detection limit was 0.02 mg of protein/mL of solution. OSSP did not dissolve in aqueous buffers such as 0.1 M succinate (pH 4.25), 0.1 M phosphate (pH 7.0), 0.1 M glycylglycine (pH 8.5), or 0.1 M carbonate (pH 9.8), or in 0.1 M sodium chloride. It was also insoluble in 1:1 methanol–water and in nonpolar solvents such as hexane and benzene. OSSP was somewhat soluble (0.4 mg/mL or less) in 0.1% $NaDodSO_4$, 28% ammonium hydroxide, ethanol, methanol, and propionic acid. Solvents in which OSSP was significantly soluble are shown in Table II.

Carbohydrate Content. Using the phenol–sulfuric acid assay (Dubois et al., 1951), a carbohydrate content of 1.5 g per 100 g of *R. rubrum* OSSP was found. This value was reproducible to within ±20% for several different preparations of OSSP.

Amino Acid Analyses. The results of 20, 24, 30, 48, and 72 h acid hydrolyses of the chloroform–methanol soluble polypeptide isolated from chromatophores were averaged and are expressed as mole percent and as number of residues in columns three and four of Table III. Threonine showed very little degradation with time and was left uncorrected. Serine degradation was significant and was corrected by extrapolation to zero time. Leucine and isoleucine showed 25 and 35% increases respectively from 20 to 72 h, and values at 72 h were chosen as the best values. The polarity of this protein was found to be 42% using the method of Capaldi and Vanderkooi (1972). This is higher than the polarity of the reaction center proteins isolated from *R. rubrum* G-9 (Steiner et al., 1974) which apparently do not exhibit such unique solubility properties. Based on the amino acid composition and assuming one histidine residue, the minimal molecular weight of the organic solvent soluble polypeptide was calculated to be 18 800.

Three 24-h amino acid analyses were performed on protein extracted from photoreceptor complexes. In the first two columns of Table III are compared the uncorrected results for 24-h hydrolyses of the organic solvent soluble proteins isolated from photoreceptor complexes and from chromatophores. Tryptophan was not determined for the protein isolated from photoreceptor complexes, so the mole percent values for the protein isolated from both preparations were calculated to total 100% omitting tryptophan. Cysteine and tyrosine were absent from both protein preparations. Most of the amino acid contents were equal in the two preparations, within the experi-

TABLE II: Solubility of Purified Protein from *R. rubrum* Chromatophores.[a]

	Solubility (mg/mL)
Aqueous solvents	
1.0% $NaDodSO_4$	8.3
1.0% Triton X-100	5
1.0 M Quadrol buffer[b]	2
1:1 pyridine–water	~20–25
1:1 dioxane–water	2.1
6 M urea	5 (solution cloudy)
Nonaqueous solvents	
Glacial acetic acid	0.6
98% formic acid	5.1
Trifluoroethanol	22
1:1 trifluoroethanol–methanol	3.6
Hexafluoro-2-propanol–methanol	16
Hexafluoro-2-propanol	17.1
1:1 petroleum ether–methanol	0.6
1:1 chloroform–methanol	4.0
Methanol + 0.25 mg of phosphatidylcholine/mg of protein	4.2
1:1 methanol–petroleum ether + 0.25 mg of phosphatidylcholine/mg of protein	1.0

[a] The solubility was determined using [^{14}C]phenylalanine-labeled protein. [b] Quadrol buffer (Pierce) is *N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine–trifluoroacetic acid buffer in 1-propanol–water, 3:4 (v:v), pH 9.0.

mental error. Thus, we conclude that the proteins isolated from *R. rubrum* chromatophores and from photoreceptor complexes by chloroform–methanol extraction are identical.

After chloroform–methanol extraction of chromatophores, there was a band remaining at 10 K when $NaDodSO_4$–PAGE was run on the residue left after extraction (Figure 3, bottom). By electrophoresing several gels of the residue and cutting off the 10 K band, we determined that the amino acid content of the band was very different from the chloroform–methanol soluble protein (Tonn, 1976). The polarity of the 10 K protein from the residue was 56%, and tyrosine was present, while it was completely absent from the chloroform–methanol soluble polypeptide.

Amino-Terminal Analysis. An amino-terminal residue was not found using dansylation, automated Edman degradation, or leucine aminopeptidase cleavage. The amount of polypeptide used was at least 25-fold higher than the detection level for each assay. It was concluded that the amino terminal of the polypeptide was blocked.

Carboxyl-Terminal Analysis. Treatment of OSSP with hydrazine for 24 h released the free amino acids glycine (1.31 nmol), serine (0.49 nmol), and alanine (0.28 nmol). Theoretically about 20 nmol should have been recovered. Part of the cause of the low yield could have been due to incomplete hydrazinolysis. Hydrazinolysis for shorter times released smaller amounts of the same amino acids, while hydrazinolysis for 40 to 60 h yielded broad peaks from the amino acid analyzer, unidentifiable as amino acids.

Samples of OSSP were incubated with carboxypeptidase A for periods of time varying from 1 min to 12 h. Two aliquots were removed at 1, 3, 5, 10, 20, and 35 min and 1, 7, and 12 h. After lyophilization, one sample at each time was acid hydrolyzed for amide determination, and the other sample was submitted for amino acid analysis. This allowed determination of asparagine and glutamine by difference. Table IV shows the amino acids released by carboxypeptidase A treatment. The numbers in parentheses were amino acids found only after acid hydrolysis and were not present in the samples subjected only to enzymatic digestion. Their presence is unexplained and is undergoing further investigation.

TABLE III: Amino Acid Composition of the Organic Solvent Soluble Polypeptide Isolated from *R. rubrum* Chromatophores and Photoreceptor Complexes.

	Photoreceptor Complexes (mol %)	Chromatophores (mol %)	(mol %)	No. of Residues[c]
Polar residues (42.3%)				
Acidic				
Asp	6.0[a]	4.8[a]	4.3[b]	7.4
Glu	13.2	13.2	11.7	20.3
Total amide	ND[d]	ND[d]	1.8	3.2
Basic				
Lys	2.4	2.0	1.7	3.0
His	0.9	0.6	0.6	1.0
Arg	6.6	7.8	6.9	12.0
Hydroxyl				
Ser	8.2	8.4	9.3	16.0
Thr	9.0	8.6	7.8	13.4
Nonpolar residues (57.7%)				
Aliphatic				
Gly	6.5	6.3	5.6	9.6
Ala	8.1	8.7	7.6	13.1
Val	4.9	4.8	4.3	7.4
Ile	3.7	3.7	4.0	6.8
Leu	14.6	16.2	20.8	36.0
Pro	6.0	6.0	5.2	9.0
Met	2.1	2.0	1.8	3.0
Cys	0.0	0.0	0.0	0.0
Aromatic				
Tyr	0.0	0.0	0.0	0.0
Phe	8.0	7.3	6.7	11.6
Trp	ND[d]	ND[d]	1.7	3.0

[a] The values given in the first two columns are 24-h averages, uncorrected for time-dependent loss or increase of amino acids. The independent tryptophan determination for the polypeptide isolated from chromatophores is not included in the mole percent calculation, and the chromatophore analysis was normalized to 100% omitting it. This allows direct comparison of the data for chromatophores and photoreceptor complexes. [b] Corrected for serine hydrolysis losses by extrapolation to zero time, and for incomplete hydrolysis for leucine and isoleucine. [c] Calculated from mol % data (corrected), assuming one histidine per polypeptide. [d] ND, not determined.

When a sample of OSSP was incubated with carboxypeptidase B for 5 h, no amino acids were released. When both carboxypeptidases A and B were added to a protein sample and incubated 5 h, the amino acids released were the same as those released with carboxypeptidase A alone.

Polypeptide Cleavage with Cyanogen Bromide. When the cyanogen bromide digest was examined by peptide mapping and developed with ninhydrin, four spots were visible (Figure 5, left). This is consistent with three methionine residues in the polypeptide.

Polypeptide Cleavage with N-Bromosuccinimide. When the *N*-bromosuccinimide digest was examined by peptide mapping, four spots were visible (Figure 5, middle). This is consistent with three tryptophan residues in the polypeptide.

TABLE IV: Hydrolysis with Carboxypeptidase A.[a]

	1′	3′	5′	10′	20′	35′	1 h	7 h	12 h
His	0.0	0.0	0.0	0.0	0.08	0.00	0.00	0.20	0.31
Lys	0.0	0.0	0.0	0.0	0.05	0.07	0.05	0.05	0.05
Asn	0.0	0.0	0.0	0.08	0.10	0.12	0.19	0.08	0.08
Asp	0.0	0.0	0.0	0.0	0.02	0.02	0.02	0.02	0.02
Thr	0.08	0.22	0.24	0.30	0.30	0.24	0.20	0.28	0.28
Ser	0.10	0.23	0.21	0.32	0.37	0.36	0.40	0.42	0.40
Gln	0.0	0.08	0.11	0.19	0.36	0.44	0.38	0.29	0.26
Gly	0.47	0.83	1.10	2.10	2.63	2.63	2.60	2.64	2.60
Ala	(0.09)	(0.11)	(0.21)	(0.17)	(0.28)	(0.68)	(0.29)	(0.23)	(0.20)
Val	(0.09)	(0.09)	(0.09)	(0.11)	(0.11)	(0.65)	(0.60)	(0.11)	(0.11)
Met	0.0	0.0	0.0	0.0	(0.03)	(0.03)	(0.03)	(0.05)	(0.06)
Leu	0.0	0.0	0.0	0.02	0.04	0.04	0.24	0.17	0.12
Tyr	0.0	0.0	0.0	0.0	(0.03)	(0.04)	0.0	(0.03)	(0.03)
Phe	0.0	0.0	0.0	0.0	(0.10)	(0.12)	(0.11)	(0.10)	(0.10)

[a] In nmol of amino acid released/20 μL.

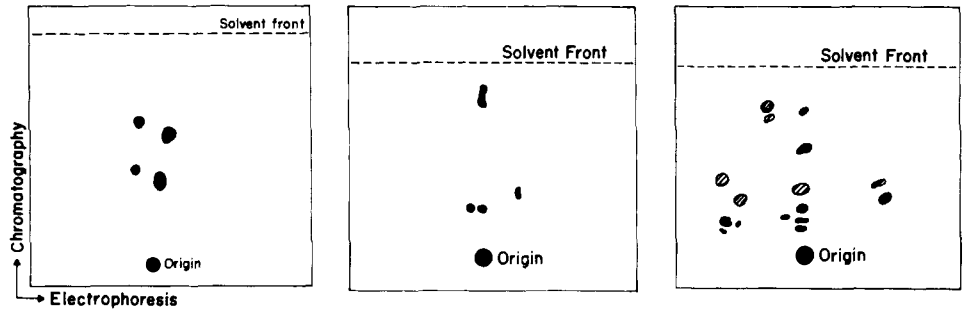

FIGURE 5: (Peptide map on left) Treatment of the organic solvent soluble polypeptide with 40-fold excess of cyanogen bromide for 24 h (23 °C). The peptides were mapped by electrophoresis at pH 6.5, followed by chromatography with butanol-pyridine-acetic acid-water (10:15:3:12). In all cases the fingerprints were developed with ninhydrin. (Peptide map in middle) Treatment of the organic solvent soluble polypeptide with a 60-fold excess of *N*-bromosuccinimide for 2 h (23 °C). The peptides were mapped by electrophoresis at pH 6.5, followed by chromatography in butanol-acetic acid-water (3:1:1). (Peptide map on right) Treatment of the organic solvent soluble polypeptide with 0.1 mg of trypsin over a 15-h total time period. The resulting peptides were mapped by electrophoresis at pH 6.5, followed by chromatography in butanol-acetic acid-water (3:1:1).

Polypeptide Cleavage with Trypsin. After trypsin treatment, the resulting peptides were examined by peptide mapping (Figure 5, right). When 80 μg of digest was spotted on the plate, 17 spots were visible. This is consistent with a total of 16 lysine and arginine residues in the polypeptide. Actual amino acid analysis gave 15 lysine and arginine residues.

Discussion

Three techniques were used to determine the degree of polypeptide purity of the organic solvent soluble polypeptide isolated from *R. rubrum* chromatophores. When examined by $NaDodSO_4$-PAGE and stained with Coomassie blue, a single band with an apparent molecular weight of 10 000–12 000 was visible. Isoelectric focusing in 1% Triton X-100 also resulted in a single band at pH 7.1. When the polypeptide was subjected to electrophoresis followed by chromatography, a single spot was visible (for chromatography, R_f 0.2). These results are consistent with the conclusion that we have isolated a single polypeptide.

The results of $NaDodSO_4$-PAGE coupled with those of amino acid analyses indicate that the same polypeptide is extracted from chromatophores and from photoreceptor complexes. This allows several steps to be omitted in the isolation of this photoreceptor complex polypeptide, permitting the isolation of a much larger amount of protein.

The amount of $NaDodSO_4$ bound under electrophoresis conditions is lower than the reported amount bound by standard water soluble polypeptides. On one hand, one might expect that the charge-to-mass ratio would be reduced, and that the polypeptide would move more slowly through the gel. On the other hand, the polypeptide might well retain its native conformation to a greater extent; the polypeptide would probably not assume the flexible extended rod found for water soluble polypeptides interacting with $NaDodSO_4$ (Tanford, 1973), so it would pass through the gel more quickly. Other integral membrane proteins have been shown to run at anomalously low molecular weights on $NaDodSO_4$-PAGE (Bridgen and Walker, 1976; Simons and Kääriäinen, 1970; Rubin and Tzagoloff, 1973; Spatz and Strittmatter, 1971, 1973; Kyte, 1972). These results suggest that one should consider the possibility of an anomalously low molecular weight determined by $NaDodSO_4$-PAGE for an integral membrane polypeptide. Clearly, the molecular weight should also be determined by other methods such as analytical ultracentrifugation, amino acid composition, and chemical cleavage, before it is viewed as the correct molecular weight.

Chemical and enzymatic cleavages support the minimum molecular weight of 18 800 determined from the amino acid composition. In the case of cyanogen bromide cleavage, the

four peptides formed indicate the presence of three methionine residues in the polypeptide. Our tryptophan determinations indicated that there are three residues of this amino acid per polypeptide with a molecular weight assumed to be 18 800. Consistent with this analysis was the result of *N*-bromosuccinimide cleavage which also gave four smaller peptides.

Organic solvent soluble polypeptides have also been isolated in our laboratory from *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata* (Tonn, 1976), and characterization of these has begun. Initial results show that the amino acid compositions of the organic solvent soluble polypeptides isolated from *Rps. sphaeroides* and *Rps. capsulata* are similar to each other but are quite different from the organic solvent soluble polypeptide isolated from *R. rubrum* (Tonn, 1976). The polypeptides isolated from *Rps. sphaeroides* and *Rps. capsulata* are more hydrophobic than the one isolated from *R. rubrum*, both of the former with polarities of about 37%.

The isolation of bacteriochlorophyll-protein complexes from *Rps. sphaeroides* whose role seems to be that of an antenna complex has also been reported by Clayton and Clayton (1972) and by Fraker and Kaplan (1971, 1972). Whereas each of these studies reported finding only one such antenna complex, our work with *Rps. sphaeroides* has indicated that there appear to be two different antenna complexes (Hall et al., 1973; Tonn, 1976). We feel that it will probably not be possible to meaningfully relate work from different laboratories on the various polypeptides of reaction center and photoreceptor preparations until each of the polypeptides being studied is completely characterized, most likely including amino acid sequence information.

Organic solvent soluble polypeptides have recently been extracted from two other photosynthetic organisms, *Chromatium vinosum* (Halsey and Byers, 1975), and spinach chloroplast membranes (Henriques and Park, 1976). In their work with *Chromatium*, Halsey and Byers used organic solvents to extract a preparation of photoreactive particles. They found that several polypeptides could be extracted from these particles into chloroform-methanol, among which was the component of apparent molecular weight of 12 000, one of 22 000, and one of 25 000. No further isolation or characterization of these components was reported. In the extraction from spinach chloroplast membranes, about one-third of the total membrane protein was solubilized with a 1:2 mixture of chloroform-methanol. Several polypeptide components seem to dissolve. Of these, one had an apparent molecular weight of 25 000 (on $NaDodSO_4$-polyacrylamide gels) and appeared to account for about 25% of the total chloroplast lamellar protein. On the basis of its apparent molecular weight, it was thought to be the main protein component of the spinach chloroplast membrane light-harvesting chlorophyll-protein complex (Henriques and Park, 1975; Genge et al., 1974; Thornber and Highkin, 1974). Further purification and characterization of this component have not been reported.

It should be emphasized that OSSP of *R. rubrum* has been isolated from a well-defined, pigment-protein complex of small molecular weight which still has complete physiological activity for converting absorbed light energy into chemical potential. In addition, because it may constitute about 50% of the total chromatophore membrane protein (Table I), it seems probable that it plays an important role in structure as well as function in the photosynthetic membrane. At this juncture it would appear that its likely function is in the organization of the light harvesting bacteriochlorophyll and carotenoid pigments. This is supported by its presence in photoreceptor complexes where light harvesting bacteriochlorophyll is present, and its absence in reaction center preparations, where the bulk bacteriochlorophyll is also absent. A calculation of stoichiometry based on the content of OSSP in chromatophores shows that there are between 3 and 7 bacteriochlorophyll molecules and between 1 to 2 carotenoid molecules per organic solvent soluble polypeptide. We suggest that this polypeptide functions in the organization of light harvesting bacteriochlorophyll into a functioning red-shifted unit. This does not necessarily imply that these many pigment molecules are bound to one OSSP, but rather we feel it is likely that several OSSP interact and bind a correspondingly larger aggregate of bacteriochlorophyll and carotenoid (Loach, 1976). Determination of the primary structure of this polypeptide would open the way to studying the structure-function relationships of protein, bacteriochlorophyll, and carotenoid pigments.

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