Rhodopsin in Reconstituted Phospholipid Vesicles. 1. Structural Parameters and Light-Induced Conformational Changes Detected by Resonance Energy Transfer and Fluorescence Quenching†

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ABSTRACT: The structure of purified rhodopsin was investigated by steady-state resonance energy transfer and fluorescence quenching techniques: (1) Fluorescence parameters and relative distances between rhodopsin sites labeled with fluorescent probes and the endogenous chromophore 11-*cis*-retinal were measured in micellar detergent solution and in reconstituted phospholipid vesicles. (2) The accessibility of the labeled rhodopsin sites in reconstituted vesicles to *N*-methyl- and *N*-benzylpicolinium was studied in the dark and subsequent to rhodopsin bleaching. Fluorescent-labeled rhodopsin was affinity purified in octyl glucoside from rod outer segments which were previously reacted with either the sulfhydryl-specific reagents, pyrenylmaleimide or monobromobimane, or reagents specific to amino groups, dansyl chloride or fluorescein isothiocyanate. The purified protein was recombined with phospholipids, and vesicles were formed by detergent dialysis. All four fluorophores appear to react ≥30 Å away from the endogenous chromophore as estimated from the efficiency of energy transfer and presumably probe rhodopsin domains exposed at the membrane surface. The maximal fraction of quenchable fluorescence and the mean quenching constant were determined in dark and bleached vesicles: bleaching did not affect the quenching of the fluorophores attached to sulfhydryl groups but markedly decreased the quenching constants of the fluorophores coupled to amino groups. The apparent collisional rate constant decreased by 20- and 4-fold for dansyl and fluorescein, respectively. The results suggest that bleaching reduced the accessibility of these sites which, in turn, may reflect light-induced displacements of rhodopsin domains at the membrane surface. Such structural changes may regulate rhodopsin–rhodopsin as well as rhodopsin–enzyme interactions.

Rhodopsin is the visual pigment in retinal rods [for reviews, see Montal & Korenbrot (1976), Hubbell & Bownds (1979), and Pober & Bitensky (1979)]. It is the major protein component in the disk membrane and consists of a protein moiety, M_r ~38 000, and an endogenous chromophore, 11-*cis*-retinal. The primary event in vision is the absorption of a photon by rhodopsin leading to the photoisomerization of the chromophore to the trans configuration. Visual transduction proceeds from the light-excited rhodopsin to reduce the Na^+ conductance of the rod plasma membrane by an intermediate process yet unknown. The role suggested for rhodopsin in this process is as either an ion translocator (Wald et al., 1963; Hagins, 1972; Montal et al., 1977) or an enzyme activator (cf. Pober & Bitensky, 1979; Bownds, 1981; Fung et al., 1981; Kühn et al., 1981), or both.

Photoexcitation of rhodopsin in the native membrane is known to induce significant conformational changes in the protein [see Hubbell & Bownds (1979)] which are presumably involved in its function. To study the nature of the light-induced structural changes in rhodopsin, we applied fluorescence techniques to a model system consisting of purified rhodopsin reconstituted in phospholipid vesicles. The adequacy of this system has been documented with a wide range of assays: Rhodopsin can be purified in a variety of detergents without affecting its spectral characteristics [see Stubbs et al. (1976)]. Bleaching in most detergents, however, modifies the protein, as it cannot be regenerated with 11-*cis*-retinal (Stubbs et al., 1976), suggesting that the lipid bilayer is required to stabilize the bleached form of the protein. Reconstitution of purified rhodopsin in phospholipid vesicles restores its capability to regenerate (Hong & Hubbell, 1972), to photoactivate the GTPase (Shinozawa et al., 1980), the nucleotide exchange process (Fung & Stryer, 1980), and the cGMP phosphodiesterase (Hurley, 1980) from rod outer segments (ROS),[1] and to express a light-induced increase in membrane permeability (Darszon et al., 1977; Hubbell et al., 1977; O'Brien et al., 1977b; Tyminski et al., 1982). Thus, purified rhodopsin reconstituted in phospholipid vesicles reasonably reproduces some known activities of the visual pigment in the photoreceptor cell, and therefore, light-induced structural events detected in the former may be interpreted in terms of structure–function relationships of rhodopsin in vivo.

Here, we describe the preparation and spectral properties of four fluorescent derivatives of rhodopsin, two of which are labeled at sulfhydryl groups and two at free amino groups. The fluorescence properties of the bound fluorophores as well as the absorption spectrum of the endogenous chromophore were used to investigate the conformation of rhodopsin in micellar solution and when reconstituted in phospholipid vesicles. Light-induced conformational changes in reconstituted rhodopsin were detected by changes in the accessibility of the fluorophores to amphiphatic quenchers. The effect of bleaching on the conformation of the reconstituted protein in the vicinity of the labeled sulfhydryl groups seems to be minor; in contrast, sites labeled via amino groups undergo marked

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[1] Abbreviations: ROS, rod outer segments; PM, *N*-(3-pyrenyl)maleimide; DNSCl, (dimethylamino)naphthalenesulfonyl chloride; FITC, fluorescein isothiocyanate; mBB, monobromobimane; OG, octyl β-D-glucopyranoside; α-mm, methyl α-mannoside; Con A–S4B, concanavalin A–Sepharose 4B; PC, phosphatidylcholine; PE, phosphatidylethanolamine; MP^+, *N*-methylpicolinium; BP^+, *N*-benzylpicolinium; P_i, sodium phosphate; P-Rho, pyrene-labeled rhodopsin; B-Rho, bimane-labeled rhodopsin; D-Rho, dansylated rhodopsin; F-Rho, fluorescein-labeled rhodopsin.

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structural changes, suggesting an increased protein packing around them. In the following paper (Borochov-Neori et al., 1983) we measured the efficiency of fluorescence energy transfer between these fluorescent rhodopsin conjugates to establish the aggregation state of the protein in reconstituted phospholipid vesicles and its sensitivity to light. A preliminary account of this research has appeared elsewhere (Borochov-Neori & Montal, 1981).

Experimental Procedures

Materials. *N*-(3-Pyrenyl)maleimide (PM) was from Molecular Probes. (Dimethylamino)naphthalenesulfonyl chloride (dansyl chloride, DNSCl) and 4,4′-dithiodipyridine were from Aldrich Chemical Co. Fluorescein isothiocyanate (FITC), 10% (w/w) on Celite, monobromobimane (mBB), and octyl β-D-glucopyranoside (octyl glucoside, OG) were from Calbiochem-Behring Corp. Celite, methyl α-mannoside (α-mm), concanavalin A–Sepharose 4B (Con A–S4B), egg PC, and dithiothreitol were from Sigma Chemical Co. Diphytanoylphosphatidylcholine (PC) was obtained from Avanti-Biochem. Bacterial phosphatidylethanolamine (PE) was from Supelco. *N*-Methylpicolinium perchlorate and *N*-benzylpicolinium perchlorate (MP^+ and BP^+, respectively) were a gift from Dr. M. Shinitzky. All chemicals were of analytical grade, and the organic solvents were of spectroscopic grade.

Preparation of Fluorescent-Labeled Rhodopsin. All procedures were carried out under dim red light at 4 °C unless otherwise stated.

(*A*) *Isolation of Rod Outer Segment Membranes.* ROS were isolated from frozen dark-adapted bovine retinas (American Stores Packing Co.) following the procedure of Papermaster & Dreyer (1974). The purified membranes had a typical absorbance ratio between 278 and 498 mm of 2.8–3.4.

(*B*) *Fluorescence Labeling of ROS.* To label with pyrene, we added a 3 mM stock solution of PM in acetone to give a final concentration of 10 μM to ROS (0.35 mg of rhodopsin/mL) resuspended in 10 mM NaCl and 10 mM sodium phosphate (P_i), pH 6.5. The suspension was stirred 5 h at room temperature. The reaction with mBB followed a similar protocol: 50 mM mBB in acetonitrile was added to a final concentration of 0.5 mM, and the reaction suspension was at pH 8.0. To label with dansyl or fluorescein, we stirred ROS suspensions (0.8 mg of rhodopsin/mL) in 67 mM P_i, pH 8.0, for 2 h at room temperature with either 0.2 mM DNSCl, 1% (w/w) on Celite, or 2 mM FITC, 10% (w/w) on Celite. For removal of the Celite, the membranes were floated on 35% (w/v) sucrose. The labeled membranes were washed 3 times with 40 volumes of 10 mM NaCl and 10 mM P_i, pH 7.0, and used immediately.

(*C*) *Rhodopsin Purification.* All rhodopsin conjugates were purified according to the same protocol: Pellets of washed labeled membranes were solubilized with 1.5% (w/v) OG in 0.1 M NaCl and 10 mM P_i, pH 6.5, to give 1 mg/mL rhodopsin. The suspensions were incubated for 30 min and centrifuged in an Eppendorf centrifuge (Model 5412) to remove undissolved material. The extract was slowly applied to a Con A–S4B column equilibrated with the detergent–buffer solution (approximately 1 mg of protein/0.6 mL of the packed resin). The column was washed with 20 volumes of detergent–buffer, and then 0.75 M α-mm in the same solution was added. After at least 3 h of equilibration the column was eluted with the α-mm solution. Most of the protein (>60%) eluted in the void volume. Rhodopsin purified according to this procedure exhibited a typical ratio of optical absorbances at 278 and 498 nm in the range 1.67–1.72 after subtraction of the fluorophore absorbances when applicable. This value is in agreement with the highest degree of purification reported for rhodopsin (Van Breugel et al., 1977). The preparations were used immediately following purification.

Rhodopsin concentration was determined with a Perkin-Elmer Model 555 spectrophotometer from the light-sensitive absorbance at 498 nm by using $\epsilon_{498} = 37\,000\ M^{-1}\ cm^{-1}$ (Wald & Brown, 1953). The concentration of the probes was calculated, after subtracting the protein absorbance, by using the following extinction coefficients: ϵ_{337}(dansyl) = 4000 M^{-1} cm^{-1} (Chen & Kernohan, 1967); ϵ_{495}(fluorescein) = 42 500 M^{-1} cm^{-1} (Gennis et al., 1972; Mercola et al., 1972); ϵ_{347}(pyrene) = 34 000 M^{-1} cm^{-1} (Betcher-Lange & Lehrer, 1978); ϵ_{383}(bimane) = 4500 M^{-1} cm^{-1} (Kosower et al., 1979).

(*D*) *Reconstitution Procedure.* The desired amount of lipid, dissolved in chloroform, was dried with nitrogen to form a thin film on the bottom of a test tube. Purified rhodopsin in OG, supplemented with 1.5% (w/v) cholate, was added, and the lipid was suspended by vortexing. The mixture was incubated 1 h and transferred to a dialysis bag (Arthur H. Thomas Co.). Vesicles formed upon dialysis against 200 volumes of 10 mM NaCl, 10 mM P_i, and 1 mM dithiothreitol, pH 7.0, for 48 h with five changes. The final vesicle preparations were immediately used for fluorescence measurements. The lipid composition of the reconstituted vesicles was routinely a mixture of PC and PE (1:1 w/w), which are the major phospholipids of bovine rod disk membranes (Daemen, 1973; O'Brien et al., 1977a). Diphytanoyl-PC [a saturated yet fluid phospholipid (Lindsey et al., 1979)] and bacterial PE were used in most experiments; however, similar results were obtained with egg PC/bacterial PE (1:1 w/w), diphytanoyl-PC alone, or asolectin [partially purified as described by Kagawa & Racker (1971)]. The latter, however, is inconvenient for fluorescence studies due to a fluorescent contaminant (Jesaitis & Fortes, 1980). During dialysis a fraction (≤10%) of rhodopsin absorbance was lost. This phenomenon was independent of either the presence of exogenous lipid during rhodopsin purification, the use of ROS lipids [purified according to Miljanich et al. (1979)] in the reconstitution, or the performance of dialysis under a constant flow of argon. Rhodopsin reconstituted in lipid vesicles was ≥80% regenerable upon incubation with 11-*cis*-retinal.

Steady-State Fluorescence Measurements. All fluorescence measurements were performed in a Perkin-Elmer, MPF-4, spectrofluorometer equipped with a differential corrected spectra unit, polarization attachment, and a thermostated cell holder with a magnetic stirrer. In all the measurements the absorbances of the different species were kept below 0.06 to prevent inner filter effects. The excitation slit was 1 nm to minimize rhodopsin bleaching, and shutters were closed between measurements. Samples of unlabeled rhodopsin in detergent and reconstituted vesicles were used to correct for scattering and background contributions to the measured fluorescence intensities. Measurements were performed at 22 °C unless otherwise stated.

The quantum yields, ϕ, of the bound fluorophores were determined by comparison with the quantum yields of quinine sulfate (0.70 in 0.1 N H_2SO_4; Scott et al., 1970) and ANS (0.37 in ethanol; Stryer, 1965). Equation 1 describes the ratio

$$\phi_1/\phi_2 = (F_1/F_2)(A_2/A_1)(1 - r_1/4) \quad (1)$$

of quantum yields, ϕ_i, as a function of the area under the emission spectra, F_i, and the absorbances at the exciting wavelength, A_i, for two fluorophores, where compound **1** is not freely rotating during the excited-state lifetime. The term $1 - r_1/4$ corrects for polarized emission (Shinitzky, 1972) and r_1 is the fluorescence anisotropy of compound **1**.

Fluorescence anisotropies were determined after correction for instrumental polarization. The degree of polarization, P, was calculated from the relationship $P = 3r/(2 + r)$. In micellar solution the fluorescence anisotropy, r_0, of the bound fluorophores in the absence of macromolecular rotation was evaluated by extrapolating the linear portion of a Perrin plot, $1/r$ vs. $(T/\eta)(F_0/F)$, at relatively low viscosity (η) to infinite viscosity as described by Dale & Eisinger (1975). The viscosity was varied by changing the temperature, and the excited-state lifetime was assumed to be proportional to the fluorescence intensity, F. In reconstituted vesicles, the rotation of rhodopsin within the membrane at 22 °C was ignored when r was evaluated. r was measured only in bleached samples, since the presence of the polarizers required excitation slits of 4–5 nm, resulting in partial bleaching during the measurement. r was measured at the same excitation wavelengths used to obtain the spectra in Figure 5. Cutoff filters were used instead of the emission monochromator to increase the detected signal: >390 nm for pyrene and >430 nm for the other probes.

The efficiency of energy transfer, E, from the various fluorophores to 11-*cis*-retinal was calculated from the donor quenching [for reviews see Stryer (1978) and Fairclough & Cantor (1978)]. The intensity of donor fluorescence in the presence of the endogenous chromophore (dark sample), F_{DA}, and in its absence (bleached sample), F_D, was measured, and E was calculated according to eq 2:

$$E = 1 - F_{DA}/F_D \tag{2}$$

To detect the energy transfer from pyrene to 11-*cis*-retinal in vesicles, it was necessary to reconstitute the labeled protein at high lipid to protein ratio (~600 phospholipid molecules per rhodopsin) to minimize the surface concentration of *trans*-retinal, which is generated by bleaching and binds tightly to the membranes. *trans*-Retinal has an absorption maximum at 380 nm, and it is an excellent acceptor for pyrene with $R_0(^2/_3) = 45$ Å so that significant quenching of pyrene by this photoproduct in bleached rhodopsin vesicles occurred at lower lipid to protein ratios. Distances, R, between the fluorophores and the endogenous chromophore in rhodopsin were estimated from the efficiency of energy transfer and the values of R_0, the distance at which $E = 50\%$, by using the following relationship derived by Förster (1959):

$$R = R_0(1/E - 1)^{1/6} \tag{3}$$

The definition of R_0 is given by eq 4:

$$R_0 = [(8.75 \times 10^{-5})n^{-4}\phi_D J\kappa^2]^{1/6} \text{ (Å)} \tag{4}$$

where n is the refractive index of the intervening medium, usually given a value of 1.4 in biological systems, and ϕ_D is the quantum yield of the donor in the absence of the acceptor. The overlap integral, J, for the donor emission, $F_D(\lambda)$, and the acceptor absorption, $\epsilon_A(\lambda)$, was calculated according to eq 5

$$J = \frac{\sum F_D(\lambda)\epsilon_A(\lambda)\lambda^4\Delta\lambda}{\sum F_D(\lambda)\Delta\lambda} \tag{5}$$

with the summation being carried out over 2- or 5-nm intervals ($\Delta\lambda$). The orientation factor, κ^2, depends on the relative orientation of the transition dipoles of the donor and acceptor and the vector joining them. It has a value of $^2/_3$ when both the donor and the acceptor are freely rotating during the excited-state lifetime but can, in theory, have a value between 0 and 4. This range, however, is narrower when the fluorophores have some degree of rotation and/or transitions of mixed polarizations are involved in the process of energy transfer (Dale & Eisinger, 1974; Haas et al., 1978). We calculated the distances between the coupled fluorophores and the endogenous chromophore using $\kappa^2 = ^2/_3$, $R(^2/_3)$, and estimated the most probable ranges of distances from the fluorescence polarization of the fluorophores and the values at the two half-heights of the probability density function for $R(^2/_3)/R$ listed in Table III of Haas et al. (1978).

Fluorescence Quenching. In a typical quenching experiment two samples were prepared with identical concentrations of the fluorescent rhodopsin conjugate. Sample A contained no quencher, and sample B contained either 0.1 M MP^+ or 0.042 M BP^+. An aliquot of sample A was removed and replaced by an identical amount of sample B, thus maintaining a constant concentration of the fluorophore while gradually increasing the quencher concentration. Prior to measuring the fluorescence intensity, the sample was well stirred and allowed to equilibrate until the intensity reached a constant value. Both quenchers equilibrated fast across the membrane (<2 min) as was evident by their fast and efficient quenching of carboxyfluorescein trapped in rhodopsin–asolectin vesicles. The quenching did not increase when the latter were dissolved in detergent (H. Borochov-Neori, unpublished results).

The quenching data were analyzed according to the treatment of Lehrer (1971) for collisional quenching in heterogeneous systems: at low quencher concentrations the dependence of the fluorescence intensity on the quencher concentration can be approximated by eq 6:

$$\frac{F_0}{\Delta F} = \frac{1}{[Q]\sum f_i K_{Qi}} + \frac{\sum K_{Qi}}{\sum f_i K_{Qi}} \tag{6}$$

where F_0 and F are the fluorescence in the absence and presence of quencher Q, $\Delta F = F_0 - F$, and f_i is the fraction of fluorescence contributed by the ith population of fluorophores which is also characterized by the quenching constant K_{Qi}. A plot of $F_0/\Delta F$ vs. $1/[Q]$ exhibits a linear region, from the slope and intercept of which the values of $\sum f_i K_{Qi}$, the mean quenching constant (K_Q^m), and $\sum f_i K_{Qi}/\sum K_{Qi}$, the upper limit of the quenchable fraction (fa), can be derived.

Results and Discussion

Fluorescence Labeling of ROS. The main criteria specified in choosing the conditions for the labeling reactions were (i) rhodopsin labeling with no more than a single fluorophore moiety per protein on the average, (ii) preservation of native spectral characteristics in the unbleached state, and (iii) capacity to regenerate with 11-*cis*-retinal after bleaching when in a membrane environment.

(*A*) *Labeling with Sulfhydryl Reagents.* Both PM and mBB were introduced as sulfhydryl-specific reagents (Weltman et al., 1973; Kosower et al., 1979). As such, the efficiency and specificity of their reactions should be pH dependent (Brewer & Riehm, 1967). As evident from Figure 1 labeling of ROS with PM only slightly increased when the pH was raised from 6.0 to 7.5. Since the specificity of maleimides to thiols is higher at pH <7 (Brewer & Riehm, 1967), we chose to conduct the reaction of ROS with PM at pH 6.5. Labeling with mBB, on the other hand, was independent of the pH in the range 6.5–7.5 but sharply increased at pH 8.0. Labeling was linear with reagent concentration in the range 1–10 μM PM and 0.1–1 mM mBB (results are not shown). We adopted the concentration and time of incubation which resulted in approximately a single fluorescent moiety bound per rhodopsin molecule. Titration of the free–SH groups with 4,4′-dithiodipyridine (Chen & Hubbell, 1978) showed that both fluorophores were coupled to sulfhydryl residues of the protein. At the studied ranges of pH and reagent concentration, the

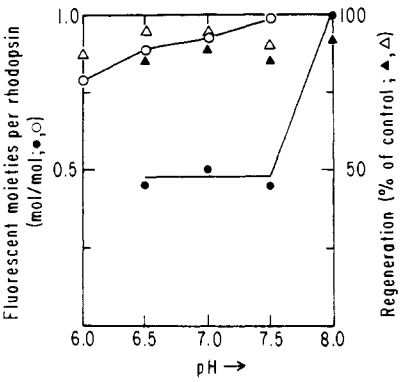

FIGURE 1: Effect of pH on the labeling of ROS with PM (○, △) and mBB (●, ▲). ROS labeling was carried out as described under Experimental Procedures varying the pH of the reaction buffer. Rhodopsin regeneration was performed by incubating bleached ROS with a 4-fold excess of 11-*cis*-retinal (mol/mol) in the dark at 4 °C overnight. The extent of regeneration obtained with ROS which were maintained at 4 °C (control) was taken as 100%. The number of fluorescent moieties bound to rhodopsin was determined from the absorption spectrum of rhodopsin purified from the labeled membranes.

labeled membranes maintained the characteristic spectral properties of rhodopsin in the unbleached state; regeneration after bleaching was 85–95% of that obtained with unreacted membranes (Figure 1). This indicates that the reaction protocol and rhodopsin labeling did not introduce significant perturbations. There are two reactive SH groups per rhodopsin in the dark. Under the stoichiometric conditions of reaction used, labeling with PM could lead to either selective labeling with high affinity to one SH group or a distribution of label between the two sites. Since labeling with mBB did not affect the reaction with PM and since the simplest way to analyze the results is assuming labeling of discrete sites, we tend to favor the first possibility. This is valid to a first approximation, but further work is necessary to establish this contention more definitively.

(*B*) *Labeling with Amino Reagents.* Both DNSCl and FITC are amino-specific reagents that most likely react with free ϵ-amino groups of protein lysine residues (Gennis et al., 1972). As evident from Figure 2A DNSCl labeled ROS at submillimolar concentrations. When on the average more than two dansyl moieties were coupled to rhodopsin, the capability of the membranes to regenerate was reduced without affecting the spectral properties of unbleached rhodopsin. Labeling with FITC required millimolar concentrations (Figure 2B). The reaction did not affect either the dark absorption spectrum of rhodopsin or its regenerability. Accordingly, in subsequent experiments 0.2 mM DNSCl and 2 mM FITC were adopted to obtain regenerable rhodopsin labeled with a single fluorescent moiety on the average. Introducing both reagents together in the reaction mixture did not affect the efficiency of labeling with either reagent, suggesting that they react with different amino groups of rhodopsin.

Spectral Characteristics of Fluorescent-Labeled Rhodopsin. The absorption spectra of the fluorescent conjugates of rhodopsin and of unlabeled rhodopsin are illustrated in Figures 3 and 4. The difference between the two spectra yields the absorption spectrum of the bound fluorophore. Since the extinction coefficients of bimane and dansyl are relatively small, the spectra of bimane-labeled rhodopsin (B-Rho) (Figure 3) and dansylated rhodopsin (D-Rho) (Figure 4) are of rhodopsin labeled at a ratio of two fluorescent moieties per protein. The fluorescence emission spectra of bleached rhodopsin adducts are also shown in Figures 3 and 4. The emission of each bound fluorophore partially overlaps the

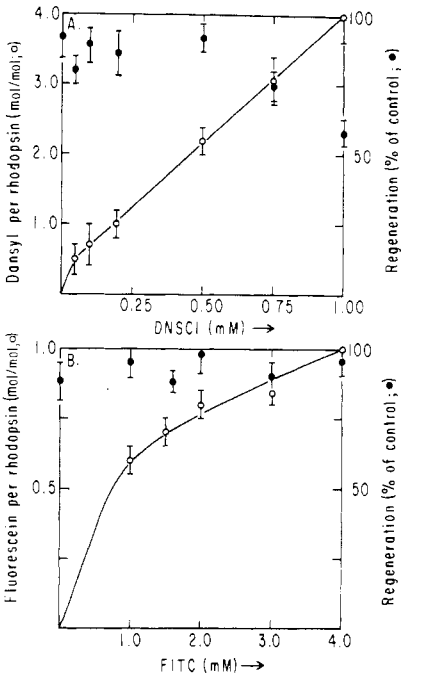

FIGURE 2: Effect of reagent concentration on the labeling of ROS with DNSCl and FITC. ROS labeling, regenerability of rhodopsin in the labeled ROS, and labeling stoichiometry were determined as described in the legend to Figure 1.

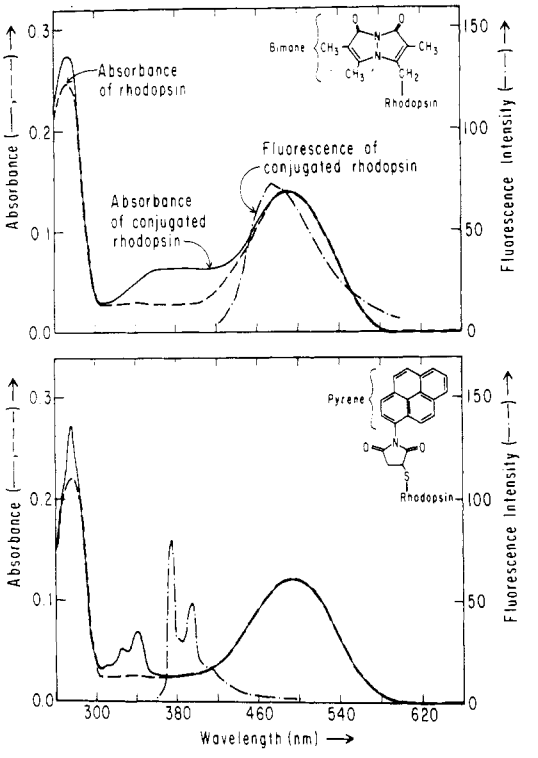

FIGURE 3: Spectral characteristics of purified rhodopsin labeled via -SH residues. Absorption (—) and fluorescence emission (---) spectra of labeled rhodopsin (~0.12 mg/mL) in 1.5% OG, 0.75 M α-mm, 0.1 M NaCl, and 10 mM P_i, pH 6.5; (---) absorption spectrum of purified unlabeled rhodopsin in the same buffer. The emission spectra were obtained with excitation at 327 nm using slits of 1 and 5 nm for the excitation and emission, respectively.

absorption spectrum of native rhodopsin. Hence, all fluorophores are potential energy donors to the endogenous chromophore, 11-*cis*-retinal.

The emission spectra of purified rhodopsin conjugates in micellar solution,in the dark and after bleaching, are presented

Table I: Fluorescence Parameters of Rhodopsin Conjugates

conjugate[a]	P-Rho		B-Rho		D-Rho		F-Rho	
system	detergent	vesicles	detergent	vesicles	detergent	vesicles	detergent	vesicles
λ_{exc}^{max} [b] (nm)	347	347	386	383	337	337	495	495
λ_{em}^{max} [c] (nm)	375	375	477	477	530	535	525	520
ϕ[d]	0.20	0.20	0.22	0.22	0.15	0.15	0.45	0.45
$R_0(^2/_3)$[e] (A)	31	31	42	42	40	39	41	42
E[f]	0.07	0.08	0.62	0.70	0.69	0.69	0.46	0.60
$R(^2/_3)$[g] (A)	48	47	39	36	35	34	42	39
r[h]	0.12	0.18	0.17	0.24	0.26	0.23	0.17	0.19
$\langle R \rangle$[i] (A)	42-55	41-54	34-45	32-42	30-41	29-40	36-48	34-45

[a] No more than a single fluorophore bound per rhodopsin. [b] Wavelength of maximal excitation. [c] Wavelength of maximal emission. [d] The quantum yield after bleaching. [e] The distance of 50% transfer efficiency calculated for random dipole orientation. [f] Transfer efficiency from the fluorophores to the endogenous chromophore. [g] The distance between the fluorophores and the endogenous chromophore assuming random dipole orientation. [h] Measured in bleached samples. See Experimental Procedures for excitation and emission setups. [i] The range of most probable values of R (see text for details).

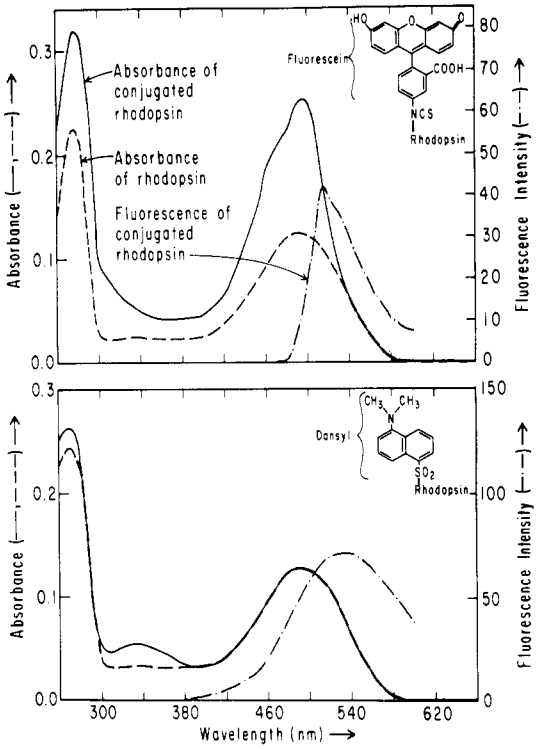

FIGURE 4: Spectral characteristics of purified rhodopsin labeled via amino groups. The symbols and the experimental description are the same as in the legend to Figure 3 except that excitation was performed at 330 nm.

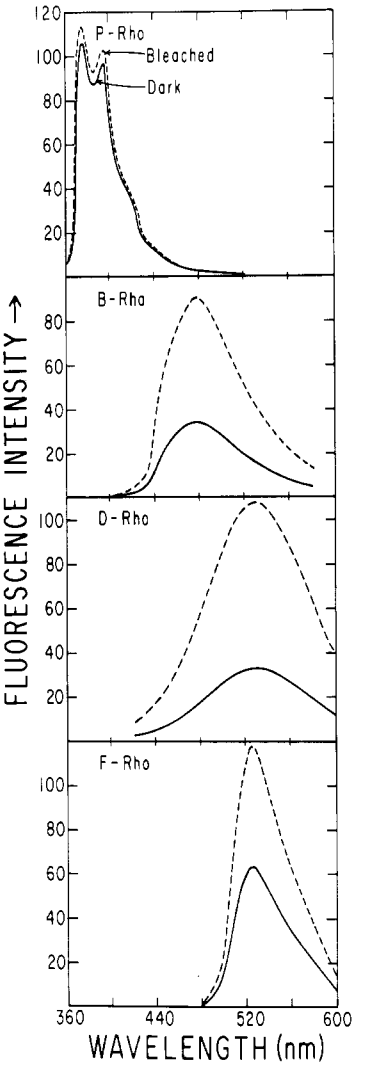

FIGURE 5: Effect of rhodopsin bleaching on the emission of the bound fluorophores. Emission spectra of dark (—) and bleached (---) suspensions of purified labeled rhodopsin in 1.5% OG, 0.75 M α-mm, 0.1 M NaCl, and 10 mM P_i, pH 6.5. Excitation was performed at either 327 (P-Rho and B-Rho) or 330 nm (D-Rho and F-Rho), and slits of 1 and 5 nm were used for the excitation and the emission, respectively. Rhodopsin was bleached by exposure to a microscope illuminator (Nicholas, Bausch & Lomb) for 5 min at room temperature.

in Figure 5. The fluorescence intensity of all the fluorophores increased upon bleaching, but no changes in the shape and maxima of the excitation and emission spectra were detected (it should, however, be noted that the wide emission slit used in these measurements, 15 nm, may have obscured small spectral changes). These results, and similar findings of Wu & Stryer (1972) on rhodopsin labeled with other fluorophores, suggest that the lower quantum yields measured in the dark result from singlet–singlet energy transfer from the bound fluorophores to the endogenous chromophore.

The spectroscopic characteristics of the rhodopsin conjugates in detergent and in reconstituted vesicles are summarized in Table I. The salient features which are worth noting are the following: (1) The emission spectrum of pyrene-labeled rhodopsin (P-Rho) is typical of a pyrene-labeled protein where neither an excimer nor a secondary reaction with a neighboring amino group occurs (Betcher-Lange & Lehrer, 1978). (2) The emission maximum of D-Rho is typical of dansyl located in a hydrophilic environment (Borochov & Shinitzky, 1976). (3) The fluorescence spectra of the rhodopsin conjugates reconstituted in phospholipid vesicles and the effect of rhodopsin bleaching on the fluorescence intensity were very similar to those exhibited in micellar solution. These results suggest that the sites of rhodopsin probed by the fluorophores do not undergo major conformational and environmental changes when

the solubilized protein is reconstituted in phospholipid vesicles.

Estimation of Distances by Resonance Energy Transfer. The apparent relative distances from the fluorophores to retinal and the relevant parameters of the energy transfer measurements are shown in Table I. Distance evaluation is meaningful only if the labeling is limited to a single site. We have no definitive evidence as to the validity of this condition in our system. We recognize that certain untested assumptions are implied in the analysis and, therefore, the conclusions advanced are only tentative. The uncertainty in the spatial relationships cannot be relieved until the assignment of probe location is umambiguously established. However, assuming that the labeled sites are distinct, we proceed to estimate the apparent distances between the fluorophores and 11-*cis*-retinal.

The polarization values of most of the fluorophores are well below the theoretical limiting value 0.5, which corresponds to an immobilized probe with electronic transitions characterized by single transition dipole moments and parallel absorption and emission dipoles. This indicates that the majority of the probes exhibit some motional freedom during the excited-state lifetime. The polarization of pyrene bound to rhodopsin (Table I) is comparable to the limiting polarization exhibited by the adduct of pyrenylmaleimide with β-mercaptoethanol (Shepherd & Hammes, 1977). However, the multiple electronic transitions of pyrene should significantly reduce the uncertainty in distance estimates with this probe (Hass, et al., 1978). The acceptor, 11-*cis*-retinal, on the other hand, was shown to be highly immobilized within its binding site on the protein (Liebman, 1962; Wald et al., 1963). Therefore, the error in the distances calculated assuming a random orientation, $R_0(^2/_3)$, (see Table I) may be significant. Considering that retinal is relatively frozen and is characterized by a single transition dipole in the wavelength range of interest and using the r values of the fluorophores, we derived a range of most probable distances ($\langle R \rangle$ in Table I) with a maximum uncertainty of ±20% (Haas et al., 1978; see Experimental Procedures).

As evident from Table I, reconstitution was associated with small increases in the efficiency of energy transfer compared with rhodopsin in detergent, the most significant being that of the fluorescein conjugate. The calculated values of $R_0(^2/_3)$ and the ranges of the apparent distances between the fluorophores and 11-*cis*-retinal are similar to the values estimated in detergent—they are only 1–3 Å smaller.

The dansyl and the fluorescein moieties are ~29–41 and 34–48 Å away from the endogenous chromophore, respectively. The sites labeled with pyrene and bimane are ~41–55 and 32–45 Å from the endogenous chromophore, respectively. Energy transfer measurements on rhodopsin labeled with both bimane and pyrene suggest that their loci are ~30 Å apart, in agreement with Wu & Stryer (1972) for the distance between the two reactive sulfhydryl groups of rhodopsin.

The sulfhydryl group labeled with pyrene is presumably the reactive sulfhydryl reported by Wu & Stryer (1972) to be >70 Å from the chromophore. This discrepancy appears not to stem from the choice of fluorophores; we obtained similar results when dansylaziridine [a fluorescent sulfhydryl reagent (Scouten et al., 1974)] was used instead of PM: the range of distances was 40–55 Å both in detergent and in reconstituted vesicles as well as in the native membrane. The difference may be accounted for, in part, by the fact that their measurements were performed on membranes containing peripheral proteins [see Kühn (1981)] as well as most probably a substantial fraction of bleached rhodopsin.

In summary, the intramolecular energy transfer measurements are consistent with the elongated shape model suggested for rhodopsin (Wu & Stryer, 1972; Sardet et al., 1976; Pober et al., 1978; Yeager et al., 1980; Corless et al., 1982): considering a molecular weight of ~38 000, if rhodopsin were to assume a spherical shape, its diameter would be less than 45 Å. With the uncertainty introduced by the assumption of discrete labeled sites, the approximate distances between the different fluorophores and 11-*cis*-retinal are schematically illustrated in Figure 6.

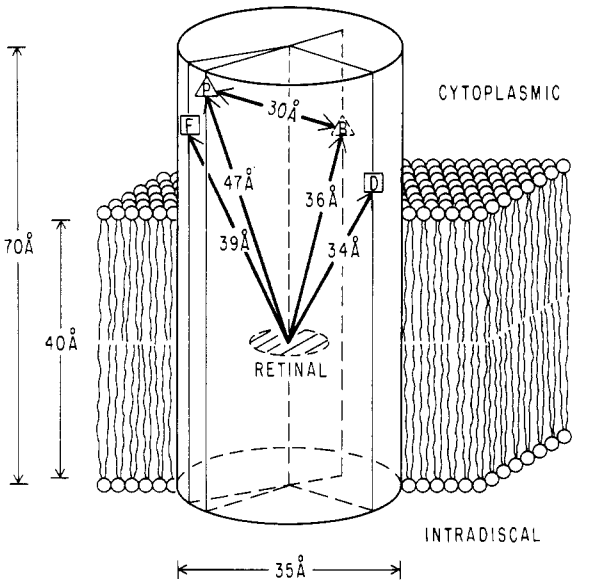

FIGURE 6: Schematic representation of the spatial relationships between fluorophore-labeled sites and the endogenous chromophore in rhodopsin. Several features of rhodopsin organization in the photoreceptor membrane are illustrated. The protein has an elongated shape—it is 70–80 Å long (Wu & Stryer, 1972; Sardet et al., 1976; Yeager et al., 1980; Corless et al., 1982) and 31–41 Å in diameter (Sardet et al., 1976). Rhodopsin transverses the membrane (Fung & Hubbell, 1978) with more of its polypeptide chain exposed at the cytoplasmic face of the membrane than at its intradiscal face (Hargrave et al., 1980). The transmembrane segment is highly oriented (Michel-Villaz et al., 1979), with the endogenous chromophore parallel to the membrane surface (Liebman, 1962; Wald et al., 1963) and most likely equidistant from the two planes of the lipid head groups (Thomas & Stryer, 1982). The assignment of location of the four conjugated fluorophores (P, pyrene; B, bimane; D, dansyl; F, fluorescein) within the protein is approximate yet consistent with the resonance energy transfer and fluorescence quenching results while recognizing that certain untested assumptions are implied.

Fluorescence Quenching of Rhodopsin Conjugates Reconstituted in Phospholipid Vesicles—Effect of Light on Site Accessibility. The availability of the fluorescence of bound probes to quenchers of different hydrophobicity may be used to study protein structure (Lehrer, 1971; Borochov & Shinitzky, 1976). For our studies two quenchers were selected: MP^+, hydrophilic, and BP^+, more hydrophobic. Both exhibited greater efficiency than I^- and Cs^+ in quenching the fluorescence of *N*-acetyl-L-tryptophanamide (Shinitzky & Rivnay, 1977). Their partition into the membrane is different, and therefore they are expected to quench with different efficiencies probes bound to membrane proteins (Borochov & Shinitzky, 1976; Shinitzky & Rivnay, 1977).

The fluorescence of the different rhodopsin conjugates reconstituted in vesicles was studied as a function of quencher concentration in the dark and after rhodopsin bleaching. The quenching curves are presented in Figures 7 and 8. The quenching parameters are summarized in Table II.

Figure 7A shows the quenching of the pyrene fluorescence by MP^+. The experimental results obtained both in the dark and after bleaching did not obey the Stern–Volmer law. By use of the analysis of Lehrer, the maximal fraction of

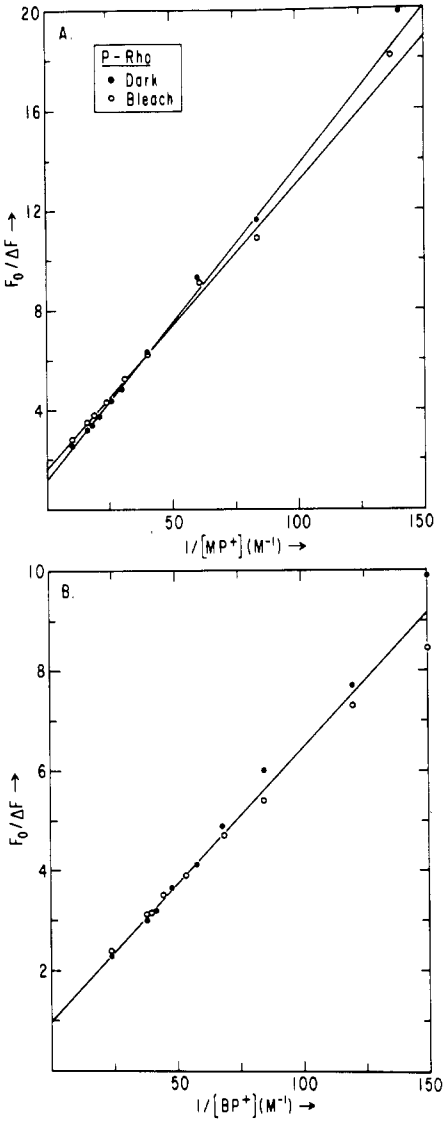

FIGURE 7: Quenching of P-Rho reconstituted in phospholipid vesicles by MP^+ (A) and BP^+ (B). Vesicle suspensions, 0.05 mg of rhodopsin/mL reconstituted with a 4-fold weight excess of phospholipids, were excited at 327 nm, and the fluorescence intensity at 375 nm was measured.

Table II: Quenching Parameters of Rhodopsin Conjugates in Reconstituted Phospholipid Vesicles

conjugate[a]	Q[b]	fa[c]		K_Q^m[d] (M^{-1})	
		D[e]	B	D	B
P-Rho	MP^+	0.83	0.63	7.9	8.6
	BP^+	1.0	1.0	18.4	18.4
B-Rho	MP^+	*f*	*f*	*f*	*f*
	BP^+	*f*	*f*	*f*	*f*
D-Rho	MP^+	*g*	*g*	*g*	*g*
	BP^+	0.67	1.0	17.1	2.8
F-Rho	MP^+	*g*	*g*	*g*	*g*
	BP^+	0.71	1.0	26.1	16.7

[a] Not more than a single fluorophore moiety was coupled per rhodopsin. [b] Q stands for quencher compound. [c] fa is the upper limit for the fraction of fluorescence available for quenching and equals $\Sigma f_i K_{Qi}/\Sigma K_{Qi}$. [d] K_Q^m is the mean quenching constant and equals $\Sigma f_i K_{Qi}$. [e] D and B stand for dark and bleached samples. [f] No quenching. [g] Quenching <10%.

fluorescence available for quenching, fa, and the mean quenching constant, K_Q^m, were calculated to be 0.83 and 7.9 M^{-1} in the dark and 0.63 and 8.6 M^{-1} after bleaching. This suggests the presence of more than a single population of

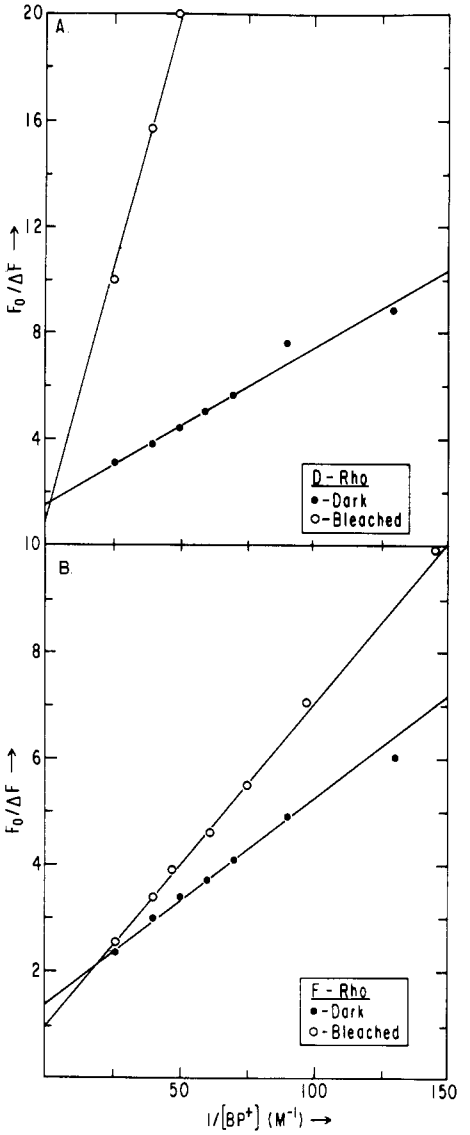

FIGURE 8: Quenching of D-Rho (A) and F-Rho (B) reconstituted in phospholipid vesicles by BP^+. Vesicle suspensions, 0.05 mg of rhodopsin/mL reconstituted with a 4-fold weight excess of phospholipid, were excited at 330 nm. The total emission intensity of D-Rho above 430 nm and the fluorescence intensity of F-Rho at 525 nm were measured.

fluorophores: it may reflect some heterogeneity either in the labeling of rhodopsin with PM or in the exposure of equivalent labeled sites. Heterogeneity in the latter could stem from the presence of rhodopsin aggregates of different sizes [evidence in favor of the presence of rhodopsin aggregates in reconstituted phospholipid vesicles is presented in the following paper (Borochov-Neori et al., 1983)].

Quenching with BP^+ (Figure 7B) was more efficient and, in the concentration range of 0–41 mM, obeyed the Stern–Volmer (1919) law both in the dark and after bleaching. No light effect was detected, and the quenching constant was 19.0 M^{-1}. A Lehrer analysis yields values of fa = 1.0 and K_Q^m = 18.4 M^{-1}. The 2-fold increase in the quenching constant observed with BP^+ compared to MP^+ suggests that the pyrene group is located in a rather hydrophobic pocket of the protein.

The fluorescence of bimane was not accessible to quenching by either MP^+ (up to 100 mM) or BP^+ (up to 41 mM) both in the dark and after bleaching. This is surprising since relative distances of the bimane moiety to the pyrene binding site and to the endogenous chromophore, which is embedded in the membrane bilayer (Thomas & Stryer, 1982), would more

likely place it close to the membrane surface. The complete inaccessibility of the bimane moiety to quenching was not charge dependent; acrylamide (up to 0.1 M) was as inefficient as the positively charged quenchers. It should be noted that all three quenchers efficiently quenched the fluorescence of the bimane conjugate when bleached rhodopsin was dissolved in detergent. These results suggest that in reconstituted phospholipid vesicles, the bimane binding site is shielded in a protein cleft which does not allow the penetration of small molecules. The existence of packed regions in rhodopsin domains exposed at the membrane surface is also consistent with the few sites available for proteolysis in situ (Saari, 1974; Hargrave et al., 1980).

The fluorescence of the dansyl moiety was virtually unquenchable by MP^+. Only few percent quenching was observed with up to 100 mM MP^+, and we did not analyze these results. This observation is puzzling since the emission spectrum of the dansyl moiety indicated that the probe was sensing a polar environment. The failure of MP^+ to reach the labeled sites may reflect a physical barrier (e.g., the protein is aggregated with the dansyl moieties being shielded by protein chains). The fluorophore was, however, quenchable with BP^+: the titration data are presented in Figure 8A, and the quenching parameters are summarized in Table II. A significant light effect was detected. Upon bleaching the mean quenching constant markedly decreased from 17.1 to 2.8 M^{-1}, whereas fa increased from 0.67 to 1.0. Since the excited-state lifetime of the fluorophore was longer after bleaching, the effect of light on the quenching kinetics was even larger: a 20-fold decrease in the rate of quenching.

The quenching of the fluorescein moiety was similar to that observed for dansyl. MP^+ was a very poor quencher, and only marginal quenching was observed with up to 100 mM MP^+. The quenching with BP^+ is presented in Figure 8B, and the calculated parameters are summarized in Table II. In analogy to the results with dansyl, upon bleaching, fa increased from 0.71 to 1.0 and K_Q^m decreased from 26.1 M^{-1} to 16.7 M^{-1}; this corresponds to a 4-fold decrease in the rate of fluorescein quenching by BP^+ induced by light. The K_Q^m values obtained for fluorescein were higher than for dansyl. This is consistent with the effect of the fluorophore charge on quenching by ions (Lehrer, 1971) since fluorescein is negatively charged at neutral pH.

In summary, the results of the fluorescence quenching experiments show that while the sites labeled with the sulfhydryl reagents do not detect marked changes in their vicinity, the probes attached to amino groups of rhodopsin undergo significant conformational changes in their vicinity upon bleaching. This light effect appears not to reflect conformational changes which result in a more hydrophobic environment in the vicinity of the probes: the emission maximum of dansyl, which is very sensitive to the medium polarity [see Borochov & Shinitzky (1976)], indicated a relatively polar environment and did not significantly change upon rhodopsin bleaching. The estimated differences in K_Q^m between the dark and light states are too large to arise from changes in environment polarity sufficiently small to pass undetected by the dansyl emission [for comparison see Borochov & Shinitzky (1976)]. Similarly, the excitation and emission spectra of fluorescein were not affected by rhodopsin bleaching. This charged moiety is most likely to be exposed at the membrane surface. Therefore, we are led to interpret the marked decrease in K_Q^m after bleaching as a result of conformational/structural changes which increased the protein packing around the probe sites, thus reducing the quencher diffusion in their vicinity.

Since the labeled sites are far from the retinal and the location of the retinal chromophore of rhodopsin appears to be near the center of the hydrophobic core of the membrane (Thomas & Stryer, 1982), the fluorophores used in this study probed rhodopsin domains which are either close to or exposed at the membrane surface. The quenching experiments suggest that these domains belong in two classes with respect to the extent of conformational changes experienced subsequent to rhodopsin photolysis. The domains which undergo substantial light-induced structural modifications are likely to participate in the activation of the GTP-binding protein triggered by rhodopsin photoisomerization (Fung et al., 1981; Kühn et al., 1981). If the mechanism of rhodopsin action were to involve rhodopsin–rhodopsin interactions, it would be reasonable to expect these domains to participate in the process. In the following paper we have tested this notion by following the light dependency of the proximity between the fluorescent conjugates of rhodopsin in reconstituted phospholipid vesicles.

Conclusions. (1) Intramolecular energy transfer data are consistent with the view that all the rhodopsin loci here studied are at least 30 Å away from the retinal chromophore and are supportive of the structural model that considers rhodopsin an elongated molecule (Wu & Stryer, 1972; Sardet et al., 1976; Pober et al., 1978; Yeager et al., 1980; Corless et al., 1982). (2) Fluorescence quenching data suggest that the rhodopsin domains exposed at the membrane surface and probed by the amino-reactive fluorophores undergo significant conformational changes upon bleaching and may be involved in the intermediate transduction events triggered by rhodopsin photoisomerization.

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