

# Rhodopsin Forms a Dimer with Cytoplasmic Helix 8 Contacts in **Native Membranes**

Adam M. Knepp, Xavier Periole, Siewert-Jan Marrink, Thomas P. Sakmar, and Thomas Huber\*,

Supporting Information

ABSTRACT: G protein-coupled receptors form dimers and higher-order oligomers in membranes, but the precise mode of receptor-receptor interaction remains unknown. To probe the intradimeric proximity of helix 8 (H8), we conducted chemical cross-linking of endogenous cysteines in rhodopsin in disk membranes. We identified a Cys316-Cys316 cross-link using partial proteolysis and liquid chromatography with mass spectrometry. These results show that a symmetric dimer interface mediated by H1 and H8 contacts is present in native membranes.

G protein-coupled receptors (GPCRs) are versatile signaling machines that constitute the largest group of membrane proteins in the human genome. GPCRs activate and modulate numerous intracellular signaling pathways, including catalysis of nucleotide exchange in the  $\alpha$  subunit of heterotrimeric G proteins. Like many other membrane receptors, GPCRs are known to form dimers and higher-order oligomers in membranes. Atomic force microscopy (AFM) images of native rod outer segment (ROS) disk membranes showing rows of rhodopsin dimers provide a striking demonstration of their possible supramolecular organization. Like those of most class A (rhodopsin-like) GPCRs, the functional role of rhodopsin dimers is unknown. In fact, it has been shown that isolated monomers can activate G proteins when segregated in membrane nanoparticles.<sup>2</sup>

Recently, the GPCR field has witnessed a surge in structural information, including the first crystal structure of a receptor complexed with heterotrimeric G protein.3 Despite these advances, and ever mounting evidence of oligomerization, the precise interface(s) mediating receptor-receptor contacts remains controversial.<sup>4</sup> Spatial constraints from the AFM images were used to predict that the primary rhodopsin dimer interface involved transmembrane (TM) helices 4 (H4) and 5 (H5). However, two-dimensional and three-dimensional<sup>7</sup> densities obtained from electron microscopy (EM) as well as X-ray data for packing of rhodopsin crystals<sup>8</sup> show that dimer contacts involve TM H1 and cytoplasmic H8. In addition, a recent cross-linking study suggests this interface exists for dopamine D2 receptors heterologously expressed at physiological densities in membranes.9 These results pointed to an oligomeric model in which both the H4/H5 and H1/H8 symmetrical interfaces are simultaneously present.

We set out to demonstrate the possibility of the H1/H8 dimer in native disk membranes by cross-linking the endogenous cysteines of rhodopsin and identifying the site(s) involved. Here we go beyond previous studies of rhodopsin in that we identify additional intermolecular cross-linking sites. 10 Working with receptors in the native membrane environment eliminates artifacts associated with dimerization in detergent.

Rhodopsin contains two primary reactive cysteines (positions 140 and 316), and two additional cysteine residues in H8 (positions 322 and 323) may also be reactive because of incomplete palmitoylation. Structural evidence suggests that the Cys316-Cys316 distance in an H1/H8 interface would be 2-3 nm.<sup>7,8</sup> In contrast, these side chains are on opposite faces and thus farther from each other in an H4/H5 dimer arrangement. We hypothesized that a Cys316-Cys316 cross-link using a reagent with a spacer length close to the predicted distance would support the presence of the H1/H8-H1/H8 dimer.

Dark state ROS membranes were first cross-linked with two homobifunctional bis-maleimide reagents containing polyethylene glycol (PEG) spacers of different lengths (Figure 1A,B). The cross-linker:rhodopsin stoichiometry was controlled to optimize cross-linking. If an overly large excess is used, the reactive cysteines saturate with the reagent rather than crosslinking to a neighboring receptor. We found that a 5:1 excess of cross-linker resulted in appreciable formation of dimers and higher-order oligomers that could be observed on a gel. Addition of either BM(PEG)<sub>3</sub> (2.1 nm, the sulfur-sulfur distance in the extended conformation of the cross-link) or Bis-MAL-dPEG<sub>3</sub> (3.1 nm) reduced the amount of monomer present relative to a negative control.

To identify the region of the cross-link, we employed a partial proteolysis procedure with thermolysin. Thermolysin cleaves rhodopsin at several C-terminal sites in addition to a primary cut site in the third intracellular loop. The C-terminal cuts result in a slightly shorter peptide, R'. Further proteolysis results in two predominant fragments: an ~28 kDa N-terminal peptide (F1) and an ~12 kDa C-terminal peptide (F2). The F1 peptide contains Cys140, and the F2 peptide contains Cys316, Cys322, and Cys323. Because these fragments are easily resolved on a gel, the region of the cysteine cross-link can be determined by observing which oligomerizes upon addition of

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<sup>&</sup>lt;sup>†</sup>Laboratory of Molecular Biology and Biochemistry, The Rockefeller University, 1230 York Avenue, New York, New York 10065, United States

<sup>&</sup>lt;sup>‡</sup>Biomolecular Sciences and Biotechnology Institute and Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

Biochemistry Rapid Report

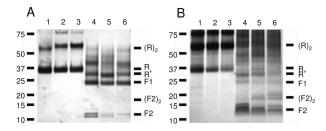


Figure 1. Chemical cross-linking of rhodopsin in ROS disk membranes followed by limited proteolysis and SDS-PAGE analysis with silver (A) and Coomassie (B) staining. Samples were analyzed before (lanes 1–3) and after (lanes 4–6) thermolysin treatment (proteolysis) in the absence of cross-linker (lanes 1 and 4), after cross-linking with BM(PEG) $_3$  (lanes 2 and 5), and after cross-linking with Bis-MAL-dPEG $_3$  (lanes 3 and 6). The cross-linkers are noncleavable homobifunctional cysteine-reactive reagents with different spacer lengths. R' results from cleavage of short C-terminal peptides; F1 is an ~28 kDa N-terminal peptide, and F2 is an ~12 kDa C-terminal peptide. The appearance of the cross-linker-dependent band (F2) $_2$  in lanes 5 and 6 of panel B demonstrates the proximity of H8 in adjacent rhodopsin monomers in ROS, as described in the text. This result is consistent with an H1/H8–H1/H8 dimer model.

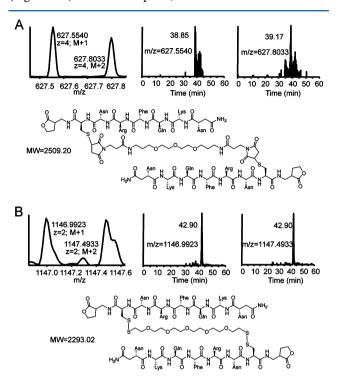
the bis-maleimide reagent. As shown in lane 4 of panels A and B of Figure 1, R', F1, and several forms of F2 (depending on the extent of C-terminal proteolysis) are the only lower-molecular mass bands observed in a control sample. The F1 band stains relatively poorly with Coomassie, likely because it is glycosylated, so it is clearer in the silver-stained gel. On the other hand, the modified F2 bands are almost invisible with silver staining, but prominent with Coomassie. Upon addition of either cross-linker (Figure 1B, lanes 5 and 6), the intensities of the F2 bands decrease and new bands appear near 20 kDa. These bands can correspond to only an F2 dimer, so we conclude that the H8 regions cross-link to each other and are thus in the proximity of each other in neighboring receptors in ROS.

Next, we demonstrated the chemical specificity of the Cys cross-linking with two methanethiosulfonate (MTS) reagents, MTS-O4-MTS (2.2 nm) and MTS-O5-MTS (2.6 nm). The MTS groups are extremely reactive, and cross-linking is observed after just 5 min (Figure S1 of the Supporting Information). These reagents have an advantage in the fact that the covalent linkage formed can be cleaved with a reducing agent. Treatment of cross-linked samples with dithiothreitol (DTT) before running a gel collapsed the higher-molecular mass bands back down to primarily monomer, as observed in negative controls. This demonstrates that the cross-linker-dependent bands are the result of thiol reactions and not nonspecific oligomerization.

We used liquid chromatography and mass spectrometry (LC-MS) to demonstrate definitively the presence of a Cys316-Cys316 dimer cross-link. ROS samples were solubilized, alkylated, and precipitated with trichloroacetic acid. The precipitate was then digested with cyanogen bromide (CNBr), which cleaves after methionines at positions 309 and 317 to yield a small peptide that can be detected by LC-MS. The C-terminal methionine is modified to a homoserine lactone as a result of the chemical cleavage, and iodoacetamide treatment adds a carbamidomethyl group to Cys316 if it has not been substituted with a cross-linker.

In cross-linked samples, the carbamidomethyl-modified 310-317 peptide was identified first (Table T1 of the

Supporting Information). It appears as several isotopes, and its mass is accurate to several parts per million with respect to theoretical monoisotopic masses. For Bis-MAL-dPEG<sub>3</sub> samples, the cross-linked product is simply two 310-317 peptides plus the mass of the reagent. This product appeared as an z = 4 ion (Figure 2A). For MTS peaks, we identified the 310-317



**Figure 2.** LC–MS analysis of Cys316–Cys316 cross-linked peptides. (A) Two 310–317 peptides cross-linked with Bis-MAL-dPEG<sub>3</sub>. The monoisotopic peaks are z=4 ions. Liquid chromatograms integrated across the two MS peaks show that the species elute at similar times. (B) Two 310–317 peptides cross-linked with MTS-O5-MTS. The monoisotopic peaks are z=2 ions and disappear from samples treated with DTT prior to precipitation. Liquid chromatograms integrated across the MS peaks show that they elute simultaneously.

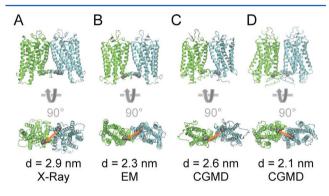
peptide with carbamidomethyl and N-ethylmaleimide (NEM) (resulting from reaction quenching) and then found the peptide substituted with a cross-linker. Monosubstituted MTS reagents have their second reactive group hydrolyzed to a thiol, which can then be modified with NEM, and this peak was also present. Finally, the cross-linked product was found, again with roughly parts per million accuracy (Figure 2B). Crucially, in samples treated with DTT prior to precipitation and digestion, the peaks corresponding to the peptide substituted with the MTS cross-linker were not observed. This additional control confirms the peak assignments. The mass spectrometry data conclusively demonstrate the existence of Cys316-Cys316 cross-links. It is notable that this cross-link was only observed with the longer reagents (≥2.6 nm) but not with the shorter reagents ( $\leq$ 2.2 nm), suggesting the distance between the side chains is less than 2.6 nm. However, the thermal mobility and other factors limit the accuracy of such estimates.

The partial proteolysis and LC–MS data are complementary, highlighting the importance of integrating diverse experimental data to identify cross-linking sites. The gels in Figure 1 and Figure S1 of the Supporting Information show extensive oligomerization, so more than one dimerization interface must be relevant. The presence of the (F2)<sub>2</sub> band demonstrates that

Biochemistry Rapid Report

one of them involves the proximity of H8 regions on adjacent receptors. The LC-MS experiments allow precise identification of the Cys316-Cys316 cross-linking site. The results are especially convincing because the assigned MTS peaks disappear upon addition of DTT, which cleaves the cross-link disulfide bond. Further, because the MTS reagent hydrolyzes so quickly, it is likely that the cross-links result from prearranged dimers and not from random collisions over extended periods of time. Interestingly, the partial proteolysis data show H8 cross-links for all four reagents employed, but the Cys316-Cys316 peaks were identified with only the two longer (Bis-MAL-dPEG<sub>3</sub> and MTS-O5-MTS) cross-linkers. It is possible that either Cys322 or Cys323 cross-links are responsible for the gel shift with the shorter reagents. As with Cys140-containing fragments, we were not able to identify these sites with our CNBr cleavage strategy.

We also probed dimerization interfaces in coarse-grained molecular dynamics (CGMD) studies of spontaneous rhodopsin assembly (X. Periole et al., manuscript in preparation). The second most frequently populated dimer cluster involved an H1/H8 interface similar to structures observed via X-ray crystallography and EM (Figure 3). The Cys316–Cys316 side



**Figure 3.** Structural and CGMD analysis of H1/H8 dimer orientations. (A) Two symmetry-related promoters from the crystal structure of photoactivated rhodopsin [Protein Data Bank (PDB) entry 2I35]. (B) Crystal structure of rhodopsin (PDB entry 1GZM) fit to the electron density of a metarhodopsin I dimer observed in an EM image. (C and D) Crystal structure of rhodopsin (PDB entry 1U19) fit to two distinct H1/H8–H1/H8 dimer clusters observed via CGMD analysis.

chain bead distance of this cluster (Figure 3C) was 2.3 nm (2.6 nm when fitting residues 310-322 of PDB entry 1U19; see the Supporting Information for a discussion of side chain distances). Notably, a similar but less populated cluster (Figure 3D) showed a Cys316-Cys316 distance closer to 1.9 nm (2.1 nm from the local fit of 1U19). It should be noted that the analysis of the CGMD self-assembly simulations on a microsecond time scale does not predict thermodynamic stability, and consequently, cluster populations do not reflect equilibrium distributions. The two structures are spatiotemporally mutually exclusive, but it is difficult to determine which of these arrangements is responsible for the cross-links presented here. If the monomer-dimer exchange is slow on the time scale of the cross-linking experiment, the more stable interface may predominate and thereby exclude the other possible orientation. On the other hand, if the dimers are more transient, then the structure that brings Cys316 residues closer and thus increases the likelihood of cross-linking could account for the data presented.

In summary, we report biochemical cross-linking experiments that conclusively identify a Cys316—Cys316 cross-linked dimer and demonstrate the proximity of these residues between rhodopsins in native disk membranes. These data suggest a rhodopsin dimer interface mediated by H1/H8 contacts exists, as suggested by previous cross-linking, EM, and crystallography data. Because cross-linking caused the formation of oligomers, a second interface, perhaps involving H4/H5 contacts, must also be present. Despite studies that have demonstrated a single receptor is sufficient for full G protein activation, the extremely high density of rhodopsin in disk membranes hints that a dimer may be the primary structural unit that interacts with the heterotrimer. The high degree of homology in class A GPCRs suggests that the results reported here might be relevant for other receptors that are known to oligomerize.

#### ASSOCIATED CONTENT

## S Supporting Information

Complete materials and methods, additional cross-linking gels, and mass spectrometry data. This material is available free of charge via the Internet at http://pubs.acs.org.

## AUTHOR INFORMATION

## **Corresponding Author**

\*E-mail: hubert@rockefeller.edu. Phone: (212) 327-8284.

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## Notes

The authors declare no competing financial interest.

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#### REFERENCES

- (1) Fotiadis, D., Liang, Y., Filipek, S., Saperstein, D. A., Engel, A., and Palczewski, K. (2003) *Nature* 421, 127–128.
- (2) Banerjee, S., Huber, T., and Sakmar, T. P. (2008) J. Mol. Biol. 377, 1067–1081.
- (3) Rasmussen, S. G. F., DeVree, B. T., Zou, Y. Z., Kruse, A. C., Chung, K. Y., et al. (2011) *Nature* 477, 549–555.
- (4) Palczewski, K. (2010) Oligomeric forms of G protein-coupled receptors (GPCRs). Trends Biochem. Sci. 35, 595-600.
- (5) Liang, Y., Fotiadis, D., Filipek, S., Saperstein, D. A., Palczewski, K., and Engel, A. (2003) *J. Biol. Chem.* 278, 21655–21662.
- (6) Schertler, G. F. X., and Hargrave, P. A. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 11578–11582.
- (7) Ruprecht, J. J., Mielke, T., Vogel, R., Villa, C., and Schertler, G. F. X. (2004) *EMBO J.* 23, 3609–3620.
- (8) Salom, D., Lodowski, D. T., Stenkamp, R. E., Le Trong, I., Golczak, M., et al. (2006) *Proc. Natl. Acad. Sci. U.S.A.* 103, 16123–16128.
- (9) Guo, W., Urizar, E., Kralikova, M., Mobarec, J. C., Shi, L., et al. (2008) EMBO J. 27, 2293-2304.
- (10) Jastrzebska, B., Maeda, T., Zhu, L., Fotiadis, D., Filipek, S., et al. (2004) J. Biol. Chem. 279, 54663-54675.