G Protein-Coupled Receptor Activation: Analysis of a Highly Constrained, "Straitjacketed" Rhodopsin†

Mary Struthers, Hongbo Yu, and Daniel D. Oprian*

Department of Biochemistry and Volen Center for Complex Systems, Brandeis University, Waltham, Massachusetts 02454

Received April 4, 2000

ABSTRACT: G protein-coupled receptor (GPCR) activation is generally assumed to result in a significant structural rearrangement of the receptor, presumably involving the rigid body movement of transmembrane helices. We have investigated the activation of the GPCR rhodopsin by the construction and analysis of a mutant which contains a total of four disulfide bonds connecting the cytoplasmic ends of helices 1 and 7, and 3 and 5, and the extracellular ends of helices 3 and 4, and 5 and 6. Despite the constraints imposed by four disulfides, this "straitjacketed" receptor retains the ability to activate the G protein transducin and, therefore, provides insight into the molecular mechanism of the initial step in signal transduction of this important class of receptors.

G protein-coupled receptors (GPCRs)[1] serve as important mediators in many critical biological functions by responding to diverse extracellular signals including hormones, neurotransmitters, and sensory stimuli such as light. Central to the function of this important class of transmembrane proteins is the ability to transduce the extracellular signal into an intracellular event through a structural rearrangement of the receptor. Understanding the nature of this structural change is essential to deciphering the mechanism of this initial step in signal transduction.

Rhodopsin, the dim-light photoreceptor of rod cells in the retina, is the structural prototype of GPCRs. Cyroelectron microscopy of rhodopsin has produced an electron density map with a resolution of 7.5 Å × 16.5 Å (*1*). Although this map and derivative models (*2*) reveal the overall architecture of the seven transmembrane helical bundle in the ground state of the protein, they shed no light on the molecular mechanism of signal transduction.

Inherent difficulties in the direct structural analysis of membrane proteins have led to the development of biochemical strategies to probe the structure of this class of proteins. Receptor cross-linking that exploits the construction of metal ion binding sites or disulfide bonds has been used to investigate the structure and dynamics of numerous membrane proteins (*3*−*7*, *32*, *33*), including rhodopsin (*4*, *8*−*12*) and other GPCRs (*3*, *13*). Cross-linking between two residues in the ground state indicates spatial proximity of these positions. In addition, cross-linking can be accompanied by inactivation of the protein which is often interpreted to suggest that the cross-link prevents a rigid body helical movement required for receptor activation (*8*, *9*, *13*). In our experience with rhodopsin, it has been difficult to elucidate the mechanism for receptor activation from cross-links which inactivate the protein, as further investigation has sometimes revealed related cross-links, which connect the same helical regions, but do not inactivate the receptor (*10*). As a consequence, it has not been possible to make a definitive statement about the structure of the active state based on data from inactivating cross-links.

In contrast, strong conclusions readily emerge from the analysis of cross-linked receptors that retain activity; the relative position of the cross-linked residues must be similar in both the ground and the active state of the receptor. Therefore, we have formulated an approach in which several active cross-links connecting various regions of the receptor are combined into a single construct, creating a highly constrained or "straitjacketed", yet active, receptor. Extensive cross-linking in such a construct restricts the conformational dynamics of the protein and thereby provides insight into the nature of the structural rearrangements required for receptor activation.

We describe here the construction and analysis of a bovine rhodopsin mutant containing multiple cysteine substitutions designed to create a total of four disulfide bonds. This straitjacketed rhodopsin (Rho-SJ, Figure 1) has two disulfides on the cytoplasmic side of the protein and two on the extracellular side. In the oxidized state of this receptor, disulfide cross-links connect residues that are thought to be part of a transmembrane helix (TM) or within a few residues of it. Specifically, on the cytoplasmic side of the receptor, a 65−316 disulfide connects TM1 and TM7 (*14*−*17*) and a 140−225 disulfide connects TM3 to TM5 (*10*). On the extracellular side, a 204−276 disulfide joins the ends of TM5 and TM6 (*4*, *12*), and the native 110−187 disulfide connects TM3 to the second extracellular loop near TM4. Each of these disulfide bonds has been investigated in mutant receptor constructs and demonstrated to yield active proteins after

† This work was supported by National Institutes of Health Grant EY07965. M.S. was supported by individual National Research Service Award EY06670. We also acknowledge support for the Volen Center for Complex Systems by the W. M. Keck Foundation.

* To whom correspondence should be addressed. Phone: (781) 736-2322; FAX: (781) 736-8487; E-mail: oprian@brandeis.edu.

[1] Abbreviations: GPCR, G protein-coupled receptor; TM, transmembrane helix; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; DM, dodecyl β-D-maltoside; ConA, concanavalin A; SJ, straightjacketed; 4PDS, 4,4-dithiopyridine.

10.1021/bi000771f CCC: $19.00
Published on Web 06/16/2000

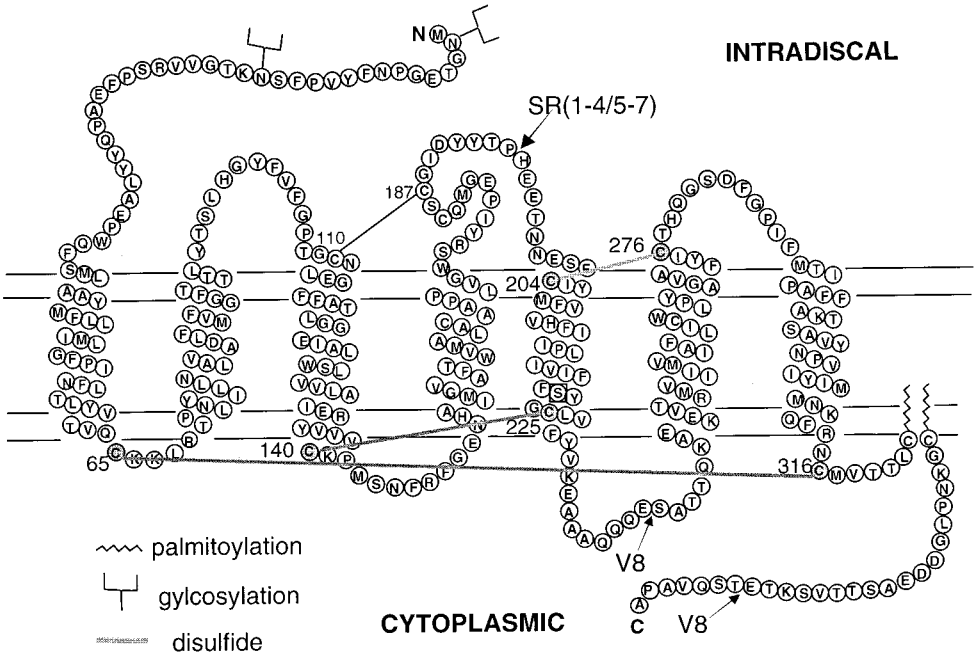

FIGURE 1: Schematic representation of the proposed secondary structure of "straitjacketed" rhodopsin illustrating the engineered and native disulfide bonds. Shaded circles indicate the positions of the cysteines involved in the engineered disulfides. The mutation of a native cysteine at position 222 to serine is indicated by a square. Single-letter abbreviations are used for amino acid. Sites of glycosylation (N2 and N15) and palmitoylation (C322 and C323) are also highlighted. Arrows indicate the sites of V8 protease cleavage. Segmentation in the split rhodopsin construct SR(1−4/5−7) is also indicated by an arrow. This split receptor consists of the first four transmembrane helices noncovalently associated with the last three transmembrane helices.

spontaneous cross-linking (without the addition of exogenous oxidant) (*10*, *12*, *18*, *19*).

EXPERIMENTAL PROCEDURES

Mutagenesis, Expression, and Purification of Rhodopsin. Construction of all rhodopsin mutants was carried out using a synthetic rhodopsin gene in a pMT3-based vector as has been previously described (*20*). The genes were expressed in COS-1 cells by transient transfection using DEAE-dextran (*21*). The mutant proteins were reconstituted with 11-*cis*-retinal in whole cells, solubilized with 1% dodecyl maltoside (DM), and purified by immunoaffinity chromatography using a 1D4 antibody-Sepharose 4B matrix essentially as described (*21*). The resin was washed extensively with 0.1% DM in buffer A (2 mM sodium phosphate, pH 6.0, 150 mM NaCl) followed by 0.1% DM in buffer B (2 mM sodium phosphate, pH 6.0). Correctly folded full-length proteins which bound 11-*cis*-retinal were separated from incorrectly folded opsin proteins by the selective elution of the pigment from the antibody column using a low-salt buffer (2 mM sodium phosphate, pH 6.0, 0.1% DM) containing peptide I (D-E-A-S-T-T-V-S-K-T-E-T-S-Q-V-A-P-A) (0.18 mg/mL) (*22*).

Oxidation of Cysteines. Oxidation of rhodopsin samples by ambient oxygen was accomplished by increasing the pH of the detergent-solubilized, purified protein from 6.0 to 7.0 by the addition of dibasic sodium phosphate (from a solution of 100 mM disodium phosphate, 150 mM NaCl) followed by incubation in the dark at 25 °C for 18−20 h.

Quantitation of Free Cysteines. Reaction of 4PDS with a free cysteine yields 1 equiv of 4-thiopyridone which can be detected and quantified by its absorbance at 324 nm (*23*). The total free sulfhydryl content of the proteins was determined essentially as described by Khorana and coworkers (*24*). The final reaction contained 0.5% SDS, 25 μM 4,4-pyridyldithiol (Aldrithiol-4, Aldrich), 0.5 μM protein, and 0.1% DM in 2 mM sodium phosphate buffer, pH 6.0, in a final volume of 150 μL. The reference cuvette contained all components of the reaction excluding protein. The absorbance at 324 nm was monitored by UV−vis spectroscopy for at least 2.5 h and the plateau value used to determine the quantity of 4-pyridonethione released. The initial absorbance at 324 nm attributable to the protein was subtracted from the plateau value before calculation of the concentration of 4-thiopyridone released using an extinction coefficient of 19 000 M^{-1} cm^{-1} (*24*).

Reduction of Disulfides. To obtain a reduced sample of Rho-SJ, the protein was treated with 30 mM DTT overnight at 4 °C while bound to the 1D4-Sepharose 4B matrix. The protein-bound matrix was then washed in buffers which had been sparged with argon to eliminate oxygen, and the protein was then eluted in 0.1% DM in buffer B containing peptide I as described (*22*). The eluted protein contained no DTT and was analyzed by reaction with 4PDS immediately. A sample of Rho-wt was similarly treated as a control.

Split Rhodopsin Mutants. Split rhodopsins are assembled through the coexpression in COS-1 cells of genes encoding complementary C- and N-terminal fragments (*4*). The nomenclature for split rhodopsin follows that previously described by Yu et al. (*4*): SR(1−4/5−7) refers to an N-terminal fragment of the receptor consisting of the first four transmembrane helices (residues 1−194) noncovalently associated with a C-terminal fragment consisting of the remaining three transmembrane helices (residues 195−348 with an added initiator methionine).

V8 Digestion and SDS−PAGE Analysis. Detergent-solublized, purified, and oxidized receptors were incubated in the dark with 25 ng/mL V8 protease at 25 °C for 4.5 h. Alternatively, the oxidized receptors were incubated with

Table 1: Quantitation of the Sulfhydryl Group in Wild-Type and Mutant Rhodopsins under Denaturing Conditions by Reaction with 4PDS

rhodopsin	equiv of thiopyridone[a]	no. of Cys[b]
Rho-wt	5.6 (0.2)	6
Rho-SJox	2.8 (0.6)	9
Rho-SJred[c]	9.0 (0.2)	9

[a] The value given is per mole of rhodopsin and represents the average of two or more measurements. The standard error is given in parantheses. [b]Cysteines present in the receptor not including C110 and C187, which are involved in a native disulfide bond and can only be reduced in the denatured state (*25*), and C322 and C323, which are palmitoylated (*26, 27*). [c]Sample was reduced prior to denaturation and analysis by 4PDS.

DTT (100 mM, 25 °C, 20 h) prior to V8 digestion. The reactions were quenched by the addition of SDS−PAGE loading buffer containing the protease inhibitor PMSF, and immediately loaded on a nonreducing SDS−PAGE gel and analyzed by Western blotting using ConA as previously described (*12*).

RESULTS AND DISCUSSION

Five cysteine substitutions were required to create the straitjacketed receptor: H65C, V204C, C222S, Q225C, and F276C. Following purification of the straightjacketed mutant, the cysteines were oxidized to form the desired disulfide as described under Experimental Procedures. Under similar conditions, each of the engineered disulfide bonds has been shown to form in mutant receptor constructs in which they have been individually studied (*10, 12, 18*). Therefore, if cross-linking in the straitjacketed receptor mimics that observed in these constructs, then Rho-SJ should contain four disulfides after air oxidation (termed Rho-SJox). To verify cross-linking in Rho-SJox, the number of disulfide bonds was determined by quantitation of the free thiols present using 4,4-dithiopyridine (4PDS). Rho-SJ contains 13 cysteines, 4 of which are unavailable for reaction: 2 cysteines are linked in the native disulfide 110−187, which can only be reduced under denaturing conditions (*25*), and 2 are palmitoylated (*26, 27*). Rho-SJ that was reduced by treatment with DTT during purification (Rho-SJred) contained approximately nine free thiols as determined by reaction with 4PDS (Table 1), indicating that the native disulfide bond is the only disulfide present. In contrast, only 2.8 free thiols are detected by reaction of Rho-SJox with 4PDS, consistent with the presence of all four disulfides after oxidation (Table 1). In addition to the indirect determination of disulfides with 4PDS, at least one disulfide was directly observed by digestion of the oxidized protein with V8 protease followed by nonreducing SDS−PAGE and Western blot analysis (Figure 2). As illustrated in Figure 1, V8 protease cleaves rhodopsin at two sites, removing the seven C-terminal amino acids and cleaving between the fifth and sixth transmembrane helices, producing two major fragments of ∼27 and ∼13 kDa mass (*8, 28*). Nonreducing SDS−PAGE and Western blots of the digests probed with ConA (which binds the mannose-containing carbohydrates at glycoslated residues Asn2 and Asn15) reveal the N-terminal ∼27 kDa fragment for Rho-wt (Figure 2, lane 2). In contrast, V8 protease treatment of Rho-SJox results in only a small mobility change (corresponding to cleavage of the seven C-terminal amino acids), consistent with one or more disulfide bonds cross-linking the two major proteolytic fragments (lane 4). In further support of the presence of disulfide cross-links in Rho-SJox, incubation of Rho-wt and Rho-SJox with DTT followed by digestion with V8 protease produced similar digestion patterns for the two proteins (Figure 2, lanes 8 and 10).

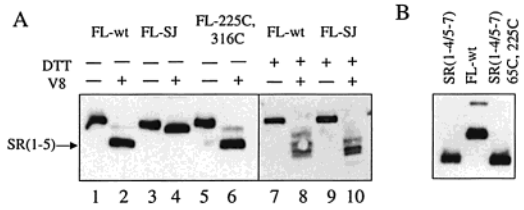

FIGURE 2: V8 protease and Western analysis using ConA of mutant receptors. (A) V8 protease digestion of FL-wt, FL-SJ, and FL-225C,316C after oxidation (pH 7.0, 25 °C, ON), using 25 ng/mL V8 protease for 4.5 h at 25 °C. The mobility of an N-terminal fragment, SR(1−5), consisting of residues 1−240 is indicated by the arrow as a molecular weight marker. V8 protease cleaves rhodopsin at two sites, removing the seven C-terminal amino acids and cleaving between the fifth and sixth transmembrane helices, producing two major fragments of ∼27 and ∼13 kDa mass (*8, 28*). The N-terminal ∼27 kDa fragment generated by V8 protease digestion of Rho-wt is detected by ConA (lane 2). In contrast, V8 digestion of Rho-SJox results in only a small mobility change (lane 4) which is not observed if the samples are treated with DTT (lanes 7−10). Samples have been treated with V8 protease and/or DTT as described under Experimental Procedures. (B) Nonreducing SDS−PAGE and Western analysis of a split receptor with 65C and 225C as the only cytoplasmic cysteines. The mutant split receptor contains the following mutations: H65C, C140S, C222S, Q225C, C316S. The split receptor was analyzed after treatment with the same conditions (pH 7.0, 25 °C, 20 h) used to oxidize the straitjacketed receptor. The absence of a disulfide is demonstrated by the mobility of the N-terminal fragment mutant receptor which matches that of the wild-type split receptor and is significantly faster than that of a full-length receptor.

Although the 4PDS assay indicates that Rho-SJox contains the desired number of disulfide bonds, this experiment does not address the possibility of incorrect pairing of cysteines, especially on the cytoplasmic face. For this purpose, a series of rhodopsin mutants, each containing pairwise combinations of the cytoplasmic cysteines (C65, C140, C225, and C316) as the only cytoplasmic cysteines, were examined for disulfide bond formation. Analysis of these mutants by V8 digestion, Western blotting, and reaction with PDS suggests that incorrect disulfide pairing of cysteines on the cytoplasmic surface does not occur under the conditions used to oxidize Rho-SJ. Four rhodopsin mutants were constructed, each of which contained two cytoplasmic cysteines (140−316, 225−316, 65−225, or 65−140) which were not expected to cross-link in the straitjacketed receptor. These mutants were analyzed for disulfide bond formation to assess the possibility of incorrect disulfide bond formation in Rho-SJ. The results are as follows. 140−316: Reaction with PDS and V8 digestion followed by Western blot of Rho-wt (containing the native cysteines at 140 and 316) indicate the absence of a 140−316 disulfide (Table 1 and Figure 2). 225−316: V8 digestion and Western blot analysis of Rho-225C,-316C (containing C140S, C222S, and Q225C mutations) produced a cleavage pattern similar to Rho-wt, indicating the absence of a 225−316 disulfide (Figure 2A, lanes 5 and 6). 65−225: Potential cross-linking between 65C and 225C was investigated in a split-rhodopsin construct, SR(1−4/5−

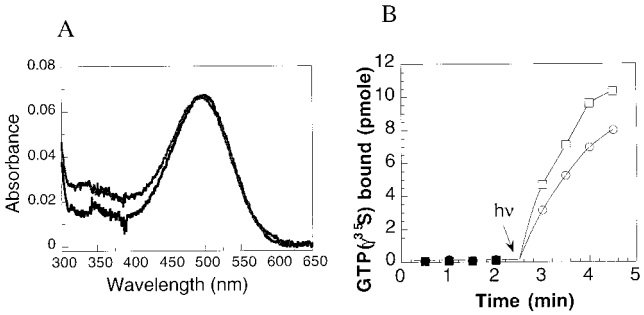

FIGURE 3: Spectra and activity of Rho-SJ. (A) Absorption spectra of the reduced form (black) and the oxidized form (gray) of Rho-SJ. (B) Catalysis of exchange of guanosine diphosphate for radiolabeled guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-^{35}S) in transducin was monitored in detergent solution (0.01% dodecyl maltoside) using a filter-binding assay as has been described previously (*30*, *31*). Final concentrations were the following: rhodopsin, 5 nM; transducin, 2.5 μM; GTP-γ-S, 3.0 μM. Rho-SJox (circles) and Rho-wt (squares) were assayed after incubation at pH 7.0 and 25 °C overnight. Solid symbols, reaction in the dark; open symbols, reaction after exposure to light (>490 nm, 300 W, for 15 s; conditions expected to produce complete bleaching of the pigment).

7)-65C,225C (containing the H65C, C140S, C222S, and Q225C mutations). Nonreducing SDS−PAGE and Western blot analysis of this split receptor showed no cross-linking between the two fragments, confirming the absence of 65−225 (Figure 2B). 65−140: The 65−316 disulfide forms selectively even in the presence of the native cysteine at 140 (*18*), suggesting that the incorrect pairing of 65C and 140C in Rho-SJox is unlikely. Additionally, treatment of Rho-65C,-140C (a rhodopsin mutant containing H65C, C316S mutations) with 4PDS indicated the presence of 5.3 free cysteines in the mutant, and this value did not decrease with extended incubation at pH 7.0. Although the number of free thiols determined by the PDS assay is slightly less than the expected six free thiols, it is significantly more than would be anticipated if disulfide bond formation had occurred (four free thiols).

These data in combination with the determination of the correct number of disulfides by analysis of Rho-SJox with 4PDS strongly suggest that the desired disulfide bonds are present in Rho-SJox after oxidation.

The straitjacketed rhodopsin has a near-wild-type absorption spectrum, and although there is a slight (<4 nm) blue shift observable upon oxidation of the protein, the absorption spectrum of Rho-SJ is very similar in both the oxidized and reduced states (Figure 3). The observation of a characteristic absorbance maximum at ~500 nm indicates that the disulfide bonds do not significantly distort the overall tertiary structure of the protein, and that the integrity of the 11-*cis*-retinal binding pocket is maintained.

Despite the presence of four disulfides, Rho-SJox activates the G protein transducin in a light-dependent manner with 60% of the specific activity of the wild-type protein (Rho-wt). The slight reduction in specific activity is most likely due to the amino acid substitutions rather than disulfide bond formation since a similar reduction in specific activity has been observed in a mutant containing only the C222S and Q225C substitutions (*10*). Furthermore, Rho-SJ that has been treated with DTT displays specific activity that is similarly reduced relative to DTT-treated Rho-wt (data not shown).

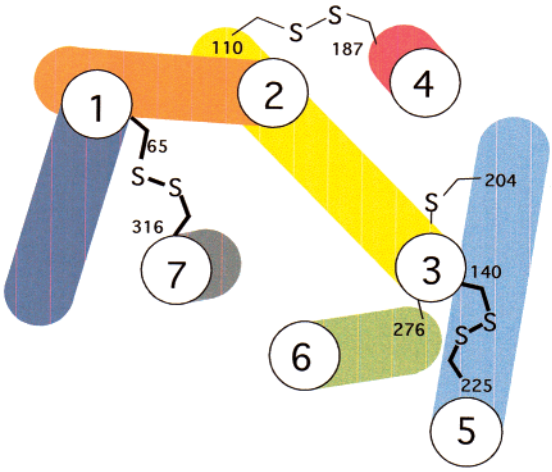

FIGURE 4: Model of the three-dimensional arrangement of the helices of rhodopsin illustrating the four disulfide bonds present in the straitjacketed receptor. The figure is based on the model by Baldwin et al. (*2*) derived in part from the cryoelectron microscopy data from Schertler and co-workers (*1*).

Therefore, the reduced activity most likely results from the amino acid substitutions and not the disulfide cross-links.

It is clear from these data that the straitjacketed receptor readily undergoes the conformational rearrangements required for activation despite the constraint of four disulfide cross-links. The distance between Cα atoms in a pair of disulfide-bonded cysteines is <7 Å. Therefore, the disulfides in Rho-SJox significantly restrict the relative positions of the two residues in both the dark and active states of the protein. The activity of Rho-SJox suggests that simultaneous proximity of the cytoplasmic ends of helices 1 and 7, the cytoplasmic ends of helices 3 and 5, and the extracellular ends of helices 5 and 6 is permitted in both active and dark states of the protein. Both the extracellular and the cytoplasmic ends of helix 5 are tethered to other helices via disulfides in Rho-SJox. A large rigid body helical movement of TM5 is therefore unlikely to be a part of the molecular mechanism of activation, unless helices 3, 5, and 6 move in a concerted manner. A similar analysis can be applied to helix 3 which is tethered to helix 5 on the cytoplasmic side by the 140−225 disulfide and is simultaneously tied on the extracellular side near helix 4 via the native disulfide bond between residues 110 and 187.

The network of disulfide bonds present in Rho-SJ should significantly restrict movement of the helices involved (helices 1, 3, 5, 6, and 7), and proposed structural mechanisms of activation can be evaluated in the context of these constraints. Based on site-directed spin labeling and disulfide cross-linking, an outward tilt of the cytoplasmic end of helix 6 has been proposed by Khorana, Hubbell, and co-workers to be critical to the mechanism of photoactivation (*8*). In our cross-linked receptor, the extracellular end of helix 6 is tethered to helix 5, which is in turn disulfide bonded to helix 3 on the cytoplasmic side (Figure 4). Therefore, our data suggest that the pivot point of such a tilt must lie close to the extracellular end of helix 6.

Previous studies using site-directed spin labeling have indicated that the side chain of cysteine 316 experiences an increase in mobility upon photoactivation and that positions 65 and 316 move apart after photoactivation (*18*). We have previously shown that 316C is involved in a light-dependent

cross-link with 140C in the presence of the oxidant Cu(phen)$_3^{2+}$, also suggesting an increase in mobility of this residue upon photoactivation (*11*). The activity of Rho-SJox as well as a rhodopsin mutant containing the 65−316 disulfide (*19*) demonstrates that although an increase in the mobility of residue 316 may accompany photoactivation, it is not a *requirement* for activation. Similarly, light-dependent changes in the environment of position 140 have been suggested by site-directed spin-labeling studies (*29*). The activity of the cross-linked form of Rho-SJ as well as a rhodopsin mutant containing the 140−225 disulfide (*10*) suggests that although this residue may experience a change in environment as a consequence of photoactivation, large relative movements of the cytoplasmic end of this helix, especially with respect to helix 5, are not essential to activity. The construction of constrained receptors such as Rho-SJ that retain activity allows for definitive statements to be made concerning the requirements for relative movements of certain regions of the protein during activation. Therefore, analysis of such straitjacketed receptors can be used in conjunction with complementary methods to differentiate regions of the protein that are directly involved in movements necessary for activation.

The straitjacketed rhodopsin, Rho-SJ, contains two disulfides on the cytoplasmic surface, which is the region of rhodopsin that interacts directly with transducin and is expected to exhibit a major portion of the conformational change upon activation. Surprisingly, these disulfides, either individually or collectively, exert no inhibitory effect on the activity of the protein. Although significant movement of one or more helices has been proposed to accompany GPCR activation, the high specific activity of Rho-SJ hints that the overall structural changes involved in receptor activation, particularly on the cytoplasmic surface of the protein, may be more subtle than originally anticipated, such that they can be readily accommodated within the conformational restrictions of multiple disulfides.

ACKNOWLEDGMENT

We thank Professor Chris Miller for suggestions on the manuscript.

REFERENCES

1. Unger, V. M., Hargrave, P. A., Baldwin, J. M., and Schertler, G. R. X. (1997) *Nature 389*, 203−206.
2. Baldwin, J. M., Schertler, G. F., and Unger, V. M. (1997) *Mol. Biol. 272*, 144−164.
3. Elling, C. E., and Schwartz, T. W. (1996) *EMBO J. 15*, 6213−6219.
4. Yu, H., Kono, M., McKee, T. D., and Oprian, D. D. (1995) *Biochemistry 34*, 14963−14969.
5. Pakula, A. A., and Simon, M. I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4144−4148.
6. Lee, G. F., Burrows, G. G., Lebert, M. R., Dutton, D. P., and Hazelbauer, G. L. (1994) *J. Biol. Chem. 269*, 29920−29927.
7. Falke, J. J., and Koshland, D. E., Jr. (1987) *Science 237*, 1596−1600.
8. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Korana, H. G. (1996) *Science 274*, 768−770.
9. Sheikh, S. P., Znyaga, T. A., Lichtarge, O., Sakmar, T. P., and Bourne, H. R. (1996) *Nature 383*, 347−350.
10. Yu, H., and Oprian, D. D. (1999) *Biochemistry 38*, 12033−12040.
11. Yu, H., Kono, M., and Oprian, D. D. (1999) *Biochemistry 38*, 12028−12032.
12. Struthers, M., Yu, H., Kono, M., and Oprian, D. D. (1999) *Biochemistry 38*, 6597−6603.
13. Elling, C. E., Møller Nielsen, S., and Swartz, T. W. (1995) *Nature 374*, 74−77.
14. Klein-Seetharaman, J., Hwa, J., Cai, K., Altenbach, C., Hubbell, W. L., and Khorana, H. G. (1999) *Biochemistry 38*, 7938−7944.
15. Altenbach, C., Klein-Seetharaman, J., Hwa, J., Khorana, H. G., and Hubbell, W. L. (1999) *Biochemistry 38*, 7945−7949.
16. Altenbach, C., Cai, K., Khorana, H. G., and Hubbell, W. L. (1999) *Biochemistry 38*, 7931−7937.
17. Cai, K., Klein-Seetharaman, J., Farrens, D., Shang, C., Altenbach, C., Hubbell, W. L., and Khorana, H. G. (1999) *Biochemistry 38*, 7925−7930.
18. Yang, K., Farrens, D. l., Altenbach, C., Farahbakhsh, Z. T., Hubbell, W. L., and Khorana, H. G. (1996) *Biochemsitry 35*, 14040−14046.
19. Cai, K., Klein-Seetharaman, J., Hwa, J., Hubbell, W. L., and Khorana, H. G. (1999) *Biochemistry 38*.
20. Ferretti, L., Karnik, S. S., Khorana, H. G., Nassal, M., and Oprian, D. D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 599−603.
21. Oprian, D. D. (1993) *Methods Neurosci. 15*, 301−306.
22. Ridge, K. D., Lu, Z., Liu, X., and Khorana, H. G. (1995) *Biochemistry 34*, 3261−3267.
23. Grassetti, D. R., and Murray, J. F., Jr. (1967) *Arch. Biochem. Biophys. 119*, 41−49.
24. Cai, K., Langen, R., Hubbell, W. L., and Khorana, H. G. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 14267−14272.
25. Karnik, S. S., and Khorana, H. G. (1990) *J. Biol. Chem. 265*, 17520−17524.
26. Karnik, S. S., Ridge, K. D., Bhattacharya, S., and Khorana, H. G. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 40−44.
27. Ovchinnikov, Y. A., Abdulaev, N. G., and Bogachuk, A. S. (1988) *FEBS Lett. 230*, 1−5.
28. Pappin, D. J., and Findlay, J. B. (1984) *Biochem. J. 217*, 605−613.
29. Farahbakhsh, Z. T., Ridge, K. D., Khorana, H. G., and Hubbell, W. L. (1995) *Biochemistry 34*, 8812−8819.
30. Wessling-Resnick, M., and Johnson, G. L. (1987) *J. Biol. Chem. 262*, 3697−3705.
31. Zhukovsky, E. A., Robinson, P. R., and Oprian, D. D. (1991) *Science 251*, 558−560.
32. He, M. M., Voss, J., Hubbell, W. L., and Kaback, H. R. (1995) *Biochemistry 34*, 15667−15670.
33. Wu, and Kaback, H. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 14498−14502.

BI000771F