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# Spatial Relationships between the Photochemical Reaction Center and the Light-Harvesting Complexes in the Membrane of *Rhodopseudomonas capsulata*†

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ABSTRACT: The topographical relationships between the photochemical reaction center (RC) and the light-harvesting antenna complexes in the membrane of *Rhodopseudomonas capsulata* were investigated by using reversible chemical cross-linking in conjunction with immunofractionation methods and by using mild detergent fractionation. Cross-linking with the three different reagents 3,3′-dimethyldithiobis(propionimidate) dihydrochloride, dithiobis(succinimidyl propionate), and the photoactivatable compound [(4-azidophenyl)dithio]-succinimidyl propionate yielded essentially identical patterns on two-dimensional polyacrylamide electrophoretograms. In addition to cross-linking between monomer RC subunits H and M and also H and L, close neighbor relationships between H and both polypeptide subunits of the B870 antenna complex as well as the $M_r$ 10000 subunit of the B800-850 complex were implied. A pivotal structural role of H in the coordination of RC-antenna complex interactions is suggested. Mild detergent fractionation was carried out with lithium dodecyl sulfate and Triton X-100. Discrete native pigmented complexes were obtained on polyacrylamide gels containing dodecyl sulfate or Triton X-100, respectively, and also on isoelectric focusing gels containing Triton X-100. The RC was found to be attached to the B870 complex. An RC fragment containing mainly subunit H was found associated with a major part of the B800-850 fraction. In contrast to B800-850, virtually all of the B870 complex was found bound to the RC, probably via both subunits.

The reaction center (RC)[1] and two different light-harvesting antenna complexes are the pigment-protein components of the photosynthetic apparatus of *Rhodopseudomonas capsulata* (*R. capsulata*) (Drews & Oelze, 1981). Light energy is gathered by bacteriochlorophyll *a* (Bchl) and carotenoid bound to polypeptides in the antenna complexes designated B870 and B800-850 according to their near-infrared absorption maxima and transferred to the RC for energy conversion. Biosynthetic investigations and energy-transfer studies with *R. capsulata* and *R. sphaeroides* have led to the idea of the antenna complex B870 being formed at a constant ratio to the RC and being closely associated with it in the membrane (Aagaard & Sistrom, 1972; Monger & Parson, 1977; Feick et al., 1980) whereas the B800-850 complex is synthesized in a variable ratio to the RC depending on growth conditions (Niederman et al., 1976; Schumacher & Drews, 1979; Kaufmann et al., 1982). Energy transfer from B800-850 to the RC is thought to occur via B870 (Monger & Parson, 1977; Feick et al., 1980).

The two pigment-binding polypeptides of the B870 complex of *R. capsulata* have apparent molecular weights of 12000 (12K) and 7000 (7K) (Peters & Drews, 1983a). The B800-850 complex contains three different polypeptides of $M_{r,app}$ 14000 (14K), 10000 (10K), and 8000 (8K), the smaller two of which bind pigment (Feick & Drews, 1979). All four pigment-binding polypeptides of B870 and B800-850 have been isolated and identified by N-terminal sequences (Tadros et al., 1982, 1983; unpublished results) by using the mutant

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[1] Abbreviations: DTSP, dithiobis(succinimidyl propionate); DTBP, dimethyl 3,3′-dithiobis(propionimidate) dihydrochloride; APDP, [(4-azidophenyl)dithio]succinimidyl propionate; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; $LiDodSO_4$, lithium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Bchl, bacteriochlorophyll *a*; RC, reaction center; $M_{r,app}$, apparent relative molecular weight; $Me_2SO$, dimethyl sulfoxide; TEMED, tetramethylethylenediamine.

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strains Y5 ($RC^-$, $B870^-$, $B800–850^+$) and $A1a^+$ ($RC^+$, $B870^+$, $B800–850^-$). Like B800–850, the RC of *R. capsulata* also contains a polypeptide, designated H ($M_r$ 28 000), which does not bind pigment and whose role in the photosynthetic apparatus is unknown. The other two subunits of $M_r$ 24 000 (M) and 20 500 (L) form a photochemically active particle, together with photosynthetic pigment and cofactors (Nieth et al., 1975). The elucidation of topographical relationships of the light-harvesting and RC polypeptides would help to understand the mode of energy transfer and the organization of the bacterial photosynthetic apparatus. Our present knowledge is restricted to information derived from excitation energy-transfer studies (Monger & Parson, 1977; Feick et al., 1980) and information about the transverse arrangement of RC subunits in the membrane of *R. sphaeroides* (Bachmann et al., 1981; Valkirs & Feher, 1982, and references therein). Little is known about the lateral topographical relationships of RC polypeptides with the light-harvesting complexes.

In situ topographical analysis by reversible chemical cross-linking is an emerging approach in structural investigations of biomembranes. Using reversible cross-linking in conjunction with immunofractionation and two-dimensional mapping on sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels, we have recently shown cross-linking of RC subunit H with subunits M and L as well as with a 7K polypeptide possibly associated with the B870 complex (Takemoto et al., 1982). Here, we present a more comprehensive study of the topographical relationships between the RC subunits and the light-harvesting polypeptides in the membrane of *R. capsulata*, using three different reversibly cleavable reagents, including the novel photoactivatable compound [(4-azidophenyl)dithio]succinimidyl propionate (APDP).

To complement the cross-linking studies, we have used mild detergent fractionation of chromatophores and analysis of the protein complexes. Treatment and polyacrylamide gel electrophoresis with lithium dodecyl sulfate ($LiDodSO_4$) in the cold have been recognized as a valuable technique in structural investigations of pigment–protein complexes (Delepelaire & Chua, 1979) and have been successfully applied to membranes of *R. sphaeroides* (Broglie et al., 1980). In addition to the ionic agent $LiDodSO_4$ we have employed the nonionic detergent Triton X-100 to fractionate the photosynthetic membrane of *R. capsulata*.

## Materials and Methods

*Chemicals.* All reagents employed were analytical grade. [(4-Azidophenyl)dithio]succinimidyl propionate (APDP), dithiobis(succinimidyl propionate) (DTSP), and dimethyl 3,3′-dithiobis(propionimidate) dihydrochloride (DTBP) were purchased from Pierce Eurochemie, Rotterdam, Holland. $NaDodSO_4$, $LiDodSO_3$, dithioerythritol (DTE), glycine, agarose, and ampholytes were bought from Serva, Heidelberg. Trizma base and Sepharose CL-4B–protein A were from Sigma, Munich. L-[$^{35}$S]Methionine was from Amersham (Buchler, Braunschweig), Triton X-100 was bought from Merck, Darmstadt, fluorochemicals were from Roth, Karlsruhe, and protein standards were from Boehringer, Mannheim, all West Germany.

*Organisms and Growth Conditions. Rhodopseudomonas capsulata*, wild-type strain St. Louis (ATCC 23782), and the mutant strain $A1a^+$ ($B870^+$, $B800–850^-$, $RC^+$, $Crt^-$, $Bchl^+$) (Drews et al., 1971) were grown anaerobically in the light (2000 lx) at 30 °C in 5-mL screw-capped bottles (Schumacher & Drews, 1979). For in vivo labeling with L-[$^{35}$S]methionine casamino acids (0.05%) were added instead of yeast extract. Radiolabel was added (100 μCi/5 mL) during logarithmic growth and cells harvested approximately 5 h later at $A^{1cm}_{680nm}$ of about 1. More than 95% of the label was incorporated by the organisms. The cells were sonicated (10-s bursts) 10 times with intermittent 1-min intervals in an ice–water bath. Pigmented membranes were purified on sucrose gradient as described by Garcia & Drews (1980). Membranes were suspended in 50 mM triethanolamine hydrochloride, pH 8.3, at 1 mg of protein/mL as estimated according to Lowry et al. (1951) and stored at 4 °C in the presence of 2 mM sodium azide.

*Reaction Center and Immunoglobulin G (IgG) Preparation.* RCs were purified from *R. capsulata*, strain $Ala^+$ as described by Nieth et al. (1975) and Feick (1980). Rabbit antisera directed against RCs were obtained as described by Dierstein et al. (1981). IgG fractions were prepared by ammonium sulfate fractionation and stored at 4 °C in 10 mM sodium phosphate, pH 8.0, and 5 mM sodium azide.

*Cross-Linking Procedures.* Cross-linking was carried out with APDP (very hydrophobic), DTSP (hydrophobic), and DTPB (hydrophilic). Membrane permeation of all reagents was indicated by cross-linking of the 10K subunit of B800–850 (Peters & Drews, 1983b) which is probably not exposed on the chromatophore surface (Feick & Drews, 1980; J. Peters and G. Drews, unpublished data).

Cross-linking with DTSP was conducted as described by Peters & Drews (1983b), except that the reaction took place at pH 8.3. Cross-linking with DTBP was performed in the same manner except that the material was added as a solid in 5–10 increments and that the reagent concentration was 7.5 mM. For cross-linking with APDP the reagent was dissolved in dimethyl sulfoxide ($Me_2SO$) and added to the membrane sample adjusted to 1 mg of protein/mL in 50 mM triethanolamine, pH 8.3. All operations involving APDP were carried out in the dark. Final concentrations were 0.25–1 mM APDP and 10 μL/mL $Me_2SO$. After incubation at room temperature for 30 min, the samples were exposed to light. For short-term illumination samples were irradiated with an Osram HBO 200 lamp for 10–30 s in quartz tubes. Long-term exposure to either daylight or white neon light (Osram L65W/25) was performed in ordinary glass tubes. Because termination of the reaction as with conventional reagents is not possible, samples were solubilized in the dark at room temperatures with 1 volume of buffer containing 150 mM Tris-HCl, pH 6.7, 20 mg of $NaDodSO_4$/mL, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 100 μL/mL glycerol. After 30 min samples were heated to 60 °C for further 10 min and subsequently applied to analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis. For immunofractionation, solubilization was performed as described below.

*Immunofractionation Procedure.* Two different methods of detergent treatment were adopted: (1) Membranes were treated with a solution containing 160 mM Tris, 80 mM sodium acetate, pH 8.8, 2 mM EDTA, and 20 mg/mL Triton X-100 (TA buffer) for 5 h at room temperature. (2) Membranes were treated with an equal volume of TA buffer containing 10 mg of $NaDodSO_4$/mL instead of Triton, heated at 60 °C for 10 min, and immediately diluted with 15 volumes of cold (4 °C) TA buffer containing Triton X-100.

Samples containing APDP were kept in the dark. After detergent treatment, samples containing 100 μg of membrane protein were spun in an Eppendorf centrifuge (12000*g*) for 15 min, and 10 μL of anti-RC IgG was added to the supernatant. All steps were performed in Eppendorf reaction vials at 4 °C. After incubation for 15 min, 60 μL of swollen protein A–Sepharose gel suspended in an equal volume of TA buffer

was added and the samples gently agitated by placing the vials in a beaker of water on a magnetic stirrer. After 2 h the Sepharose gel was sedimented by spinning in an Eppendorf centrifuge for 2 min and washed 3 times with TA buffer and another 3 times with TA buffer containing no Triton. After addition of 100 μL of solubilization buffer containing 150 mM Tris-HCl, pH 6.7, and 20 mg of $NaDodSO_4$/mL, the samples were heated at 37 °C for 2 h with frequent agitation. The supernatant was removed and after addition of 10 μL of glycerol immediately subjected to electrophoresis.

*Two-Dimensional Polyacrylamide Gel Electrophoresis.* Two-dimensional (diagonal) mapping was conducted as described by Peters & Drews (1983b), except that the presence of RCs in the samples necessitated immediate application of first-dimension gel strips to diagonal mapping, thus avoiding storage involving freezing. Unlabeled RCs and commercially available protein standards were coelectrophoresed for molecular weight calibration in the first dimension. Spots on diagonal maps were detected by staining with Coomassie Brilliant Blue R250 and by fluorographic methods as described by Bonner & Laskey (1974), with exposure times ranging between 2 h and 4 weeks.

*Detergent Fractionation Studies.* (*1*) *$LiDodSO_4$ Fractionation.* Chromatophores of *R. capsulata*, strain St. Louis, were adjusted to 0.5 mg of Bchl/mL and treated dropwise with $LiDodSO_4$ to a final concentration of 10 mg/mL at 0 °C for 15 min. The samples were spun in an Eppendorf centrifuge for 15 min, and the supernatant was applied to polyacrylamide gels prepared as described by Schumacher & Drews (1978), with the following alterations: Electrophoresis was conducted at 4 °C in the dark. Wells of 1-mm gels were loaded with samples containing 5–10 μg of Bchl. Separation was effected on 7–18% acrylamide gradients. For electrophoresis in the second dimension single strips were excised and treated with 150 mM Tris-HCl, pH 6.7, 30 mg of $NaDodSO_4$/mL, 1 mM PMSF, 10 mM DTE, and 100 μL/mL glycerol at 37 °C for 1 h. The gel strip was then applied to electrophoresis as described above the diagonal mapping. Gels were stained with Coomassie Blue. In situ spectra were recorded on an Uvikon 810 Kontron spectrophotometer.

(*2*) *Triton X-100 Fractionation.* Chromatophores were adjusted to 0.3 mg of Bchl/mL, treated dropwise with 100 mg of Triton X-100/mL to a final concentration of 15 mg/mL Triton at 0 °C for 30 min, and subsequently subjected to electrophoresis as described for $LiDodSO_4$ fractionation except that $NaDodSO_4$ in the gel buffers was replaced by 0.75 mg of Triton/mL and only 2–4 μg of Bchl/mL was loaded into a single well. Separation was effected on 5–9% acrylamide gradients. For analysis of single bands under denaturing conditions by $NaDodSO_4$–polyacrylamide gel electrophoresis, half of the respective gel slice was treated with 150 mM Tris-HCl, pH 6.7, 30 mg of $NaDodSO_4$/mL, 10 mM DTE, and 1 mM PMSF, placed on acrylamide gels that contained a stacking layer without wells, and melted onto the gel with 15 mg of agarose/mL in 100 mM Tris-HCl, pH 6.7, and 1 mg of $NaDodSO_4$/mL at 50 °C at a lateral distance to other gel pieces of ca. 1 cm.

*Isoelectric Focusing.* For isoelectric focusing, membrane samples (100–200 μL containing 10–20 μg of Bchl/mL) were solubilized as described above. After one-fourth of the sample volume of 250 mM Tris, 80 mM glycine, pH 9.3, and 500 mg of sucrose/mL was added, samples were applied to tube gels containing 5% acrylamide, 2% ampholytes, pH range 5–7, 0.5 mg of Triton X-100/mL, 200 mg of sucrose/mL, 1 μg/mL sodium peroxydisulfate, and 0.5 μL of TEMED/mL by using 10 mM $H_3PO_4$ as the anolyte and 20 mM NaOH as the catholyte. Prefocusing was carried out for 30 min at 1 mA/gel at 4 °C in the dark. Isoelectric focusing of membrane samples was conducted at a maximum of 50 mW/gel (200–400 V) to prevent joule heating. Gels were completely immersed in catholyte agitated with a magnetic stirrer. Focusing was terminated as soon as equilibrium was attained. Analysis in the second dimension was performed by incubating excised gel slices in 150 mM Tris-HCl, pH 6.7, 30 mg of $NaDodSO_4$/mL, 1 mM PMSF, and 10 mM DTE at 37 °C for 24 h and subjecting the extracts to electrophoresis as above after addition of 10 μL of glycerol/100 μL.

## Results

*Cross-Linking Studies.* (*1*) *Immunofractionation.* In studies of the lateral topographical relationships of reaction center polypeptides we have placed particular emphasis on in situ near-neighbor analysis using reversible chemical cross-linking. To investigate the validity of this approach, we employed three cross-linking agents differing in functional groups and in their solubility in water. The complexity of the polypeptide patterns received upon cross-linking of intact membrane vesicles and two-dimensional (diagonal) mapping on $NaDodSO_4$–polyacrylamide gels necessitated fractionation of membrane polypeptides prior to analysis. Efficient separation of species containing RC polypeptides from other membrane constituents was achieved by using immunofractionation with purified anti-RC IgG. Whereas $NaDodSO_4$ treatment leads to specific recognition by the antibody of only H and very little L [see also Takemoto et al. (1982)], all three RC subunits are recovered with Triton. With both methods, a small amount of an unidentified $M_r$ 40 000 polypeptide was also precipitated. With respect to contamination with other membrane polypeptides, both fractionation methods were roughly equivalent. Triton extraction for periods shorter than 3 h led to increased levels of contamination.

(*2*) *Cross-Linking Conditions.* For reversible cross-linking, reagent concentrations were chosen so as to give significant yields of cross-linking without causing perturbation to the membrane as monitored by absorption spectral measurements. The native infrared absorption represents a sensitive probe of protein–pigment complex integrity. Reversible bleaching of the RC absorption peak at 865 nm with actinic light is an accepted assay for photochemical activity of the reaction center [see Feher & Okamura (1978)]. At reagent concentrations of 20 mM DTSP and DTBP, small spectral changes could be detected, whereas at the concentrations employed no effect was observed and reversible bleaching at 865 nm was unaffected (error margin ±5%) with all three reagents both with membranes and with isolated RCs. The yield of cross-linking was maximal when the reagents were added incrementally in 5–10-min intervals which also minimized the formation of high molecular mass species with apparent molecular weights above the exclusion limit of acrylamide gels used (ca. $5 \times 10^5$). The electrophoretic diagonal maps received with APDP (see below) after long-term illumination of samples with daylight as well as with white neon light did not differ significantly from those obtained with 10–30-s intense irradiation with a mercury vapor lamp. However, polypeptide spots were generally found to be more distinct with weak white light and glass tubes virtually excluding UV radiation.

(*3*) *Cross-Linking Involving RC Polypeptides.* When membranes were cross-linked with DTSP and immunofractionated by using the $NaDodSO_4$ treatment, maps such as shown in Figure 1 typically resulted. Spots below the diagonal corresponding to light-harvesting polypeptides stained much

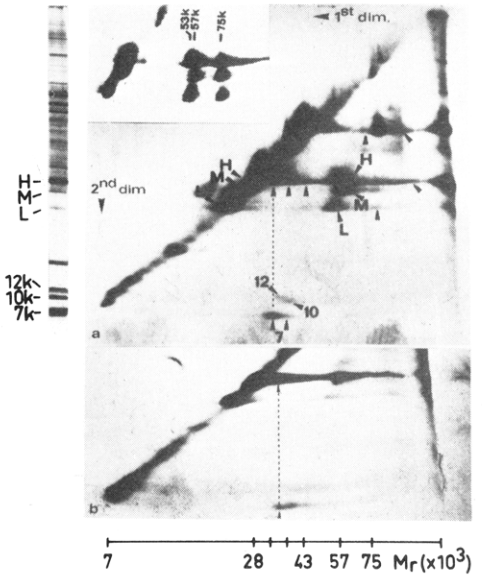


FIGURE 1: Diagonal mapping of DTSP cross-linked chromatophores fractionated with anti-RC IgG ($NaDodSO_4$ treatment; see Materials and Methods). Part a (top) shows a fluorogram. Cross-linking was conducted with DTSP for 30 min. Spots vertically related to RC subunit H are denoted with arrows, and the vertical alignment corresponding to 7K–H is demonstrated with a dashed line. The insert in (a) shows the two-dimensional pattern of lauryldimethylamine oxide solubilized RCs (1 mg of protein/mL) at 0.2 mM DTSP for 2 min. Part b shows the cross-linking pattern of strain A1a$^+$ chromatophores obtained under the same conditions.

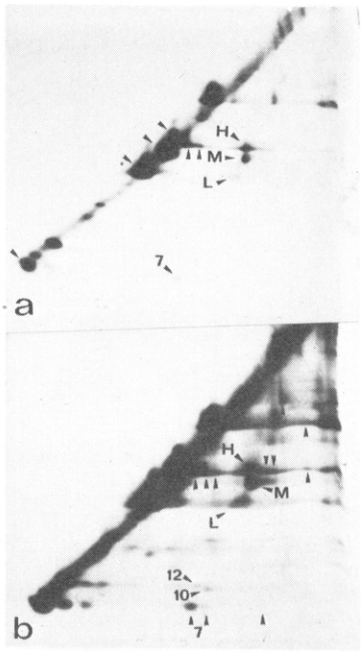


FIGURE 2: Diagonal mapping of APDP cross-linked chromatophores, immunofractionated with anti-RC IgG (Triton X-100 treatment; see Materials and Methods). Two fluorograms of the same gel (exposure time 2 and 20 days) are shown. Cross-linking was conducted at 1 mM APDP; photoactivation of the reagent was effected with white neon light for 1 h. Arrows at 63K and 70K (64K denoted) indicate M–H–7K and M–H–(2 × 7K). Possible vertical alignments of H with other polypeptides are indicated by arrows.

more lightly than RC polypeptides. This is to a large extent due to the lower specific labeling rates of light-harvesting polypeptides obtained under the experimental conditions used.

The incorporation of the $^{35}S$ label was found not to be proportional to the methionine content with the light-harvesting polypeptides (unpublished results). Another reason for weaker staining is the increased broadening of spots with the distance of migration.

Apparent molecular weight values of cross-linked species were determined with protein standards coelectrophoresed in the first dimension. The monomer constituents of cross-linked species were identified according to their respective migration distance in the second dimension. Vertical relationships at $M_{r,app}$ 53 000 and 57 000 match roughly with the values expected for monomer copies of H–L and H–M, respectively. A comparison of results obtained with Triton X-100 and $NaDodSO_4$ immunofractionation procedures (not shown) rules out the possibility that spots below the diagonal corresponding to M and L are derived from dimers of the respective polypeptides: species not containing H would not appear in maps obtained after $NaDodSO_4$ treatment. The agreement of the data received with both fractionation procedures pertains to all reagents used. Thus, vertical relationships of M and L to polypeptides other than H were not observed.

For comparison, the insert in Figure 1a shows the two-dimensional cross-linking pattern of purified RCs received with the same reagent. Apart from H–L and H–M at 53K and 57K, H–M–L at 75K is also seen. This reflects cross-linking of both M and L to H, as both H–M and H–L occur at significant yields with purified RCs whereas H–L is generally faint with chromatophores. Control experiments without cross-linking did not reveal any spots below the diagonal [see also Takemoto et al. (1982)].

It is important to note that cross-links within isolated RCs are formed at much higher yields than with chromatophores, even if the reaction time is 15 times shorter and the reagent concentration 5 times lower as in this case. This discrepancy was generally observed and has also been found with the isolated B800–850 complex (Peters & Drews, 1983b). As hydrolysis of the reagent is a competing reaction, prolonged incubation did not elevate the yields of cross-linking appreciably.

Spots belonging to H and 7K vertically aligned at $M_{r,app}$ 33 000, 38 000, and 43 000 in the first dimension (indicated by arrows in Figure 1a) are attributable to cross-linking of one, two, and three copies of 7K to H, respectively. With the maximal concentration of DTSP used (2.5 mM) up to four copies of 7K binding to H have been resolved, whereas three was a minimal number consistently found. Polypeptides 10K and 12K are also found to cross-link to H. The yields, however, are much lower than with 7K. As 7K of B870 and 8K of B800–850 migrate very closely on gels, cross-linking was also carried out on chromatophores of the mutant strain A1a$^+$, which contains no B800–850. Figure 1b shows that the cross-linking pattern of 7K obtained with this mutant may be superimposed on the one in Figure 1a, thus confirming the identification of this polypeptide on diagonal maps.

With membranes, we have not detected any cross-linked species containing RC subunits in more than one copy. To demonstrate the potential of RC polypeptides to form inter-RC cross-links, DTSP cross-linking was also carried out with an RC preparation that had previously been extensively dialyzed.

RCs thus treated aggregate and eventually precipitate out of solution. Indeed, considerable inter-RC cross-linking was found. Taking these data into account, it appears unlikely that oligomers of the RC are present in the chromatophore membrane.

Figure 2 shows a map obtained with the photoactivatable reagent APDP. This pattern was qualitatively independent of the illumination conditions used. Although the azido site of APDP is supposed to react unspecifically with proteins, no

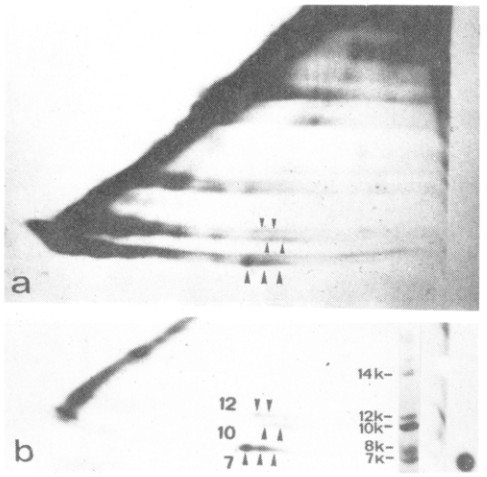


FIGURE 3: Diagonal mapping of DTBP cross-linked chromatophores. Cross-linking was conducted at 7.5 mM DTBP for 30 min. Maps obtained from an identical sample without (a) and with (b) immunofractionation (anti-RC IgG, Triton X-100) are shown. Spots indicated by arrows represent cross-linking to H, as in Figures 1 and 2. Part b reveals spots corresponding to 7K, 10K, and 12K, indicating cross-linking of these polypeptides to H (compare with Figures 1 and 2).

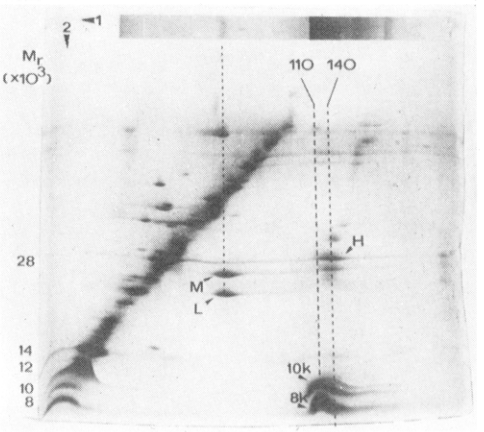


FIGURE 4: Diagonal mapping of $LiDodSO_4$-fractionated chromatophores. Fractionation and two-dimensional electrophoresis are described under Materials and Methods. The horizontal track was excised from the first-dimension gel; visible bands are native pigmented. The two-dimensional gel below was stained with Coomassie. The dashed lines indicate the vertical relationships L–M, 8K/10K, H–(8K/10K), and M–(8K/10K).

additional species with respect to DTSP and DTBP are resolved. Spots at $M_{r,app}$ 63 000 and 70 000 (arrows) may be attributed to M–H–7K and M–H–(2 × 7K), respectively, as both H–M and H–7K are formed in significant amounts.

Figure 3 shows a comparison between maps obtained from an identical sample with and without immunofractionation prior to cross-linking with DTBP. Figure 3a shows that cross-linking of 7K to H prevails over intra-RC cross-linking, in contrast to the results obtained with APDP (Figure 2). Intra-B800–850 and –B870 cross-linking is more pronounced than binding of these complexes to the RC. To resolve these complex patterns, studies with mutant strains lacking either of the two antenna complexes such as by Peters & Drews (1983b) are required. A comparison between the two parts of Figure 3 shows that the immunofractionation procedure leads to a virtually complete removal from the map of polypeptides other than those cross-linked to H. No 14K or 8K are detected below the diagonal. Spots corresponding to 12K and 10K are faint but are specifically immunoadsorbed from a fraction of cross-linked polypeptides.

By comparing Figure 3b (DTBP) with the corresponding mapping patterns shown in Figure 1 (DTSP) and Figure 2 (APDP), it is evident that the three different reagents give comparable results. However, with APDP, cross-linking of H to 7K occurs at similar yields as H–M, whereas with DTBP, H–7K is much more pronounced.

In summary, specific vertical relationships of RC subunit H with M and L as well as with light-harvesting polypeptides 7K and much less pronounced 12K of B870 and 10K of B800–850 have been observed. Maps obtained with three different cross-linking agents were essentially congruent. RCs are likely to occur in monomer copies in the membrane.

*Detergent Fractionation Studies.* (*1*) *$LiDodSO_4$ Fractionation.* Chromatophores of *R. capsulata*, strain St. Louis, were treated with $LiDodSO_4$ and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis as described under Materials and Methods. Two major native pigmented bands could be discerned: A red-pigmented band at $M_{r,app}$ 110 000 gave a spectrum identical with that of the native B800–850 [refer to Shiozawa et al. (1982)]. This band migrated to the same position in the gel as the one obtained with identically treated chromatophores of the mutant strain Y5 containing B800–850 as the only pigmented complex. Minor pigmented bands (see top of Figure 4) at apparent molecular weights higher than that of the major band were also identical with both strains, indicating that these bands represent aggregational states of native B800–850. As described for the mutant strain Y5 (Shiozawa et al., 1982), the major band was found to contain the two pigment-binding polypeptides of B800–850, as revealed by the diagonal map shown in Figure 4.

A violet pigmented band at $M_{r,app}$ 50 000 exhibited a native RC spectrum, including carotenoids. Bleaching of the absorption at 865 nm could be demonstrated. The same result has been reported for the RC of *R. sphaeroides* (Broglie et al., 1980). Diagonal mapping also revealed a relationship between the RC and the B800–850 complex: At $M_{r,app}$ 140 000 there appeared a spot corresponding to subunit H and, less pronounced, M (Figure 4). Analogous studies with the B800–850 defective mutant strain A1a$^+$ revealed no RC subunit at a high apparent molecular weight. Although spots of 8K and 10K were not resolved from the components of the unmodified complex at $M_{r,app}$ 110 000, the position of the high molecular weight spots of H and M leaves no other likely explanation than an association of these polypeptides with B800–850. The apparent molecular weight values determined in this system serve to show that the high molecular weight spots of H and M are skewed off with respect to the unmodified B800–850 complex by a distance expected for a complex containing the native B800–850 unit and H or M. We are aware that with this gel system the apparent molecular weight depends on the acrylamide gradient used as demonstrated by Shiozawa et al. (1982) for B800–850. Spots above the diagonal probably represent polypeptides aggregated during harsh $NaDodSO_4$ treatment.

Under the prevailing conditions only small amounts of 12K appear at high apparent molecular weight, indicating destruction of B870. The major portion of H also appears as a monomer on the diagonal. It should be noted, that gels have to be slightly overloaded with protein to ensure complete preservation of the pigment–protein complexes.

(*2*) *Triton X-100 Fractionation.* The nonionic detergent Triton X-100 was used in addition to $LiDodSO_4$ to study the relationships between the RC and the light-harvesting polypeptides. With this detergent H is not cleaved from L–M

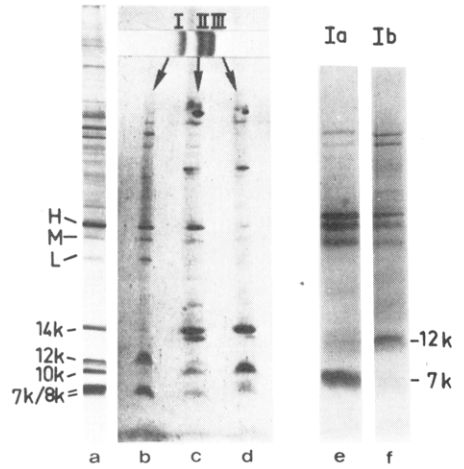


FIGURE 5: Separation of pigment–protein complexes on Triton X-100–polyacrylamide gels. Tracks b–f represent polypeptide patterns of pigmented protein complexes on denaturing $NaDodSO_4$ gels. (a) Reference track of strain St. Louis chromatophores; (b–d) band patterns of the three native pigmented bands obtained by Triton X-100 polyacrylamide (5–9%) gel electrophoresis at pH 8.8. The section of the Triton gel containing the three red pigmented bands (I–III) is horizontally superimposed on tracks b–d. The bands were excised and subjected to electrophoresis in the second dimension after the denaturing $NaDodSO_4$ treatment as described under Materials and Methods. Tracks e and f show subfractions (Ia and Ib) of the RC–B870 complex (I, track b) obtained from strain $A1a^+$ chromatophores by isoelectric focusing as described under Materials and Methods.

(Nieth & Drews, 1974) and 14K of B800–850 is not removed from 8K/10K (Feick & Drews, 1978).

Electrophoretic separation of chromatophores extracted with Triton X-100 and electrophoresed under mild conditions on 5–9% polyacrylamide gels was conducted as described under Materials and Methods. Three distinct pigmented bands resulted, as shown in Figure 5. As bands II and III were too close to be resolved by diagonal mapping, they were excised and analyzed separately on $NaDodSO_4$–polyacrylamide gels. Band I contained B870 polypeptides 7K and 12K and the three subunits of RC (Figure 5). Band II was comprised of RC (mainly H) and the B800–850 subunits 14K, 10K, and 8K, and band III contained mainly the polypeptides of the B800–850 complex. Band I showed the native spectral characteristics of the constituent pigment–protein complexes (absorption maxima at 802 and 870 nm), whereas bands II and III gave a spectrum of B800–850, in which the absorption peak ratio $A_{850}$:$A_{800}$ was reduced to 1:1 as opposed to 1.55:1 for native B800–850 (Feick & Drews, 1979). Band II contained no detectable native RC–Bchl. Free RC, which migrates to a lower position in the gel, was not detected, but some L was found at a longer migration distance by two-dimensional mapping (not shown). Thus, virtually the total amount of RC is likely to be bound to the light-harvesting complexes in the photosynthetic membrane of *R. capsulata*. To establish that the pigmented complexes observed did not arise from the fractionation procedure itself, chromatophores surface iodinated in situ were fractionated in the same manner. Specific labeling of polypeptide H in band II (Figure 5) was 7 times higher than in band I (J. Peters and G. Drews, unpublished data). Thus, bands I and II may be assumed to represent topographically distinct fractions.

At pH 8.8 separation in 5–9% polyacrylamide gradient gels containing Triton X-100 took place largely according to size as the apparent isoelectric points of the pigment–protein complexes investigated are very similar and lie about 3 units below the running pH of the gel (unpublished results) and as the migration distances observed depend strongly on the acrylamide gradient used. Native isoelectric focusing using nonionic detergents is an alternative tool for the resolution of native proteins (Helenius et al., 1976), although artifactual aggregation may occur (Simon et al., 1973). We found that under the conditions of isoelectric focusing the native RC complex of strain $A1a^+$ obtained by Triton fractionation on chromatophores is partially subfractionated into two separate species consisting of the RC and, alternatively, one of the two B870 polypeptides, as shown in Figure 5 (tracks e and f). Spectra indicated association of partially degraded B870 with native RC.

Isoelectric focusing experiments with lauryldimethylamine oxide as detergent (not shown here) using the wild-type strain as well as the mutant strain $A1a^+$ also revealed RC-7K as a distinct band, whereas 12K migrated separately as a partially denatured B870 complex comparable to the one isolated by Feick & Drews (1978).

In summary, detergent fractionation with $LiDodSO_4$ and Triton X-100 yielded discrete and largely native pigment–protein complexes. $LiDodSO_4$ treatment cleaved H from L–M of RC and 14K from 8K/10K of B800–850 and destroyed most of the B870 complex. These interactions were not affected by Triton. However, both approaches revealed a considerable amount of H and less M in association with the B800–850 complex.

## Discussion

In this study we have used two independent, complementary approaches to identify the lateral topographical relationships of the reaction center in the membrane of *Rhodopseudomonas capsulata*. Reversible chemical cross-linking revealed associations of polypeptides having a distance of about 1 nm or less within the membrane. As the penetration of the reagent to the site of reaction and the availability of reactive sites are prerequisites for the formation of covalent bridges, three reagents varying widely in their solubility in water and having different functional groups were employed. The results presented above show that the solution behavior of reagents is not critical in the system investigated. However, reagents much more hydrophobic than APDP such as dithiobis(phenyl azide), which does not require any specific reactive sites, could not be applied in aqueous solutions containing less than 5% $Me_2SO$. The reagent APDP was used not only to detect possible relationships not captured by DTSP and DTBP because of their requirement for properly oriented primary amino groups but also to investigate whether the cross-links observed might represent artifacts caused by diffusion-controlled random collisions of proteins in the membrane. Similar photoactivatable reagents have been used by Mikkelsen & Wallach (1976), Huang & Richards (1976), Ji (1977), and Kiehm & Ji (1977). The reactive azidonitrene formed upon illumination may be estimated to have a lifetime of about 1 ms in a membrane environment (Kiehm & Ji, 1977).

As diffusion coefficients of photosynthetic polypeptides have, to our knowledge, not been reported, random cross-linking may not be entirely ruled out by using a photoactivatable reagent. It is, however, instructive to compare the results obtained with this reagent with the data received with the cross-linkers DTSP and DTBP which have half-lifes of minutes in aqueous solution, are, thus, active for several orders longer than APDP, and should yield considerably more random cross-linking, if the polypeptides investigated were diffusible. The agreement of the data obtained with the three reagents, however, indicates that the interactions between photosynthetic polypeptides in the membrane are so strong that only specific, permanent close-neighbor relationships are observed.

The absence of certain vertical relationships which are to be expected because of the occurrence of the respective polypeptides within the same detergent-solubilized protein–pigment complex suggests that the sterical requirements for cross-linking to occur are quite stringent.

The complete absence of cross-links between polypeptides containing abundant lysine provides additional evidence for the rigidity of the bacterial photosynthetic apparatus and the validity of the cross-linking approach with this system: RC subunit H (13 lysine residues with the closely related organism *R. sphaeroides*) (Steiner et al., 1974) and 14K of B800–850 (8 lysine residues) (Shiozawa et al., 1980) cross-link at the highest relative yields; however, not with each other. Thus, if the occurrence of 14K–H were defined as the artifactual background of randomly cross-linked polypeptides, random cross-linking is nonexistent on the levels detected in the present investigation. Disulfide exchange may be ruled out for the same reason. As both H (Webster et al., 1980) and 14K (Feick & Drews, 1979) are exposed on the cytoplasmic surface of the membrane, and absence of 14K–H also renders intervesicular cross-linking most unlikely.

We have already emphasized that comparatively low yields of cross-linking are obtained with chromatophores. This may to some extent be attributable to the presence of phospholipids which might also hinder access of the reagent to the specific reactive sites. Phosphatidylethanolamine is a major constituent of the photosynthetic membrane of *R. capsulata* (Steiner et al., 1970), and the amino groups thus present in great abundance compete for reaction with the reagents employed.

The cross-linking data indicate that the B870 light-harvesting complex and, possibly, the B800–850 complex are associated with RC subunit H, although the low yields of cross-linking do not permit conclusions on the extent of these interactions. Consistent with previous suggestions (Takemoto et al., 1982) H appears to assume an important structural role in the membrane in the coordination of RC–antenna complex interactions. Speculations that H provides binding sites for proteins associated with energy transduction or with the electron transport chain were expressed earlier by Feher & Okamura (1978). Unidentified vertical relationships of H with high molecular weight polypeptides as indicated above (Figure 4) might reflect such interactions.

Detergent fractionation and analysis represent complementary and quantitative means for investigating the lateral membrane topography. The fact that only three pigmented and discrete bands are obtained on polyacrylamide gels when separation is effected according to charge and size supports the idea that the relationships observed reflect actual topographical relationships as opposed to micellar artifacts. As the RC–B870 complex migrates as a single band when solubilized with Triton X-100, a fixed stoichiometry of this complex, which has previously been implied by Schumacher & Drews (1978) and Drews et al. (1981) on the basis of biosynthetic investigations, is suggested. The detergent fractionation data also indicate that a considerable amount of B800–850 is attached to RC subunit H in a stoichiometrically defined manner. Taking together the evidence obtained with the two approaches, it appears that monomer RC particles are surrounded by B870 in a fixed stoichiometry whereas the B800–850 complex is to a large extent bound to RC or a fragment of RC containing mainly subunit H. Whereas RC–B870 interactions may be reconciled with currently discussed variations of the lake model (Monger & Parson, 1977; Drews et al., 1981), the relationship of the B800–850 complex with the RC cannot be accommodated in currently favored models of the bacterial photosynthetic apparatus and will require further investigation. Most recent studies in our laboratory involving reconstitution of B800–850 and RC into liposomes have revealed spontaneous aggregation of both pigment–protein complexes, and fluorescence emission data indicate energy transfer between the B800–850 complex and reaction center which is, however, less efficient than with B870–RC (T. Schonhardt, unpublished results).

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# Prelysosomal Divergence of Transferrin and Epidermal Growth Factor during Receptor-Mediated Endocytosis†

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ABSTRACT: The routes followed by epidermal growth factor and transferrin during their endocytosis by human epithelial cells were compared in double-label studies by using density gradient centrifugation of cell homogenates and fluorescence microscopy with intact cells. Gradient centrifugation studies of cells incubated with radioactively labeled epidermal growth factor and transferrin indicated that both ligands initially were associated with a class of vesicles having a density of 1.037 g/mL and then were rapidly transferred to a membrane compartment having a slightly higher density (1.039 g/mL). Subsequently, the two ligands diverged. Epidermal growth factor ultimately was transferred to a membranous compartment containing lysosomal enzymes (density 1.08 g/mL) where it was degraded. Transferrin was released intact from the cells; very little was transferred to lysosomes. Using fluorescently labeled ligands, it was observed that after cells were warmed to 37 °C for 5 min, transferrin and epidermal growth factor gave coincident, punctate fluorescent patterns, strongly suggesting they were localized within the same endocytic vesicles. Subsequently, the epidermal growth factor signal was observed in lysosomes whereas the transferrin signal became weaker and diffuse and did not coincide with the punctate epidermal growth factor fluorescence. The time course of the divergence of the radioactive and fluorescent ligands coupled with the previous morphologic studies on the pathway of epidermal growth factor internalization [Willingham, M. C., & Pastan, I. (1982) *J. Cell Biol. 94*, 207–212] suggests that the sorting process is prelysosomal and possibly Golgi associated.

Receptor-mediated endocytosis is the process involved in the cellular entry of certain hormones, plasma proteins, viruses, and bacterial toxins (Pastan & Willingham, 1981a,b; Goldstein et al., 1979). Ligands enter cells via clathrin-coated pits in the plasma membrane and are transferred to receptosomes, a term used to describe these endocytic vesicles to emphasize their role in receptor-mediated endocytosis (Pastan & Willingham, 1981a,b; Willingham & Pastan, 1980). Receptosomes are acidic (Tycko & Maxfield, 1982) and devoid of a clathrin coat Willingham & Pastan, 1980). Similar structures also have been termed endocytic vacuoles (Wall et al., 1980) or endosomes (Helenius et al., 1980). In double-label studies, many different ligands have been visualized entering cells together in the same coated pits and receptosomes, indicating that a common initial route of cellular entry exists for many receptor-bound ligands (Pastan & Willingham, 1981a,b; Dickson et al., 1981b; Via et al., 1982; Willingham et al., 1981, 1983a).

At present, two intracellular destinations for ligands entering cells by receptor-mediated endocytosis have been defined. Low-density lipoproteins, $\alpha_2$-macroglobulin ($\alpha_2$M),[1] asialo-

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[1] Abbreviations: $\alpha_2$M, $\alpha_2$-macroglobulin; EGF, epidermal growth factor; EGF–HRP, epidermal growth factor–horseradish peroxidase conjugate; PBS, Dulbecco's phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; TES buffer, triethanolamine-, EDTA-, and sucrose-containing buffer; IgG, immunoglobulin G; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ER, endoplasmic reticulum; UDP-galactose, uridine diphosphogalactose; RH, rhodamine; FL, fluorescein.