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Alkylated Hydroxylamine Derivatives Eliminate Peripheral Retinylidene Schiff Bases but Cannot Enter the Retinal Binding Pocket of Light-Activated Rhodopsin

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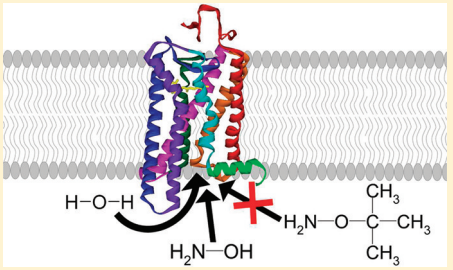
ABSTRACT: Besides Lys-296 in the binding pocket of opsin, all-*trans*-retinal forms adducts with peripheral lysine residues and phospholipids, thereby mimicking the spectral and chemical properties of metarhodopsin species. These pseudophotoproducts composed of nonspecific retinylidene Schiff bases have long plagued the investigation of rhodopsin deactivation and identification of decay products. We discovered that, while hydroxylamine can enter the retinal binding pocket of light-activated rhodopsin, the modified hydroxylamine compounds *o*-methylhydroxylamine (mHA), *o*-ethylhydroxylamine (eHA), *o*-*tert*-butylhydroxylamine (*t*-bHA), and *o*-(carboxymethyl)hydroxylamine (cmHA) are excluded. However, the alkylated hydroxylamines react quickly and efficiently with exposed retinylidene Schiff bases to form their respective retinal oximes. We further investigated how *t*-bHA affects light-activated rhodopsin and its interaction with binding partners. We found that both metarhodopsin II (Meta II) and Meta III are resistant to *t*-bHA, and neither arrestin nor transducin binding is affected by *t*-bHA. This discovery suggests that the hypothetical solvent channel that opens in light-activated rhodopsin is extremely stringent with regard to size and/or polarity. We believe that alkylated hydroxylamines will prove to be extremely useful reagents for the investigation of rhodopsin activation and decay mechanisms. Furthermore, the use of alkylated hydroxylamines should not be limited to in vitro studies and could help elucidate visual signal transduction mechanisms in the living cells of the retina.



Rhodopsin (λ_{max} = 500 nm) consists of the chromophore 11-*cis*-retinal covalently linked via a protonated Schiff base to Lys-296 of the G-protein-coupled receptor opsin.[1,2] The retinylidene Schiff base in rhodopsin is extraordinary in its chemical stability,[3] which is bestowed by the protein structure.[4] Light catalyzes the isomerization of 11-*cis*-retinylidene to all-*trans*-retinylidene, which induces conformational changes in the protein structure and the evolution of a series of metarhodopsin species.[4] Within milliseconds, metarhodopsin II (Meta II; λ_{max} = 380 nm) evolves in equilibrium with its precursor Meta I (λ_{max} = 480 nm). Notably, the retinylidene Schiff base in Meta II is deprotonated, and Meta II can couple to and activate the G-protein transducin. Meta II is not stable and decays within minutes as the retinylidene Schiff base is hydrolyzed and all-*trans*-retinal is released.[5] In addition, light-activated metarhodopsin can decay via Meta III (λ_{max} = 470 nm). Meta III results from *anti* → *syn* isomerization of the retinylidene Schiff base, which can occur spontaneously in Meta I[6,7] or can be induced in Meta II by blue light.[8] Meta III is relatively long-lived (minutes to hours) but eventually decays to opsin and free all-*trans*-retinal (for a review, see ref 9).

Historically, the investigation of the rhodopsin activation and decay cycle has been complicated by the chemical reactivity of retinal. Retinal reacts with amines to form Schiff bases, particularly those found in phosphatidylethanolamine,[10,11] phosphatidylserine,[12] and cytoplasmically exposed lysine residues of opsin.[13,14] On the basis of the reported pK_a of 7.3 for model retinylidene Schiff bases in solution,[3] approximately half of these peripheral Schiff bases would be protonated at physiological pH. Because the absorbance of a protonated Schiff base (λ_{max} = 440 nm)[15] is similar to those of many metarhodopsin species, these nonspecific Schiff bases have often been mistaken for genuine photoproducts (and vice versa). Thus, we call these nonspecific retinylidene Schiff bases pseudophotoproducts. For instance, the existence of Meta III was long debated, with many investigators believing it was simply a pseudophotoproduct (for a review, see ref 16).

The strong nucleophile hydroxylamine (HA) (Figure 1A) has long been used to rapidly cleave the retinylidene Schiff base in Meta II and convert retinal to retinal oxime. In addition, HA has been useful in probing the relative stability of rhodopsin mutants[17,18] and in elucidating how the bulk solvent enters Meta II to hydrolyze the retinylidene Schiff base.[19] The alkylated hydroxylamine derivatives *o*-methylhydroxylamine (mHA) and *o*-ethylhydroxylamine (eHA) (Figure 1A) have been used for some time to convert retinal to alkylated retinal oxime for high-performance liquid chromatography (HPLC) analysis.[20−22] We report here that these alkylated hydroxylamines, in addition to *o*-*tert*-butylhydroxylamine (*t*-bHA) (Figure 1A), can be used to preferentially cleave peripheral Schiff bases while not affecting Meta II. Furthermore, we show that *t*-bHA does not affect Meta III or transducin and arrestin binding to Meta II. This biochemical tool allows for the straightforward discrimination of pseudophotoproducts and

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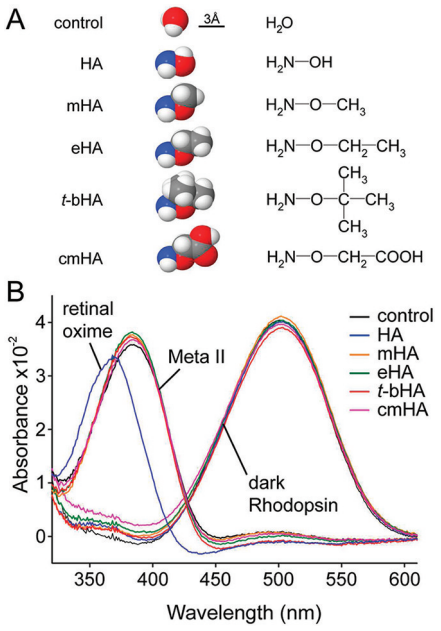

Figure 1. Influence of HA and HA derivatives on the absorption properties of rhodopsin and Meta II. (A) Molecular structures of water (control), hydroxylamine (HA), *o*-methylhydroxylamine (mHA), *o*-ethylhydroxylamine (eHA), *o*-*tert*-butylhydroxylamine (*t*-bHA), and *o*-(carboxymethyl)hydroxylamine (cmHA). Space-filling models were rendered in Jmol (http://www.jmol.org/) using published van der Waals radii.[61] Atoms are colored as follows: gray for carbon, red for oxygen, blue for nitrogen, and white for hydrogen. (B) Absorbance spectra of ROS membranes containing 1 μM rhodopsin were measured in the absence (black) or presence of 25 mM HA (blue), mHA (orange), eHA (green), *t*-bHA (red), or cmHA (magenta). Illumination in the absence of hydroxylamine resulted in the characteristic 380 nm absorbance peak of Meta II. The presence of mHA, eHA, *t*-bHA, or cmHA had no effect on Meta II formation. In contrast, illumination in the presence of HA resulted immediately in a 360 nm peak indicating retinal oxime. Spectra were recorded as described in Experimental Procedures, and rhodopsin was illuminated in the presence of 300 μM $G_t\alpha$ peptide to prevent Meta I formation.

metarhodopsin species. Furthermore, HA and *t*-bHA together represent a tool kit that can be used to easily discriminate among Meta II, Meta III, and opsin. We discuss how the application of this tool kit to living cells might help shed light on the molecular mechanisms of vision.

EXPERIMENTAL PROCEDURES

Materials. Bovine retinas were obtained either from a local slaughterhouse or from W. L. Lawson Co. (Omaha, NE). 11-*cis*-Retinal was created in house using commercially available all-*trans*-retinal and purified by HPLC.[23] The high-affinity analogue peptide derived from the α-subunit of transducin ($G_t\alpha$ peptide), VLEDLKSCGLF, was synthesized by P. Henklein (Institut für Biochemie, Charité). Chromatography supplies for arrestin purification were purchased from GE Healthcare, and the fluorescent probe IANBD [*N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-ethylenediamine] was from Invitrogen. All other reagents were from Sigma or Fluka.

Membrane and Protein Preparations. Rod outer segments (ROS) were prepared from frozen retinas using the previously described discontinuous sucrose gradient method.[24] ROS isolated from retinas originating from the United States were used to prepare phosphorylated rhodopsin using the native rhodopsin kinase, following an earlier protocol[25] that was later modified.[26] After regeneration of phosphorylated ROS membranes with 11-*cis*-retinal, free retinal was removed with extensive BSA washes as described previously.[26] ROS membranes that were not phosphorylated were prepared from locally obtained retinas, and soluble and membrane-associated proteins were removed by washing the membranes several times with low-ionic strength buffer [5 mM Pipes (pH 7) and 1 mM EDTA] as previously described.[27,28] Opsin was prepared by bleaching these ROS in the presence of hydroxylamine, followed by repeated washes as previously described.[29] Transducin was prepared from locally obtained retinas following an established protocol.[30] Recombinant mutant arrestin I72C, which lacked native cysteine and tryptophan residues, was expressed, purified, and labeled with the IANBD fluorophore as recently described.[26] Note that labeled arrestin I72C is termed I72NBD.

Preparation of Meta III. Meta III was formed in washed ROS membranes according to a published protocol,[28,31] and Meta III yields were typically ~20% of the total receptor yield. Membranes were subsequently solubilized in 0.1% *n*-dodecyl β-D-maltopyranoside (receptor concentration of approximately 3 μM) for 5 min at room temperature, followed by high-speed centrifugation (100000*g* for 10 min). Detergent-solubilized Meta III was stored on ice, and under these conditions, decay was slow enough that the sample could be used for experiments over several hours.

Absorbance Spectroscopy. UV−visible absorbance spectra of samples were recorded in 1 cm path length quartz microcuvettes (90 μL) using a Varian Cary 50 spectrometer (2 nm resolution, 11 nm/s scan rate). For samples of washed ROS membranes, opsin membranes with light scattering properties that closely matched those of the ROS were used as a reference. Absorbance spectra were subsequently modified by subtracting an arbitrarily calculated curve that approximated the sloping baseline. Because the calculated baseline did not perfectly match the sample, the apparent absorbance maxima were wavelength-shifted. However, these shifts in absorbance do not affect the interpretation of our results. For samples of detergent-solubilized Meta III, sample buffer was used as the reference, and absorbance spectra were automatically recorded every 30 s. Difference spectra were calculated by manually subtracting spectra in SigmaPlot 10.0 (SysStat Software).

Fluorescence Spectroscopy. Fluorescence measurements were taken on a SPEX Fluorolog (1680) instrument in the front-face mode. Membrane suspensions were stirred in a 1 cm path length cuvette (750 μL) equipped with a stir bar, and the temperature of the cuvette holder was controlled with a circulating water bath. Excitation slits were generally set between 0.1 to 0.2 nm, while emission slits were set at 4 nm. Meta II decay was monitored as an increase in opsin tryptophan fluorescence (λ_{ex} = 295 nm; λ_{em} = 330 nm).[31−33] For measuring steady-state emission spectra (500−660 nm) of I72NBD, we placed samples in a 0.3 cm path length microcuvette (80 μL) and excited them at 360 nm (2 nm step, 0.5 s integration per point). For time-based fluorescence acquisition, stirred samples (750 μL) were excited at 500 nm, and emission was measured at 550 nm. Because this excitation wavelength overlaps with the absorption of rhodopsin, excitation slits were reduced to 0.05 nm. Samples were exposed

to a bright light source (<495 nm) to activate rhodopsin (see below). During this step, the fluorescence detector was protected by manually flipping a mirror on the instrument, which diverted the bright light away from the detector.

Kinetic Light Scattering. Transducin binding to washed ROS membranes was assessed as a change in the scattered near-infrared light exactly as previously described.[34,35] Arrestin binding was assessed as described recently.[26] For experiments with arrestin, membranes were sonicated briefly in a bath sonicator (5−10 s) before they were used to reduce the level of aggregation inherent to regenerated membranes.

Illumination Protocol. For the full photoactivation of ROS membranes, samples were illuminated for 10 s with a 150 fiber optic light source filtered through a heat filter (Schott KG2) and a >495 nm long-pass filter. For light scattering measurements, transducin and arrestin binding to the membrane was initiated with a flash of light (<2 ms) from a commercial photoflash as described previously, which photoactivated 35% of the rhodopsin.[34,35]

Experimental Conditions. Except where noted below, experiments were conducted at 20 °C in isotonic buffer consisting of 20 mM bis-tris-propane (pH 7.5), 130 mM NaCl, and 1 mM $MgCl_2$. Experiments involving Meta III were performed in the same buffer at pH 8. Fluorescence experiments involving I72NBD were performed in 50 mM HEPES (pH 7). Stock solutions of hydroxylamine and the different hydroxylamine derivatives were prepared in water, and the pH was adjusted to 7 using NaOH. All hydroxylamine derivatives were easily soluble in water up to 800 mM. Aliquots of these stock solutions were stored at −40 °C, and thawed aliquots were discarded after being used.

Mathematical Analysis of Rates. The rates of Meta II decay reported in Figure 3 were determined by dividing the initial slope by the final amplitude. The light scattering N signal (Figure 5A) was fit to the simple monoexponential $A(1 - e^{-kt})$, where k is the rate constant and A is the maximum amplitude. Nonlinear regression algorithms within Statgraphics Plus (Statistical Graphics Corp.) were used for the fitting procedures.

RESULTS

The Absorption Properties of Rhodopsin and Meta II Are Unaffected by mHA, eHA, *t*-bHA, and cmHA. We first examined the effects of HA and various modified forms of HA (see Figure 1A) on the UV−visible absorbance spectra of rhodopsin and light-activated Meta II. Dark-state rhodopsin had a characteristic absorbance peak at 500 nm (Figure 1B), which was unaffected by HA, mHA, eHA, *t*-bHA, or cmHA. Light activation of rhodopsin led to the formation of the 380 nm light-absorbing Meta II (Figure 1B). In the presence of mHA, eHA, *t*-bHA, and cmHA, identical absorbance spectra were recorded (Figure 1B), suggesting that these modified hydroxylamine compounds did not affect the formation of Meta II. In contrast, light activation of rhodopsin in the presence of HA immediately resulted in a 360 nm absorbance peak (Figure 1B), which is characteristic of retinal oxime.[23]

mHA, eHA, and *t*-bHA Rapidly Cleave Peripheral Retinylidene Schiff Bases. When all-*trans*-retinal (λ_{max} = 380 nm) was added to a suspension of opsin membranes at pH 7.5, the absorbance around 460 nm increased over time at the expense of the 380 nm absorbance (Figure 2A). These

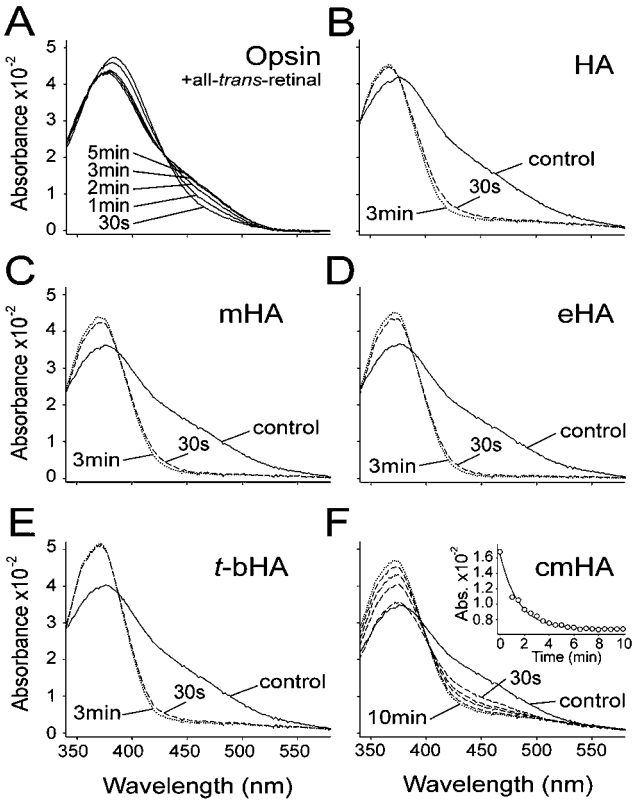

Figure 2. Reactivity of HA and HA derivatives with pseudophotoproducts. (A) All-*trans*-retinal (2 μM) was added to opsin membranes (1 μM), and the formation of pseudophotoproducts resulted in an increase in absorbance around 460 nm and a simultaneous decrease in that at 380 nm over time. (B) Addition of 25 mM HA to the opsin/all-*trans*-retinal mixture (—, "control") eliminated the 460 nm absorbance within seconds and resulted in a 360 nm peak (---). (C−E) As in panel B, addition of the alkylated hydroxylamine derivative mHA, eHA, or *t*-bHA quickly eliminated the 460 nm absorbance. (F) Addition of cmHA to the opsin/all-*trans*-retinal mixture also eliminated the 460 nm absorbance, although much more slowly than with the other HA compounds. The inset is a plot of the loss of 440 nm absorbance over time after the addition of cmHA. Spectra were recorded as described in Experimental Procedures.

absorbance changes were due to the reaction of retinal with the phospholipid and/or protein components of the membranes to form protonated Schiff bases (PSB), yielding an absorbance in the 460 nm range. The absorbance of the PSB was red-shifted from the expected value of 440 nm because of the simultaneous decrease in retinal absorbance. The subsequent addition of HA, mHA, eHA, or *t*-bHA quickly eliminated the PSB absorbance and resulted in the 360 nm absorbance peak of retinal oxime (Figure 2B−E). In contrast to the other hydroxylamine compounds, the addition of cmHA resulted in a much slower conversion of PSB to *o*-(carboxymethyl)retinal oxime (Figure 2F). The carboxy group in cmHA may withdraw electrons from the amine group and thus make it a less effective nucleophile.

Meta II Decays Slightly Faster in the Presence of Alkylated Hydroxylamine Derivatives. We measured Meta II decay in washed ROS membranes using the standard fluorescence assay based on the retinylidene-dependent quenching of opsin tryptophan fluorescence.[31−33] In the absence of any hydroxylamine (control), retinal was released

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from rhodopsin at a rate of ~1.6 × 10^{-3} s^{-1} ($t_{1/2}$ = 7.2 min) (Figure 3A), which is similar to what has previously been observed at this pH and temperature.[31] The alkylated hydroxylamine compounds mHA, eHA, and *t*-bHA increased the rate of Meta II decay by factors of 3.6, 2.2, and 1.6, respectively, and cmHA had no effect on Meta II decay (Figure 3A). In stark contrast, HA increased the rate of Meta II decay by nearly 90-fold. Light activation of rhodopsin in the presence of HA resulted in a fast and substantial increase in fluorescence, followed by a slow and much smaller increase in fluorescence (inset of Figure 3A, blue trace). We noted that the final fluorescence amplitude was much higher in the presence of HA, most likely because Meta III formation was prevented.[31]

We observed that the rate of Meta II decay increased with an increasing HA concentration, and a fit of the data suggested a maximal rate at saturation of 0.5 s^{-1} and an apparent affinity (K_D) of 60 mM (Figure 3B). Compared to HA, *t*-bHA had an insubstantial effect on Meta II decay (Figure 3B), although an expanded *y*-scale shows that the rate of Meta II decay increased linearly with an increasing *t*-bHA concentration (Figure 3B, inset).

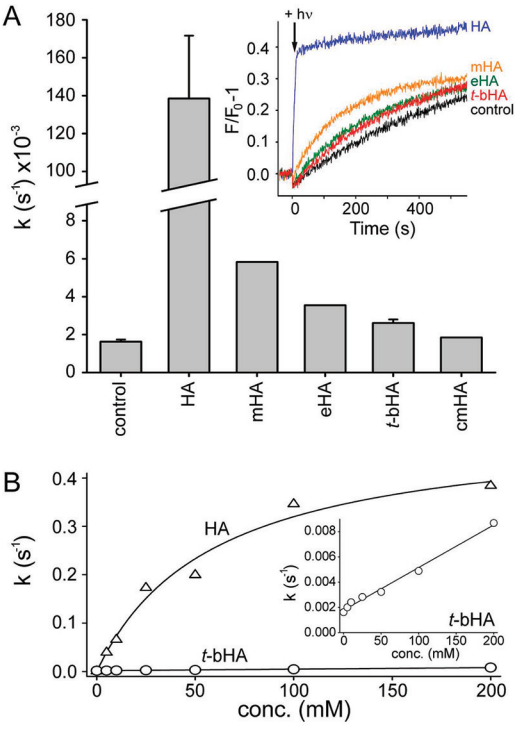

Figure 3. Influence of HA and HA derivatives on the decay of Meta II. (A) ROS membranes containing 1 μM rhodopsin were illuminated (arrow, *hν*) in the absence (black) or presence of 25 mM HA (blue), mHA (orange), eHA (green), or *t*-bHA (red). The release of all-*trans*-retinal from light-activated rhodopsin was monitored as an increase in opsin tryptophan fluorescence (inset). The rates of Meta II decay (initial slope divided by final amplitude) were determined to be 1.6 × 10^{-3} s^{-1} for the control (n = 3; SD = 0.11 × 10^{-3}), 0.14 s^{-1} in the presence of HA (n = 3; SD = 0.033), 5.8 × 10^{-3} s^{-1} in the presence of mHA (n = 2), 3.5 × 10^{-3} s^{-1} in the presence of eHA (n = 2), 2.6 × 10^{-3} s^{-1} in the presence of *t*-HA (n = 3; SD = 0.19 × 10^{-3}), and 1.8 × 10^{-3} s^{-1} in the presence of cmHA (n = 2). Note that the fluorescence in each experiment shown in the inset did not reach the same final amplitude because of varying effects of HA and the HA derivatives on Meta III formation. (B) Meta II decay was measured as described for panel A in the presence of increasing amounts of HA or *t*-bHA. A hyperbolic fit of the HA titration data yielded a rate at saturation of 0.5 s^{-1} and an apparent affinity (K_D) of 60 mM. The inset shows the *t*-bHA data on an expanded *y*-scale.

Meta III Is Stable in the Presence of *t*-bHA. In isolated ROS membranes, the photoproduct Meta III decays slowly over time to opsin and free all-*trans*-retinal.[28,31] We observed similar decay kinetics when Meta III was solubilized in detergent [Figure 4 (▼)]. As we previously observed in membranes,[28] detergent-solubilized Meta III was depleted by $G_t\alpha$ peptide, the high-affinity analogue peptide derived from the α-subunit of transducin (●). HA similarly induced the decay of Meta III (△). However, Meta III decay was not accelerated by *t*-bHA (○). The inset of Figure 4 shows the difference spectra measured 5 min after the addition of $G_t\alpha$ peptide, HA, or *t*-bHA. Note that before the addition of any reagent, the sample consisted of Meta III, opsin, ROS phospholipids, free all-*trans*-retinal, and all-*trans*-retinylidene Schiff base linked to peripheral lysine groups and phospholipids, all solubilized in detergent. In the presence of $G_t\alpha$ peptide, the 470 nm light-absorbing Meta III was converted to an ~380 nm light-absorbing Meta II-like species.[28,36] In the presence of HA, the 470 nm light-absorbing Meta III was also depleted, and the 360 nm light-absorbing retinal oxime evolved. The negative shoulder in the range of ~440 nm was likely due to the cleavage of peripheral protonated retinylidene Schiff bases. While minimal Meta III depletion was observed in the presence of *t*-bHA, a similar negative shoulder at ~440 nm and

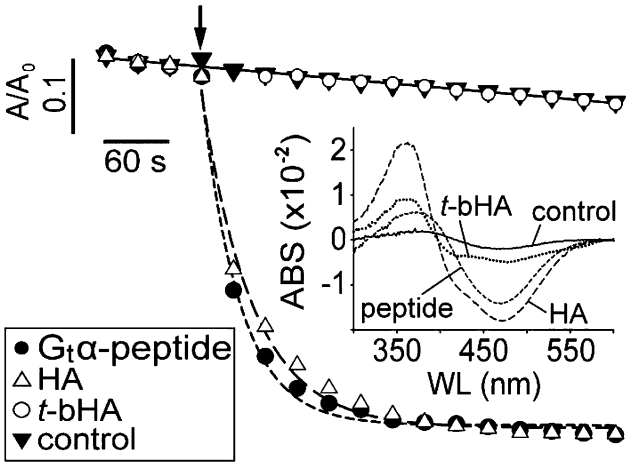

Figure 4. Influence of HA and *t*-bHA on the decay of Meta III. The absorbance of detergent-solubilized Meta III (3 μM total receptor) at 470 nm was measured over time (▼). A fast depletion of Meta III was observed upon addition of $G_t\alpha$ peptide (●). The decay of Meta III in the presence of HA (△) occurred at nearly the same rate. Both plots were fitted with a monoexponential equation (−−−; k = 0.019 s^{-1} for $G_t\alpha$ peptide, and k = 0.015 s^{-1} for HA). The decay of Meta III in the presence of *t*-bHA (○) occurred at the same rate as the control (▼). The arrow indicates the time point of addition of $G_t\alpha$ peptide, HA, or *t*-bHA. After 20 min, $G_t\alpha$ peptide was added to each sample to fully deplete Meta III and determine the baseline absorbance (not shown). This value was used to normalize the absorbance plots to the maximum. Absorbance values are corrected for dilution effects. The inset shows difference spectra taken 5 min after the addition of the various reagents described above. Note that the difference spectra are not normalized.

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positive peak at 360 nm indicated the cleavage of peripheral retinylidene Schiff bases by *t*-bHA.

Transducin and Arrestin Binding Are Unaffected by *t*-bHA. Transducin (G_t) and arrestin are binding partners of Meta II and are responsible for signal propagation and termination, respectively. We assessed the influence of *t*-bHA on G_t and arrestin binding by light scattering (Figure 5).

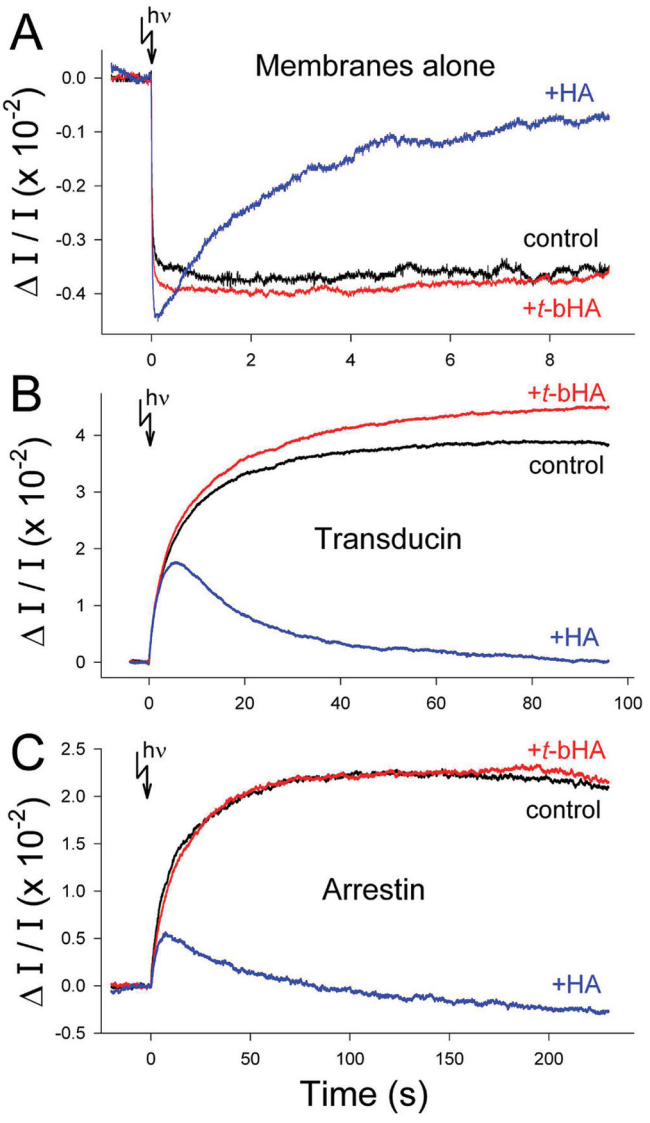

Figure 5. Influence of HA and *t*-bHA on light scattering signals. (A) Photoactivation of ROS results in a light scattering signal with a negative amplitude (*N* signal). *N* signals were recorded for a suspension of ROS membranes in the absence (black) or presence of 25 mM *t*-bHA (red) or 25 mM HA (blue). Reaction of HA with Meta II could be observed as a change in the *N* signal (blue), and a monoexponential fit yielded a rate constant (k) of 0.4 s^{-1}. (B) Light scattering signals were measured for ROS membranes and transducin in the absence or presence of *t*-bHA or HA, as described for panel A. (C) Light scattering signals were measured for ROS membranes and arrestin in the absence or presence of *t*-bHA or HA, as described for panel A. For each experiment, a flash of light at time zero ($h\nu$) photoactivated 35% of the rhodopsin. Samples contained 3 μM rhodopsin with or without 0.6 μM G_t or arrestin. The appropriate *N* signals have been subtracted from the signals shown in panels B and C. Note the different *x*- and *y*-scales for each panel.

We first measured light scattering signals for ROS membranes alone, which must be subtracted from G_t and arrestin binding signals. The so-called *N* signal is a decrease in light scattering arising from conformational changes in rhodopsin that change the refractive index of the sample.[37] Interestingly, we observed the reaction of HA with Meta II as an extra component superimposed on the *N* signal (Figure 5A, blue trace). A monoexponential fit to this signal yielded a rate of 0.4 s^{-1} ($t_{1/2}$ = 1.73 s) for the HA-induced decay of Meta II at 20 °C using 25 mM HA, which is similar to that reported previously (0.1 s^{-1} using 10 mM HA)[33] and what we determined above.

Light-induced binding of transducin to the ROS membrane was observed as an increase in light scattering (Figure 5B). In these experiments, approximately half of the 0.6 μM G_t was bound to the ROS membranes in the dark,[34] and the binding signal reflected the binding of the soluble portion to the membrane after light activation. Compared to the control experiment with no *t*-bHA (black trace), G_t bound to light-activated Meta II in the presence of *t*-bHA with similar kinetics (red trace). The slightly higher amplitude of binding in the presence of *t*-bHA may have been due to the influence of *t*-bHA on the dark binding of G_t. In the presence of HA, only a transient binding signal was observed (blue trace).

Similarly, arrestin binding was unaffected by the presence of *t*-bHA, and a transient binding signal was observed in the presence of HA (Figure 5C). Note that the relative differences in the amplitude of the HA-induced transient binding signals for G_t and arrestin are due to the different rates with which G_t and arrestin bind the ROS membrane.

Arrestin binding to phosphorylated Meta II was also assessed using the fluorescently labeled arrestin mutant I72NBD. The fluorescence of I72NBD increases when arrestin binds the activated receptor (Figure 6A, black traces), which is likely due to the solvent-sensitive fluorophore being buried in a hydrophobic protein−protein interface.[38,39] No difference in the fluorescence binding signal was observed in the presence of *t*-bHA (red traces). To assess what effect *t*-bHA might have on arrestin release, we monitored the fluorescence over time (Figure 6B). In the absence of any hydroxylamine (black trace), arrestin bound after light activation and was then released as Meta II decayed. The fluorescence plateaued higher than the starting-state fluorescence, suggesting that some arrestin remained bound to a photodecay product as previously described.[39] In the presence of *t*-bHA (red trace), arrestin also bound after light activation and was released as Meta II decayed. The relative rates of arrestin release in the absence or presence of *t*-bHA appeared very similar, especially when fluorescence signals were normalized between the maximum and minimum (Figure 6B, top inset). After decay, the fluorescence in the presence of *t*-bHA plateaued at a level closer to the starting state, indicating that all photodecay products capable of binding arrestin were removed[a]. This effect was also seen when *t*-bHA was added after decay to the control sample (black trace). In the presence of HA (blue trace), only a transient binding signal was observed (inset), similar to what was observed in light scattering experiments (Figure 5C).

DISCUSSION

In this study, we showed that the alkylated hydroxylamine derivatives mHA, eHA, and *t*-bHA react quickly and efficiently with peripheral retinylidene Schiff bases, while rhodopsin and

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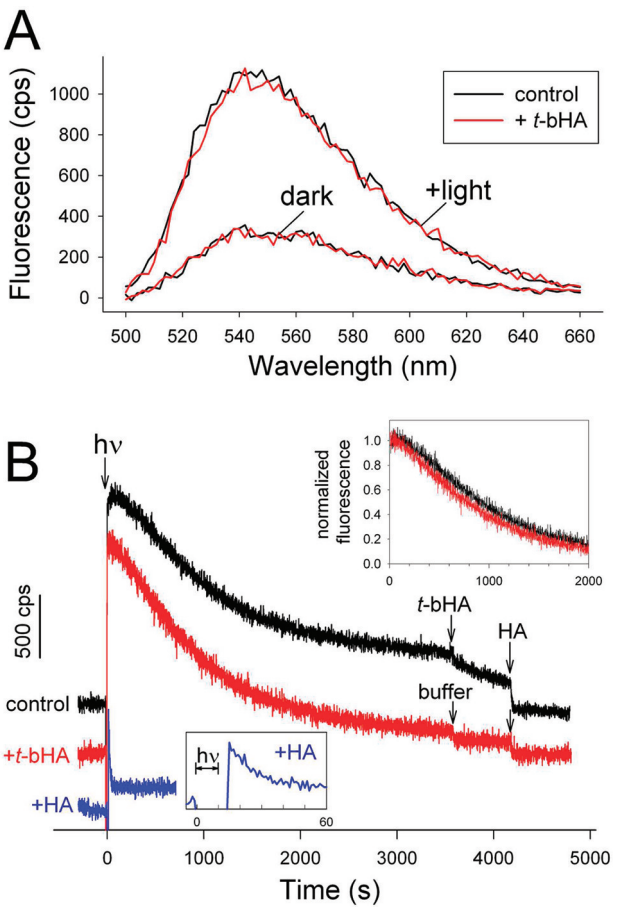

Figure 6. Binding of arrestin to and release of arrestin from phosphorylated Meta II. (A) I72NBD (1 μM) was mixed with an excess of phosphorylated rhodopsin in ROS membranes (4 μM). The fluorescence was measured before (dark) and after (+*hν*) light activation. The experiment was performed in the absence (black) and presence (red) of 25 mM *t*-bHA. (B) Arrestin binding and release were observed as fluorescence changes over time. Samples contained 2 μM I72NBD and 2 μM rhodopsin in the presence of 25 mM t-bHA (red) or 25 mM HA (blue). The control experiment (black) contained no hydroxylamine. The samples were photoactivated at time zero, and binding signals could not be observed during the 10 s illumination (see Experimental Procedures for details). At 3600 s, 25 mM *t*-bHA or an equal volume of buffer was added to the control or the +*t*-bHA experiment, respectively. At 4200 s, 25 mM HA was added to both samples. The additions resulted in <5% sample dilution, and fluorescence signals are not corrected for this dilution. Note that the traces are offset from one another for the sake of clarity. The top inset shows the rate of arrestin release in the presence of *t*-bHA (red) was nearly the same as that in its absence (black). Signals are normalized such that the maximum fluorescence equals 1 and the minimum fluorescence (1 h after photoactivation) equals 0. The bottom inset shows that in the presence of HA, a transient binding signal was recorded. Note the expanded time scales of the insets.

its light-activated forms, Meta II and Meta III, are not significantly affected. Likewise, binding of G_t and arrestin to and release of G_t and arrestin from Meta II are not affected by *t*-bHA. These findings have several implications for both the understanding and the investigation of rhodopsin structure and function.

Metarhodopsin Species Are Sensitive to HA. The retinylidene Schiff base in dark-state rhodopsin is insensitive to HA, presumably because bulk solvent is excluded from the retinal binding pocket.[17,40] At which point in the photoactivation sequence does the retinylidene Schiff base become susceptible to HA? It has recently been reported that Meta I is likely insensitive to HA,[41] although a much earlier study reported the opposite.[33] However, the susceptibility of Meta I to HA is difficult to address experimentally because of the fast Meta I−Meta II equilibrium. In addition, the low temperatures required for substantial Meta I formation may inhibit HA reactivity. This possibility is supported by the fact that at pH 8 and 2 °C, where ∼8% of light-activated rhodopsin exists as Meta II,[42] we observed no retinal oxime formation, even with high levels of HA (500 mM) (data not shown). Thus, it is difficult to conclude whether Meta I is truly sensitive to HA.

Meta II, the active form of the receptor that couples with transducin and is phosphorylated by rhodopsin kinase and then bound by arrestin, has long been known to be susceptible to HA.[5] The binding of either transducin or arrestin to Meta II restricts access of HA to the retinylidene Schiff base, as shown by the slow HA-induced dissociation of arrestin and transducin (Figure 5B,C).[43,44] This observation is consistent with the recent finding that HA likely enters the helical bundle of the receptor from the cytoplasmic side.[19]

We observed that detergent-solubilized Meta III is susceptible to HA, as has been reported for membrane-bound Meta III.[45] However, the rate of HA-induced Meta III decay was significantly slower than the rate of HA-induced Meta II decay. This result suggests that the retinylidene Schiff base in Meta III is less susceptible to HA, because either the protein structure excludes HA better or the isomerization and/or protonation state of the Schiff base affects the reaction with HA.[9] This idea is consistent with the notion that Meta III is more similar to dark-state rhodopsin than Meta II.[46]

Meta II and Meta III Are Insensitive to *t*-bHA. We observed that Meta III appeared to be completely insensitive to *t*-bHA, while the rate of Meta II decay was slightly increased in the presence of 25 mM *t*-bHA at 20 °C. The most likely explanation for the relative insensitivities of Meta II and Meta III to *t*-bHA is that the addition of the bulky *tert*-butyl group to the hydroxylamine molecule (Figure 1A) excluded it from the retinylidene binding pocket in opsin (see below). It is unlikely that metarhodopsin species were nonreactive with *t*-bHA because the hydrophobic *tert*-butyl group partitioned the molecule to the hydrophobic membrane and thus made it unavailable to enter opsin from the aqueous solvent. The low concentration of ROS membranes we employed in our experiments (1 μM) would have been unable to sequester much of the 25 mM *t*-bHA, and all hydroxylamine derivatives, including *t*-bHA, were quite soluble in water (see Experimental Procedures).

If the alkylated hydroxylamine derivatives were able to enter the membrane, it would theoretically be possible for these molecules to enter opsin via openings between the transmembrane helices, which have been proposed to form the gates of a continuous channel through which retinal accesses the binding pocket.[47] However, our data suggest that none of the alkylated hydroxylamine derivatives were sufficiently hydrophobic to enter the membrane and access the relatively large retinal channel.

The small increase in the rate of Meta II decay in the presence of *t*-bHA might be related to destabilizing effects of the hydroxylamine moiety on the secondary structure of opsin,[41] specifically in extracellular loop 2 that forms the "lid" of

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the retinal binding pocket.[48] In any case, the influence of *t*-bHA on Meta II is small enough to establish its usefulness when studying Meta II, and it is not surprising that neither transducin nor arrestin binding was affected by *t*-bHA.

As previously reported,[39,44,49] we observed that a fraction of arrestin remained bound to opsin long after Meta II decay (Figure 6B, black trace). When present before light activation, *t*-bHA prevented the formation of this complex (Figure 6B, red trace). Both *t*-bHA and HA dissociated this complex when added after decay (Figure 6B), although *t*-bHA acted much more slowly than HA. Because the arrestin-bound photodecay product was sensitive to *t*-bHA, it is likely not Meta III as suggested previously.[39] The identity of this arrestin-bound photodecay product is currently being investigated by us.

Implications for the Hypothetical Solvent Channel. The opening of the helical bundle that occurs upon Meta II formation[50,51] allows solvent molecules to gain access to the retinylidene Schiff base,[40,52] most likely from the cytoplasmic side of the receptor.[19] Although tightly bound structural water molecules are present within the helical bundle of dark-state rhodopsin,[53] water molecules from the bulk solvent are likely responsible for retinylidene Schiff base hydrolysis.[19,52] Only small molecules like water, hydroxylamine, and borohydride are known to be able to traverse the length of the helical bundle to the retinal binding pocket.[19,52,54] The solvent channel must be selective with regard to size and/or polarity, because the addition of a single methyl group to the hydroxylamine molecule inhibits its penetration into the helical bundle (Figures 1B and 3A). The restricted access through the solvent channel is also reflected in the hyperbolic shape of the hydroxylamine titration curve. The reaction of hydroxylamine with the retinylidene Schiff base is a two-step process: a pre-equilibrium in which hydroxylamine enters the retinal binding pocket followed by aminolysis of the retinylidene Schiff base. The high apparent K_D of 60 mM (Figure 3B) means hydroxylamine cannot easily enter the helical bundle, and thus, hydroxylamine may represent the upper size limit of allowable molecules with regard to size. These properties may explain why no continuous solvent channel was reported in the recent crystal structures of Meta II.[50,51]

Alkylated Hydroxylamine Derivatives as Molecular Tools for Investigating Visual Signal Transduction Mechanisms. While peripheral retinylidene Schiff bases (pseudophotoproducts) are cleaved quickly by both HA and *t*-bHA, Meta II and Meta III have different sensitivities to HA and *t*-bHA. Meta II decay is ~50 times faster in the presence of HA than in the presence of *t*-bHA. Meta III decay in the presence of HA is much slower than HA-induced Meta II decay, and Meta III appears to be completely resistant to *t*-bHA. Considering these differences, HA and *t*-bHA represent a tool kit that can be used to easily discriminate between peripheral retinylidene Schiff bases and metarhodopsin, as well as between Meta II and Meta III.

The ability of *t*-bHA to discriminate between peripheral retinylidene Schiff bases and Meta III represents an enormous improvement over previously employed methods. For example, different groups have identified Meta III on the basis of the chromophore orientation relative to the membrane, which required magnetically oriented rod outer segment membranes[45] or immobilized single cells[16] to be measured by dichroic absorbance spectroscopy. While such techniques are generally out of reach for most laboratories, the application of *t*-bHA represents a readily accessible biochemical method in helping to identify Meta III and other photodecay products of rhodopsin.

This method can be applied not only to purified components as we have done here but also to isolated rod cells as is routinely done in electrophysiology.[55,56] For instance, it has recently been shown that, in the absence of arrestin, Meta III decay likely determines the rate of dark current recovery of the mouse rod after exposure to light.[57] Although it has been reported that HA has a minimal effect on the recovery of wild-type rods,[56,58] it would be interesting to test the effects of HA and *t*-bHA on the recovery of arrestin-lacking rod cells. In fact, the investigation of Meta III in arrestin knockout mice[59] is especially tempting, because arrestin binding has been suggested to play a major role in regulating Meta III.[12,60] One would expect HA to induce the decay of Meta III and hence speed the rate of rod cell recovery, while *t*-bHA would not affect Meta III and would serve as a valuable control. *t*-bHA still contains the reactive hydroxylamine group but, as we have shown in this paper, does not react with metarhodopsin species.

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Notes

[a]Note that after the complete release of arrestin, the fluorescence of I72NBD was elevated compared to the starting-state fluorescence in the presence of dark-state rhodopsin. This effect was likely due to the fluorescence of IANBD being quenched by the transfer of energy to the chromophore of rhodopsin, because a significant portion of the arrestin was bound to the ROS membrane in the dark.

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ABBREVIATIONS

cmHA, *o*-(carboxymethyl)hydroxylamine; eHA, *o*-ethylhydroxylamine; G_t, transducin; $G_t\alpha$ peptide, high-affinity peptide analogue derived from the C-terminus of the α-subunit of transducin; HA, hydroxylamine; I72NBD, IANBD-labeled arrestin mutant I72C; IANBD, *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine; λ_{em}, wavelength of emission; λ_{ex}, wavelength of excitation; λ_{max}, wavelength of maximal absorbance; Meta II, metarhodopsin II; mHA, *o*-methylhydroxylamine; PSB, protonated Schiff base; ROS, rod outer segment membranes; *t*-bHA, *o*-*tert*-butylhydroxylamine; SD, standard deviation.

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