Tertiary Interactions between Transmembrane Segments 3 and 5 near the Cytoplasmic Side of Rhodopsin†

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ABSTRACT: Previous studies [Yu, H., Kono, M., and Oprian, D. D. (1999) *Biochemistry 38*, xxxx−xxxx] using split receptors and disulfide cross-linking have shown that native cysteines 140 and 222 on the cytoplasmic side of transmembrane segments (TM) 3 and 5 of rhodopsin, respectively, can cross-link to each other upon treatment with the oxidant $Cu(phen)_3^{2+}$. In this paper we show that although the 140−222 cross-link does not affect the spectral properties of rhodopsin, it completely and reversibly inactivates the ability of the receptor to activate transducin. Following on this lead we further investigate the cytoplasmic region of TM3 and TM5 and identify three additional pairs of residues that when changed to Cys are capable of forming disulfide cross-links in the protein: 140/225, 136/222, and 136/225. These disulfides are able to form without addition of the $Cu(phen)_3^{2+}$ oxidant. Similar to the 140−222 cross-link, none of the additional disulfides affect the spectral properties of rhodopsin. Also like the 140−222 bond, the 136−222 disulfide completely and reversibly inactivates the light-dependent activation of transducin by the receptor. In contrast, the 140−225 and 136−225 disulfides have no effect on the ability of rhodopsin to activate transducin. The pattern of cross-linking observed in Cys and disulfide scans of the protein is consistent with helical secondary structure in TM3 from 130 to 142 and in TM5 from 218 to 225.

In an investigation of the state-dependent formation of disulfide bonds in rhodopsin presented in the preceding paper in this issue (*1*), we identified a disulfide bond that forms between Cys140 of TM3 and Cys222 of TM5 upon treatment of the protein with a $Cu(phen)_3^{2+}$ oxidant. This observation led us to further investigate the secondary structure of and tertiary contacts between these TM[1] segments. In studies presented here we used a milder and more selective method of disulfide bond formation involving oxidation by ambient oxygen (*2*) for Cys-scanning mutagenesis and cross-linking of the cytoplasmic ends of TM3 and TM5. We identify three additional cross-links between these two segments and further analyze the effect of each of these disulfide bonds on the spectral properties and catalytic activity of the pigments.

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). DE-52, 1,-10-phenanthroline (phen), Sepharose 4B, and biotinylated Con A were from Sigma (St. Louis, MO). *N*-Ethylmaleimede (NEM) was from Aldrich. [^{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).

The monoclonal antibody rho 1D4, which is specific for the C-terminus of rhodopsin, has been described previously (*3*). The antibody was coupled to Sepharose 4B by the method of Cuatrecasas (*4*). 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 nomenclature and construction of the split receptors have been described previously (*5*). The split opsin construct used in this study is SR(1−4/5−7) (Figure 1). 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

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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.

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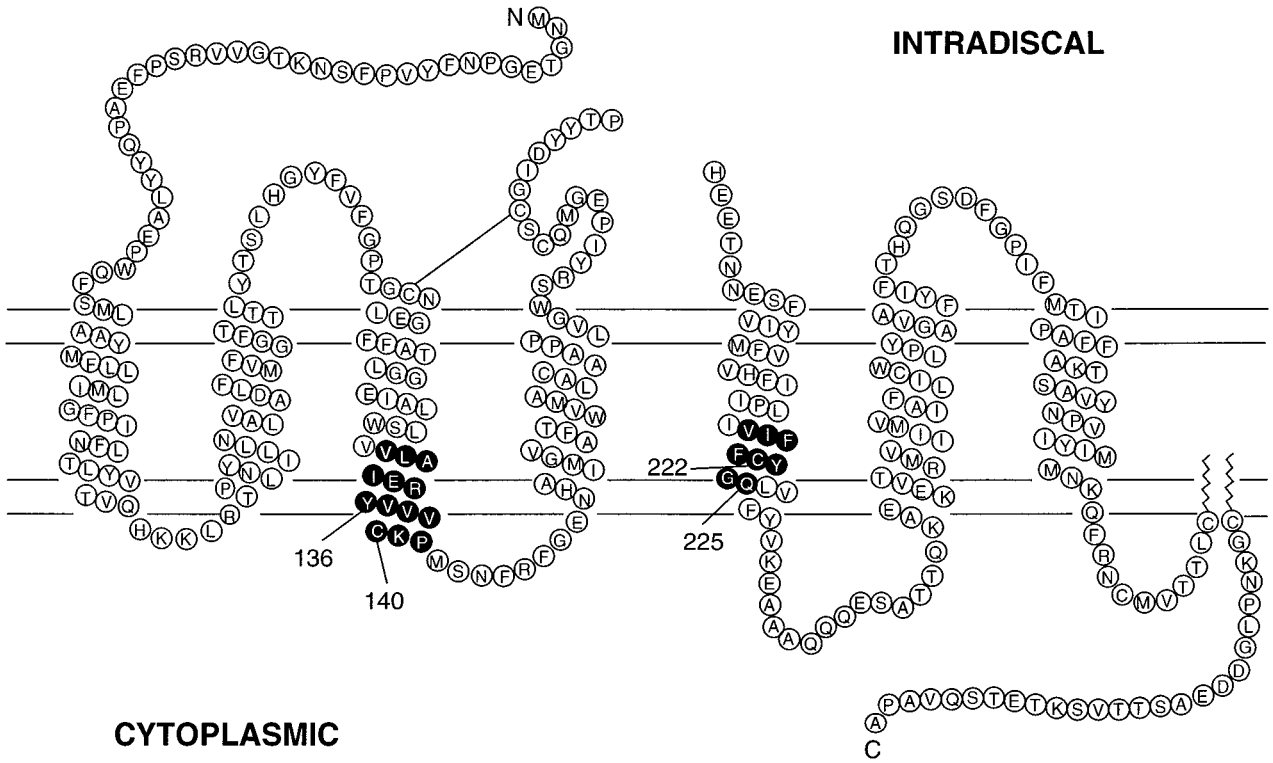

FIGURE 1: Schematic representation of the structure of the split rhodopsin SR(1−4/5−7) used in this study. The protein is split between Pro194 and His195 in the loop connecting TM4 and 5. The C-terminal fragment SR(5−7) contains an initiator Met residue (not shown in the figure) in front of the N-terminal His195. Amino acids replaced by single cysteine substitutions are indicated in black (residues 130−142 on TM3 and 218−225 on TM5).

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).

The N- and C-terminal fragments of each split construct are encoded on separate pMT3 (*6*) 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 (*5, 7*). Transfected COS cells were harvested 72 h after initial exposure to DEAE-dextran and DNA. Absorption spectra were recorded on samples of pigment reconstituted and purified from 10 100-mm plates of cultured cells. 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 (*7*−*12*). These procedures were modified for single-plate transfections (*13, 14*) in the analysis of Cys scans by Western blot.

Absorption Spectroscopy. UV/visible absorption spectra were recorded from the samples on a Hitachi Model U-3210 spectrophotometer that was specifically modified by the manufacturer for use in a darkroom. Data were collected with an IBM-compatible microcomputer using Spectra Calc software from Galactic Industries Corp. (Salem, NH). All spectra were recorded on samples of 1.0 cm path length.

Assay for Activation of Transducin. Transducin was purified from bovine retinas according to the procedure of Wessling-Resnick and Johnson (*15*) and then subjected to ion-exchange chromatography on DE−52 as described by Baehr et al. (*16*). As a final step, the protein was dialyzed against 10 mM Tris buffer (pH 7.5) containing 50% (v/v) glycerol and 2 mM $MgCl_2$ and then stored at −20 °C. The wild-type and mutant rhodopsins were assayed for their ability to catalytically activate transducin by following the binding of [^{35}S]GTPγS as has been described previously (*11*). The assays contained 5 nM rhodopsin (as determined by visible absorption spectroscopy assuming the mutations had no effect on extinction coefficient of the pigments) in 0.01% (w/v) DM.

Disulfide Cross-Linking and Reduction of the Disulfide Bonds. 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. (*17*) for the time and temperature indicated in the figure legends. Alternatively, spontaneous disulfide bond formation (i.e., without exogenously added oxidant) was promoted by incubation at pH 7.0 and 25 °C for the indicated time. 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.

To obtain spectra of the split receptors treated with $Cu(phen)_3^{2+}$, samples were passed through 5 mL Sephadex G-50 spin columns to remove $Cu(phen)_3^{2+}$, and absorption spectra were immediately recorded. Bleached difference spectra were obtained as follows: the sample was exposed to light for 1 min using a 300-W slide projector with a 490 nm cut-on filter, and the bleached spectrum was recorded

and presented as a bleached minus dark-state difference spectrum. Acid-trapped difference spectra were obtained as follows: 10 μL of concentrated HCl was added to the bleached sample and the spectrum was recorded again and presented as the acid-trapped minus bleached difference spectrum.

Cross-linked samples prepared for transducin assays were similarly passed over G-50 spin columns to remove $Cu(phen)_3^{2+}$. If indicated, the samples were reduced by 25−50 mM DTT (final concentration) at 25 °C for 19−22 h.

Western Blot Analysis. The purified proteins were visualized on Western blots (*18*) by probing for the N-terminal fragments with biotinylated Con A (*19*), similar to the method of Azen and Yu (*20*). After cross-linking, the samples were separated by SDS−PAGE on nonreducing 12.5% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes using a Bio-Rad trans-blot SD semidry electrophoretic transfer cell. 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. The blots were then treated with 3 μL of alkaline phosphatase-conjugated streptavidin (Promega) in 15 mL of the Con A buffer with NP-40 for 1 h at room temperature. The membranes were then washed three times with Con A buffer containing 0.05% NP-40. 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 recognize the N-terminal fragment. Thus, only the N-terminal fragments that copurified with C-terminal fragments were detected.

RESULTS

Effect of the Cys140−222 Disulfide Bond on Activity of Rhodopsin. In previous studies the split rhodopsin SR(1−4/5−7) has been shown to have spectral properties and specific activity for light-dependent activation of transducin that are essentially indistinguishable from those of wild-type rhodopsin (*5*, *21*, *22*). In the preceding paper (*1*) we show that native cysteines at positions 140 and 222 in SR(1−4/5−7) form a disulfide bond upon treatment with $Cu(phen)_3^{2+}$. To assess the effect of the 140−222 disulfide bond on the spectral properties and activity of the protein, we treated purified SR(1−4/5−7) with $Cu(phen)_3^{2+}$ for 30 min at 37 °C in the dark to form the 140−222 disulfide bond and then passed the sample through a Sephadex G-50 spin column to remove the copper oxidant. As shown in Figure 2, the absorption spectrum of the cross-linked protein has a 500 nm absorption maximum characteristic of the wild-type pigment. Furthermore, when the sample was exposed to light, the spectrum shifted to that with a maximum at 380 nm, characteristic of the activated intermediate metarhodopsin II or MII (inset, curve a), which in turn could be trapped with acid as a protonated Schiff base with maximum at 440 nm (inset, curve b). Thus, the disulfide bond did not affect the spectral properties of the protein.

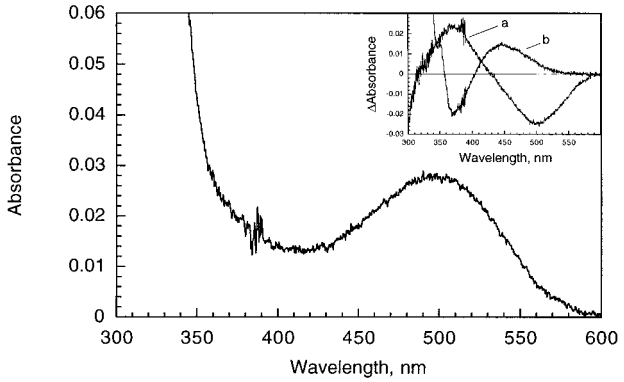

FIGURE 2: Absorption spectrum of the cross-linked form of wild-type SR(1−4/5−7) containing a 140−222 disulfide bond. Following purification, wild-type SR(1−4/5−7) was treated with $Cu(phen)_3^{2+}$ at 37 °C for 30 min (cross-linking verified in Figure 3). Inset: (a) Bleached difference spectrum with characteristic maximum at about 380 nm and minimum at 500 nm. (b) Acid-trapped difference spectrum with characteristic maximum at 440 nm and minimum at about 380 nm.

In contrast to the spectroscopic properties of the protein, the ability of SR(1−4/5−7) to activate transducin was dramatically affected by the 140−222 cross-link. As shown in Figure 3, treatment with $Cu(phen)_3^{2+}$ completely suppressed the light-dependent activation of transducin by SR(1−4/5−7). Subsequent treatment of the inactive, cross-linked protein with DTT resulted in restoration of the original electrophoretic mobility and full recovery of the activity, demonstrating that inactivation was (1) reversible and (2) due to the formation of a disulfide bond. The two single mutants, C140S and C222S, were not cross-linked or inactivated upon treatment with $Cu(phen)_3^{2+}$ (Figure 3). Therefore, inactivation of SR(1−4/5−7) upon treatment with $Cu(phen)_3^{2+}$ is specifically attributable to the disulfide between Cys140 and 222.

The Cys140−222 disulfide bond also forms in full-length, wild-type rhodopsin upon treatment with $Cu(phen)_3^{2+}$, as shown by the transducin activation data in Figure 4. Similar to SR(1−4/5−7), the spectral properties of full-length rhodopsin are unaffected (not shown) and the ability to activate transducin is lost upon treatment with the copper oxidant (Figure 4). Activity of the full-length rhodopsin is completely restored upon subsequent treatment with DTT. Since neither the C140S nor the C222S single mutant is inactivated by treatment with $Cu(phen)_3^{2+}$, it is clear that the Cys140−222 disulfide bond is also responsible for loss of activity in the full-length protein.

Cross-Linking between Cysteines 140 and 225. To further study the structure of TM3 and TM5 near the cytoplasmic side of rhodopsin, we introduced a series of single cysteine mutations into each fragment of SR(1−4/5−7) (Figure 1). Residues 130−142 on TM3 were individually changed to cysteines in a C140S background in SR(1−4). Residues 218−225 on TM5 were also individually changed to cysteines in a C222S background in SR(5−7).

We first tested the ability of Cys140 on TM3 to cross-link with Cys substitutions for residues 218−225 on TM5. Purified samples of protein were incubated for 2 h at pH 7.0 and 25 °C, after which the reactions were quenched with NEM and loaded onto a nonreducing SDS−PAGE gel. As shown in Figure 5A, the Cys substitution at position 225 is

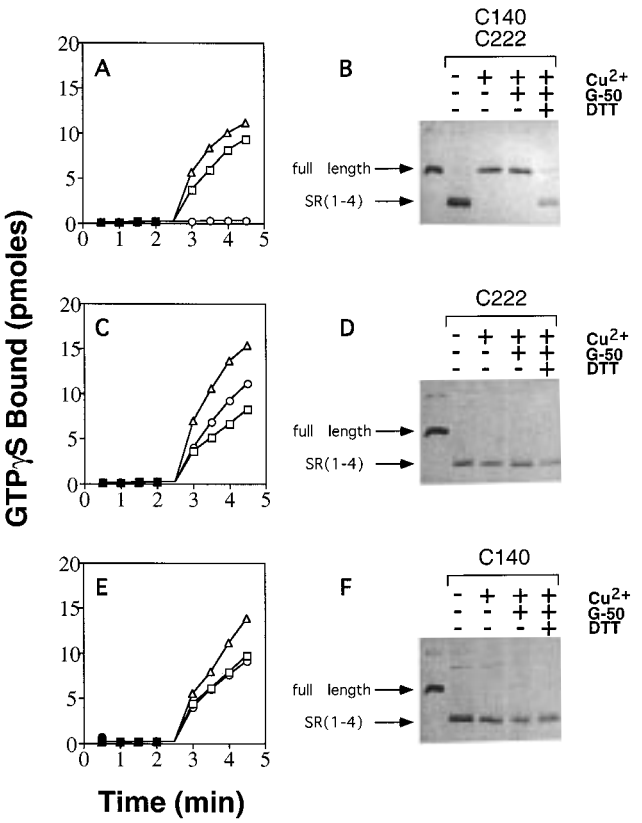

FIGURE 3: Effect of the 140−222 cross-link on the ability of SR(1−4/5−7) to activate transducin. The purified split rhodopsin mutants were treated with $Cu(phen)_3^{2+}$ at 37 °C for 30 min and then passed through a G-50 spin column to remove the $Cu(phen)_3^{2+}$. Reduced samples were prepared by treatment of the cross-linked sample with 25 mM DTT at room temperature for 19 h. (A, C, E) Time course for the activation of transducin by the cross-linked and reduced forms of each protein. (squares) Sample before treatment of receptors with the copper oxidant; (circles) after oxidation with and removal of $Cu(phen)_3^{2+}$; (triangles) oxidized samples following treatment with DTT. (solid symbols) Reaction in the dark; (open symbols) dark-reaction samples after exposure to light. (B, D, F) Western blots for each sample assayed in the left-hand panels of the figure. (A, B) Wild-type SR(1−4/5−7). (C, D) SR(1−4:C140S/5−7) mutant, which contains a Ser substitution at position 140 in fragment SR(1−4) and the wild-type Cys at position 222 in fragment SR(5−7). (E, F) SR(1−4/5−7:C222S) mutant, which contains the wild-type Cys at position 140 and a Ser substitution at position 222.

the only residue in this region of TM5 that cross-links with Cys140. Note that these cross-linking reactions were performed in the absence of $Cu(phen)_3^{2+}$. Thus the conditions under which the 140−225 disulfide forms are different from those for the 140−222 disulfide: the 140−225 disulfide forms spontaneously, whereas formation of the 140−222 bond requires the $Cu(phen)_3^{2+}$ oxidant. These data imply that 225 is in a more favorable position to react with 140 than is 222.

We next tested the spectral properties of the mutant receptor containing the 140−225 disulfide (Figure 5B). The spectrum for the cross-linked protein in the dark displays the 500 nm maximum characteristic of wild-type rhodopsin. Upon exposure to light, the spectrum shifts to the blue and exhibits a new maximum at 380 nm characteristic of the active intermediate MII in wild-type rhodopsin. The covalently bound chromophore was trapped with acid to give a protonated Schiff base with maximum at 440 nm, indicating

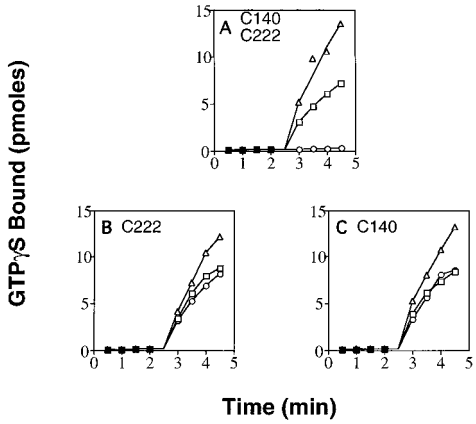

FIGURE 4: Effect of the 140−222 cross-link on the ability of full-length rhodopsin to activate transducin. The figure shows time courses for the activation of transducin by the cross-linked and reduced forms of wild-type rhodopsin and the two single Cys mutants C140S (C222) and C222S (C140). Samples were treated as described in the legend to Figure 3. (Squares) Protein samples before treatment with the $Cu(phen)_3^{2+}$; (circles) after oxidation and removal of copper on the G-50 column; (triangles) oxidized samples following treatment with DTT. (Solid symbols) Reaction in the dark; (open symbols) dark-reaction samples after exposure to light.

that the bleached spectrum represents an intermediate in the photocascade. Therefore, the disulfide cross-link between 140 and 225 does not affect the spectral properties of the protein.

The effect of the 140−225 disulfide on transducin activation was also tested. As shown in Figure 5C, the cross-linked and reduced forms of SR(1−4/5−7:Q225C,C222S) activate transducin in a light-dependent manner with essentially identical specific activity. Thus, the disulfide cross-link between Cys140 and 225 affects neither the spectral properties of the protein nor the ability to activate transducin.

Cross-Linking between Cysteines 136 and 222. We next tested the ability of Cys222 on TM5 to cross-link with Cys substitutions for residues 130−142 on TM3. As shown in Figure 6A, Cys136 cross-links to completion with Cys222 after incubation for 8 h at pH 7.0 and 25 °C. No other residue in this region of TM3 shows any significant ability to cross-link with Cys222 under these conditions.

The 136−222 cross-link has no effect on the spectral properties of the pigment (Figure 6B) but significantly inhibits the ability of the protein to activate transducin (Figure 6C). Although Cys136 and 222 cross-link spontaneously, the reaction is slow, and therefore, $Cu(phen)_3^{2+}$ was used to facilitate disulfide bond formation in the experiments presented in Figure 6B,C. Cys136 and 222 are not cross-linked immediately following elution of the protein from the immunoaffinity resin, and the reduced form of the protein has wild-type specific activity for activation of transducin. Following treatment with $Cu(phen)_3^{2+}$, the cross-linked protein is unable to activate transducin. Upon subsequent treatment with DTT, activity of the split receptor is restored (Figure 6C). Only about 70% of the original activity was recovered in this experiment, but this level of activity is consistent with the Western analysis, which indicates that reduction of the disulfide was not complete. These data demonstrate that the 136−222 disulfide bond is responsible for loss of activity.

Cross-Linking between Cysteines 136 and 225. Cys substitutions at positions 136−142 of TM3 in SR(1−4) were

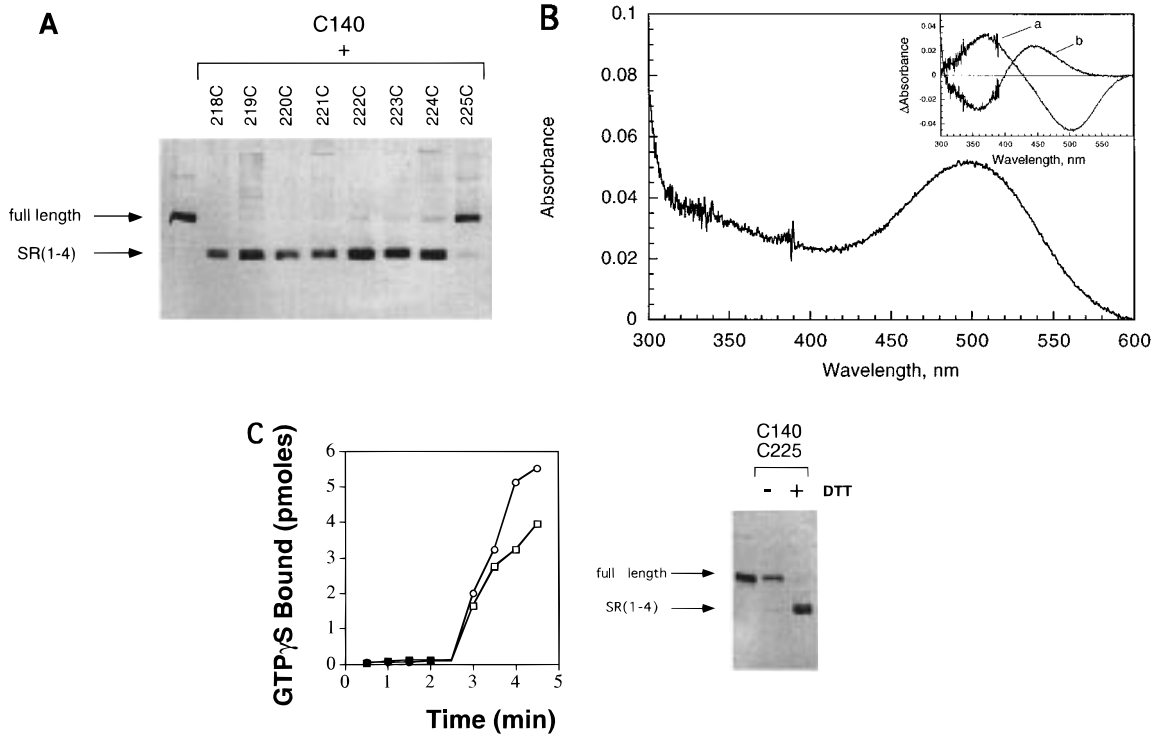

FIGURE 5: Disulfide cross-linking between 140 and 225. (A) Disulfide cross-linking reactions between single cysteine substitutions at positions 218−225 on TM5 and Cys140 on TM3. Each mutant receptor was purified from a single plate of COS cells transfected with the wild-type SR(1−4) and the indicated SR(5−7) mutants and then incubated at 25 °C for 2 h before addition of SDS−PAGE load buffer containing 12.5 mM NEM. The samples were then analyzed for disulfide cross-links by Western blot. From the left: full-length wild-type rhodopsin followed by split receptors with the indicated Cys substitutions in TM5. With the exception of Cys222, which contains the native cysteine at position 222, all of the other samples have the indicated mutations in a C222S background. The positions of full-length rhodopsin and the SR(1−4) fragment are indicated by arrows. (B) Absorption spectrum of the cross-linked form of SR(1−4/5−7:Q225C,C222S) containing a 140−225 disulfide bond. Following purification, the sample was incubated for 2 h at 25 °C to allow disulfide bond formation. The absorption spectrum for the dark state of the cross-linked receptor is shown in the main body of the figure. Inset: (a) Bleached difference spectrum with characteristic maximum at about 380 nm and minimum at 500 nm. (b) Acid-trapped difference spectrum with characteristic maximum at 440 nm and minimum at about 380 nm. (C) Effect of the 140−225 cross-link on the ability of SR(1−4/5−7:Q225C,C222S) to activate transducin. Following immunoaffinity purification, the split receptor was incubated at 25 °C for 2 h to allow the formation of a disulfide bond between Cys140 and Cys225. After removal of an aliquot for assay, the sample was reduced with DTT and again assayed for ability to activate transducin. Left panel, time course for activation of transducin by the cross-linked and reduced forms of SR(1−4/5−7:Q225C,C222S). (squares) Cross-linked form of SR(1−4/5−7:Q225C,C222S); (circles) reduced form of SR(1−4/5−7:Q225C,C222S). (Solid symbols) Reaction in the dark; (open symbols) reaction after exposure to light at 2.5 min. (Right panel) Western blot of samples used in the transducin assays shown on the left. From left to right, lanes on the Western blot contain full-length wild-type rhodopsin; disulfide cross-linked SR(1−4/5−7:Q225C,C222S); and the SR(1−4/5−7:Q225C,C222S) sample after reduction with DTT. The positions of full-length rhodopsin and N-terminal SR(1−4) fragment are indicated by arrows.

next tested for ability to cross-link with Cys225 of TM5 in SR(5−7). When disulfide bond formation was allowed to proceed at 25 °C for 2 h in the absence of $Cu(phen)_3^{2+}$, cysteines at positions 136, 139, and 140 were all found to cross-link with Cys225 (Figure 7A). The rates of cross-linking to Cys225 varied in the order 140 > 139 > 136 (not shown). The cross-linking reactions in this region of TM3 demonstrate a clear (*i*, *i* + *3*) and (*i*, *i* + *4*) pattern with respect to position 225 on TM5 and suggest that these residues (136−142) on TM3 form a helix.

Similarly, in data not shown, a clear pattern of helical secondary structure was evident when residues 218−225 of TM5 were tested for ability to cross-link with Cys136 of TM3 in SR(1−4/5−7). In this experiment, Cys222 and 225 form a disulfide bond with Cys136 at 25 °C for 20 h. Cys223 also reacts with Cys136, but much more slowly than either Cys222 or 225. Cys218, 219, 220, 221, and 224 show no significant reaction with Cys136 at all.

The Cys 136−225 disulfide bond forms more rapidly than does the 136−222 cross-link, and like the 136−222 bond, the 136−225 bond has no effect on the spectral properties of the protein (Figure 7B). However, unlike the 136−222 disulfide, the 136−225 cross-link has no effect on the ability of the protein to activate transducin (Figure 7C).

DISCUSSION

In the preceding paper (*1*) we showed that the native cysteines 140 of TM3 and 222 of TM5 in the split rhodopsin construct SR(1−4/5−7) form a disulfide cross-link after treatment with $Cu(phen)_3^{2+}$. Following this lead we further examined TM3 and TM5 near the cytoplasmic surface of the protein by Cys-scanning mutagenesis and identified three additional pairs of positions that can form disulfide bonds. In contrast to formation of the 140−222 disulfide, which had a strict requirement for the oxidant $Cu(phen)_3^{2+}$, these disulfides formed spontaneously, without the addition of $Cu(phen)_3^{2+}$. Disulfides 136−225 and 140−225 were identified from a scan of cysteine mutations from 130 to 142 in TM3 paired with a cysteine mutation at position 225 on TM5. This scan displayed a clear (*i*, *i* + *3*) and (*i*, *i* + *4*) pattern of reactivity consistent with an α-helical structure at the cytoplasmic end of TM3. A cysteine scan of positions from

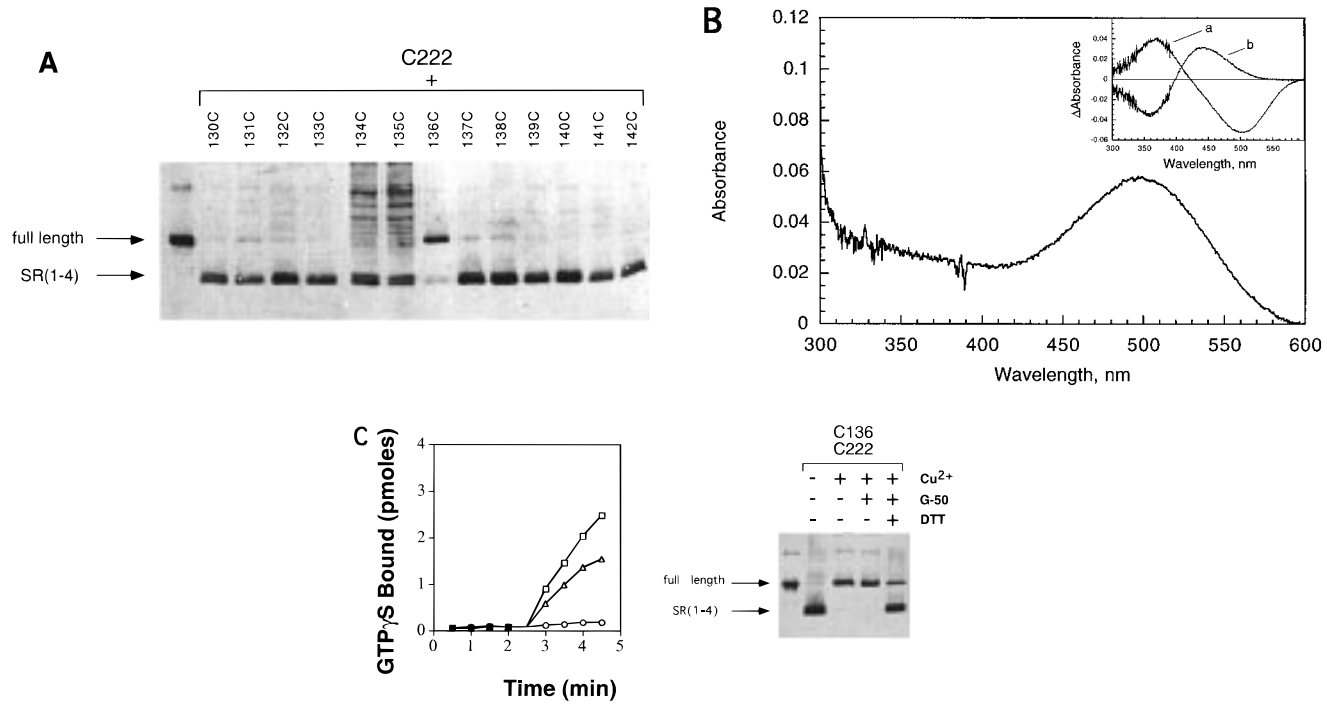

FIGURE 6: Disulfide cross-linking between 136 and 222. (A) Disulfide cross-linking reactions between single cysteine substitutions at positions 130−142 on TM3 and Cys222 on TM5. Purified receptors were incubated at 25 °C for 8 h before addition of SDS−PAGE load buffer containing 12.5 mM NEM. The samples were then analyzed for disulfide cross-links by Western blot. From the left: full-length wild-type rhodopsin followed by split receptors with various Cys substitutions in TM3 as indicated. With the exception of sample 140C, which contains the native cysteine at position 140, all of the other samples have the indicated mutations in a C140S background. Mutants 134C [SR(1−4:E134C,C140S/5−7)] and 135C [SR(1−4:R135C,C140S/5−7)] had low levels of expression, which required larger volumes of the purified samples for Western blot analysis. As a consequence, these samples have a higher proportion of Con A-reactive impurities. (B) Absorption spectrum of the cross-linked form of SR(1−4:Y136C,C140S/5−7) containing a 136−222 disulfide bond. Following purification, SR(1−4:Y136C,C140S/5−7) was treated with $Cu(phen)_3^{+2}$ at 25 °C for 2 h (although the 136−222 cross-link forms spontaneously, we found it convenient to catalyze formation of the disulfide for preparative-scale reactions). Inset: (a) Bleached difference spectrum with characteristic maximum at about 380 nm and minimum at 500 nm. (b) Acid-trapped difference spectrum with characteristic maximum at 440 nm and minimum at about 380 nm. (C) Effect of the 136−222 cross-link on the ability of SR(1−4:Y136C,C140S/5−7) to activate transducin. Following immunoaffinity purification, the split receptor mutant was treated with $Cu(phen)_3^{2+}$ at 25 °C for 2 h. Reduced samples were prepared by treatment with DTT. (Left panel) Time course for the activation of transducin by the cross-linked and reduced forms of the mutant protein. (Squares) Before treatment with $Cu(phen)_3^{2+}$; (circles) after treatment with $Cu(phen)_3^{2+}$ and passage through the G-50 column; (triangles) after reduction of the sample with DTT. (Solid symbols) Reaction in the dark; (open symbols) reaction after exposure to light at 2.5 min. (Right panel) Western blot of samples used in the transducin activation assays shown in the right panel of the figure. From left to right: full-length wild-type rhodopsin; SR(1−4:Y136C,C140S/5−7) before treatment with $Cu(phen)_3^{2+}$; after treatment with $Cu(phen)_3^{2+}$; after passage through the G-50 spin-column; and finally, after reduction with DTT. The positions of full-length rhodopsin and the N-terminal fragment SR(1−4) are indicated by arrows.

218 to 225 on TM5 paired with cysteine mutation at 136 on TM3 revealed cross-links between 136 and 222 and between 136 and 225. In this case, the (i, $i + 3$) pattern of reactivity of cysteine residues on TM5 suggests a discrete secondary structure that is most likely helical.

None of the disulfide bonds identified in this study had any effect on the spectral properties of the mutant receptors. The cross-linked mutants all displayed the 500 nm visible absorption maximum characteristic of the wild-type chromophore, which upon exposure to light shifted to 380 nm characteristic of the active MII intermediate. Thus all four disulfides (136−222, 136−225, 140−222, and 140−225) are compatible with an overall nativelike dark-state structure. These results indicate that the elements of secondary structure and tertiary contacts for TM3 and TM5 suggested by the cross-linking data are found in the native rhodopsin structure.

On the basis of electron micrographs of frozen-hydrated two-dimensional crystals of both frog and bovine rhodopsin, Schertler and co-workers (*23*, *24*) have produced an electron density map of the protein which indicates that helix 3 and helix 5 tilt toward each other on the cytoplasmic side of the protein. In fact, the electron density for helix 5 merges with that of helix 3 toward the cytoplasmic surface of the protein roughly 16 Å above the middle of the lipid bilayer (*23*). Using the electron density map, mutagenesis data, and sequence analysis for many G protein-coupled receptors, Baldwin et al. (*25*) have constructed an α-carbon atom model of rhodopsin in which helix 3 extends to residue 142 and helix 5 extends to residue 233 on the cytoplasmic side. Both the electron density map and the derivative model are in substantial agreement with the cross-linking data presented here and summarized in the helical wheel diagram for TM3 and TM5 in Figure 8.

Our cross-linking studies are also in good agreement with previous studies from Hubbell and Khorana and co-workers using site-directed EPR spin-labeling which suggest that positions 136 and 140 belong to the helical region of TM3 near the cytoplasmic surface (*26*) and that position 225 is also in a helical segment of protein near the cytoplasmic end of TM5 (*27*, *28*). However, our data indicate that residue

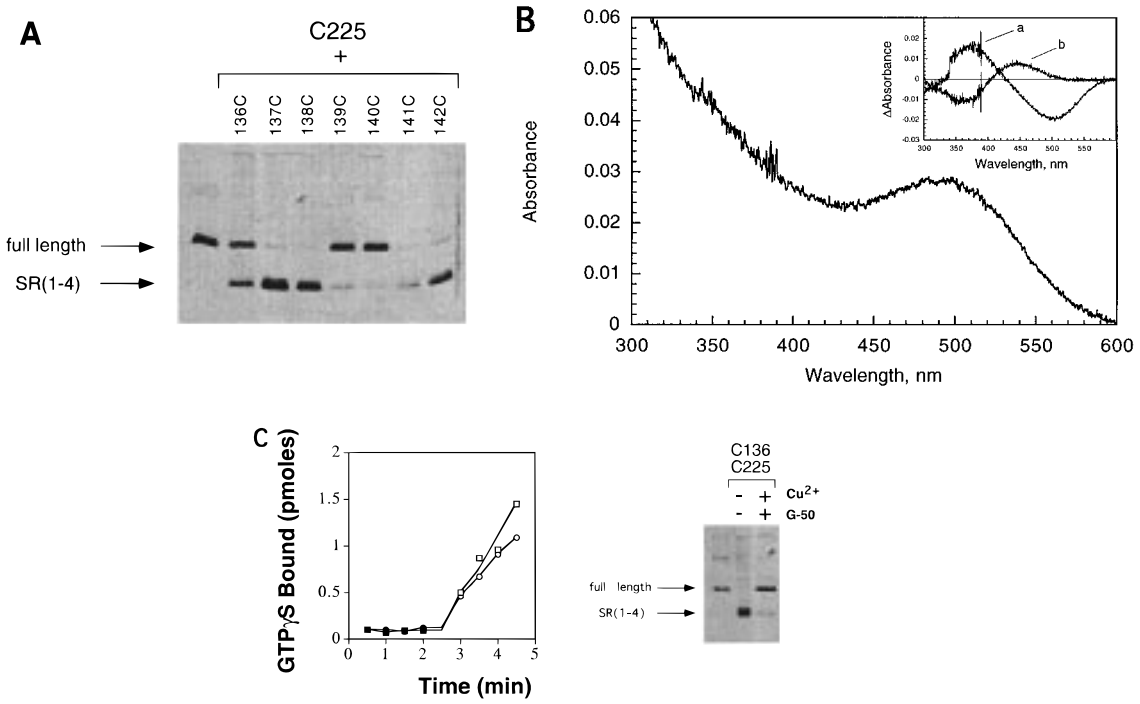

FIGURE 7: Disulfide cross-linking between 136 and 225. (A) Disulfide cross-linking reactions between single cysteine substitutions at positions 136−142 on TM3 and Cys225 on TM5. Purified receptors were incubated at 25 °C for 2 h before addition of SDS−PAGE load buffer containing 12.5 mM NEM. The samples were then analyzed for disulfide cross-links by Western blot analysis as shown in the figure. The leftmost lane of the blot contains full-length wild-type rhodopsin as a marker. The remaining lanes contain split receptors with various single Cys substitutions in TM3 of SR(1−4) (as indicated at the top of the blot) cotransfected with SR(5−7:Q225C,C222S). The positions of full-length rhodopsin and the SR(1−4) fragment are indicated by arrows. (B) Absorption spectrum of the cross-linked form of SR(1−4:Y136C,C140S/5−7:Q225C,C222S) containing a 136−225 disulfide bond. Following purification, SR(1−4:Y136C,C140S/5−7:Q225C,C222S) was treated with $Cu(phen)_3^{2+}$ (for convenience) at 25 °C for 19 h. Inset: (a) Bleached difference spectrum with characteristic maximum at about 380 nm and minimum at 500 nm. (b) Acid-trapped difference spectrum with characteristic maximum at 440 nm and minimum at about 380 nm. (C) Effect of the 136−225 cross-link on the ability of SR(1−4:Y136C,C140S/5−7:Q225C,C222S) to activate transducin. Following immunoaffinity purification, the split receptor mutant was treated with $Cu(phen)_3^{2+}$ (for convenience) at 25 °C for 19 h. (Left panel) Time course for the activation of transducin by the cross-linked and reduced forms of the mutant receptor. (Squares) Reduced form of SR(1−4:Y136C,C140S/5−7:Q225C,C222S) before treatment with $Cu(phen)_3^{2+}$; (circles) cross-linked form obtained after treatment with the copper oxidant. (Solid symbols) Reaction in the dark; (open symbols) reaction after exposure to light at 2.5 min. (Right panel) Western blot of samples used in the transducin activation assays shown in the right panel of the figure. From left to right: full-length wild-type rhodopsin; SR(1−4:Y136C,C140S/5−7:Q225C,C222S) before treatment with $Cu(phen)_3^{2+}$; and after treatment with $Cu(phen)_3^{2+}$ and passage through the G-50 column. The positions of full-length rhodopsin and the N-terminal fragment SR(1−4) are indicated by arrows.

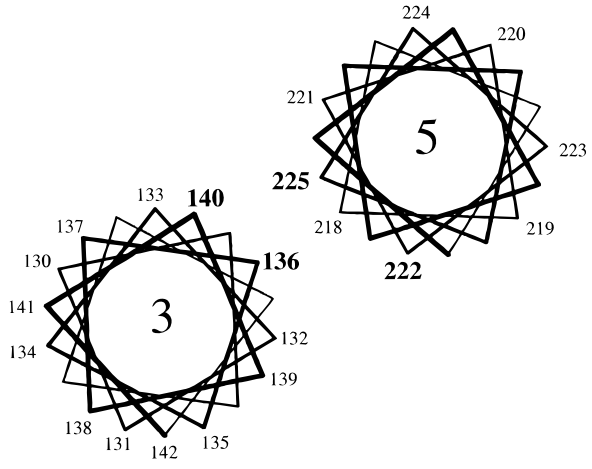

FIGURE 8: Helical wheel model of TM3 and 5 viewed from the cytoplasmic surface of rhodopsin. Model shows register and orientation of the two helices as suggested by the disulfide cross-linking data presented in this paper.

225 forms part of the face of helix 5, which is oriented more toward helix 3 than is indicated in models based on the EPR experiments.

The wild-type spectral properties of all four cross-linked split receptors led us to investigate the effect of these disulfides on the light activation of the receptors by determining the ability of the cross-linked proteins to activate the G protein transducin. Although none of the disulfides affected the spectral properties, formation of either the 136−222 or the 140−222 disulfide resulted in complete and reversible inhibition of transducin activation by the split receptor mutants. In contrast, disulfides between these same cysteines at 136 and 140 on TM3 with cysteine 225 on TM5 had no effect on transducin activation. Thus, closely spaced disulfides can have very different effects on receptor activation; cross-links to 222 inhibit activation, while cross-links to 225 do not. Therefore, it is difficult to clearly interpret these results in terms of the structural rearrangement that occurs upon receptor activation. Since this region of rhodopsin has been implicated in a direct interaction with transducin (*29*−*32*), the inhibition by the 140−222 and 136−222 disulfides might result from disruption of this interaction. It is clear from these data that inhibition of G protein coupling by receptor cross-linking should be interpreted with care. While inhibition of receptor activation by an engineered disulfide may result from restricting structural rearrangements

in the protein, a complete investigation of the region of interest to identify possible cross-links that produce active proteins should also be undertaken. On the other hand, when receptor activation is not inhibited by a disulfide cross-link, a clear and unambiguous interpretation may be made that movement of those two residues away from each other is not required for activation of the protein.

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