MODELING THE ROD OUTER SEGMENT BIREFRINGENCE CHANGE CORRELATED WITH METARHODOPSIN II FORMATION

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ABSTRACT A rapid birefringence loss associated with metarhodopsin II formation, $\delta(\Delta n)_{MII}$, is produced when frog rod outer segments are exposed to a bleaching light flash. To analyze the nature of the underlying structure change, measurements of $\delta(\Delta n)_{MII}$ were made in rod outer segments perfused with glycerol solutions to increase the refractive index of the cytoplasmic and intradisk spaces. Comparisons of experimental results with computed changes in the form birefringence component using two- and three-dielectric outer segment models for several putative structure changes were made. It is concluded that $\delta(\Delta n)_{MII}$ can be due to either a change in the intrinsic birefringence component caused by the reorientation of anisotropic molecules, or to a change in the form birefringence component caused by small changes in the cytoplasmic and/or intradisk volumes.

INTRODUCTION

Previous studies have shown that a bright light flash produces a complex sequence of birefringence ($\Delta n \equiv n_{\parallel} - n_{\perp}$) changes in *Rana pipiens* rod outer segments (ROS) (1, 2). For wavelengths >650 nm, the initial Δn change is a decrease $\delta(\Delta n) \approx -7 \times 10^{-5}$ that is correlated with the formation of metarhodopsin II (MII). Because $\delta(\Delta n)_{MII}$ is fast enough to be associated with the biochemical changes of visual transduction, it is of interest to identify the responsible structure change.

The net $\Delta n \approx 0.0008$ of dark-adapted ROS is the sum of a negative form birefringence, Δn_F, produced by the regular layering of different dielectric materials (disk membranes, cytoplasm, intradisk spaces) and a positive intrinsic birefringence, Δn_I, produced by the net alignment of dielectrically anisotropic molecules (presumably in the disk membranes) (1, 3, 4). In the wavelength region of the rhodopsin absorption band ($\lambda_{max} = 502$ nm for frog ROS), asymmetric anomalous dispersion of the refractive index causes the intrinsic component to have a complicated wavelength dependence (5). However, it has been shown previously that the $\delta(\Delta n)_{MII}$ signal measured at $\lambda > 650$ nm is caused by structure changes that alter either Δn_F or the chromophore-independent part of Δn_I (1).

The refractive index of ROS cytoplasmic spaces (and presumably the intradisk spaces) can be raised by perfusing with isotonic glycerol-containing saline solutions (1, 3). The consequent reduction of the differences in optical frequency dielectric constant between the membrane and interstitial spaces reduces the form birefringence magnitude. Using an improved micro-retardometer that separates Δn changes from possible light-scattering changes (6), this study confirms an earlier report by Liebman et al. (1) that $\delta(\Delta n)_{MII}$ is independent of perfusate refractive index, n_p. This experimental result has been interpreted to mean that the $\delta(\Delta n)_{MII}$ signal is caused by a reordering of anisotropic molecules in the disk membranes that reduces Δn_i (1, 2). However, earlier analyses only considered qualitative arguments and a two-dielectric ROS model to dismiss the possibility that $\delta(\Delta n)_{MII}$ is a change in Δn_F. This report shows that it is possible to model a form birefringence change that is appropriately independent of n_p if a three-dielectric model is used. The glycerol perfusion technique is therefore not definitive in separating form from intrinsic birefringence changes in ROS.

The effect the putative light-induced structure changes would have upon Δn_F can be calculated by varying the appropriate parameters in the equations describing the ROS as a lamellar dielectric system. For a given supposed structure change, the change in $\delta(\Delta n)_{MII}$, if it is a change in Δn_F, can also be computed as a function of n_p. If the experimentally determined dependence of $\delta(\Delta n)_{MII}$ upon n_p does not correspond to the calculated dependence of $\delta(\Delta n_F)$, it can be concluded that the putative structure change is probably not responsible for $\delta(\Delta n)_{MII}$.

In this study two classes of structure changes that would affect $\delta(\Delta n_F)$ are tested using a two- and a three-dielectric ROS model. For one class it is supposed that during MII formation, rhodopsin has a net motion into or out of the disk membrane to cause an increase or decrease in the membrane bulk refractive index and a concomitant opposite change in the cytoplasmic bulk refractive index (7–11). For the other class it is assumed that the relative volume fractions of the dielectric layers change due to

osmotically-induced contractions or expansions (10–13). One model works on the assumption that light causes the intradisk space to either contract or expand accompanied by a compensatory change in the cytoplasmic space so that disk-to-disk period and overall ROS length remain constant. For the other model it is assumed that the intradisk volume is constant, but the cytoplasmic spaces expand or contract with a consequent change in ROS length.

A partial account of the results and computations in this report has appeared previously (14).

METHODS

Experimental

ROS were isolated from dark-adapted *R. pipiens* retinas in a perfusion chamber as described previously (3). The procedure produces a large population of osmotically intact ROS embedded in a thin agar film that is permeable to glycerol-containing perfusates.

Simultaneous measurements of $\delta(\Delta n)$ and forward-direction light-scattering changes (δS) were made using a micro-retardometer/nephelometer (MR/N) described elsewhere (6). Dark-adapted ROS samples were positioned on the microscope stage of the MR/N using an infrared-sensitive television camera so that a 2 μm × 17 μm measuring beam passed through a single ROS in a direction normal to the cell axis. The MR/N was first placed in its microspectrophotometer (MSP) mode, and an initial visual pigment absorption spectrum measured. Time scans of $\delta(\Delta n)$ and δS were then begun using a deep-red measuring beam (λ_{max} = 708 nm, half-peak bandwidth = 48 nm). After recording baseline values, the ROS was exposed to a saturating 1.2 ms xenon flash, filtered to remove the red wavelengths used in the monitoring beam. The $\delta(\Delta n)$ and δS signals were monitored for 30 min to allow metarhodopsin III (which has an absorption spectrum that overlaps that of rhodopsin) to decay completely (15). A second absorption spectrum was then measured, and the difference spectrum calculated so that $\delta(\Delta n)_{MII}$ measurements could be normalized for the amount of rhodopsin photolyzed per unit path length.

Cells were perfused with isotonic glycerol-containing saline solutions as described previously (1–3). Perfusate glycerol concentration was varied from 0% (by vol) (n_p = 1.3350) to 40% (n_p = 1.3902). Between 8 and 23 cells were measured for each data point in Figs. 3 and 4. Outer segments perfused with glycerol remain osmotically responsive to 25% (wt/vol) sucrose, and do not stain with *N,N'*-didansyl cystine, indicating that the plasma membrane is intact.

Calculations

Intradisk and cytoplasmic volume fractions (f_i, f_c) and solids concentrations (s_i, s_c), membrane volume fraction (f_m) and bulk refractive index (n_m), and the contributions of Δn_I and Δn_F to the net Δn can be calculated from plots of net Δn as a function of perfusate refractive index, n_p. Previously determined values for these parameters were used in the calculations of this study (3, 4).

Because the intradisk volume fraction is small compared with the cytoplasmic and membrane volume fractions, a simple two-dielectric layer model of the ROS was used in previous studies (1, 3). Based upon the formulation of Wiener (16), the lamellar body in Fig. 1 *A* has a form birefringence (see Fig. 1) given by

$$\Delta n_F = \frac{-f_m f_c (\kappa_m - \kappa_c)^2}{2\,(f_m\kappa_c + f_c\kappa_m)\,(f_m\kappa_m + f_c\kappa_c)^{1/2}} \qquad (1)$$

where $f_m = D_m/(D_m + D_c)$, $f_c = 1 - f_m$, $\kappa_m = n_m^2$, and $\kappa_c = n_c^2 = (n_p + r_c)^2$. The refractive increment r_c is related to the cytoplasmic solids concentration by $r_c = 0.0018\ s_c$ (17). It is also assumed that $[f_m\ \kappa_m + (1.335 + r_c)^2]^{1/2} = 1.4106$, the bulk refractive index of frog ROS in 100% water Ringer solution (n_p = 1.335) measured using microscopic index matching (18). An increase in n_m, due, for example, to rhodopsin sinking into the disk membranes, would cause a concurrent decrease in s_c and therefore n_c. An osmotically induced change in f_c would cause compensatory change in f_m and s_c.

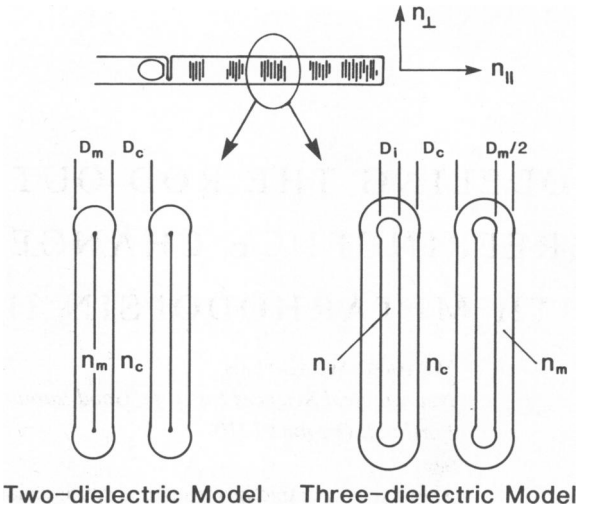

FIGURE 1 Diagrams showing ROS topology. Birefringence, Δn, is defined as the refractive index for light linearly polarized along the ROS axis, $n_\parallel$, minus the refractive index for orthogonal linearly polarized light, $n_\perp$. In the two-dielectric model on the left, the disk membrane pair has a refractive index n_m and spans a distance D_m. The interstitial cytoplasm has a refractive index n_c and spans a distance D_c. The volume occupied by the intradisk space is assumed to be negligible. In the three-dielectric model on the right, the intradisk volume is taken into consideration. An intradisk span D_i and refractive index n_i are assumed in the computer models of light-induced form Δn changes.

The three-dielectric lamellar body form birefringence, modified from a general expression (see Fig. 1 *B*) derived by Thornburg (19), is given by

$$\Delta n_F = \frac{-[f_i f_m \kappa_c(\kappa_i - \kappa_m)^2 + f_m f_c \kappa_i(\kappa_m - \kappa_c)^2 + f_i f_c \kappa_m(\kappa_i - \kappa_c)^2]}{2\,(f_i\kappa_m\kappa_c + f_m\kappa_i\kappa_c + f_c\kappa_i\kappa_m)\,(f_i\kappa_i + f_m\kappa_m + f_c\kappa_c)^{1/2}} \qquad (2)$$

where $f_i = D_i/D$, $f_m = D_m/D$, $f_c = D_c/D$, $D = D_i + D_m + D_c$, $\kappa_i = n_i^2 = (n_p + r_i)^2$, $\kappa_m = n_m^2$, $\kappa_c = n_c^2 = (n_p + r_c)^2$, $r_i = 0.0018\ s_i$ and $r_c = 0.0018\ s_c$. In this case the n_p = 1.335 boundary condition is

$$[f_i(1.335 + r_i)^2 + f_m\kappa_m + f_c(1.335 + r_c)^2]^{1/2} = 1.4106.$$

As with the two-dielectric case, it is assumed that n_m and r_c would increase and decrease respectively for the model in which rhodopsin sinks into the disk membrane from the cytoplasmic side (and conversely for rhodopsin extrusion into the cytoplasm). In the first changing-volume fraction model, it is assumed that any expansion or shrinkage of the intradisk space is compensated by an equal change in the dimension of the cytoplasm between the disks so that the disk-to-disk spacing and overall ROS length remain constant (13). In the second changing-volume fraction model it is assumed that the intradisk space remains constant while the cytoplasmic space shrinks or expands with a consequent change in disk-to-disk period and ROS length (10–12). In both of the volume-change models, intradisk and cytoplasmic refractive indices are assumed to change, because the constant solids contents become more concentrated or diluted. The disk membrane span and refractive index are assumed to remain constant.

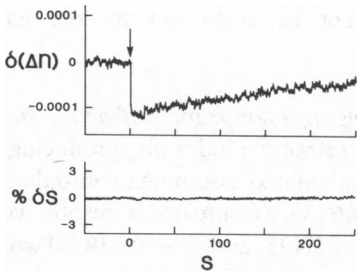

FIGURE 2 Upper trace, birefringence change induced in a single isolated frog rod outer segment by a saturating flash (arrow). The initial rapid birefringence loss, $\delta(\Delta n)_{MII}$, is correlated with the formation of metarhodopsin II. Lower trace, concurrent measurement of forward light scattering. No δS changes related to metarhodopsin II formation were detected for any of the perfusate refractive indices tested (6).

RESULTS

For ROS perfused with normal aqueous frog Ringer solution ($n_p = 1.335$), the mean $\delta(\Delta n)_{MII} = -6.7 \times 10^{-5}$. (Fig. 2). All structure change calculations use this as the criterion $\delta(\Delta n_F)$.

Normalized birefringence change computations for the six perfusate n_p values tested ($1.335 \leq n_p \leq 1.3902$) show that $\delta(\Delta n)_{MII}$ is nearly independent of the refractive index of the imbibed glycerol-containing perfusate (see data points and regression line in Figs. 3 and 4). This result agrees with an earlier study by Liebman et al. (1).

No forward light-scattering changes correlated with MII formation were seen for any of the n_p values tested (6). Light-scattering measurement resolution was $\delta I/I = 0.2\%$, 10 times smaller than scattering changes reported in ROS fragments and disk membrane vesicles (20–23).

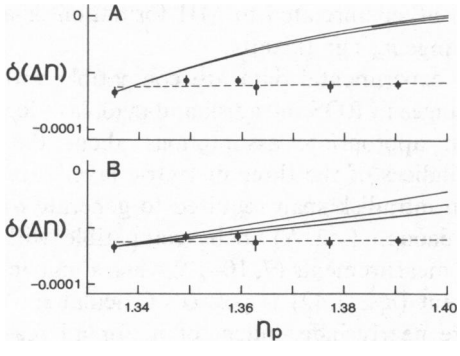

FIGURE 3 Comparison of normalized experimental data, $\delta(\Delta n)_{MII}$, with two-dielectric model calculations of ROS $\delta(\Delta n_F)$ as a function of perfusate refractive index, n_p, for different putative structure changes. Linear regression analysis (dashed lines) shows that $\delta(\Delta n)_{MII}$ is nearly independent of n_p. *A*, Changing cytoplasmic volume fraction model. For the example shown, computed plots of $\delta(\Delta n_F)$ (solid lines) were made assuming that $n_m = 1.495$, that (top to bottom) $f_c = 0.65$, 0.735, and 0.85, and that $\delta f_c = -0.0198$ (−16 Å), −0.0142 (−15 Å) and −0.0098 (−18 Å), respectively. Computed plots using $n_m = 1.510$ and $n_m = 1.475$ were qualitatively similar to the ones shown. *B*, Changing membrane refractive index model. In this example it was assumed that $f_c = 0.735$ and that (top to bottom) $n_m = 1.475$, 1.495, and 1.510, and that $\delta n_m = 0.00071$, 0.00055, and 0.00047, respectively. Results using the assumption that $f_c = 0.65$ and 0.85 were qualitatively similar.

Two-Dielectric Model

Changing Cytoplasmic Volume Fraction. Values of δf_c (and the equivalent change in membrane-pair spacing) yielding the criterion $\delta(\Delta n_F)$ were calculated using the two-dielectric model. There are two negative δf_c solutions (all unreasonably large) for each combination of n_m and initial f_c.

Typical computed changes in form Δn as a function of imbibed perfusate refractive index for several fixed δf_c values are shown in Fig. 3 *A*. Lack of correspondence between the computed plot and the experimental data points means that if it is appropriate to model ROS as a two-dielectric lamellar system, then $\delta(\Delta n)_{MII}$ cannot be caused by a light-induced shrinkage of the cytoplasmic space between the disk membranes.

Changing Membrane Refractive Index. Changes in n_m that produce the criterion $\delta(\Delta n_F)$ were also computed using the two-dielectric model. A pair of solu-

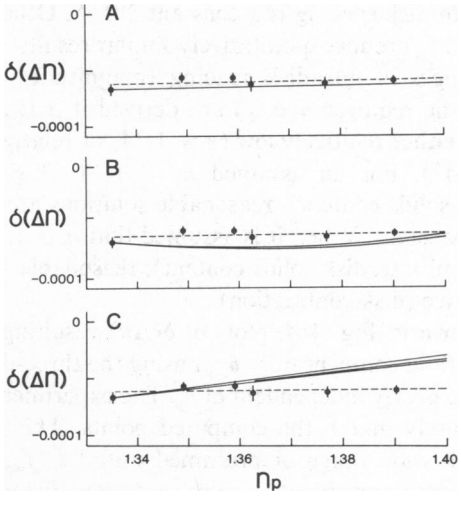

FIGURE 4 Comparison of normalized experimental data, $\delta(\Delta n)_{MII}$, with three-dielectric model calculation of ROS $\delta(\Delta n_F)$ as a function of n_p for different putative structure changes. *A*, Changing intradisk and cytoplasmic spaces with fixed disk-to-disk period. For the example shown, computed plots of $\delta(\Delta n_F)$ (solid line) were made assuming that $n_m = 1.510$, $f_m = 0.224$ ($D_m = 66$ Å) and $n_i = 1.46$; traces for $f_i = 0.034$, 0.119, and 0.203 ($d_i = 10$, 35, and 60 Å) superimpose. A value of $\delta f_i = -0.0032$ (−1 Å) gave the criterion $\delta(\Delta n_F)$ for all three cases. For all reasonable initial refraction indices and volume fractions, computed $\delta(\Delta n_f)$ plots approximated experimental results. *B*, Changing cytoplasmic space and disk-to-disk period. In this example it was assumed that $n_m = 1.495$, $f_m = 0.224$, and $n_i = 1.46$ (Eq. 2 has no solution for many parameter combinations when $n_m = 1.510$ is assumed). In the upper trace it was assumed that $D_i = 10$ Å; in the lower trace $D_i = 60$ Å. Values computed for δf_c were 0.0133 (16 Å) and 0.0203 (15 Å) respectively. Plots assuming other initial parameters using this model do not fit the experimental data as well as the plot shown. *C*, Changing membrane refractive index. For the example shown, it was assumed that $n_m = 1.510$, $f_m = 0.224$ ($D_m = 66$ Å), and $n_i = 1.34$; plots are shown (top to bottom) for an assumed $D_m = 60$, 35 and 10 Å respectively. Computed values for dn_m were 0.01075, 0.000850, and 0.000697, respectively. Qualitatively similar plots were derived using $n_i = 1.38$, 1.42, and 1.46.

tions, one small and positive, one large and negative, satisfy the two-dielectric model equation. Only the small, positive solutions, ranging from 0.00035 to 0.00126, are reasonable. Positive solutions imply a movement of material into the disk membranes from the cytoplasmic spaces.

Plots of computed form Δn as a function of n_p for fixed δn_m values (Fig. 3 *B*) also do not correspond to experimental results, suggesting that, if the model for ROS as a two-dielectric lamellar system is adequate, $\delta(\Delta n)_{MII}$ is not due to light-induced solids migration into or out of the disk membranes.

Three-Dielectric Model

Changing Intradisk and Cytoplasmic Spaces with Fixed Disk-to-Disk Period. For the three-dielectric lamellar model, changes in intradisk volume fraction δf_i (accompanied by compensatory changes in cytoplasmic volume fraction δf_c) yielding the criterion $\delta(\Delta n_F)$ were computed assuming that the $n_m = 1.510, f_m = 0.224$ (two membrane thicknesses of one disk occupy 66 Å), that and the disk-to-disk spacing is a constant 295 Å. Other values for n_m and f_m produce qualitatively similar results. Reasonable changes in intradisk spacing (magnitudes < 10 Å) yielding the required $\delta(\Delta n_F)$ are derived if it is assumed that n_i is either relatively low ($n_i = 1.34$) or relatively high ($n_i \geq 1.42$). For an assumed $n_i = 1.34$ (3 g/100 ml intradisk solids content), reasonable solutions are positive (disk expansion). When it is assumed that $n_i \geq 1.42$ (≥47 gm/100 ml intradisk solids content), reasonable solutions are negative (disk contraction).

As shown in Fig. 4 *A*, plots of $\delta(\Delta n)$ resulting from a fixed δf_i (and compensatory δf_c) using the three-dielectric model are nearly independent of n_p. The experimental data points closely match the computed points. This result is true for a wide range of presumed initial f_i, f_m, and n_m values. Such results indicate a change in Δn_F due to a small light-induced expansion or contraction of intradisk spaces cannot be ruled out as a cause for $\delta(\Delta n)_{MII}$.

Changing Cytoplasmic Space and Disk-to-Disk Period. Changes in cytoplasmic volume fraction (and the equivalent changes in disk-to-disk period) producing the criterion $\delta(\Delta n_F)$ were calculated assuming a range of n_m values. For all initial parameter combinations attempted, a substantial change in disk-to-disk spacing of more than 6 Å was required to produce the criterion $\delta(\Delta n_F)$.

Fig. 4 *B* compares typical plots of computed $\delta(\Delta n_F)$ values using the three-dielectric shrinking cytoplasm model with the experimentally determined $\delta(\Delta n)_{MII}$ as a function of n_p. In some cases (such as $n_i = 1.46$ chosen for Fig. 4 *B*) the computed plots approximate the experimental data points fairly well. Therefore, the shrinking cytoplasm model could be compatible with an MII-dependent form birefringence change if the disk membrane refractive index is relatively low, and if the sizable dimension changes required cannot be ruled out on the basis of other evidence.

Changing Membrane Refractive Index. Changes in membrane refractive index δn_m producing the criterion $\delta(\Delta n_F)$ were calculated assuming an initial $n_m = 1.510$ and $f_m = 0.224$ (66 Å). (Comparable results were found for $n_m = 1.475$ and 1.495, and $f_m = 0.339$.) Two δn_m solutions were found for each set of parameters, one large magnitude negative solution ($\delta n_m < -0.04$), and one smaller magnitude positive solution ($\delta n_m < 0.002$).

An example of a computed plot of $\delta(\Delta n)$ as a function of n_p for a fixed change in n_m using the three-dielectric model is shown in Fig. 4 *C*. As was the case with the two-dielectric model, the computed plots all have slopes significantly higher than the regression line through the experimental data points. The conclusion that $\delta(\Delta n)_{MII}$ cannot be due to a membrane refractive index change caused by light-induced solids migration (e.g., rhodopsin sinking or extrusion) is therefore model independent.

DISCUSSION

Improved measurements using glycerol perfusion to reduce the Δn_F contribution confirm the previous finding by Liebman et al. (1) that $\delta(\Delta n)_{MII}$ is independent of perfusate refractive index. Comparisons of computed form birefringence changes as a function of glycerol perfusate refractive index with the measured values of $\delta(\Delta n)_{MII}$ indicate the following.

(*a*) The $\delta(\Delta n)_{MII}$ signal is not due to a change in membrane refractive index. Small protein displacements measured in x-ray (8, 10, 11) and neutron (9) diffraction studies are either unrelated to MII formation or are too small to change n_m significantly.

(*b*) The experimental data are compatible with MII-related changes in ROS intradisk and cytoplasmic volume fractions if appropriate assumptions about the initial refractive indices of the three dielectric layers are made. Changes in intradisk span required to generate $\delta(\Delta n)_{MII}$ are small enough (<2 Å) to be compatible with x-ray diffraction measurements (7, 10–13) when a high intradisk solids content ($n_i \geq 1.42$) is posited. Calculated $\delta(\Delta n_F)$ values were nearly independent of n_p for all reasonable combinations of ROS component refractive indices and volume fractions. The shrinking/expanding cytoplasm model did not fit the experimental data as well as the shrinking/expanding disk model. For many reasonable combinations of component parameters, the criterion $\delta(\Delta n_F)$ could not be achieved by postulating a change in cytoplasmic volume fraction. In addition, for those combinations in which the criterion $\delta(\Delta n_F)$ could be achieved, the change in cytoplasmic spacing was at least 6 Å. This is well above the maximum change in disk-to-disk repeat period measured by Chabre and Cavaggioni (10) using rapid (50 s resolution) x-ray diffraction measurements in isolated ROS. In addition, Falk and Fatt (24) found that

light caused no changes in ROS length above the 2% resolution limit of their measurements (equivalent to a 6 Å change in disk-to-disk period).

Because results of the glycerol-imbibition experiments can be simulated using three-dielectric changing-volume-fraction models, it is possible to attribute $\delta(\Delta n)_{MII}$ to a change in either the form birefringence component or the intrinsic birefringence component. Contrary to the conclusions of earlier studies (1, 2), the glycerol perfusion method is not definitive in discriminating between the two types of birefringence changes. A change in Δn_i is supported by several other physical studies. A significant change in entropy during the metarhodopsin I → metarhodopsin II reaction suggests that membrane component configuration becomes considerably more random (25). Linear dichroism measurements show a substantial (5°–15°) reorientation of the retinaldehyde chromophore group of MII when compared with rhodopsin (26). Rotation of opsin aromatic residues has been postulated on the basis of light-induced changes in ROS diamagnetic anisotropy (27) and ultraviolet linear dichroism (28, 29).

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