Rhodopsin in Reconstituted Phospholipid Vesicles. 2. Rhodopsin–Rhodopsin Interactions Detected by Resonance Energy Transfer†

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ABSTRACT: The interactions between rhodopsin molecules in a micellar detergent solution (octyl glucoside) and in reconstituted phospholipid vesicles were studied in the dark and after bleaching. Resonance energy transfer measurements were used to monitor the proximity between rhodopsin monomers conjugated with a fluorescent donor or a fluorescent acceptor. Reactive sulfhydryl groups of rhodopsin were labeled with pyrenylmaleimide (donor) or monobromobimane (acceptor), whereas amino groups were labeled with dansyl chloride (donor) or fluorescein isothiocyanate (acceptor). The results suggest that in the micellar solution rhodopsin was monomeric in the dark and aggregated after bleaching. If the aggregate were to be a dimer, the labeled sulfhydryl groups of the monomers would be approximately 40 Å apart, while the labeled amino groups would be at least 68 Å distant from each other. Rhodopsin reconstituted in phospholipid vesicles appeared aggregated both in the dark and after bleaching. The proximity between the sulfhydryl groups of the monomers was not influenced by illumination. In contrast, the labeled amino groups seemed to be largely separated in the dark and closer to each other once the vesicles were bleached. If the aggregate were to be a dimer, the labeled sulfhydryl groups would be approximately 40 Å apart both in the dark and after bleaching, whereas the labeled amino groups would be >60 Å apart in the dark and approximately 44 Å from each other after bleaching. These findings are discussed in the context of rhodopsin structure, its ability to regenerate after bleaching, and the light-induced events initiated by rhodopsin photoexcitation.

Recent studies in visual transduction have suggested that rhodopsin, in addition to its role as light detector, may act as an ion translocator, an enzyme activator, or, perhaps, both [for reviews see Hubbell & Bownds (1979), Pober & Bitensky (1979), Bownds (1981), Stryer et al. (1981), and Saibil (1982)]. These putative functions presumably proceed via direct protein–protein interactions. Investigation of the interactions between rhodopsin monomers within the membrane bilayer in the dark and after bleaching is, therefore, of interest.

Several studies have provided evidence suggestive of changes in the aggregation of rhodopsin upon illumination, both in micellar solutions (Hubbard, 1954; Osborne et al., 1974; Sardet et al., 1976; McCaslin & Tanford, 1981) and in reconstituted phospholipid vesicles (Chen & Hubbell, 1973). In rod outer segments (ROS),[1] studies on the rotational relaxation time of rhodopsin failed to detect changes in rhodopsin–rhodopsin interactions upon partial bleaching (Cone, 1972) or shortly after total bleaching (Baroin et al., 1979). These techniques, however, in their present state cannot establish unambiguously whether the protein is monomeric or oligomeric, nor detect local changes in protein–protein interactions. In fact, recent saturation-transfer ESR studies on purified rhodopsin reconstituted in phospholipid vesicles suggest that the rotational correlation time of rhodopsin measured in the native ROS membrane may correspond to that of a rhodopsin dimer (Kusumi & Hyde, 1982). Such structural parameters can be investigated, in principle, with better resolution by resonance energy transfer measurements [for reviews see Stryer (1978) and Fairclough & Cantor (1978)]. The technique has been used in two-dimensional systems to study the surface distribution of lectin receptors (Fernandez & Berlin, 1976), the structure of serum lipoproteins (Sklar et al., 1980), the oligomeric structure of gramicidin A (Veatch & Stryer, 1977), the anion transport system of human red cells (Dissing et al., 1979) and of the Ca-ATPase (Vanderkooi et al., 1977), the binding of hemoglobin to red blood cell ghost membranes (Shaklai et al., 1977), the surface density of phospholipids in lipid vesicles (Fung & Stryer, 1978), and the surface density and proximity between protein sites and lipids in (Na,K)-ATPase (Moczydlowski & Fortes, 1981).

In the preceding paper the preparation and properties of four fluorescent derivatives of rhodopsin were described. Here, energy transfer between these derivatives is used to investigate the interactions between rhodopsin monomers in a micellar solution (octyl glucoside, OG) and in reconstituted phospholipid vesicles, both in the dark and after bleaching. Preliminary accounts of this work were presented elsewhere (Borochov-Neori et al., 1981, 1982).

Experimental Procedures

A full account on the materials used in this study, the preparation of the fluorescent labeled derivatives of rhodopsin, their purification in OG, and their reconstitution in phospholipid vesicles was presented in the preceding paper (Borochov-Neori & Montal, 1983).

Analysis of Vesicle Composition. For determination of the phospholipid to rhodopsin ratio in the reconstituted vesicles, the vesicles were applied to discontinuous sucrose density gradients, 5–35% (w/v) prepared in the dialysis buffer, 20 mM imidazole, pH 7.0, and subjected to centrifugation at 100000*g* for 24 h at 4 °C. Bands containing rhodopsin were collected, dialyzed to remove the sucrose, and analyzed for phospholipid and protein contents. Protein was determined according to

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[1] Abbreviations: ROS, rod outer segments; OG, octyl glucoside; P-Rho, pyrene-labeled rhodopsin; B-Rho, bimane-labeled rhodopsin; D-Rho, dansylated rhodopsin; F-Rho, fluorescein-labeled rhodopsin.

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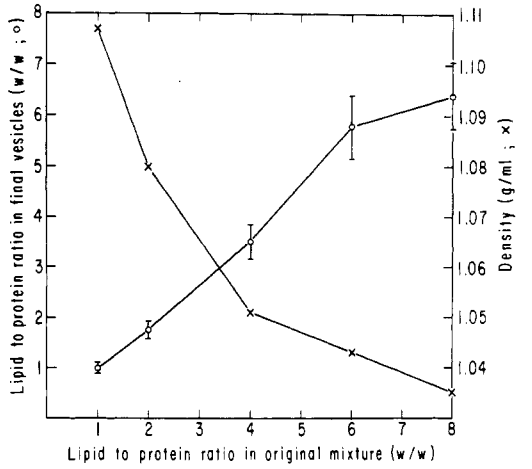

FIGURE 1: Preparation of reconstituted vesicles with a defined phospholipid to rhodopsin ratio. The dependence of the lipid to protein ratio (O) and the density (×) of the reconstituted vesicles on the original lipid to protein ratio in detergent prior to dialysis (see text for experimental details). The experimental values of the lipid to protein ratio are the average of triplicates.

Lowry et al. (1951). Phospholipids were extracted following the method of Renkonen et al. (1963), and the phosphorus content was measured according to Bartlett (1959) with the modification of Böttcher et al. (1961). The density along the gradient was measured in control tubes (vesicles omitted) with a refractometer (Bausch & Lomb, Model Abbe 3-L).

When vesicle preparations, originating from mixtures of different ratios of phospholipid to rhodopsin in detergent, were subjected to centrifugation on sucrose gradients, sharp bands corresponding to rhodopsin appeared. Each preparation exhibited a single band; its position along the gradient varied as a function of the original lipid to protein ratio.

Figure 1 shows the dependence of the final composition of the vesicles on the initial phospholipid to rhodopsin ratio. It is clear that up to a weight ratio of 6:1 (i.e., ~300 phospholipid molecules per rhodopsin) the reconstituted vesicles maintained the original lipid to protein ratio introduced in the detergent. When preparation of vesicles with a higher lipid to protein ratio was attempted, a fraction of the lipid assembled into vesicles devoid of rhodopsin; as a result, the rhodopsin-containing vesicles had a lower lipid to protein ratio than in the original mixture. Thus, an excess of lipid was required to generate weight ratios higher than 6:1. It is worth emphasizing that to obtain vesicles with lipid to rhodopsin weight ratio higher than 2:1, it was necessary to include cholate (1.5% w/v) before the dialysis step. A similar protocol was independently developed by Kusumi et al. (1980), presumably for similar reasons.

Steady-State Resonance Energy Transfer Measurements. The basic theory, formulations, and instrumentation were described in detail in the preceding paper (Borochov-Neori & Montal, 1983).

(A) Determination of the Resonance Energy Transfer Efficiency. The measurements were first performed on dark vesicle preparations by using an excitation slit of 1 nm to avoid bleaching. The same samples were then bleached for 5 min at room temperature with a microscope illuminator (Nicholas, Bausch & Lomb) and measured again. Small cuvettes of 3 × 3 mm were used in order to reduce the optical density to less than 0.06 and, thus, minimize inner filter effects.

The efficiency of dipole–dipole energy transfer can be determined either from the quenching of the donor or from the sensitized fluorescence of the acceptor [for reviews see Stryer (1978) and Fairclough & Cantor (1978)].

The transfer efficiency, E, determined from the sensitized fluorescence of the acceptor is given by

$$E = \frac{\Delta F_D^A / F_A^A}{A_D / A_A} \quad (1)$$

in which ΔF_D^A is the increase in the fluorescence intensity of the acceptor when a mixture of donors and acceptors is excited at the donor absorption band, A_D is the absorbance of the donor at the excitation wavelength, F_A^A is the fluorescence intensity of the acceptor when excited at the acceptor absorption band, and A_A is the absorbance of the acceptor at the excitation wavelength.

In each experiment, four types of reconstituted vesicles were prepared: "D" vesicles contained only donor-labeled rhodopsin; "A" vesicles contained only acceptor-labeled rhodopsin; "DA" vesicles contained both rhodopsin derivatives; "C" vesicles contained unlabeled rhodopsin. The four preparations had the same phospholipid to rhodopsin ratio, and their fluorescence spectra were recorded under identical experimental conditions. The vesicle concentrations, however, differed from one preparation to another. The spectra of "C" vesicles, normalized to the vesicle concentrations in the other preparations, were subtracted from the corresponding spectra of "D", "A", and "DA" vesicles to correct for background and scattering.

For determination of the efficiency of energy transfer, the fluorescence intensities at two excitation and two emission wavelengths were recorded. In the following, each fluorescence intensity is described by the general term F_λ^λ("X"), where the subscript denotes the excitation wavelength, the superscript denotes the emission wavelength, and "*X*" is the vesicle type. Excitation was performed at either λ = D, a wavelength within the donor absorption band (where the acceptor also has a residual absorbance), or λ = A, a wavelength at which only the acceptor absorbs. Emission was recorded at either λ = D, a wavelength where only the donor emits, or λ = A, a wavelength enriched with emission of the acceptor (but contains some contribution of the donor). The following set of values of fluorescence intensity was established for each measurement of fluorescence energy transfer: F_D^D("D"), F_D^A("D"), F_A^A("A"), F_D^A("A"), F_D^D("DA"), F_A^A("DA"), and F_D^A("DA"). To evaluate the extent of the sensitized fluorescence of the acceptor (ΔF_D^A in eq 1), it was necessary to subtract from F_D^A("DA") the contributions of the donors and the acceptors excited directly. These contributions are F_D^D("DA")[F_D^A("D")/F_D^D("*D*")] for the donor and F_A^A("DA")[F_D^A("A")/F_A^A("A")] for the acceptor. After these terms were substituted in eq 1, E was evaluated from

$$E = \{[F_D^A(\text{"DA"}) - F_D^D(\text{"DA"})[F_D^A(\text{"D"})/F_D^D(\text{"D"})] - F_A^A(\text{"DA"})[F_D^A(\text{"A"})/F_A^A(\text{"A"})]]/F_A^A(\text{"DA"})\}/(A_D/A_A) \quad (2)$$

where A_D is the net absorbance of the donor at the excitation wavelength λ = D and A_A is the net absorbance of the acceptor at the excitation wavelength λ = A, both determined in the "DA" vesicles. The absorbances of the acceptors were determined directly from the absorption spectra of the samples. The absorbances of the donors were corrected for the contribution of the acceptors to the measured values.

(B) Analysis of the Energy Transfer Results with a Model of Randomly Distributed Rhodopsin Monomers. The problem of the Förster energy transfer in two-dimensional arrays was theoretically analyzed by several groups recently (Fung & Stryer, 1978; Estep & Thompson, 1979; Wolber & Hudson, 1979). It was shown, both theoretically and experimentally, that unassociated donors and acceptors which are randomly

Table I: Fluorescence and Energy Transfer Parameters of Rhodopsin Conjugates Reconstituted in Phospholipid Vesicles

conjugate[a]	λ_{exc}^{max} [b] (nm)	λ_{em}^{max} [c] (nm)	ϕ[d]	r[e]	$R_0(^2/_3)$[f] (Å)	$\langle R_0 \rangle$[g] (Å)
P-Rho	347	375	0.19-0.21[h]	0.18	30	(27-33)[j]
B-Rho	383	477	0.07-0.22[i]	0.24		
D-Rho	337	535	0.05-0.15[i]	0.23	31-36[i]	(32-40)[j]
F-Rho	495	520	0.18-0.45[i]	0.19		

[a] No more than a single fluorophore bound per rhodopsin. [b] Wavelength of maximal excitation. [c] Wavelength of maximal emission. [d] Quantum yield. [e] Fluorescence anisotropies in bleached vesicles were measured by using the following settings: P-Rho, exc. 327 nm/em. > 390 nm; B-Rho, exc. 383 nm/em. > 420 nm; D-Rho, exc. 330 nm/em. > 420 nm; F-Rho, exc. 470 nm/em. 520 nm. [f] The distance of 50% transfer efficiency calculated for random dipole orientation. [g] The range of most probable values of R_0 estimated as described in Borochov-Neori & Montal (1983). [h] Range of values obtained at different lipid to rhodopsin ratios, in the dark and after bleaching. [i] The lower value corresponds to dark vesicles, and the higher, to bleached vesicles. [j] Range of most probable values in bleached vesicles [see Borochov-Neori & Montal (1983)].

distributed within a membrane bilayer may exhibit significant energy transfer. The efficiency of energy transfer in such a system depends on the R_0 of the donor–acceptor couple, the distance of closest approach between donors and acceptors (radius of the excluded volume, R_e, approximated by the sum of the radii of the donor and acceptor entities), and the surface concentration of the acceptor, but not of the donor. This dependence is described by the following approximation explicitly derived by Wolber & Hudson (1979):

$$E \approx 1 - [A_1 \exp(-k_1 C) + A_2 \exp(-k_2 C)] \quad (3)$$

where C is the acceptor surface concentration per R_0^2 and the parameters A_1, A_2, k_1, and k_2 are determined by the ratio of R_e to R_0 in the system. This approximation is accurate to better than 1% for $C \leq 0.5$ Å^{-2}.

This function was used to generate theoretical curves of E vs. C for a model of rhodopsin monomers unassociated and randomly distributed within the membrane bilayer. R_0 was calculated assuming a random dipole orientation, $R_0(^2/_3)$ [see Table I for values of $R_0(^2/_3)$ and the range of most probable values of R_0). The R_e values used are the published estimates of the rhodopsin diameter, 31–41 Å (Sardet et al., 1976). The corresponding values for A_1, A_2, k_1, and k_2 were taken from Table I of Wolber & Hudson (1979).

To compare the experimental results with the theoretical analysis, we calculated the acceptor surface concentration in our systems using the values of 60 Å^2 and 1000 Å^2 for the phospholipid (Shah & Schulman, 1967) and rhodopsin (Sardet et al., 1976) average surface area, respectively, and the following assumptions: (a) Rhodopsin is symmetrically oriented in the membrane (Fung & Hubbell, 1978). (b) Each monolayer consists of an equal number of phospholipid molecules [the average diameter of the vesicles was ~1000 Å (H. Borochov-Neori, M. H. Ellisman, and M. Montal, unpublished results); hence, the difference between the surface areas of the two monolayers is negligible (Chan et al., 1973)]. (c) Energy transfer between donors and acceptors in opposite leaflets of the bilayer is insignificant [the bound fluorophores are all ≥30 Å from the endogenous chromophore (Borochov-Neori & Montal, 1983), which appears to be located near the center of the bilayer (Thomas & Stryer, 1982); thus, the fluorophores are most probably close to the membrane surface; for R_0 < 40 Å and a membrane thickness = 50 Å, the contribution of transmembrane energy transfer is ≤11% (Fung & Stryer, 1978)]. (d) Contribution of residual detergent to the surface area can be ignored [experiments with [^{14}C]OG showed less than four detergent molecules present per rhodopsin molecule (C. Vandenberg, personal communication)].

Results and Discussion

Fluorescence Energy Transfer between Rhodopsin Monomers in a Micellar Solution. (A) Rhodopsin Labeled with Sulfhydryl Reagents. Figure 2 shows the absorption and the fluorescence emission spectra of pyrene and bimane coupled to rhodopsin. It is evident that the fluorophores are a suitable couple for fluorescence energy transfer measurements, with pyrene–rhodopsin (P-Rho) as a donor and bimane–rhodopsin (B-Rho) as an acceptor.

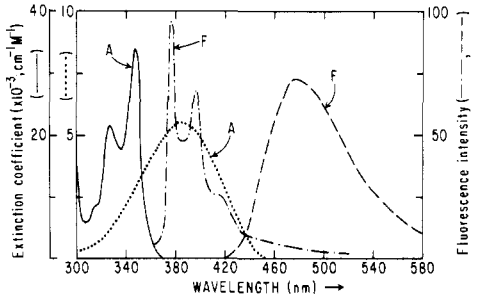

FIGURE 2: Pyrene and bimane are a suitable couple for resonance energy transfer. Absorption (—) (···) and fluorescence emission (- · -) (- - -) spectra of pyrene and bimane, respectively, conjugated to rhodopsin. Spectra of bleached purified rhodopsin conjugates in OG were recorded. The absorption spectrum of an analogous preparation of bleached-purified unlabeled rhodopsin was subtracted to produce the net absorption spectra of the bound fluorophores. Emission spectra were obtained with excitation at 342 and 380 nm for pyrene and bimane, respectively, using slits of 4 nm.

The fluorescence emission and excitation spectra of a mixture of purified and delipidated P-Rho and B-Rho in a micellar solution are illustrated in Figure 3. These spectra are compared to the sum of the spectra of each derivative in the absence of the other, normalized to their final concentration in the mixture. In the dark, both the emission (Figure 3A) and the excitation (Figure 3B) spectra of the mixture were indistinguishable from the corresponding sums of the individual spectra even after 24-h incubation. This indicates that no energy transfer occurred in the dark. In contrast, the fluorescence spectra of a bleached mixture of P-Rho and B-Rho clearly deviated from the corresponding sums of the individual spectra. When the bleached mixture was excited at 327 nm (Figure 3A), where the absorbance of pyrene predominates, the fluorescence intensity of pyrene at 375 and 395 nm decreased. A concomitant increase in the fluorescence of bimane at 480 nm was observed. Analogously, when the emission of bimane at 477 nm was monitored (Figure 3B), increased intensities were observed at excitation wavelengths coinciding with the absorption maxima of pyrene, i.e., 327 and 347 nm. These observations reflect the occurrence of energy transfer. At such low protein concentrations (≤2.5 μM) and high detergent to protein molar ratio (~2 × 10^4:1), the average distance between rhodopsin monomers would be too large (>400 Å) for energy transfer unless interactions between monomers occur. The results thus indicate that after bleaching

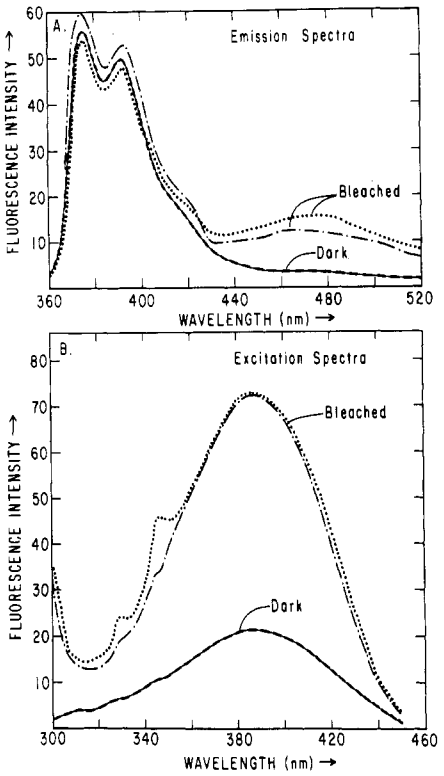

FIGURE 3: Emission (A) and excitation (B) spectra of a mixture of P-Rho and B-Rho in OG. Spectra of the mixture in the dark (—), and after bleaching (···). Sum of the spectra of P-Rho and B-Rho in the dark (---) and after bleaching (-·-). The preparations consisted of P-Rho, 0.06 mg of rhodopsin/mL, ~0.8 pyrene moiety per rhodopsin, and B-Rho, 0.1 mg of rhodopsin/mL, ~1 bimane moiety per rhodopsin. Emission spectra were obtained with excitation at 327 nm. Excitation spectra were recorded by monitoring the emission at 477 nm. The excitation and emission slits were 1 and 15 nm, respectively.

rhodopsin was aggregated in the micellar solution. In this aggregate the –SH groups of rhodopsin monomers labeled with pyrene are likely to be more than 10 Å apart, since no evidence for excimer formation (Birks, 1970) was observed in the emission spectrum of a bleached solution of P-Rho. The efficiency of energy transfer from P-Rho to B-Rho was 10% when calculated from pyrene quenching and 7% when calculated from the sensitized fluorescence of bimane. The stoichiometry of the aggregate was unknown; When a minimum size aggregate, a dimer, was assumed and when the fraction of hybrid dimers (Veatch & Stryer, 1977) was calculated, the distance between the labeled –SH groups was estimated to be approximately 40 Å.

The failure to detect energy transfer in the dark does not necessarily mean that the protein was monomeric; it could have been aggregated, yet the separation between the pyrene and the bimane moieties was too large (>60 Å) or their relative orientation was unfavorable for energy transfer to occur. However, it was previously suggested that rhodopsin in other detergents, in particular detergents which prevent rhodopsin regeneration, is monomeric in the dark and aggregated after belaching (Osborne et al., 1974). Our results in OG, under conditions which led to loss of the rhodopsin ability to regenerate after bleaching, are consistent with such light-dependent aggregation states.

(B) Rhodopsin Labeled with Amino Reagents. Figure 4 illustrates the emission spectrum of a mixture of purified and delipidated rhodopsin labeled with either dansyl (D-Rho) or fluorescein (F-Rho) in a micellar solution, as well as the sum

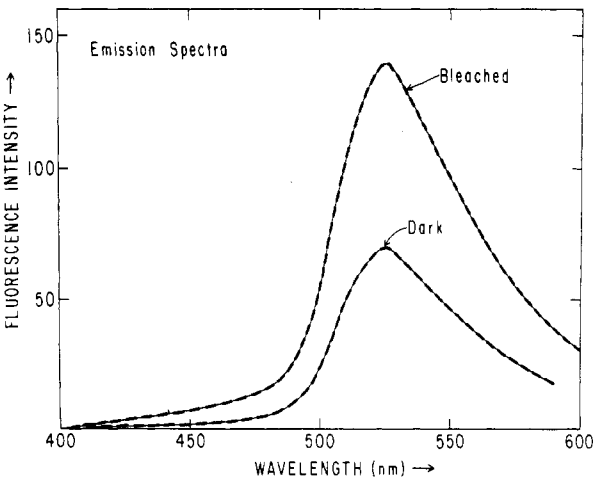

FIGURE 4: Emission spectra of D-Rho and F-Rho in OG. Emission spectrum of the mixture (—) and the sum of the emission spectra of D-Rho and F-Rho (---). The preparations consisted of D-Rho, 0.2 mg of rhodopsin/mL, ~1 dansyl moiety per rhodopsin, and F-Rho, 0.2 mg of rhodopsin/mL, ~0.8 fluorescein moiety per rhodopsin. Excitation was at 330 nm, and the excitation and emission slits were 1 and 20 nm, respectively.

of the individual spectra of D-Rho and F-Rho. In the dark, no difference was detected between the spectrum of the mixture and the corresponding sum of the spectra of D-Rho and F-Rho, i.e., no energy transfer occurred. This is in agreement with the results obtained with the previous couple. Unlike the results with P-Rho and B-Rho, bleaching the mixture of D-Rho and F-Rho did not lead to detectable energy transfer. The absence of energy transfer, both in the dark and after bleaching, persisted even when the conjugates were incubated for 24 h prior to the measurements. On the other hand, bleaching the mixture of D-Rho and F-Rho produced the same small increase in light scattering (measured as the absorbance change at 670 nm) which was observed after bleaching any other preparation of rhodopsin, labeled or unlabeled, in OG. This increase in light scattering was interpreted as reflecting the aggregation of opsin. It seems, therefore, that the lack of energy transfer after bleaching could be due to a large separation (i.e., >68 Å if a dimer is considered) within the aggregate between monomer sites labeled with dansyl and fluorescein, rather than to the maintenance of the monomeric form after bleaching.

Fluorescence Energy Transfer Measurements on Rhodopsin Reconstituted in Phospholipid Vesicles. (A) Rhodopsin Labeled with Sulfhydryl Reagents. To measure energy transfer between P-Rho and B-Rho in a membrane system we compared the fluorescence spectra of vesicles reconstituted with both derivatives ("DA" vesicles) to the spectra of an analogous mixture of vesicles containing either P-Rho ("D" vesicles) or B-Rho ("A" vesicles). Typical results are presented in Figure 5. The excitation spectra of bimane in these two classes of vesicles were clearly different; the fluorescence intensity in the range of excitation wavelengths which corresponds to the absorption spectrum of pyrene was higher in "DA" vesicles. This was observed both in the dark and after rhodopsin bleaching, indicating that energy transfer occurred in these vesicles in both states.

We attempted to establish whether the observed energy transfer reflected the presence of rhodopsin aggregates or merely originated from randomly distributed rhodopsin monomers in close proximity. To do so, the efficiency of energy transfer was measured as a function of the phospholipid to rhodopsin ratio, the mole fraction of the unlabeled rhodopsin,

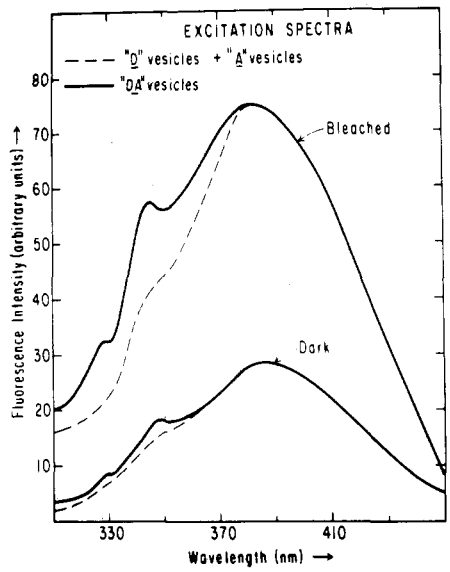

FIGURE 5: Fluorescence excitation spectra of vesicles reconstituted with P-Rho and B-Rho. Spectra of vesicles containing both derivatives (—) and an analogous mixture of vesicles containing either conjugate (---). Preparations consisted of 0.2 mg of total rhodopsin/mL. The ratio between P-Rho and B-Rho was 1.5:1. The lipid to protein ratio in the vesicles was 6:1 (w/w). The emission at 477 nm was monitored, and slits of 1 and 15 nm were used for excitation and emission, respectively.

and the mole fraction of the acceptor, B-Rho, in the vesicles. The results obtained with bleached vesicles are shown in Figure 6 (the results in the dark were very similar and, therefore, are not presented). In Figure 6A rhodopsin was diluted in the membrane by increasing the number of phospholipid molecules per rhodopsin from 50 to 400, while the ratio of P-Rho to B-Rho and the mole fraction of unlabeled rhodopsin were maintained constant. The transfer efficiency was not significantly affected by varying the lipid to protein ratio in the vesicles. In Figure 6B the donor and acceptor concentrations were diluted by introducing increasing amounts of unlabeled rhodopsin in the vesicles. The lipid to protein ratio and the ratio of P-Rho to B-Rho were kept constant in the different preparations. As the mole fraction of unlabeled protein increased from 10 to 50%, the efficiency of energy transfer decreased 5-fold and was no longer detectable when more than 60% of rhodopsin was unlabeled. The dependence of the transfer efficiency on the mole fraction of the acceptor is shown in Figure 6C. The mole fraction of B-Rho was varied by changing the ratio of P-Rho to B-Rho in the vesicles while the lipid to protein ratio and the mole fraction of unlabeled rhodopsin were maintained constant. The efficiency of energy transfer was highly dependent on the donor to acceptor ratio: doubling the mole fraction of B-Rho more than doubled the transfer efficiency.

Of the three sets of results presented in Figure 6 only those of Figure 6A can be regarded as conclusive evidence indicative of aggregation. If rhodopsin were monomeric in the bilayer, dilution with lipid should have increased the estimated average distance between the centers of monomers from ~63 to ~158 Å in the experiment of Figure 6A. This should have reduced markedly the efficiency of energy transfer. However, this was not observed. A decrease in the transfer efficiency accompanied a decrease in the surface concentration of acceptors only when B-Rho was substituted with either unlabeled rhodopsin (Figure 6B) or P-Rho (Figure 6C), but not when B-Rho was diluted with lipid (Figure 6A). Thus, the decrease in transfer efficiency observed in Figure 6B,C appears to have

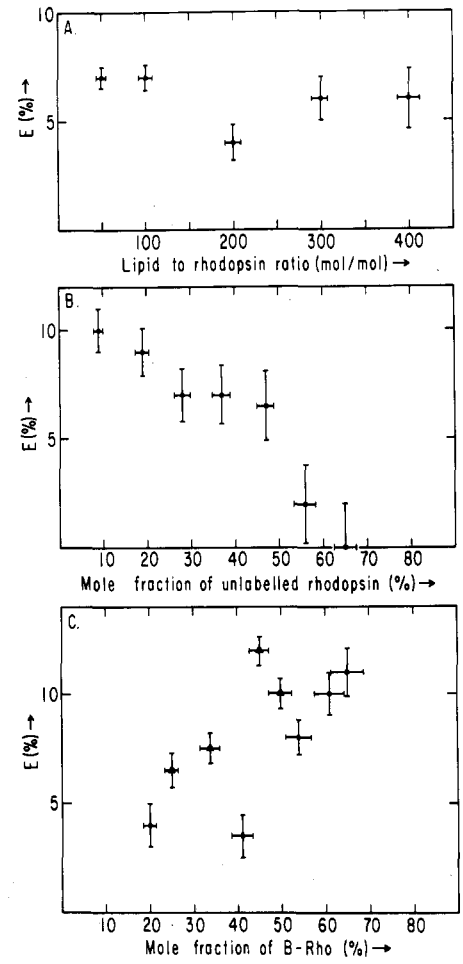

FIGURE 6: Dependence of the efficiency of energy transfer between P-Rho and B-Rho on the composition of the reconstituted vesicles. All the values are for bleached vesicles. The experimental points are the average of three measurements. The total rhodopsin concentration was 0.2 mg/mL. (A) Dependence on lipid to protein ratio. The ratio of P-Rho to B-Rho was 1:1. Approximately 35% of the protein was unlabeled. (B) Dependence on unlabeled rhodopsin. The ratio of P-Rho to B-Rho was maintained at 0.8:1. The ratio of lipid to protein was 6:1 (w/w). (C) Dependence on donor to acceptor ratio. The mole fraction of B-Rho was varied by changing the ratio of P-Rho to B-Rho in the range 1.7–0.14:1. (▲) The lipid to protein ratio was 4:1 (w/w), and the mole fraction of unlabeled rhodopsin was 0.15. (●) The lipid to protein ratio was 6:1 (w/w), and the mole fraction of unlabeled rhodopsin was 0.3.

resulted from the decreased probability of forming aggregates containing both donors and acceptors, rather than a decreased surface concentration of acceptors. These conclusions were tested quantitatively by comparing the results with the predictions of a model of energy transfer between randomly distributed rhodopsin monomers in the bilayer. Figure 7 shows the experimental values of transfer efficiency as a function of the acceptor surface concentration and the theoretical curves generated from the model using R_0 = 30 Å and R_e = 31–41 Å (see Table I and Experimental Procedures). If rhodopsin were monomeric and randomly distributed within the membrane, the experimental values would be expected to fall within the shaded area. This is evidently not the case: the results consistently deviated from the predicted behavior toward higher values, suggesting that rhodopsin was aggregated with the pyrene- and bimane-labeled sites in sufficient proximity for energy transfer to occur.

In summary, the results on vesicles containing P-Rho and B-Rho indicate that the protein was aggregated both in the dark and after bleaching. In the aggregate, pyrene-labeled

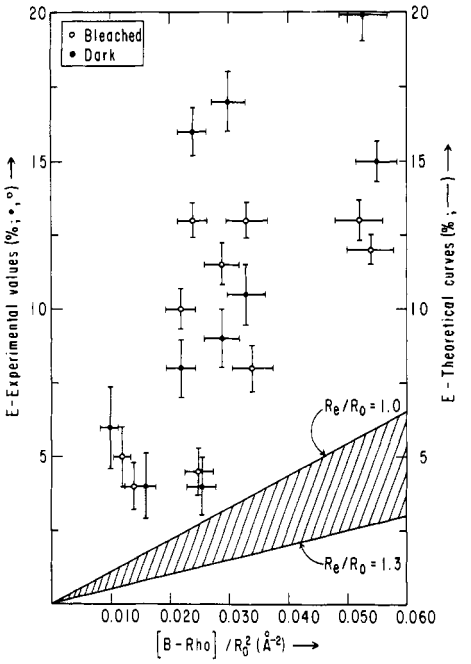

FIGURE 7: Comparison between the experimental values of the efficiency of energy transfer between P-Rho and B-Rho in reconstituted vesicles and the theoretical curves generated for a model of randomly distributed rhodopsin monomers (see text for details).

sites of different monomers were more than 10 Å apart, since no excimer band was observed in the fluorescence emission spectrum of vesicles containing P-Rho only. When the minimal aggregate, a dimer, was assumed, the sites labeled with pyrene and bimane were approximately 40 Å apart. The actual number of monomers in the aggregate cannot be deduced from the available data; however, the aggregation was not massive, since freeze–fracture electron microscopy showed isolated intramembrane particles of ~80–90-Å diameter in similar vesicles reconstituted with unlabeled rhodopsin (H. Borochov-Neori, M. H. Ellisman, and M. Montal, unpublished observations).

(B) Rhodopsin Labeled with Amino Reagents. The fluorescence emission spectra of vesicles containing D-Rho and F-Rho are shown in Figure 8. The spectra of vesicles reconstituted with both derivatives ("DA" vesicles) are compared to spectra of an analogous mixture of vesicles containing either conjugate ("D" + "A" vesicles). In the dark the two preparations exhibited undistinguishable spectra, indicating the absence of energy transfer. After bleaching, however, dansyl fluorescence decreased and fluorescein fluorescence increased in "DA" vesicles relative to "D" + "A" vesicles. Thus, energy transfer between D-Rho and F-Rho was detected after bleaching.

The transfer efficiency (calculated from the sensitized fluorescence of fluorescein) was studied as a function of the lipid to protein ratio and the mole fraction of F-Rho in the vesicles. The results for dark and bleached vesicles are shown in Figure 9. Dilution of rhodopsin with lipid (Figure 9A) at constant donor to acceptor ratio and mole fraction of unlabeled rhodopsin did not affect the transfer efficiency. The apparent energy transfer in the dark was insignificant, whereas after bleaching it was ~15% and virtually constant in the range 100–400 phospholipid molecules per rhodopsin. Figure 9B shows that at a constant lipid to protein ratio the efficiency of energy transfer was highly sensitive to the mole fraction of F-Rho in bleached vesicles. As in the case of P-Rho and B-Rho these results clearly indicate that the protein was aggregated in these vesicles. In the dark, however, energy transfer remained insignificant even at a high acceptor concentration. The absence of energy transfer in the dark could be due to the protein being either monomeric or aggregated in a way which restricted energy transfer. Analysis of these results in terms of the model of randomly distributed rhodopsin monomers (plots similar to those in Figure 7) led to consistent deviations of the experimental values from the theoretical curves: in the dark, the experimental values of transfer efficiency were lower than the values predicted by the model. Bleached vesicles, on the other hand, exhibited more energy transfer than predicted by the model (results are not shown). These results are consistent with a situation where rhodopsin is aggregated within the membrane with the sites labeled with dansyl and fluorescein well separated in the dark but closer in bleached vesicles. Considering a dimer, the labeled amino groups were at least 61 Å apart in the dark and approximately 44 Å apart after bleaching.

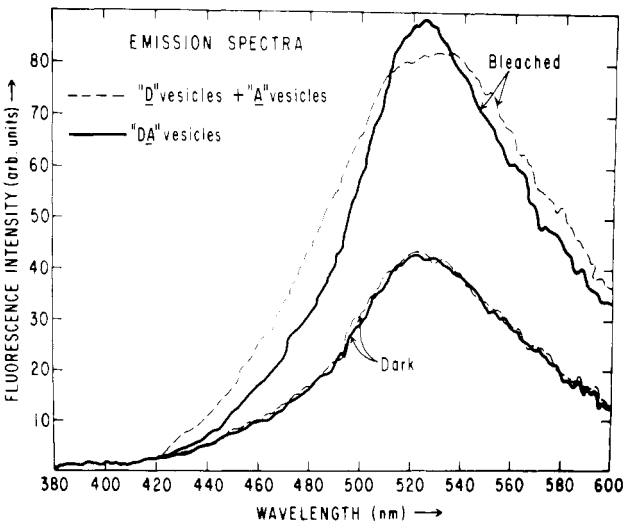

FIGURE 8: Fluorescence emission spectra of vesicles reconstituted with D-Rho and F-Rho. Emission spectra of vesicles containing both derivatives (—) and an analogous mixture of vesicles containing either conjugate (---). The total concentration of rhodopsin was 0.2 mg/mL. The ratio between D-Rho and F-Rho was 2:1, and the lipid to protein ratio was 6:1 (w/v). Excitation was at 330 nm using slits of 1 and 20 nm for excitation and emission, respectively.

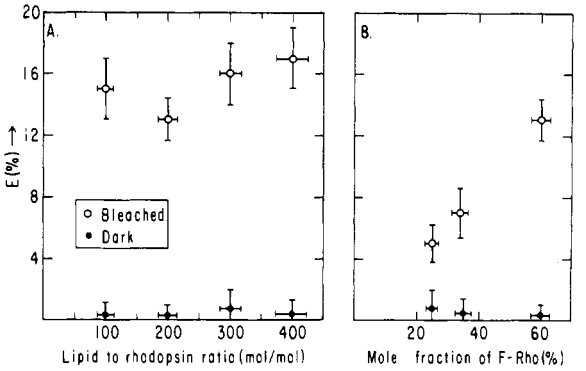

FIGURE 9: Dependence of the efficiency of energy transfer between D-Rho and F-Rho on light and on vesicle composition. The experimental values are the average of three measurements. The total rhodopsin concentration was 0.2 mg/mL. (A) Dependence on lipid to protein ratio. The ratio of D-Rho to F-Rho was 0.5:1. The mole fraction of unlabeled rhodopsin was 0.15. (B) Dependence on donor to acceptor ratio. The mole fraction of F-Rho was varied by changing the ratio of D-Rho to F-Rho in the range 2.5–0.5:1. The lipid to protein ratio was 4:1 (w/w), and the mole fraction of unlabeled rhodopsin was 0.15.

Table II: Energy Transfer between Fluorescent-Labeled Rhodopsin Monomers[a]

system	donor/acceptor couple	energy transfer[b]		aggregation		distance[e] (Å)	
		dark	bleached	dark	bleached	dark	bleached
micellar solution	P-Rho/B-Rho	−	+	−	+	>60	~40
	D-Rho/F-Rho	−	−	−	+[c]	>59	>68
reconstituted phospholipid vesicles	P-Rho/B-Rho	+	+	+	+	~40	~40
	D-Rho/F-Rho	−	+	+[d]	+	>60	~44

[a] See text for experimental details. [b] The symbols (−) and (+) denote the absence or the occurrence of energy transfer, respectively. [c] Detected as an increase in light scattering. [d] See text. [e] Distance between labeled sites assuming a dimer and employing the experimental parameters in Figures 3, 4, 6A, and 9A (see text for details).

It is worth noting that the sites labeled with dansyl and fluorescein, which in reconstituted vesicles increased in proximity after bleaching, were separated in OG by a distance larger than 68 Å. Since rhodopsin regeneration occurred in the vesicles but not in OG, it is conceivable that these discrete local interactions between bleached rhodopsin (opsin) monomers are required to maintain the bleached protein in a conformation capable of regeneration [see also McCaslin & Tanford (1981)].

The results of this study are summarized in Table II. The structural basis for the different behavior of rhodopsin with the two sets of probes is unclear. Further work is necessary to determine whether perturbations induced by labeling or variable sensitivity of the probes arising from their location in the protein are responsible for the observed effects.

These studies and those described in the preceding paper (Borochov-Neori & Montal, 1983) reveal several new structural features of rhodopsin in reconstituted vesicles: (1) Intramolecular fluorescence energy transfer measurements indicated that all the labeled sites of rhodopsin were far from the endogenous chromophore (>30 Å) [see Figure 6 in Borochov-Neori & Montal (1983)]. Thus, the monitored events were likely to occur in domains confined to the membrane surface. (2) Intermolecular energy transfer measurements demonstrated that rhodopsin–rhodopsin interactions within the membrane occurred both in the dark and after exposure to light. The effect of bleaching was to change the interactions between discrete domains of rhodopsin monomers, bringing increased proximity between certain sites. In the dark, these sites were probably exposed at the exterior of the rhodopsin aggregate. In the bleached state, these sites appear to displace toward the interior of the aggregate and approach each other, thereby becoming buried within the protein assembly. The latter is suggested by their reduced accessibility to amphiphatic quenchers (Borochov-Neori & Montal, 1983). Such light-induced structural changes may provide a mechanism by which a photosensitive ion channel acts: Monomer rotations and displacements within an aggregate of homologous subunits could lead to the opening or the closing of a hollow channel (cf. Montal, 1979; Unwin & Zampighi, 1980). The salient finding that rhodopsin in reconstituted vesicles occurs as an aggregate strongly suggests that similar entities may exist in photoreceptor membranes as structural assemblies that provide a larger surface for the interaction between rhodopsin and the GTP-binding protein. In fact, recent optical diffraction analysis of micrographs of negatively stained two-dimensional rhodopsin crystals show them to be associated as dimers (Corless et al., 1982). Moreover, the observed conformational changes in rhodopsin domains exposed at the membrane surface may be involved in the mechanism of enzyme photoactivation which is initiated by rhodopsin (Stryer et al., 1981; Bownds, 1981; Kuhn et al., 1981). While recognizing that certain untested assumptions are implied, we proceed to suggest that the measured changes in rhodopsin–rhodopsin interactions and in rhodopsin conformation after photoisomerization may be crucial events in the physiological regulation of the rod disk membrane permeability and of the activity of the cyclic nucleotide enzymes. These suggestions are testable.

The observations described here were obtained with purified rhodopsin reconstituted in phospholipid vesicles. In such preparations rhodopsin was shown to retain many of the known properties it exhibits in the native retinal ROS membranes. However, the possibility that the observed aggregation may be a consequence of the experimental approach cannot, at present, be discarded.

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Interactions of Thyroid Hormone, Growth Hormone, and High Carbohydrate, Fat-Free Diet in Regulating Several Rat Liver Messenger Ribonucleic Acid Species†

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ABSTRACT: Pleiotropic effects of thyroid hormone, growth hormone, and high carbohydrate, fat-free diet in regulating the intracellular levels of rat liver mRNA were examined. Total hepatic poly(A)-containing RNA, isolated from appropriately treated animals, was translated in the mRNA-dependent reticulocyte lysate system. Two-dimensional gel electrophoresis of the ^{35}S-labeled translational products allowed separation of approximately 200 different mRNA-encoded products. Computerized video densitometry was utilized to quantitate the relative proportion of any individual product in the total population. Previous work [Seelig, S., Liaw, C., Towle, H. C., & Oppenheimer, J. H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4733–4737] has shown that administration of triiodothyronine (T_3) to hypothyroid rats leads to specific alterations in approximately 8% of the mRNA species detected by this technique. We now show that a subset of these T_3-responsive mRNA species is indirectly affected by T_3 through its action on pituitary production of growth hormone. Administration of growth hormone to hypothyroid rats leads to specific alterations in the levels of eight different mRNA species. The relative levels of four mRNA species are diminished, and those of four mRNA species are increased by growth hormone. Thus, this hormone which presumably binds to a membrane receptor can act at a pretranslational level to augment and attenuate several specific mRNA sequences. For two of these mRNA species, the combined action of T_3 and growth hormone is necessary for maintenance of normal levels. In addition, switching rats from standard laboratory chow to a diet high in carbohydrate and fat free resulted in changes in the levels of 10 different mRNA-encoded products. Interestingly, all but one of these mRNA species was also influenced by the thyroidal status of the animal. Thus, a high degree of overlap exists between mRNA species regulated by T_3 and a high carbohydrate, fat-free diet. Most T_3-responsive mRNA species appear to be regulated in a complex multifactorial pathway.

Many, if not all, of the cellular actions of thyroid hormone are initiated by binding of triiodothyronine (T_3)[1] to a specific chromatin-bound receptor in target cells [for reviews, see Samuels (1978), Latham et al. (1978), and Oppenheimer (1979)]. This hormone–receptor interaction is postulated to cause specific alterations in the nuclear production of RNA (Tata & Widnell, 1966) and consequently in the pattern of proteins synthesized in the tissue. In no case has the expression of a gene product been definitively demonstrated to be directly responsive to hormone–receptor binding; however, the finding of increased mRNA levels for at least three specific proteins induced by T_3 provides correlative evidence in favor of this pathway. Thus, the inductions of growth hormone in rat pituitary tumor cell lines (Martial et al., 1977a; Seo et al., 1977; Shapiro et al., 1978), α_{2u}-globulin in rat liver (Roy et al., 1976; Kurtz et al., 1976), and malic enzyme in avian and rat liver (Towle et al., 1981; Siddiqui et al., 1981) are all accompanied by parallel changes in the cellular levels of mRNA encoding these proteins.

Recently we have examined more fully the qualitative and quantitative range of the effects of T_3 on the mRNA popu-

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[1] Abbreviations: T_3, L-triiodothyronine; CHO diet, high carbohydrate, fat-free diet; Gdn·HCl, guanidine hydrochloride.

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