2-Hydroxypropyl- β -Cyclodextrin Removes All-*Trans* Retinol from Frog Rod Photoreceptors in a Concentration-Dependent Manner

Daniel Johnson,¹ Chunhe Chen,² and Yiannis Koutalos²

Abstract

Purpose: To determine whether a nonprotein lipophilic carrier, 2-hydroxypropyl- β -cyclodextrin (HP- β -CD), can remove all-*trans* retinol from rod photoreceptor outer segments. All-*trans* retinol is generated in rod outer segments after light exposure. It is highly insoluble, and its efficient transport across extra- and intracellular aqueous space requires specialized carriers.

Methods: Experiments were carried out with isolated frog rod photoreceptor cells. The removal of all-*trans* retinol by different concentrations of this carrier was measured by imaging its fluorescence in single-rod photoreceptors.

Results: HP- β -CD concentrations >0.3 mM significantly increased the rate of all-*trans* retinol removal. The rate of removal increased linearly with carrier concentration, with a slope of $0.0058 \text{ min}^{-1}/\text{mM}$.

Conclusions: The effectiveness of HP- β -CD shows that a specialized interaction with the cell membrane is not necessary for the efficient transfer of all-*trans* retinol between the cell membrane and the carrier. The transfer occurs through a collision-based mechanism, as indicated by the linear increase of the rate of removal with the carrier concentration.

Introduction

VITAMIN A (ALL-*TRANS* RETINOL), as well as other retinoids, is highly insoluble¹ and requires the mediation of specialized carriers for its efficient transport across aqueous space.² In the retina, retinoid transport is a required component for the regeneration of the visual pigment that follows light detection. A retinoid essential for vision is the 11-*cis* retinal chromophore of vertebrate visual pigments, the primary light detectors.^{3,4} Visual pigments are present in large concentrations in photoreceptor outer segments,^{5,6} the organelles that convert incoming light to an electrical signal. The detection of light by the photoreceptor cells involves the photoisomerization of the 11-*cis* chromophore of the visual pigment to all-*trans*, necessitating pigment regeneration.^{7–10} The regeneration process encompasses removal of the all-*trans* chromophore from the photoreceptor outer segment and supply of fresh 11-*cis* isomer. Removal of the all-*trans* isomer begins with the release of all-*trans* retinol from the pigment and its reduction to all-*trans* retinol.^{11,12} All-*trans* retinol is then removed from the photoreceptor outer segment by the interphotoreceptor retinoid-binding protein (IRBP),¹³ a specialized carrier present in the interphoto-

receptor space,¹⁴ and transferred to neighboring cells,¹⁵ where it is converted to the 11-*cis* isomer.^{7–10} IRBP is also involved in the transport of fresh 11-*cis* chromophore to the photoreceptor outer segments.^{16,17}

The transfer of all-*trans* retinol between cell membranes and its carriers is not well understood. One possibility is that all-*trans* retinol transfers by first hydrating into aqueous solution; another is that it transfers directly through collisions, which would be facilitated by membrane receptors for the carriers. In rod photoreceptors, the removal of all-*trans* retinol has been examined using its intrinsic fluorescence.^{12,13} This fluorescence provides a good measure of retinol outer segment concentration.¹⁸ Fluorescence imaging experiments with single-rod photoreceptors have shown that IRBP and the lipophilic carrier protein bovine serum albumin (BSA) can remove retinol from the outer segment.^{13,18} The rate of retinol removal increases with the carrier protein concentration, indicating that these carriers operate through a collision-dependent mechanism. These previous experiments further suggested that the transfer of retinol from the membrane to the carrier might be facilitated by a specialized interaction between the carrier and the cell membrane, perhaps mediated by a receptor.

¹Department of Physics, College of Charleston, Charleston, South Carolina.

²Department of Ophthalmology, Medical University of South Carolina, Charleston, South Carolina.

The present work examines the removal of retinol by the nonprotein carrier 2-hydroxypropyl- β -cyclodextrin (HP- β -CD). Cyclodextrins are cyclic oligosaccharides with a hydrophilic shell enclosing a hydrophobic cavity, and are widely used to increase the aqueous solubility and stability of hydrophobic drugs.¹⁹ HP- β -CD is composed of 7 dextrose molecules with a hydroxypropyl substitution at the 2-hydroxyl position of the dextrose unit. Because of its low toxicity,²⁰ it is being extensively used to solubilize drugs, including retinoids. A generic lipophilic carrier like HP- β -CD is unlikely to have any specialized interactions with cell membranes, and so its effect on the rate of retinol removal would clarify whether a receptor is required for the efficient transfer of retinol between membrane and carrier.

In the rest of the text, all-*trans* retinol is referred to simply as retinol.

Methods

Frogs (*Rana pipiens*) were obtained from The Sullivan Company (Nashville, TN). Animals were kept and sacrificed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association; the authors adhere to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Dark-adapted isolated rod photoreceptor cells were obtained in amphibian Ringer's exactly as described.¹⁸ Briefly, retinol fluorescence measurements on isolated cells were carried out on the stage of an inverted Zeiss Axiovert 100 microscope (Carl Zeiss, Thornwood, NY), following the published procedures.¹⁸ Fluorescence images (360 nm excitation and >420 nm emission) were acquired at different times after bleaching an isolated cell with long-wavelength light (1 min; >530 nm from a 150 W halogen lamp illuminator). Fluorescence intensity was measured over defined regions of interest in the outer segments and background. After correcting for background, the outer segment fluorescence due to retinol was obtained by subtracting the initial outer segment fluorescence before bleaching.

Different concentrations of HP- β -CD were prepared in amphibian Ringer's and added to the chambers containing the cells 40 min after the bleaching of rhodopsin. The values of retinol fluorescence after HP- β -CD addition were normalized to the value at 40 min immediately after the addition. The rate of retinol removal, R , was determined by fitting the normalized fluorescence values, $N(t)$, with single-exponential functions of unitary amplitude at $t = 40$ min

$$N(t) = e^{-R \cdot (t - 40)} \quad (1)$$

The reported rate of retinol removal for a given concentration of HP- β -CD is the average of the rates measured from individual cells.

HP- β -CD was from Sigma-Aldrich Chemical Company (St. Louis, MO); all other reagents were of analytical grade. Experiments were carried out at room temperature. Error bars represent standard errors.

Results

Exposure of an isolated dark-adapted frog rod photoreceptor to light results in the formation of retinol in the outer

segment, which can be monitored from its fluorescence.^{12,13} In the absence of lipophilic carriers in the solution bathing the isolated cell, the fluorescence signal reaches a maximum about 30–40 min after light exposure and declines very slowly thereafter (Fig. 1A, B). Addition of 10 mM of the lipophilic carrier HP- β -CD at 40 min after light exposure speeds up the removal of retinol, as indicated by the faster decay of the fluorescence signal (Fig. 1C, D). Figure 2A shows the removal of retinol by different concentrations of HP- β -CD. The rate of removal in the absence of any carrier, $0.006 \pm 0.001 \text{ min}^{-1}$, is the same as reported previously.¹⁸ The effect of the carrier becomes noticeable at concentrations higher than $\sim 0.3 \text{ mM}$, and becomes more pronounced as its concentration increases. The removal of retinol follows first-order kinetics, consistent with the previous report of 1:1 binding between retinol and HP- β -CD.²¹ Only with 10 mM HP- β -CD does retinol removal appear to deviate from first-order kinetics at later times. Although the reason for this apparent slow-down is not clear, it might reflect the contribution of higher order complexes of retinol with the carrier at these lower retinol-to-carrier ratios. The rate of retinol removal increases linearly with the HP- β -CD concentration (Fig. 2B), with a slope of $0.0058 \text{ min}^{-1} / \text{mM}$.

FIG. 1. 2-Hydroxypropyl- β -cyclodextrin removes all-*trans* retinol from frog rod photoreceptor outer segments. (A) Formation of all-*trans* retinol in the outer segment of an isolated rod photoreceptor. In the absence of lipophilic carriers, the all-*trans* retinol that forms after bleaching does not readily leave the outer segment. (a) Infrared image of a dark-adapted frog rod photoreceptor cell (scale bar 10 μm). (b–e) Fluorescence images of the cell, shown at the same intensity scaling, at different times after bleaching: (b) dark-adapted cell, before bleaching; (c) 40 min; (d) 50 min; (e) 90 min after bleaching. (B) Rod outer segment retinol fluorescence intensity at different times after rhodopsin bleaching for the cell shown in (A). Rhodopsin was bleached for 1 min, beginning at $t = -1$ min and ending at $t = 0$. (C) Same experimental protocol as in (A), with 10 mM HP- β -CD added in the bath at 40 min after bleaching. (a) Infrared image of the cell (scale bar 10 μm). (b–e) Fluorescence images acquired at the same times as for the cell in (A). (D) Rod outer segment retinol fluorescence at different times after bleaching for the cell in (C).

FIG. 2. The rate of retinol removal increases with the concentration of 2-hydroxypropyl- β -cyclodextrin (HP- β -CD). (A) Fraction of the remaining retinol fluorescence after the addition of different concentrations of HP- β -CD at 40 min after rhodopsin bleaching. The fluorescence intensities have been normalized over the value at 40 min. Symbols represent experimental data points; the lines are simple exponentials (Eq. 1) with rate constants determined from the individual cells. HP- β -CD concentrations (in mM) were 0 ($\diamond$, $n=8$), 0.3 ($\bullet$, $n=8$), 1 ($\triangle$, $n=9$), 3 ($\blacktriangledown$, $n=5$), and 10 ($\circ$, $n=5$). (B) Dependence of the rate of retinol removal on the concentration of HP- β -CD ($\circ$). The straight line is a linear least squares fit with slope $0.0058 \pm 0.0001 \text{ min}^{-1}/\text{mM}$.

Discussion

The increase in the rate of retinol removal with carrier concentration suggests that the transfer of retinol from the cell membrane to HP- β -CD proceeds via a collision-based mechanism. That is, retinol combines with the carrier while it is still in the membrane, where it is moving unrestrictedly.²² If instead retinol were transferring by first hydrating into aqueous solution, the rate of transfer would be independent of the carrier concentration.^{23–25} This mechanism of transfer is the same as in the case of the protein carriers IRBP and BSA, for which the rate of retinol removal also increases with carrier concentration. In that case, it was suggested that the removal of retinol by these protein carriers was mediated by a specialized interaction between the carriers and the plasma membrane. The effectiveness of HP- β -CD suggests that such a specialized interaction, for example, via a receptor for the carrier, is not required for the efficient transfer of retinol.

IRBP and BSA are far more effective than HP- β -CD. For BSA, the rate of retinol removal increases linearly with the BSA concentration,¹⁸ with a slope of $0.32 \text{ min}^{-1}/\text{mM}$. For IRBP, the rate of retinol removal saturates at higher concentrations, but below saturation the rate of removal increases linearly with the IRBP concentration¹⁸ with a slope of $5.0 \text{ min}^{-1}/\text{mM}$. That is, BSA is $\sim 55\times$ more effective and IRBP $\sim 860\times$ than HP- β -CD. One likely reason for these large differences in effectiveness is the differences in affinity for retinol. The affinity of IRBP for retinol is $\sim 100 \text{ nM}$,²⁶ of BSA is $\sim 2 \mu\text{M}$,²⁴ while that of HP- β -CD, on the basis of its affinity for retinoic acid,²⁷ is in the order of 1 mM .

The results presented here appear to be inconsistent with previous studies demonstrating that retinol transfers between protein carriers, liposomes, and cell membranes by first hydrating into aqueous solution.^{23–25} This discrepancy can be reconciled by taking into account the differences in the concentrations of retinol: these previous experiments were carried out with μM concentrations of retinol, whereas the levels of retinol generated in the rod photoreceptor outer segment are in the order of mM (similar to the concentration

of the visual pigment^{5,6}). A collision-based mechanism will make a larger contribution to the rate of retinol transfer at higher concentrations because of the higher rate of collisions and the low aqueous solubility of retinol.

In summary, we find that the transfer of retinol from cell membranes to a nonspecialized carrier molecule occurs through a collision-based mechanism, similar to the case of protein carriers, including the specialized protein carrier IRBP. A membrane receptor for the carrier does not appear to be required for efficient transfer of retinol, although the presence of a receptor on the cell membrane could increase the rate of collisions and further increase the retinol transfer rate. Because of the limited aqueous solubility of retinol, the collision-based transfer mechanism is likely to be generally relevant at high retinol concentrations. It remains unclear whether the high effectiveness of IRBP is due not only to its high affinity for retinol, but also to the presence of a receptor on the rod outer segment plasma membrane. Such a receptor could also facilitate the removal of retinol by IRBP at low retinoid concentrations.

Acknowledgments

The authors thank Dr. Linda Jones for support and encouragement and Dr. Rosalie K. Crouch for helpful discussions. The work was supported by NIH grant EY14850 (Y.K.), and an unrestricted grant to MUSC Storm Eye Institute from Research to Prevent Blindness, Inc., New York, NY.

Author Disclosure Statement

The authors state that no competing financial interests exist.

References

1. Szuts, E.Z., and Harosi, F.I. Solubility of retinoids in water. *Arch. Biochem. Biophys.* 287:297–304, 1991.
2. Moise, A.R., Noy, N., Palczewski, K., and Blaner, W.S. Delivery of retinoid-based therapies to target tissues. *Biochemistry* 46:4449–4458, 2007.
3. Ebrey, T., and Koutalos, Y. Vertebrate photoreceptors. *Prog. Retin. Eye Res.* 20:49–94, 2001.
4. Palczewski, K. G protein-coupled receptor rhodopsin. *Annu. Rev. Biochem.* 75:743–767, 2006.
5. Harosi, F.I. Absorption spectra and linear dichroism of some amphibian photoreceptors. *J. Gen. Physiol.* 66:357–382, 1975.
6. Liebman, P.A., and Entine, G. Visual pigments of frog and tadpole (*Rana pipiens*). *Vision Res.* 8:761–775, 1968.
7. Saari, J.C. Biochemistry of visual pigment regeneration: the Friedenwald lecture. *Invest. Ophthalmol. Vis. Sci.* 41:337–348, 2000.
8. McBee, J.K., Palczewski, K., Baehr, W., and Pepperberg, D.R. Confronting complexity: the interlink of phototransduction and retinoid metabolism in the vertebrate retina. *Prog. Retin. Eye Res.* 20:469–529, 2001.
9. Lamb, T.D., and Pugh, E.N., Jr. Dark adaptation and the retinoid cycle of vision. *Prog. Retin. Eye Res.* 23:307–380, 2004.
10. Imanishi, Y., Lodowski, K.H., and Koutalos, Y. Two-photon microscopy: shedding light on the chemistry of vision. *Biochemistry* 46:9674–9684, 2007.
11. Futterman, S., Hendrickson, A., Bishop, P.E., Rollins, M.H., and Vacano, E. Metabolism of glucose and reduction of

- retinaldehyde in retinal photoreceptors. *J. Neurochem.* 17: 149–156, 1970.
12. Chen, C., Tsina, E., Cornwall, M.C., Crouch, R.K., Vijayaraghavan, S., and Koutalos, Y. Reduction of all-trans retinal to all-trans retinol in the outer segments of frog and mouse rod photoreceptors. *Biophys. J.* 88:2278–2287, 2005.
 13. Tsina, E., Chen, C., Koutalos, Y., Ala-Laurila, P., Tsacopoulos, M., Wiggert, B., et al. Physiological and microfluorometric studies of reduction and clearance of retinal in bleached rod photoreceptors. *J. Gen. Physiol.* 124:429–443, 2004.
 14. Chader, G.J. Interphotoreceptor retinoid-binding protein (IRBP): a model protein for molecular biological and clinically relevant studies. Friedenwald lecture. *Invest. Ophthalmol. Vis. Sci.* 30:7–22, 1989.
 15. Okajima, T.I., Pepperberg, D.R., Ripps, H., Wiggert, B., and Chader, G.J. Interphotoreceptor retinoid-binding protein: role in delivery of retinol to the pigment epithelium. *Exp. Eye Res.* 49:629–644, 1989.
 16. Edwards, R.B., and Adler, A.J. IRBP enhances removal of 11-cis-retinaldehyde from isolated RPE membranes. *Exp. Eye Res.* 70:235–245, 2000.
 17. Okajima, T.I., Pepperberg, D.R., Ripps, H., Wiggert, B., and Chader, G.J. Interphotoreceptor retinoid-binding protein promotes rhodopsin regeneration in toad photoreceptors. *Proc. Natl. Acad. Sci. U. S. A.* 87:6907–6911, 1990.
 18. Wu, Q., Blakeley, L.R., Cornwall, M.C., Crouch, R.K., Wiggert, B.N., and Koutalos, Y. Interphotoreceptor retinoid-binding protein is the physiologically relevant carrier that removes retinol from rod photoreceptor outer segments. *Biochemistry* 46:8669–8679, 2007.
 19. Stella, V.J., and He, Q. Cyclodextrins. *Toxicol. Pathol.* 36:30–42, 2008.
 20. Gould, S., and Scott, R.C. 2-Hydroxypropyl-beta-cyclodextrin (HP-beta-CD): a toxicology review. *Food Chem. Toxicol.* 43:1451–1459, 2005.
 21. Angelova, A., Ringard-Lefebvre, C., and Baszkin, A. Drug-cyclodextrin association constants determined by surface tension and surface pressure measurements. *J. Colloid Interface Sci.* 212:280–285, 1999.
 22. Wu, Q., Chen, C., and Koutalos, Y. All-trans retinol in rod photoreceptor outer segments moves unrestrictedly by passive diffusion. *Biophys. J.* 91:4678–4689, 2006.
 23. Ho, M.T., Massey, J.B., Pownall, H.J., Anderson, R.E., and Hollyfield, J.G. Mechanism of vitamin A movement between rod outer segments, interphotoreceptor retinoid-binding protein, and liposomes. *J. Biol. Chem.* 264:928–935, 1989.
 24. Noy, N., and Xu, Z.J. Interactions of retinol with binding proteins: implications for the mechanism of uptake by cells. *Biochemistry* 29:3878–3883, 1990.
 25. Noy, N., and Xu, Z.J. Kinetic parameters of the interactions of retinol with lipid bilayers. *Biochemistry* 29:3883–3888, 1990.
 26. Tschanz, C.L., and Noy, N. Binding of retinol in both retinoid-binding sites of interphotoreceptor retinoid-binding protein (IRBP) is stabilized mainly by hydrophobic interactions. *J. Biol. Chem.* 272:30201–30207, 1997.
 27. Lin, H.S., Chean, C.S., Ng, Y.Y., and Chan, S.Y., Ho, P.C. 2-Hydroxypropyl-beta-cyclodextrin increases aqueous solubility and photostability of all-trans-retinoic acid. *J. Clin. Pharm. Ther.* 25:265–269, 2000.

Received: February 5, 2010

Accepted: March 12, 2010

Address correspondence to:

Dr. Yiannis Koutalos

Department of Ophthalmology

Medical University of South Carolina

167 Ashley Ave.

Charleston, SC 29425

E-mail: koutalo@musc.edu