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SLOW BLEACH-INDUCED COOPERATIVE FLUORESCENCE CHANGES IN BOVINE PHOTORECEPTOR DISK MEMBRANES

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Summary: The increase of protein fluorescence in suspensions of bovine photoreceptor disk membrane fragments was investigated under various conditions. The increment of fluorescence on bleaching was dependent on temperature, being about 10% at 10°C and 50% at 40°C. The time course of fluorescence increase also depended on temperature, and the activation energy was estimated to be about 14 kcal/mole. The relationship between the extent of fluorescence increase and the degree of bleaching was not stoichiometric. It was concluded that the environment of tryptophan residues of unbleached rhodopsin molecule(s) located near a bleached rhodopsin molecule is cooperatively modified upon bleaching (to a more hydrophobic environment).

Introduction: Cooperative phenomena play important roles in biological systems. As regards photoreception, Fein and Charlton (1) reported that the late receptor potential enhancement in Limulus ventral photoreceptor cells was cooperative, and chromophore-chromophore interactions in the purple membrane also appeared to show cooperativity (2). However, few data are available on cooperative conformational or environmental changes in vertebrate photoreceptor disk membranes in relation to photoreception. Our previous report demonstrated a bleach-induced cooperative turbidity change in the fragments of bovine rod outer segment (ROS) disk membranes (3). Turbidity measurements are indicative of the shape and size of the disk membrane fragments, but do not give any detailed information concerning the molecular structure or environment of rhodopsin molecules in the membrane. Ebrey (4) has reported fluorescence enhancement of the tryptophan (Trp) residues of rhodopsin on bleaching, and Guzzo (5) observed a light-induced rapid decrease followed by a slow increase in the protein fluorescence of rhodopsin.

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Thus, we attempted to confirm that cooperative conformational changes in fact occur at the molecular level, employing measurements of protein fluorescence as a probe. The cooperative changes in the membrane conformation reported in the previous paper (3) and those in the rhodopsin structure of ROS reported in this study may well be closely related to the mechanism of light and dark adaptation.

Materials and Methods: Fresh bovine eyes were obtained from the Tokyo Shibaura Intestine Co. The eyes were collected in a black polyethylen bag immediately after slaughter, kept in the dark at room temperature for 2-3 hr, then cooled with ice. ROS was isolated from the retinas by a slight modification of the method of Asai et al. (6). Fifty retinas were gently shaken instead of homogenized in 50% (W/V) sucrose dissolved in Ringer's solution A containing 115 mM NaCl, 3 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$ and 20 mM Tris-HCl buffer (pH 7.4). The shaken suspension was poured into a centrifugation tube and then overlaid with Ringer's solution A. After centrifugation at 15,000 x g for 25 min, crude ROS floating at the interface between Ringer's solution and 50% sucrose was collected. The collected ROS was centrifuged in Ringer's solution A at 6,000 x g for 15 min, then resuspended in 34% sucrose solution and centrifuged at 15,000 x g for 20 min. This partially purified ROS was sedimented in 28% sucrose solution and the supernatant was discarded. Purified ROS was obtained by washing the resulting pellet with Ringer's solution A by centrifugation at 6,000 x g for 15 min. ROS disk membranes were prepared by a slight modification of the low salt dialysis method of Raubach et al. (7). The washed ROS was suspended in a solution containing 115 mM KCl, 3 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$ and 20 mM Tris-HCl buffer (pH 7.4) and dialyzed against 200 volumes of cold (4°C) low salt solution (1.15 mM KCl, 0.03 mM NaCl, 0.03 mM $MgCl_2$, 1 mM $CaCl_2$ and 2 mM Tris-HCl buffer, pH 7.4) for 24-36 hr. After dialysis the membranes were centrifuged at 15,000 x g for 20 min and the supernatant was discarded The pellet of purified ROS disk membranes was suspended in Ringer's solution B containing 135 mM KCl, 5 mM NaCl and 50 mM Tris-HCl buffer (pH 7.4) and stored at 0°C until use. Preparation was carried out shortly before use, and all operations were carried out under dim red light at 4°C. The absorbance ratio A_{280}/A_{498} of the disk membrane preparation was in the range of 2.2-2.4. The rhodopsin of disk membrane fragments thus prepared was largely unbleached, judging from spectrophotometric measurements.

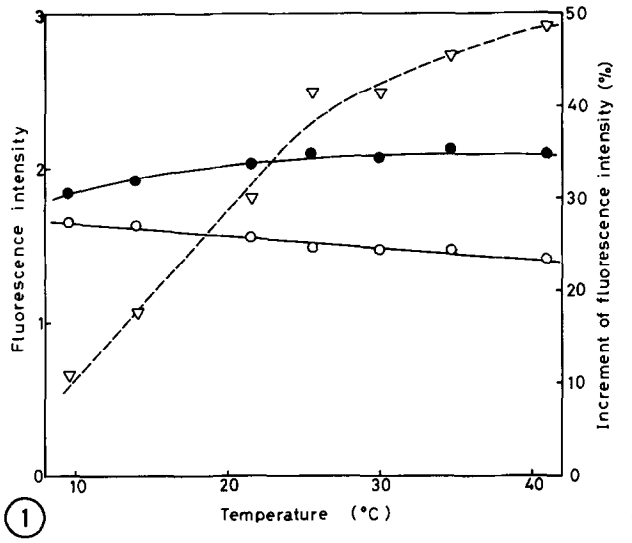
A double-beam difference spectrofluorophotometer (Shimazu model RF-503) was used for measurements of fluorescence and light scattering. The temperature of cells was controlled to $\pm$0.5°C at the set temperature using a Taiyo Coolnit CL-15 circulating water system. The fluorescence spectra were corrected for the absorbances of samples at the excitation and emission wavelengths by the method of Förster (8). The monochromator bandwidths for excitation and emission were 4 nm and 5 nm, respectively. To measure difference fluorescence, a fully bleached sample was used as a reference. ROS disk membranes were suspended (0.21-0.25 mg of protein per ml) in Ringer's solution B.

Rhodopsin in the ROS disk membrane fragments was partially bleached by illumination with white light and the extent of bleaching was measured in terms of the difference absorbances of the samples against a fully bleached sample at 498 nm using a Hitachi 356 spectrophotometer

Results and Discussion: The fluorescence intensity of rhodopsin in ROS disk membrane fragments increased upon bleaching, with a small blue shift of the emission peak from 340 nm to 338 nm. Both the blue shift and the increase in quantum yield are indicative of a decrease in the polarity of the environment of Trp residues, as suggested in the case of the protein fluorescence of heavy meromyosin by Werber et al. (9). These results are in partial conflict with those of Ebrey (4), who reported that the fluorescence yield increased but the emission peak shifted toward longer wavelength upon bleaching. It is generally known that as the polarity of the solvent decreases, the emission peak of Trp residues shifts toward shorter wavelength and the quantum yield increases (10,11). We believe that the environments of most Trp residues in unbleached rhodopsin in the disk membrane fragments are rather hydrophobic, and become more hydrophobic on bleaching.

The temperature dependences of the corrected fluorescence intensities before and after bleaching are shown in Fig. 1. The broken line in Fig. 1 shows the increment of fluorescence intensity at 338 nm. The increment of fluorescence intensity due to bleaching was about 10% at 10°C and increased at higher temperatures. In contrast with this result, we (3) previously reported that the turbidity change on bleaching was almost constant over the temperature range 12-27°C.

The time courses of fluorescence increase at various degrees of bleaching are shown in Fig. 2. The use of a Shimazu RF-503 dual-cell spectrofluorophotometer means that the difference fluorescence is unaffected by background fluorescence, Raman scattering from the solvent and cell, and stray light. The changes in absorbance at 290 nm and 338 nm after complete bleaching were about 0.32% and 6.6%, respectively. The light scattering intensities at the wavelengths of excitation (290 nm) and emission (338 nm) after complete bleaching



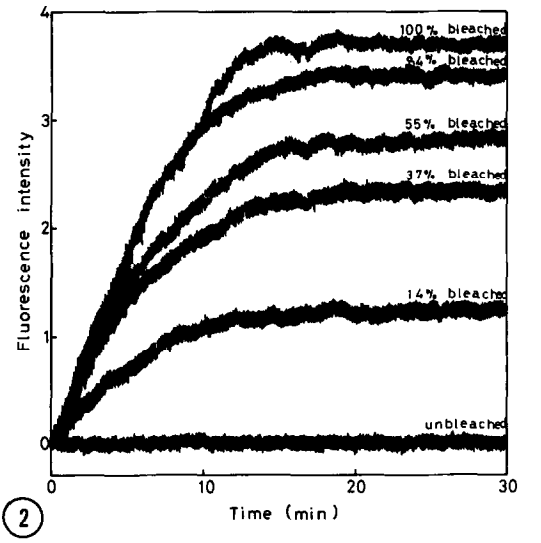


Fig. 1. Temperature dependences of the corrected fluorescence intensities (o, unbleached; ●, bleached) and of the increment of fluorescence intensity (▽) at 338 nm with excitation at 290 nm.

Fig. 2. Time courses of difference fluorescence change at 338 nm with excitation at 290 nm for various extents of bleaching, measured at 22°C. Samples were bleached to various extents by exposure to white light at zero time.

decreased by about 7.5% and 2.7%, respectively. Stirring the samples did not affect the time courses or the extents of the fluorescence changes. This indicates that the fluorescence increases shown in Fig. 2 are not due to aggregation or sedimentation; rather, they suggest that the environment of Trp residues in rhodopsin has become more nonpolar (hydrophobic). At 22°C it took about 5 min to reach half-saturation and about 20 min for complete saturation of the fluorescence increase. The time courses of fluorescence change were also measured at 32°C. Comparison of the plots at the two temperatures gave a value for the temperature coefficient, Q_{10}, of the fluorescence increase of 2.2, corresponding to an activation energy, E_a, of 14 kcal/mole. The activation energy was found to be independent of the extent of bleaching. The activation energy of the hydrolysis of metarhodopsin II to all-trans retinal and opsin has been reported

to be 20 kcal/mole for cattle rhodopsin solution (12). Kaplan and Liebman (13) reported similar slow bleach-induced birefringence changes in ROS. They suggested that the birefringence increase might be partially correlated with the formation of retinol, though they did not study the relationship between the extent of birefringence change and the degree of bleaching. However, our previous report (3) showed a nonlinear relationship between the turbidity change in a suspension of ROS disk membrane fragments and the degree of bleaching.

The relationship between the extent of corrected fluorescence change (F) and the degree of bleaching (p) is not stoichiometric, as can be seen in Fig. 3, where p can also be regarded as the probability that a given rhodopsin molecule is bleached. It is considered that the bleaching of one rhodopsin molecule in a disk membrane fragment cooperatively causes a concomitant fluorescence change in some of the neighboring unbleached rhodopsin molecules. If it is assumed that each of the n neighboring unbleached rhodopsin molecules contributes to the fluorescence change to an extent α, the following equation can be obtained (3): $F=p+\alpha(1-p)\{1-(1-p)^n\}$. The solid lines in Fig. 3 show theoretical curves based on the above equation for various values of n and α. The data in Fig. 3 suggest that the number of neighbors is one or two. It is worth noting here that formation of the metarhodopsin II conformation in one bleached molecule of the rhodopsin dimer induces the analogous conformation in the unbleached rhodopsin molecule, and that the rhodopsin dimer constitutes a Ca^{2+} channel across the disk membrane (14). With respect to the enzymatic reaction in ROS disk membranes, small amounts of light are up to ten times more effective in initiating phosphorylation than larger amounts (15), and the light-sensitive phosphodiesterases are fully activated by only 0.1% bleaching of rhodopsin (16).

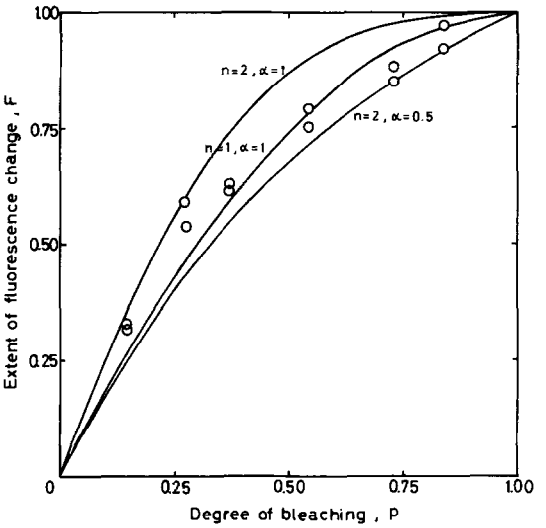


Fig. 3. The relationship between the extent of fluorescence change (F) and the degree of bleaching of rhodopsin (p). The fluorescence increments of unbleached and fully bleached samples are taken as F=0 and 1.0, respectively, and the states in which all rhodopsin molecules in the disk membranes are unbleached and bleached are taken as corresponding to p=0 and 1.0, respectively. The solid lines are theoretical curves calculated from the equation $F=p+\alpha(1-p)\{1-(1-p)^r$ (see the text).

We believe that this slow bleach-induced cooperative fluorescence change in the disk membrane is due either to a local environmental change of Trp residues of rhodopsin or to a local conformational change of modified rhodopsin, such as would be produced by the phosphorylation of opsin. This cooperative fluorescence change may well be related to the dark adaptation of visual cells.

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