Induced Optical Activity of the Metarhodopsins*

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ABSTRACT: The 11-*cis*-retinal chromophore in rhodopsin is known to exhibit induced optical activity which disappears on bleaching to opsin and retinal (Crescitelli, F., Mommaerts, W. F. H. M., and Shaw, T. I. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1729). We measured the induced circular dichroism of physiologically pertinent intermediates in the bleaching of bovine rhodopsin to ascertain the extent of conformational change following illumination. Metarhodopsins I and II in digitonin solution and in sonicated particles of rod outer segment both exhibit strong induced circular dichroism in their longest wavelength absorption band. The induced rotational strengths (in Debye magnetons) are 0.52 for the 500-nm band of rhodopsin, 0.73 for the 480-nm band of metarhodopsin I, and 0.77 for the 380-nm band of metarhodopsin II. The similarity of the induced circular dichroism of rhodopsin and the metarhodopsins suggests that the dissymmetric interactions at the retinal binding site are nearly the same in these three species. Thus, a significant portion of the local environment of the bound retinal is conserved in the transition from rhodopsin to metarhodopsin I to metarhodopsin II.

A retinal rod cell can be excited by a single photon (Hecht *et al.*, 1942). The first step in this exquisitely sensitive transduction mechanism is the isomerization by light of the 11-*cis*-retinal chromophore in rhodopsin (Wald, 1968). In vertebrates, the outer membrane of the rod outer segment becomes hyperpolarized 1 msec later (Tomita, 1970). The molecular events that link the isomerization of the chromophore in the disk membrane to the hyperpolarization of the outer membrane are not yet known. One approach to this challenging problem is to investigate the spectroscopic properties of the bound retinal to obtain information about the immediate environment of the photoreceptor chromophore.

We report here studies of the induced circular dichroism of intermediates in the bleaching of rhodopsin. These measurements provide information about the dissymmetric character of the environment of the bound retinal following illumination. The basis of this experimental approach is that symmetric chromophores can acquire optical activity by interacting with dissymmetric macromolecules (Blout and Stryer, 1959; Ulmer and Vallee, 1965). It is known that the 11-*cis*-retinal chromophore in rhodopsin exhibits strong induced optical activity which disappears on bleaching to opsin and retinal (Crescitelli and Shaw, 1964; Williams, 1966; Crescitelli *et al.*, 1966; Takezaki and Kito, 1967; Mommaerts, 1969; Shichi, 1970). Our studies were carried out to determine the step at which the induced optical activity is lost.[1] The significant intermediates are metarhodopsin I and metarhodopsin II (Figure 1), since they are the only ones detected in the intact eye in times short enough to allow participation in visual excitation (Hagins, 1956; Cone and Cobbs, 1969). In contrast, opsin is formed much too slowly. A comparison of the conformational properties of rhodopsin, metarhodopsin I, and metarhodopsin II is thus pertinent in elucidating the primary events in visual excitation.

We have measured the circular dichroism of sonicated bovine rod outer segment particles and of solutions of rhodopsin solubilized by digitonin. Metarhodopsin I and II are sufficiently stable at 3° during the time required for obtaining spectra and their relative proportions can be varied by altering the pH (Matthews *et al.*, 1963). We find that metarhodopsins I and II both exhibit induced optical activity of nearly the same magnitude as that of rhodopsin. Thus a significant portion of the local environment of the bound retinal is preserved in going from rhodopsin to metarhodopsin I and then to metarhodopsin II.

Experimental Section

Rod outer segments were isolated by a method similar to that of Matthews *et al.* (1963), except that treatment with alum was omitted. All operations were carried out in the dark or under dim red light unless otherwise specified. Aqueous buffer refers to 0.067 M potassium phosphate buffer at pH 6.5, whereas sucrose buffer refers to 40% (w/v) sucrose in 0.067 M potassium phosphate buffer at pH 6.5. Frozen bovine retinas (100; G. Hormel Co., Austin, Minn.) were thawed and then suspended in 60 ml of sucrose buffer. After stirring for 1 hr, the mixture was centrifuged at 35,000*g* for 20 min. The supernatant was discarded, and the pellet was suspended in 70 ml of sucrose buffer and homogenized with a Thomas tissue grinder. The homogenate was poured into four 40-ml centrifuge tubes, and 3 ml of aqueous buffer was layered on top of each tube. The tubes were centrifuged at 35,000*g* for 15 min. The outer segments at the interface between the aqueous buffer and the sucrose buffer layers were removed with a no. 17 needle. The combined outer segments were suspended in 70 ml of sucrose buffer. The interface flotation procedure was repeated four times. The outer segments were then suspended in 70 ml of aqueous buffer and centrifuged at 35,000*g* for 15 min. The outer segments were washed three more times with aqueous buffer and then stored at −20°.

Particles of rod outer segments suitable for absorption and circular dichroism spectroscopy were obtained by sonication (Fukami, 1962; Shichi, 1970). Frozen rod outer segments from 20 retinas were thawed and suspended in 6 ml of glass-distilled

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[1] Horwitz and Heller (1971) have recently reported an absorption and circular dichroism study of illuminated rhodopsin at liquid nitrogen temperature.

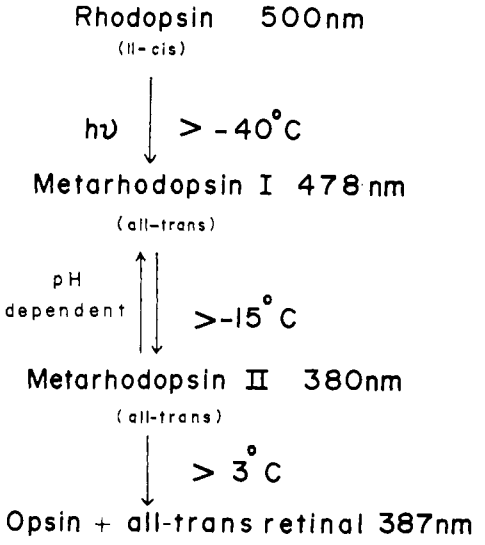

FIGURE 1: Intermediates in the photolysis of bovine rhodopsin (Matthews *et al.*, 1963).

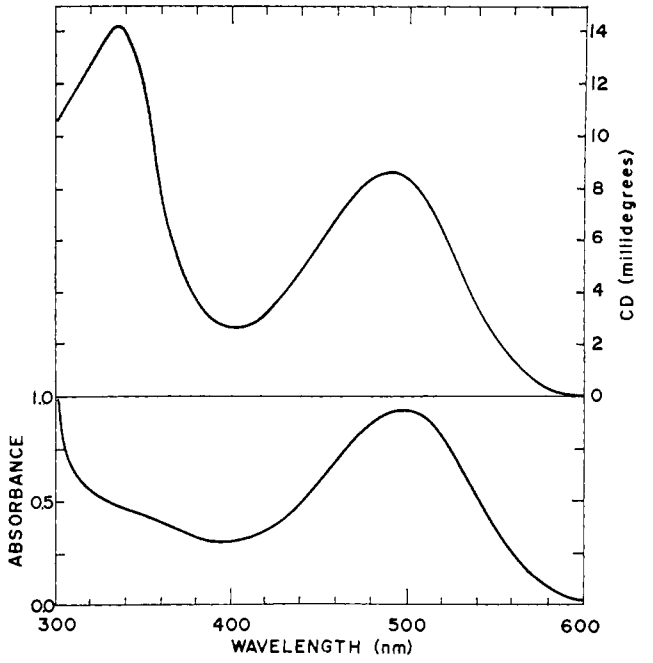

FIGURE 2: Absorption and circular dichroism spectra of rhodopsin in digitonin solution at pH 7.7, 3°. The path length was 1 cm and the concentration of rhodopsin was 2.23×10^{-5} M.

H_2O in a 30-ml stainless steel centrifuge tube. The suspension was sonicated with a Branson Sonifier (Model W 185D). The power was set at 3 and a standard tip was used. The stainless steel tube was immersed in an ice bath which was rapidly stirred. The suspension of rod outer segments was sonicated four times for 2.5 min each, with 1-min intervals between sonications to minimize heating. The resulting sonicate of rod outer segments had an A_{500} of 1.85 and an A_{600} of 0.25. The pH of an aliquot of the sonicate was adjusted from 6.6 to 8.0 with 0.1 N NaOH. Spectroscopic measurements on the sonicated particles were performed within 3 hr of their formation.

Rhodopsin was extracted from rod outer segments with digitonin (Sigma). Rod outer segments from 40 retinas were suspended in 10 ml of 2% digitonin in 0.005 M (pH 6.8) potassium phosphate buffer. The mixture was stirred gently overnight at 3°, and then centrifuged at 35,000*g* for 15 min. This supernatant solution of rhodopsin was used without further purification. The pH of aliquots of the solution was adjusted to 5.4 and 7.7 with 1 M KH_2PO_4 and 1 M K_2HPO_4. The final buffer concentration was 0.067 M. After adjustment of pH, the samples were divided into two aliquots, one for absorption spectroscopy, the other for circular dichroism spectroscopy.

A Honeywell Strobonar flash source filtered by a Corning CS 3-67 filter was used to convert rhodopsin into the metarhodopsins. Six flashes were delivered in about 1 min. Absorption spectra were obtained on a Cary Model 15 recording spectrophotometer equipped with a thermostated cell holder. Circular dichroism spectra were obtained on a Cary Model 61 instrument. A 1-cm path-length thermostated cell with quartz windows was used. The settings on the circular dichroism spectrometer were time constant 3 and slit multiplier 1. For circular dichroism spectra of rhodopsin, the scan rate was 1 nm/sec. Less than 5% of the rhodopsin was photolyzed during a scan from 600 to 300 nm. A scan of about 0.5 nm/sec was used to obtain circular dichroism spectra of the metarhodopsins, which are much less photolabile than rhodopsin.

The molecular ellipticity, $[\theta]$ (in deg cm^2 dmole^{-1}), was calculated from the observed circular dichroism θ (in deg) using the relationship $[\theta] = 100\theta/lc$, where l is the path length (in cm) and c is the concentration (in moles/l.). The molar concentration of rhodopsin was determined from the absorbance at 500 nm, assuming an extinction coefficient at 42,000 cm^{-1} M^{-1} (Matthews *et al.*, 1963). The rotational strength, R, (in cgs units), of a circular dichroism band was calculated from

$$R = 1.23 \times 10^{-42} [\theta]_{max} \frac{\Delta}{\lambda_{max}}$$

where $[\theta]_{max}$ is the maximum ellipticity, Δ is the wavelength interval in which $[\theta]$ falls from $[\theta]_{max}$ to $0.368[\theta]_{max}$, and λ_{max} is the wavelength of maximum ellipticity (Moscowitz, 1960). It is convenient to express the rotatory strength in units of a Debye magneton which is equal to 0.927×10^{-38} cgs unit.

Results

Induced Circular Dichroism of Rhodopsin and the Metarhodopsins in Solution. The absorption and circular dichroism spectra of rhodopsin in solution at 3° at pH 7.7 are shown in Figure 2. As previously observed (Takezaki and Kito, 1967; Shichi, 1970), bovine rhodopsin exhibits strong induced optical activity in the visible absorption bands of the bound 11-*cis*-retinal. The molecular ellipticity peaks at 490 and 340 nm are 38,600 and 62,700 (deg cm^2) dmole^{-1}, respectively.

Metarhodopsins I and II were trapped by illuminating solutions of rhodopsin at 3°. At this temperature, metarhodopsins I and II are stable for hours and are in equilibrium with each other (Matthews *et al.*, 1963). The relative proportions of these intermediates depend on pH. The absorption and circular dichroism spectra of flash-illuminated solutions of rhodopsin at 3° are shown in Figure 3. The absorption spectrum of the pH 5.4 solution exhibits peaks at 380 and 480 nm, whereas that of the pH 7.7 solution shows a single peak at 480 nm. The 380-nm peak corresponds to metarhodopsin II and the 480-nm peak corresponds to metarhodopsin I (Matthews *et al.*, 1963). The pH 5.4 solution is predominantly metarhodopsin II, whereas the pH 7.7 solution is mainly metarhodopsin I. These

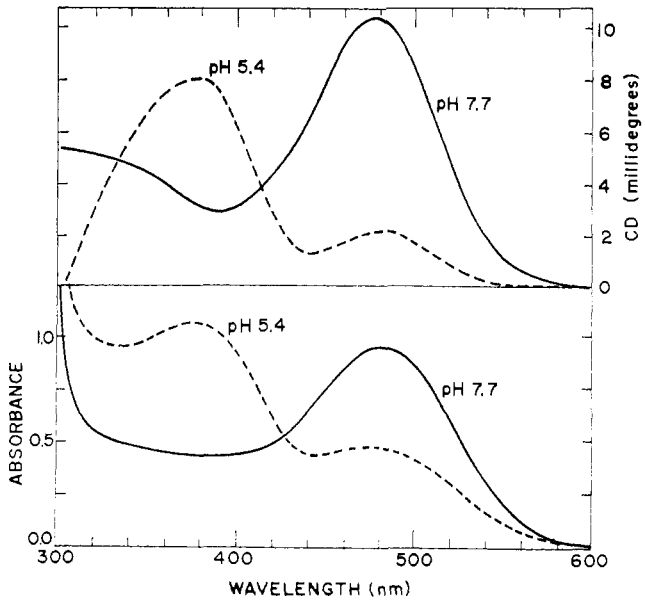

FIGURE 3: Absorption and circular dichroism spectra of flash illuminated rhodopsin in digitonin solution at 3°. The pH's of the solutions were 5.4 (dashed line) and 7.7 (solid line). The path length was 1 cm and the concentration of rhodopsin was 2.23×10^{-5} M. The peak near 480 nm is from metarhodopsin I, whereas that near 380 nm is from metarhodopsin II.

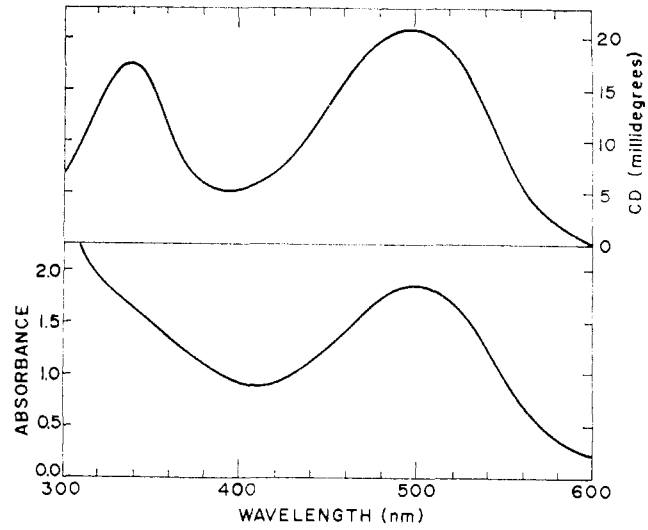

FIGURE 4: Absorption and circular dichroism spectra of rhodopsin in sonicated particles of rod outer segments at pH 6.6, 3°. The path length was 1 cm and the concentration of rhodopsin was 4.41×10^{-5} M.

solutions of metarhodopsin I and II also contain rhodopsin and isorhodopsin, which are formed by the action of light on photoreversible intermediates in the bleaching sequence (Hubbard and Knopf, 1958; Williams, 1970). The flash and filter combination used in our experiments produced a mixture in which the proportion of rhodopsin plus isorhodopsin was between 20 and 25% as determined by the method of Hubbard and Kropf (1958). There was considerably more isorhodopsin than rhodopsin in the photolyzed mixture.

The circular dichroism spectra of these flash-illuminated solutions of rhodopsin at 3° are shown in Figure 3. The pH 7.7 solution exhibits a large circular dichroism peak at 478 nm, whereas the pH 5.4 exhibits a large circular dichroism peak at 380 nm and a smaller one at 478 nm. The peak wavelengths of these circular dichroism bands correspond to the peak wavelengths of the absorption bands of metarhodopsins I and II. Hence, it is evident that *metarhodopsins I and II both exhibit induced circular dichroism in their longest wavelength absorption bands.* The observed molecular ellipticity at 478 nm of the pH 7.7 solution is 46,700, whereas that of the pH 5.4 solution at 380 nm is 38,600 (deg cm^2) dmole^{-1}.

The molecular ellipticities of the longest wavelength circular dichroism bands of metarodopsin I and metarhodopsin II can be estimated from the observed circular dichroism spectra. We take the proportions of metarhodopsin I, metarhodopsin II, isorhodopsin, and rhodopsin to be 0.60, 0.15, 0.20, and 0.05, respectively at pH 7.7 and 0.15, 0.60, 0.20, and 0.05, respectively, at pH 5.4. The relative proportions of metarhodopsins I and II in digitonin at 3° at these pH values are based on the analysis of Matthews *et al.* (1963). The calculated circular dichroism contributions of isorhodopsin (Takezaki and Kito, 1967) and of rhodopsin were subtracted from our observed circular dichroism spectra. The molecular ellipticities derived in this way are 58,500 (deg cm^2) dmole^{-1} for metarhodopsin I at 478 nm, and 55,000 (deg cm^2) dmole^{-1} for metarhodopsin II at 380 nm. These estimates are subject to considerable error because of the overlap of the circular dichroism bands of metarhodopsins I and II, and uncertainty as to the relative proportions of these intermediates and of rhodopsin and isorhodopsin.

Induced Circular Dichroism of Rhodopsin and the Metarhodopsins in Sonicates of Rod Outer Segments. Similar results were obtained with sonicated particles of rod outer segments. The absorption and circular dichroism spectra of rhodopsin in sonicated particles at 3° are shown in Figure 4. The induced optical activity of rhodopsin in the rod outer segment particles is like that of rhodopsin in digitonin solution, except that the circular dichroism band at 340 nm is reduced in magnitude relative to the 500-nm band, as previously noted by Shichi (1970). The sonicated particles were flash illuminated at 3° to produce a mixture of metarhodopsins I and II (Figure 5). Circular dichroism peaks occur at 480 nm, corresponding to metarhodopsin I, and 378 nm, corresponding to metarhodopsin II (Figure 5). The proportions and molecular ellipticities of metarhodopsins I and II are uncertain because it is difficult to quantitatively correct for the effects of light scattering. However, it is evident that the relative heights of these circular dichroism peaks qualitatively agree with the relative proportions of metarhodopsins I and II as reflected in the absorption spectra. Thus, metarhodopsins I and II in rod outer segment particles exhibit induced circular dichroism similar to that found in digitonin solution. The induced optical activity of the bound retinal was lost after the sonicated particles were warmed to 21° (Figure 6), resulting in the formation of opsin and *all-trans*-retinal.

Discussion

The significant finding is that bovine metarhodopsin I and metarhodopsin II exhibit induced optical activity in their longest wavelength absorption bands. The sign of the induced circular dichroism of these intermediates is the same as for rhodopsin, and the magnitudes of their induced rotational strengths are similar. The induced rotational strengths (in Debye magnetons) in digitonin solution are approximately 0.52 for the 500-nm band of rhodopsin, 0.73 for the 480-nm band of metarhodopsin I, and 0.77 for the 380-nm band of

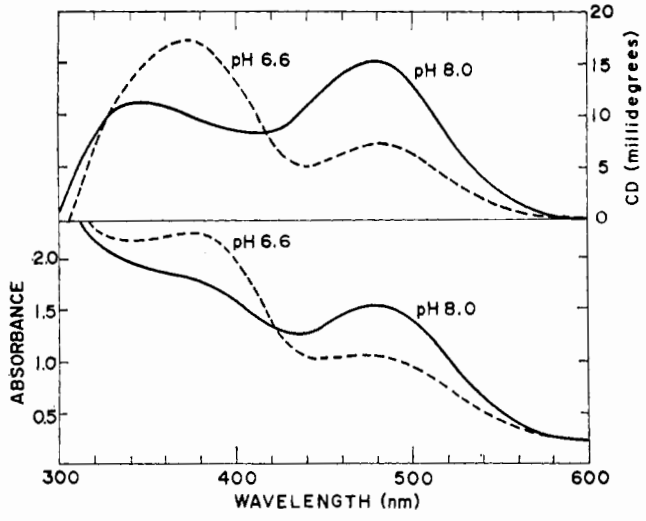

FIGURE 5: Absorption and circular dichroism spectra of flash illuminated rhodopsin in sonicated particles of rod outer segments at 3°. The pH's of the suspensions were 6.6 (dashed line) and 8.0 (solid line). The path length was 1 cm and the concentration of rhodopsin was 4.41×10^{-5} M. The peak near 480 nm is from metarhodopsin I, whereas that near 380 nm is from metarhodopsin II.

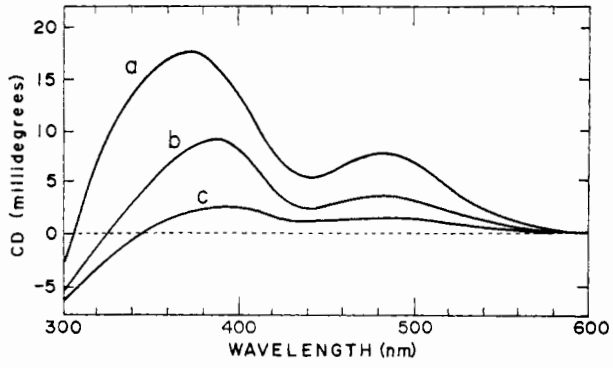

FIGURE 6: Disappearance of the induced circular dichroism of metarhodopsin I and II on warming a suspension of sonicated particles of rod outer segments. A: pH 6.6, 3° (same conditions as in Figure 5). B: 21°, after 2.5 min. C: 21°, after 30 min.

metarhodopsin II. The magnitudes of these rotational strengths are indicative of very strong induced optical activity. These results on bovine rhodopsin contrast with the reported absence of induced circular dichroism in the metarhodopsins of squid (Kito *et al.*, 1968).

The induced optical activity arises from interactions of the bound retinal with the dissymmetric environment of the protein moiety. In principle, two types of interactions are possible. (1) *The bound retinal may be inherently dissymmetric.* The classical example of an inherently dissymmetric chromophore is hexahelicene (Moscowitz, 1960). Mommaerts (1969) noted that 11-*cis*-retinal can have chirality, and suggested that the induced optical activity of rhodopsin arises because one of the screw senses of a twisted form of 11-*cis*-retinal is preferentially stabilized by opsin. It also possible that a twisted form of 11-*cis*-retinal is produced by interaction with opsin. Our finding of induced circular dichroism in metarhodopsins I and II renders these hypotheses less plausible, since the retinal chromophore in these intermediates is in the all-trans form. Also, Takezaki and Kito (1967) found that isorhodopsin, which contains 9-*cis*-retinal, exhibits strong induced circular dichroism. Thus, the presence of an 11-cis configuration is not a necessary condition for induced optical activity of the bound retinal. Induced dissymmetry resulting for a twist elsewhere in the retinal, say near C_7 or C_{13}, remains a possibility. (2) Alternatively, *the bound retinal may be symmetric and acquire optical activity by interacting with a dissymmetric arrangement of charges and dipoles in its immediate environment.* A calculation of the order of magnitude of the induced rotational strength can be made using the coupled oscillator theory (Kirkwood, 1937). The maximal rotatory strength (in Debye magnetons) produced by the interaction of two optimally oriented transition dipoles (Schellman, 1968; Kägi *et al.*, 1971) is

$$|R| = 1.71 \times 10^{54} \times \frac{D_i D_j}{r^2} \frac{\nu_i \nu_j}{\nu_j^2 - \nu_i^2}$$

where D_i and D_j are the dipole strengths (in cgs units) of the interacting transitions, ν_i and ν_j are their frequencies, and r is the distance (in cm) between the dipoles. This equation assumes point dipoles. For rhodopsin, $D_i = 74 \times 10^{-36}$ cgs units and $\nu_i = 20{,}000$ cm^{-1}. Suppose that the perturbing dipole is located 4 Å away and that it is associated with a transition in the ultraviolet, so that $D_j = 20 \times 10^{-36}$ cgs unit and $\nu_j = 50{,}000$ cm^{-1}. The induced rotatory strength would then be 0.75 Debye magneton, which is comparable to the observed values. This order of magnitude calculation shows that the induced optical activity of rhodopsin and the metarhodopsins might arise from the interaction of retinal with a suitably oriented chromophore on the protein that is in close proximity.

The similarity of the induced circular dichroism of rhodopsin and the metarhodopsins suggests that the dissymmetric interactions at the retinal binding site are nearly the same in these three species. It seems likely that *a significant portion of the local environment of the retinal is conserved in the transition from rhodopsin to metarhodopsin I to metarhodopsin II.* The dissymmetric interactions are evidently different from those involved in the bathochromic shift and protonation of the Schiff's base linkage in rhodopsin and metarhodopsin I. The dominant dissymmetric interactions between retinal and opsin may be preserved either near the ionone ring or in the vicinity of the Schiff's base linkage. Our circular dichroism results suggest that the conformational changes occuring near the retinal following its cis–trans isomerization may not be large.

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Solute Perturbation of Protein Fluorescence. The Quenching of the Tryptophyl Fluorescence of Model Compounds and of Lysozyme by Iodide Ion*

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ABSTRACT: The effect of iodide on the tryptophyl fluorescence of model compounds and of lysozyme was studied in order to evaluate the factors that determine the use of iodide as a selective quencher of the fluorescence of tryptophyl side chains of proteins exposed to solvent. The results with the model compounds indicate the involvement of a collisional quenching mechanism due to the agreement with the Stern–Volmer law and the proportionality of the quenching constant with T/η for indole-3-acetamide. Bimolecular rate constants, k_3, calculated from measured quenching constants using available lifetime data are equal to, greater than, or less than 4–6 $\times$ 10^9 M^{-1} sec^{-1} for uncharged, positively charged, and negatively charged tryptophyl compounds, respectively. A modified version of the Stern–Volmer law was calculated for a fluorophor population with different quantum yields and quenching constants. This formulation allows the calculation of the effective quenching constant from the intercept and the slope at low iodide concentration of a $F_0/\Delta F$ *vs.* $1/(I^-)$ plot. Data obtained for lysozyme indicate that for the native protein about one-half the tryptophyl fluorescence is accessible at pH 5.3 whereas all of the tryptophyl fluorescence is accessible in 6 M Gdn·HCl. Information regarding the presence of charged groups near tryptophyl side chains was obtained for lysozyme by studying the dependence of the quenching on pH.

In a preliminary study it was shown that a large fraction of the tryptophyl fluorescence of lysozyme in aqueous solution was quenched by low concentrations of iodide ion (Lehrer, 1967). It was concluded from a study of the magnitude of the quenching of fluorescence and the character of the difference fluorescence spectrum produced in the presence and absence of substrate that the fluorescence of tryptophyls exposed to solvent and located in the substrate binding site was preferentially quenched by iodide. It appeared that this technique, which can be called solute perturbation of protein fluorescence, could be used as a probe of fluorophor exposure in proteins in a manner analogous to the technique of solvent perturbation of protein absorption (Herskovits and Laskowski, 1960; Laskowski, 1966).

More recently, studies by other workers have used bromate (Winkler, 1969) and iodide (Arrio *et al.*, 1970) to quench extrinsic fluorescence (Teale and Badley, 1970). Oxygen has also been used as a quencher of pyrenebutyric acid bound to proteins (Vaughan and Weber, 1970). Burstein (1968a) has also independently studied the quenching of tryptophyl fluorescence in model compounds by iodide.

In order to learn more about the quenching mechanism and the factors which determine fluorophor exposure, various tryptophyl model compounds and a model protein, lysozyme, were used in the present study. The results of the model compound study provide evidence for a mechanism that follows the classical Stern–Volmer law (1919), predominantly involving collisional quenching, and illustrate the importance of local charge and solvent viscosity. The quenching of lysozyme fluorescence by iodide also appears to follow a similar mechanism because of the agreement obtained with a modified version of the Stern–Volmer law which was calculated for a heterogeneous distribution of fluorophors in a protein. Effective Stern–Volmer quenching constants and values for the fractional accessible fluorescence were obtained for lyso-

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