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24. We thank S. Henikoff for helpful discussions and S. Frank for technical assistance. Supported by USDA grants to Z.A. (93-37300-8769) and to J.L.B. (94-37300-0299).

31 May 1996; accepted 23 August 1996

Requirement of Rigid-Body Motion of Transmembrane Helices for Light Activation of Rhodopsin

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Conformational changes are thought to underlie the activation of heterotrimeric GTP-binding protein (G protein)–coupled receptors. Such changes in rhodopsin were explored by construction of double cysteine mutants, each containing one cysteine at the cytoplasmic end of helix C and one cysteine at various positions in the cytoplasmic end of helix F. Magnetic dipolar interactions between spin labels attached to these residues revealed their proximity, and changes in their interaction upon rhodopsin light activation suggested a rigid body movement of helices relative to one another. Disulfide cross-linking of the helices prevented activation of transducin, which suggests the importance of this movement for activation of rhodopsin.

G protein–coupled receptors (GPCRs) form a superfamily that mediates the actions of extracellular signals as diverse as light, odorants, peptide hormones, and neurotransmitters (*1*). Activation of these receptors is assumed to require protein conformational changes. Understanding the nature of these structural changes is central to understanding the molecular mechanism of GPCR activation.

Rhodopsin is one of the best characterized GPCR systems. Secondary structure models have been proposed on the basis of biochemical, biophysical, and mutagenic data (Fig. 1) (*2*), and the location of the membrane-aqueous interfaces have been determined for some of the helices (*3*). A model for the packing of the transmembrane helices has been derived from cryoelectron microscopy (*4*), and schemes for mapping the rhodopsin sequence onto the resolved helices have been proposed (*3, 5*). Site-directed spin labeling (SDSL) studies reveal that isomerization of the 11-*cis*-retinal chromophore by light leads to reorganization of the tertiary contact surfaces of helices C and F, as well as to changes in the structure of cytoplasmic interhelical loops that are known to interact with transducin (*3*). These results were interpreted in terms of a possible rigid body motion of helices C and F upon light activation.

Rhodopsin mutants containing two reactive cysteines have been used together with disulfide–cross-linking and SDSL studies to probe the spatial proximity of Cys^{65}, which replaced His at that position, (helix A) and Cys^{316} (helix G) and their relative movement after photoactivation in rhodopsin (*6*). We used this approach to explore the proximity of the cytoplasmic ends of helices C and F in rhodopsin and light-induced alteration in their conformation. Six double cysteine mutants were constructed in a mutant of rhodopsin in which the native cysteines at positions 140, 316, 322, and 323 were replaced by serine (*7*). In each mutant, the position of one cysteine (Cys^{139}) was kept constant, whereas the location of the second cysteine was varied among amino acid positions 247 through 252 in helix F (Fig. 1). The mutant opsin proteins were expressed in COS-1 cells, regenerated with 11-*cis*-retinal, and purified in dodecyl maltoside (DM) detergent with an immunoaffinity procedure (*8, 9*). All mutants bound 11-*cis*-retinal to form pigments with properties similar to those of native rhodopsin [$\lambda_{max} \approx$ 498 nm and ratio of absorbances at 280 and 500 nm (A_{280}/A_{500}) between 1.6 and 1.8]. Upon illumination with light of wavelength (λ) greater than 496 nm, all showed an absorbance shift to 380 nm and subsequent retinal release characteristic of the native protein (*10*).

The sulfhydryl groups in the double cysteine mutants were derivatized with a methanethiosulfonate spin label (Fig. 2) (*11*). The double cysteine mutants so derivatized were designated as 139R1-248R1 through 139R1-252R1.

Static magnetic dipolar interactions between two nitroxide labels in an unoriented sample lead to spectral line broadening, and thus a decrease in signal intensity, for separations less than about 25 Å. Electron para-

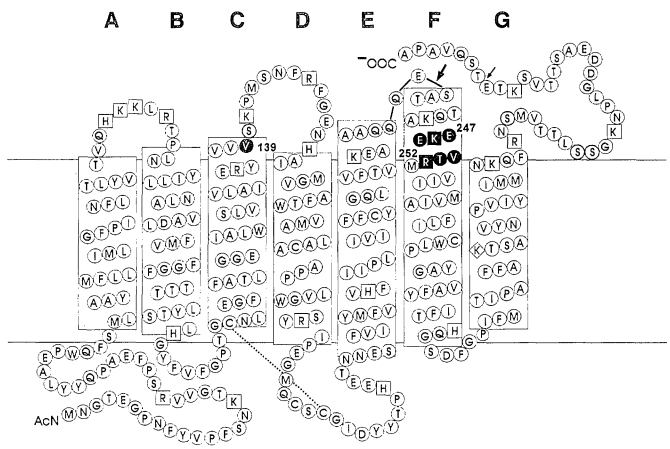

Fig. 1. A secondary structure model of rhodopsin showing the positions of cysteine substitutions (139 and 247 through 252; black residues). In all mutants, the native cysteines Cys^{140}, Cys^{316}, Cys^{322}, and Cys^{323} were substituted with serines (shaded residues). Single-letter amino acid abbreviations are used (*20*). The sites of V-8 proteolysis are indicated by arrows. Note that after V-8 digestion, rhodopsin was cleaved primarily into two large fragments, F1 (~27 kD) and F2 (~13 kD) (*21*). Ac indicates acetyl.

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magnetic resonance spectra of the doubly labeled mutants were obtained in the dark and after photoactivation to produce the metarhodopsin II state. In each case, the sample was frozen after preparation of the desired photochemical state at room temperature. The spectra were recorded in frozen solutions to eliminate effects resulting from differences in nitroxide mobility. Decreases in spectral linewidth were observed after photoactivation for 139R1-248R1, 139R1-251R1, and 139R1-252R1. An increase in linewidth after photoexcitation was observed with 139R1-250R1, whereas relatively little change was seen for 139R1-249R1. No such changes were observed for the single mutants after photoactivation. These changes upon rhodopsin photoactivation indicate an increase in distance between nitroxides in the pairs 139R1-248R1, 139R1-251R1, and 139R1-252R1; a decrease in distance between those in 139R1-250R1; and little change in distance between those in 139R1-249R1 (*12*).

Interspin distances between pairs of nitroxides can be estimated by simulation techniques, and that approach was used here to obtain approximate ranges of interspin distances before and after photobleaching (*13*). In the dark state, the average position of the nitroxide in 139R1 lies in the range of 12 to 14 Å from those in 248R1 and 251R1 and 15 to 20 Å from those in 249R1, 250R1, and 252R1. After photoactivation, the distances from 139R1 to 248R1, 251R1, and 252R1 increased to near the limit for detection of interaction (23 to 25 Å), the distance from 139R1 to 250R1 decreased to 12 to 14 Å, and that from 139R1 to 249R1 changed little. These results demonstrate the proximity of the cytoplasmic ends of helices C and F in the rhodopsin structure and indicate a relative displacement of the helices after photoactivation.

Further support for proximity between cysteine pairs in the double cysteine mutants was obtained from their ability to form disulfide bonds. Oxidation of the mutants was done with the mild oxidizing reagent copper-phenanthroline, followed by V-8 proteolytic digestion and analysis with SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 3). Disulfide cross-links were formed by all double mutants except 139C-252C and the wild-type rod outer segment (ROS) rhodopsin (*14*).

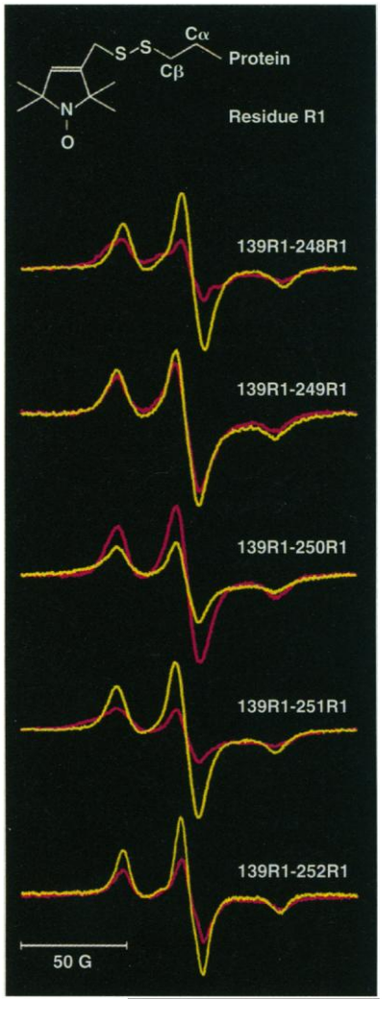

Fig. 2. Rigid lattice (183 K) X-band electron paramagnetic resonance spectra for the spin-labeled double cysteine mutants. Spectra are shown for the dark state (red trace) and for the metarhodopsin II state (yellow trace), produced by photoactivation. Microwave power was 50 μW in a loop-gap resonator with a field modulation of 1 G.

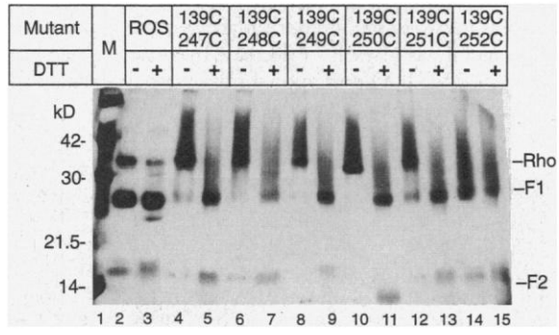

Fig. 3. V-8 proteolysis and SDS-PAGE analysis of oxidized double cysteine rhodopsin mutants. Samples were oxidized with Cu-(phenanthroline)$_3$ (*22*). If linked by a disulfide, the fragments F1 and F2 do not separate on an SDS-PAGE gel. Thus, disulfide formation in a mutant is indicated when, after the V-8 digestion, a single fragment is detected in the absence of DTT and two fragments are present after reduction with DTT. For each mutant, 1 μg of oxidized rhodopsin sample was proteolyzed in the dark (rhodopsin-to-V-8 ratio of 10:1) for 3 hours at room temperature. Before SDS-PAGE (13% gel), samples were solubilized for 1 hour in an SDS gel loading buffer containing either 2.5 mM *N*-ethylmaleimide (even lanes) or 2.5 mM DTT (odd lanes). Proteins were visualized by silver staining (*23*). The smear observed for the mutant rhodopsins results from heterogeneous glycosylation. Molecular size standards are indicated by M.

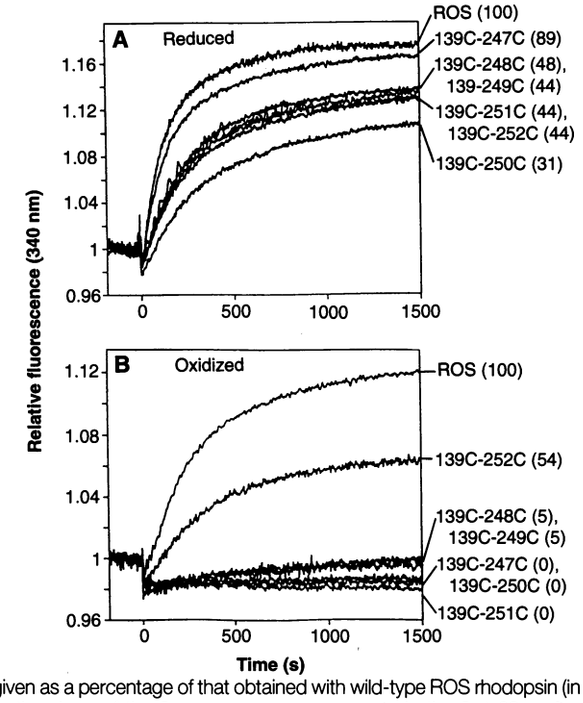

Fig. 4. Transducin activation by double cysteine mutants in the reduced state [1 mM DTT (pH 6)] and after oxidation with copper-phenanthroline. Transducin activation was measured by the rate of complex formation between $G_{T(\alpha)}$ and guanosine 5′-*O*-(3′-thiotriphosphate) (GTP-γ-S) in a fluorescence assay (*24*) at 20°C. (**A**) Transducin activation by reduced double cysteine mutants. Samples were incubated with 5 mM DTT for 1.5 to 2.5 hours and the assay buffer contained 1 mM DTT. (**B**) Transducin activation by oxidized double cysteine mutants of rhodopsin (*22*). For each measurement, rhodopsin samples were bleached and added, to a final concentration of 5 nM, to a stirred cuvette containing 700 μl of 250 nM transducin in 0.01% DM, 10 mM tris (pH 7.1), 100 mM NaCl, and 2 mM $MgCl_2$. After 5 min, GTP-γ-S was added to a final concentration of 5 μM (at time = 0). The initial rates of transducin activation for each mutant are given as a percentage of that obtained with wild-type ROS rhodopsin (in parenthesis) and were obtained from the slope of the fluorescence measurements in the first 60 s after addition of GTP-γ-S.

To investigate the functional consequences of disulfide bond formation, we studied activation of transducin (G_T). All double cysteine mutants activated G_T under reducing conditions [1 mM dithiothreitol (DTT), pH 6.0]. However, after oxidation, only those samples that did not form disulfide cross-links activated G_T (Fig. 4) (*15*). The inability of the disulfide cross-linked mutants to activate G_T implies a functional importance for the helix movement detected by the spin labels.

Our results can be interpreted in terms of a simple rigid body motion of helix F relative to helix C (Fig. 5). The light-activated conformational change appears to involve rigid body motion of helix F relative to C, rather than secondary structural changes, because the mobility of the spin labels on the outer surfaces of helices C and F change little after activation, whereas those on the interior surface become more mobile as a result of decreased tertiary interactions (*3*). The rotational-translational motion indicated in the model moved 250R1 closer to 139R1, but increased the distance between all other pairs, as was observed.

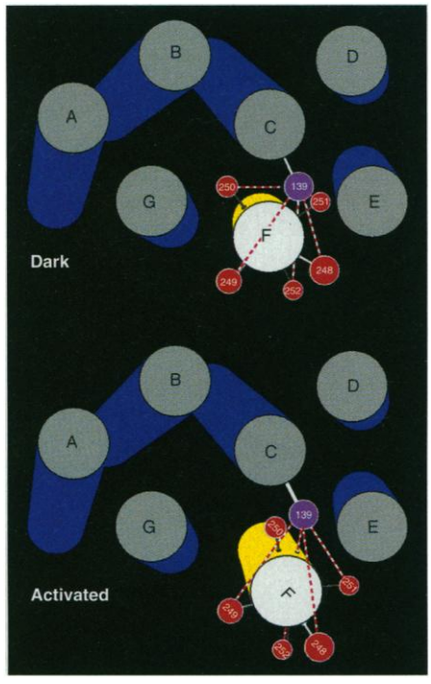

Fig. 5. A model for the structure of inactive rhodopsin (dark) and light-dependent changes (activated) in the relative positions of the C and F helices, viewed from the cytoplasmic face. The model for rhodopsin in the dark is based on the helix packing scheme of Schertler and co-workers (*4*), results from SDSL studies of rhodopsin (*3*), and the five approximate distance constraints provided by our spin labeling results. Relevant nitroxide side chains are indicated with circles sized roughly according to their vertical positions and are placed as described (*3*). In the light-activated model, helix F was tilted and rotated to satisfy the change in distance constraints discussed in the text. Possible motions in other helices are not excluded.

Helices C and F flank the ionone ring of retinal, which makes contact with the bulky, highly conserved residue Trp^{265} in helix F (*16*). Trp^{265} may serve to transmit chromophore motions to helix F, resulting in the structural change at the cytoplasmic face reported here. It is noteworthy that an outward movement of helix F would result in an increased exposure of Arg^{135}, a residue conserved throughout the GPCR family and required for G_T binding and activation (*17*).

As a consequence of F helix motion, changes would be expected in the important E-F interhelical loop recognized by G_T (*17*) and rhodopsin kinase (*18*). Movement in the topologically similar membrane protein bacteriorhodopsin is also largely confined to helix F (*19*), which suggests that motion of this helix upon activation may be a common trait shared among membrane-bound retinal proteins and perhaps in all GPCRs.

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7. Double cysteine mutants were constructed in a synthetic rhodopsin gene by combining appropriate plasmid fragments from single-cysteine mutants [K. D. Ridge, C. Zhang, H. G. Khorana, *ibid.* **34**, 8804 (1995); K. Yang, D. L. Farrens, W. L. Hubbell, H. G. Khorana, *ibid.* **35**, 12464 (1996).
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9. Mutant purifications were as described (*6, 7*), with some modifications. All buffers were argon purged. After harvesting, transfected COS cells were solubilized (4°C for 1 hour) in phosphate-buffered saline containing 0.5% DM, 0.5 mM phenylmethylsulfonylfluoride, 3 mM DTT, and 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6). The cells were then bound to 1D4 antibody beads (4°C for 3 hours; antibody supplied by the National Cell Culture Center, Minneapolis, MN) washed five times with 10 ml of 5 mM MES (pH 6), 0.025% DM, 1 mM EDTA and then washed two times with 5 mM MES (pH 6), 0.05% DM. The samples were eluted in the latter buffer containing 300 μM competing nine–amino acid peptide oligomer and stored at 4°C in the dark. Yields of the expressed mutants were similar to that of the wild-type rhodopsin.
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12. A similar pattern of changes was observed at room temperature, but under these conditions, both changes in spin label mobility (*3*) and changes in distance affect the spectral amplitudes.
13. Least squares fitting of spectral lineshapes [A. H. Beth *et al.*, *J. Biol. Chem.* **259**, 9717 (1984); Z. T. Farahbakhsh *et al.*, *Biochemistry* **34**, 509 (1995)] and convolution techniques [M. R. Rabenstein and Y. K. Shin, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8239 (1995)] have both been used to obtain interspin distances between pairs of nitroxides in rigid lattices. In the present case, the approach described by Farahbakhsh *et al.* was used, but the presence of a small population of noninteracting R1 side chains at the native cysteines at positions 167, 185, 222, and 264 limits the determination to an estimate for probable ranges of interspin distances.
14. Disulfide cross-linking probability is apparently strongly dependent on low-frequency conformational fluctuations [C. L. Careaga and J. J. Falke, *J. Mol. Biol.* **226**, 1219 (1992)], whereas distances between nitroxide groups at low temperature are determined in part by the conformation of the nitroxide side chain in the protein. Thus, it is not unexpected that, in particular cases, proximity will be detected by one method but not the other. For 139C-252C, no cross-linking occurred, but nitroxide interactions were detectable.
15. Transducin activation by at least one of the cross-linked mutants (139C-250C) could be restored by treatment with DTT (D. L. Farrens, C. Altenbach, K. Yang, W. L. Hubbell, H. G. Khorana, unpublished data).
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20. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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22. Oxidation with Cu-phenanthroline [K. Kobashi, *Biochim. Biophys. Acta* **158**, 239 (1968)] was initiated by addition of $CuSO_4$ and phenanthroline (to a final concentration of 50 and 150 μM, respectively) to 1 μM rhodopsin in 0.2% DM, 5 mM MES, 50 mM KPO_4, and 150 mM NaCl (pH 7.2). The mixture was incubated at 4°C for 7 min, and the reaction was halted by adding EDTA to a final concentration of 12.5 mM.
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25. In rhodopsin, a series of histidine substitutions at the cytoplasmic ends of helices C and F, but not C and G, created high-affinity metal-ion binding sites. Activation of transducin was blocked when metal was bound to the high-affinity sites, presumably because of effective cross-linking of helices C and F, consistent with results presented here (S. Sheikh, T. Zvyaga, O. Lichtarge, T. Sakmar, H. Bourne, *Nature*, in press).
26. This is paper 19 in the series "Structure and Function in Rhodopsin." Paper 18 is C. Altenbach *et al.*, *Biochemistry*, in press. We thank U. L. RajBhandary and C. Zhang for many helpful discussions and members of the H.G.K. and W.L.H. laboratories and K. Ridge (Center for Advanced Research in Biotechnology, University of Maryland) for the $Val^{139} \rightarrow Cys^{139}$ mutant gene. Supported by NIH grants EY05216 (W.L.H.) and GM28289 (H.G.K.), NIH National Research Service Award EY06465 (D.L.F.), and the Jules Stein Professorship endowment (W.L.H.).

14 June 1996; accepted 23 August 1996