CONFORMATIONAL ASPECTS OF RHODOPSIN AND RETINAL DISC MEMBRANES

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We have used two experimental approaches – fluorescence spectroscopy and lectin binding – to obtain information about the structure of rhodopsin and its position in retinal disc membranes. Energy transfer was used as a spectroscopic ruler to deduce proximity relationships in rhodopsin and in disc membranes. Concanavalin A and wheat germ agglutinin served as specific macromolecular probes of the accessibility of the carbohydrate unit of rhodopsin in disc membranes. We infer from these studies that: (1) The rhodopsin molecule is at least 75 Å long and has an elongated shape. (2) There may be distinct hydrophobic and hydrophilic domains in rhodopsin. (3) The carbohydrate moiety of rhodopsin is on the surface of the disc membrane, probably only on the external face. (4) The disc membrane has an asymmetric structure. (5) The external surface of the disc membrane is closer to site A (a sulfhydryl residue on rhodopsin) than to 11–cis retinal.

The molecular basis of visual excitation is a challenging and attractive area of inquiry for several reasons (for reviews, see refs. 1–3): (1) Vertebrate retinal rod cells are exquisitely sensitive detectors. A rod cell can be stimulated by a single photon. (2) The sensitivity of a rod cell depends on the ambient light level. Regulatory mechanisms enable rods to function over a wide range of incident light intensity. (3) Appreciable amounts of rhodopsin (~100 mg) can readily be purified. (4) The excitation process can be investigated at different levels of organization: the isolated rhodopsin molecule, disc membrane fragments, intact discs, whole rod outer segments, and the intact retina. (5) The magnitude, timing, and localization of the exciting light pulse can be precisely controlled.

The major functions of a retinal rod cell are compartmentalized in a very distinctive way (4). The outer segment is devoted to photorecption, whereas other parts of the cell are concerned with energy generation, protein synthesis, and communication with other neurons. A mammalian outer segment is typically 2 μm in diameter and 30-μm long. It contains a stack of about 1000 discs. These closed flattened membranous sacs are densely packed with rhodopsin molecules. X-ray diffraction studies have revealed that lipid bilayer regions are major structural elements of disc membranes (5, 6). However, the shape of rhodopsin and its location in the disc membrane have not yet been defined by X-ray studies. Moreover, the insolubility of rhodopsin in aqueous media in the absence of detergents has rendered it difficult to study. Indeed, even its molecular weight has not been unequivocally established. Estimates have ranged from 27,000 to 40,000 daltons (7, 8).

Wald showed that the primary event in visual excitation is the isomerization of the 11-

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cis retinal chromophore of rhodopsin to all-trans retinal (9). Further insight into the mechanism of visual excitation has come from the laboratories of Tomita, Hagins, and Cone (10–12). The next defined event following illumination is a decrease in the permeability of the plasma membrane to sodium ions. This change in Na^+ permeability leads to a hyperpolarization of the plasma membrane. A key question is this: What are the links between the cis-trans isomerization in a rhodopsin molecule (the primary event) and the change in Na^+ permeability of the plasma membrane (the amplified response)? It is evident that the elucidation of this remarkable transduction process will require a knowledge of the detailed structure and dynamics of the disc and plasma membranes of the rod outer segment. We have used two experimental approaches – fluorescence spectroscopy and lectin binding – to obtain information about the structure of rhodopsin and its position in disc membranes. We summarize here some of our previously published work in this regard and present some new experimental findings.

Energy Transfer as a Spectroscopic Ruler

Energy transfer can be used as a spectroscopic ruler to estimate distances in biological macromolecules and assemblies (13). The basis of this experimental approach is that electronic excitation energy can be efficiently transferred between a fluorescent energy donor and a suitable energy acceptor over distances as large as 70 Å. The transfer efficiency is critically dependent on distance. Förster proposed a theory of energy transfer and derived explicit expressions for the rate and efficiency of energy transfer in terms of geometric and spectroscopic variables (14). The transfer efficiency E is related to the distance r between the donor and acceptor by

$$E = r^{-6} / (r^{-6} + R_0^{-6}) \tag{1}$$

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

$$R_0 = (J K^2 Q_0 n^{-4})^{1/6} / (9.79 \times 10^3) \tag{2}$$

where K^2 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, expresses, the overlap of the emission spectrum of the energy donor and the absorption spectrum of the energy acceptor. Förster's theory has been tested in well-defined model systems, where it has been shown that the transfer rate is in fact proportional to r^{-6} and J (15, 16). Energy transfer has recently been used to deduce proximity relationships in a variety of biological macromolecules in addition to rhodopsin: transfer RNA, carboxypeptidase A, adrenocorticotrophic hormone, transferrin, immunoglobulin G, and erythrocyte ghost membranes (17–22).

Proximity Relationships in Rhodopsin

We have used energy transfer as a spectroscopic ruler to deduce proximity relationships in bovine rhodopsin in digitonin solution (23). The first step in these experiments was to specifically label rhodopsin with a fluorescent energy donor. The donors were chosen so that their fluorescence emission spectra overlapped the 500 nm absorption band of 11-cis retinal, the energy acceptor. Three sites were specifically labeled (Table I). Site A, a

Table I. Labeling Reagents

	Fluorescent Probe	Site
1	N–(iodoacetamidoethyl) -1-amino-naphthalene-5-sulfonic acid	A
2	N–(iodoacetamidoethyl)-1-amino-naphthalene-8-sulfonic acid	A
3	5–Iodoacetamido salicylic acid	A
4	Di-dansyl cystine	B
5	Di–fluorescein isothiocarbamido-cystamine	B
6	9–Hydrazinoacridine	C
7	Proflavin	C

sulfhydryl residue, was alkylated by fluorescent derivatives of iodoacetamide. Site B, a different sulfhydryl, was labeled by fluorescent disulfides by a disulfide–sulfhydryl interchange reaction. Acridine derivatives were tightly bound to site C by noncovalent interactions. The insertion of these fluorescent chromophores did not appear to grossly alter the conformation of rhodopsin, as evidenced by the retention of the 500 nm absorption band and of regenerability after bleaching.

The transfer efficiency E was determined from measurements of the quantum yield (Q) and the excited state lifetime (τ) of the energy donor in the presence and absence of 11-cis retinal energy acceptor [i.e., in the dark (d) and after bleaching (b), respectively]:

$$E_Q = 1 - (Q_d / Q_b) \tag{3}$$

$$E_\tau = 1 - (\tau_d / \tau_b) \tag{4}$$

The distance between a donor and an acceptor can be calculated from the transfer efficiency if J, Q_0, K^2, and n are known (eqs. 1 and 2). J. and Q_0 are experimentally accessible, and n can be taken to be 1.4 with relatively little uncertainty. K^2 cannot be measured directly. However, if the donor–acceptor orientation is rapidly and completely randomized, K = 2/3. The nanosecond emission anisotropy for the donors attached to sites A, B, and C indicate that the orientations of these probes are partially randomized during the excited-state lifetime. Furthermore, apparent distances obtained with different probes (hence different orientations) at a given site agree closely (Table II). We therefore think it likely that the apparent distances between 11-cis retinal and sites A, B, and C closely approximate the actual distances.

Table II. Energy Transfer to 11-Cis Retinal in Digitonin–Solubilized Rhodopsin

Energy Donor	Site	E_Q	E_τ	R'_0 (Å)	r' (Å)
1	A	.09	.09	51	75
2	A	.06	.04	45	77
3	A	.03	.04	41	73
4	B	.39	.36	52	57
5	B	.20	.22	42	52
6	C	.20	.12	33	46
7	C	.21	.23	41	49

We found that the apparent distances between sites A, B, and C and 11-cis retinal are 75, 55, and 48 Å, respectively (Table II). Estimates of the distances between sites A, B, and C were obtained from energy transfer measurements of rhodopsin labeled at two of these sites. The apparent distances are 35Å for A to B, 32 Å for A to C, and 30 Å for B to C. The observed apparent distance between site A and 11-cis retinal shows that the rhodopsin molecule is at least 75 Å long. If rhodopsin were sperical, its diameter would be 45 Å for a molecular weight of 40,000 daltons (or 40 Å for a molecular weight of 28,000 daltons.) Thus, our energy transfer results reveal that the rhodopsin molecule has an elongated shape. The clustering sites A, B, and C in a region that is far from 11-cis retinal may not be fortuitous. This labeling pattern suggests that the rhodopsin molecule may consist of a highly hydrophobic domain (containing 11-cis retinal) and a relatively hydrophilic one (containing sites A, B, and C). A model of rhodopsin based on these proximity relationships (measured in digitonin solution) is shown in Fig. 1.

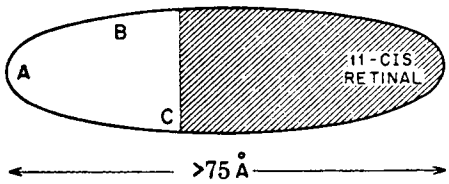

Fig. 1. A model of the rhodopsin molecule based on the observed proximity relationships in digitonin solution (from ref. 23).

Proximity Relationships in Disc Membranes

We then turned from detergent-solubilized rhodopsin to rhodopsin in its native biological environment. Energy transfer and lectin binding studies were carried out to determine the length of the rhodopsin molecule and its orientation in the disc membrane. We summarize here our preliminary findings. We found that site A can be labeled in the intact disc membrane, whereas site B is unreactive. The transfer efficiency between energy donor 1 or 2 at site A and 11-cis retinal was less than 5%. These results showed that the apparent distance between site A and 11-cis retinal in the disc membrane is greater than 75 Å. Thus, the rhodopsin molecule has an elongated shape in the disc membrane, as in digitonin solution.

The external surface of the disc membrane was labeled to gain information about the orientation of rhodopsin. Specifically, we wanted to determine whether the external surface is closer to site A or to 11-cis retinal. Pyridoxal phosphate and fluorescamine were used to label the amino groups of phosphatidyl ethanolamine and phosphatidyl serine molecules. These reagents reacted almost entirely with the amino groups of phospholipids rather than of rhodopsin when the extent of labeling was low ($\sim$ 1 per retinal). The Schiff base formed on addition of pyridoxal phosphate was reduced by $NaBH_4$ to give a fluorescent pyridoxamine derivative. Pyridoxal phosphate does not readily traverse biological membranes (24). Unreacted fluorescamine in aqueous solution is hydrolyzed within a few seconds (25). We therefore think it likely that pyridoxal phosphate and fluorescamine labeled only the external surface of the disc membranes. Cytochrome c and fluorescent-labeled lysozyme also were used as probes. These small basic proteins bind to phospholipid molecules of disc membranes primarily by electrostatic interactions, as has been

Table III. Energy Transfer in Disc Membranes

Energy Donor	Energy Acceptor	N*	<E>	R'_0 (Å)	<r' > (Å)
1 at site A	11-cis retinal	1.0	~ 0	47	>75
2 at site A	11-cis retinal	1.0	~ 0	45	>75
1 at site A	Heme of cytochrome c	1.0	0.17	42	53
1 at site A	Fluorescein-lysozyme	1.0	0.25	46	56
Fluorescein-lysozyme	11-cis retinal	1.0	0.06	41	64
Fluorescamine-lysozyme	11-cis retinal	1.0	0.06	43	67
Fluorescamine-lipids	11-cis retinal	1.0	0.03	43	74
Fluorescein-lysozyme	Heme of cytochrome c	0.5	0.13	34	47
Fluorescamine-lipids	Heme of cytochrome c	3.5	0.31	30	35
Pyridoxamine-lipids	Heme of cytochrome c	3.5	0.70	32	25

*N is the number of energy acceptors per rhodopsin. <E> is the mean transfer efficiency calculated from the mean excited-state lifetimes according to eq. 4. <r'> is the mean apparent distance.

found for other membranes (26). At saturation, 3.5 cytochrome c (or lysozyme) molecules are bound for each rhodopsin molecule. These 3.5 cytochrome c molecules would cover a surface area of about 3000 $Å^2$, which is about half of the available phospholipid surface calculated on the basis of 80 lipid molecules per rhodopsin and 70 $Å^2$ area per lipid molecule. The binding constant in 10^{-5} M KCl is 2×10^{-7} for cytochrome c and 6.5×10^{-8} M for lysozyme. The apparent distance between bound fluorescein-lysozyme (0.5 per rhodopsin) and cytochrome c (0.5 per rhodopsin) is 47 Å (Table III). It seems likely from this result and from the stoichiometry at saturation that cytochrome c and lysozyme are bound only to the external surface of the disc membrane.

We found that the apparent distance between site A and a surface probe was about 55 Å, whereas the apparent distance between 11-cis retinal and a surface probe was about 68 Å (Table III). The molar ratio of bound or covalently attached surface probe to rhodopsin was 1:1 in these experiments. Thus, site A is closer to the external surface than is 11-cis retinal. Furthermore, site A is closer to the heme group of bound cytochrome c (or to the fluorescein group of bound labeled lysozyme) than to the 11-cis retinal group on the same rhodopsin molecule. This finding reinforces our previous conclusion that rhodopsin has an elongated shape.

Accessibility of the Carbohydrate Moiety of Rhodopsin

Rhodopsin is known to contain a covalently attached carbohydrate moiety consisting of three N-acetylglucosamine and three mannose residues (27). We have investigated the location of this carbohydrate unit in intact disc membranes by using concanavalin A (Con A) and wheat germ agglutinin as specific macromolecular probes (28). Con A, a lectin from jack beans, specifically binds α-D-glucosyl residues at the nonreducing termini of mono- and polysaccharides. We found that Con A binds tightly to disc membranes. The dissociation constant is 2×10^{-7} M. At saturation, the stoichiometry is 0.96 Con A monomer per retinal. The binding involves the sugar recognition site of Con A since it is competitively inhibited by α -methyl-D-mannoside. Vesicles formed from disc membrane lipids do not bind Con A. The carbohydrate unit of rhodopsin is almost certainly the binding site for

Con A in disc membranes. This conclusion is supported by the finding that rhodopsin solubilized in cetyltrimethylammonium bromide binds to Con A that is covalently attached to agarose. We think it likely that the carbohydrate unit of rhodopsin is located on the external surface of disc membranes where it is accessible for interaction with Con A. We have recently obtained similar results with wheat germ agglutinin, which is specific for N–acetylglucosamine. Con A and wheat germ agglutinin compete in binding to disc membranes. As anticipated, cytochrome c diminishes the binding of Con A to disc membranes.

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