TRANSIENT DICHROISM IN PHOTORECEPTOR MEMBRANES INDICATES THAT STABLE OLIGOMERS OF RHODOPSIN DO NOT FORM DURING EXCITATION

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ABSTRACT If a photoexcited rhodopsin molecule initiates the formation of rhodopsin oligomers during the process of visual excitation, the rate of rotational diffusion of the rhodopsin molecules involved should change markedly. Using microsecond-flash photometry, we have observed the rotational diffusion of rhodopsin throughout the time period of visual excitation and found that no detectable change occurs in its rotational diffusion rate. Partial chemical cross-linking of the retina yields oligomers of rhodopsin and causes a significant decrease in the rotational diffusion rate of rhodopsin even when as little as 20% of rhodopsin is dimeric. Moreover, the pattern of oligomers formed by cross-linking, taken together with the magnitude of decreases in rotational diffusion rate accompanying the cross-linking reaction, suggests that rhodopsin is a monomer in the dark-adapted state. The experiments reported here show that photoexcited rhodopsin molecules do not irreversibly associate with unbleached neighbors during the time course of the receptor response. Hence, it is not likely that stable oligomers of rhodopsin trigger the excitation of the photoreceptor cell.

INTRODUCTION

One important and widespread function of membrane proteins is to mediate ion translocation through membranes. In spite of the diversity of biological pores, channels, and transport systems being investigated, the structural and mechanistic details of the translocation process remain to be elucidated in virtually every case. Some of the basic questions concerning how proteins control ion movement in membranes are exemplified in photoreceptors. Photoexcited rhodopsin molecules in disk membranes of vertebrate receptors trigger the generation of the receptor potential. It has long been proposed that the primary role of rhodopsin is to release an ionic transmitter (Ca^{++}) from disk membranes (1, 2), presumably by acting as a light-activated Ca^{++} channel, and there is now strong evidence for both the release of Ca^{++} from disk membranes and its transmitter role in vertebrate outer segments (3–6). Moreover, purified rhodopsin has been shown to mediate changes in ion permeability for Ca^{++} and H^{+} as well as other cations in recombinant phospholipid vesicles in response to light (7–10) and Ca^{++} can be released from preloaded disk membrane vesicles (11) by photoexciting rhodopsin. If rhodopsin functions as a light-activated ion channel,[1] a fundamental question is whether a rhodopsin monomer forms the ion channel or whether the channel is formed by the lateral association, within the membrane, of several rhodopsin molecules. Oligomeric configurations of this kind have been proposed as a general feature of membrane transport systems for ions (12, 13). Moreover, both the size and density of particles in some freeze-fracture images of dark-adapted disk membranes raise the possibility of oligomeric rhodopsin (14).

In the excitatory response of the photoreceptor cell, such an oligomeric channel might exist only transiently. Given the rapid rotational and translational diffusion of rhodopsin in photoreceptor membranes (15–17), there is more than adequate time available during the latent phase of the excitatory process for a photoexcited molecule of rhodopsin to collide and combine with other rhodopsin molecules (or conversely, for a preformed oligomer to dissociate and the proteins to diffuse apart). Lipid bilayers containing rhodopsin do in fact respond to light with a pattern of conductance changes suggesting the formation of transmembrane channels by the lateral aggregation of bleached rhodopsin molecules (18).

The studies we report here were undertaken to test whether or not light causes rhodopsin molecules in the receptor membrane to form oligomers that could function as ion channels in the excitatory process. Should an oligomer be the native association state of rhodopsin, its dissociation could also be detected in our experiments. However, since flash photometry limits observations to changes occurring in a large fraction of the rhodopsin molecules, our results do not provide any information about interactions of rhodopsin with other proteins in the

[1]The possibility of a carrier mechanism cannot be ruled out, although the transmembrane structure of rhodopsin does not require it. Similar questions can be asked concerning a carrier.

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rod outer segment (e.g., the GTP-binding protein and opsin kinase), since these proteins are present at such low concentrations that they could affect the rotational diffusion of only a small fraction of rhodopsin molecules.

METHODS

Rotational diffusion was observed using the flash photometer described by Cone (15) with the following modifications. A continuous-wave krypton ion laser (CR-500K, Coherent Radiation Inc., Palo Alto, CA) was used in place of the original Xenon flashtube. Measuring pulses 1 ms in duration were delivered to the retina by opening an electronic shutter (Vincent Associates, Rochester, NY). The measuring light was focused onto the retina and refocused onto the photomultiplier tube (4526, RCA Electro-Optics and Devices, Lancaster, PA), using a pair of ×10, NA 0.25 objectives (Nikon Inc., Garden City, NY). The maximum intensity of the measuring light delivered to a circular patch of retina ~100 μ in diameter was on the order of 1 W/cm^2 and bleached <4% of the rhodopsin in 1 ms. The actinic (bleaching) flash, ~5 ns in duration, was provided by a pulsed nitrogen laser (AVCO C-950; Everett Research Laboratories, Everett, MA) dye-tuned to emit at 470 nm and was timed to occur during the first 10% of the measuring pulse. The actinic light was passed through a 480-nm cut-off filter (Ditric Optics, Inc., Hudson, MA) and neutral density filters to adjust the intensity so that bleaches of 10–40%/flash were obtained with the actinic light encompassing the entire area of the measuring beam. Additional interference filters protected the photomultiplier from the actinic flash. For double flash experiments, the measuring and bleaching illumination sequence was activated twice, at various intervals, using waveform and pulse generators (Tektronix, Inc., Beaverton, OR) together with an additional gating switch (Uniblitz, Vincent Associates, Rochester, NY). Thus the geometry of actinic and measuring light, as well as their position with respect to the retina, were identical for the two flashes.

Retinas were dissected from eyecups of dark-adapted *Rana Catesbeiana* under dim red light in a physiological saline buffer (111 mM NaCl, 2.5 KCl, 5 mM $CaCl_2$, 3 mM CDTA (*trans*-1,2-diaminocyclohexanetetraacetic acid), 5 mM D-glucose, 3 mM TES (*N*-tris [hydroxymethyl] methyl-2-aminoethanesulfonic acid), pH 7.4. Pieces of retina (3 mm × 3 mm) were placed in a small covered chamber on a microscope slide with the rod outer segments oriented upward. The slide was placed in a temperature-controlled stage with the chamber in the optical path of the microscope. The sample was scanned under dim red illumination to locate regions in which rod outer segments were well aligned parallel to the optical path. The decay of photoinduced transient dichroism was then measured by recording the absorbance changes induced by actinic flashes polarized either parallel to or perpendicular to the polarization of the measuring light. Alternate parallel and perpendicular flashes were delivered consecutively to the same patch of retina or to closely spaced patches as indicated.

Cross-linking of bullfrog retinas, electrophoresis, and analysis of the electrophoretic patterns were performed as described in the accompanying paper (19). If cross-linked retinas were to be used for measurement of rotational diffusion rates, small pieces (25%) of retina were removed, rinsed twice in phosphate-buffered physiological saline, then observed in the microspectrophotometer as described above. The remainder of the retina was used to determine the extent of cross-linking by gel electrophoresis.

The osmolarity of solutions used in cross-linking experiments was determined using a vapor pressure osmometer (Wescor, Inc., Logan, UT). The osmolarity of the 250 mM glutaraldehyde stock (250 ± 2 mOsm) was close to that of physiological saline solutions (TES saline, 251 ± 4; phosphate saline, 226 ± 4 mOsm) used in cross-linking and/or measurements of transient dichroism. Therefore, retinas were not subjected to hypoosmotic shock during cross-linking or subsequent measurement of transient dichroism. Hypoosomtic medium can cause an increase in the rotational relaxation time for rhodopsin in retinal membranes (Downer N. W., and R. A. Cone, unpublished observations).

[2]Qualitatively similar results were seen with dimethyl suberimidate and dimethyl adipimate at concentrations ranging from 20 to 100 mM. However, the degree of reaction obtained within the relatively short lifetime (29) of the imidates in solution at pH 8.5 was less than with the other reagents. For this reason, cross-linking with diimidates was not pursued.

RESULTS AND DISCUSSION

Rotational Diffusion

The rate of rotational diffusion of rhodopsin around an axis perpendicular to the disk membrane can be measured by observing the decay of transient dichroism induced by partially bleaching retinas with polarized light (15). All available structural information (summarized in reference 15) indicates that the chromophore maintains an essentially fixed orientation with respect to opsin, at least during the early photointermediate states. Thus, the decay of the induced dichroism probably reflects the rotational motion of the entire rhodopsin molecule.

For a cylinder undergoing Brownian motion in a membrane, the Stokes-Einstein relationship (20) gives the diffusion coefficient for rotational motion around the axis perpendicular to the membrane $D_R = 1/T = (kT/4\pi\eta r^2 h)$, where r is the radius of the cylinder and h is the thickness of the membrane. The relaxation time, which is proportional to the square of the radius, is therefore expected to be quite sensitive to changes in the radius of a roughly cylindrical transmembrane protein. The formation or dissociation of rhodopsin oligomers could involve twofold or even greater changes in the effective radius of the rotating molecules, and such changes should be easily detected by measurements of rotational relaxation times.

In these studies we sought to detect specifically those changes in rotational diffusion rates that might reflect events having a direct role in excitation. Since rhodopsin in disk membranes regulates sodium channels in the plasma membrane, located some microns away, changes in rhodopsin that trigger excitation must precede the onset of the receptor potential in the plasma membrane by an interval at least as great as diffusional delay times (~10 ms or longer, depending on the diffusion coefficient for small molecules or ions in the cytoplasm). The half-time for the rise of the receptor response in frog rod outer segments is in the range of 250 ms at 20°C (21). During the interval between photon absorption and the appearance of the receptor potential, several identifiable photointermediates of rhodopsin form and rapidly decay, leading finally to the production of metarhodopsin II with a half-time of ~5 ms at 10°C (22). Metarhodopsin II is then stable for the duration of the receptor response under physiological conditions. The formation of metarhodopsin II is accompanied by changes in protein and/or lipid conformation in photoreceptor membranes (22, 23) that might induce a change in rhodopsin's association state or in its interactions

with other proteins. Taking these observations into account, we made measurements of rotational diffusion rates during the time interval from 4 ms out to 800 ms after an initial bleaching flash; that is, from the time at which a significant amount of metarhodopsin II accumulates until the peak of the receptor response is passed.

A second important consideration for detecting changes relevant to excitation is the great sensitivity of the photoreceptor cell. Since a single photon can excite the photoreceptor (24, 25), any oligomer of rhodopsin that functions in excitation must consist of one photoexcited rhodopsin (R*) combined with other unexcited molecules. Conversely, if excitation involves dissociation of a preexisting oligomer, then a single photoexcited molecule must be capable of dissociating the oligomer. In either case, if a flash bleaches a major fraction of the photopigment, both the photoexcited molecules and their unexcited neighbors should exhibit marked changes in their rotational diffusion rates. We chose in these experiments to monitor the unexcited molecules, since it is experimentally difficult to observe rotational diffusion of metarhodopsin II. An initial polarized bleaching flash converted >25% of the rhodopsin molecules to metarhodopsin II. A second polarized flash was then delivered at various intervals to determine the relaxation time for the unexcited neighbors of the metarhodopsin II molecules generated by the initial flash.

The rotational relaxation time for rhodopsin can be determined from the rate of decay of absorbance changes at 568 nm recorded with the measuring light polarized either parallel or perpendicular to the actinic flash. Values of the dichroic ratio are calculated from the records (oscilloscope traces) and plotted as a function of time to show the decay of transient dichroism. Data from a retina at 10°C are shown in Fig. 4. Points (x) on the solid curve are the mean values of the dichroic ratio from three sets of records taken on different patches of the retina. A rotational relaxation time of 47 ± 8 μs was determined from the decay curve as described previously (15). For retinas in which the rod outer segments are uniformly oriented with their long axes parallel to the path of the measuring light, an initial dichroic ratio close to 3 was often observed when no more than 10–15% of the photopigment was bleached per flash. This indicates that under optimal conditions for measuring rotational diffusion rate, as utilized for this control experiment, little depolarization of the actinic and measuring light occurred (26).

The decay of transient dichroism was then examined under conditions such that light-induced changes in the association state of rhodopsin could be detected. Fig. 1 shows records of parallel and perpendicular absorbance changes produced by two successive actinic flashes. The third (bottom trace) shows the final absorbance with no actinic flash. In these experiments, each actinic flash bleached 25–40% of the rhodopsin present, as could be measured from the decreasing baseline absorbance. We used such intense actinic flashes to ensure that we could

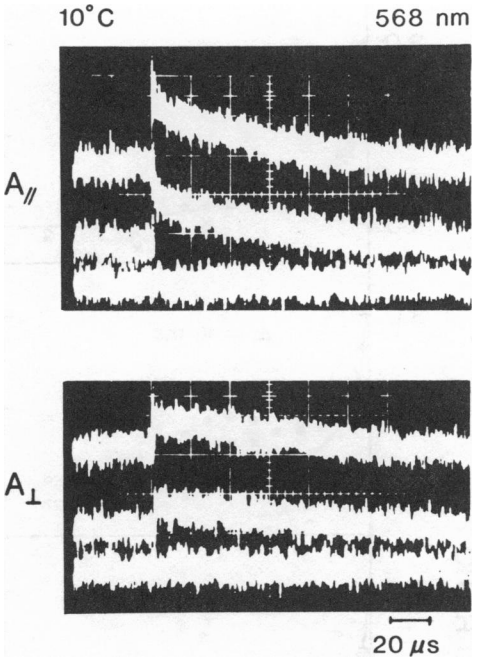

FIGURE 1 Transient dichroism from double flashes to bullfrog retinas. The *upper* trace in each set is the response to the first actinic flash. The *middle* trace shows the response to a second actinic flash delivered 40 ms later, and the *lower* trace was obtained 10 s later by exposing the same patch of retina to measuring light alone. Each set of three recordings was obtained from the same patch of retina. The two sets shown, with measuring light polarized either parallel or perpendicular to the polarized actinic flash, were obtained on closely spaced patches. The first flash bleached 38% of the rhodopsin corresponding to a change in transmission of 5.8%. The second flash bleached less, as shown by comparison of the absorbance changes resulting from the two flashes. In control records on the same retina, actinic flashes bleaching 10–15% were delivered to measure decay of transient dichroism under conditions optimal for observing high dichroic ratios. In these control experiments, the dichroic transients obtained from parallel and perpendicular records made on the same patch were similar to those from the experimental (double-flash) records on neighboring patches with higher percentages of rhodopsin bleached per flash (see also Fig. 2).

detect an effect on remaining rhodopsin even for the case where a single R* might associate with as few as one to three other molecules.

Experiments like that shown in Fig. 1 were performed using the following intervals between first and second flashes: 4, 5, 10, 20, 40, 100, 160, 200, 250, 320, 400, 500, 600 and 800 ms. In Fig. 2, the rates of decay of transient dichroism are shown for first (●) and second flashes (x) recorded at several representative intervals. The consecutive flashes allow a comparison of the behavior of molecules in the same population of rod outer segments before and after the production of the photointermediate metarhodopsin II. In no case was there a significant difference between the time courses of transient dichroism recorded from the first and second flashes. The noise in the dichroic ratio for the double-flash experiments was considerable, especially in the later part of the decay when both parallel and perpendicular absorbance changes were small. Nev-

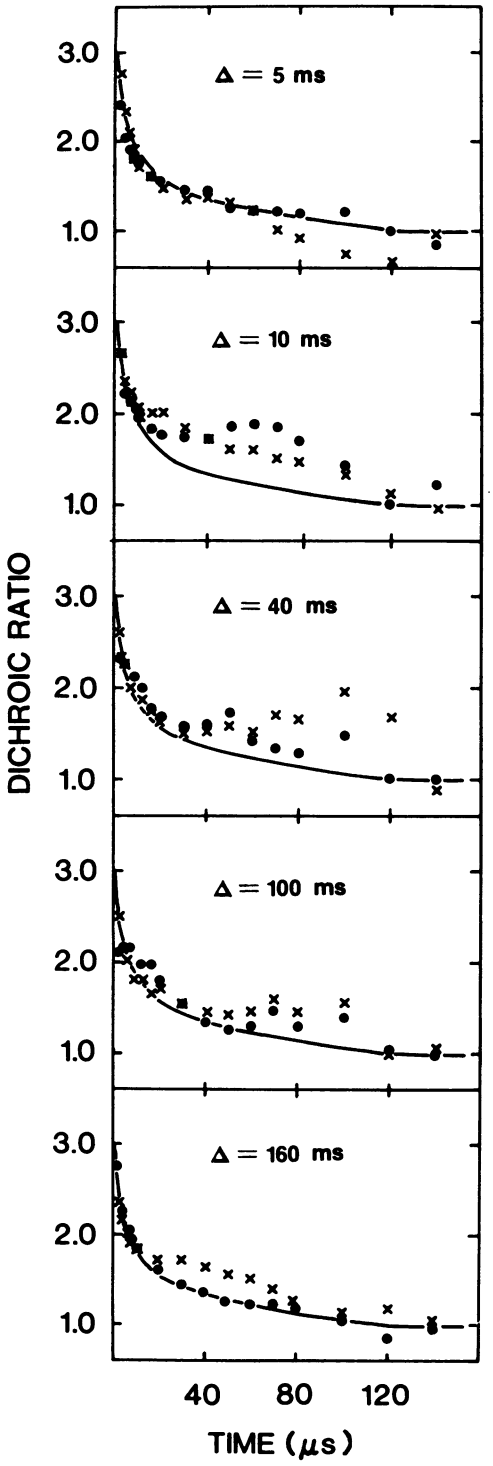

FIGURE 2 Decay of dichroic ratio for double flashes. Values of the dichroic ratio at different times were calculated using records from first (●) or second (x) flashes. Time intervals between the first and second flash are indicated in each panel. Dichroic ratio = $\Delta A_{\parallel}/\Delta A_{\perp}$ where ΔA is taken as the change in signal relative to the initial baseline for parallel and perpendicular records, respectively. The solid curve, shown for reference in each panel, indicates the decay of the dichroic ratio determined as the mean from three sets of traces recorded using actinic flashes that bleach 10–15% of rhodopsin. Dichroic ratios in the double-flash experiments were measured on different patches of the same retina using higher intensity actinic flashes (see Fig. 1) that resulted in initial values of dichroic ratio somewhat <3. All records were obtained at 10°C on bullfrog retinas. The data are representative of results obtained for all intervals noted in the text. For intervals <200 ms, data were obtained from several different retinas over a period of 1 y.

ertheless, the variability in decay half-times between first and second flashes was less than that between first flashes to different patches in the same retina. Furthermore, the time at which the parallel and perpendicular traces became indistinguishable (which was clear from visual inspection after superimposing traces) was similar for first and second flashes, confirming that the overall time course for decay of transient dichroism was the same. Thus, there is no evidence to suggest that a photoexcited rhodopsin molecule either associates with neighboring molecules to form stable oligomers or that it causes an existing, stable oligomer to dissociate. These measurements should have detected any change in oligomeric state persisting for the lifetime of metarhodopsin II.

Cross-linking of Rhodopsin in Photoreceptor Membranes

Cross-linking with bifunctional protein modifying reagents provides a general method for investigating the oligomeric state of proteins. This method has been used successfully to demonstrate the subunit structure for several enzymes in solution (27–29) and has been applied to many membrane systems (29; for a review, see reference 30). Here, the formation of covalently cross-linked oligomers of rhodopsin in retinas not only provided information on the oligomeric state of dark-adapted rhodopsin, but also enabled us to demonstrate that small changes in oligomeric state do indeed cause significant changes in the rotational relaxation time for rhodopsin.

Treatment of retinas with glutaraldehyde (25–250 mM) led to formation of covalent oligomers of rhodopsin that were identified on SDS (sodium dodecyl sulfate) polyacrylamide gels of solubilized rod outer segments. In Fig. 3, the protein composition of rod outer segments from glutaraldehyde cross-linked retinas is compared with that from an unreacted control. Whereas the major component in the untreated rod outer segments (Fig. 3 *A*) is a protein of ~35,000 dalton molecular weight (opsin), cross-linked retinas exhibited additional polypeptides that migrated to positions expected for oligomers ranging from dimer to hexamer (Fig. 3 *B*, *C*, *D*). Increasing either the concentration of glutaraldehyde or the reaction time at 0°C generally increased the fraction of rhodopsin that became cross-linked. A characteristic distribution of oligomers (*n*-mers) was observed in which the yield of oligomer decreased as *n* increased. This pattern has been observed in sheep photoreceptor membranes (31) and was interpreted to be indicative of monomeric rhodopsin. Extension of the glutaraldehyde cross-linking studies reported here (19) indicates that dark-adapted rhodopsin is indeed monomeric and that

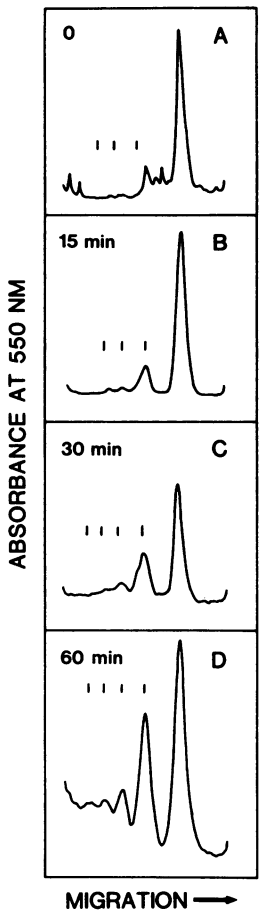

FIGURE 3 Glutaraldehyde cross-linking of bullfrog retinas. Rod outer segments (ROS) isolated from glutaraldehyde-treated retinas were analyzed by SDS-gel electrophoresis. Electrophoretic patterns are shown for unreacted controls (*A*) and for ROS after cross-linking with 125 mM glutaraldehyde for the times indicated (*B–D*). The unreacted control has a major protein component with an apparent molecular weight of 35,000 daltons, estimated from the migration of standard proteins. Tick marks indicate the expected migration for oligomers of opsin as determined from the standard curve for each gel shown. Several minor protein components are present in controls. Although migration of these proteins is different from that expected for oligomers, the presence of some dimer in control rod outer segment cannot be ruled out.

cross-linked oligomers are produced as a result of collisions between the closely spaced monomers in the fluid photoreceptor disk membrane (see also below).

The decay of transient dichroism was slowed significantly in retinas that had been partially cross-linked by glutaraldehyde (125 mM) for times ranging from 15 to 105 min. The effects of different degrees of cross-linking on the rotational relaxation rate of photopigment molecules are shown in Fig. 4. Table I gives the distribution of oligomers present in the retinas on which these measurements were made. These data allow us to evaluate the sensitivity of our method for detecting oligomers through measurement of rotational relaxation rates. Cross-linking for 15 min with 125 mM glutaraldehyde led to the appearance of a barely detectable slow component in the decay of transient dichroism (Fig. 4). Only a limited fraction of the diffusing molecules appeared to be involved and the half-time for decay of the slow phase was roughly 2–3 times longer than the decay of dichroism in control retinas (–x–). The uncertainty in the value of the dichroic ratio for each of the cross-linked retinas is similar to that shown for the control retina in Fig. 4. The change observed after 15 min of reaction barely exceeds the uncertainty in the measurement. The retina in this experiment was found to have 15% of the opsin molecules present as covalent dimers with only a few percent in higher oligomers (Table I). Replicate determinations of oligomeric distribution from a given sample gave values that agreed to ±5%. We therefore conclude that as little as 20% dimeric rhodopsin could have been detected by our measurements of rotational relaxation rates in untreated retinas; for larger oligomers the method should be even more sensitive.

The observed degree of slowing in rotational diffusion rates can be accounted for reasonably well by the formation of covalent oligomers as detected by electrophoresis. As the proportion of cross-linked molecules was increased, an increasing fraction of molecules exhibited rotational half-times as much as 10 times greater than those seen in controls. The decay of dichroism in cross-linked retinas was biphasic. There was general quantitative agreement between the fraction of oligomers and the amplitude of the slow phase. For reaction times longer than 30 min, the faster phase of the decay was slowed at least 2–3-fold, even though as much as 50% of the rhodopsin remained monomeric (Table I). Monomeric rhodopsin might be expected to have nearly the same rotational relaxation rate in these retinas as in control retinas, and initial decays of the dichroic transients might therefore coincide. However, glutaraldehyde can react with amino-containing phospholipids, and it would not be surprising if it were to have a general effect on the apparent viscosity of the membrane or to cross-link phospholipids to proteins (see reference 32). In this regard, it is important that conditions (15 min of reaction) could be found in which oligomers were detected at the same time that the relaxation time for most rhodopsin was unchanged, indicating that the viscosity of the membrane was not altered markedly. Small molecule EPR probes that sense the microviscosity of the membrane bilayer (Downer, N. W., and C. Wey, unpublished data) indicated that the effect of glutaraldehyde cross-linking on microviscosity of retinal membranes was less than twofold and remained essentially constant under conditions where the rotational relaxation rates of rhodopsin changed significantly (reaction times up to 60 min). The observed changes in relaxation times for the slow phase (2–10-fold slower relative to controls) are consistent with an increase of from 1.5–3-fold in the effective radius of the diffusing species. This is a reasonable value for the change in radius given the oligomers (n = 2, 3, 4) found to be present in significant amounts in the cross-linked retinas. Collectively, these results suggest that oligomer formation is the

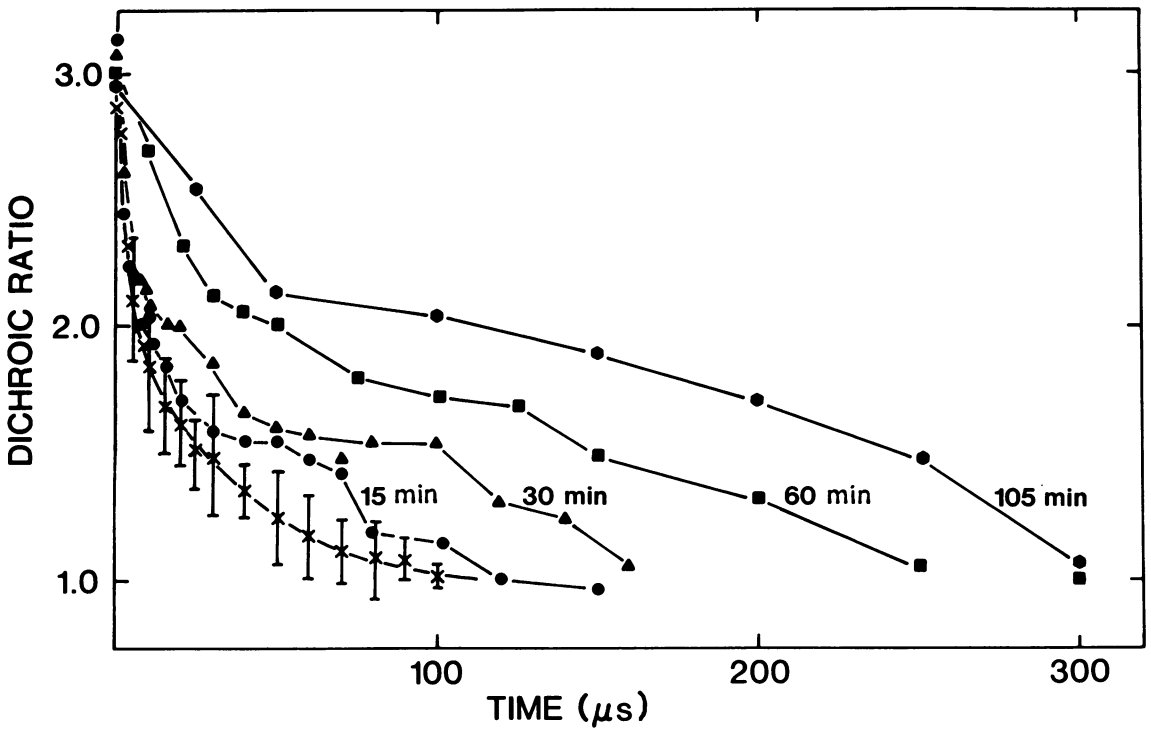

FIGURE 4 Decay of the dichroic ratio in glutaraldehyde cross-linked retinas. Cross-linking was performed as described (19). At the times indicated, retinas were transferred through several changes of phosphate-buffered physiological saline to dilute the glutaraldehyde reagent. Pieces of retina were cut out and mounted for measurements of transient dichroism as described. Decay of the dichroic ratio is shown for retinas cross-linked for the times indicated and is compared to that for unreacted controls (x). All measurements of transient dichroism were made at 10°C in phosphate saline, pH 7.5 using actinic flashes that bleached 10–15% of the rhodopsin. Dichroic ratios for the control retina are plotted as the mean ±SD from three sets of records.

dominant phenomenon affecting rotation diffusion rates in cross-linked retinas.

The behavior observed for the chemically cross-linked oligomers allows us to conclude with some certainty that oligomers of rhodopsin (R^*R_n) are not present in significant amounts in the doubly flashed retinas (Fig. 2). Even if R* forms only dimers ($R + R^* \rightleftharpoons RR^*$), a first flash should have produced at least 50% dimeric rhodopsin, since at least 25% of the rhodopsin was excited in the first flash. This would be a minimum value because larger changes are expected for $n > 2$. 50% dimerization corresponds to a degree of cross-linking midway between that seen in the 30 and 60 min glutaraldehyde-treated retinas (Fig. 4). The data in Fig. 2 comparing the decay of dichroism for two consecutive flashes clearly exclude a change of this magnitude. On this basis, we conclude that a single R* molecule does not irreversibly change its oligomeric state prior to, or during, the receptor potential. In light of the evidence that rhodopsin is monomeric in dark-adapted retinas (for summary, see reference 19), we can further conclude that a monomer of photoexcited rhodopsin is the species most likely to initiate the receptor response.

TABLE I
OLIGOMERIC DISTRIBUTION IN CROSS-LINKED RETINAS

Reaction time with 125 mM glutaraldehyde at 2°C	Fraction of opsin as *n*-mer					
	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$
15	0.83	0.15	0.02	<0.01	—	—
30	0.68	0.24	0.06	0.01	—	—
60	0.55	0.30	0.09	0.04	0.02	<0.01
105	not determined					

Transient Oligomers

The results reported here demonstrate that <20% of rhodopsin molecules are oligomeric, on the average, during a time interval in which excitation is likely to be triggered. Although we cannot categorically rule out short-lived oligomeric species involving R*, their existence is limited to the following possibilities: (*a*) oligomeric forms of meta I and (*b*) transient oligomers of meta II.

To consider the first case, the time window for rotational relaxation measurements is compared to the sequence of rhodopsin photointermediates. During the latent period of the photoreceptor response, photointermediates form and decay according to the scheme rhodopsin → bathorhodopsin → lumirhodopsin → meta I → meta II. In our experiments, meta I forms from lumirhodopsin during the dichroic transient used to measure the rotational relaxation time T_R of the photopigment, but it has largely been

replaced by meta II at 4 ms when the earliest second flash is delivered to the retina (Fig. 2). Thus T_R was not directly observed during the entire lifetime of meta I and the possibility cannot be excluded that association of meta I with neighboring rhodopsin triggers excitation. However, all available evidence on conformational changes in rhodopsin indicates that major alterations first occur at the stage of meta II. This evidence includes an increase in accessibility of the chromophore binding site (33), birefringence changes measured in rod outer segments (22), hydrogen exchange measurements directly reflecting protein conformation (23), and charge movements reflected in the ERP (34). To the extent that some alteration in protein conformation would necessarily accompany a change in oligomeric state, the absence of positive evidence that meta I is significantly altered relative to lumirhodopsin makes it seem unlikely that the oligomeric state of meta I changes. The rotational diffusion rate of lumirhodopsin has been investigated previously (15) and has been shown to be indistinguishable from that of rhodopsin. Formation or dissociation of oligomers involving lumirhodopsin can therefore be ruled out.

It is possible, in principle, that meta II molecules participate in oligomeric association reactions that are in rapid equilibrium with respect to the time scale of the receptor response. Thus transient complexes with lifetimes sufficient for significant ion release (should the complex form an ion channel) could be present, but at levels too low to detect in the double-flash experiments. The presence of such transient complexes would have to be evaluated by more sensitive methods.

Correlation of the Cross-linking Reaction and Changes in Rotational Diffusion

Although it is not the primary aim of this paper to assess the oligomeric state of rhodopsin in dark-adapted photoreceptor membranes, the parallel observations of the chemical cross-linking reaction and alterations of rotational diffusion rates reported here provide valuable insight into the glutaraldehyde cross-linking process. If oligomers of rhodopsin were to exist in the membrane, we would expect intraoligomeric cross-linking to proceed more rapidly than reactions between oligomers. Indeed, this is the basis for the use of bifunctional cross-linking reagents to assess oligomeric state of proteins in dilute solution. As the proteins in question become concentrated, the rate of interoligomeric cross-linking should increase, but even at high densities, as occur for rhodopsin in disk membranes, a rate differential should exist favoring covalent cross-linking within stable oligomers. Thus if rhodopsin were oligomeric, the initial cross-links would be intraoligomeric, but they would not affect the rotational relaxation time of rhodopsin because the size of intramembranous particles would not be altered. There should be a lag between the onset of cross-linking as assayed by electrophoresis and an observed change in rotational relaxation rate. In contrast, our experiments demonstrated that a detectable change in the relaxation rate of a fraction of the rhodopsin molecules was observed to accompany even a very limited extent of cross-linking detected by SDS gel electrophoresis (Fig. 4 and Table I). Furthermore, the changes in degree of cross-linking and rotational behavior continue more or less in parallel, as if most of the initial covalent cross-linking events we detect by electrophoresis are causing a change in the size of diffusing membrane particles. This is consistent with the notion that initial glutaraldehyde cross-linking occurs between diffusing particles.

The conditions used here for cross-linking retinas (0.5–2.5% glutaraldehyde, 15–60 min) are comparable to those employed for cross-linking crystals of carboxypeptidase (35). In the latter case, complete modification of lysine residues and cross-linking of protein molecules within crystals had occurred after ~1 h of treatment with 1% glutaraldehyde. It is difficult to assess the effect of membrane components when comparing cross-linking within retinal membranes to that of closely packed proteins in a crystal. However, it seems unlikely that the membrane environment would render the subunit contacts of rhodopsin oligomers, should oligomers exist, markedly less reactive to cross-linking by glutaraldehyde than crystalline proteins (or colliding oligomers). Only in this unlikely event would the specific intraoligomeric cross-linking reaction for membrane-bound rhodopsin be sufficiently slowed to not contribute to the observed cross-linking of rhodopsin reported here. The best interpretation of our data, therefore, is that the initial cross-linking of membrane-bound rhodopsin is between rhodopsin monomers that represent the native form of the protein in the dark-adapted retina.

Implications for Vision

How are our findings on the oligomeric state of rhodopsin related to biochemical events in rod outer segments? Photoexcitation of rhodopsin causes both release of Ca^{++} into the photoreceptor cytoplasm (6) and a decrease in cytoplasmic cGMP resulting from activation of a cGMP phosphodiesterase (36). The activation of the phosphodiesterase is mediated by association of a GTP-binding protein with R* on a time scale that implicates meta II (37, 38). The interaction of the two proteins, which has been measured by a light-scattering transient (38), would not be seen in our measurements of rotational diffusion because we directly observed only unbleached rhodopsin molecules. Thus, our results do not address the question of whether several meta II molecules associate to form $(\text{meta II})_n$, and it is possible that an oligomeric form of meta II might be involved in activation of cGMP phosphodiesterase.

The primary excitatory role of R* may be the regulation of Ca^{++} release to the rod outer segment cytoplasm (4–6). Results from studies of rhodopsin in phospholipid vesicles indicate that single R* molecules are capable of triggering Ca^{++} release and that the release from vesicles is correlated with the meta II photointermediate (39, 40). Our

observations of rotational diffusion show that single meta II molecules do not irreversibly associate with unbleached rhodopsin during the interval corresponding to Ca^{++} transients seen in intact photoreceptors (4, 5). Thus a photoexcited monomer of rhodopsin is the species most likely to be regulating Ca^{++} release into the cytoplasm during the photoreceptor response.

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