State-Dependent Disulfide Cross-Linking in Rhodopsin†

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ABSTRACT: In previous studies, we developed a new method for detecting tertiary interactions in rhodopsin using split receptors and disulfide cross-linking. Cysteines are engineered into separate fragments of the split opsin, the disulfide bond can be formed between the juxtaposed residues by treatment with Cu(phen)$_3^{2+}$, and then disulfide cross-links can be detected on the gel by an electrophoretic mobility shift. In this study, we utilized this method to examine the cross-linking reactions between native cysteines in the ground state and after photoexcitation of rhodopsin. In the dark, Cys140 on transmembrane segment (TM) 3 cross-links to Cys222 on TM5. After photobleaching, Cys140 cross-links to Cys316 and Cys222, and the rate of the cross-linking reaction between Cys140 and Cys222 significantly increases.

We have recently developed a biochemical method using Cys and disulfide scanning (*1*) in split-receptor constructs of rhodopsin to map tertiary contacts in the protein (*2*). In this method, a Cys residue is engineered into each fragment of the split receptor, and disulfide bond formation is detected by a mobility shift observed on a nonreducing SDS−polyacrylamide gel as a consequence of covalent coupling of the two fragments. We have used this approach to probe the structure of the extracellular ends of TM5[1] and 6 in rhodopsin (*2*). Cu(phen)$_3^{2+}$ has been frequently employed as an oxidant to promote disulfide bond formation in the reaction (*3*, *4*), and for this reason we decided to investigate the effect of this reagent on the native Cys residues in rhodopsin. As presented here, treatment of dark-state rhodopsin with this reagent leads to the formation of a disulfide cross-link between Cys140 of TM3 and Cys222 of TM5 at the cytoplasmic surface of rhodopsin. In the course of this work we discovered that photoactivation of the receptor led to the formation of a different cross-link from that observed in the dark state. After exposure to light and Cu(phen)$_3^{2+}$, Cys140 cross-links preferentially with a native cysteine at the cytoplasmic surface of TM7, Cys316, to form a disulfide bond not observed in the dark-state protein. We present here an investigation of the state-dependent formation of disulfide bonds in rhodopsin. In the following paper in this issue (*5*), we further investigate the structure of the cytoplasmic ends of TM3 and TM5 and determine the effects of the disulfide cross-links in this region of the protein on the spectral properties and activity of rhodopsin.

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[1] Abbreviations: TM, transmembrane segment; Con A, concanavalin A; NEM, *N*-ethylmaleimide; phen, 1,10-phenanthroline; BSA, bovine serum albumin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; DM, dodecyl β-D-maltoside; N-terminal, amino-terminal; C-terminal, carboxy-terminal.

EXPERIMENTAL PROCEDURES

Materials. 11-*cis*-Retinal was supplied by Dr. Rosalie Crouch of the Medical University of South Carolina and the National Institutes of Health. Dodecyl β-D-maltoside (DM) was from Calbiochem (La Jolla, CA). Bovine retinas were obtained from J. A. Lawson Co. (Lincoln, NE). 1,10-Phenanthroline (phen), Sepharose 4B, and biotinylated Con A were from Sigma (St. Louis, MO). [^{35}S]GTPγS (1156 Ci/mmol) was from NEN, and nonradiolabeled GTPγS (tetralithium salt) was from Boehringer Mannheim. Nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and the alkaline phosphatase-conjugated streptavidin were from Promega (Madison, WI). *N*-Ethylmaleimide (NEM) was from Aldrich.

The monoclonal antibody rho 1D4, which is specific for the C-terminus of rhodopsin, has been described previously (*6*). The antibody was coupled to Sepharose 4B by the method of Cuatrecasas (*7*). Peptide I (Asp Glu Ala Ser Thr Thr Val Ser Lys Thr Glu Thr Ser Gln Val Ala Pro Ala), used for the elution of opsin from the 1D4−Sepharose 4B matrix, was purchased from American Peptide Co., Inc. (Santa Clara, CA).

Construction and Mutagenesis of the Split Opsins. The split opsin constructs used in this study are SR(1−4/5−7) and SR(1−5/6−7). The nomenclature and construction of the split receptors have been described previously (*1*). For example, SR(1−4) refers to an N-terminal fragment of the protein containing the first four transmembrane segments, and SR(5−7) refers to the C-terminal complement containing the last three transmembrane segments. SR(1−4/5−7) refers to an opsin that has been assembled by coexpression of two plasmids encoding fragments SR(1−4) and SR(5−7). Point mutations are indicated by beginning with the name of the fragment, followed by a colon, followed by the single-letter code for the wild-type amino acid, followed by the number of the amino acid, followed by the single-letter code for the new amino acid. For example, the single mutant in which Cys140 is changed to Ser in SR(1−4) and then combined with wild-type SR(5−7) is designated as SR(1−4:C140S/5−7).

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The N- and C-terminal fragments of each split construct are encoded on separate pMT3 (*8*) expression vectors. The mutations in the split opsins were introduced by either cassette or PCR mutagenesis.

Expression, Reconstitution, and Purification of the Proteins. The wild-type and mutant opsins used in this study were expressed in COS cells according to previously published procedures (*1*, *9*). Transfected COS cells were harvested 72 h after initial exposure to DEAE-dextran and DNA. Procedures for reconstitution with 11-*cis*-retinal, solubilization of the COS cells with 1% (w/v) DM, and purification of the proteins by immunoaffinity chromatography on the 1D4−Sepharose 4B matrix have been described (*9*−*14*). These procedures were modified for single-plate transfections (*15*, *16*) in the analysis of Cys scans by Western blot.

Disulfide Cross-Linking Reaction. Disulfide formation in the rhodopsin split receptors was catalyzed by treatment of the purified samples with 3 mM $CuSO_4$ and 9 mM 1,10-phenanthroline in 10 mM sodium phosphate buffer (pH 7.0) containing 2% (v/v) glycerol as described by Lee et al. (*4*) at room temperature for the time indicated in the figure legends. Reactions were performed either in the dark or after exposure of the protein to light. Exposure to light was for 30 s, using a 300-W slide projector with a 490 nm cut-on filter, followed immediately by treatment with $Cu(phen)_3^{2+}$. The reactions were quenched by addition of (final concentrations) 12.5 mM NEM and 12.5 mM EDTA in SDS−PAGE load buffer [60 mM Tris buffer (pH 6.8) containing 2% (w/v) SDS, 6% (w/v) sucrose, and 0.005% (w/v) bromophenol blue] and the samples were loaded on 12.5% polyacrylamide gels for nonreducing SDS−PAGE and Western blot analysis.

Western Blot Analysis. The purified proteins were visualized on Western blots (*17*) by probing for the N-terminal fragments with biotinylated Con A (*18*) similar to the method of Azen and Yu (*19*). After cross-linking, the samples were separated by SDS−PAGE on nonreducing 12.5% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes by using a Bio-Rad trans-blot SD semidry electrophoretic transfer cell (Bio-Rad). The membranes were subsequently blocked with 5% (w/v) BSA in 50 mM HEPES (pH 7.0), 100 mM NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ (Con A buffer) for 1 h at 37 °C. The blots were then incubated at room temperature overnight in a solution of 0.001% (w/v) biotinylated Con A in 5% (w/v) BSA dissolved in Con A buffer. The membranes were washed three times for 15 min in Con A buffer with 0.05% (v/v) NP-40 (wash buffer). The blots were then treated with 3 μL of alkaline phosphatase-conjugated streptavidin (Promega) in 15 mL of wash buffer for 1 h at room temperature. The membranes were then washed three times with wash buffer. Protein bands were visualized by reaction with NBT and BCIP according to directions supplied by the manufacturer (Promega). In this method, the C-terminal antibody 1D4 was used to purify the split receptors and biotinylated Con A was used to probe for the N-terminal fragment. Thus only the N-terminal fragments that co-purified with C-terminal fragments were detected.

RESULTS

State-Dependent Formation of Disulfide Bonds in Rhodopsin. Two different split receptor mutants of rhodopsin were used in this study, both of which have wild-type spectral properties as has been described previously (*1*). SR(1−4/5−7) is split within the intradiscal loop connecting TM4 and 5, and SR(1−5/6−7) is split within the cytoplasmic loop connecting TM5 and 6 (see Figure 1A). When SR(1−4/5−7) is treated with 3 mM $Cu(phen)_3^{2+}$ in the dark, the N- and C-terminal fragments cross-link through the formation of a

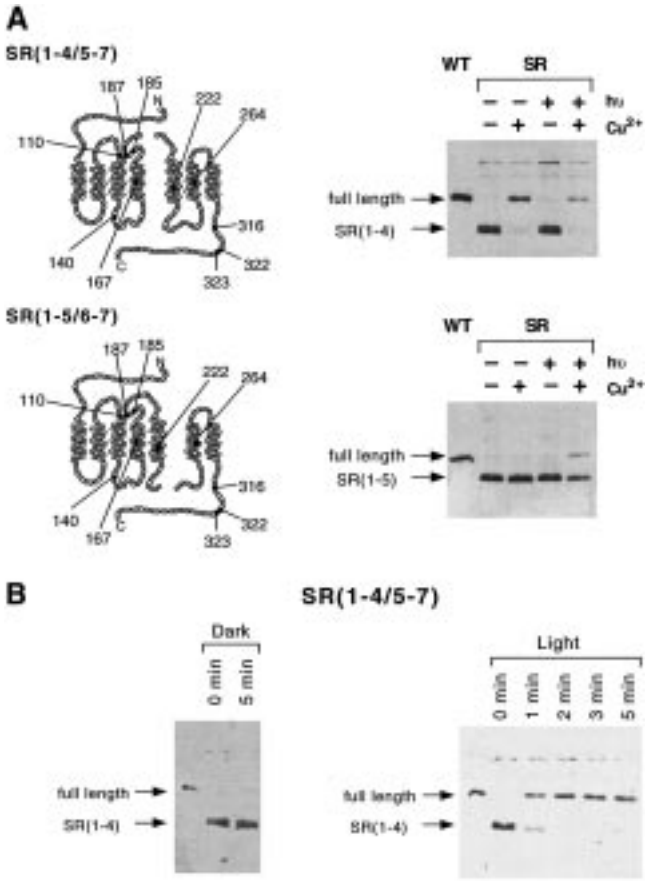

FIGURE 1: State-dependent disulfide cross-linking of split receptors SR(1−4/5−7) and SR(1−5/6−7). (A) Disulfide cross-linking of split receptors SR(1−4/5−7) and SR(1−5/6−7). The figure shows the dark- and light-dependent disulfide cross-linking results for the split rhodopsin mutants SR(1−4/5−7) and SR(1−5/6−7) in the top and bottom panels, respectively. In each case a schematic diagram of the split rhodopsin is shown on the left. Cys residues are indicated by shaded circles and identified by amino acid number in the polypeptide chain. Con A-Western blots from the cross-linking reactions are shown on the right. Cross-linking reactions were conducted in the dark or after exposure of the protein to a 30 s pulse of light and in the absence or presence of $Cu(phen)_3^{2+}$ oxidant, as indicated in the figure and detailed under Experimental Procedures. The migration positions of full-length rhodopsin and the SR(1−4) and SR(1−5) fragments are indicated by arrows on the left-hand side of the Western blots. The WT and SR labels at the top of each Western blot indicate columns containing wild-type rhodopsin and split rhodopsin, respectively. The cross-linking of dark-state receptor was for 40 min [SR(1−4/5−7)] or 45 min [SR(1−5/6−7)], while cross-linking after exposure of the protein to light was for 3 min [SR(1−4/5−7)] or 5 min [SR(1−5/6−7)]. (B) Cross-linking of SR(1−4/5−7) in the dark and after exposure to light. Cross-linking reactions in the dark are shown on the left and cross-linking reactions after exposure to light are shown on the right. The reactions were conducted for the times indicated. Full-length, wild-type rhodopsin was loaded in the first lane of each gel as a marker. The migration positions of full-length rhodopsin and the SR(1−4) fragment are indicated by arrows on the left-hand side of the Western blots.

disulfide bond in a reaction that requires about 40 min for completion (Figure 1A, top panel). If SR(1−4/5−7) is first exposed to light and then treated with $Cu(phen)_3^{2+}$, cross-linking of the two fragments is rapid; the fragments are essentially completely cross-linked in 3 min. SR(1−5/6−7), on the other hand, shows no cross-linking of the N- and C-terminal fragments after 45 min of incubation in the dark but undergoes rapid but partial cross-linking after exposure to light (Figure 1A, bottom panel). The reaction does not go to completion even if allowed to proceed for longer periods of time (not shown).

The effect of light activation on the rate of cross-linking in SR(1−4/5−7) is shown in Figure 1B. There is no significant cross-linking of the two fragments in the dark after incubation for 5 min at room temperature. In contrast, the cross-linking reaction goes to completion in 2 min after exposure to light.

In summary, the split rhodopsin mutants show pronounced state-dependent cross-linking of N- and C-terminal fragments. SR(1−4/5−7) cross-links both in the dark and in the light, but the fragments cross-link about 10-fold more rapidly after exposure of the protein to light. The fragments of SR(1−5/6−7) do not cross-link at all in the dark but rapidly form a cross-link in a fraction of the molecules after exposure of the protein to light.

In the sections to follow, (1) we identify which of the native cysteines are involved in the state-dependent cross-linking reactions and (2) we account for the incomplete cross-linking of fragments in light-activated SR(1−5/6−7).

Cys140 and Cys222 Form a Disulfide Bond in the Dark State. On the basis of the fact that both split receptors exhibit properties that are essentially identical to those of full-length, wild-type rhodopsin (*1*), we assume that the same Cys residues that form a disulfide bond in one split receptor also form a disulfide bond in the other split receptor. If Cys222 is one of the cysteines involved in the dark-state disulfide bond, this would explain simply why the two fragments of SR(1−5/6−7) do not cross-link in the dark. Cys222 is the only Cys residue that belongs to different fragments in the two split receptors; it belongs to the C-terminal fragment of SR(1−4/5−7) and to the N-terminal fragment of SR(1−5/6−7). A disulfide bond between Cys222 and another Cys in the first four transmembrane segments would cross-link the two fragments of SR(1−4/5−7). The same disulfide bond would still be formed in SR(1−5/6−7), but both Cys residues would be in the N-terminal fragment. The disulfide bond would not cross-link the two fragments and, therefore, would not be detected in our analysis.

To test this hypothesis, we constructed three mutants of SR(1−4/5−7) consisting of a wild-type N-terminal fragment paired with C-terminal fragments containing various Cys to Ser mutations. As is shown in Figure 2A, when Cys222 is changed to Ser (mutant C222S), SR(1−4) and SR(5−7) no longer cross-link; if all C-terminal Cys residues except Cys222 (i.e., Cys264, 316, 322, 323) are changed to Ser (mutant C222/Cys$^-$), the two fragments still cross-link; and finally, if all Cys residues in the C-terminal fragment are changed to Ser (mutant Cys$^-$), the fragments do not cross-link (the expression level for this mutant was low compared to C222S and C222/Cys$^-$). It is clear from these data that Cys222 is one of the two Cys residues that cross-link SR(1−4/5−7) in the dark.

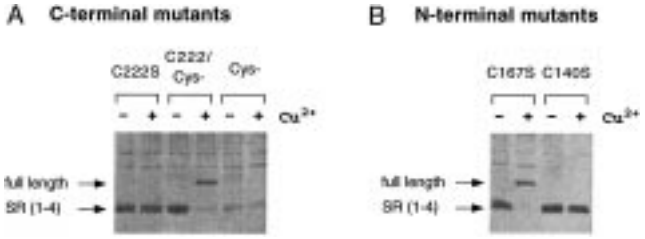

FIGURE 2: Cross-linking of SR(1−4/5−7) mutants in the dark. Con A-Western blots of SR(1−4/5−7) mutants were used to identify the Cys residues involved in cross-linking of SR(1−4) and SR(5−7) in the dark. (A) Cross-linking reactions of mutants of the C-terminal fragment SR(5−7) paired with a wild-type SR(1−4) N-terminal fragment. Mutants are C222S, SR(1−4/5−7:C222S); C222/Cys$^-$, SR(1−4/5−7:C222,Cys$^-$); and Cys$^-$, SR(1−4/5−7:Cys$^-$), as indicated above each column by the single-letter code for amino acid and by the designation Cys$^-$ for cysteine-free C-terminal fragment. C222/Cys$^-$ or C222,Cys$^-$ signifies that residue 222 is a Cys while the rest of the cysteines in the C-terminal fragment are mutated to serines. (B) Cross-linking reactions of mutants of the N-terminal fragment SR(1−4) paired with a wild-type SR(5−7) C-terminal fragment. From left to right, the lanes contain SR(1−4:C167S/5−7) and SR(1−4:C140S/5−7). $Cu(phen)_3^{2+}$ was added as indicated above each lane. The positions of full-length rhodopsin and the N-terminal SR(1−4) fragment are indicated by arrows on the left of each Western blot. All cross-linking reactions were for 40 min.

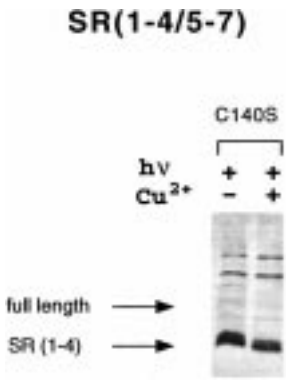

FIGURE 3: Cross-linking of SR(1−4:C140S/5−7) in the light. The cross-linking reactions were performed for 3 min after the samples were exposed to light for 30 s. The protein samples were treated without or with $Cu(phen)_3^{2+}$ as indicated above each lane. The positions of full-length rhodopsin and the N-terminal SR(1−4) fragment are indicated by arrows on the left of the Western blot.

We next identified the N-terminal Cys residue involved in the cross-linking of dark SR(1−4/5−7). Of the five Cys residues located in the N-terminal fragment SR(1−4) (i.e., Cys110, 140, 167, 185, 187), Cys110, 185, and 187 are unlikely to be involved because they are located in the extracellular domain of the protein, and Cys110 and 187 are known to be in a disulfide linkage with each other and unavailable for reaction (*18*, *20*). Therefore, we focused our attention on the remaining Cys140 and 167. As is shown in Figure 2B, when Cys167 is changed to Ser (mutant C167S), SR(1−4) and SR(5−7) are still able to cross-link. However, when Cys140 is changed to Ser (mutant C140S), the fragments no longer cross-link. Therefore, Cys140 forms a disulfide bond with Cys222 in the dark.

Disulfide Bond Formation in Photoactivated Rhodopsin. As shown in Figure 1, the two fragments of SR(1−4/5−7) are completely cross-linked within 3 min by treatment with 3 mM $Cu(phen)_3^{2+}$ if the protein is first exposed to light for 30 s. In contrast, the two fragments of the C140S mutant of

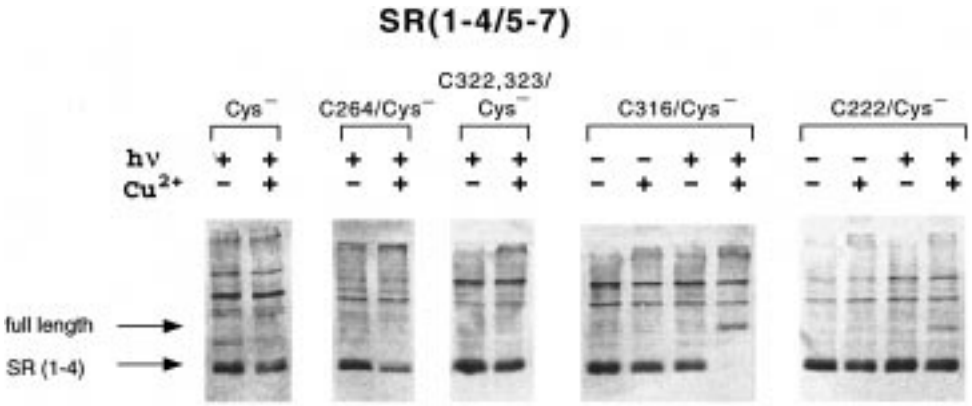

FIGURE 4: Cross-linking of SR(1−4/5−7) mutants in the light and in the dark. All the split receptors shown here contain a wild-type N-terminal fragment and mutant C-terminal fragments. From left to right, mutants are Cys$^-$, C264/Cys$^-$, C322,323/Cys$^-$, C316/Cys$^-$, and C222/Cys$^-$, as indicated above each column. The cross-linking reactions were done for 1 min either in the dark or after the samples were exposed to light for 30 s. For each mutant, samples were treated without or with $Cu(phen)_3^{2+}$ as indicated above each lane. The migration positions of full-length rhodopsin and the SR(1−4) fragment are indicated on the left of the Western blots.

SR(1−4/5−7) do not cross-link under the same conditions (Figure 3). Therefore, Cys140 is the N-terminal cysteine involved in the formation of a disulfide bond in the light-activated conformation of rhodopsin.

Identification of the C-terminal cysteine involved in the cross-linking reaction proved more difficult for two reasons. First, there are two cysteines that react with Cys140 in the photoactivated state of the protein, and second, mutation of Cys316 in rhodopsin results in incomplete palmitoylation of Cys322 and 323 (*21*), which then enables these residues to participate in cross-linking reactions that are not possible in the wild-type protein. As a result, single point mutations were not very informative, and the participating Cys were most readily identified from mutant fragments in which all but one of the Cys residues were removed. As can be seen in Figure 4, mutants in which only Cys264 (C264/Cys$^-$) or Cys322 and 323 (C322,323/Cys$^-$) were present are not cross-linked efficiently, eliminating Cys264, 322, and 323 from consideration as the residues responsible for cross-linking the two fragments of photoactivated SR(1−4/5−7). In contrast, mutants in which only Cys316 (C316/Cys$^-$) or Cys222 (C222/Cys$^-$) is present in the C-terminal fragments are still able to cross-link to the N-terminal fragments; Cys316 cross-links to completion, whereas cross-linking with Cys222 is partial under these conditions. These data demonstrate that Cys316 and 222 can participate in cross-linking reactions with Cys140 in the photoactivated state and suggest that Cys316 and 222 may compete for reaction with Cys140 in wild-type SR(1−4/5−7) and SR(1−5/6−7).

Competitive formation of the Cys140/222 and Cys140/316 disulfide bonds would nicely account for the observation that the fragments of SR(1−5/6−7) only partially cross-link after exposure to light and $Cu(phen)_3^{2+}$. Presumably the remaining population of split receptor not cross-linked as judged by the mobility on nonreducing SDS−polyacrylamide gels contains a disulfide bond between Cys140 and 222, which is internal to the N-terminal fragment in SR(1−5/6−7) and, therefore, not detected in our assay. In agreement with this explanation, when Cys222 is changed to Ser in SR(1−5/6−7) the fraction of cross-linked fragments dramatically increases with essentially complete cross-linking after 5 min under these conditions (Figure 5). Therefore, we conclude that Cys140 reacts with both Cys316 and Cys222 in the photoactivated state of rhodopsin.

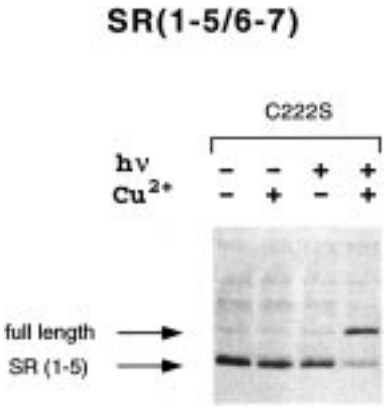

FIGURE 5: Cross-linking of SR(1−5:C222S/6−7) in the dark and in the light. Split receptor SR(1−5:C222S/6−7) has a C222S mutation in its N-terminal fragment and a wild-type C-terminal fragment. The Western blot has four lanes: in the dark without $Cu(phen)_3^{2+}$, in the dark with $Cu(phen)_3^{2+}$, in the light without $Cu(phen)_3^{2+}$, and in the light with $Cu(phen)_3^{2+}$. The cross-linking reactions in the dark were for 45 min and the cross-linking reactions in the light were for 5 min after exposure to light for 30 s. The positions of full-length rhodopsin and SR(1−5) fragment are indicated on the left of the Western blot.

DISCUSSION

There are two main conclusions to be drawn from these studies. First, Cys140 and 222 form a disulfide bond in the dark state of rhodopsin upon treatment with $Cu(phen)_3^{2+}$, and, therefore, these two residues must be relatively close to each other in the three-dimensional structure of the protein. Second, upon light activation and treatment with $Cu(phen)_3^{2+}$, the reactivity of the native cysteine residues changes. Cys140 and 222 still form a disulfide in the light-activated state but with a significantly faster rate than in the dark. In addition, a new disulfide bond, not seen in the dark state, is formed between Cys140 and 316.

These data are the first to show state-dependent formation of disulfide bonds in rhodopsin. Although a detailed structural interpretation cannot be made from this limited data set, it is clear that significant structural changes take place

upon photoactivation, and a signature of the photoactivated state is (1) the increased reactivity of Cys140 and 222 and (2) the new disulfide between Cys140 and 316, which is not seen in the dark state. It is possible that the increased reactivity of Cys316 results from structural changes in the seventh transmembrane segment of rhodopsin; perhaps the release of structural constraints increases flexibility of the polypeptide chain surrounding Cys316 such that the thiol can sample a larger reactive volume in the activated state. Recently, Abdulaev and Ridge (*22*) have shown that an antibody specific for amino acids 304−311 of rhodopsin binds to the native protein only after exposure to light and consequent formation of the activated state metarhodopsin II. Their results and ours are consistent with a significant structural change in the cytoplasmic portion of the seventh transmembrane segment of rhodopsin upon light activation, as is also the observation of Khorana and Hubbell and co-workers (*23*) that under some conditions an electron paramagnetic resonance (EPR) spin-label attached to Cys316 experiences increased mobility after formation of metarhodopsin II.

It is clear from these studies that this method can be used not only to probe the dark-state structure of rhodopsin but also to investigate the structural changes that occur upon receptor activation. The dark-state cross-linking identified between Cys140 and 222 suggests the spatial proximity of the cytoplasmic ends of TM3 and TM5, which we further investigate in the following paper (*5*).

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REFERENCES

1. Yu, H., Kono, M., McKee, T. D., and Oprian, D. D. (1995) *Biochemistry 34*, 14963−14969.
2. Struthers, M., Yu, H., Kono, M., and Oprian, D. D. (1999) *Biochemistry 38*, 6597−6603.
3. Falke, J. J., and Koshland, D. E., Jr. (1987) *Science 237*, 1596−1600.
4. Lee, G. F., Burrows, G. G., Lebert, M. R., Dutton, D. P., and Hazelbauer, G. L. (1994) *J. Biol. Chem. 269*, 29920−29927.
5. Yu, H., and Oprian, D. D. (1999) *Biochemistry 38*, 12033−12040.
6. Molday, R. S., and MacKenzie, D. (1983) *Biochemistry 22*, 653−660.
7. Cuatrecasas, P. (1970) *J. Biol. Chem. 245*, 3059−3065.
8. Franke, R. R., Sakmar, T. P., Oprian, D. D., and Khorana, H. G. (1988) *J. Biol. Chem. 263*, 2119−2122.
9. Oprian, D. D., Molday, R. S., Kaufman, R. J., and Khorana, H. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8874−8878.
10. Oprian, D. D., Asenjo, A. B., Lee, N., and Pelletier, S. L. (1991) *Biochemistry 30*, 11367−11372.
11. Oprian, D. D. (1993) *Methods Neurosci. 15*, 301−306.
12. Zhukovsky, E. A., and Oprian, D. D. (1989) *Science 246*, 928−930.
13. Zhukovsky, E. A., Robinson, P. R., and Oprian, D. D. (1991) *Science 251*, 558−560.
14. Zhukovsky, E. A., Robinson, P. R., and Oprian, D. D. (1992) *Biochemistry 31*, 10400−10405.
15. Struthers, M., and Oprian, D. D. (1999) *Methods Enzymol.* (in press).
16. Kono, M., and Oprian, D. D. (1999) in *Structure−Function Analysis of G Protein-Coupled Receptors* (Wess, J., Ed.) John Wiley & Sons, New York (in press).
17. Burnette, W. N. (1981) *Anal. Biochem. 112*, 195−203.
18. Kono, M., Yu, H., and Oprian, D. D. (1998) *Biochemistry 37*, 1302−1305.
19. Azen, E. A., and Yu, P. L. (1984) *Biochem. Genet. 22*, 1−19.
20. Karnik, S. S., and Khorana, H. G. (1990) *J. Biol. Chem. 265*, 17520−17524.
21. Karnik, S. S., Ridge, K. D., Bhattacharya, S., and Khorana, H. G. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 40−44.
22. Abdulaev, N. G., and Ridge, K. D. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 12854−12859.
23. Resek, J. F., Farahbakhsh, Z. T., Hubbell, W. L., and Khorana, H. G. (1993) *Biochemistry 32*, 12025−12032.

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