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Investigation of the Organization of Rhodopsin in the Sheep Photoreceptor Membrane by using Cross-Linking Reagents

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The organization of rhodopsin in the photoreceptor membrane of sheep rod outer segments was investigated by using a variety of bifunctional reagents. Of the nine reagents used, seven gave oligomeric opsin species, whereas two, copper phenanthroline and dithiobisphenyl azide, failed to cross-link the protein. In general, the cross-linked species obtained showed diminishing yields from dimer to tetramer, together with some higher-molecular-weight aggregates. It is proposed that the patterns of cross-linking arise as a result of collision complexes and best describe a monomeric organization for native rhodopsin. No significant differences between the patterns obtained with dark-adapted bleached or regenerated protein states were observed. This interpretation is discussed in relation to the postulated mechanism of action of rhodopsin.

The transduction of light energy into an electrical signal is believed to be mediated by rhodopsin, a protein–chromophore molecular complex located in the membranes of the stacked intracellular discs that make up the outer segments of vertebrate rod cells. Absorption of incident light by the chromophore ultimately causes the protein moiety, opsin, to undergo conformational changes that are thought to catalyse the movement of intradiscal Ca^{2+} cations across the membrane and into the cytosol (Deaman, 1973; Montal & Korenbrot, 1973).

X-ray-diffraction (Blasie & Worthington, 1969; Blasie, 1972*a*; Worthington, 1973), electron-microscopic (Chen & Hubbell, 1973) and chemical-modification studies (Steinemann *et al.*, 1973) suggest that rhodopsin penetrates deeply into, and probably completely through, the phospholipid bilayer. Other than the highly orientated nature of the molecule, however, little is known about its organization and the disposition of the polypeptide chain in the membrane.

As a starting point for the definition of such protein–protein interactions, we have made use of a number of bifunctional cross-linking agents, which vary in their chemical specificities and the distance between their reactive groups. The molecular species that result from such treatments under a variety of experimental conditions have been analysed by SDS/polyacrylamide-gel disc electrophoresis. The results of these experiments are interpreted in terms of the geometrical relationships that exist in the membrane and with respect to the postulated mechanism of action of rhodopsin.

Abbreviation used: SDS, sodium dodecyl sulphate.

Experimental

Materials

All the chemicals used in this work were of AnalaR-reagent grade. Ficoll, SDS and digitonin were obtained from Sigma Chemical Co., Poole, Dorset BH17 7NH, U.K.; acrylamide, *NN'*-methylenebisacrylamide, *NNN'N'*-tetramethylethylenediamine, dimethyl pyrocarbonate, Triton X-100, *o*-phenanthroline, β-mercaptoethanol and sodium deoxycholate were from BDH; 1,5-difluoro-2,4-dinitrobenzene, dimethyl suberimidate dihydrochloride, dimethyl adipimidate dihydrochloride, dimethyl 3,3'-dithiobispropionimidate dihydrochloride, 4,4-dithiobisphenyl azide and dithiobissuccinimidyl propionate were from Pierce and Warriner (U.K.), Chester CH1 4EF, Cheshire, U.K.

Methods

Preparation of rod outer-segment discs. Rod outer-segment discs from sheep retina were prepared by using mild homogenization, centrifugation in a sequence of sucrose solutions of decreasing density and treatment with a hypo-osmotic Ficoll solution by a combination of the procedures described by Daeman (1973) and Smith *et al.* (1975). All procedures were carried out at 4°C and for dark-adapted photoreceptor membrane preparations in dim red light.

Retinas were dissected from sheep eyes within 4h of the animal's slaughter, frozen rapidly on a block of solid CO_2 and stored at −20°C until required. About 70 thawed retinas were homogenized in a total volume of 90ml of 0.1M-Tris/HCl buffer,

pH7.2, made 45% (w/v) with respect to sucrose. The homogenate was centrifuged for 1h at 65000*g*. The floating material was carefully removed, diluted 10-fold with 0.1M-Tris/HCl buffer and spun for 10min at 45000*g*. The pellet was resuspended in 90ml of 0.1M-Tris/HCl buffer, pH7.2, made 40% (w/v) with respect to sucrose and again centrifuged for 1h at 65000*g*. The floating material was removed and washed twice with centrifugation at 45000*g* for 10min in 0.1M-Tris/HCl buffer. These rod outer segments were resuspended in 45ml of water, an equal volume of 10% (w/v) Ficoll solution was added and the mixture left overnight at 4°C. The suspension was then centrifuged for 1h at 65000*g*. The floating material, consisting of pure sheep rod photoreceptor membranes, was removed and washed twice in 0.1M-Tris/HCl buffer.

Treatment of rod disc membranes with cross-linking reagents. The disc preparation was suspended to a concentration of about 1.0mg/ml (estimated by the normal ninhydrin method; Hirs, 1967) in the appropriate buffer as described below.

Dimethyl suberimidate dihydrochloride and dimethyl adipimidate dihydrochloride were prepared as fresh stock solutions of 20mg/ml in ice-cold 25mM-Na_2HPO_4/1mM-$MgCl_2$, made pH8.0 with HCl, and portions added to disc membrane preparations suspended in the same buffer to give working concentrations of 0.5, 2.0 and 10mg/ml. The reactions were allowed to proceed at room temperature for 1h and quenched by the addition of 50μl of 1.0M-ammonium acetate/ml of the reaction mixture. Reaction conditions for dimethyl dithiobispropionimidate, prepared as a 20mg/ml stock solution in 25mM-Na_2HPO_4/1mM-$MgCl_2$ buffer, pH8.0, were identical with those for dimethyl adipimidate dihydrochloride and dimethyl suberimidate dihydrochloride.

Dithiobis-succinimidyl propionate was added from a freshly prepared stock solution of 20mg/ml in dimethyl sulphoxide to give a working concentration of 0.5mg/ml. The reaction time was 5min, but otherwise was the same as cross-linking with dimethyl suberimidate dihydrochloride and dimethyl adipimidate dihydrochloride.

Copper phenanthroline, prepared by dissolving 6.8mM-*o*-phenanthroline in 3.4mM-$CuSO_4$ solution, was added to a photoreceptor-membrane preparation suspended in 50mM-triethanolamine/1mM-$MgCl_2$ adjusted to pH8.0 with concentrated HCl to give working concentrations of 0.135 and 0.25mM. Samples were incubated for 30 min at 24°C and the reaction was quenched by the addition of 50μl of iodoacetamide (20mg/ml) and 100μl of 10mM-EDTA/ml of suspension.

Glutaraldehyde was added in batches from a 0.2M stock solution to a membrane suspension in 25mM-Na_2HPO_4/1mM-$MgCl_2$ adjusted to pH7.0 with concentrated HCl, to give working concentrations of 1–5mM-glutaraldehyde. The reaction was allowed to proceed at room temperature for 15min before being terminated by the addition of 50μl of 1.0M-ammonium acetate/ml of mixture.

Difluorodinitrobenzene was prepared as a 1.0M stock solution in acetone and added to the disc suspension in 25mM-Na_2HPO_4/1mM-$MgCl_2$ adjusted to pH8.0 with concentrated HCl, to give a final concentration of difluorodinitrobenzene of 5.0mM. The reaction was terminated after 30min by dialysis against a large volume of 0.1M-ammonium acetate adjusted to pH7.0 with acetic acid.

Cross-linking with 4,4′-dithiobisphenyl azide was carried out by addition from a 10mM stock solution in ethanol to give a final concentration of 200μM. The reagent was stored in the dark at −20°C and added to a 1.0mg/ml membrane suspension in 25mM-Na_2HPO_4/1mM-$MgCl_2$ adjusted to pH8.0. The mixture was incubated for 10min at room temperature in the dark and then illuminated with a Phillips Ultraphil 300W lamp for 30min at 25°C.

Dimethyl pyrocarbonate was prepared freshly as a 20% (v/v) solution in ethanol and added in 2–50μl batches to a membrane suspension (1.0mg/ml) in 25mM-Na_2HPO_4/1mM-$MgCl_2$, pH8.0, such that the final concentration of reagent was 0.25–1.0% (v/v). The mixture was incubated for 5min and the reaction terminated by the addition of 100μl of 10% (w/v) SDS.

Cross-linking in the presence of detergents. To determine the molecular species resulting from dissolution of the disc membrane, a variety of detergents were introduced into the reaction mixture before the addition of the bifunctional reagents. Triton X-100, sodium deoxycholate, digitonin and SDS were all prepared as 5% (w/v) solutions in the appropriate buffer and added to the membrane suspension to a final concentration of 0.5% (w/v). The solutions almost immediately clarified, which was taken as an indication of solubilization of the membrane. After incubation at room temperature for 20min the cross-linking reagents were added and the reactions allowed to proceed as described above.

SDS/polyacrylamide-gel disc electrophoresis. All samples were prepared for electrophoresis by the addition of 100μl of 10% (w/v) SDS and 100μl of Pyronin Y marker dye solution containing 10% (v/v) glycerol to 0.5ml of cross-linked membrane suspension. Where appropriate β-mercaptoethanol was added to the sample to give a 1% (v/v) solution.

Electrophoresis was carried out as described by Findlay (1974) in 5% acrylamide gels. Gels were stained for at least 4h in 0.25% Coomassie Blue dissolved in a solution of 7.5% (v/v) acetic acid and 40% (v/v) methanol.

Apparent molecular weights of bands detected on gels were obtained from plots of log(molecular

weight) against mobility for the following standards: β-galactosidase (130000), bovine serum albumin (69000), ovalbumin (45000), pepsin (35000) and trypsin (23800).

Results were recorded as densitometric traces by using a Unicam SP. 1800 u.v. spectrophotometer.

Results

When sheep rod disc photoreceptor membranes prepared as described are dissolved in SDS and subjected to electrophoresis on a 5% acrylamide gel a single major band is seen of mol.wt. 39000 (S.E.M. ±1000) together with a faint variable second band of mol.wt. 78000 (Fig. 1). These bands comprise the rhodopsin monomer and dimer respectively. The band pattern is not affected by bleaching the membrane preparation or by treatment of the solubilized membrane with 1% β-mercaptoethanol before electrophoresis.

Cross-linking with copper o-phenanthroline

Despite the presence of at least two available thiol groups on the molecule (P. L. Kam & J. B. Findlay, unpublished work), no oligomers of rhodopsin were observed with this reagent when used at a final working concentration of 0.135 or 0.25mM. This was true for both bleached and dark-adapted membrane preparations and was not altered by sonication with a type 7530 Branson–Dawe Soniprobe of the rod disc preparation for 3×10s (power level 3) at 4°C in the presence of copper *o*-phenanthroline.

Cross-linking with glutaraldehyde

Both dark-adapted and bleached membrane preparations when treated with glutaraldehyde gave bands of mol.wt. 39×10^3, 78×10^3 and 117×10^3 together with higher-molecular-weight material that failed to enter the gel (Fig. 2). An increased concen-

Fig. 1. *SDS-gel electrophoresis of purified rhodopsin*
Rhodopsin purified as described under 'Methods' when electrophoresed on a 5% gel at a loading of 150μg shows one major band of mol.wt. 39000.

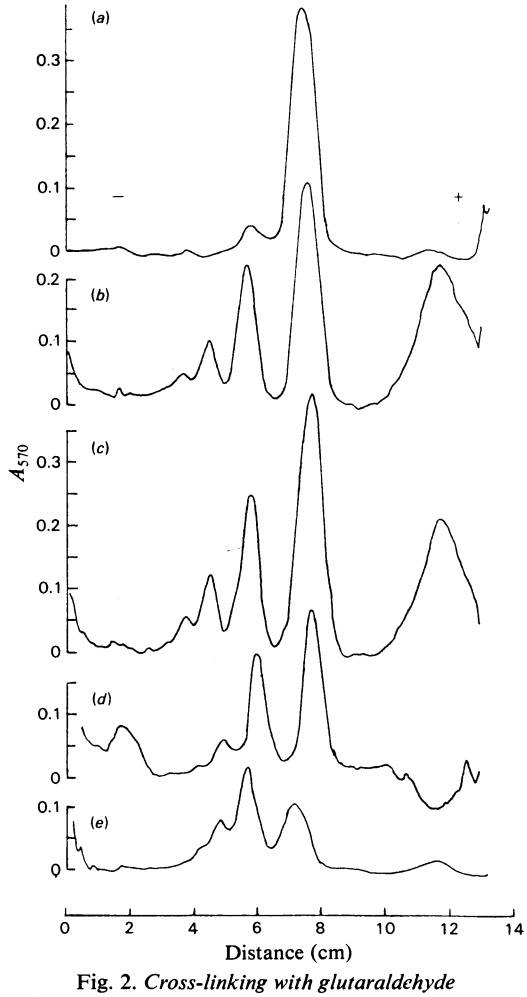

Fig. 2. *Cross-linking with glutaraldehyde*
Discs (1.0mg/ml) suspended in 25mM-Na_2HPO_4 pH7.0, were treated with 5mM-glutaraldehyde for 15min. (*a*) Control (loading 10μg); (*b*) dark-adapted (loading 50μg); (*c*) bleached (loading 50μg); (*d*) solubilized in 0.5% (w/v) sodium deoxycholate (loading 50μg); (*e*) solubilized in 0.5% (w/v) Triton X-100 (loading 50μg).

tration of glutaraldehyde (5mM) or longer incubation times (up to 1h) did not qualitatively alter the result, but gave a quantitative shift to the higher-molecular-weight species. The observed bands were designated as monomer, dimer, trimer and tetramer. The decreasing yield evident in band intensity from monomer to tetramer was a consistent feature of the results and independent of the concentration of glutaraldehyde or the incubation time.

Cross-linking with 1,5-difluoro-2,4-dinitrobenzene

This bifunctional reagent can react with closely spaced lysine amino and tyrosine phenolic groups (Cuatrecasas *et al.*, 1968). At pH9.0 in 0.1M-$NaHCO_3$/NaOH buffer complete aggregation of sheep rhodopsin occurred without the addition of cross-linker. The reaction was therefore carried out in the 25mM-sodium phosphate/HCl buffer, pH8.0, as described under 'Methods'. Treatment with 5mM-difluorodinitrobenzene resulted in a marked decrease in the amount of material able to enter the gel (Fig. 3). The top of the gel was heavily stained, suggesting that the diminution in band intensity of the monomer resulted from formation of highly aggregated material unable to enter the gel. Illumination of the disc suspension before treatment with difluorodinitrobenzene gave similar results.

Cross-linking with imido-esters

Successful cross-linking with the imido-ester bifunctional reagents depended critically on the concentration of the reagent, but the effective concentration varied between the reagents. At 0.5mg/ml, dimethyl adipimidate dihydrochloride and dimethyl dithiobispropionimidate gave no evidence of cross-linking, but dimethyl suberimidate dihydrochloride-treated membranes gave oligomers that had molecular weights characteristic of dimers, trimers and tetramers. A diminishing yield with increasing molecular weight was observed. At 2.0mg/ml both dimethyl adipimidate dihydrochloride and dimethyl dithiobispropionimidate showed the pattern of cross-linking comparable with that observed with dimethyl suberimidate dihydrochloride at 0.5mg/ml. An increase in concentration of dimethyl suberimidate dihydrochloride to 2.0mg/ml or for dimethyl adipimidate dihydrochloride and dimethyl dithiobispropionimidate to 10.0mg/ml gave a pattern of cross-linking in which the dimeric species predominated, and decreasing yields of higher oligomers were observed (Figs. 4, 5 and 6).

When 1% β-mercaptoethanol was added to dimethyl dithiobispropionimidate-treated membranes before electrophoresis, the oligomers were not observed, demonstrating unambiguously the specificity of the cross-linked products arising from treatment with imido-esters (Fig. 6).

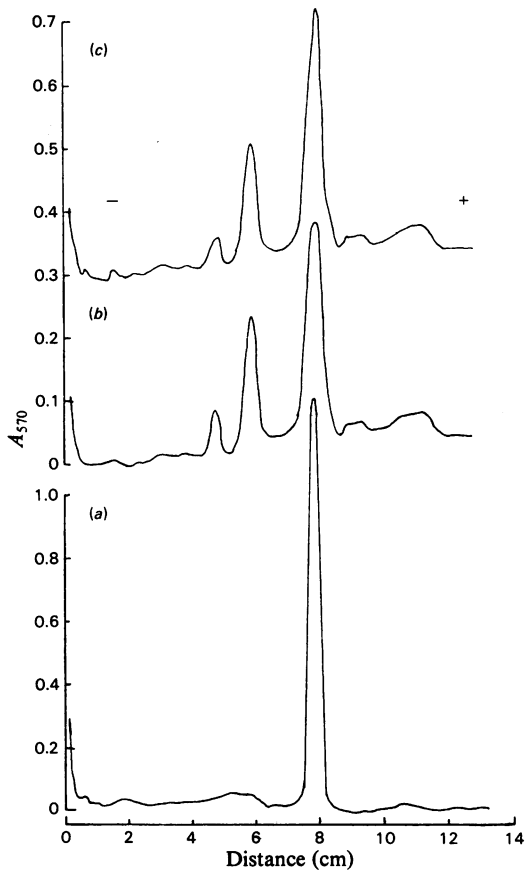

Fig. 3. *Cross-linking with difluorodinitrobenzene* Discs (1.0mg/ml) suspended in 25mM-Na_2HPO_4, pH8.0, were treated with 5mM-difluorodinitrobenzene for 30min. (*a*) Control (loading 100μg); (*b*) dark-adapted (loading 100μg); (*c*) bleached (loading 100μg).

Bleached membrane preparations gave cross-linking patterns that were indistinguishable from those obtained with dark-adapted preparations. The addition of 11-*cis*-retinaldehyde (Akhtar *et al.*, 1968) to ensure complete regeneration of rhodopsin gave better than 90% regeneration on the basis of spectrophotometric evidence (D. J. C. Pappin & J. B. C. Findlay, unpublished work). The addition of dimethyl suberimidate dihydrochloride to the regenerated preparation did not noticeably alter the cross-linking patterns (Fig. 4).

Cross-linking with dithiobis-succinimidyl propionate

This reagent was presented to the membrane preparation as a solution in dimethyl sulphoxide, the final concentration being 5% (v/v) dimethyl

sulphoxide. Control samples treated with this concentration of dimethyl sulphoxide only were unaffected.

However, both dark-adapted and bleached disc membrane preparations treated with dithiobis-succinimidyl propionate showed a marked loss of species entering the gel, as evidenced by band intensity, the top of the gel being heavily stained with oligomeric complexes that failed to enter the gel. The species entering the gel had molecular weights characteristic of monomer and dimer, the latter often showing a slight predominance in staining intensity, whereas trimeric and tetrameric species were present in low yield (Fig 7). Complete regeneration of rhodopsin, obtained by the addition of 11-*cis*-retinaldehyde, gave the same results as dark-adapted preparations.

When dithiobis-succinimidyl propionate-treated membranes were incubated with 1% β-mercaptoethanol before electrophoresis the oligomeric species that failed to enter the gel were not observed and the monomeric protein was obtained in good yield (Figs. 7*g* and 7*h*). Some non-cleavable dimeric species always remained after this treatment.

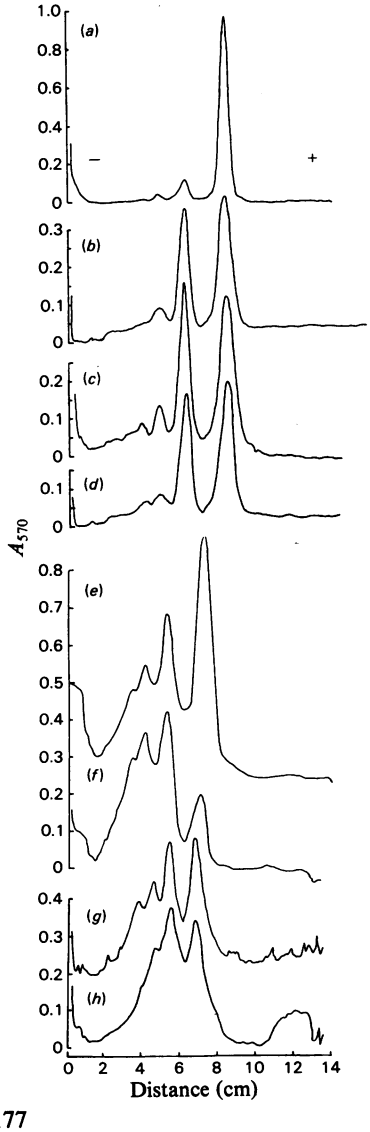

Fig. 4. *Cross-linking with dimethyl suberimidate dihydrochloride*

Discs (1.0mg/ml) suspended in 25mM-Na_2HPO_4, pH8.0, were treated with 0.5–2.0mg of dimethyl suberimidate dihydrochloride/ml for 1h. (*a*) Control (loading 50μg); (*b*) bleached (loading 100μg; 1.0mg of dimethyl suberimidate dihydrochloride/ml); (*c*) regenerated with 11-*cis*-retinaldehyde (loading 100μg); 1.0mg of dimethyl suberimidate dihydrochloride/ml; (*d*) dark-adapted (loading 100μg; 1.0mg of dimethyl suberimidate dihydrochloride/ml); (*e*) dark-adapted (loading 100μg; 0.5mg of dimethyl suberimidate dihydrochloride/ml); (*f*) dark-adapted (loading 100μg, 2.0mg of dimethyl suberimidate dihydrochloride/ml); (*g*) solubilized in 0.5% (w/v) Triton X-100 (loading 100μg; 2.0mg of dimethyl suberimidate dihydrochloride/ml); (*h*) solubilized in 0.5% (w/v) sodium deoxycholate (loading 100μg; 2.0mg of dimethyl suberimidate dihydrochloride/ml).

Cross-linking with dithiobisphenyl azide

Since cross-linking with dithiobisphenyl azide is initiated by photoactivation the photoreceptor membrane preparations were of necessity bleached. At reagent concentrations of 200μM no cross-linking of the opsin moiety was observed under the experimental conditions described.

Cross-linking with dimethyl pyrocarbonate

Membrane preparations were not adversely affected by concentrations of ethanol up to 5% (v/v) of suspension. Incubation with dimethyl pyrocarbonate for 5 min gave a decreasing yield of monomeric species with increasing concentration of reagent and the appearance of aggregated material that failed to penetrate the gel (Fig. 8). Oligomeric species characteristic of the dimer, trimer and tetramer were observed after dimethyl pyrocarbonate treatment. These experiments have identical results in dark-adapted or bleached membrane suspensions.

Cross-linking in the presence of detergents

Membrane suspensions completely solubilized with one of the following detergents, digitonin,

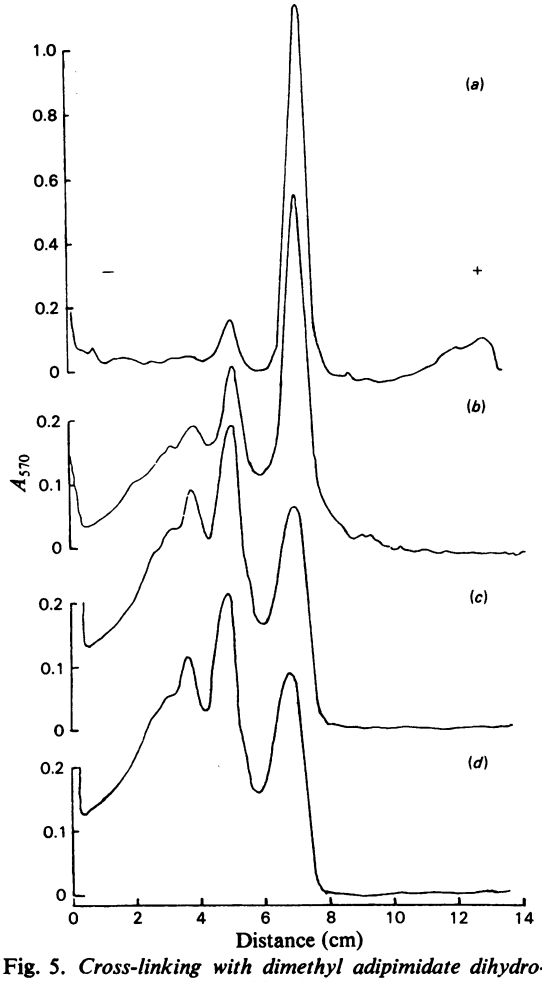

Fig. 5. *Cross-linking with dimethyl adipimidate dihydrochloride*

Discs (1.0mg/ml) suspended in 25mM-Na_2HPO_4, pH8.0, were treated with 2.0–10mg of dimethyl adipimidate dihydrochloride/ml for 1h. (*a*) Control (loading 50μg); (*b*) dark-adapted (loading 100μg, 2.0mg of dimethyl adipimidate dihydrochloride/ml); (*c*) dark-adapted (loading 100μg; 10mg of dimethyl adipimidate dihydrochloride/ml); (*d*) bleached (loading 100μg; 10mg of dimethyl adipimidate dihydrochloride/ml).

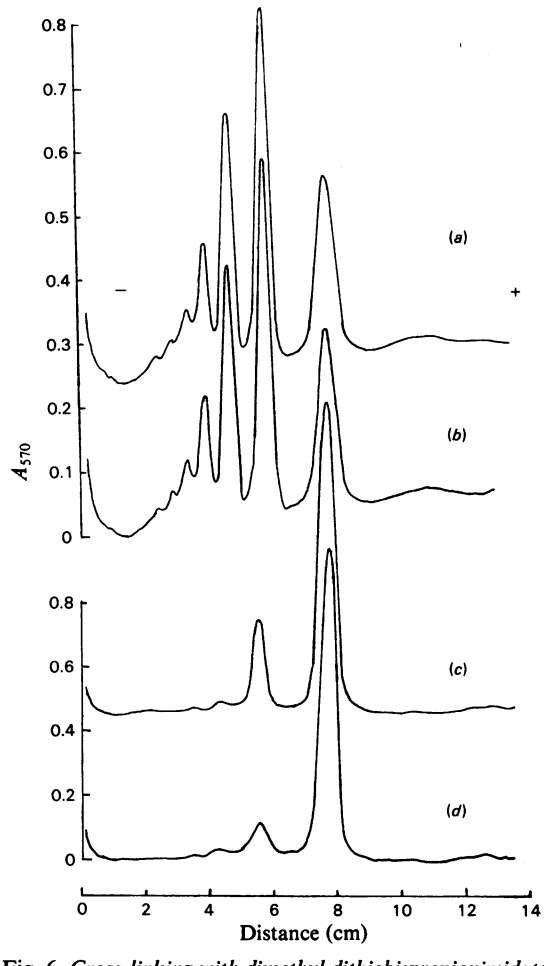

Fig. 6. *Cross-linking with dimethyl dithiobispropionimidate*

Discs (1.0mg/ml) suspended in 25mM-Na_2HPO_4, pH8.0, were treated with 10mg of dimethyl dithiobispropionimidate/ml for 1h. (*a*) Dark-adapted (loading 100μg); (*b*) bleached (loading 100μg); (*c*) cross-linked sample treated with 1% (v/v) β-mercaptoethanol (loading 50μg); (*d*) control (loading 50μg).

sodium dodecyl sulphate, sodium deoxycholate or Triton X-100, as described under 'Methods', were exposed to the cross-linking reagents glutaraldehyde, dithiobis-succinimidyl propionate or dimethyl suberimidate. No cross-linking with any of these reagents was observed in membrane suspensions completely solubilized in digitonin or sodium dodecyl sulphate. In contrast, sodium deoxycholate- or Triton X-100-solubilized preparations both continued to show patterns of cross-linking for each bifunctional reagent comparable with those observed in the native photoreceptor membrane (Figs. 2*d*, 2*e*, 4*g*, 4*h*, 7*e*, and 7*f*). Dithiobis-succinimidyl propionate still gave a marked decrease in the amount of protein able to enter the gel, and those species entering the gel were primarily monomer and dimer in similar yields, with a trace of trimer and no observable tetramer. Membrane preparations treated with glutaraldehyde after solubilization in Triton X-100 or sodium deoxycholate yielded predominantly

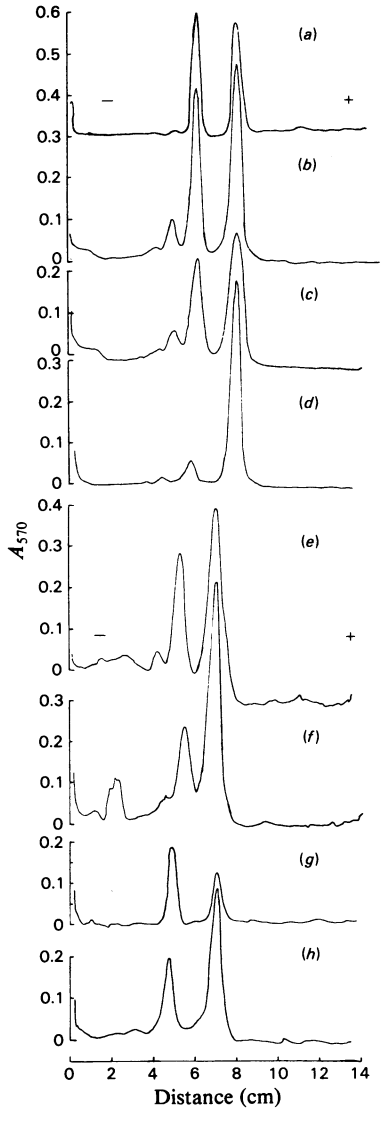

Fig. 7. *Cross-linking with dithiobis-succinimidyl propionate* Discs (1.0mg/ml) suspended in 25mM-Na_2HPO_4, pH8.0, were treated with 0.5mg of dithiobis-succinimidyl propionate/ml for 5min. (*a*) Bleached (loading 100μg); (*b*) regenerated with 11-*cis*-retinaldehyde (loading 100μg); (*c*) dark-adapted (loading 100μg); (*d*) control (loading 10μg); (*e*) solubilized in 0.5% (w/v) sodium deoxycholate (loading 100μg); (*f*) solubilized in 0.5% (w/v) Triton X-100 (loading 100μg); (*g*) bleached dithiobis-succinimidyl propionate cross-linked sample before treatment with β-mercaptoethanol (loading 50μg); (*h*) samples as for (*g*), but treated with 1% β-mercaptoethanol (loading 50μg).

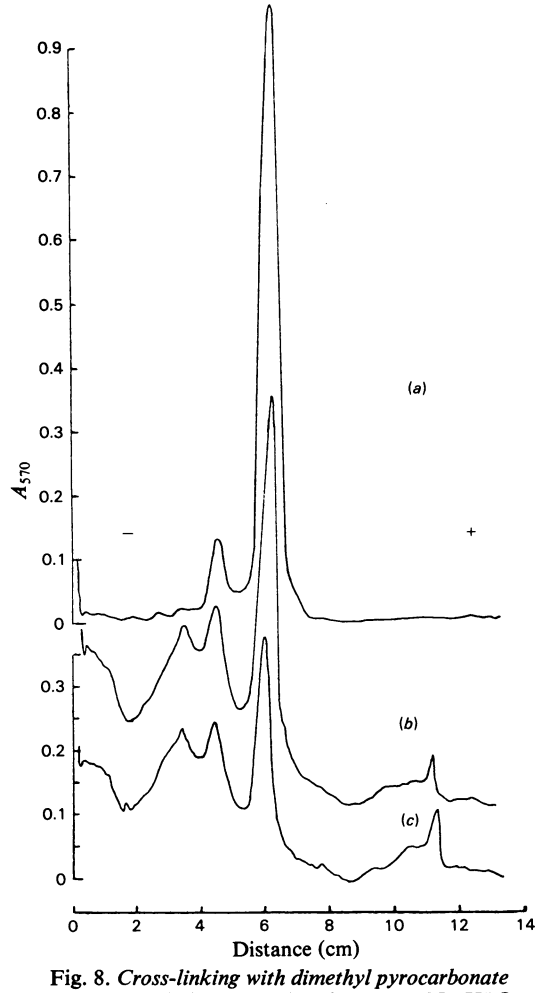

Fig. 8. *Cross-linking with dimethyl pyrocarbonate* Discs (1.0mg/ml) suspended in 25mM-Na_2HPO_4, pH8.0, were treated with dimethyl pyrocarbonate (0.25–1.0%, v/v) dissolved in ethanol for 5min. (*a*) Control (loading 100μg); (*b*) dark-adapted (loading 100μg; dimethyl pyrocarbonate 0.5%, v/v); (*c*) bleached (loading 100μg; dimethyl pyrocarbonate 1.0%, v/v).

the monomer and dimer species, and the trimer was also present, but in a significantly lower yield. Species with a molecular weight characteristic of the tetramer were not seen. However, tetrameric species were clearly detected after treatment of Triton X-100-solubilized membranes with dimethyl suberimidate dihydrochloride, but were not observed in sodium deoxycholate-solubilized samples treated with dimethyl suberimidate, although in both detergents similar yields of the trimer and dimer cross-linked species were observed.

Discussion

Freeze-fracture electron microscopy of vertebrate rod outer-segment disc membranes has revealed particles 5–6nm in diameter, reasonably presumed to contain the visual pigment, since rhodopsin comprises 85% of the photoreceptor membrane protein (Chen & Hubbell, 1973; Saari, 1974; Leeson, 1971). The present study attempted to investigate the subunit structure of these intramembranous particles.

In general, the pattern of cross-linking for bleached, dark-adapted and fully regenerated preparations comprised a progressively diminishing yield of cross-linked products from dimer to tetramer and occasionally higher oligomers together with some aggregated material excluded from the 5% acrylamide-gel matrix. This pattern could result from cross-linking between random collision complexes of monomeric rhodopsin molecules or alternatively from a tetrameric assembly (Peters & Richards, 1977).

The former interpretation provides the most plausible model for the organization of rhodopsin consistent with the fluid nature of the photoreceptor membrane (Träuble & Sackman, 1973). The protein is free to rotate and diffuse in the plane of the membrane, allowing an estimated collision frequency between molecules of 10^5–10^6/s (Cone, 1972, 1974; Poo & Cone, 1973, 1974). If rhodopsin is a monomer in the membrane and cross-linking takes place between collision complexes then the yield of a cross-linked species will reflect the probability of specific collision events and will decrease with the increase in molecular weight of cross-linked oligomers as is observed in these experiments.

The patterns of cross-linking observed for rhodopsin would satisfy a tetrameric assembly in the membrane only if it is assumed that the various contact domains show little preferential reactivity towards the bifunctional reagents under all the experimental conditions used in this study (Hucho *et al.*, 1975). Such a lack of symmetry in an oligomeric structure would seem unusual. Hucho *et al.* (1975), for example, in a survey of eight tetrameric proteins found only one, yeast alcohol dehydrogenase, whose pattern of cross-linking failed to indicate any symmetrical organization in the quaternary structure.

The former interpretation is further supported by the results after cross-linking detergent-solubilized protein. Dissolution of the membrane significantly lowers the effective protein concentration, with a consequent diminution in the frequency of random collisions between protein molecules. Bleached rod disc membranes solubilized with SDS or digitonin showed complete abolition of cross-linking with bifunctional reagents. On the other hand, Triton X-100- or deoxycholate-solubilized bleached membranes gave patterns of cross-linking similar to those obtained in native membrane preparations. These results are explained by the irreversible denaturation of opsin that occurs in certain detergents (Hubbard, 1958; Snodderly, 1967; Johnson & Williams, 1970; Ebrey, 1971; Shichi, 1971; Osborne *et al.*, 1974; Stubbs *et al.*, 1976). Bleached opsin is aggregated in Triton X-100 or deoxycholate and hence liable to cross-linking. SDS effects a complete denaturation and disaggregation of rhodopsin, thereby preventing cross-linking. In digitonin, regenerability and, by inference, the native protein structure of opsin is largely retained (Hubbard, 1954, 1958). The failure to cross-link opsin in digitonin suggests therefore that the bleached protein is monomeric, in agreement with sedimentation and diffusion measurements in the detergent (Hubbard, 1954).

The failure of copper phenanthroline to cross-link either rhodopsin or opsin supports the argument that the protein is a monomer. Sheep rhodopsin has two free cysteine residues that can be carboxyamidomethylated in the membrane (P. L. Kam & J. B. C. Findlay, unpublished work). Presumably collision complexes fail to cross-link with copper phenanthroline because of the transience of the complex lifetime and/or an unfavourable spatial orientation of thiol groups.

The failure of dithiobisphenyl azide to cross-link the opsin molecule may also reflect a monomeric structure of rhodopsin. This reagent is lipid-soluble, partitions into the bilayer, and it was of interest to compare the patterns of cross-linking between hydrophobic domains of the protein with those produced by the hydrophilic bifunctional reagents. The reason for its failure to cross-link opsin remains to be identified, but may have something to do with the lifetime of the nitrene radical and the transience of rhodopsin collision complexes.

These experiments did not show any differences between dark-adapted, regenerated or bleached photoreceptor membrane preparations. Possible conformational changes in the opsin molecule when illuminated have been suggested by changes in the reactivity of thiol groups (de Grip *et al.*, 1973) and alteration of the net electric charge of the molecule on bleaching (Blasie, 1972*b*). Montal *et al.* (1977), to explain the latency of the photoresponse after illumination of rhodopsin incorporated into planar lipid bilayers, have suggested that rhodopsin is a monomer that, when bleached, aggregates to form an ion channel through the membrane. We can detect no preferential increase in any cross-linked oligomers of opsin at high or low concentrations of bifunctional reagents that would suggest such an aggregation phenomenon after illumination. Therefore if rhodopsin does indeed function as a light-sensitive ion channel then the pore must exist within the rhodopsin monomer.

To recapitulate, the results most simply and con-

sistently satisfy a monomeric organization of rhodopsin in the photoreceptor membrane both before and after illumination. Cross-linked oligomers arise from random collision of monomers in the highly fluid rod disc membrane. If rhodopsin alters the plasma-membrane ionic conductance by forming a transmembrane channel, then this functional unit must be the monomer, since no detectable generation of quaternary structure is observed on illumination.

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