CROSS-LINKING OF DARK-ADAPTED FROG PHOTORECEPTOR DISK MEMBRANES

Evidence for Monomeric Rhodopsin

NANCY W. DOWNER
Department of Biochemistry, University of Arizona, Tucson, Arizona 85721

ABSTRACT A model for random cross-linking of identical monomers diffusing in a membrane was formulated to test whether rhodopsin's cross-linking behavior was quantitatively consistent with a monomeric structure. Cross-linking was performed on rhodopsin both in intact retinas and in isolated rod outer segment (ROS) membranes using the reagent glutaraldehyde. The distribution of covalent oligomers formed was analyzed by SDS-polyacrylamide gel electrophoresis and compared to predictions for the random model. A similar analysis was made for ROS membranes cross-linked by diisocyanatohexane and retinas cross-linked by cupric ion complexed with *o*-phenanthroline. Patterns of cross-linking produced by these three reagents are reasonably consistent with the monomer model. Glutaraldehyde was also used to cross-link the tetrameric protein aldolase in order to verify that cross-linking of a stable oligomer, under conditions comparable to those used for ROS, yielded the pattern predicted for a tetrameric protein having D2 symmetry. This pattern is markedly different from the one for a random-collision model. Moreover, a comparison of rates showed that aldolase cross-linking with glutaraldehyde is significantly faster than cross-linking of membrane-bound rhodopsin. It is concluded that rhodopsin is monomeric in dark-adapted photoreceptor membranes and that the observed cross-linking results from collisions between diffusing rhodopsin molecules.

INTRODUCTION

Rhodopsin is the predominant protein of vertebrate photoreceptor membranes. The suggestion that rhodopsin is monomeric was first made on the basis of x-ray diffraction studies (1). An investigation of rhodopsin in sheep photoreceptor membranes by cross-linking provided further evidence supporting this view (2). However, in some images of disk membranes made by freeze-fracture electron microscopy, the density of intramembranous particles is consistent with the existence of rhodopsin oligomers (3). Finally, the observation that there is a correlation between detergents that solubilize rhodopsin as an oligomeric complex and those that support regeneration after bleaching has been put forward to suggest that native membrane-bound rhodopsin may be oligomeric (4). Cross-linking studies here now provide strong evidence for the monomeric nature of rhodopsin in dark-adapted membranes. Changes in association state initiated by photoexciting rhodopsin in native retinal membranes have been addressed in separate studies reported in the preceding paper (5).

The application of chemical cross-linking to native membrane systems is, in general, complicated by the fact that membrane-bound proteins exist at sufficiently high densities that random collisional cross-linking between monomeric proteins or between oligomeric complexes cannot be ruled out. Therefore, covalent cross-links formed do not necessarily reflect the stable, naturally occurring association of proteins. One solution to this problem has been to utilize photochemically activated bifunctional reagents with very short lifetimes (6) to reduce the probability of observing cross-linking within random collisional complexes. In contrast, the approach used here involves a comparison of observed patterns of cross-linking in ROS membranes with appropriate theoretical and experimental models to distinguish cross-linking within stable oligomers from cross-linking within transient complexes. A related quantitative approach has been used in structural studies on actin (7, 8), in which cross-link formation within a long actin filament is described by a model for random cross-linking.

This paper reports the use of several cross-linking reagents to investigate the oligomeric state of rhodopsin in dark-adapted photoreceptor membranes from frog. Previous cross-linking studies on sheep photoreceptors (2) have been interpreted qualitatively in terms of a monomeric organization of rhodopsin. In the studies reported here, freshly dissected frog retinas and isolated ROS[1]

[1] *Abbreviations used in this paper:* EDTA, ethylenediaminetetraacetic acid; MES, 2-[*N*-morpholino] ethane sulfonic acid; NEM, *N*-ethylmaleimide; ROS, rod outer segment; SDS, sodium dodecyl sulfate; PE, phosphatidylethanolamine.

membranes were cross-linked using various reagents and conditions, and the resulting distributions of covalent oligomers of opsin were quantitatively analyzed. The experimental results were compared with distributions of oligomers predicted if cross-linking were mediated by random collisional events in the membrane. Aldolase was utilized as a model oligomer, to verify that oligomeric proteins yield a pattern of cross-linked oligomers distinguishable from the random model under the experimental conditions used here. Consideration of the rate, extent, and pattern of cross-linking is required to make the distinction between a monomeric vs. oligomeric organization of membrane-bound rhodopsin.

MATERIALS AND METHODS

All experiments were performed in darkness or dim red light. Whole retinas used in cross-linking experiments were dissected from eye cups of bullfrogs (*Rana catesbeiana*) and placed in a frog Ringer's buffer containing 5 mM D-glucose and 3 mM phosphate at pH 7.5. Cross-linking reactions were initiated within 15 min of dissection. ROS were isolated from *Rana pipiens* as described (9). Rhodopsin concentrations were determined from absorbance at 500 nm using an E_{500} of 40,000 M^{-1} cm^{-1} and a molecular weight of 39,000 daltons. Absorbance ratios A_{280}/A_{500} for the ROS preparations varied from 2.4 to 2.8.

Chemical Cross-linking

Reagents for glutaraldehyde cross-linking were prepared immediately before use by the following procedure. 25% aqueous glutaraldehyde was diluted 10-fold in distilled water to make a glutaraldehyde stock (pH 3.4) that was nominally 250 mM. The pH was raised to 8.5 by addition of 0.5 M NaOH. After 10 min the stock was diluted with the appropriate Ringer's buffer to give reagents with nominal concentrations calculated on the basis of the dilutions; actual concentrations of the effective cross-linking species must be lower since glutaraldehyde undergoes aldol condensation reactions at alkaline pH to form polymers of heterogenous size (10). The protocol for preparing glutaraldehyde reagents was designed to minimize variation in the condensation process and hence the resulting mixture of polymers. For cross-linking of retinas, the Ringer's solution used to dilute the stock was 111 mM NaCl, 2.5 mM KCl, 2.0 mM $CaCl_2$, and 3 mM NaH_2PO_4, pH 7.5. For cross-linking ROS membranes, 10 mM borate, pH 8.5, was used instead of phosphate with the same Ringer's salt solution.

For each assay of cross-linking on intact retinas, a retina (two or three in experiments at higher reagent concentrations) was soaked in glutaraldehyde reagent (20–250 mM as indicated) at 0°C for 20–200 min. At the end of the reaction the retina was removed using forceps and shaken vigorously in 1.5 ml distilled water. The outer segments released by shaking were pelleted in a microfuge (Beckman Instruments, Inc, Cedar Grove, NJ) and washed once with 1.5 ml distilled water. The final pellet was solubilized immediately in 20–40 μl dissociating buffer (2% SDS, 10% sucrose, 2 mM EDTA, 80 mM dithiothreitol, and 20 mM Tris HCl, pH 8.0).

Cross-linking of ROS membranes was initiated by diluting suspensions of ROS into glutaraldehyde reagent (0.1–10 mM) to a final rhodopsin concentration ≤0.05 mg/ml. Reactions were carried out at 0–4°C. Cross-linking was terminated by dilution or addition of 0.2 M glycine. The ROS membranes were pelleted and washed with distilled water. 25–40 μl of dissociating buffer without dithiothreitol was added to solubilize the pellets. Final samples contained 100–200 μg of rhodopsin (calculated assuming quantitative recovery of membranes during washing).

Rabbit muscle aldolase (0.3 mg/ml) was cross-linked by reaction with 1 mM and 5 mM glutaraldehyde in borate Ringer's buffer, pH 8.5. Reactions were terminated by addition of 0.2 M glycine. Samples for electrophoresis were concentrated to 25 μl using a Minicon B-15 concentrator (Amicon Corp., Danvers, MA) and solubilized by addition of an equal volume of dissociating buffer.

Cross-linking catalyzed by cupric *o*-phenanthroline was performed by incubating whole retinas in the presence of Ringer's solution (buffered with 50 mM MES, pH 7.5) containing *o*-phenanthroline and $CuSO_4$ in a molar ratio of 3:1. Reactions were carried out at 25°C for 30 min with the concentration of *o*-phenanthroline varying from 0.3 to 15 mM as indicated. ROS were collected and solubilized as for glutaraldehyde cross-linking on ROS, except that no β-mercaptoethanol was added prior to electrophoresis.

Reactions of ROS (0.05 mg/ml) with diisocyanatohexane (Aldrich Chemical Co., Milwaukee, WI) were carried out at room temperature in 10 mM NaCl, 10 mM NaH_2PO_4, pH 7.1. Freshly prepared stock solution of 0.5% diisocyanathohexane in DMSO was diluted into the ROS suspension to give a final concentration of 0.01%. Reactions were terminated by addition of 0.2 M glycine.

Labeling with NEM

Available sulfhydryl groups in ROS were labeled with [^{14}C] NEM (specific activity = 5.7×10^{11} cpm/mol) for 12 h using the procedure of Fung and Hubbell (11). Labeled ROS prepared in this way had incorporated slightly more than 3 mol NEM/mol rhodopsin. This value did not take into account the possible contribution of opsin, which may have been produced during labeling procedures. Therefore the ratio of NEM to total photopigment may have been lower.

Polyacrylamide Gel Electrophoresis in SDS

ROS from cross-linking experiments on intact retinas were electrophoresed on 5.6% polyacrylamide gels prepared according to Fairbanks et al. (12). All other samples were analyzed on 7.5% SDS polyacrylamide (acrylamide: *N,N*-methylene-bis acrylamide = 37.5) mini-slab gels (5 cm × 10 cm × 0.2 cm) in the presence of 0.1 M Tris-bicine and 0.1% SDS. Samples from cross-linking on isolated ROS were prepared for electrophoresis by adding one-quarter volume of a bromophenol blue dye solution containing 5% β-mercaptoethanol and 50% glycerol to cross-linked ROS that had been exposed to dissociating buffer for at least 1 h. Gels were stained for protein with Coomassie blue as described by Fairbanks et al. (12). Densitometric traces of tube gels or lanes sliced from gels were obtained by scanning at 550 nm. The area under each protein peak in densitometric traces was determined by cutting out and weighing the peaks. Apparent molecular weights of proteins and protein oligomers were determined using a series protein molecular weight standards.

Gels containing [^{14}C] NEM-labeled rhodopsin were analyzed by slicing a sample lane into 1 mm segments. Each segment was solubilized by incubation with 0.5 ml 30% H_2O_2, 70°C for >5 h. Samples were neutralized with 0.2 ml of 0.025 M NaOH and counted in the presence of a toluene/Triton X-100 (2:1) scintillation cocktail containing 4 gm/l of Omnifluor (New England Nuclear, Inc., Boston, MA).

Model for Random Cross-linking of Rhodopsin Monomers

The distribution of oligomers expected to result from random, diffusional cross-linking was predicted from a model based on the assumption that the probability of forming a cross-link within any encounter complex in the membrane remains constant during the course of the cross-linking experiment. The cross-linking can be seen as a series of independent (Bernoulli) trials in which a positive outcome, the formation of an initial cross-link between two species, can be detected as the formation of an *n*-mer containing $n - 1$ initial cross-links. (Further intra-oligomeric reactions are not detected in these experiments.) A variable μ, which gives

the overall degree of cross-linking, is the mean number of initial cross-links present per diffusing species and can be calculated from the equation $\mu = \Sigma_n (n - 1)$ (fraction n-mer). The Poisson approximation to the binomial distribution can be used to calculate the expected distribution of n-mers if the probability of forming an initial cross-link is very small and remains constant during a cross-linking experiment. The frequency of collisions between rhodopsin molecules in frog photoreceptor membranes can be estimated from the rate of lateral diffusion at $\sim 10^5\ s^{-1}$ (13). Since the observed value for μ does not exceed 1 even after several hours of reaction with glutaraldehyde, the probability of cross-link formation in a given collision complex must be very low, $<10^{-9}$. Therefore, the fractions of monomer and various n-mers expected for a given value of μ measured in a cross-linking experiment can be calculated by use of the Poisson distribution

$$\text{fraction of } n\text{-mer} = \frac{e^{-\mu} \cdot \mu^r}{r!},$$

where $r = n - 1$ is the number of initial cross-links per n-mer. This relationship provides a reasonable approximation to the oligomeric distribution for the random collision case.

RESULTS

Cross-linking of Retinas

Treatment of freshly dissected frog retinas with 20–250 mM glutaraldehyde produced oligomers of rhodopsin. Fig. 1, *B* shows the electrophoretic pattern of SDS-solubilized ROS from a cross-linked retina. For samples treated with the highest concentration of glutaraldehyde, species with apparent molecular weights corresponding to oligomers of opsin ranging from dimer to hexamer could be resolved on gels. These conditions also gave rise to some higher molecular weight material that did not enter the gel. Even under the most stringent conditions used, however, a considerable fraction of the opsin remained as a monomer. Intermediate degrees of cross-linking could be achieved by varying the glutaraldehyde concentration or the reaction time at 0°C. (This is illustrated by data in Fig. 3 of the preceding paper). Increasing the temperature to 25°C slightly increased the degree of cross-linking but had no effect on the pattern of cross-linked oligomers observed. Virtually all of the opsin in untreated controls (Fig. 1 *A*) migrates as a monomer with an apparent molecular weight of 35,000 daltons. Small amounts of proteins other than opsin are observed in the controls. Although migration of the minor proteins does not correspond to that expected for oligomers, as shown in Fig. 1 *A*, it is impossible to rule out the presence of some dimer in unreacted ROS. The contaminating proteins are not evident in ROS from glutaraldehyde-treated retinas.

Reaction of retinas with the complex of cupric ion and *o*-phenanthroline produced a pattern of oligomers similar to that seen for glutaraldehyde treated retinas (Fig. 1 *C*). In addition to oligomers of opsin, there was a band migrating close to the opsin monomer. This may indicate formation of an intramolecular disulfide bridge that alters the migration of the opsin polypeptide. The satellite band

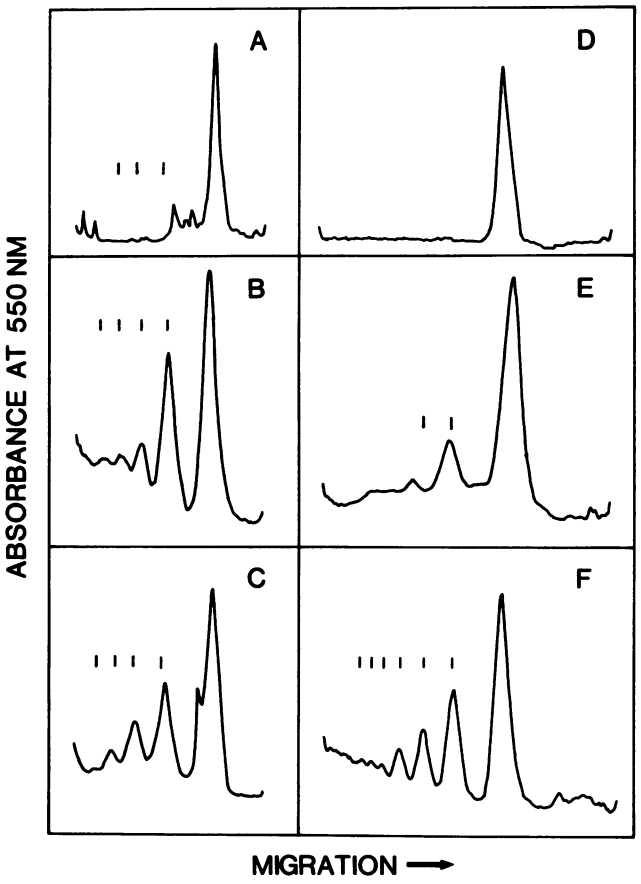

FIGURE 1 Cross-linking of rhodopsin in intact retinas and isolated ROS membranes. Samples of unreacted ROS containing 20–50 μg rhodopsin or cross-linked ROS containing 100–200 μg rhodopsin were solubilized and electrophoresed as described in Materials and Methods. Material from intact retinas (*A–C*) was run on 5.6% gels; material from isolated ROS membranes (*D–F*), on 7.5% gels. Densitometric traces are shown for unreacted control, *A*; 60 min at 125 mM glutaraldehyde, *B*; 30 min at 5 mM $CuSO_4$, 15 mM *o*-phenanthroline, *C*; unreacted control, *D*; 3.5 h at 0.1 mM glutaraldehyde, *E*; 65 min at 10 mM glutaraldehyde, *F*. Absorbance is in arbitrary units. The expected migrations of polypeptides with molecular weights that are multiples of opsin were determined from the appropriate standard curve and are indicated by tick marks.

was not present in untreated controls.[2] As with glutaraldehyde, the degree of cross-linking increased with increasing reagant concentration. The disulfide cross-links produced by the cupric *o*-phenanthroline complex are labile to reducing agents. For this reason, dithiothreitol was not present in the SDS-dissociating buffer used to solubilize the ROS, and controls for these experiments contained as much as 20% aggregated opsin, mostly dimer.

A consistent feature of the oligomeric distributions obtained by cross-linking with both reagents is the decreasing yield of oligomeric species (n-mers) with increasing n.

[2]The multiple forms of frog rhodopsin observed by Molday and Molday (14) under reducing conditions were not resolved in either of the gel systems used here when membrane samples were solubilized in the presence of β-mercaptoethanol.

These observations are in general agreement with the studies of Brett and Findlay (2) who investigated isolated sheep photoreceptor membranes using a variety of bifunctional cross-linking reagents.[3]

Distribution of Cross-linked Oligomers

Data on the oligomeric distribution resulting from glutaraldehyde cross-linking of retinas are displayed as the open symbols in Fig. 2. The fraction of different *n*-mers observed is plotted vs. the value of μ measured in each experiment. The fraction of opsin present as each *n*-mer was determined from the areas under each protein peak in densitometric traces. For a given sample, the Coomassie-blue staining for each monomer and each oligomer peak was linear with the total amount of ROS protein on the gel. In replicate determinations on the same sample, the fraction of *n*-mers measured agreed to ± 0.05. The solid lines in Fig. 2 represent values predicted for various *n*-mers from the model for random cross-linking. The data are in good agreement with the model predictions for the range of cross-linking tested.

Patterns that would be expected if oligomeric membrane proteins were cross-linked and then examined by SDS gel electrophoresis are illustrated in Fig. 3. For the simplest case of a dimer, depletion of monomer is paralleled by formation of covalent dimer until μ reaches a maximum value of 1 (Fig. 3 *A*). Hucho et al. (15) have developed models for the cross-linking of stable, planar tetramers in solution. Oligomeric distributions predicted from their theoretical models were recalculated in terms of the variable μ for comparison with the random (Fig. 3 *D*) and dimeric models. A planar configuration best approximates the arrangement of a transmembrane oligomer. The case of a tetramer in which the probability of cross-linking is equivalent for all four contact domains (Fig. 3 *B*) is compared to a tetramer with D2 symmetry, in which the molecule behaves as two pairs of subunits. For the case shown in Fig. 3 *C*, there was a fourfold difference between the probabilities of cross-linking at the two different interfaces. An implicit assumption in applying the oligomeric models to membranes is that specific intra-oligomeric cross-linking would dominate (by virtue of being faster) over inter-oligomeric collisional cross-linking even at high concentration of membrane-bound oligomers (see Discussion).

The pattern of cross-linking that resulted from glutaraldehyde treatment of retinas is clearly inconsistent with rhodopsin being dimeric. Although the distribution is in good agreement with the random collision model, cross-linking of a planar tetramer (such as that shown in Fig. 3 *B*) would give rise to very similar distributions of species for $\mu < 1$. The data from cross-linking of retinas alone are therefore not adequate to distinguish a monomeric organization from oligomers larger than a dimer.

[3]Cupric *o*-phenanthroline did not catalyze the formation of oligomers in isolated sheep photoreceptor membranes, as reported by Brett and Findlay (2). This observed difference could result either from a species difference in rhodopsin or from differences in rhodopsin's sulfhydryl chemistry between freshly isolated retinas and purified disk membranes.

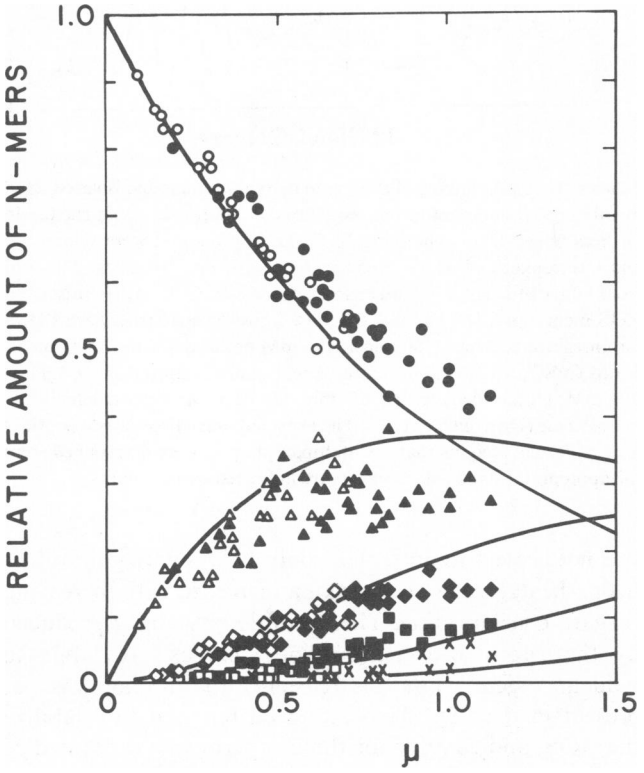

FIGURE 2 Oligomeric distributions resulting from glutaraldehyde cross-linking of intact retinas and isolated ROS membranes. The mass fraction of opsin migrating as monomer and various oligomers was determined from the electrophoretic patterns as described in Experimental Procedures. μ was calculated as described in the text. Each distribution was plotted vs. the corresponding μ value using open (retinas) or closed (ROS) symbols for *n*-mers as follows: $n = 1$, ●; 2, ▲; 3, ◆; 4, ■; 5, *X* (ROS only). The solid lines show the distribution predicted for the random model. The data from isolated ROS membranes include time series at 0.1, 1, 2, 5, and 10 mM glutaraldehyde.

Glutaraldehyde Cross-linking of Isolated ROS Membranes

Attempts to extend the range of the analysis by increasing the extent of cross-linking in retinas were thwarted because the yield of ROS isolated by the simple shaking procedure decreased significantly as glutaraldehyde concentration or reaction time was increased. Furthermore, neither the effective glutaraldehyde concentration, nor the fraction of total membrane-bound rhodopsin contributing to the distributions measured from gels could be estimated from experiments on retinas. These problems could be solved by cross-linking of isolated ROS membranes. Moreover, the results on isolated ROS allowed an estimation of initial cross-linking rates for rhodopsin that could be compared to rates for cross-linking of oligomeric protein subunits.

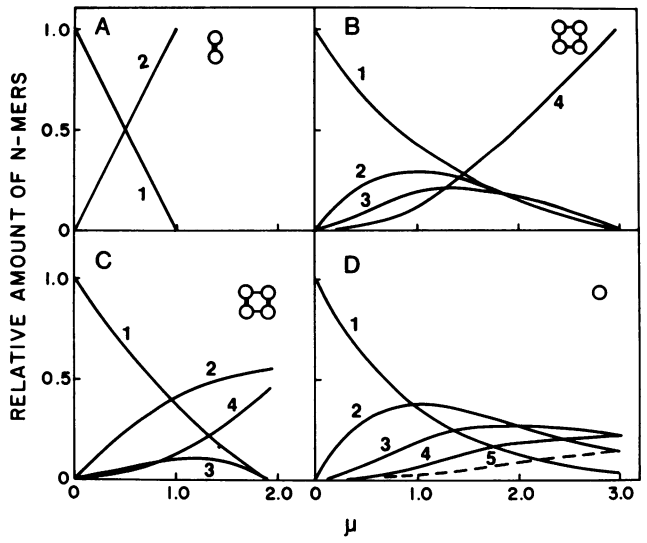

FIGURE 3 Patterns of oligomeric distribution predicted for planar arrays of monomeric vs. oligomeric proteins. Curves represent the depletion of monomer and formation of respective *n*-mers as the degree of cross-linking increases. Degree of cross-linking is designated by μ as described in text. (*A*) dimer; (*B*) tetramer with identical binding domains; (*C*) tetramer with D2 symmetry and probability ratio for cross-linking between two binding domains of 1:4; (*D*) random Poisson model (see text). The tetrameric models are derived from those of Hucho et al. (1975) as described in the text.

Glutaraldehyde concentrations in the range 0.1–10 mM were found to be suitable for investigating the pattern of cross-linking of rhodopsin in isolated ROS membranes. Glutaraldehyde treatment of isolated ROS led to distributions of oligomeric species that were experimentally indistinguishable from those observed in freshly dissected retinas. Electrophoretic patterns representing different degrees of cross-linking are shown in Fig. 1 *E* and *F*. In contrast to unreacted control samples from retinas (Fig. 1 *A*), similarly treated controls for isolated ROS solubilized in SDS with dithiothreitol or β-mercaptoethanol often exhibited a tendency to form opsin aggregates, predominantly dimers. This problem could be minimized by solubilizing cross-linked ROS in SDS-dissociating buffer immediately after cross-linking and adding the β-mercaptoethanol (<2%) along with tracking dye 10–30 min before the samples were electrophoresed. Under these conditions, the amount of dimer in control samples (Fig. 1 *D*) was ≤7%. The distribution of covalently cross-linked oligomers was calculated without any correction for this small, variable fraction of noncovalent aggregates.[4]

Data on the distribution of oligomers from glutaraldehyde cross-linking of the isolated ROS membranes are plotted (closed symbols, Fig. 2) as fraction of *n*-mer vs. μ. Higher values of μ were achieved by increased reaction time at lower reagent concentration. At the lowest concentration of glutaraldehyde used (0.1 mM) and reaction times extending to several days, it was possible to increase the observed values for μ to ~1.2. The data clearly begin to deviate from the random model as μ increases beyond 0.7. In particular, the amount of dimer is less than expected, and falls between the values predicted for the random and planar tetramer models. It is important to note that, although there is deviation from the random model, the data do not fit tetrameric models in two significant respects. There is no enrichment of the cross-linked tetramer as expected on the basis of both tetrameric models (see Fig. 3), and there is accumulation of pentamer (Fig. 2) and hexamer (data not plotted) as predicted for collisional cross-linking but not for tetrameric models.

Kinetics of Glutaraldehyde Cross-linking

The progress of cross-linking for the reaction of ROS with 0.1 mM glutaraldehyde was investigated for up to 36 h. These conditions were chosen because they generated values of μ that spanned the entire range of data in Fig. 2. The progress of cross-linking at 0.1 mM glutaraldehyde is shown in Fig. 4, which indicates that cross-linking is roughly linear with time, i.e., appears to be zero order, for about 5 h. At longer times and values of $\mu > 0.5$, the cross-linking reaction begins to slow and eventually saturates. This presumably occurs when all available reactive sites on rhodopsin molecules have reacted with glutaraldehyde. One can conclude that the experimental data on rhodopsin cross-linking are predicted very well by the simple random model in the range where the apparent cross-linking rate is zero-order and the Poisson approximation applies, i.e., for $\mu < 0.5$.

Cross-linking of Radioactively Labeled Rhodopsin

Cross-linking of ^{14}C-NEM labeled rhodopsin was used to verify the quantitative analysis of the oligomeric distributions and determine the fraction of the total protein that contributed to the analysis. ROS membranes that had been reacted with ^{14}C-NEM to quantitatively label available sulfhydryl groups on rhodopsin were treated with 0.1 mM glutaraldehyde as in the experiments presented in Fig. 4 and analyzed on SDS-polyacrylamide slab gels. The observed peaks correspond to monomeric opsin or *n*-mers with $n = 2$–4. (Larger oligomers were not resolved because of slice thickness.) Some highly cross-linked opsin was found at the top of the gel. Oligomeric distributions and corresponding μ values were calculated from the radioactivity in each protein peak and distributions plotted as a function of μ (not shown) were in agreement with the results derived from analysis of Coomassie-blue staining (Fig. 2). The agreement between the two methods indicates that Coomassie-blue staining was proportional to the

[4]A contribution of GTP-binding protein to the observed oligomeric distributions is also possible. However, the amount bound to isolated, dark-adapted ROS and cross-linkable by limited reaction is likely to be within the error of the analysis.

amount of opsin in a given gel regardless of whether the protein was monomeric or cross-linked.

Interpretations of the oligomeric distribution are meaningful only if a significant fraction of the total protein is measured. The proportion of the total protein (cpm) remaining at the top of the gel was determined at several reaction times. The values are plotted in Fig. 4, which also shows the progress of cross-linking in terms of μ. In the range where the cross-linking rate appears zero order, more than 75% of the protein enters the gel and hence contributes to the analysis of oligomer distribution. Thus in the range where the random model applies, the cross-linking patterns reported here represent the behavior of the majority of rhodopsin molecules.

Glutaraldehyde Cross-linking of Aldolase

The results of rhodopsin cross-linking in ROS membranes were compared with the cross-linking pattern for a known oligomeric protein reacted under similar conditions. Aldolase was chosen because it is a tetramer containing subunits (40,000 daltons) comparable in size to rhodopsin. Structural information from x-ray crystallography reveals that the tetramer possesses D2 symmetry (16). Preliminary cross-linking results using diimidates (15) were also consistent with an oligomeric structure having D2 symmetry.

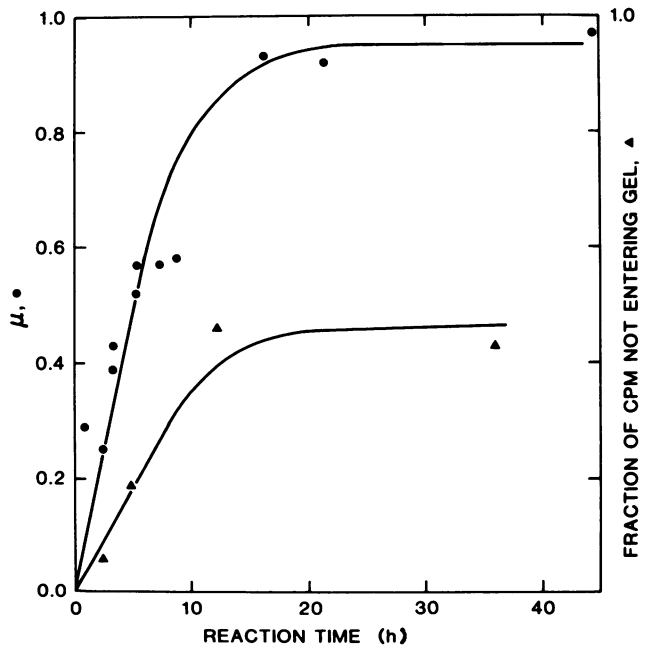

FIGURE 4 Progress of glutaraldehyde cross-linking of isolated ROS membranes. Suspensions of ROS membranes (±^{14}C-NEM label) at 50 μg rhodopsin/ml were cross-linked by incubation with 0.1 mM glutaraldehyde at 4°C. Samples were taken from the suspension at various times, ROS were solubilized and analyzed for oligomeric distribution as described in Experimental Procedures. For unlabeled ROS, μ (●) determined from densitometry at 550 is plotted vs. time. For ^{14}C-NEM labeled ROS, the fraction of total cpm in the top 1 mm slice of the gel (▲) is plotted vs. reaction time.

Aldolase was cross-linked with 1 and 5 mM glutaraldehyde for times ranging from 2 to 200 min. A difference between aldolase and rhodopsin cross-linking was readily apparent from inspection of gels, in that dimer and tetramer predominated over monomer for aldolase even at relatively short reaction times. The data from glutaraldehyde cross-linking of aldolase are plotted in Fig. 5 and compared to the model prediction for a planar tetramer[5] with D2 symmetry (solid lines are from Fig. 3 *C*). Cross-linking with glutaraldehyde for the times indicated led to almost complete depletion of monomer with the concomitant appearance of three higher molecular-weight bands having apparent molecular weights corresponding to dimer, trimer and tetramer, respectively. In agreement with the model, dimer and tetramer predominated strongly over monomer and trimer at higher degrees of cross-linking. Most of the aldolase time points yield values of $\mu > 1$. It is evident from these results that the cross-linking of aldolase proceeds more rapidly than that of membrane-bound rhodopsin; monomer is virtually depleted in 2 h for aldolase whereas for ROS, 50% of the rhodopsin remains monomeric even after 24 h of reaction at the same concentration of glutaraldehyde. Thus rhodopsin's cross-linking was distinguished from that of an oligomer model by (*a*) having a slower overall rate and (*b*) displaying a pattern different from oligomeric models in that there was no preferential accumulation of oligomeric species reflecting a particular oligomeric structure under any of the conditions investigated.

Cross-linking of Rhodopsin with Other Bifunctional Reagents

Interpretation of cross-linking results is complicated by the fact that it is possible to obtain negative results, i.e., no formation of covalent oligomers, even with proteins known to be oligomeric (15, 17). Thus it is possible that rhodopsin oligomers might exist, but intra-oligomeric cross-linking would be prevented because reactive groups are not sufficiently close or are sterically hindered. Although this is unlikely to be the case for glutaraldehyde (see Discussion), two other bifunctional reagents with different properties have been employed to evaluate this possibility in the case of rhodopsin. Data on the oligomeric distribution of ROS reacted with the more lipid-soluble amino group reagent diisocyanatohexane (18) are shown in Fig. 6. The data are in reasonable agreement with the random model and again show no preferential accumulation of dimer, trimer or tetramer. For the cross-linking of intact retinas with cupric *o*-phenanthroline (see Fig. 1 *C*) the presence of some nonspecific oligomers, presumably resulting from air oxidation, prevents a rigorous interpretation of cross-linking

[5]Although aldolase is most likely tetrahedral (16), its cross-linking would be equivalent to that of a planar molecule if one pair of contacts were unreactive.

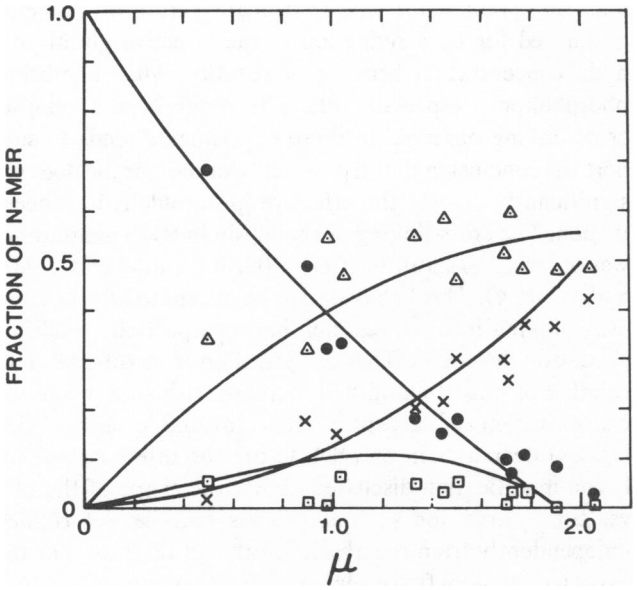

FIGURE 5 Oligomeric distribution of glutaraldehyde cross-linked aldolase. Aldolase was cross-linked in the presence of 1 mM and 5 mM glutaraldehyde at 0°C. Samples containing 150 μg protein were withdrawn at times ranging from 2 to 200 min. Oligomeric distributions were determined from densitometric traces of solubilized samples run on 7.5% SDS-polyacrylamide gels. Distributions are plotted as mass fraction of *n*-mers vs. μ. *n* = 1, ●; 2, △; 3, □; 4, *X*.

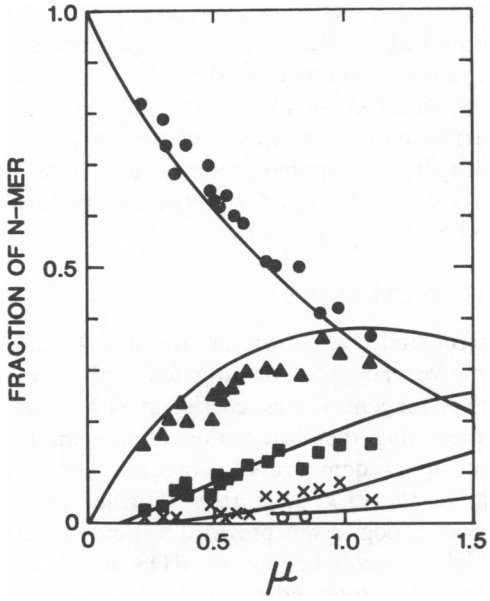

FIGURE 6 Oligomeric distributions of ROS membranes cross-linked with diisocyanatohexane. ROS suspensions at 50 μg rhodopsin/ml were treated with 0.01% diisocyantohexane at room temperature. Samples containing 100–200 μg were withdrawn at times ranging from 5 to 80 min. Distributions were determined from densitometric traces of samples run on 7.5% SDS-polyacrylamide gels. Mass fraction of *n*-mers was plotted vs. μ. *n* = 1, ●; 2, ▲; 3, ■; 4, *X*; 5, *o*.

patterns. Nevertheless, it can be noted that distributions of oligomeric species produced by this reagent (data not shown) fit the random model very well for μ values up to 1.2. For higher levels of cross-linking at least, the relative contribution from air oxidation is not likely to be significant. In summary, the overall patterns of cross-linking obtained with three different reagents are similar and consistent with predictions of a model for random cross-linking of rhodopsin monomers.

DISCUSSION

Monsan et al. (9) have shown that at alkaline pH, glutaraldehyde forms variable length polymers in which aldehyde groups are conjugated with ethylenic double bonds. These molecules form the acid stable linkages responsible for the observed covalent cross-linking of proteins by glutaraldehyde. In the predominant species of the cross-linking reagent (trimer), the reactive aldehyde groups are separated by one double and five single carbon-carbon bonds. However, greater separations will occur in larger polymers. Because of the widely variable distance between its functional groups, glutaraldehyde should be an optimal reagent for random cross-linking of amino groups in proteins.

An oligomeric distribution characteristic of monomeric rhodopsin was observed after cross-linking with two other bifunctional cross-linking reagents as well as with glutaraldehyde. At neutral pH, diisocyanatohexane cross-links proteins by reaction with amino groups (18). However, the fact that it is not water soluble, suggests that this reagent might have access to and react with a different set of amino groups than glutaraldehyde. Results obtained with amino specific reagents were compared with those for a sulfhydryl reagent. Cupric ion complexed with *o*-phenanthroline catalyzes the oxidation of protein sulfhydryl groups (17). This reagent led to significant enhancement of rhodopsin oligomers in retinal membranes relative to untreated controls.

Given the failure to observe rhodopsin cross-linking characteristic of a stable oligomeric protein with three diverse reagents, it seems unlikely that this negative result could have arisen as an artifact attributable to the lack of reactive groups with appropriate proximity and orientation. The experiments on aldolase demonstrate, by contrast, that the distribution of oligomers predicted for a planar tetramer is observed when aldolase is cross-linked by glutaraldehyde under conditions that approximate as closely as possible those used for studying rhodopsin in membranes. The ratio of glutaraldehyde to protein (~130-fold molar excess) was the same in both series. Rhodopsin cross-linking at 0.1 mM glutaraldehyde was observed for times as long as 40 h (Fig. 2) compared to the approximately 3 h that are required to deplete aldolase monomers using 1 mM glutaraldehyde (Fig. 5). To the extent that oligomeric rhodopsin in membranes would present a configuration similar to a planar tetramer in solution, its

cross-linking in these experiments would be expected to proceed at a comparable rate and produce, as for aldolase, a distribution of covalent oligomers enriched in species reflecting the structure of the stable oligomers.

Kinetic Considerations of Rhodopsin Cross-linking

Two lines of evidence indicate that the cross-linking occurring in ROS reflects random collisions between molecules rather than specific intra-oligomeric reactions. The first involves reaction rates. Glutaraldehyde has been shown to react almost exclusively with lysine residues of proteins (17, 19, 20). Quiocho and Richards (20) measured the progress of lysine modification in crystals of carboxypeptidase during glutaraldehyde cross-linking. They found that cross-linking proceeds at a rate roughly comparable to lysine modification. Hence to a first approximation the rate of chemical reaction appears to be rate limiting for cross-linking in a protein crystal. Using the cross-linking of protein crystals as an experimental model for glutaraldehyde cross-linking of oligomeric proteins, and taking the pH difference between the two experiments into consideration, a second-order rate constant of $\sim 50 \times 10\ M^{-1}\ min^{-1}$ is predicted for the formation of monovalent glutaraldehyde/lysine adducts for the aldolase experiments carried out at pH 8.5. The rate of cross-link formation is expected to be similar.

The observed rate constant for initial cross-link formation per aldolase monomer at 1 mM glutaraldehyde was $20 \times 10\ M^{-1}\ min^{-1}$. Thus, the cross-linking of aldolase is kinetically consistent with cross-linking behavior expected on the basis of observations on carboxypeptidase. For ROS membranes, initial rate of cross-link formation is linear in glutaraldehyde concentration up to ~5 mM and the second-order rate constant for initial cross-link formation between rhodopsin molecules is $2.6\ M^{-1}\ min^{-1}$, i.e., an order of magnitude slower than that observed for aldolase. The comparison of rates, although consistent with expectation for intra-oligomeric vs. collisional cross-linking, is affected by the number of lysines that participate in cross-linking between the respective monomers of aldolase and rhodopsin.

Direct experimental evidence that the initial rhodopsin cross-linking observed is a result of collisions between diffusing molecules comes from the parallel observations of transient dichroism and cross-linking of retinas (5). These show that the earliest detectable covalent cross-linking is accompanied by an increase in the size of diffusing species. Taken together, the results indicate that cross-linking of rhodopsin is slower than would be expected if stable oligomers existed in the membrane and that the slower rate is most likely accounted for because diffusion mediated cross-linking is slower than intra-oligomeric cross-linking.

A problem that has to be considered is whether the rate difference between the observed cross-linking of rhodopsin and the predicted lysine-modification rate is partially accounted for by a reduction in the effective glutaraldehyde concentration because of reaction with membrane phospholipids, especially PE. The progress of rhodopsin cross-linking observed in these experiments tends to support the conclusion that the presence of membrane does not significantly change the effective glutaraldehyde concentration. The cross-linking of rhodopsin in ROS membranes was observed to saturate after ~20 h at 0.1 mM glutaraldehyde (Fig. 4). This behavior can be accounted for because cross-linking involves reaction between partially modified rhodopsin molecules. The concentration of modified lysines capable of cross-linking will increase with time while the concentration of acceptor sites (available unmodified lysines) decreases in parallel. Using the rate constant for lysine modification discussed above, the shape of the rate vs. time curve for such a process can be determined independently from the absolute value of the rate. For the expected pseudo–first-order modification rate ($5 \times 10^{-3}\ min^{-1}$ at 0.1 mM glutaraldehyde), the rate of cross-linking would increase rapidly to a plateau. Decay from the plateau would be half complete at 6.7 h and reactive lysines would be saturated at 21 h. The observed progress of cross-linking for ROS membranes corresponds reasonably well with the simulated rate behavior. From data in Fig. 4, the phase with apparently constant rate was estimated to last ~5 h and saturation occurred at 20 h. These results support the argument that modification of lysine residues on membrane-bound rhodopsin is proceeding at a rate comparable to that predicted from carboxypeptidase results and observed for aldolase. Thus, there is no reason to suspect that the effective concentration of glutaraldehyde is markedly different for the experiments with the membrane-bound protein vs. the soluble protein. Apart from possible effects of PE on rate, the similarity in size and distribution of cross-linked oligomers observed with both sulfhydryl and amino group reagents indicates that cross-linking of PE is not significantly perturbing the pattern of cross-linking.

CONCLUSIONS

The current study has shown that the cross-linking behavior of native rhodopsin investigated with a variety of bifunctional reagents is most consistent with a quantitative model predicting the distribution of oligomeric species produced by random cross-linking between monomers diffusing in the disk membrane. Further evidence for monomeric rhodopsin was provided by demonstrating that glutaraldehyde cross-linking of ROS disk membranes results in both a rate and a pattern for rhodopsin cross-linking that can be distinguished from a reasonable experimental model for a transmembrane oligomeric protein. In addition to ruling out planar oligomers of the usual kind, the results reported here also argue against large circular oligomers ("donuts"), such that the rhodopsin aggregates

would be equivalent to an infinitely long filament. As demonstrated by Knight (7), this configuration would result in a distribution of oligomers very close to that of the random model used here. However, the cross-linking should proceed at a rate characteristic of oligomers and the range of conditions used should have produced larger values of μ, as seen when actin filaments were cross-linked (7). Fatt (21) has postulated the existence of oligomers ($n = 12$) that might have properties in common with both of the oligomeric alternatives discussed above. The results reported here argue against the existence of these large rhodopsin aggregates in dark-adapted photoreceptor membranes.

In native membranes virtually all observations of rhodopsin's size, shape, and mobility (1, 22) are compatible with a monomeric structure although these studies did not specifically address the issue of oligomeric state. Most reported evidence for oligomeric rhodopsin (with the exception of freeze-fracture electron microscopy) has been derived from detergent-solubilized rhodopsin (4, 23) or rhodopsin that has been purified using detergent or organic solvents (24, 25). It therefore appears that in environments other than the native membrane, rhodopsin may be stabilized by protein/protein interactions that are not characteristic of its native dark-adapted state.

The form of rhodopsin that regulates the Ca^{++} flux into the ROS cytoplasm is most likely a monomer. This follows from the fact that rhodopsin is monomeric in the dark-adapted state and that single R* molecules are not incorporated into oligomers stable over the time course of the Ca^{++} response (5). The question remains open whether or not formation of stable oligomers of photoexcited rhodopsin is involved in other light-regulated functions in ROS, for example, the activation of phosphodiesterase. This question is amenable to study by cross-linking methods similar to those reported here.

I thank Drs. Hamutal Neori-Borochov and Maurice Montal for suggesting the reagent diisocyanatohexane and for useful discussions.
This work was supported by U.S. Public Health Service Research grant EY 03105.

Received for publication 17 January 1984 and in final form 6 July 1984.

REFERENCES

1. Blasie, J. K., C. R. Worthington, M. M. Dewey. 1969. Molecular localization of frog retinal receptor photopigment by electron microscopy and low-angle x-ray diffraction. *J. Mol. Biol.* 39:407–416.
2. Brett, M., and J. B. C. Findlay. 1979. Investigation of the organization of rhodopsin in the sheep photoreceptor membrane by using cross-linking reagents. *Biochem. J.* 177:215–223.
3. Chen, Y. S., and W. L. Hubbell. 1973. Temperature- and light-dependent structural changes in rhodopsin-lipid membranes. *Exp. Eye Res.* 17:517–532.
4. McCaslin, D. R., and C. Tanford. 1981. Different states of aggregation for unbleached and bleached rhodopsin after isolation in two different detergents. *Biochemistry.* 20:5212–5221.
5. Downer, N. W., and R. A. Cone. 1985. Transient dichroism in photoreceptor membranes indicates that oligomers of rhodopsin do not form during excitation. *Biophys. J.* 47:277–284.
6. Kiehm, D. J., and T. H. Ji. 1977. Photochemical cross-linking of cell membranes. A test for natural and random collisional cross-links by millisecond cross-linking. *J. Biol. Chem.* 252:8524–8531.
7. Knight, P., and G. Offer. 1978. p-*NN'*-phenylenebismaleimide, a specific cross-linking agent for F-actin. *Biochem. J.* 175:1023–1032.
8. Knight, P. 1979. Hydrolysis of p-*NN'*-phenylenebismaleimide and its adducts with cysteine. *Biochem. J.* 170:191–197.
9. Downer, N. W., and S. W. Englander. 1977. Hydrogen exchange study of membrane-bound rhodopsin. I. Protein structure. *J. Biol. Chem.* 252:8092–8100.
10. Monsan, P., G. Puzo, and H. Mazarguil. 1975. Etude du mecanisme d' etablissement des liasons gluteraldehyde-proteines. *Biochemie.* 57:1281–1292.
11. Fung, B. K.-K, and W. L. Hubbell. 1978. Organization of rhodopsin in photoreceptor membranes. 1. Proteolysis of bovine rhodopsin in native membranes and the distribution of sulfhydryl groups in the fragments. *Biochemistry.* 17:4396–4402.
12. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major proteins of the human erythrocyte membrane. *Biochemistry.* 10:2606–2617.
13. Poo, Mu-m, and R. A. Cone. 1974. Lateral diffusion of rhodopsin in the photoreceptor membrane. *Nature (Lond.).* 247:438–441.
14. Molday, R. S., and L. L. Molday. 1979. Identification and characterization of multiple forms of rhodopsin and minor proteins in frog and bovine rod outer segment disc membranes. *J. Biol. Chem.* 254:4653–4660.
15. Hucho, F., H. Mullner, and H. Sund. 1975. Investigation of the symmetry of oligomeric enzymes with bifunctional reagents. *Eur. J. Biochem.* 59:79–87.
16. Eagles, P. A. M., L. N. Johnson, M. A. Joynson, C. H. McMurray, and H. Gutfreund. 1969. Subunit structure of aldolase: Chemical and crystallographic evidence. *J. Mol. Biol.* 45:533–544.
17. Peters, K., and F. M. Richards. 1977. Chemical cross-linking: reagents and problems in studies of membrane structure. *Annu. Rev. Biochem.* 46:523–551.
18. Wold, F. 1972. Bifunctional reagents. *Methods Enzymol.* 25:623–651.
19. Steck, T. L. 1972. Cross-linking the major proteins of the isolated erythrocyte membrane. *J. Mol. Biol.* 66:295–305.
20. Quiocho, F. A., and F. M. Richards. 1966. The enzymatic behavior of carboxypeptidase-A in the solid state. *Biochemistry.* 12:4062–4076.
21. Fatt, P. 1981. Proteins of vertebrate rod outer segments: a possible role for multiple forms of rhodopsin. *Exp. Eye Res.* 33:31–46.
22. Blaurock, A. E. 1977. What x-ray and neutron diffraction contribute to understanding the structure of the disc membrane. *In* Vertebrate Photoreception. H. Barlow and P. Fatt, editors. Academic Press, Inc., London. 61–76.
23. Montal, M. 1979. Rhodopsin in model membranes. *Biochim. Biophys. Acta.* 559:231–257.
24. Corless, J. M., D. R. McCaslin, and B. L. Scott. 1982. Two-dimensional rhodopsin crystals from disk membranes of frog retinal rod outer segments. *Proc. Natl. Acad. Sci. USA.* 79:1116–1120.
25. Borochov-Neori, H., P. A. G. Fortes, and M. Montal. 1983. Rhodopsin in reconstituted phospholipid vesicles. 2. Rhodopsin-rhodopsin interactions detected by resonance energy transfer. *Biochemistry.* 22:206–213.