Disulfide Bond Exchange in Rhodopsin†

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ABSTRACT: Rhodopsin contains two cysteines (Cys110 and Cys187) that are highly conserved among members of the G protein coupled receptor family and that form a disulfide bond connecting helixes 3 and 4 on the extracellular side of the protein. However, recent work on a rhodopsin mutant split in the cytoplasmic loop connecting helixes 3 and 4 has shown that the amino- and carboxy-terminal fragments of this split protein do not comigrate on nonreducing SDS−PAGE gels, suggesting that the native Cys110−Cys187 disulfide bond is not present in this mutant [Ridge et al. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3204−3208; Yu et al. (1995) *Biochemistry 34*, 14963−14969]. We show here that the inability to observe the disulfide bond on SDS gels is the result of a disulfide bond exchange reaction which occurs when this split rhodopsin is denatured in preparation for SDS−PAGE. Cys185 reacts with the native disulfide, displacing Cys110 and forming a new disulfide with Cys187. If the sulfhydryl-specific reagent *N*-ethylmaleimide is included in the sample during preparation for electrophoresis or if Cys185 is changed to Ser, the two fragments do comigrate with full-length rhodopsin on SDS gels and, therefore, are connected by the native Cys110−Cys187 disulfide bond. In related experiments, we find no evidence that the Cys110−Cys187 disulfide bond is broken upon formation of the active intermediate metarhodopsin II.

Rhodopsin is a light-activated member of the G protein coupled receptor (GPCR) superfamily. It contains 10 native cysteines, two of which, Cys110 and Cys187, are highly conserved among GPCRs (*1*) and form a native disulfide between helixes 3 and 4 on the extracellular side of the membrane (*2*).

Recently, our group (*3*) and Ridge et al. (*4*) described reconstitution of a rhodopsin mutant that was expressed from two separate gene fragments split in the cytoplasmic loop connecting helixes 3 and 4 [SR(1−3/4−7)] (Figure 1). Spectrally and functionally, this two-fragment rhodopsin behaved like wild-type. Surprisingly, when this split protein was subjected to SDS−PAGE under nonreducing conditions, the two fragments did not comigrate with the full-length protein as would have been expected if the two fragments were linked by the Cys110−Cys187 disulfide bond.

We show here that the reason the fragments were not linked on SDS gels is that a disulfide bond exchange reaction takes place upon denaturation of the protein. Thus, SR(1−3/4−7) does indeed contain the same Cys110−Cys187 disulfide bond found in wild-type rhodopsin. Upon denaturation, Cys185 reacts with Cys187 to form a new disulfide bond, displacing Cys110 and releasing the amino-terminal fragment SR(1−3) such that the amino-terminal and carboxy-terminal fragments do not comigrate on SDS gels. Reaction with the sulfhydryl-specific reagent *N*-ethylmaleimide (NEM) or mutation of Cys185 preserves the native disulfide bond in the denatured protein, and under these conditions, the split protein comigrates with the full-length protein. We also find no evidence to support the postulate that the native disulfide in rhodopsin is broken upon formation of the active intermediate metarhodopsin II (MII).

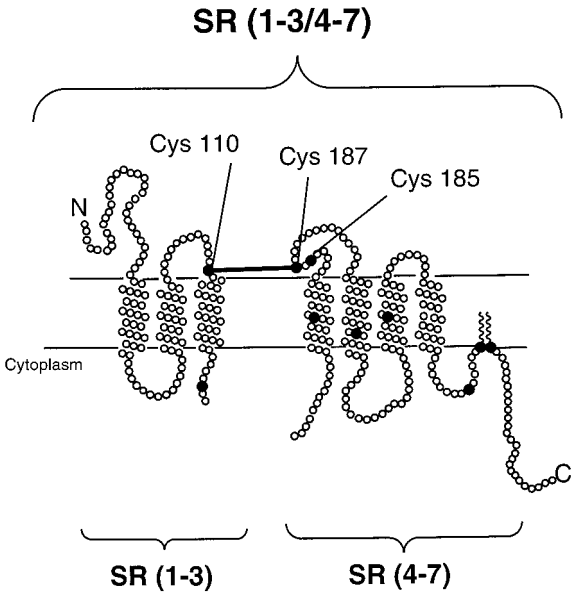

FIGURE 1: Schematic diagram of split rhodopsin SR(1−3/4−7). The locations of the 10 native cysteines are indicated in the figure as solid circles. The native disulfide bond is between the highly conserved residues Cys110 and Cys187. SR(1−3/4−7) is split between residues Pro142 and Met143 in the cytoplasmic loop connecting transmembrane helixes 3 and 4.

EXPERIMENTAL PROCEDURES

Materials. 11-*cis*-Retinal was kindly supplied by the National Institutes of Health and Dr. Rosalie Crouch of the

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Medical University of South Carolina. Biotinylated concanavalin A (Con A) was from Sigma (St. Louis, MO). [^{35}S]-GTPγS (1156 Ci/mmol) was from NEN. NEM was from Aldrich. Nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and conjugated streptavidin alkaline phosphatase used for Con A blot analysis were from Promega (Madison, WI).

Mutagenesis and Expression of the Rhodopsin and Split Rhodopsin Genes. Our nomenclature for the split rhodopsin construct was adapted from Kobilka et al. (*5*) as described in Yu et al. (*3*). SR(1−3) refers to the amino-terminal split receptor fragment containing the first 3 helixes (Met1 to Pro142); SR(4−7) refers to the carboxy-terminal split receptor fragment containing the last 4 helixes (Met143 to Ala348); and SR(1−3/4−7) refers to the reconstituted split protein containing SR(1−3) and SR(4−7) (Figure 1). The genes for SR(1−3) and SR(4−7) were contained in separate plasmids and were used to transiently cotransfect COS cells (*3*, *6*). The proteins were reconstituted and purified in 0.1% dodecyl maltoside as described in detail previously (*3*, *6*−*10*). These samples were then used for absorption spectroscopy, determination of light-dependent activation of transducin, and SDS−PAGE as described (*3*).

Con A Blots. Immunopurified rhodopsin and split rhodopsin were subjected to electrophoresis on 12% SDS−polyacrylamide gels essentially as described by Laemmli (*11*) except that the gel load buffer was as specified in the text and figure legends. The proteins were then transferred to nitrocellulose filters and probed with Con A in a procedure modified from those described by Azen and Yu (*12*) and Clegg (*13*). Briefly, the nitrocellulose blot was treated with a solution of 50 mM HEPES buffer, pH 7.0, containing 2% (w/v) bovine albumin, 100 mM NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ for 1 h at 37 °C and then incubated overnight with 0.001% (w/v) biotinylated Con A. After removal of Con A, the blot was treated with streptavidin alkaline phosphatase and visualized by reaction with NBT and BCIP according to directions supplied by the manufacturer (Promega). Con A reacts with N-linked oligosaccharyl chains at positions Asn2 and Asn15 in the amino terminus of rhodopsin (*14*). Thus, only SR(1−3) is detected on Con A blots of the split rhodopsin mutant SR(1−3/4−7). Two closely migrating bands are observed on the blots for SR(1−3), reflecting presumably heterogeneous glycosylation of the amino terminus in COS cells (*4*).

RESULTS

When the split rhodopsin mutant SR(1−3/4−7) is denatured in gel load buffer (final concentration of 2% SDS, 6% sucrose, 0.005% bromophenol blue, and 60 mM Tris, pH 6.8) and subjected to nonreducing SDS−PAGE, the amino-terminal fragment migrates with a mobility characteristic of free SR(1−3) and not that of full-length rhodopsin. This result was reported previously by our group (*3*) and Ridge et al. (*4*) and is reproduced in Figure 2, lane 2. If, however, the sample is denatured in gel load buffer containing NEM (12.5 mM final concentration), the protein runs on nonreducing SDS gels with a mobility identical to that of full-length rhodopsin (Figure 2, lane 3), as would be expected if the native disulfide bond were intact. That the linkage between fragments is indeed a disulfide bond is shown by

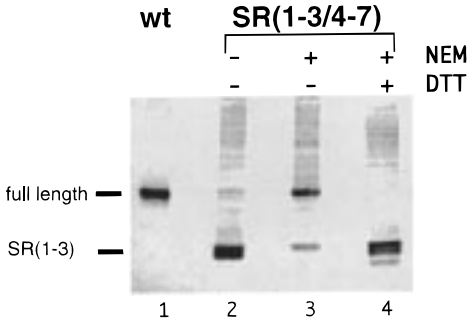

FIGURE 2: Con A blot of SR(1−3/4−7): lane 1, full-length rhodopsin (wt) used as a molecular size marker; lane 2, SR(1−3/4−7) solubilized in gel load buffer (final concentration of 2% SDS, 6% sucrose, 0.005% bromophenol blue, and 60 mM Tris, pH 6.8); lane 3, SR(1−3/4−7) solubilized in gel load buffer containing NEM (final concentration of 12.5 mM); lane 4, SR(1−3/4−7) solubilized in gel load buffer containing NEM (final concentration of 12.5 mM) and then treated with 50 mM DTT at room temperature for 30 min before loading the sample onto the gel. The residual material migrating with free SR(1−3) in lane 3 is likely due, at least in part, to incomplete quenching of Cys185 with NEM before it has had a chance to react with Cys187 in the disulfide bond exchange reaction (see text and Figure 3). The high molecular weight materials observed in lanes 2−4 are impurities in this SR(1−3/4−7) preparation that react with Con A.

the fact that the band migrates with the mobility of free SR(1−3) after treatment with 50 mM DTT (Figure 2, lane 4).

The preservative effect of NEM on the Cys110−Cys187 disulfide bond in rhodopsin suggests that the thiolate of another Cys residue reacts in a disulfide exchange reaction with either Cys110 or Cys187 upon denaturation of the protein. A likely candidate for the thiolate of the exchange reaction is Cys185, which lies two residues from Cys187 in the carboxy-terminal fragment SR(4−7). The thiol of Cys185 is buried and inaccessible in native rhodopsin (*15*) but may become exposed and modified by NEM upon denaturation of the protein before the exchange reaction can occur. If this model for disulfide exchange is correct, then mutation of Cys185 should also preserve the Cys110−Cys187 disulfide bond. In accord with this prediction, the split receptor mutant SR(1−3/4−7:C185S), in which Cys185 is changed to Ser, comigrates with full-length rhodopsin on SDS−PAGE gels even when the sample is prepared for electrophoresis in gel load buffer not containing NEM (Figure 3, lane 2). That the observed mobility is due to a disulfide bond connecting the amino- and carboxy-terminal fragments is demonstrated by the fact that treatment with 50 mM DTT increases mobility of the band to that observed for the free amino-terminal fragment SR(1−3) (Figure 3, lanes 3 and 5).

From an analysis of absorption spectra and activity of the rhodopsin mutants, we conclude that the Cys185 to Ser mutation in SR(1−3/4−7) does not globally disrupt the native structure of the protein (Figure 4). Rhodopsin has a distinctive absorption maximum at 500 nm. Upon illumination, the maximum shifts to 380 nm (*16*), characteristic of the active intermediate MII (*17*). Like SR(1−3/4−7) (Figure 4a), the Cys185 mutant SR(1−3/4−7:C185S) displays a wild-type absorption spectrum with a maximum at 500 nm, a wild-type photoconversion to MII, and a wild-type specific activity for light-dependent activation of transducin (Figure 4b).

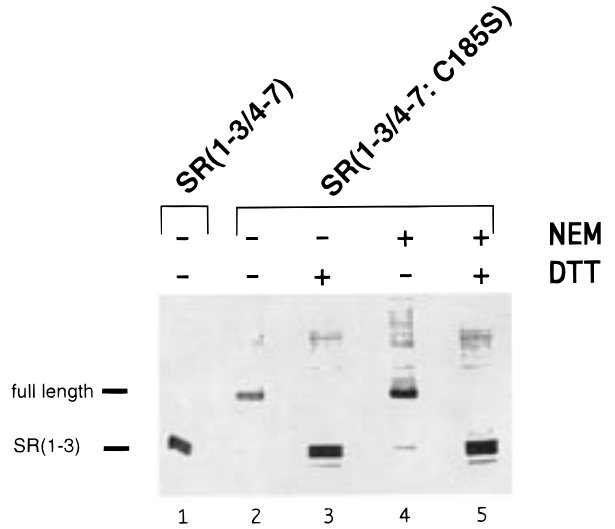

FIGURE 3: Con A blot of the SR(1−3/4−7:C185S) mutant: lane 1, SR(1−3/4−7) solubilized in gel load buffer; lane 2, SR(1−3/4−7:C185S) solubilized in gel load buffer; lane 3, SR(1−3/4−7:C185S) solubilized in gel load buffer and then treated with 50 mM DTT; lane 4, SR(1−3/4−7:C185S) solubilized in gel load buffer containing NEM (final concentration of 12.5 mM); lane 5, SR(1−3/4−7:C185S) solubilized in gel load buffer containing NEM (final concentration of 12.5 mM) and then treated with 50 mM DTT.

We also investigated whether the Cys110−Cys187 disulfide bond breaks upon formation of MII. When SR(1−3/4−7) is illuminated with >490 nm light for 1 min and then incubated for various lengths of time before quenching in gel load buffer (in the presence of NEM), the protein comigrates on SDS gels with full-length rhodopsin, suggesting that the native disulfide bond is not broken in this reaction (Figure 5). At 20 and 60 min some free SR(1−3) fragment does appear if illumination is performed in the absence of NEM (Figure 5, lanes 3 and 4). However, this is clearly not due to the formation of MII, which is complete within the time of illumination in this experiment (Figure 4; *3*). Free SR(1−3) does not appear in the later time points when illumination and incubation are carried out in the presence of NEM (Figure 5, lanes 7 and 8).

DISCUSSION

Our group (*3*) and Ridge et al. (*4*) have previously demonstrated that SR(1−3) and SR(4−7) are not linked through a disulfide bond when run on SDS−PAGE gels. This surprising result suggested that the native disulfide connecting Cys110 and Cys187 was not present in the SR(1−3/4−7) split rhodopsin mutant. However, we show here that the Cys110−Cys187 disulfide is indeed present in SR(1−3/4−7) but undergoes an exchange reaction upon denaturation of the protein in the SDS−PAGE gel load buffer. We propose that the thiolate of Cys185 attacks the sulfur of Cys187 in a reaction that displaces Cys110 and forms a new disulfide bond between Cys185 and Cys187. This reaction severs the disulfide bond connecting the SR(1−3) and SR(4−7) fragments, and as a result, SR(1−3) is detected on Con A blots as a band migrating with the mobility of the free amino-terminal fragment and not that of full-length rhodopsin. In agreement with this model, SR(1−3/4−7) comigrates with full-length rhodopsin if NEM is included in the gel load buffer or if Cys185 has been changed to Ser, both of which would remove 185 from participation in the disulfide bond exchange reaction.

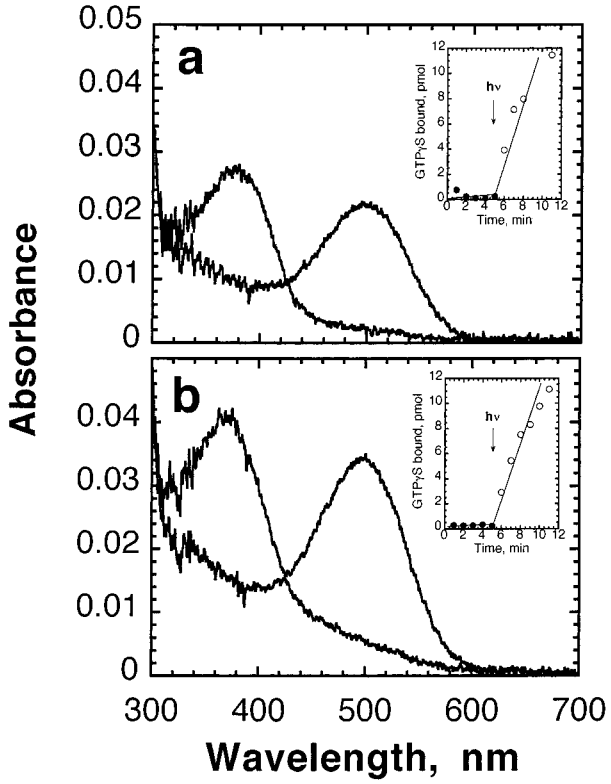

FIGURE 4: Absorption spectra and transducin activity of (a) SR(1−3/4−7) and (b) SR(1−3/4−7:C185S). There are two spectra shown for each pigment: one with a maximum at 500 nm which was recorded in the dark, and one with a maximum at 380 nm which was recorded from the same sample after illumination for 30 s by light from a 300-W slide projector filtered through a 490-nm cut-on filter. The 380-nm species is MII (*3*), the photointermediate capable of activating transducin. (Inset) Light-dependent activation of transducin. Activity was measured by following the binding of [^{35}S]GTPγS with time as described previously (*3*): in the dark (solid circles) and after exposure to light (open circles) . The specific activities of SR(1−3/4−7) and SR(1−3/4−7:C185S) were essentially the same. SR(1−3/4−7) has been shown to activate transducin with wild-type specific activity (*3*).

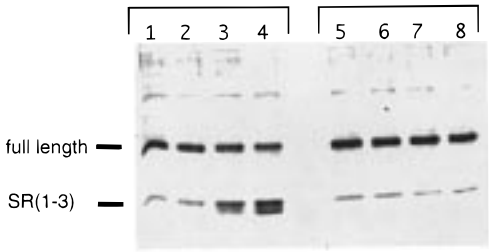

FIGURE 5: Con A blot of SR(1−3/4−7) showing the effect of light on the native Cys110−Cys187 disulfide bond. Lanes 1−4: SR(1−3/4−7) was illuminated for 1 min in the absence of NEM and denatured with gel load buffer containing NEM after 0 (lane 1), 5 (lane 2), 20 (lane 3), and 60 (lane 4) min of incubation. Lanes 5−8: SR(1−3/4−7) was illuminated for 1 min in the presence of NEM and denatured with gel load buffer after 0 (lane 5), 5 (lane 6), 20 (lane 7), and 60 (lane 8) min of incubation. The final concentration of NEM in both experiments was 12.5 mM.

We also investigated whether the Cys110−Cys187 disulfide bond is broken and then re-formed upon formation and decay of MII. This mechanism has been proposed by Rath et al. (*18*) as a possible explanation for the transient appearance of a positive band near 2550 cm^{-1} (assigned to the S−H stretching mode of one or more cysteine residues in the protein) in FTIR difference spectra of rhodopsin. The

intensity of this band followed the kinetics of formation and decay of MII. Here we find no evidence for loss of the Cys110−Cys187 disulfide upon formation of MII in SR-(1−3/4−7). Illumination of SR(1−3/4−7), both in the absence and in the presence of NEM, followed by quenching with gel load buffer (final concentration of 12.5 mM NEM) demonstrated that at early time points, when the sample is quantitatively converted to MII (Figure 4), the protein comigrates on SDS gels with full-length rhodopsin, indicating that the native Cys110−Cys187 disulfide bond has not been broken (Figure 5, lanes 1 and 2). We cannot rule out formation of a Cys110-Cys185 disulfide in this reaction, but the fact that the C185S mutant has wild-type specific activity for activation of transducin (Figure 4) demonstrates that, were such a mechanism to occur, it would not be essential to photoactivation of rhodopsin. At longer time points (20−60 min), some of the sample is observed to migrate with free SR(1−3) (Figure 5, lanes 3 and 4), but by this time a significant decay of MII has occurred and the limited disulfide bond breakage in these samples is unlikely to be related to activation of the protein. Similar results were also obtained with the C185S mutant (not shown).

We have shown that a non-native Cys185−Cys187 disulfide bond forms as a result of a disulfide interchange reaction upon denaturation of rhodopsin with SDS−PAGE load buffer. The same non-native disulfide may form in some rhodopsin mutants that do not fold properly. Khorana and co-workers have suggested that the specific tertiary structure on the extracellular side of rhodopsin is important in determining proper overall folding of the protein (*19*), and that improperly folded forms of wild-type and mutant rhodopsins have non-native disulfide bonds (*15*). In particular, Garriga et al. (*20*) and Liu et al. (*21*) suggested that a Cys110−Cys185 or Cys185−Cys187 disulfide bond forms in certain improperly folded mutant rhodopsins found in patients with retinitis pigmentosa. Our work suggests that the proposed non-native disulfide may be between Cys185 and Cys187.

As a final comment, we note that the identification of the disulfide exchange reaction may be of practical benefit for investigators studying the structure and function of rhodopsin. Despite significant efforts by a number of different laboratories, successful refolding of rhodopsin from a denatured state in vitro has never been achieved. We suggest that the entropic advantage of the Cys185−Cys187 disulfide over the native Cys110−Cys187 bond may present a significant obstacle to refolding of the protein in vitro. If this is the case, the C185S mutant may be a better choice for refolding studies.

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