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Supporting information for:

Rhodopsin forms a dimer with cytoplasmic helix 8 contacts in native membranes

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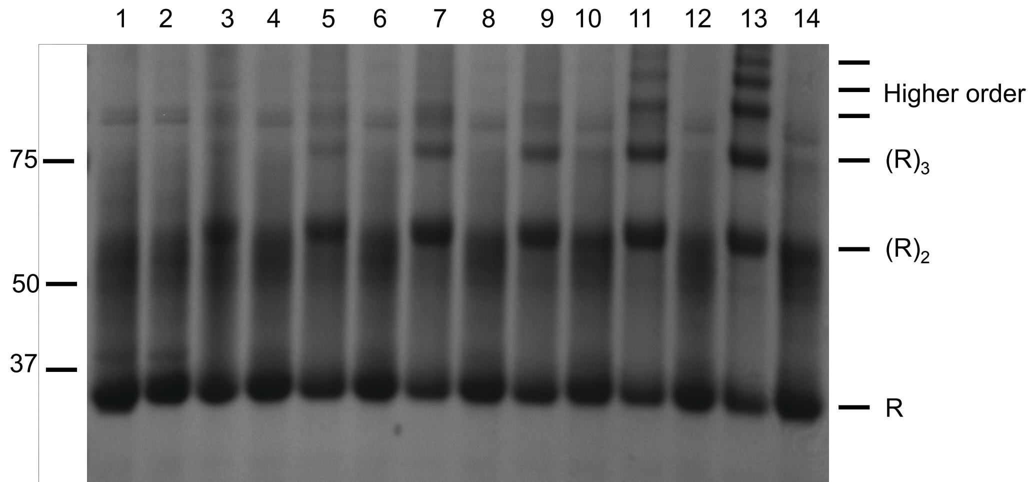
SUPPORTING FIGURE



SUPPORTING FIGURE S1: Chemical crosslinking of rhodopsin in ROS disc membranes with a cleavable reagent. Samples were analyzed by SDS-PAGE with Coomassie staining in the absence of crosslinker (lanes 1 and 2), in the presence of MTS-O4-MTS for 5 (lanes 3 and 4), 15 (lanes 5 and 6), and 60 min (lanes 7 and 8), and in the presence of MTS-O5-MTS for 5 (lanes 9 and 10), 15 (lanes 11 and 12), and 60 min (lanes 13 and 14). The even numbered lanes were treated with DTT before running the gel. The reduction of crosslinked samples to profiles resembling the negative control in lane 1 demonstrates the chemical specificity of the crosslink.

SUPPORTING TABLE

Sample	Obs. Mass	Theor. mass	Error (ppm)	Sequence ID	Ion
Bis-MAL-dPEG$_3$	1050.508	1050.5029	4.85	310-316-H+C	M+2H
Bis-MAL-dPEG$_3$	1051.512	1051.5029	8.65	310-316-H+C	M+2H+1
Bis-MAL-dPEG$_3$	1052.514	1052.5029	10.55	310-316-H+C	M+2H+2
Bis-MAL-dPEG$_3$	1053.514	1053.5029	10.54	310-316-H+C	M+2H+3
Bis-MAL-dPEG$_3$	2510.216	2510.1954	8.21	2*(310-316-H)+B	M+4H+1
Bis-MAL-dPEG$_3$	2511.212	2511.1954	6.61	2*(310-316-H)+B	M+4H+2
MTS-O5-MTS	1048.4836	1048.4872	3.43	310-316-H+C	M
MTS-O5-MTS	1118.5264	1118.5291	2.41	310-316-H+C+N	M+2H
MTS-O5-MTS	1119.5276	1119.5291	1.34	310-316-H+C+N	M+2H+1
MTS-O5-MTS	1120.5276	1120.5291	1.34	310-316-H+C+N	M+2H+2
MTS-O5-MTS	1121.529	1121.5291	0.09	310-316-H+C+N	M+2H+3
MTS-O5-MTS	1304.5999	1304.5801	15.18	310-316-H+M	M+H
MTS-O5-MTS	1305.6111	1305.5801	23.74	310-316-H+M	M+H+1
MTS-O5-MTS	1428.6198	1428.6199	0.07	310-316-H+M+N	M+H
MTS-O5-MTS	1429.6204	1429.6199	0.35	310-316-H+M+N	M+H+1
MTS-O5-MTS	1430.6214	1430.6199	1.05	310-316-H+M+N	M+H+2
MTS-O5-MTS	1431.6228	1431.6199	2.03	310-316-H+M+N	M+H+3
MTS-O5-MTS	2293.9846	2294.0223	16.43	2*(310-316-H)+M	M+1
MTS-O5-MTS	2294.9864	2295.0223	15.64	2*(310-316-H)+M	M+2

SUPPORTING TABLE T1: Mass spectrometry peaks identified in analysis. The masses were calculated by multiplying the peak m/z ratio by its assigned charge. The abbreviations are the following. H: homoserine lactone (position 317 modified by CNBr treatment); C: carbamidomethyl; B: Bis-MAL-dPEG$_3$; N: N-ethylmaleimide; M: MTS-O5-MTS

SUPPORTING DISCUSSION

Previous rhodopsin crosslinking studies. The crosslinking and site identification methods used here were adapted from the work of earlier groups. A study by Jastrzebska, *et al*. (*1*) also used homobifunctional cysteine-reactive reagents, including $BM(PEG)_3$ to trap rhodopsin dimers and higher-order oligomers, but crosslinking sites were not identified. Reports by Suda, *et al.* (*2*) and Jacobsen, *et al*. (*3*) crosslinked rhodopsin and successfully identified sites by partial proteolysis and mass spectrometry, respectively. These two studies used heterobifunctional reagents to generate intramolecular crosslinks between Cys316 and Lys residues of the receptor. Here we extend this work and report a Cys316-Cys316 intermolecular crosslink between adjacent receptors.

Side chain distances. Several distances between Cys316 residues in dimer structures are noted in the text. The Cys316-Cys316 distances generally refer to the distances measured between the Sγ atoms in the corresponding residue in the two protomers. For the CGMD dimers, we report distances of the side chain beads in the coarse grained model together with fits of crystallographic structures to the coarse grained representations. The first distances mentioned correspond to the distance between Cys316-Cys316 side chain beads in the CGMD dimer clusters: 2.3 nm for the cluster in 3C and 1.9 nm for the cluster in 3D. The dimer images in the figure panels were generated from a complete fit of Cα 1-326 of PDB 1U19 to the CGMD beads. The Cys316-Cys316 distances of the Sγ atoms in these two dimer structures are 2.8 nm and 1.9 nm for 3C and D, respectively. The local fits were generated in a similar manner, except that only Cα 310-322 of PDB 1U19 was fit to the CGMD beads. This resulted in distances of 2.6 nm and 2.1 nm for 3C and D, respectively, which are the distances noted in the figure. Local fitting reduces the effects of distortions in other regions of the receptor that otherwise might result in unsatisfactory fits.

MATERIALS AND METHODS

Chemicals. The detergent n-dodecyl β-D-maltoside (DM) was obtained from Anatrace, Inc. (Maumee, OH). Cyanogen bromide (CNBr), trichloroacetic acid (TCA), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis). $BM(PEG)_3$ was purchased from Pierce, Bis-MAL-$dPEG_3$ from Quanta Biodesign, and MTS-O4-MTS/MTS-O5-MTS from Toronto Research Chemicals, Inc. The MTS reagents were initially solubilized in chloroform, aliquoted, and dried with a gentle stream of argon. When ready for use, DMSO was added to the dry residue. Buffer C: 100 mM Na_2HPO_4, pH 7.0, with 150 mM NaCl. Buffer P: 10 mM Tris-HCl, pH 7.5, with 5 mM $CaCl_2$.

Biochemical crosslinking. Bovine ROS membranes were prepared as described (*4*). The membranes were suspended at a concentration of 1.6 mg/mL in Buffer C. The crosslinking reagents were solubilized in DMSO and added to a final concentration of 200 μM (5-fold molar excess) in the dark. The bis-maleimide reactions proceeded for 24 h at room temperature before quenching with 10 mM cysteine. The MTS reactions were allowed to proceed for 5, 15, or 60 min at room temperature before quenching with 20 mM N-ethylmaleimide. In control samples, the MTS reactions were treated with 100 mM DTT after crosslinking to cleave the disulfide linkages.

SDS-PAGE and immunoblot analysis. Samples were resolved on NuPage Novex 4-12% Bis-Tris SDS-PAGE gels. Coomassie Blue and silver staining were carried out with standard protocols. Immunoblotting (Immobilin membrane, Millipore) was performed with 1D4 primary antibody (National Cell Culture Center; 1:3,000) and horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (GE Healthcare; 1:10,000). Blots were visualized by enhanced chemiluminescence treatment (Pierce) and exposed to HyBlot CL autoradiography film (Denville Scientific, Inc.).

Partial proteolysis. The site of the crosslink was determined, in part, by partial proteolysis with thermolysin in the dark. Crosslinked ROS membranes were resuspended in Buffer P. Thermolysin was added at a rhodopsin-to-thermolysin ratio of 50:1 by mass. The digestion proceeded for 6 h at 37°C and was stopped by adding 5 mM EDTA and placing the tubes on ice. The samples were solubilized in 1% DM before SDS-PAGE analysis.

Liquid chromatography-mass spectrometry. Sample preparation for LC-MS was based on a previous study (*5*). Crosslinked ROS membranes were solubilized in 1% DM and bleached. Bis-maleimide samples and the MTS negative controls were treated with 15 mM DTT for 1 h, and all samples were treated with iodoacetamide (100 mM) for 3 h. Rhodopsin was precipitated with 10% TCA and spun at 14k rpm for 5 min to produce a pale yellow pellet. The pellet was washed with 95% ethanol in a bath sonicator three times to remove TCA and lipids. The pellet was then dissolved in 100% TFA containing 500 M excess CNBr per rhodopsin methionine in the sample. Water was added to give a final concentration of 80% TFA, and the digestion proceeded overnight. Samples were dried with argon and desiccation. When ready for LC-MS analysis, the dried precipitate was resuspended in 20 μL ddH_2O. The samples were loaded onto a C18 5 μm trap column (LC Packings) and run at 30 μL/min, then on an in-house made C18 analytical column (75 μm diameter beads) at 0.2 μL/min. Solvent A was water + 0.1% formic acid and solvent B was acetonitrile + 0.1% formic acid, and elution proceeded from 5-40% B over 35 min. MS was performed with 5 μL injections onto a LTQ Orbitrap XL (Thermo Scientific) with ion trap. The mass range was 400-1600 m/z. Charges were automatically calculated by the software (Xcalibur, Thermo).

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