Role of Bulk Water in Hydrolysis of the Rhodopsin Chromophore*

Rhodopsin (Rho), the visual pigment in retinal rod photoreceptors, is a G protein-coupled receptor responsible for dim light vision (1, 2). Rho is composed of its apoprotein, opsins, and a retinylidene chromophore in an 11-cis-conformation covalently linked by a Schiff base to Lys296 of the opsin (Fig. 1A). Photoactivation of Rho is initiated by isomerization of the retinylidene ligand to its all-trans-configuration (3), allowing the photoreceptor after deprotonation of the Schiff base to adopt the active Meta II state required for G protein (transducin) activation. The atomic structure of Rho led to the identification of crystallographically ordered water molecules adjacent to functionally important conserved protein residues (Fig. 1A) (4), supporting the conclusion that these waters are essential not just for structural stabilization of the receptor but also for the activation process. One of these tightly bound waters positioned close to the chromophore-binding pocket has been postulated to play a key role in the counterion switch between ground state (dark; Glu113) and activated states (Glu181) of Rho (5, 6). Moreover, Glu113 and Glu181 are also likely involved in the hydrolytic process via the carbinol ammonium ion and in a regeneration reaction between 11-cis-retinal and opsin. To form the Schiff base, Lys296 must be deprotonated, the carbonyl group must be polarized, and water must be 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 exchange of ordered water molecules indicated that ordered internal waters do not exchange with bulk solvent in ground state Rho, Meta II, or opsin (7, 8). Thus, ordered waters function as noncovalent 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 G protein-coupled receptors, and a high level of conservation of polar residues in close proximity to these waters is also found in all G protein-coupled receptor sequences (9, 10).

Rho does not react with hydroxylamine (NH₂OH) in the dark, but changes in the Rho structure upon photoactivation allowing this small nucleophile to penetrate inside the protein and accelerate chromophore hydrolysis (Fig. 1B) (11). Molecular dynamic simulations and ¹H magnetic spinning NMR indicate a dramatic increase in interior Rho hydration after 11-cis-retinylidene isomerization (12). This large increase in intratransmembrane region hydration is characteristic of the Meta I state and appears to be directly related to the Schiff base counterion interaction that occurs immediately after breaking the Glu113-Schiff base salt bridge. Water influx is not accompanied by a significant change in the overall structure of this receptor, but most likely, it plays a functional role in the transition of dark state Rho to its Meta I intermediate (13).

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 ¹⁸O and ¹⁵N labeling methodologies combined with modern mass spectrometry techniques. This approach allowed the experimental determination of which side of the

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3 The abbreviations used are: Rho, rhodopsin; at-RAL, all-trans-retinal; RO5, rod outer segment(s); Bistris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.
photoreceptor NH₂OH (and presumably, bulk water) enters the chromophore-binding pocket upon Rho photoactivation.

EXPERIMENTAL PROCEDURES

Materials—Fresh bovine eyes were obtained from a local slaughterhouse (Mahan’s Packing, Bristolville, OH). WT and Lrat-deficient (Lrat⁻/⁻) mice (15) were housed in the animal facility at the School of Medicine of 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). at-RAL was obtained from TRC (Toronto, Canada), and H₂¹⁸O (97 atom %) and ¹⁵NH₂OH (98 atom %) was purchased from Sigma.

Mouse Retina and Bovine Rod Outer Segment (ROS) Isolation and 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 Bistris propane, pH 7.4, and 100 mM NaCl. Retinas were then washed

FIGURE 1. Water molecules present in the crystal structure of bovine Rho and 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 (Protein Data Bank 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 magnified 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 the 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).
twice with the same buffer, followed by centrifugation at 1000 × g to remove contaminants. Bovine ROS membranes were prepared from frozen retinas under dim red light according to the procedure of Papermaster (16). Isolated ROS membranes were washed five times with hypotonic buffer (5 mM Bistris propane, 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-nonyleglyco-
pyranoside and purified from ROS by ZnCl$_2$/opsin precipitation (17). ZnCl$_2$ was removed by dialysis in the presence of 0.5% NH$_2$OH. Pelleted proteoliposomes were washed with 10 mM NH$_2$OH and to minimize derivatization upon extraction, samples were supplemented with 40 mM formaldehyde just prior to hexane addition. The efficiency of rhodopsin photoactivation in the above-described light conditions was ~98% as deduced from the 11-cis/all-trans-retinal oxime ratio after retinoid extraction in the presence of hydroxylamine and methanol.

**LC/MS of Retinoids**—Retinoid composition was analyzed with an Agilent 1100 series HPLC system attached to an LXQ mass spectrometer (Thermo Scientific). Retinoids extracted with hexane were injected onto a normal phase HPLC column (5-μm ZORBAX SIL, 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 of 1.4 ml/min. The eluate 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-visible spectra with absorbance maxima at 368 and 357 nm, respectively. The ratio between at-RAL and its oximes was determined based on their characteristic UV-visible spectra with absorbance maxima at 368 and 357 nm, respectively. The ratio between at-RAL and its oximes was determined based on their characteristic UV-visible spectra with absorbance maxima at 368 and 357 nm, respectively.

**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 pmol of at-[18O]RAL (70% enriched) delivered in 1 μl of N,N-dimethylformamide was incubated in 0.2 ml of 10 mM Bistris propane, pH 7.0, and 100 mM NaCl containing 1% BSA and added to liposomes composed of soybean lipid extract (250 μM), homogenized retinas isolated from Lrat$^{-/-}$ mice (two retinas/sample), or 50% methanol. Samples were vigorously vortexed and incubated under conditions identical to those used for Rho bleaching for various time periods as indicated under “Results.” at-RAL was then extracted with 0.4 ml of hexane. The isotopic composition of at-RAL was analyzed by MS. Analogous experiments were performed in buffers composed of H$_2$O in which the rate of 18O 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.
Hydrolysis of the Rhodopsin Retinylidene Schiff Base

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 water isotopically labeled with $^{18}$O. Because the tightly bound water of Rho does not exchange with bulk solvent in the ground state, unlabeled 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 $\sim$10% of the total at-RAL found in a methanol-treated sample (protein denaturing conditions) and corresponded closely to the amount predicted by Meta II decay kinetics (25). Phototactivation of Rho in the presence of $H_2^{18}$O followed by rapid organic extraction revealed a pool of at-RAL that was highly enriched in the $^{18}$O 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 $H_2^{18}$O—origin of the oxygen exchange into at-RAL released from photobleached Rho. Purified bovine ROS washed and resuspended in buffers containing various ratios of $H_2^{18}$O were extracted with hexane, and the retinoid composition was examined by MS. The pool of analyzed at-RAL was significantly enriched in $^{18}$O. The ratio between at-RAL and at-$^{18}$O corresponded closely to the water composition present in the experimental samples (gray bars). The theoretical percentage of at-$^{18}$O that could be produced from unlabeled at-RAL due to oxygen back-exchange into buffers present in the samples is shown (white bars). D, Retinal composition and oxygen exchange in isolated mouse retina. At-RAL or at-$^{18}$O-RAL (300 pmol) was added to homogenized retinas isolated from $L_{rat}^{-/-}$ mice and washed with buffers containing $H_2^{18}$O or $H_2O$. Samples were incubated on ice for 5 min and extracted with hexane. Oxygen back-exchange did not exceed 15% in the examined samples (light gray and white bars). WT mouse retinas washed with 90% $H_2^{18}$O-containing buffer and exposed to light under similar experimental conditions revealed the presence of at-RAL highly enriched in $^{18}$O (dark gray bar). All data represent averaged values of three independent experiments, each performed in duplicate.
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![Image of a diagram showing the orientation of Rho in proteoliposomes]

**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 to indicate Meta II state formation. The difference spectrum was calculated by subtracting the spectrum recorded after and before light exposure. AU, absorbance units. C, determination of Rho molecular orientation by proteolytic digestion with Asp-N endoprotease, which specifically cleaves the C terminus of Rho. Lanes 1 and 3 represent washed intact proteoliposomes prepared in the presence or absence of NH$_4$OH. Lanes 2 and 4 (protease-treated samples) reveal dominant protein bands corresponding to the Rho proteolytic fragment. D, densitometric quantification of protein bands shown in C indicate that at least 85% of Rho molecules preferentially adopt an orientation in which the N termini face the lumen of proteoliposomes.

The retinal morphology is preserved in these animals, the retinoid pool is dramatically reduced due to metabolic blockade of the uptake, storage, and production of visual chromophore (15). Thus, the isotopic composition of externally added atRAL cannot be diluted by internal retinoids. Experimentally determined oxygen back-exchange in retinas isolated from Lrat$^{-/-}$ mice that were supplemented with synthetic atRAL was comparable to previous observations made in the presence of soybean lipids and indicated an ~12% loss of label during the 5-min incubation (Fig. 2D). Consistent with the data obtained from bovine ROS, atRAL liberated from WT mouse retinas resuspended in buffer containing H$_2$^{18}$O and exposed to light contained predominantly $^{18}$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 the lipo- somal lumen (Fig. 3A). The functional integrity of the preparation was examined spectrophotometrically by record-
the spectrum of the synthetic $^{15}\text{N}\text{RAL}$ standard. However, it did not exceed 10% of the $m/z$ 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 $^{15}\text{NH}_2\text{OH}$ contributed to retinylidene Schiff base cleavage.

**DISCUSSION**

Photoisomerization of the chromophore bound to Rho leads to activation of this G protein-coupled receptor. 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 $\text{NH}_2\text{OH}$ or $\text{NaBH}_4$ 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-retinal 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

**FIGURE 4. Side of NH$_2$OH entry into light-activated Rho.** Two types of Rho-containing proteoliposomes (prepared in either the absence or presence of NH$_2$OH) were exposed to light while they were maintained in NH$_2$OH-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 traces correspond to proteoliposomes loaded with NH$_2$OH, and blue traces represent data obtained from the empty vesicles. Panel a, extraction with hexane; panel b, disruption of proteoliposomes with methanol prior to extraction; panel c, incubation with NH$_2$OH added after light exposure and 5 min prior to extraction. B, identification of detected retinoids. Peaks corresponding to at-RAL (left panel) and its oxime (syn) (right panel) were assigned based on the characteristic shapes and absorbance maxima of their UV-visible spectra. mAU, milliabsorbance units.

**FIGURE 5. Isotopic composition of at-RAL oximes.** Proteoliposomes containing NH$_2$OH were exposed to light in the presence of $^{15}\text{NH}_2\text{OH}$ 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 the at-RAL oxime ([M + H]$^+$) charged state. Upper and middle panels, unlabeled and $^{15}\text{N}$ isotope-containing synthetic standards, respectively; lower panel, isotopic composition of at-RAL oxime (syn) extracted from proteoliposomes exposed to light. Robust incorporation of the $^{15}\text{N}$ isotope into at-RAL oxime released from photoactivated Rho indicates that $^{15}\text{NH}_2\text{OH}$ derived from the C-terminal side of Rho is solely involved in retinylidene Schiff base cleavage.
driving force for this protein’s transitions to new thermodynamically stable subconfigurations (31). The concomitant influx of water molecules upon phototransduction appears to remodel the network of internal water molecules relative to the ground state. This increase in hydration elevates the rate of deuteration 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 FTIR spectroscopy (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 intertransmembrane 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^{296} 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 the N- and C-terminal sides of Rho, which most likely acts as a conduit for the activation signal through the membrane. Increased hydration of Rho upon light exposure poses the question as to 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, filled with unlabeled NH_{2}OH, and its C termini, located on the liposome exterior, exposed to solvent enriched in ^{2}H_{2}OH. This experiment revealed that the NH_{2}OH involved in Schiff base breakdown arose exclusively from the extravesicular space. 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 III and IV, located on the extracellular side of Rho, are connected by an antiparallel β-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 photocceptor 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 Ga_{i} 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 I and VII and helices V and VI (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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