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Energy Transfer in Rhodopsin, *N*-Retinyl-Opsin, and Rod Outer Segments

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ABSTRACT ***N*-retinyl, the chromophore of bleached and reduced rhodopsin, *N*-retinyl-opsin, was used as a covalently attached fluorescence probe to examine the structure of *N*-retinyl-opsin and the rod outer segment. The efficiency of energy transfer from the protein part of *N*-retinyl-opsin to the chromophore is 12 ± 5%. It is argued that this implies that the *N*-retinyl-opsin molecule is asymmetrical. Kropf has estimated the efficiency of energy transfer from the protein to the chromophore in native rhodopsin to be about 50%. This difference of efficiencies seems to imply a large movement of the chromophore away from the tryptophans of the opsin after rhodopsin is bleached.**

From excitation spectrum measurements, it has been found that light absorbed by the protein of the rod outer segments has more action in sensitizing the fluorescence of the chromophore than does light absorbed by the protein part of pure *N*-retinyl-opsin. Thus, some other tryptophans or tyrosines in either another *N*-retinyl-opsin molecule or another protein must be close enough (about 28 Å) to the chromophore to transfer energy to it. Measurements of the polarization of the fluorescence of the chromophore suggest, however, that the chromophores of neighboring *N*-retinyl-opsin molecules are more than 20 Å apart. Moreover, these neighboring chromophores do not transfer energy to each other, tending to rule out any clustering of chromophores of different *N*-retinyl-opsin molecules and suggesting that rhodopsin chromophores do not transfer energy to each other.

The mechanism of visual excitation can not be understood without a fuller understanding of the structure and function of the proteins in the membranes of the photoreceptors, particularly rhodopsin. The significance of rhodopsin goes beyond the immediate problems of visual excitation. Of the best-known membrane proteins and membrane structures, rhodopsin and the rod outer segments (r.o.s.) are among the most suitable for extensive study. Structurally, rhodopsin is quite important since it constitutes about 50% of the protein of the bovine r.o.s. (unpublished data). Functionally, it is important since it is the pigment that absorbs the light used in vision. Rhodopsin can be isolated in fairly large quantities in a pure form, and the purity of rhodopsin and r.o.s. samples can be assayed (1). Much is known already about the photochemistry of rhodopsin; it is almost certain that the first step in visual excitation is the isomerization of the chromophore of rhodopsin, retinal, from the 11-*cis* to the all-*trans* form (2). Because rhodopsin is arranged in a systematic array in the r.o.s., much work utilizing the techniques of both electron microscopy and x-ray diffraction has been possible (3, 4). Because the rod outer segment is an electrically active membrane (5), a number of electrophysiological findings may prove useful to limit hypotheses on what possible changes in the r.o.s. may account for visual excitation.

I would like to present evidence here on the structure of the native rhodopsin molecule, the bleached rhodopsin molecule, and the r.o.s. This evidence is based on measurements of the polarization of, and the excitation spectrum for, the fluorescence of the chromophore of *N*-retinyl-opsin. In my studies, I have used *N*-retinyl-opsin, rather than rhodopsin, because its chromophore has a much higher fluorescence yield than the chromophore of rhodopsin. Bownds and Wald (6, 7) and Ahktar *et al.* (8) have shown that the chromophore of rhodopsin can be reduced by $NaBH_4$ while the rhodopsin is being bleached. They have also shown that after reduction the chromophore is still covalently attached to the protein, and have argued convincingly that it probably is still attached to its original binding site. These properties make *N*-retinyl an ideal fluorescence probe.

I have measured the fluorescence excitation spectrum of purified *N*-retinyl-opsin in solution and have compared the efficiency of light absorbed by the protein in exciting the chromophore fluorescence with the efficiency of light absorbed by the protein in bleaching native rhodopsin. Furthermore, I have measured the fluorescence excitation spectrum for exciting *N*-retinyl fluorescence in sonicated but unsolubilized r.o.s. I have also measured the fluorescence polarization of the reduced chromophore of rhodopsin to determine whether there is transfer of energy from one *N*-retinyl-opsin molecule to another.

The theoretical and experimental bases of energy transfer have recently been reviewed (9). The efficiency of energy transfer, E, is predicted by Förster's theory to be dependent on the sixth power of the distance from the donor to the energy acceptor, R.

$$E = \frac{R_0^6}{R_0^6 + R^6} \qquad (1)$$

R_0 can be calculated from $R_0 = 8.8 \cdot 10^{-25}\, \phi_D \cdot K^2 J$, where J is the spectral overlap integral, ϕ_D is the quantum yield of the donor fluorescence, and K^2 is the dipole–dipole orientation factor.

Theories to explain the depolarization of fluorescence due to energy transfer are not as well-developed (10). However, a detailed examination of the theory is not necessary, since the data to be presented in this paper give straightforward results. Briefly, there are two mechanisms that can reduce the extreme value of the polarization of the fluorescence: rotation of the molecule during the excited state, and transfer of the energy before emission to another molecule whose transition

Abbreviation: r.o.s., rod outer segments.

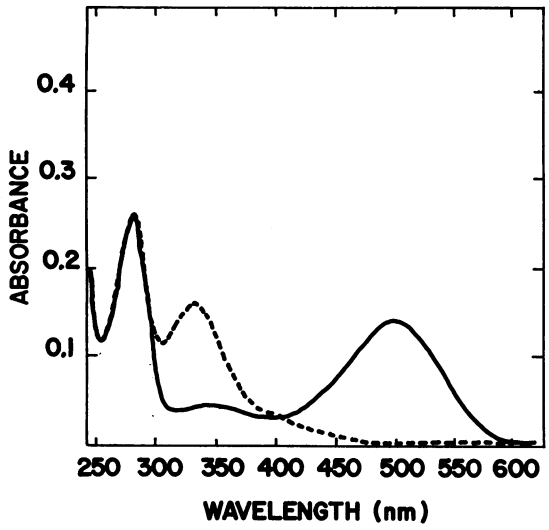

FIG. 1. The absorption spectrum of pure rhodopsin before (——) and after (- - -) bleaching and reduction.

moment is not parallel to that of the original molecule. It will be argued below that no large amount of rotation occurs during the lifetime of the fluorescence. Thus, as long as the chromophores are not parallel, the difference in the polarization of the fluorescence between the transferring and the nontransferring situation can be used to predict the number of transfers of an absorbed quantum before elimination by such processes as fluorescence.

MATERIALS AND METHODS

Rod outer segments were prepared by a slight modification of the method of Shichi *et al.* (1, 20). Purification was by a series of flotations of the r.o.s. in 37% sucrose, and subsequent washings with buffer. At any step during the purification procedure, the purity of the r.o.s. could easily be assayed by solubilizing a drop of the r.o.s. in a 1.5% solution of the detergent Ammonyx LO (Onyx Chemical Co., Jersey City, N.J.) in 0.067 M sodium phosphate buffer, pH = 7. The Ammonyx LO seems to completely dissolve the membrane so that the absorption spectrum of the rods can be measured without interference from scattering. The criterion of purity for both the rhodopsin and the r.o.s. used here is the ratio of the absorption at 280 nm, due to the tyrosines and tryptophans of the protein, to the absorption at 500 nm, due to the chromophore. The lower is this ratio, the purer is the sample, because less extraneous protein is present. As far as I know, this is the only reliable criterion for the purity of r.o.s. My best preparations of r.o.s. have ratios of 3.0, although more typically the ratio is 3.5. After purification, to obtain unsolubilized r.o.s. of good optical quality, the r.o.s. were sonicated for 1 min and then centrifuged in a Spinco centrifuge (SW 65 head) for 2 hr to pellet the larger (more scattering) fragments (1).

From r.o.s. prepared as outlined above, pure rhodopsin is obtained by chromatography of Ammonyx LO-solubilized r.o.s. on a calcium phosphate column (11). The purity (ratio) of the rhodopsin was typically 1.7–1.8, with my best samples having a purity of 1.65.

$NaBH_4$-reduced rhodopsin, *N*-retinyl-opsin, was prepared by the method of Shields *et al.* (12). The fluorescence of the chromophore of *N*-retinyl-opsin has its emission maximum at 470 nm, and its excitation maximum at 340 nm. The fluorescence yield is about 1%. The absorption spectra of rhodopsin and *N*-retinyl-opsin are shown in Fig. 1.

As a control, rhodopsin was first completely bleached with yellow light (tungsten lamp with a Corning 3-68 filter). After the chromophore came off the opsin, it was reduced with $NaBH_4$. This yielded what is essentially a mixture of retinol and opsin. In order to demonstrate that the chromophore was no longer bound to the opsin, free retinol was extracted according to the procedure of Bownds.

Procedures identical to these were used to prepare *N*-retinyl-opsin *in situ* in sonicated r.o.s. However, because of scattering problems, as well as increased absorption at 280 nm compared to 330 nm, the 280- and 330-nm absorption bands were not as well separated in the absorption spectrum as in the case of pure *N*-retinyl-opsin. The absorption spectra of unbleached, and bleached and reduced, sonicated r.o.s. are shown in Fig. 2.

For comparison with these sonicated r.o.s., sonicated r.o.s. containing *N*-retinyl-opsin were dispersed in a 1.5% solution of the detergent Ammonyx LO to separate the proteins from each other.

Fluorescence excitation and polarization spectra were measured with a Perkin-Elmer MPF-2A fluorescence spectrophotometer. For correcting fluorescence excitation spectra, a Rhodamine B (3 g/l) quantum counter was used to obtain quantum fluxes on the sample. These values were checked against the fluorescence excitation spectrum of quinine bisulfate.

All absorption spectrum measurements were made with a Cary 14 recording spectrofluorometer.

RESULTS

The fluorescence excitation spectrum of *N*-retinyl-opsin is shown in Fig. 3. It is superimposed on the excitation spectrum

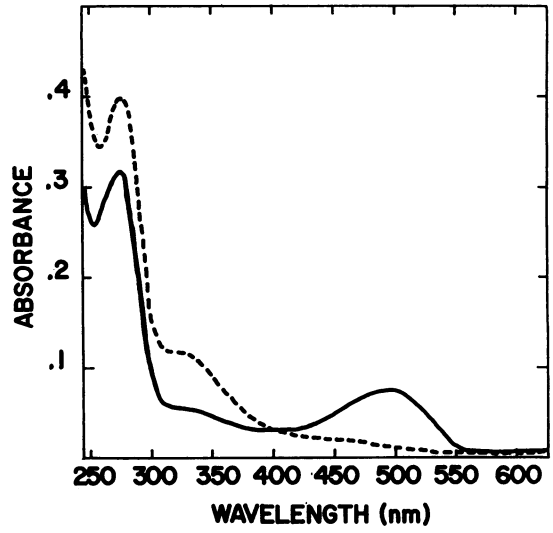

FIG. 2. The absorption spectrum of sonicated rod outer segments before (——) and after (- - -) bleaching and reduction. Scattering has raised the absorbance at 280 nm compared to 500 nm from the value this solution would have when solubilized of A 280/A 500 = 3.4. Moreover, with this sample, as happens quite often, increased scattering after bleaching and reduction has raised the A at 280 nm even more.

of the fluorescence at 470 nm of the control, a solution of retinol and opsin. This excitation spectrum has been moved along the wavelength axis about 4 nm, to compensate for a small difference in the absorption spectra of free and covalently bound retinol. Thus, two samples with reduced chromophores are compared, one attached to the protein opsin and the other not. As is clearly seen in Fig. 3, 280-nm light is more effective in exciting the chromophore fluorescence of *N*-retinyl-opsin than in exciting the fluorescence of retinol from the control. This difference is presumably due to energy transfer from the protein to the chromophore. When the fluorescence excitation spectrum is corrected, it is found that the efficiency of energy transfer from the tyrosines and tryptophans of *N*-retinyl-opsin to the chromophore is 12 ± 5%. When the fluorescence yield of the protein is examined, it is possible to show that not all of the energy absorbed by the tyrosines and tryptophans is quenched by the chromophore. The yield of the tryptophans of *N*-retinyl-opsin is not zero, which would indicate total transfer, but rather about 0.14. The yields of most tryptophan-containing proteins vary from 0.01 to 0.20 (13).

A striking phenomenon is observed when the fluorescence excitation spectrum of *N*-retinyl-opsin in sonicated, but unsolubilized, r.o.s. is compared to a similar preparation that has been totally solubilized and dispersed after *N*-retinyl-opsin has been formed, as shown in Fig. 4. The fluorescence excitation spectrum of the dispersed mixture of r.o.s. proteins is identical with that for purified *N*-retinyl-opsin, as might be expected. The fluorescence excitation spectra show that the efficiency of energy transfer is 20 ± 5% overall, or 40 ± 10% calculated on a basis of the amount of rhodopsin protein present. These results indicate that in sonicated r.o.s. there is much more fluorescence excited by light absorbed by the tyrosines and tryptophans in r.o.s. than by these same residues in pure *N*-retinyl-opsin.

To see if there is transfer of energy from one chromophore to another in the r.o.s., I measured the polarization of the 470-nm fluorescence from *N*-retinyl-opsin, with an exciting wavelength of 340 nm. I found that this fluorescence is highly polarized, with $p = 0.35$. This is quite close to the maximum theoretical value of 0.5, and actually higher than that of solubilized *N*-retinyl-opsin. Hence, there is little or no depolarization of the fluorescence.

DISCUSSION

Rhodopsin

These measurements of the efficiency of energy transfer between the tryptophans and tyrosines of the protein moiety of *N*-retinyl-opsin and its reduced chromophore, *N*-retinyl, can be compared with two quantities. One is the efficiency of energy transfer from the tyrosines and tryptophans to the chromophore in native rhodopsin. Kropf (14) found that the efficiency of isomerization (bleaching) of the chromophore for light absorbed at 280 nm, when compared with light absorbed at 500 nm, is 40–50%. The higher figure obtains if the corrected extinction coefficient of pure rhodopsin of Shichi *et al.* (11) is used. This compares with an efficiency of 12% for the sensitization of the chromophore fluorescence in bleached reduced rhodopsin (*N*-retinyl-opsin).

The second quantity that can be compared with the measured efficiency is the efficiency predicted by Förster's theory. In Eq. 1, the efficiency, E, is dependent on R_0, the parameter representing the distance between donor and acceptor when efficiency of energy transfer is 50%. To calculate R_0, the donor fluorescence yield was taken to be 0.04, the measured fluorescence yield of the protein fluorescence of *N*-retinyl-opsin. J, the overlap integral, was calculated from the fluorescence spectrum of the tryptophans of pure *N*-retinyl-opsin and the absorption spectrum of the chromophore of *N*-retinyl-opsin, assuming Bownd's value (7) for the extinction coefficient, 50,000 cm^{-1} M^{-1}. Finally, the transition moments of the donor, the tryptophan residues, were assumed to take all positions with respect to the fixed chromophore. This assumption can be made because I have found that when the substrate is excited with vertically polarized light, the fluorescence from the tryptophans is quite depolarized, indicating

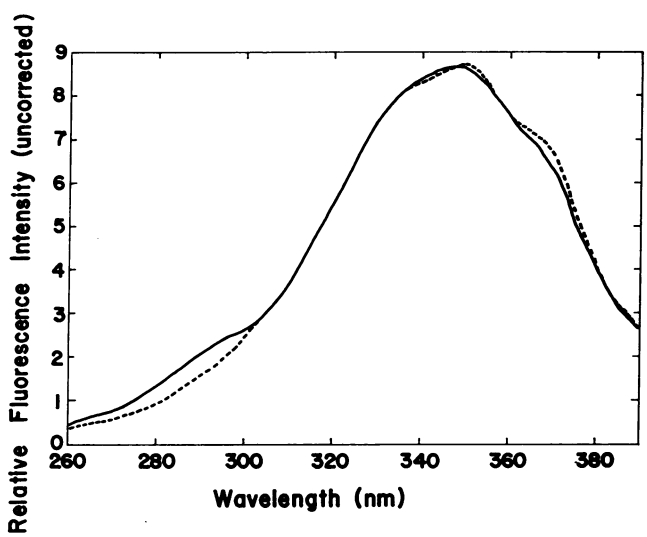

FIG. 3. Fluorescence excitation spectrum of *N*-retinyl-opsin (——) and retinol + opsin (- - -). The gain of the photomultiplier was adjusted to give equal fluorescence intensities at about 340 nm. Excitation band width, 6 nm; emission: monochromator at 470 nm with a band width of 8 nm + 430-nm cut-off filter.

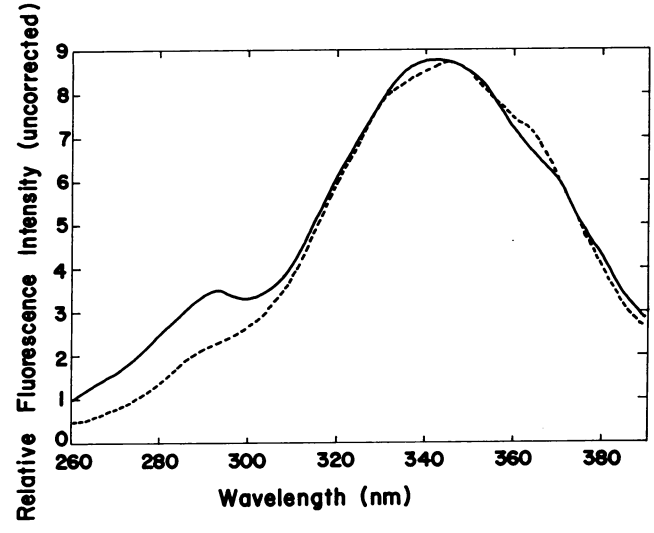

FIG. 4. Fluorescence excitation spectrum of *N*-retinyl in intact rod outer segments (——) and rod outer segments dispersed with Ammonyx LO (- - -). The gain of the photomultiplier was adjusted to give equal fluorescence intensities at about 340 nm. Other conditions as in Fig. 3.

no parallel orientation of the tryptophan transition moments. Under these conditions, $K^2 = 0.475$ (15). With these assumptions, R_0 was found to be 28 Å. In other words, if the tryptophans were clustered together 28 Å from the chromophore, the efficiency of transfer between them and the *N*-retinyl would be 50%. Since the efficiency of transfer is found to be only 10%, there must be a larger distance between most of the tryptophans and the chromophore. If *N*-retinyl-opsin were a spherical protein of 28,000 daltons, then its diameter would be about 41 Å. It is difficult to conceive of a simple model for *N*-retinyl-opsin which, when the tryptophans are distributed randomly throughout a 41 Å sphere, would lead to such a low efficiency of energy transfer. Either the tryptophans must be clustered far from the chromophore or *N*-retinyl-opsin must be a nonspherical, asymmetrical molecule. For rhodopsin, this second possibility was suggested by the x-ray studies of Blaurock and Wilkins (4).

The higher efficiency of energy transfer to the chromophore for unbleached native rhodopsin is also interesting to consider, especially because its R_0 is similar to that of *N*-retinyl-opsin. R_0 is again calculated from the parameters J, ϕ_D, and K^2. In the case of the chromophore of unbleached rhodopsin, there is again considerable overlap of the chromophore absorption and the tryptophan fluorescence (although about four times less than for *N*-retinyl-opsin). The yield, ϕ, is taken as that of pure opsin, about 0.06, and K^2 is again taken a 0.475. The resultant R_0, now slightly smaller than before, is 24 Å. This change in R_0 is in the wrong direction to explain the difference in efficiency. Moreover, because the fluorescence from the tryptophans, when the substrate is excited with vertically polarized light, is strongly depolarized in both native rhodopsin and *N*-retinyl-opsin (20), it is unlikely that a change in the orientation factor K^2 could explain the difference. Rather, the same orientation factor, $K^2 = 0.475$, for a random donor and a nonrandom acceptor (15) should be used. Therefore, the most reasonable explanation of the difference in the efficiency of energy transfer from 50 to 12% is not a change in R_0, but rather a change in the strongly dependent (sixth power) parameter, the distance R. For some distributions in space of the tryptophans, a movement of the chromophore with respect to the tryptophans of as little as 10 Å could explain the difference in efficiencies in the two cases; however, actual quantitation must depend on more work, both theoretical and experimental.

Rod outer segments

The efficiency of energy transfer between the tryptophans and the *N*-retinyl-opsin is higher in sonicated r.o.s. than either in pure *N*-retinyl-opsin or in solubilized r.o.s. This suggests, assuming the same conformation of the *N*-retinyl-opsin molecule *in situ* as in solution, that in sonicated r.o.s. some tryptophans other than the ones in the opsin molecule that bind the fluorescing chromophore must be close enough to the chromophore ($R \simeq 30$ Å) to transfer energy to it. On the other hand, in the detergent-dispersed state, these other tryptophans are far from the chromophore. There are two possible explanations for these extra tryptophans; one is that they are from another *N*-retinyl-opsin molecule and the second is that they are from some other protein. Some constraints can be put on the first possibility by also considering the data for the polarization of fluorescence from the chromophore when the chromophore itself is excited. I indicated that this polarization is quite high ($p = 0.35$), suggesting that there is very little energy transfer between chromophores. This interpretation of the polarization data assumes that the chromophores are not parallel. In fact, single-rod dichroism experiments (16, 17) suggest that the chromophores are not parallel to each other, but are randomly oriented in the plane of the r.o.s. disks. Hence, my results indicate that the chromophores do not transfer energy between each other.

How far apart could two chromophores be, randomly oriented with respect to each other, and energy transfer between them still be detected by our method? If we make generous allowances for measuring errors, the theory of Knox (10) would predict that depolarization effects due to transfer between chromophores at an average distance of greater than $1.5 \times R_0$ should be detectable. Using Hagins and Jenning's (18) data for the absorption and fluorescence of retinol, and assuming these quantities are the same for the *N*-retinyl fluorescence, we find that R_0 is 15 Å, and the distance of separation for randomly arranged chromophores must be greater than 23 Å.

Incidentally, the lack of depolarization also eliminates the possibility of *N*-retinyl chromophore rotation, either by itself or because it is attached to a rotating protein. A protein rotation effect is not expected because a rough calculation predicts the rotational diffusion time to be greater than 15 nsec, while the fluorescence lifetime of the chromophore is probably about 5 nsec (19).

Because their spectral properties are quite similar, the spectral overlap integral J, which appears in the calculation of Förster's R_0, will be similar for *N*-retinyl-opsin and rhodopsin; ϕ, the quantum yield, is less for rhodopsin than *N*-retinyl-opsin, so R_0 will be less for rhodopsin than for *N*-retinyl-opsin. Thus, the lack of energy transfer from one *N*-retinyl-opsin molecule to another in the r.o.s. strongly suggests that there can be no energy transfer between rhodopsin molecules in the r.o.s.

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