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# Lateral Organization of Proteins in the Chromatophore Membrane of *Rhodospirillum rubrum* Studied by Chemical Cross-Linking

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## Abstract

The organization of proteins in the chromatophore membrane, particularly of the reaction center and the light-harvesting polypeptide, was examined by the use of a hydrophobic and a hydrophilic cross-linking reagent, namely DSP (dithiobis-succinimidyl propionate) and glutaraldehyde. The linkage of proteins was studied by SDS polyacrylamide pore gradient electrophoresis. DSP was shown to link proteins within the core of the membrane. The subunit H of the reaction center is linked with DSP at a low concentration, either with itself or with other membrane proteins but not to the subunits M and L. In isolated reaction centers the subunits H are exclusively linked with each other. With increasing concentrations of DSP the bands of the subunits M, L, and the light-harvesting polypeptide disappear simultaneously from the gel, suggesting that these proteins are linked together. This hypothesis is supported by the finding that reaction centers isolated from chromatophores treated with DSP retain an appreciable amount of light-harvesting polypeptide. With increasing concentrations of the hydrophilic cross-linking reagent glutaraldehyde, the bands of all the three subunits of the reaction center, H, M, and L, progressively disappear from the gel, suggesting that they are linked together. The light-harvesting polypeptide remains free when this reagent is used.

**Key Words:** Chromatophore membrane; reaction center; light-harvesting polypeptide; cross-linking; dithiobis-succinimidyl propionate; glutaraldehyde; SDS-PAGE.

## Introduction[3]

In many purple bacteria such as *Rhodospirillum rubrum* the components of the photosynthetic apparatus are located in the intracytoplasmic membranes. These membranes can be isolated as vesicles: the chromatophores. The

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[3] Abbreviations used: DSP, dithiobis-succinimidyl propionate; SDS, sodium dodecyl sulfate; H,M,L, heavy, medium, and light subunit of the reaction center; PAGE, polyacrylamide pore gradient electrophoresis.



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cytoplasmic side of the membranes is exposed on the outside of these vesicles (Oelze, 1978). The photosynthetic apparatus consists of the light-harvesting complex, the reaction center, and other components of the photosynthetic electron transport chain. Much is known already about the composition and the photochemistry of the photosynthetic apparatus but little about the spatial arrangement of the individual molecules within the membrane.

The topology of membrane proteins was studied first with antisera raised against the whole reaction center or against the subunit H and subunits M–L of *Rhodopseudomonas sphaeroides*. The results indicated that all three subunits are exposed on the cytoplasmic side of the chromatophores (Steiner *et al.*, 1974; Reed *et al.*, 1975; Feher and Okamura, 1978). Enzymatic iodination of the chromatophores of *R. rubrum* indicated that subunit H is exposed to both sides of the membrane, whereas subunits L and M are embedded in the hydrophobic core of the membrane (Zürrer *et al.*, 1977). The same was indicated by experiments with proteolytic enzymes (Oelze, 1978; Hall *et al.*, 1978; Erokhin and Vasil'ev, 1978). In chromatophores of *R. sphaeroides* only subunit H and not M and L was digested by incubation with pronase (Hall *et al.*, 1978), and the same was the case in chromatophores of *R. rubrum* (Oelze, 1978) treated with trypsin or $\alpha$-chymotrypsin. In the same line is the finding of a preferential binding of the hydrophobic marker 5-iodonaphthylazide to subunits L and M as compared with subunit H (Odermatt *et al.*, 1980). The hydrophobic marker fluorescamine binds mostly with the subunit M and to a lesser extent with the subunit H. To explain the absence of binding to subunit L a shielded position of L in the membrane, precluding labeling by the very short-lived reagent fluorescamine, was postulated (Bachofen, 1979).

These studies shed light on the transverse location of proteins in chromatophores. Little information is available, however, on the lateral organization of proteins in the membrane. We approached this question by nearest-neighbor analysis using the method of chemical cross-linking. Linkage of proteins in the chromatophores by DSP (hydrophobic, chain length of 11 Å) and glutaraldehyde (hydrophilic, chain length of 7 Å) was followed by SDS–PAGE analysis.

## Materials and Methods

### *Organism and Growth Conditions*

*Rhodospirillum rubrum* (strain G-9) was grown anaerobically in the light at 30°C in the medium described by Ormerod *et al.* (1961), omitting peptone and yeast extract.

### *Preparation of the Membranes*

The cells were harvested by centrifugation and washed twice with phosphate buffer (10 mM potassium phosphate buffer, pH 7) containing 5 mM Na-EDTA. They were disrupted by two passages through a French pressure cell (Aminco) at 100 kp/cm$^2$ at 4°C. DNase I (Calbiochem, Los Angeles, USA) (0.1 mg/g wet weight) and the protease inhibitor phenylmethane sulfonyl fluoride (Merck AG, Darmstadt, Germany) (15 $\mu$l/g wet weight of cells, from a fresh stock solution, 200 mM in ethanol) were added between the two runs. In order to remove cell debris effectively the homogenate was centrifuged twice at 18,000 $\times$ $g$ for 15 min (Beckman J-21 centrifuge, rotor JA 20, 4°C). The chromatophores in the supernatant were further purified by centrifugation [230,000 $\times$ $g$ (av.) for 40 min, Kontron TGA 65 centrifuge, rotor Ti 60, 4°C], leaving the ribosomes in the supernatant. The chromatophores in the pellet were resuspended in a small volume of 10 mM phosphate buffer containing 60% (w/v) sucrose. After 5 min at 4°C the membranes were osmotically shocked by rapidly diluting the suspension with a 15-fold volume of buffer, followed by a second centrifugation as before but for 60 min. The pellet was resuspended in 60% (w/v) sucrose with Na-EDTA (pH 7) solution as a diluent. The treatment by osmotic shock, by which vesicles are ruptured repeatedly, was found to increase the degree of purification as measured by the OD at 870 nm/280 nm ratio. The sidedness remains unchanged, i.e., cytoplasmic face outside, as shown by Oelze (1978). After the third centrifugation as before and for 60 min the pellet was resuspended in 25 mM triethanolamine buffer adjusted to pH 8.

### *Isolation of Chromatophore Proteins*

The reaction center and the light-harvesting polypeptide were isolated according to published methods (Snozzi and Bachofen, 1979; Cuendet and Zuber, 1977).

### *Cross-linking with DSP or Glutaraldehyde and Labeling of the Membrane Surface with Diazobenzene Sulfonate*

The chromatophore suspension was diluted with triethanolamine buffer to an optical density of 37 at 280 nm. Of this suspension 150 $\mu$l was mixed with a solution of DSP (10–50 $\mu$l of stock solution: 40 mg DSP (Pierce Eurochemie B.V., Rotterdam, Netherlands) per milliliter of dimethyl sulfoxide). Volumes were made up to 200 $\mu$l with distilled water. After incubation for 1 min at room temperature the reaction was stopped by the addition of 200 $\mu$l of the sample buffer.

For cross-linking with glutaraldehyde 5–60 $\mu$l of 1% glutaraldehyde

(50% glutaraldehyde from Fluka AG, Buchs, Switzerland, freshly diluted with distilled water) was added to 150 μl of a suspension of chromatophores (same optical density as above) and incubated at room temperature for 20 min. The reaction was stopped by dissolving the membranes in 200 μl of sample buffer.

The surface of the chromatophores was labeled with diazobenzene sulfonate (Fluka AG, Buchs, Switzerland) according to Berg (1969).

### *Protease Treatment of Chromatophores*

Thermolysin (1 mg/ml) (Boehringer, Mannheim, Germany) was dissolved in triethanolamine buffer (pH 8) supplemented with 2 mM $CaCl_2$. Chromatophores (200 μl, optical density 72 at 280 nm) were incubated with the protease solution (200 μl) for 30 min at 30°C in the dark. Protease activity was stopped by the addition of 100 μl EDTA (50 mM, pH 8). The chromatophores were put into triethanolamine buffer (final volume 3 ml), then collected by centrifugation (200,000 × *g* for 60 min, rotor SW 50, 4°C) and resuspended for cross-linking.

### *SDS Polyacrylamide Pore Gradient Electrophoresis*

Samples for SDS–PAGE were mixed with an equal volume of sample buffer (3.6% SDS, 7.9% glycerol, 0.02% bromophenol blue, and 50 mM Tris pH 6.8) and incubated for 15 min at 60°C. Slab gels (1.5 mm thick) were prepared with a linear gradient of 8–16% acrylamide which was stabilized by a sucrose gradient (0.24–1.21 M). The stacking gel contained 3% acrylamide. For electrophoresis the discontinuous buffer system of Laemmli (1970) was used. Gels were run at a constant current of 10 mA for about 8 h, then stained at 60°C for 2 h in a solution of 0.05% Coomassie Brilliant Blue R-250, 50% methanol, and 5% acetic acid, and destained overnight in 10% methanol and 10% acetic acid.

### *Spectroscopy*

The absorption spectra were measured with an Aminco DW-2 spectrophotometer at room temperature. The gels were scanned with the scanning accessory to the same spectrophotometer developed by Broger *et al.* (1979) using the dual wavelengths mode (570–530 nm).

## Results

### *Purity Test of Isolated Chromatophores and SDS–PAGE Analysis*

The purity of the chromatophores was tested by measuring the absorption spectra from 250–900 nm. Most remarkable is that the absorption peak

at 870 nm (caused mainly by the light-harvesting polypeptide complex) is much higher relative to the absorption peak at 280 nm (proportional to total protein content) in the purified chromatophores than in the crude ones. The absorbance ratio (870/280 nm) was $\geq$ 1.4 in the purified chromatophores and $\leq$ 0.95 in the crude ones. The relative absorption at 260 nm is lower in purified chromatophores than in the crude ones, indicating that the amount of ribosomes is significantly reduced. A final equilibrium centrifugation of the purified chromatophores in a linear sorbitol density gradient (25–55%) gave no improvement of the purity according to the criteria used and was therefore omitted.

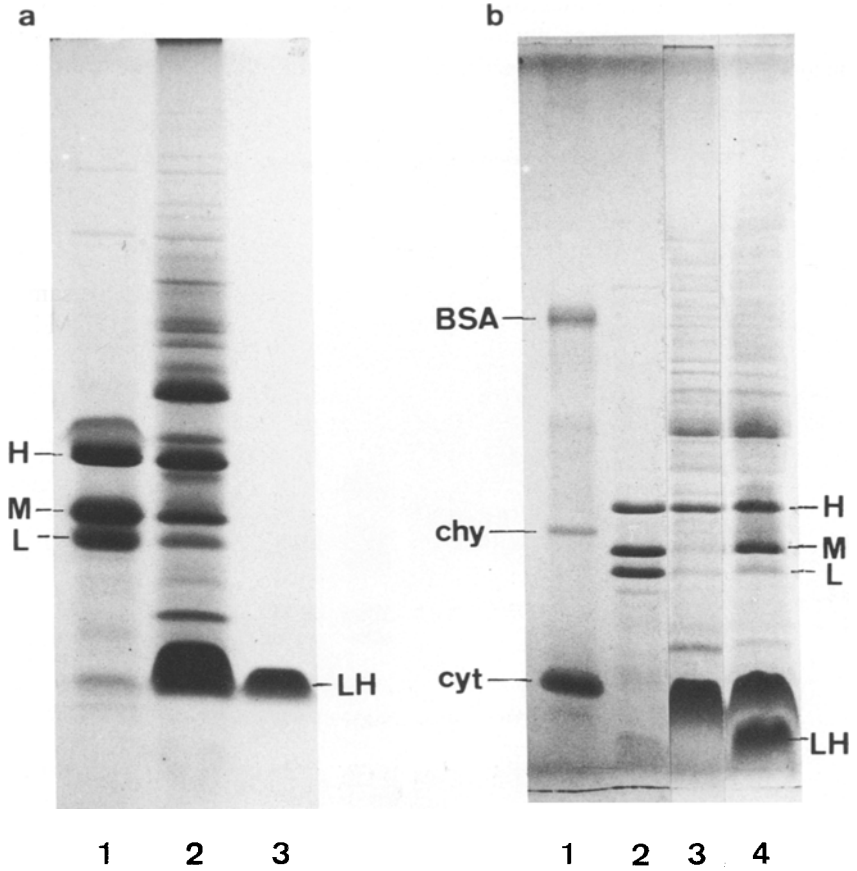


**Fig. 1.** Identification of chromatophore proteins analyzed by SDS–PAGE. (a) Reaction centers (1), purified chromatophores (2), and light-harvesting polypeptide (LH) (3). (b) Marker proteins (BSA: mol. wt. 67,000, chy is chymotrypsinogen A: mol. wt. 25,000, cyt is cytochrome c: mol. wt. 12,500) (1), isolated reaction centers (2), purified chromatophores depleted of light-harvesting polypeptide (3), and chromatophores (4).

After SDS–PAGE of chromatophores about 25 bands could be discerned in the gel (Fig. 1a, No. 2). Only few of the bands have been identified so far, among them the reaction center polypeptides H, M, and L (Fig. 1a, No. 1 and Fig. 1b, No. 2) and the light-harvesting polypeptide (Fig. 1a, No. 3, and Fig. 1b, Nos. 3, 4). The gels in Fig. 1b, Nos. 4 and 3, were loaded with whole chromatophores and chromatophores from which the light-harvesting polypeptide had been extracted with organic solvents according to Cuendet and Zuber (1977).

*Determination of the Reaction Site of Cross-Linking with DSP*

Chromatophores were cross-linked with DSP directly, and after saturation of the amino groups exposed on the surface of the membrane with diazobenzene sulfonate. The efficient reaction of diazobenzene sulfonate

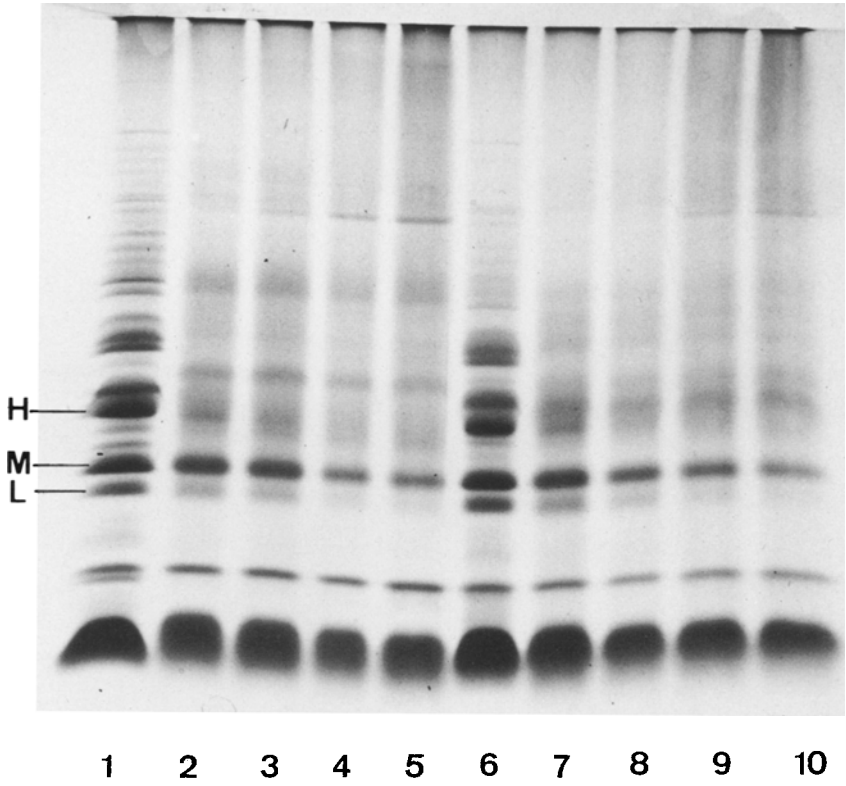


**Fig. 2.** Cross-linking of diazobenzene sulfonate labeled chromatophores with DSP. Gels of chromatophores cross-linked with 0, 10, 20, 30, and 40 μl DSP (1–5). The same, but cross-linking performed after treatment with diazobenzene sulfonate (6–10).

with the membranes was tested spectrophotometrically after washing. The banding pattern in the gel after DSP cross-linking was essentially identical in both the diazobenzene sulfonate pretreated chromatophores and the control (Fig. 2). This shows that DSP does not react with the amino groups exposed on the surface of the membranes but only with reaction sites located in the core of the membranes.

This is supported by the following finding: Upon incubation of the chromatophores with low concentration of the protease thermolysin only subunit H is affected; two new bands of approximately half the molecular weight of the intact subunit H appear, most probably fragments of H (Fig. 3). When the protease-treated chromatophores were cross-linked by DSP at a low concentration, the bands of the two fragments disappeared and products of high molecular weight were formed which did not enter into the gel. This indicates that the regions of H exposed on the surface of the membrane which are digested by protease are not involved in the cross-linking with DSP but only the regions of H located in the hydrophobic core of the membrane, inaccessible to the protease.

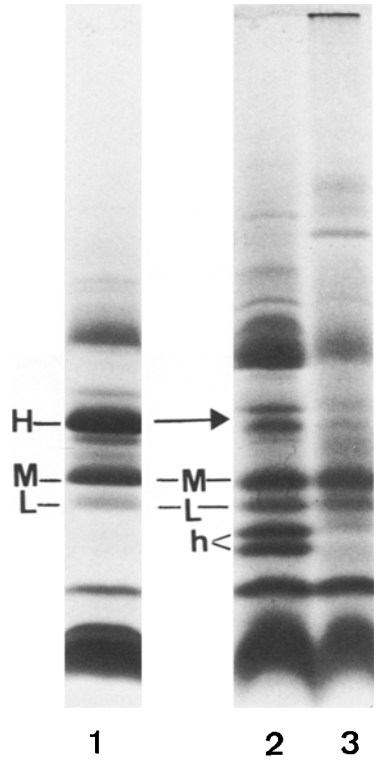


**Fig. 3.** Cross-linking of chromatophores treated with thermolysin. Chromatophores (1); chromatophores in which the subunit H is almost digested (2) were treated with 10 $\mu$l DSP (3). h refers to two cleavage products of subunit H.

*Cross-Linking with DSP*

The effect of increasing concentrations of DSP on chromatophores is illustrated in Figs. 4 and 5. With DSP at a low concentration the most obvious change was the almost complete disappearance of the bands of most proteins with apparent molecular weight higher than 28,000, including the subunit H of the reaction center. The dominant remaining bands were subunits L and M and the light-harvesting polypeptide. These three polypeptides were cross-linked only with high concentrations of DSP. The formation of a new band

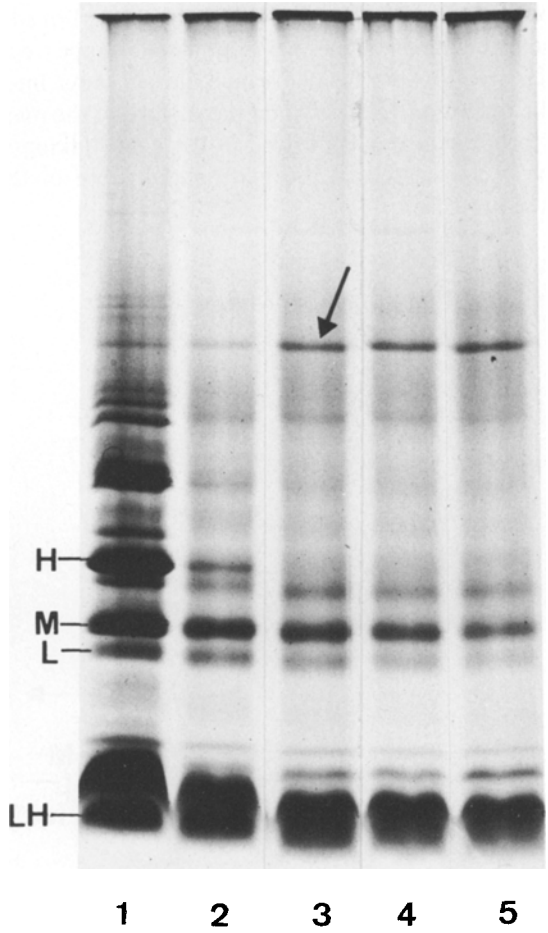


**Fig. 4.** Cross-linking of chromatophores with DSP. Gels of untreated chromatophores (1) and chromatophores treated with 10, 20, 40, and 50 $\mu$l DSP (2–5). The arrow indicates a newly appearing band.

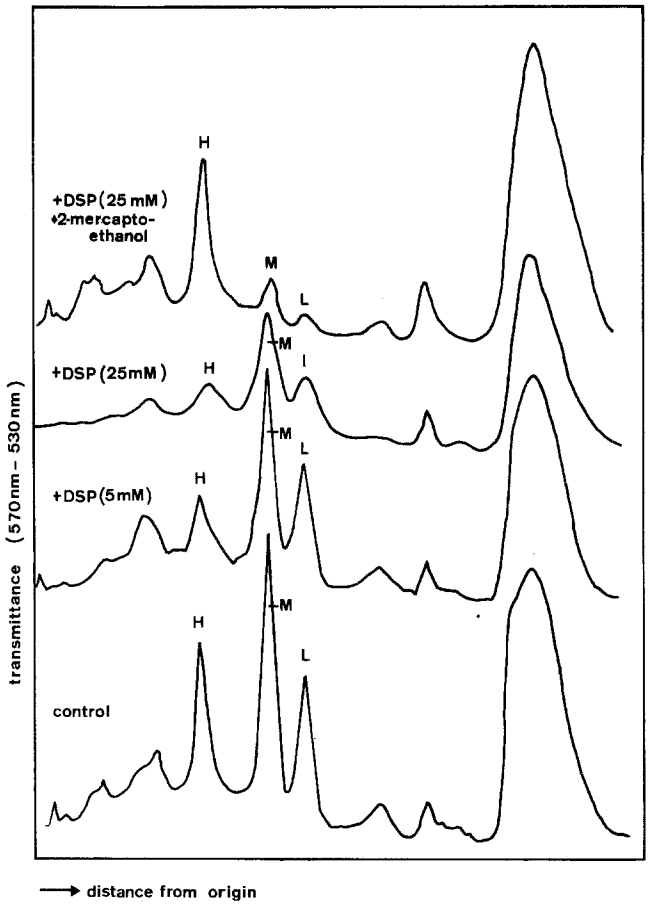


**Fig. 5.** Cross-linking of chromatophores with DSP and cleaving with 2-mercaptoethanol. Scan profile of the gels stained with Coomassie brilliant blue show the decrease of the peaks of subunits L, M, H, and the light-harvesting polypeptide after cross-linking, and the increase of the peak of H and the light-harvesting polypeptide after the cross-linker had been cleaved.

(molecular weight around 68,000) coincides with the disappearance of the bands of subunits L, M, and of the light-harvesting polypeptide. But the new band represents only a part of the complexes formed. Most of the complexes are larger and hence immobilized at the top of the gel.

An estimation of the peak areas of gel scans from an identical experiment shows that the amount of subunit H is decreased to about 50% with the lowest DSP concentration (5 mM) used, whereas only about 4% of M and L disappear (Fig. 5). With higher DSP concentration (25 mM) the decrease of subunits M and L is in the range of 70%. By cleaving the cross-linkage with

2-mercaptoethanol the subunit H can be recovered at its original position to about 85–90%. This is also the case with most other chromatophore proteins but not with L and M. These two subunits are irreversibly aggregated by cross-linking and hence all attempts to analyze the products in a two-dimensional gel were unsuccessful.

The parallel fading of the bands of subunits L, M, and the light-harvesting polypeptide on the gel upon cross-linking suggests that these proteins are linked together (for the light-harvesting polypeptide see the lower part of the front band in Fig. 4, and the change in size and shape of the front peak in Fig. 5). This is further supported by the absorption spectra of reaction centers isolated from cross-linked chromatophores (Fig. 6). In these reaction centers the absorbance ratio 870/758 nm is 1.7, whereas in the control it is 0.85. This increase of the absorption at 870 nm is due to light-harvesting pigments linked to the reaction center.

When isolated, pure reaction centers are cross-linked by low concentrations of DSP, the subunits behave similarly as in the chromatophores (Fig. 7). The intensity of the band of subunit H decreases drastically while the bands of subunits L and M show little change. A new band appears, probably containing dimers of subunit H.

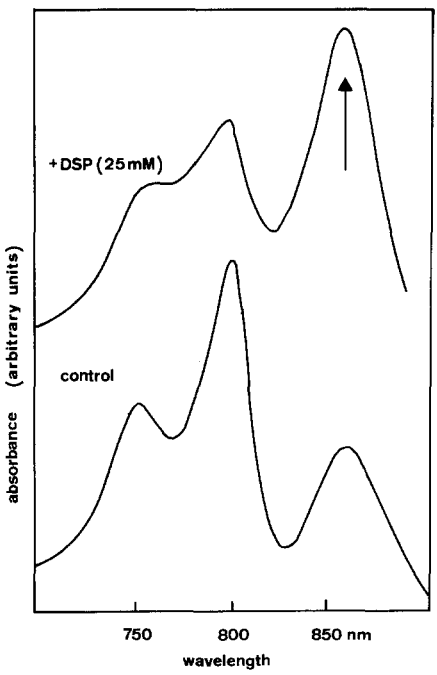


**Fig. 6.** Isolation of reaction centers of chromatophores treated with DSP. The arrow indicates the peak increase at 870 nm due to light-harvesting polypeptide covalently bound to the reaction center.

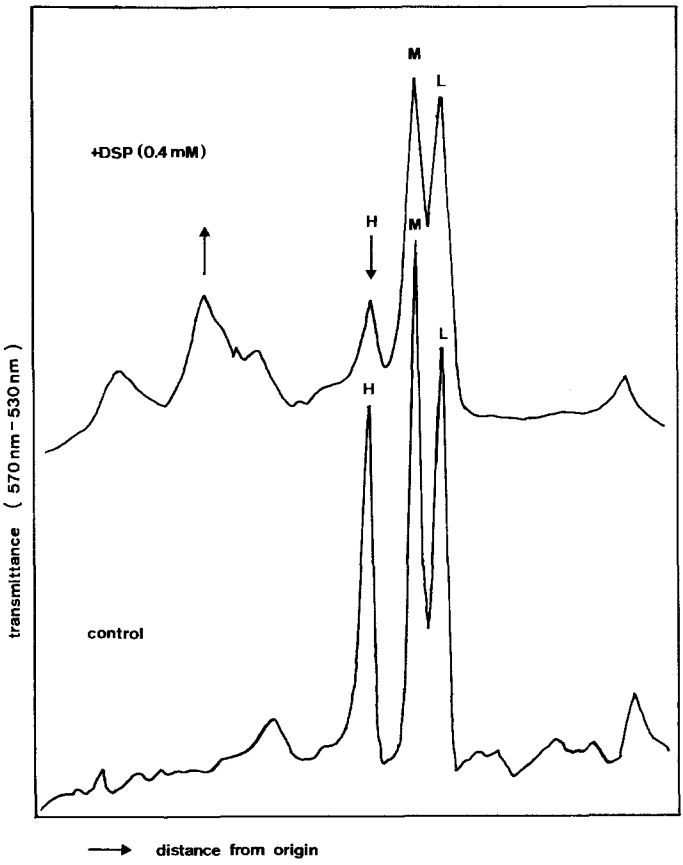


**Fig. 7.** Cross-linking of the isolated reaction centers with DSP at a low concentration. The arrows indicate the main decrease and increase, respectively, on the scan profile of isolated reaction centers.

### *Cross-Linking with Glutaraldehyde*

Glutaraldehyde is an interesting cross-linking reagent because of its variable chain length. Under the conditions used, the active species is mostly the open-chain monomer. However, the open-chain dimers or oligomers are also present (Peters and Richards, 1977). Glutaraldehyde, like DSP, reacts preferentially with free amino groups of proteins.

The effect of glutaraldehyde increases with increasing concentration of the reagent. The bands of all the three subunits of the reaction center disappear progressively (Fig. 8). The light-harvesting polypeptide is not cross-linked, but the unknown polypeptides behind the light-harvesting region

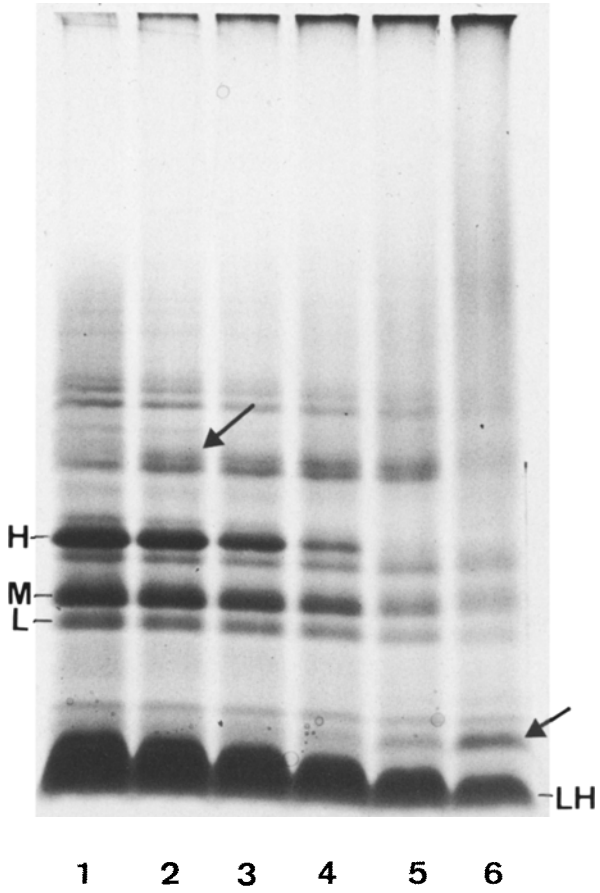


**Fig. 8.** Cross-linking of chromatophores with glutaraldehyde. Gel profiles of chromatophores treated with 0, 5, 10, 20, 40 and 60 $\mu$l glutaraldehyde (1–6). The arrows indicate the new bands.

of the gel disappear. Two conspicuous new bands appear, one in the middle region of the gel (molecular weight about 40,000) and the other in the front region behind the light-harvesting polypeptide. The latter may be a dimer of a polypeptide lost as monomer during the staining procedure or hidden in the large front band.

## Discussion

Extensive cross-linking of chromatophore proteins occurred both with a hydrophobic and with a hydrophilic cross-linking reagent.

When chromatophores are treated with mild detergent (Feher and Okamura,

1978) subunit H is always extracted together with the other subunits of the reaction center. It is therefore remarkable that the hydrophobic cross-linking reagent DSP does not link together all three subunits. Subunit H is linked either to itself (this occurred in isolated reaction centers exclusively) or to other proteins of the membrane. The reaction centers of *R. rubrum,* isolated by detergent, were dissociated into subunit H and L–M complex when the ionic strength was increased (Snozzi and Bachofen, 1979; Vadeboncoeur *et al.,* 1979). At the same time the amount of bound lipids decreased, suggesting that the lipids are responsible for the binding of the subunit H to the L–M complex in the membrane. The lipids may therefore hinder also the cross-linking between subunit H and the L–M complex. Since subunit H is not a necessary component in the reaction center for light-energy conversion (Feher and Okamura, 1978) a direct protein–protein interaction is probably not required. The chromatophores from which the subunit H had been digested were still photochemically active (Hall *et al.,* 1978).

After cross-linking L and M with DSP the subunits can no longer be dissociated by 2-mercaptoethanol. Cross-linking apparently induces a conformational change of subunits M and L so that they irreversibly aggregate. Similar observations were made by others attempting to isolate L and M separately.

## Acknowledgment

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