Proximity Relationships in Rhodopsin
(visual receptor/energy transfer/fluorescence spectroscopy/excited state/Förster theory)

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ABSTRACT Energy transfer was used as a spectroscopic ruler to deduce proximity relationships within bovine rhodopsin in digitonin solution. Rhodopsin was specifically labeled with fluorescent chromophores at three sites. Site A was alkylated by fluorescent derivatives of iodoacetamide. Site B was labeled by fluorescent disulfides, by a disulfide-sulfhydryl interchange reaction. Sites A and B are sulfhydryl residues. Acridine derivatives were tightly bound to site C by noncovalent interactions. The labeled rhodopsins retained their 500-nm absorption band and were regeneratable after bleaching, suggesting that the fluorescent probes did not grossly perturb the conformation of the protein. A fluorescent chromophore at one of these sites served as the energy donor, while 11-cis retinal was the energy acceptor. The efficiency of singlet–singlet energy transfer was determined from the quantum yield and excited-state lifetime of the donor in the presence and absence of the acceptor. By Förster’s theory, the apparent distances between 11-cis retinal and sites A, B, and C were calculated to be 75, 55, and 48 Å, respectively. Energy transfer measurements on rhodopsin labeled at two of these sites gave these apparent distances: 55 Å for A to B, 32 Å for A to C, and 30 Å for B to C.

These energy transfer studies suggest that the rhodopsin molecule has a length of at least 75 Å. Thus, the rhodopsin molecule appears to be sufficiently long to traverse the disc membrane. Rhodopsin might act as a light-controlled gate.

Rhodopsin, a photoreceptor protein, is an integral part of the disc membranes of vertebrate retinal rod cells (1). The insolubility of rhodopsin in aqueous media has posed difficulties in the purification and characterization of this protein. Indeed, even the molecular weight of rhodopsin is uncertain. Estimates for bovine rhodopsin have ranged from 27,000 to 40,000 (2). Furthermore, little is known about the shape of the molecule or about its interactions with other constituents of the disc membrane.

We report here fluorescence studies that provide information about the shape of the rhodopsin molecule and have implications concerning its role in visual excitation. The basis of this experimental approach is that energy transfer can be used as a spectroscopic ruler to estimate distances between specific sites on a macromolecule (3, 4). In Förster’s theory of dipole–dipole energy transfer (5), the transfer efficiency $E$ is related to the distance $r$ between the donor and acceptor by

$$E = \frac{r^{-4}}{r^{-4} + R_0^{-4}}$$

where $R_0$, the distance in Å at which the transfer efficiency is 50%, is given by

$$R_0 = \left(\frac{9.79 \times 10^6}{K^3} \right)^{\frac{1}{4}}$$

where $K^3$ is the orientation factor for dipole–dipole transfer, $Q_0$ is the quantum yield of the donor in the absence of transfer, and $n$ is the refractive index of the medium. $J$, the spectral overlap integral (in cm$^2$M$^{-1}$), is given by

$$J = \frac{\int f(\lambda) \epsilon(\lambda) \lambda^2 d\lambda}{\int f(\lambda) d\lambda}$$

where $f(\lambda)$ is the fluorescence intensity of the donor at wavelength $\lambda$ and $\epsilon(\lambda)$ is the extinction coefficient of the energy acceptor at that wavelength. Förster’s theory has been tested in well-defined model systems, in which it has been shown that the transfer rate is in fact proportional to $r^{-4}$ and $J$ (4, 6, 7). Singlet–singlet energy transfer has recently been used to deduce distances in various biological macromolecules (8).

We have specifically labeled rhodopsin with fluorescent energy donors at three distinct sites. The donors were chosen so that their fluorescence emission spectra overlapped the 500-nm absorption band of 11-cis retinal, the energy acceptor. The transfer efficiency was determined from measurements of the quantum yield and excited-state lifetime of the fluorescent donor in the presence and absence of acceptor. The energy acceptor was erased by bleaching rhodopsin. The distances obtained from these transfer efficiencies reveal that the rhodopsin molecule has an elongated shape, and suggest that it might traverse the disc membrane and act as a light-controlled gate.

METHODS AND MATERIALS

Rod outer segments, rhodopsin in digitonin solution, and opsin were prepared as described (9). Three kinds of fluorescent labeling reagents were used (Fig. 1): iodoacetamide derivatives, disulfides, and acridine derivatives. $N$-(iodoacetamidoethyl)-1-aminonaphthalene-5-sulfonic acid (1) and its 1, 8 isomer (2) were gifts of Dr. Earl Hudson and Dr. Gregorio Weber (10). 5-Iodoacetamido salicylic acid (3) was synthesized by Haugland (11). Di-(1-dimethylaminonaphthalene-5-sulfonate(dansyl))-L-cystine (4) was obtained from Pierce Chemical Co. Di-fluorescein isothiocarbamido-cystamine (6) was synthesized from cystamine (Aldrich) and fluorescein isothiocyanate (isomer I, Sigma). 9-Hydrazinocarbazide (6) and protoporphyrin (7) were obtained from Eastman Chemicals.

Rhodopsin labeled with I, 2, 3, 6, or 7 was prepared by addition of the fluorescent labeling reagent in the dark to a suspension of rod outer segments or to rhodopsin in digitonin solution in 0.067 M sodium phosphate buffer (pH 6.5). The
molar ratio of label to rhodopsin ranged from 10 to 40 for labels 1, 2, and 3. For labels 6 and 7, this ratio was 2. The reaction mixture was incubated for 1–2 hr at room temperature. The labeled rod outer segments were washed 5 times with buffer and then extracted with a 2% digitonin solution. The fluorescent-labeled rhodopsin in digitonin solution was purified by passage through a Sephadex G-25 column containing 0.067 M sodium phosphate buffer (pH 6.5). The fluorescence properties of rhodopsin labeled with these reagents in situ in the disc membrane appeared to be the same as that of rhodopsin labeled in digitonin solution. In contrast, 4 and 6 labeled rhodopsin only in digitonin solution. The pH of the buffer was 8.0, and the molar ratio of label to rhodopsin was 10. After incubation for 1–2 hr at room temperature, the mixture was passed through a Sephadex G-25 column equilibrated with pH 6.5 buffer.

Fluorescence emission spectra were measured on a recording spectrofluorimeter (12), and corrected for the variation with wavelength in the sensitivity of the detection system. Quantum yields were determined with 1-anilinonaphthalene-8-sulfonate in ethanol as a standard of quantum yield 0.37 (12). Peak absorbances were less than 0.05 to obviate inner-filter effects. There was no detectable bleaching of the rhodopsin in the course of the absorption or fluorescence measurements. Nanosecond emission kinetics were measured with a single-photon counting apparatus (13). Excited-state lifetimes (which took into account the finite duration of the light pulse) were reproducible to within 0.1 nsec. Corning glass filters and interference filters were used to select appropriate excitation and emission wavelengths. All spectral measurements were performed at 22 ± 1°C.

**FIG. 1.** Fluorescent labeling reagents.

**RESULTS**

**Fluorescent labeling of rhodopsin**

The stoichiometry of labeling indicated that the probes were located at specific sites, designated A, B, and C. The ratio of fluorescent probe to 11-cis retinal in the labeled rhodopsins was 0.8 for site A, 0.98 for site B, and 1.1 for site C. Prolonged incubation (2–3 days) at 4°C with a large excess (more than 40-fold) of fluorescent reagent did not significantly alter the stoichiometry of attachment of labels 1 through 5. However, binding at additional sites was observed when a 10-fold excess of 6 and 7 was added to rhodopsin.

Sodium dodecyl sulfate–acylamide gel electrophoresis (14) showed that the fluorescent labels at sites A and B were covalently attached to rhodopsin, whereas the binding of probes to site C was not covalent, as anticipated. However, rhodopsin has high affinity for 6 and 7. These fluorescent probes remained bound to rhodopsin after extensive washing of labeled rod outer segments, and also after gel filtration of a digitonin solution of labeled rhodopsin.

Sites A and B are sulfhydryl residues. Amino-acid analysis of an acid hydrolysate of rhodopsin labeled at site A yielded 0.89 residue of S-carboxymethylcysteine per retinal. A 5,5’-dithio-bis-(2-nitrobenzoic acid) titration (15) of a digitonin solution of rhodopsin at pH 8.0 in the dark showed one reactive sulfhydryl residue per retinal. Rhodopsin labeled at site A gave nearly the same value, whereas rhodopsin labeled at site B had less than 0.1 sulfhydryl residue available for reaction with this reagent. Thus, the sulfhydryl at site A is not titratable with dithio-bis-(nitrobenzoate), in contrast to the one at site B. These sulfhydryls also differ in that intact disc
membranes did not react with this sulphydryl reagent and could not be labeled at site B, but could be labeled at site A.

The insertion of fluorescent probes did not appear to grossly alter the conformation of rhodopsin. The 500-nm absorption band of the native protein was preserved in the labeled rhodopsins, which also were regenerable after bleaching.

Energy transfer from sites A, B, and C to 11-cis retinal

The fluorescent probes at the three sites are suitable energy donors to 11-cis retinal because their emission spectra overlap the intense 500-nm absorption band of rhodopsin, as shown in Fig. 2 for label 1. After bleaching, there is no energy transfer because the spectral overlap is negligible (Fig. 2). The transfer efficiency $E$ was determined from measurements of the quantum yield ($Q$) and the excited-state lifetime ($\tau$) of the energy donor in the presence and absence of the 11-cis retinal energy acceptor [i.e., in the dark ($d$) and after bleaching ($b$), respectively]:

$$E = 1 - \frac{Q_d}{Q_b} \quad [4]$$

$$E = 1 - \frac{\tau_d}{\tau_b} \quad [5]$$

The emission spectrum of 1 attached to rhodopsin is given in Fig. 3. The fluorescence intensity increased on bleaching, showing that there was energy transfer from 1 to 11-cis retinal. Also, there was a single excited-state lifetime, which increased from 19.4 nsec (Fig. 4) to 21.3 nsec after bleaching. From Eqs. [4] and [5], the transfer efficiency from 1 to 11-cis retinal is calculated to be 9%. Eqs. [4] and [5] assume that the local conformation of the energy donor is not altered by bleaching. This assumption was validated by the finding that the emission spectrum of each of the energy donors was the same before and after bleaching (Fig. 3). Furthermore, nanosecond emission anisotropy measurements showed that the rotational motions of the energy donor were unaffected by bleaching (Wu and Stryer, to be published).

The quantum yield and excited-state lifetime data for energy donors 1 through 7 before and after bleaching are given in Table 1. The quantum yield ratios are in excellent agreement with the corresponding excited lifetime ratios, indicative of a homogeneous population of donor-acceptor pairs. The exception is probe $\theta$, for which these ratios differ significantly. Also, the nanosecond emission kinetics of $\theta$, in contrast to the other probes, was a sum of two exponential decays, in which the minor component had an amplitude of about 10%. Thus, it can be concluded that a small fraction of $\theta$ occupied a site other than C. All of the other fluorescent probes appeared to be located at single sites.

Energy transfer between sites A, B, and C

Rhodopsin was labeled at two sites to obtain estimates of the distances between sites A, B, and C. Transfer efficiencies were determined by measurement of the excited-state lifetimes of the energy donor in the presence and absence of the energy acceptor. Measurements were made on bleached samples to eliminate energy transfer to 11-cis retinal. Rhodopsin was labeled with 1 at site A. An aliquot was further labeled with $\delta$ at site B. The excited-state lifetime of 1 in the bleached single-labeled protein was 21.3 nsec, whereas in the bleached double-labeled protein it was 21.1 nsec. The ratio of these lifetimes is 0.1, and so the transfer efficiency is 90%. Energy transfer between sites B and C and between sites A and C was similarly measured (Table 2).

Distance estimates

The distance $r$ between an energy donor and acceptor can be calculated from Eq. [1] from the observed transfer efficiency, if $R_0$ is known. $R_0$ depends on $J$, $Q$, $K^2$, and $n$, as given in Eq. [2]. $J$ and $Q_0$ are experimentally accessible. Though $n$ cannot be measured directly, there is relatively little uncertainty as to its value, which we assume to be 1.4. The problem arises in assigning a value to the orientation factor $K^2$. If the relative orientation of donor-acceptor pairs is completely randomized...
during the excited-state lifetime, \( K^2 = 7/9 \). We define \( R_0^* \) as the calculated distance at which the transfer efficiency is 50%, using the experimentally observed values of \( J \) and \( Q_0 \) and assuming that \( n = 1.4 \) and \( K^2 = 7/9 \). An apparent distance \( r' \) can then be calculated from \( R_0^* \) and the observed transfer efficiency.

\[
r' = \frac{R_0^*}{(E^{-1} - 1)^{1/6}} \tag{6}
\]

Apparent distances were calculated in this way (Table 1). Different probes at the same site yielded apparent distances that agreed closely. When the values obtained from different probes at the same site were averaged, the apparent distances between sites A, B, and C and 11-cis retinal are 75, 55, and 48 Å, respectively. Apparent distances between sites A, B, and C were determined from a single transfer efficiency for each pair of sites (Table 2). The observed values are 35 Å from A to B, 32 Å from A to C, and 30 Å from B to C.

Nanosecond emission anisotropy measurements show that the energy donors used in this study have local rotational mobility (Wu and Stryer, to be published). When an energy donor has complete rotational freedom and an energy acceptor is fixed, \( K^2 \) can range from \( 1/3 \) to \( 4/3 \), depending on the angle between the transition moment of the acceptor and the donor–acceptor separation vector. Energy donor \( J \) rotates over an angle of the order of 30 degrees within 5 nsec, which probably suffices to make \( K^2 \) greater than \( 1/3 \). This conclusion is supported by the finding that the apparent distances obtained with different probes at a site agree closely (Table 1). If the donor–acceptor pairs were held fixed in unique orientations, quite different apparent distances would be expected for probes 1, 2, and 3 at site A, since they would be oriented differently with respect to the rhodopsin molecule. The similarity of the observed apparent distances, namely 75, 77, and 73 Å, indicates that these donor–acceptor pairs have similar orientation factors. For these reasons, we think it likely that the apparent distance between sites A, B, and C and 11-cis retinal closely approximate the actual distances.

**DISCUSSION**

These energy transfer studies suggest that the rhodopsin molecule is at least 75 Å long. Confirmation of this finding by other experimental approaches would be desirable. Our conclusions assume that Förster's theory is quantitatively valid and applicable in our experiments. If rhodopsin were spherical, its diameter would be 45 Å for a molecular weight of 40,000 (or 40 Å for a molecular weight of 28,000). Thus, it is evident that the rhodopsin molecule has an elongated shape (Fig. 5). The width of the disc membrane is known from electron microscopy (16) to be about 75 Å. Thus, rhodopsin appears to be sufficiently long to traverse the disc membrane if it is suitably oriented.

The elongated shape of rhodopsin may be critical for its role in visual excitation. A plausible hypothesis is that rhodopsin acts as a light-controlled gate. If rhodopsin traversed the disc membrane, it could readily serve as a channel for the efflux of ions or transmitter molecules from the intradisc

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**TABLE 1. Energy transfer to 11-cis retinal**

<table>
<thead>
<tr>
<th>Site</th>
<th>Energy donor</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda_d (\text{nm}) )^*</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>B</td>
<td>B</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>( \lambda_f (\text{nm}) )^*</td>
<td>495</td>
<td>350</td>
<td>223</td>
<td>350</td>
<td>495</td>
<td>440</td>
<td>470</td>
<td>512</td>
</tr>
<tr>
<td>( q_d )</td>
<td>0.68</td>
<td>0.28</td>
<td>0.44</td>
<td>0.61</td>
<td>0.12</td>
<td>0.04</td>
<td>0.14</td>
<td>0.79</td>
</tr>
<tr>
<td>( q_f / q_d )</td>
<td>0.75</td>
<td>0.30</td>
<td>0.45</td>
<td>0.95</td>
<td>0.15</td>
<td>0.05</td>
<td>0.18</td>
<td>0.97</td>
</tr>
<tr>
<td>( r_f (\text{nsec}) )</td>
<td>19.4</td>
<td>17.8</td>
<td>2.1</td>
<td>11.5</td>
<td>2.5</td>
<td>3.5</td>
<td>3.6</td>
<td>4.7</td>
</tr>
<tr>
<td>( r_s (\text{nsec}) )</td>
<td>21.3</td>
<td>18.5</td>
<td>2.1</td>
<td>17.9</td>
<td>2.2</td>
<td>4.0</td>
<td>0.88</td>
<td>0.77</td>
</tr>
<tr>
<td>( J \times 10^{14} \text{ (cm}^3\text{ M}^{-1} )</td>
<td>1.84</td>
<td>1.75</td>
<td>0.31</td>
<td>1.29</td>
<td>2.26</td>
<td>1.38</td>
<td>1.87</td>
<td>41</td>
</tr>
<tr>
<td>( R_0^* (\text{Å}) )</td>
<td>51</td>
<td>45</td>
<td>41</td>
<td>52</td>
<td>42</td>
<td>33</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>( E(%) )</td>
<td>9</td>
<td>4</td>
<td>3</td>
<td>36</td>
<td>22</td>
<td>12</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>( r'(\text{Å}) )</td>
<td>75</td>
<td>77</td>
<td>73</td>
<td>57</td>
<td>52</td>
<td>46</td>
<td>49</td>
<td></td>
</tr>
</tbody>
</table>

* \( \lambda_d \) is the long-wavelength excitation maximum of the energy donor and \( \lambda_f \) is its fluorescence emission maximum.

† For energy donor \( S \), \( Q_0 \) refers to the quantum yield of \( S \) attached to opsin.

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**TABLE 2. Energy transfer between chromophores at sites A, B, and C**

<table>
<thead>
<tr>
<th>Distance estimated</th>
<th>A to B</th>
<th>A to C</th>
<th>B to C</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda_d (\text{nm}) )^*</td>
<td>495</td>
<td>470</td>
<td>520</td>
</tr>
<tr>
<td>( \lambda_f (\text{nm}) )^*</td>
<td>495</td>
<td>470</td>
<td>470</td>
</tr>
<tr>
<td>( r_f (\text{nsec}) )</td>
<td>21.3</td>
<td>21.3</td>
<td>17.3</td>
</tr>
<tr>
<td>( r_s (\text{nsec}) )</td>
<td>2.1</td>
<td>1.6</td>
<td>1.3</td>
</tr>
<tr>
<td>( r_f / r_s )</td>
<td>0.1</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>( Q_0 )</td>
<td>0.75</td>
<td>0.75</td>
<td>0.95</td>
</tr>
<tr>
<td>( J \times 10^{14} \text{ (cm}^3\text{ M}^{-1} )</td>
<td>1.60</td>
<td>1.14</td>
<td>0.78</td>
</tr>
<tr>
<td>( R_0^* (\text{Å}) )</td>
<td>51</td>
<td>48</td>
<td>47</td>
</tr>
<tr>
<td>( E(%) )</td>
<td>90</td>
<td>92</td>
<td>93</td>
</tr>
<tr>
<td>( r'(\text{Å}) )</td>
<td>35</td>
<td>32</td>
<td>30</td>
</tr>
</tbody>
</table>

* \( \lambda_d \) is the fluorescence emission maximum of the donor and \( \lambda_f \) is the long-wavelength absorption maximum of the acceptor.

† \( r_f \) and \( r_s \) are the excited-state lifetimes of the donor in the absence and presence of energy acceptor, respectively.

† \( Q_0 \) is the quantum yield of the donor in the absence of the acceptor.
space. The isomerization of 11-cis retinal by light could open such a channel either allosterically or directly.

The clustering of sites A, B, and C in a region that is far from the 11-cis retinal group may not be fortuitous. Indeed, we have found that numerous fluorescent reagents (e.g., dansyl chloride, fluorescein isothiocyanate, and isatoic anhydride) also label sites that are more than 40 Å away from 11-cis retinal. This labeling pattern suggests that the rhodopsin molecule may consist of two distinct domains: a highly hydrophobic one containing the 11-cis retinal site and a relatively hydrophilic region that includes sites A, B, and C (Fig. 5).

Energy transfer studies of rhodopsin in intact disc membranes are in progress. The distance between site A and 11-cis retinal is nearly the same in the intact membrane as in digitonin solution. Thus, rhodopsin probably has an elongated shape in its biological environment, as well as in digitonin solution.

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Fig. 5. A model of the rhodopsin molecule based on the observed proximity relationships.