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Rapid formation of all-*trans* retinol after bleaching in frog and mouse rod photoreceptor outer segments

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SUMMARY

All-*trans* retinol is formed in the outer segments of vertebrate rod photoreceptors from the reduction of the all-*trans* retinal released by photoactivated rhodopsin. The reduction requires NADPH and is therefore dependent on metabolic input. In metabolically intact photoreceptors, a large increase in rod outer segment fluorescence, attributed to the fluorescence of all-*trans* retinol, follows rhodopsin photoactivation. The fluorescence increase is biphasic, including a rapid and a slow component. In metabolically compromised cells, there is a much smaller fluorescence increase following rhodopsin photoactivation, but it too contains a rapid component. We have measured the fluorescence signal in single living frog and mouse rod photoreceptors, and have characterized its dependence on the wavelengths of light selected for excitation and for collecting emission. We find that in metabolically intact cells, the excitation and emission properties of both the rapid and slow components of the fluorescence signal are in close agreement with those of all-*trans* retinol fluorescence. In metabolically compromised cells however, the signal can only partially be due to all-*trans* retinol, and most of it is consistent with all-*trans* retinal. The results suggest that in the outer segments of living rod photoreceptors there is rapid release of all-*trans* retinal, which in metabolically intact cells is accompanied by rapid conversion to all-*trans* retinol.

INTRODUCTION

The active intermediate of the visual pigment of rod photoreceptors, rhodopsin, is generated through the photoisomerization of its retinyl chromophore from 11-*cis* to all-*trans* ^{1,2}. This is followed by the hydrolysis of the Schiff base bond between the chromophore and the protein, and the dissociation of all-*trans* retinal. The released all-*trans* retinal is subsequently reduced to all-*trans* retinol by the enzyme retinol dehydrogenase using metabolic input in the form of NADPH ³. All these reactions take place in the outer segment, the rod photoreceptor compartment responsible for the conversion of light to an electrical signal. The all-*trans* retinol that forms leaves the outer segment and is transported to the adjacent cells of the retinal pigment epithelium (RPE) ⁴, where it is processed into 11-*cis* retinal ^{5–7}. 11-*cis* retinal is then transported from the RPE cells to the rod outer segment (ROS) where it is used for the regeneration of rhodopsin ⁸, which has been destroyed during the formation of its active intermediate.

The intrinsic fluorescence of all-*trans* retinol has been used to characterize its formation kinetics in single rod and cone photoreceptors from several vertebrate species ^{9–14}. This is achieved by imaging the large increase in outer segment fluorescence following visual pigment bleaching. Although most of this fluorescence signal is due to all-*trans* retinol ¹⁰,

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the extent of the contributions of other fluorophores has been only partially addressed. Specifically, although it is clear on the basis of the outer segment fluorescence emission spectrum that the contributions of NADH and NADPH are minimal¹⁰, the extent of the all-*trans* retinal contribution has not been rigorously examined. Although the fluorescence of all-*trans* retinal is readily detectable at low temperature (77 °K), its quantum yield is very small (<0.001) at room temperature¹⁵. By contrast, retinol has a substantial fluorescence quantum yield, even at room temperature, in the order of 0.006 – 0.03^{16–18}. So, it is expected that all-*trans* retinal would make a minimal contribution to outer segment fluorescence. However, its fluorescence emission spectrum at room temperature is very similar to that of all-*trans* retinol¹², and there are two important situations where its contribution may be significant. First, the outer segment fluorescence increase after bleaching includes a rapid component manifesting itself as a “jump” in fluorescence intensity immediately after bleaching, evident in salamander (Figs. 3A and 4 12), frog (Fig. 1G 13) and mouse (Fig. 3B 14) rods. Perhaps a large part of this “jump” is due to all-*trans* retinal. Second, the outer segment fluorescence increase after bleaching is largely suppressed in broken off rod outer segments due to the lack of a sufficient NADPH supply¹². But there is a residual fluorescence increase in the broken off rod outer segments, which, if it were due to all-*trans* retinol, would point to the presence of a substantial NADPH-generating machinery in the rod outer segment.

We have used frog and mouse rod photoreceptors to examine the respective contributions of all-*trans* retinal and retinol to the outer segment fluorescence increase after bleaching. We have selected these photoreceptors as model systems because they contain exclusively vitamin A₁-based chromophore, greatly simplifying data interpretation. To distinguish the fluorescence signal of all-*trans* retinal from that of all-*trans* retinol, we have excited outer segment fluorescence with light of different wavelengths. Because of the large difference in the absorption maxima of all-*trans* retinal ($\lambda_{\text{max}} \sim 380$ nm) and all-*trans* retinol ($\lambda_{\text{max}} \sim 325$ nm)¹⁹, this approach has allowed us to establish that for intact rods, the fluorescence signal is consistent with all-*trans* retinol, while for broken off rod outer segments is mostly due to all-*trans* retinal. For the remainder of the paper, retinal and retinol refer to the all-*trans* isomers.

EXPERIMENTAL

Grass frogs (*Rana pipiens*; The Sullivan Company, Nashville, TN) and mice (*Mus musculus*, sv129; The Harlan Laboratories, Indianapolis, IN) were kept in cyclic light, with 12-h light cycle. Mice were 2–3 months old. All animal procedures were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina. Animals were dark-adapted overnight, sacrificed under dim red light, and the retinas were excised and placed in the appropriate Ringer's (in mM; for frogs: 110 NaCl, 2.5 KCl, 1.6 MgCl₂, 1 CaCl₂, 5 HEPES, 5 glucose, pH = 7.55; for mice: 130 NaCl, 5 KCl, 0.5 MgCl₂, 2 CaCl₂, 25 HEPES, 5 glucose, pH = 7.40). Living, isolated rod photoreceptors were obtained following procedures described previously^{13,14}. Isolated photoreceptor cells were obtained by chopping the retina with a razor blade on Sylgard elastomer and were subsequently transferred to 100 μ L chambers coated with 0.01% polyornithine.

The fluorescence of isolated cells was imaged on an inverted Zeiss Axiovert 100 microscope (Carl Zeiss, Thornwood, NY) with a 40 $\times$ Fluor oil immersion lens (NA = 1.3). The light for excitation was provided by a Xenon continuous arc light source from Sutter Instrument Company (Novato, CA), and fluorescence images were acquired with a Hamamatsu CCD camera (Hamamatsu Photonics, Hamamatsu-City, Japan). For measuring the overall kinetics of the rod outer segment fluorescence signal, fluorescence was excited with a broadband (40

nm bandwidth) 360 nm filter and the emission was collected through a longpass 420 nm filter (emission >420 nm). For measuring the dependence of the fluorescence signal on the excitation light wavelength, fluorescence was excited with narrow bandpass (10 nm bandwidth) filters centered at 340, 360, and 380 nm; emission was collected for >420 nm. For measuring the dependence of the fluorescence signal on the emitted light wavelength, fluorescence was excited with the broadband (40 nm bandwidth) 360 nm filter, and emission was measured through a 457 nm filter (50 nm bandwidth) and a 528 nm filter (38 nm bandwidth). Experiments with frog cells were carried out at room temperature, while experiments with mouse cells were carried out at 37 °C by heating the microscope stage and the oil-immersion objective (Warner Instruments, Hamden, CT). Experiments were carried out with both metabolically intact rods, and metabolically compromised broken off ROS. An experiment began with a dark-adapted cell viewed under infrared light. An initial fluorescence image was captured and the cell was bleached with long wavelength light (1 min; >530 nm) from a 150 W halogen lamp illuminator (Edmund Optics, Barrington, NJ); fluorescence images were then captured at different times after bleaching. Fluorescence intensity was measured over defined regions of interest (ROI) contained in the outer segment and surrounding background. After correcting for background fluorescence, the value of outer segment fluorescence intensity obtained before rhodopsin bleaching was subtracted from all subsequent values to obtain the rod outer segment fluorescence signal generated by bleaching. All hardware control, image acquisition, and analysis were carried out using Slidebook (Intelligent Imaging Innovations, Denver, CO).

We have defined the amplitude of the rapid component of the fluorescence signal as the increase in rod outer segment intensity immediately after bleaching, while that of the slow component as the change in fluorescence intensity between 0 and 30 mins after bleaching (see Figs. 1 and 2 for graphical definition). We have obtained reference excitation and emission spectra for retinal and retinol by loading broken off ROS with 50 – 100 μ M concentrations of the retinoids for 10 mins. To compare and average data across different cells, we have normalized fluorescence intensities for each cell as follows: for the three different excitation wavelengths (340, 360 and 380 nm) we divided the intensities by the intensity obtained with 340 nm excitation; for the two different fluorescence emission wavelengths (457 and 528 nm), we divided them by the intensity measured at 457 nm. Hence, all excitation spectra have a value of 1 at 340 nm, while the emission spectra have a value of 1 at 457 nm (Fig. 4).

Exogenous all-*trans* retinal and all-*trans* retinol were supplied to broken off ROS using 1% bovine serum albumin (BSA) as lipophilic carrier. All reagents were of analytical grade. Reported errors are SEM.

RESULTS AND DISCUSSION

A large increase in the fluorescence of the outer segment follows bleaching of the visual pigment rhodopsin in the photoreceptors of several vertebrate species, including salamander^{9,12}, gecko¹¹, frog¹³, and mouse¹⁴. Fig. 1 shows the appearance and kinetics of this fluorescence signal in an intact rod (Fig. 1A) and a broken off ROS (Fig. 1B) from a frog retina. The increase in outer segment fluorescence after bleaching is plotted as a function of time in Figs. 1A–g and 1B–g, and the definition of the rapid and slow components used for analysis is shown on the graphs, too. Fig. 2 shows the appearance of the fluorescence signal in an intact rod (Fig. 2A) and a broken off ROS (Fig. 2B) from a mouse retina. The rapid component is especially obvious in the broken off ROS (Fig. 2B–g).

To obtain information on the identity of the fluorophore(s) responsible for the rapid and slow components in intact rods and broken off ROS, we excited their fluorescence with light

of different wavelengths, while collecting the emission for >420 nm. Because retinal has a much higher absorbance than retinol at 380 nm¹⁹, we used this wavelength to preferentially excite retinal fluorescence. For comparison, we excited the fluorescence with 340 and 360 nm as well. Fig. 3 shows the results of such experiments, using 340 and 380 nm excitation, with frog (Fig. 3A) and mouse (Fig. 3B) intact rods and broken off ROS. For the intact rods (Figs. 3A-I & 3B-I), the fluorescence of both the rapid (Figs. 3A-Ib & 3B-Ib) and the slow component (Figs. 3A-Ic & 3B-Ic) is preferentially excited by the 340 nm light (top images). For the broken off ROS on the other hand (Figs. 3A-II & 3B-II), the fluorescence of the rapid component (Figs. 3A-IIb & 3B-IIb) is excited equally well by 340 (top) and 380 nm (bottom image) light, while that of the slow component (Figs. 3A-IIc & 3B-IIc) is preferentially excited by 380 nm (bottom). These results demonstrate that the fluorescence signals in intact rods and broken off ROS originate from different fluorophores. To probe the identity of these fluorophores, we loaded broken off ROS with large concentrations of retinal and retinol. Because broken off ROS lack access to the metabolic machinery of the inner segment, large concentrations of exogenously added retinal and retinol cannot be metabolized to any significant extent, and so the resultant fluorescence will be due to the retinoid loaded to the ROS. Figs. 3A-III & 3B-III show that the fluorescence of a broken off ROS loaded with retinol is preferentially excited by 340 nm light (Figs. 3A-IIIb & 3B-IIIb, top). In contrast, the fluorescence of a broken off ROS loaded with retinal is preferentially excited by 380 nm light (Figs. 3A-IVb & 3B-IVb, bottom). To allow quantitative comparisons between the excitation properties of the outer segment, the retinal, and the retinol fluorescence signals, we divided the fluorescence intensities elicited with the three different wavelengths by that obtained with 340 nm excitation.

We obtained information on the emission properties of the rapid and slow components of the outer segment fluorescence signal by carrying out similar experiments as those shown in Fig. 3 (images not shown). We excited the outer segment fluorescence with light of a fixed wavelength, 360 nm, and collected emission at two different wavelengths, 457 and 528 nm. As in the case with different excitation wavelengths, we normalized the emission values by dividing the fluorescence intensities measured at the two different wavelengths by that obtained at 457 nm.

Fig. 4 compares the excitation and emission properties of the rapid and slow components to those of retinal and retinol. In the case of frog (Fig. 4A), for intact frog rods ($n = 6$) the excitation spectra of the rapid (purple circles, ●) and slow (purple triangles, ▲) components are both indistinguishable from the spectrum of retinol (blue diamonds, ◆, from $n = 7$ broken off ROS loaded with 100 μ M retinol). For broken off ROS, the spectrum of the slow component (green triangle, ▲, $n = 16$) is in close agreement with that of retinal (red squares, ■, from $n = 5$ broken off ROS loaded with 100 μ M retinal). The rapid component (green circles, ●, from the same cells as the slow component) displays intermediate properties between those of retinal and retinol. The emission properties of the rapid and slow components were measured from $n = 7$ intact frog rods and $n = 17$ broken off frog ROS. They are indistinguishable from each other and from those of retinol (from $n = 9$ broken off ROS loaded with 100 μ M retinol) and retinal (from $n = 8$ broken off ROS loaded with 100 μ M retinal). Virtually the same spectra are obtained from mouse rods (Fig. 4B). The excitation spectra were measured from $n = 6$ intact rods and $n = 9$ broken off ROS, and are compared to the spectra of retinol (from $n = 10$ broken off ROS loaded with 50 μ M retinol), and retinal (from $n = 7$ broken off ROS loaded with 50 μ M retinal). The emission spectra, measured from $n = 16$ intact mouse rods and $n = 13$ broken off mouse ROS, are indistinguishable from each other and from those of retinol (from $n = 7$ broken off ROS loaded with 50 μ M retinol) and retinal (from $n = 6$ broken off ROS loaded with 50 μ M retinal).

The formation of retinol in the rod outer segment has attracted a lot of experimental attention for at least two reasons. First, the formation of all-*trans* retinol begins the recycling of the chromophore, supplementing the supply of 11-*cis* retinal needed for the regeneration of rhodopsin. Second, the formation of retinol achieves the removal of retinal. Because retinal is a photosensitizer²⁰ and can also generate potentially toxic adducts^{21,22}, this reduction to retinol plays a protective role. In addition, retinal can increase the activity of opsin^{23–25}, and so its removal speeds up the recovery of the cell from light stimulation¹².

The outer segment fluorescence signal has been used as a retinol surrogate for imaging and measuring the retinol formation kinetics in single photoreceptor cells. Such use has been justified by experiments showing that the kinetics of retinol formation as determined from fluorescence measurements are in good agreement with biochemical measurements from HPLC analysis of retinoid extracts^{13,14}. Furthermore, the emission spectrum of the outer segment fluorescence is consistent with that of retinol¹⁰. The appearance of retinol in the outer segment however is indicative of the presence of retinal, since retinal is the substrate of the retinol dehydrogenase reaction that has retinol as its product. Together with the reversibility of the dehydrogenase reaction, this means that accumulation of retinol is associated with a concomitant rise in the concentration of retinal. Indeed, the presence of retinal in the outer segment has been directly observed with microspectrophotometry^{9,26}. HPLC analysis of retina extracts also shows the presence of retinal at long times after bleaching, when there has been plenty of time for its release from photoactivated rhodopsin and its reduction to retinol^{13,14}.

The presence of retinal in the outer segment and the similarity of its fluorescence emission spectrum with that of retinol, raises the question of the extent of its contribution to outer segment fluorescence. This is particularly relevant for the rapid increase in fluorescence observed immediately after bleaching, which has not been well-resolved with other techniques. Our results (Fig. 4) show that in metabolically intact frog and mouse rods this fluorescence component appears to be exclusively due to retinol; the same holds for the slow component as well. As discussed previously¹⁴, the sizeable rapid component of retinol formation is consistent with the fast release of retinal from photoactivated rhodopsin²⁷ and the availability of sufficient amounts of NADPH to reduce a large portion of the released retinal to retinol.

The properties of the fluorescence of broken off ROS are clearly different from that of the outer segments of intact cells (Fig. 4). Broken off ROS are separated from the rest of the cell, lack access to its metabolic machinery and supply of NADPH, and their ability to reduce retinal to retinol is severely compromised. Consistent with this state of affairs, the slow component of fluorescence increase in broken off ROS appears to be exclusively due to retinal suggesting negligible conversion to retinol. The slow appearance of retinal is due to a slow release phase from photoactivated rhodopsin, consistent with the presence of multiple rhodopsin photoproducts after bleaching^{26–28}. The rapid component has properties intermediate to those of retinal and retinol, and a plausible interpretation is that this is because both of them contribute to it. This would suggest that there is a small pool of NADPH in the broken off ROS, which allows the conversion of some of the rapidly released retinal to retinol. Because of its comparatively much higher fluorescence quantum yield, the small amount of retinol can shift the excitation spectrum of the overall fluorescence.

The results also show that, as expected, the dependence of the retinal and retinol fluorescence on the emission wavelength is virtually identical and cannot be used to distinguish between the two. The outer segment fluorescence emission is consistent with either retinal or retinol (Fig. 4), but is clearly distinct from that of NADH or NADPH (see Fig. 3 10).

CONCLUSION

To summarize, we have corroborated the presence of a rapid component of retinol formation after rhodopsin bleaching in the outer segments of both frog and mouse rod photoreceptors. Our results are consistent with a biphasic release of retinal from photoactivated rhodopsin. In metabolically intact rod outer segments, the NADPH supply is sufficient to reduce a major portion of the released retinal to retinol, and as a result the outer segment fluorescence signal is exclusively due to retinol. In metabolically compromised broken off ROS, there appears to be a small pool of NADPH, which is used up to reduce a small portion of the rapidly released retinal to retinol, giving rise to a fluorescence signal to which both retinoids contribute. As more retinal is slowly released, and in the absence of additional NADPH input to reduce it to retinol, its fluorescence comes to dominate the outer segment fluorescence signal.

ABBREVIATIONS

ROS	rod outer segment
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
RPE	retinal pigment epithelium
BSA	bovine serum albumin

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Figure 1.

Rapid and slow phases of fluorescence increase in outer segments of frog rod photoreceptors after rhodopsin bleaching. (A) Intact frog rod photoreceptor; (B) Broken off rod outer segment, separated from the rest of the cell. For both (A) and (B), fluorescence increases in the outer segment after bleaching of rhodopsin. a, infrared image of the cell; b–f, fluorescence images (excitation 360 nm, emission >420 nm) of the cell; b, before bleaching; c, immediately after; d, 1; e, 5; and f, 30 minutes after bleaching. All fluorescence images are shown at the same intensity scaling. g, fluorescence increase in the rod outer segment after bleaching plotted as a function of time, and showing the definition of the amplitudes of the rapid and slow components. Bleaching was carried out from $t = -1$ min to $t = 0$. Bar is 10 μm . Experiments at room temperature.

Figure 2.

Rapid and slow phases of fluorescence increase in outer segments of mouse rod photoreceptors after rhodopsin bleaching. (A) Intact mouse rod photoreceptor; (B) Broken off rod outer segment, separated from the rest of the cell. For both (A) and (B), bleaching was carried out from $t = -1$ min to $t = 0$; bars are $5\ \mu\text{m}$. Fluorescence increases in the outer segment after bleaching of rhodopsin. a, infrared image of the cell; b–f, fluorescence images (excitation 360 nm, emission >420 nm) of the cell; b, before bleaching; c, immediately after; d, 1; e, 5; and f, 30 minutes after bleaching. All fluorescence images are shown at the same intensity scaling. g, fluorescence increase in the rod outer segment after bleaching plotted as a function of time, and showing the definition of the amplitudes of the rapid and slow components. Experiments at $37\ ^\circ\text{C}$.

Figure 3.

Excitation of (A) frog and (B) mouse rod outer segment fluorescence with 340 nm (top rows of fluorescence images) and 380 nm (bottom rows) light. Fluorescence emission was collected for >420 nm. For each top and bottom pair, the images are shown at the same intensity scaling. All bars are 10 μ m. (I) Intact rod photoreceptors. a, infrared image; b, fluorescence images immediately, and c, 30 minutes after bleaching. (II) Broken off rod outer segment. a, infrared image; b, fluorescence images immediately, and c, 30 minutes after bleaching. (III) Addition of exogenous all-*trans* retinol to a broken off rod outer segment. a, infrared image; b, fluorescence images 10 minutes after the addition of 100 μ M (A, frog) or 50 μ M (B, mouse) retinol. (IV) Addition of exogenous all-*trans* retinal to a

broken off rod outer segment. a, infrared image; b, fluorescence images 10 minutes after the addition of 100 μ M (A, frog) or 50 μ M (B, mouse) retinal.

Figure 4.

Excitation and emission spectra of outer segment fluorescence of (A) frog and (B) mouse rod photoreceptors. For both species, and for intact rod photoreceptors, the spectra of both the rapid (●) and the slow (▲) components of the fluorescence increase are in close agreement with the spectra of retinol (◆). For broken off rod outer segments, the spectra of the slow component (▲) are in close agreement with those of retinal (■), while the rapid component (●) has an intermediate spectrum, consistent with the presence of some retinol. Error bars represent standard errors.