Role of bulk water in the hydrolysis of rhodopsin’s chromophore

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Rhodopsin (Rho) is a prototypical G protein-coupled receptor (GPCR) that changes from an inactive conformational state to the G protein-activating state as a consequence of its retinal chromophore isomerization, 11-cis-retinal → all-trans-retinal. The photoisomerized chromophore covalently linked to Lys296 by a Schiff base is subsequently hydrolyzed but little is known about this reaction. Recent research indicates a significant role for tightly bound, transmembrane water molecules in the Rho activation process. Atomic structures of Rho and hydroxyl radical footprinting reveal ordered waters within Rho transmembrane helices which are located close to highly conserved and functionally important receptor residues, forming a hydrogen bond network. Using ¹⁸O labeled H₂O, we now report that water from bulk solvent, but not tightly-bound water, is involved in the hydrolytic release of chromophore upon Rho activation by light. Moreover, small molecules, and presumably water, enter the Rho structure from the cytoplasmic side of the membrane. Thus, this work indicates two distinct origins of water vital for Rho function.

Abbreviations used: 11-cis-RAL, 11-cis-retinal; at-RAL, all-trans-retinal; BTP, bis-tris propane; LC/MS, liquid chromatography mass spectrometry; FTIR, fourier transform infrared spectroscopy; LRAT, lecithin:retinol acyltransferase; MES, 2-(N-morpholino)ethanesulfonic acid; NG, n-nonyl-glucopyranoside; Rho, rhodopsin; ROS, rod outer segments; WT, wild type.
tightly bound waters positioned close to the chromophore-binding pocket has been postulated to play a key role in the counter ion switch between ground state (dark; Glu$^{113}$) and activated states (Glu$^{181}$) of Rho (5,6). Moreover, Glu$^{113}$ and Glu$^{181}$ are also likely involved in the hydrolysis process via the carbinol ammonium ion, and in a regeneration reaction between 11-cis-retinal and opsin. To form the Schiff base, Lys$^{296}$ must be deprotonated, the carbonyl group must be polarized, and water accommodated within the chromophore binding site (1). Hydroxyl radical footprinting revealed local conformational changes in the Rho structure following its photoactivation, presumably mediated by the dynamics of both ordered water molecules and the protein (7,8). Moreover, protein footprinting combined with rapid H$^2$O mixing methodology and deuterium-hydrogen exchange on C2-His residues indicate that these tightly bound internal waters do not exchange with bulk solvent in ground state Rho, Meta II, or opsin (7,8). Thus, ordered waters function as non-covalent cofactors that actively participate in transmitting the activation signal from the retinylidene-binding pocket to the cytoplasmic face of Rho where binding of transducin occurs. These individual waters are observed in high resolution structures of Rho as well as other GPCRs, and a high level of conservation of polar residues in close proximity to these waters is also found in all GPCR sequences (9,10).

As a consequence of photoactivation and relaxation processes in opsin, the chromophore Schiff base linkage is hydrolyzed, releasing all-trans-retinal (at-RAL) (Fig. 1B). But it is unclear if ordered water or bulk water is responsible for retinylidene Schiff base hydrolysis. Although, a role of extracellular water in this process had been suggested (14), we addressed this issue by using $^{16}$O and $^{15}$N labeling methodologies combined with modern mass-spectrometry techniques. This approach allowed the experimental determination of which side of the photoreceptor NH$_2$OH, and presumably bulk water, enters the chromophore-binding pocket upon Rho photoactivation.

Materials and Methods

Materials – Fresh bovine eyes were obtained from a local slaughterhouse (Mahan's Packing, Bristolville, OH). Wild type (WT) and Lrat-deficient (Lrat$^{-/-}$) mice (15) were housed in the animal facility at the School of Medicine, Case Western Reserve University, and all animal procedures and experiments were approved by the Case Western Reserve University Animal Care Committees. Manipulations in the dark were performed under dim red light transmitted through a filter (transmittance > 560 nm; No. 1 Safelight; Eastman Kodak, Rochester, NY). at-RAL was obtained from TRC, Toronto, Canada whereas H$_2^{18}$O (97 atom %) and $^{15}$NH$_2$OH (98 atom %) was purchased from Sigma-Aldrich, St. Louis, MO.

Mouse retina and bovine rod outer segment (ROS) isolation; Rho purification – Retinas were removed from 24 h dark-adapted WT and Lrat$^{-/-}$ mouse eyeballs through an incision in the cornea and immediately immersed in 10 mM BTP, pH 7.4, and 100 mM NaCl. Retinas were then washed twice with the same buffer followed by centrifugation at 1,000g to remove contaminants. Bovine ROS membranes were prepared from frozen retinas under dim red light according to the Papermaster procedure (16). Isolated ROS membranes were washed 5 times in hypotonic buffer (5 mM bis-tris propane (BTP),
pH 7.5) to dispose of membrane associated proteins. Washed ROS were stored at -80°C or used immediately for Rho extraction. Rho was solubilized in n-nonyl-glucopyranoside (NG) and purified from ROS by ZnCl₂-opsin precipitation (17). ZnCl₂ was removed by dialysis in the presence of 0.5% NG. Rho concentrations were determined with a Cary 50 (Varian, Palo Alto, CA) UV-visible spectrophotometer and quantified by absorption at 500 nm by using the absorption coefficient \( \varepsilon = 40,600 \text{ M}^{-1}\text{cm}^{-1} \) (18).

**Preparation of bovine Rho proteoliposomes** – Proteoliposomes were prepared by the procedure described by Niu et al. (19). A solution of 1% asolectin (commercially available soybean lipids, Sigma-Aldrich) in buffer composed of 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.8, 100 mM NaCl, and 1% NG was mixed with purified Rho. The molar ratio of phospholipids to Rho was 250:1. This solution was dialyzed against buffer composed of 10 mM MES, pH 5.8, and 100 mM NaCl in the dark at 4°C for 48 h with four changes of buffer totaling 8 liters. The proteoliposome suspension was pelleted by centrifugation at 100,000 g at 4°C for 1 h. To prepare vesicles with enclosed NH₂OH, the above procedure was performed with buffers containing 50 mM NH₂OH. Pelleted proteoliposomes were washed in 10 mM MES, pH 5.8, and 100 mM NaCl and centrifuged again at 100,000 g at 4°C for 1 h. Proteoliposomes were resuspended in the same buffer to achieve a final Rho concentration of about 2 mg/ml. The efficiency of Rho incorporation into lipid vesicles was determined by measuring the absorption spectra of the supernatant and pellet recorded after the first ultracentrifugation in a UV-visible Cary 50 spectrophotometer.

**Orientation of Rho in proteoliposomes** – A proteolysis assay with Asp-N endoprotease that specifically cleaves Rho between Gly329 and Asp330 in the C-terminus (20) was used to determine the orientation of Rho in asolectin vesicles. A total of 20 µl of proteoliposomes containing 30 µg of Rho in a buffer composed of 10 mM BTP, pH 7.4, and 100 mM NaCl was subjected to proteolysis. The sample was incubated at room temperature for 20 h and an aliquot containing 5 µg of Rho was run on 10% SDS-PAGE gel to resolve the cleaved fragments of Rho. The efficiency of digestion was determined by densitometric analysis with ImageJ software.

**Chromophore hydrolysis and extraction** – Bovine ROS membranes or mouse retinas were washed twice with 0.5 ml of buffer containing 10 mM BTP, pH 7.0, and 100 mM NaCl prepared in either H₂¹⁸O or H₂¹⁶O and resuspended in the same buffer. The final concentration of Rho in ROS was 1.5 mg/ml. From mice, each experimental tissue sample contained the equivalent of two retinas. To initiate chromophore hydrolysis, 50 µl of Rho in ROS membranes were illuminated with a 150 W bulb for 5 min during which samples were placed on ice to prevent overheating. Then, retinoids were immediately extracted with 0.3 ml of hexane. The organic phase was separated by centrifugation at 16,000 g for 1 min and retinoid composition was analyzed promptly by the LC/MS (see below). In experiments with proteoliposomes, 30 µg of Rho incorporated into liposomes and resuspended in 0.2 ml of 10 mM MES, pH 5.8, and 100 mM NaCl was illuminated with a 150 W bulb for 5 min. Both NH₂OH-loaded and "empty" proteoliposomes were exposed to light in the presence or absence of 10 mM NH₂OH. Depending on the experiment, the sample extraction procedure involved addition of either 0.3 ml of hexane or 0.2 ml of methanol prior to the hexane, followed by vigorous shaking for 2 min and centrifugation at 16,000 g for 1 min. To control the time during which at-RAL reacted with NH₂OH and to minimize derivatization upon extraction, samples were spiked with 40 mM formaldehyde just prior to hexane addition. The efficiency of rhodopsin photoactivation in described above light conditions was around 98% as deducted from 11-cis / all-trans-retinal oxime ratio after retinoids extraction in the presence of hydroxylamine and methanol.

**LC/MS of retinoids** – Retinoid composition was analyzed with an Agilent 1100 series HPLC attached to a LXQ mass spectrometer (Thermo Scientific, Waltham, MA). Retinoids extracted with hexane were injected onto a normal phase HPLC column (Zorbax Sil 5 µm, 4.6 × 250 mm, Agilent Technology) equilibrated with 10% ethyl
acetate in hexane. Retinals were separated by isocratic elution with the equilibration solvent at a flow rate 1.4 ml/min. The eluent was directed into the mass spectrometer through a diode array detector followed by an atmospheric pressure chemical ionization source working in the positive mode. Data were recorded and analyzed with Xcalibur 2.0.7 software (Thermo Scientific). Elution times for at-RAL and its oximes were determined based on their characteristic UV/Vis spectra with absorbance maxima at 368 and 357 nm, respectively. The ratio between at-RAL and at-18O-RAL present in a sample was calculated based on areas under m/z = 285.3 [M+H]⁺ and 287.3 [M+H]⁺ peaks determined from extracted ion chromatograms that corresponded to the labeled and unlabeled retinoid.

Oxygen exchange in at-RAL – at-18O-RAL was synthesized according to a previously published procedure, extracted with hexane and stored under argon at -80°C (21). To monitor oxygen back-exchange, 300 pmols of at-18O-RAL (70% enriched) delivered in 1 µl of N,N-dimethylformamide was incubated in 0.2 ml of 10 mM BTP, pH 7.0, and 100 mM NaCl containing 1% bovine serum albumin (BSA) and added either to liposomes composed of soybean lipid extract (250 µM), homogenized retinas isolated from Lrat⁻/⁻ mice (2 retinas per sample), or 50% methanol. Samples were vigorously vortexed and incubated in conditions identical with those used for Rho bleach for various time periods indicated in the Results section. Then, at-RAL was extracted with 0.34 ml of hexane. The isotopic composition of at-RAL was examined by MS. Analogous experiments were performed in buffers composed of H218O in which the rate of 16O exchange in at-RAL was recorded.

Results

Carbonyl oxygen exchange in at-RAL – Aldehydes readily exchange their carbonyl oxygen with the oxygen atom of water (22). This replacement proceeds via a hydration-dehydration mechanism that depends on the electrophilicity of the carbonyl carbon and is accelerated by both acidic and basic conditions (23). This characteristic of aldehydes that can lead to loss of the initial oxygen label represents the main obstacle to interpreting experimental studies involving the mechanism of aldehyde formation (24). To counteract the potential effect of oxygen back-exchange, rates of 18O isotope lost from synthetic at-18O-RAL were studied under aqueous conditions in the presence of additives that included methanol, BSA or lipids. Interestingly, progression of oxygen exchange, monitored by a decline of the m/z = 287.3 signal in the MS spectrum corresponding to at-18O-RAL ([M+H]⁺), strongly depended on the buffer composition and was much faster in the presence of proteins or lipids (Fig. 2A, B). This phenomenon can be explained by transient formation of Schiff base adducts of at-18O-RAL with available primary amino groups present in proteins or phospholipids. Reversibility of this reaction in the presence of water (hydrolysis) facilitates exchange of the 18O label with bulk solvent. Therefore, samples containing protein and lipids should be employed as appropriate controls for oxygen back-exchange in studies of at-RAL formation upon Rho photoactivation in ROS.

The origin of the oxygen atom in newly formed at-RAL released from light-activated Rho – To investigate the potential role of internal structural water molecules in retinylidene Schiff base hydrolysis, we washed isolated bovine ROS with buffers composed of 18O isotopically labeled water. Because in the ground state, the tightly bound water of Rho does not exchange with bulk solvent, unlabelled water molecules are preserved within the transmembrane helical bundle (7). Prepared experimental materials were exposed to light for 5 min and directly extracted with hexane. The amount of extracted at-RAL represented approximately 10% of the total at-RAL found in a methanol-treated sample (protein denaturating conditions) and corresponded closely to the amount predicted by Meta II decay kinetics (25). Photoactivation of Rho in the presence of H218O followed by rapid organic extraction revealed a pool of at-RAL that was highly enriched in the 18O isotope (Fig. 2C). The isotopic composition of oxygen in at-RAL closely imitated that in the bulk water solvent and was much higher than the isotopic content of unlabeled at-RAL exposed to H218O predicted.
from oxygen exchange rates in control experiments (Fig 2A). Thus, the contribution of unlabeled internal protein-bound water molecules to retinylidene Schiff base hydrolysis appears insignificant. Because experimental conditions might influence the rate of oxygen back-exchange, we examined retinas isolated from Lrat<sup>−/−</sup> mice to approximate the environment present in a biological sample. Although the lipid and protein composition as well as retinal morphology are preserved in these animals, the retinoid pool is dramatically reduced due to metabolic blockade in the uptake, storage, and production of visual chromophore (15). Thus, the isotopic composition of externally added at-RAL cannot be diluted by internal retinoids. Experimentally determined oxygen back-exchange in retinas isolated from Lrat<sup>−/−</sup> mice that were spiked with synthetic at-RAL was comparable to previous observations made in the presence of soybean lipids and indicated about a 12% loss of label during the 5 min incubation (Fig. 2D). Consistent with the data obtained from bovine ROS, at-RAL liberated from WT mouse retinas resuspended in buffer containing H<sub>2</sub><sup>18</sup>O and exposed to light contained predominantly <sup>18</sup>O (Fig. 2D). The above observations indicate that external water molecules penetrate the Rho photoreceptor upon light activation and contribute extensively to retinylidene Schiff base hydrolysis.

**Light-activation of Rho allows small molecules to reach the chromophore-binding pocket from the cytoplasmic side** – The N- and C-termini of Rho are oriented on opposite sides of the lipid bilayer and differ in terms of function (1). To investigate the potential asymmetrical role of these termini in the transduction of water molecules into the chromophore-binding pocket, we prepared proteoliposomes that contained preferentially oriented Rho with N-termini located inside liposomal lumen (Fig. 3A). Functional integrity of the preparation was examined spectrophotometrically by recording Meta II state formation in response to a light stimulus (Fig. 3B). The orientation of photoreceptors inserted into liposomes, as determined by proteolytic digestion with Asp-N protease, indicated that about 85% of the protein was appropriately and uniformly oriented. Moreover, at-RAL released form rhodopsin incorporated into proteoliposomes was enriched in <sup>18</sup>O to the same extent as it was observed in the experiments on bovine ROS, (data not shown). Notably, photoreceptors orientation in the liposomes was preserved during proteoliposome washes (Fig. 3C, D). Therefore, it was possible to deplete the external NH<sub>2</sub>OH concentration without changing the lumenal pool of solvent and generate proteoliposomes loaded with NH<sub>2</sub>OH (Fig. 3A). Under our experimental conditions, Rho exposed to light rapidly is converted into the Meta II state which is relatively stable at acidic pH. Moreover, hydrolysis of the retinylidene Schiff base in Meta II can be accelerated by added nucleophiles such as NH<sub>2</sub>OH. Consequently, extraction of proteoliposomes under non-denaturating conditions in the absence of NH<sub>2</sub>OH revealed the presence of only small amounts of at-RAL. Interestingly, the presence of NH<sub>2</sub>OH inside the liposomes did not provoke massive at-RAL oxime formation under these conditions (Fig. 4Aa). The small amount of oximes seen in chromatograms represented less than 10% of total extractable at-RAL oximes and may have been derived from the small fraction of Rho molecules with a reversed orientation. However, significant amounts of at-RAL oximes were detected after liberation of NH<sub>2</sub>OH trapped inside the vesicles by addition of either methanol or detergent (10 mM diheptanoyl phosphatidylcholine) following light exposure (Fig. 4Ab, data for methanol treatment shown). In this case, the amount of at-RAL derivatization depended on the amount of NH<sub>2</sub>OH released from inside the vesicles. The striking difference between these two groups of samples indicates that NH<sub>2</sub>OH was indeed trapped within the liposomal lumen and did not diffuse across the lipid membrane, at least within the time frame of this experiment. In contrast, the presence of NH<sub>2</sub>OH added externally to proteoliposomes led to the rapid break down of the retinylidene Schiff base resulting in quantitative formation of at-RAL oximes in both types of proteoliposomes (Fig. 4Ac). Thus, NH<sub>2</sub>OH molecules involved in Schiff base decomposition came from outside the vesicles. Considering the orientation of this protein in liposomes, we conclude that small molecules enter the chromophore-binding pocket of activated Rho from its C-terminal side.
For improved discrimination between internal and external pools of NH$_2$OH, proteoliposomes that contained NH$_2$OH inside the vesicles were exposed to light in the presence of 10 mM $^{15}$N isotope-labeled reagent ($^{15}$NH$_2$OH) added externally during the bleaching procedure (Fig 5A). Similar to previous experiments and to avoid potential cross contamination during extraction, the excess of unreacted NH$_2$OH was depleted by addition of 40 mM formaldehyde after the incubation period but just prior to the organic solvent. In this case, the isotopic composition of at-RAL oximes examined by MS revealed a predominant peak at m/z = 301.17 ([M+H]$^+$) corresponding to $^{15}$N-RAL oxime (Fig. 5B). The contribution of the 300.25 peak corresponding to unlabeled at-RAL oxime in the MS spectrum of extracted retinoid was slightly higher than in the spectrum of synthetic $^{15}$N-RAL standard. However, it did not exceed 10% of the 301.17 ion intensity. Again this result could be a consequence of a small pool of Rho molecules with a reversed membrane orientation (Fig. 3). Together these data indicate that only externally added $^{15}$NH$_2$OH contributed to retinylidene Schiff base cleavage.

**Discussion**

Photoisomerization of the chromophore bound to Rho leads to activation of this GPCR. Consequently, the retinylidene Schiff base is hydrolyzed followed by dissociation of at-RAL from the chromophore-binding pocket. We investigated this process in more detail and arrived at two significant conclusions: first, bulk solvent provides the source of water molecules utilized for chromophore hydrolysis following Rho activation, and second, a flux of small molecules and presumably water enters into the retinal-binding pocket from the cytoplasmic side of this photoreceptor upon attainment of the activated state.

Increased intramembranous accessibility to external small molecules and water is required for Rho activation to advance beyond the Meta I state (26,27). Moreover, the retinylidene chromophore becomes accessible to reagents such as NH$_2$OH or NaBH$_4$ only upon attainment of the Meta II state (11,28). Results obtained by NMR and hydrogen/deuterium exchange also suggest that the hydration state of Rho increases upon photoactivation (12,29). The process of retinylidene hydrolysis is complex, involving multiple protonation/deprotonation and proton transfer events. It most likely occurs in dark state Rho by employing the internal water network to mediate cleavage of the Schiff base linkage (30) but due to structural constraints provided by this state of the receptor, 11-cis-RAL release is hindered and the Schiff base reforms. Subtle structural relaxation at the retinal-binding site rather than major conformational changes upon photoactivation can be envisioned as the driving force for this protein’s transitions to new thermodynamically stable sub-configurations (31). The concomitant influx of water molecules upon photoactivation appears to remodel the network of internal water molecules relative to the ground state. This increase in hydration elevates the rate of deuterium exchange with the protonated Schiff base by several orders of magnitude (32). Such rapid proton exchange is enabled by an effective increase in water concentration near the retinal Schiff base. In D$_2$O isotopic effect studies, slower rates of Schiff base hydrolysis were measured during Meta II decay, providing evidence that external D$_2$O rather than internal water was involved in this process (30). Furthermore, exchange of the carbonyl oxygen of at-RAL released from Rho was visualized by Fourier transform infrared spectroscopy (FTIR) (14). This evidence along with our observations of $^{18}$O incorporation into at-RAL indicates a role for bulk solvent in the transition from Meta II to opsin and hydrolyzed dissociated at-RAL.

Interestingly, increased hydration *per se* does not promote rapid replacement of tightly bound inter-transmembrane waters as judged by radiolytic footprinting observations (7). Because the hydrolysis reaction requires a protonated Schiff base for efficacy, it is tempting to speculate that ordered water activated by the Glu$^{113}$ side chain contributes to the protonation event whereas the abundance of bulk solvent molecules ensures rapid and spontaneous hydrolysis of the retinylidene-Lys$^{26}$ linkage.
Thus, transient reprotonation of the Schiff base should be the rate-limiting step in overall Meta II decay and the hydrogen bond network surrounding the chromophore-binding site would play an essential role in this process.

Ordered water molecules lie in a specific channel connecting N-terminal and C-terminal sides of Rho, which most likely acts as a conduit for the activation signal through the membrane. Increases hydration of Rho upon light exposure poses the question from which side of the membrane this incoming bulk water originates. To investigate this problem, we prepared proteoliposomes containing Rho preferentially oriented with its N-termini facing the liposomal lumen which was filled with unlabeled NH$_2$OH, and its C-termini, located on the liposome exterior, exposed to solvent enriched in $^{15}$N-labeled NH$_2$OH. This experiment revealed that the NH$_2$OH involved in Schiff base breakdown arose exclusively from the extra-vesicular space. So why would small polar molecules and water take such a long detour deep into the retinal-binding pocket if the chromophore is located closer to the lumen of the disk membrane and N-terminus of Rho? The answer may relate to the fact that transmembrane helices HIII and HIV located on the extracellular side of Rho are connected by an antiparallel $\beta$-sheet that forms a “plug”, completely masking the retinal-binding pocket and retinal from extracellular bulk solvent (33). This “plug” also hinders chromophore release from dark state Rho. Mutations in this region have been shown to perturb photoreceptor function, leading to retinitis pigmentosa (34). Minor structural changes in the same location have been identified by FTIR spectroscopy after Rho photoactivation. Therefore, it appears that changes in the N-terminal face of Rho that occur after light activation are not large enough to allow massive water influx from the extracellular space. Marked changes do occur at the cytoplasmic surface of Rho, opening its structure to the entry of bulk water. In fact, crystallographic structures of opsin and opsin complexed with the C-terminal peptide of G$_{\text{t}}$ display formation of a solvent accessible cavity at the cytoplasmic site (35). Moreover, none of these structures reveal any openings on the extracellular side of this photoreceptor. However, in opsin, transmembrane bundles transiently open into the hydrophobic membrane region through two holes located between helices H1 and HVII, and HV and HVI (35). A connecting channel between these openings might provide the means for retinal passage after Schiff base hydrolysis, confirming earlier predictions based on random acceleration molecular dynamics (36,37).

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Figure legends

Figure 1. Water molecules present in the crystal structure of bovine Rho; the role of water and small molecules in retinylidene Schiff base cleavage and reduction. A. Positions of selected waters (shown as blue spheres) in structures of bovine Rho (PDB accession code 1U19). Several Rho bound waters are located within the transmembrane helical bundle of the protein. The water network extends from the region of visual chromophore-binding (red) to the N- and C-termini. A. Zoomed view of the chromophore-binding site reveals the presence of oriented water molecules near the retinylidene Schiff base. B. Schematic representation of retinylidene Schiff base chemical reactivity in ground and photoactivated states of Rho. As a consequence of photoreceptor activation by light, the retinylidene-Lys covalent bond is hydrolyzed. In this process, a water molecule donates its oxygen atom to form a new at-RAL molecule. The activated state of Rho allows a small nucleophile (NH₂OH) or reducing agents such as NaBH₄ to penetrate inside the photoreceptor and chemically modify the Schiff base. Additionally, NaBH₃CN was shown to be effective in reducing retinylidene-Lys bound in the ground state of Rho (38).

Figure 2. Incorporation of ¹⁸O into at-RAL liberated from photoactivated Rho. A. Exchange of ¹⁸O in synthetic at-¹⁸O-RAL upon incubation in an aqueous solution containing 50% methanol (closed circles), rather than 1% BSA (open circles), or liposomes (closed triangles). The 5 min time frame used for these experiments is indicated by the gray background. B. Representative MS spectra indicate changes in isotopic composition of at-¹⁸O-RAL upon incubation in 10 mM BTP buffer, pH 7.0, in 70% H₂¹⁸O with 100 mM NaCl and 1% BSA. The peak at m/z = 285.3 corresponds to at-RAL ([M+H⁺]). The two dalton shift in mass of at-¹⁸O-RAL is indicated by the ion at m/z = 287.3 ([M+H⁺]). C. Incorporation of ¹⁸O into at-RAL released from photobleached Rho. Purified bovine ROS washed and resuspended in buffers containing various ratios of H₂¹⁸O were extracted with hexane and the retinoid composition was examined by MS. The pool of analyzed at-RAL was significantly enriched in ¹⁸O. The ratio between at-RAL and at-¹⁸O-RAL closely reflects the water composition present in the experimental samples (dark bars). The theoretical percentage of at-¹⁸O-RAL that could be produced from unlabeled at-RAL due to oxygen back-exchange into buffers present in the samples is shown as white bars. D. Retinal composition and oxygen back-exchange in isolated mouse retina. Three hundred pmols of at-RAL or at-¹⁸O-RAL were added to homogenized retinas isolated from Lrat⁻/⁻ mice and washed with buffers containing H₂¹⁸O or H₂O. Samples were incubated on ice for 5 min and extracted with hexane. Oxygen back-exchange did not exceed 15% in the examined samples (grey and white bars). WT mouse retinas washed with 90% H₂¹⁸O containing buffer and exposed to light under similar experimental conditions revealed the presence of at-RAL highly enriched in ¹⁸O (dark grey bar). All data represent averaged values of three independent experiments, each performed in duplicate.

Figure 3. Characterization of Rho proteoliposomes. A. Schematic representation of Rho orientation within proteoliposomes. B. Functional characterization of incorporated Rho. Spectrophotometric analyses of changes in absorption spectra observed after proteoliposomes were exposed to light indicate Meta-II state formation. The difference spectrum was calculated by subtracting the spectrum recorded after and before light exposure. C. Determination of Rho molecular orientation by proteolytic digestion with Asp-N endoprotease that specifically cleaves the C-terminus of Rho. Lines 1 and 3 represent washed, intact proteoliposomes prepared in the presence or absence of NH₂OH. Lines 2 and 4 corresponding to protease-treated samples reveal dominant protein bands corresponding to the Rho proteolytic fragment. D. Densitometric quantification of protein bands shown in panel C indicates that at least 85% of Rho molecules preferentially adopt an orientation in which the N-termini face the lumen of proteoliposomes.

Figure 4. The side of NH₂OH entry into light-activated Rho. Two types of Rho-containing proteoliposomes, prepared either in the absence or presence of NH₂OH, were exposed to light while they were maintained in NH₂OH-free buffers. Extracted retinoids were analyzed by HPLC. A.
Chromatograms show HPLC separation of at-RAL (peak 1) and its oxime (syn) (peak 2). Red lines correspond to proteoliposomes loaded with NH₂OH whereas blue traces represent data obtained from the "empty" vesicles. Panel (a) - extraction with hexane; (b) - disruption of proteoliposomes with methanol prior to extraction; (c) - incubation with NH₂OH added after light exposure and 5 min prior to extraction. B. Identification of detected retinoids. Peaks corresponding to at-RAL and its oxime (syn) were assigned based on the characteristic shapes and absorbance maxima of their UV/Vis spectra (at-RAL - left panel; at-RAL oxime - right panel).

**Figure 5. Isotopic composition of at-RAL oximes.** Proteoliposomes containing NH₂OH were exposed to light in the presence of ¹⁵NH₂OH added to the surrounding buffer. After extraction the isotopic composition of at-RAL oxime (syn) liberated from Rho was examined by MS. A. Schematic representation of the experimental setup. B. Isotope distribution of at-RAL oxime, [M+H]⁺ charged state. Top and middle panels correspond to unlabeled and ¹⁵N isotope-containing synthetic standards, respectively. Bottom panel represents the isotopic composition of at-RAL oxime (syn) extracted from proteoliposomes exposed to light. Robust incorporation of the ¹⁵N isotope into at-RAL oxime released from photoactivated Rho indicates that ¹⁵NH₂OH derived from the C-terminal side of Rho is solely involved in retinyldiene Schiff base cleavage.
References


Figure 2

A. Graph showing the percentage of 18O-RAL (% 18O-RAL) over time (min) with a decline in concentration.

B. Mass spectrum (m/z) showing relative intensity at 287.3, 285.3, and 280.4 with annotations for 0 min, 5 min, and 30 min.

C. Bar graph comparing H218O/H216O ratios (0/100, 45/55, 90/10) with 18O-RAL percentage.

D. Comparison of 18O-RAL (%) between Lrat-/- retina and WT retina with standard conditions.
Figure 3

A. Diagram of Rho in liposomes.

B. Graph showing absorbance (AU) with wavelengths (nm) 300, 400, 500, 600.

C. Gel electrophoresis with lanes 1-4 showing bands at kDa 95, 72, 55, 43, 34, 26.

D. Bar graph showing relative band intensity (%) with lanes 1-4 showing bands for Rho, RhoA329C, Rho, RhoA329C.
Figure 4
Figure 5

A

[Diagram showing chemical structures]

B

[Graphs showing m/z values and relative intensity for different samples]

RAL oxime (standard)

15N-RAL oxime (standard)

Experimental sample

m/z

300.25

302.17

301.17