ARTICLE

OBC
www.rsc.org/obc

A2-Rhodopsin: a new fluorophore isolated from photoreceptor outer segments

Nathan Fishkin,[a] Young-Pyo Jang,[a] Yasuhiro Itagaki,[a] Janet R. Sparrow[b] and Koji Nakanishi*[a]

[a] *Departments of Chemistry, Columbia University, New York, NY 10027. E-mail: kn5@columbia.edu*

[b] *Ophthalmology, Columbia University, New York, NY 10027*

Received 9th December 2002, Accepted 13th February 2003
First published as an Advance Article on the web 5th March 2003

A2E and iso-A2E are fluorescent amphiphilic pyridinium bisretinoids involved in age-related macular degeneration (AMD). It is now shown that the presence of high exogenous concentrations of all-*trans*-retinal in photoreceptor outer segments leads to the formation of A2-rhodopsin (A2-Rh), an unprecedented fluorescent rhodopsin adduct which consists of bisretinoids (A2) linked to each of three lysine residues in rhodopsin (Rh) and which exhibits an emission spectrum similar to A2E. The fluorophore to protein ratio was determined by MALDI-TOF-MS and UV–VIS spectroscopy. Enzymatic degradation with thermolysin and cathepsin D showed that two of the A2 moieties were located in the region of the third cytoplasmic loop and 8th helix of Rh. Examination of A2-Rh and A2-PE (the precursor of A2E) fluorescence in relation to all-*trans*-retinal concentration indicated that whereas A2-PE formation is favored over that of A2-Rh, for a single rhodopsin molecule only one phosphatidylethanolamine molecule is available to react with all-*trans*-retinal; this phosphatidylethanolamine is probably tightly associated with the protein.

Introduction

As a consequence of the continual light-related vitamin A cycling in the retina, the orange-emitting fluorophore A2E (Fig. 1) is deposited in retinal pigment epithelial (RPE) cells as a constituent of the fluorescent pigments and uncharacterized lipids and proteins that constitute lipofuscin. Indeed, A2E, its slightly less polar photoisomer, iso-A2E and other minor *cis*-isomers recently identified, together constitute the most prominent hydrophobic pigments in RPE extracts assayed by reverse phase HPLC.[1,2] The amassing of lipofuscin fluorophores such as A2E is, however, not only a feature of aging RPE since excessive amounts of these fluorophores are also present in the RPE of patients with Stargardt disease, an inherited retinal disorder with juvenile onset.[3–7] Since RPE cells are essential to the health of photoreceptor cells, the demise of these cells in some retinal disorders, such as AMD and Stargardt disease, is a critical component of the disease process.

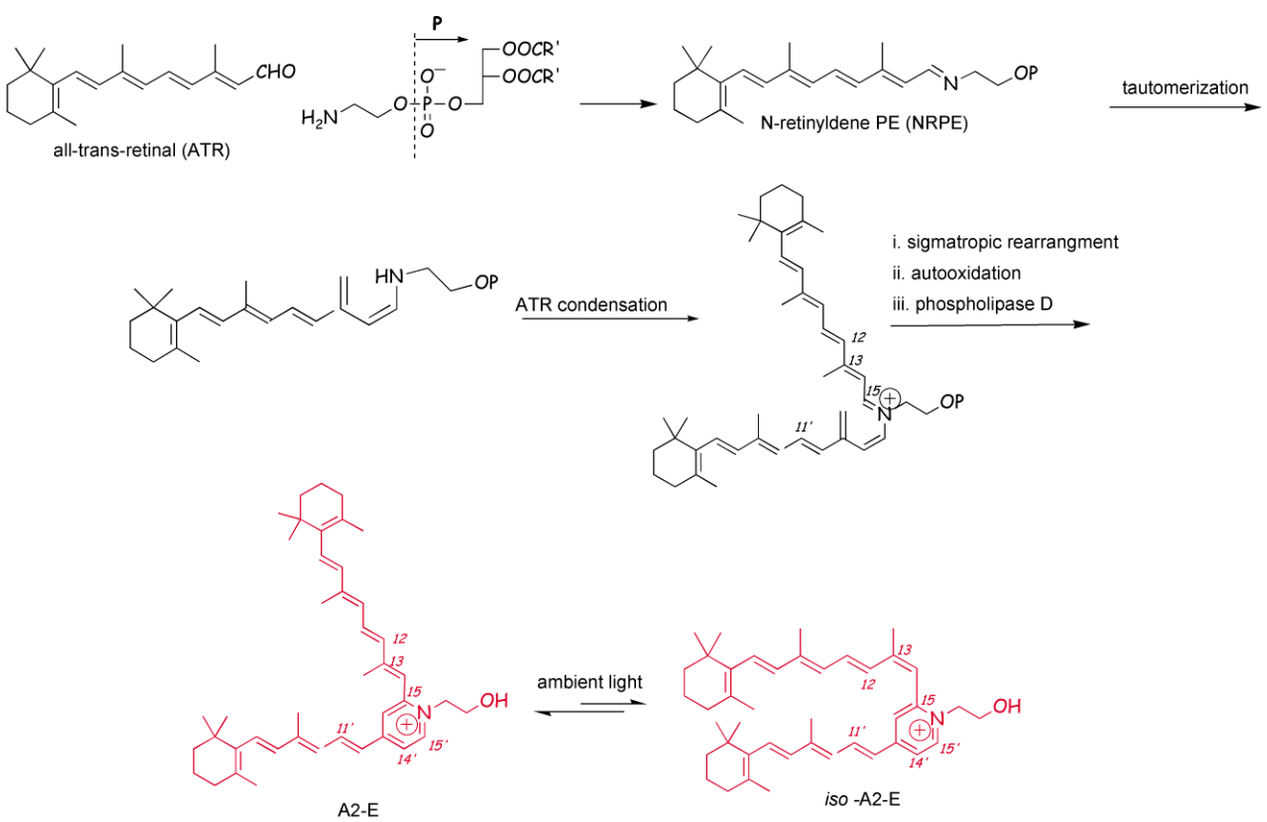

Fig. 1 Biosynthesis of A2E and iso-A2E from NRPE. It is proposed that the formation of A2-derivatized rhodopsin involves a similar step-wise condensation and cyclization with reactions of 2 molecules of all-*trans*-retinal at each of 3 lysines on rhodopsin.

DOI: 10.1039/b212213h

Evidence is mounting that a major factor contributing to the death of RPE cells, particularly in the macula, may be the accumulation of lipofuscin.[4,5,8–10]

NMR and total chemical synthesis revealed A2E to consist of an unprecedented pyridinium polar head group and two hydrophobic retinoid tails (a pyridinium bis-retinoid).[11,12] A2E is generated synthetically by the condensation of two molecules of all-*trans*-retinal adduct (vitamin A aldehyde) and a molecule of ethanolamine, hence the name A2E.[1,13,14] However, within the retina, A2E biosynthesis (see Fig. 1) is initiated in the photoreceptor outer segment (ROS) membrane by a reaction between phosphatidylethanolamine and a single molecule of all-*trans*-retinal that generates a Schiff base conjugate (*N*-retinylidenephosphatidylethanolamine, NRPE).[1,15] We proposed[1,11] that this adduct undergoes a [1,6]-proton tautomerization generating a phosphatidylethanolamine–retinyl enamine which reacts with a second molecule of all-*trans*-retinal. After an aza-6π-electrocyclization (to phosphatidyldihydropyridine bisretinoid) and autooxidation, the fluorescent pigment phosphatidyl pyridinium bisretinoid (A2-PE) is formed and following phosphate hydrolysis, A2E is generated. Phagocytosis of outer segment disk membrane causes A2E to be deposited in RPE cells. As with other lipofuscin components, A2E cannot be degraded by RPE lysosomal enzymes, and thus accumulates. There is now considerable experimental evidence that A2E is damaging to the RPE cell.[33,34]

The initial NRPE intermediate is believed to form after the release of all-*trans*-retinal from opsin during photobleaching. Rim protein (RmP), a retina specific ATP-binding cassette transporter encoded by the ABCR gene,[16–18] is thought to be involved in the active transport of NRPE across the lipid bilayer, thereby making all-*trans*-retinal available for reduction by all-*trans*-retinol dehydrogenase on the cytoplasmic side of the disc membrane.[19–21] Since the reduction of retinal to retinol is the rate-limiting step in the vitamin A cycle,[22] the sequestration of all-*trans*-retinal as NRPE and access of the latter to all-*trans*-retinol dehydrogenase, appears to be important in limiting the accumulation of all-*trans*-retinal in the outer segment. The alternative is illustrated in mice with homozygous mutations in the ABCR gene. Namely, an accumulation of NRPE in outer segments leads to increased A2-PE formation and 20-fold greater levels of A2E in RPE cells.[21]

In addition to the formation of retinylidene Schiff bases, it is possible that the ε-amino group of lysine residues of nearby proteins could condense with retinal, ultimately forming an A2-protein in a process similar to A2-PE biosynthesis. Recent work has revealed that (*E*)-3-methoxycarbonyl-2,4,6-trienals[23] are potent inhibitors of phospholipase A_2 activity due to their formation of a Schiff base with an active site lysine and subsequent irreversible cyclization to a dihydropyridine derivative. We therefore chose to investigate whether an adduct could form between all-*trans*-retinal and rhodopsin, the major membrane bound protein in rod outer segments.

Results

To determine whether an all-*trans*-retinal adduct could form covalently on dark-adapted rhodopsin, urea-washed ROS were incubated with 50 μM all-*trans*-retinal (~10-fold molar excess) for 3 days at 37 °C. This experiment yielded a protein which comigrated with rhodopsin on a polyacrylamide gel but in contrast to rhodopsin, was highly fluorescent when imaged through a >525 nm high band pass filter. The fluorescence of this all-*trans*-retinal protein adduct, hereafter referred to as A2-rhodopsin, was significantly diminished when the detected emission was modulated with either a 525 nm or 435 nm band pass filter. This fluorescence profile is consistent with that of A2E, which has a fluorescent emission spectrum centered around 600 nm. Thermolysin cleavage of the rhodopsin (Fig. 2A schematic) adduct on the C-terminal loop connecting

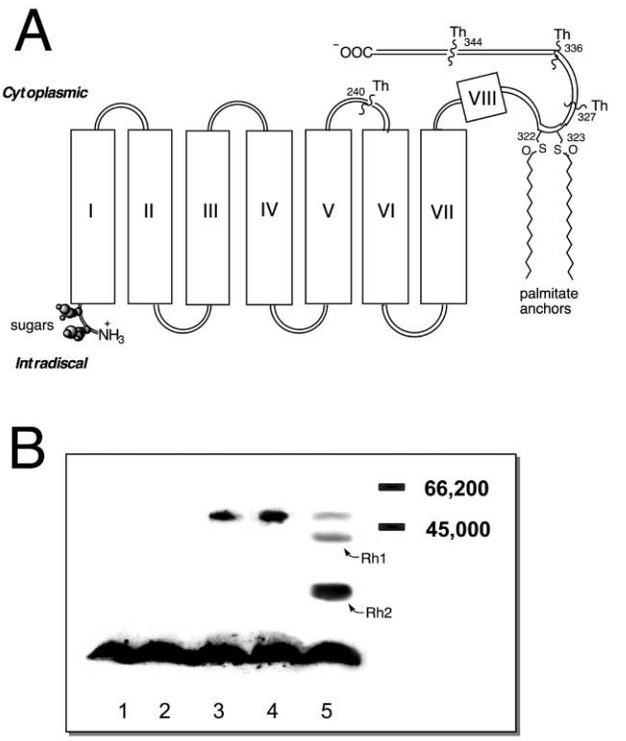

Fig. 2 A) Model of rhodopsin showing the seven trans-membrane helices typical of G-protein coupled receptors (GPCRs). Sites of thermolysin cleavage are shown (Th). B) Denaturing SDS-PAGE analysis of A2 derivatized rhodopsin using a 10% tricine gel with fluorescence detection (>525 nm emission). Lane 1, incubation of ROS with 1 eq. all-*trans*-retinal (3 days, 37 °C, in dark). Similar incubations were carried out with 2 eq. (Lane 2), 4 eq. (Lane 3), and 8 eq. (Lane 4) of retinal. The same quantity of protein was loaded in each lane. A sample equivalent to lane 4 was digested with thermolysin while still in the disk membrane, producing two new protein fragments seen in lane 5, corresponding to residues 1–240 (higher band, Rh1) and 241–327 (lower band, Rh2) of the rhodopsin sequence.

helices V and VI afforded two new protein bands[24] corresponding to residues 1–240 (helices I–V) and 241–327 (helices VI–VII) (see Fig. 2B, lane 5), both with the same fluorescence profile as described above for the intact protein. This suggested that at least one A2 moiety was formed in each fragment. After measurement of the relative fluorescence intensities in both bands under denaturing conditions, it appeared that the lower molecular weight fragment had approximately twice the intensity (1.8 : 1) as the 1–240 amino acid fragment.

Additional evidence from MALDI-TOF-MS (matrix assisted laser desorption ionization time-of-flight mass spectrometry) was needed to verify the covalent attachment of all-*trans*-retinal to rhodopsin. It was necessary to remove the N-terminal sugars from rhodopsin prior to MS analysis since a second peak corresponding to $GluNAc(Man)_3(GluNAc)_2$ (~1096 Da) was likely to obscure the mass shift due to any A2 derivatization. Glycopeptidase F[25] was therefore added to a solubilized solution of rhodopsin and A2-rhodopsin in order to cleave the various glycoforms. Attempts were made to perform glycolysis in ROS suspensions but the MS data showed no sugar removal under these conditions. The MALDI data obtained suggests that three A2 units are attached to the protein. A clean peak top shift of 1660 Da (see Fig. 3) compared to native rhodopsin corresponds to the addition of 6 retinals minus 6 water molecules to the protein and suggests that the synthesis of A2-rhodopsin, like the biosynthesis of A2E, involves step-wise condensation and cyclization of two retinals occurring on three different lysines. The sharp change in the average mass implies that the lysine residues derivatized are the same three in each protein molecule as opposed to random lysines with varying degrees of reactivity.

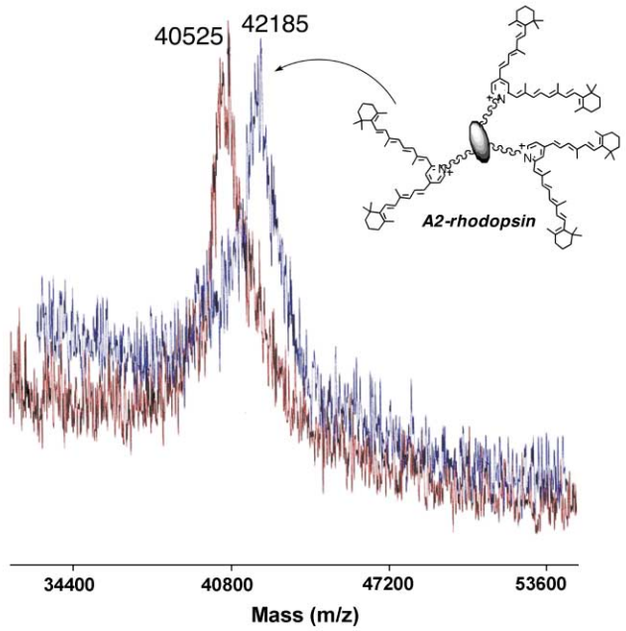

Fig. 3 MALDI-TOF spectra of regular dodecyl maltoside solubilized rhodopsin (red trace) and solubilized A2-Rh (blue trace). N-Terminal sugars on both samples were removed prior to mass spectral analysis. Both spectra were taken in linear mode using sinapinic acid and glycerol as the matrix.

Further evidence for the attachment of A2 moieties to rhodopsin was deduced from analysis of the UV–VIS spectra of A2-Rh before and after bleaching (>500 nm) of the pigment in the presence of 50 mM hydroxylamine. It was important that only yellow light be used for the bleaching, as it has been recently found that blue light can cause photo-oxidation of A2E.[26] The amount of rhodopsin present in the sample was determined from the difference spectrum (Fig. 4C, solid line – dashed line in Fig. 4B) which shows a positive band centered at 500 nm representing the amount of rhodopsin which was lost upon photo-bleaching. A negative band centered around 365 nm also shows the concomitant appearance of all-*trans*-retinal oxime which is formed upon liberation of retinal from the protein and condensation with the hydroxylamine. The remaining absorbance at 440 nm after bleaching (Fig. 4B, dashed line) was considered to arise from the A2 derivative attached to opsin since the absorbance of all-*trans*-retinal oxime is minimal at 440 nm and any A2-PE in the sample was removed by centrifugal filtration through a 30 000 MW cut-off cellulose membrane. In addition, a strong band centered at around 330 nm in the A2-Rh spectrum is not present in native rhodopsin, but is characteristic of the short wavelength transition in A2E (see Fig. 4A). The molar ratio of A2-moiety to rhodopsin was calculated using the molar absorption coefficients for rhodopsin[27] (40 600 M^{-1} cm^{-1} at 500 nm) and A2E[1] (36 900 M^{-1} cm^{-1} at 440 nm), the latter molecule having the same A2-moiety as A2-Rh. The stoichiometry was 1 : 3.3 (Rh : A2), a finding which supports the MS data quite well.

Interestingly, a fairly low threshold seems to exist for formation of A2-Rh in relation to the exogenous all-*trans*-retinal concentration. Incubation with one or two molar equivalents of retinal (relative to Rh) produced little or no fluorescence associated with the rhodopsin protein band (*i.e.* A2-Rh) by SDS-PAGE (see Fig. 2, lanes 1–2). However, a highly fluorescent species at the bottom of the gel was determined to be mainly A2-PE when independently examined by reverse-phase HPLC.[1,2,15] A2-PE fluorescence at the bottom of the gel (lanes 2–5) did not increase significantly with increasing equivalents of retinal. In contrast, lanes 3 and 4 in Fig. 2 show that at 4 and 8 molar equivalents of retinal, a significant fluorescent emission is observed for the rhodopsin protein band. Incubation of

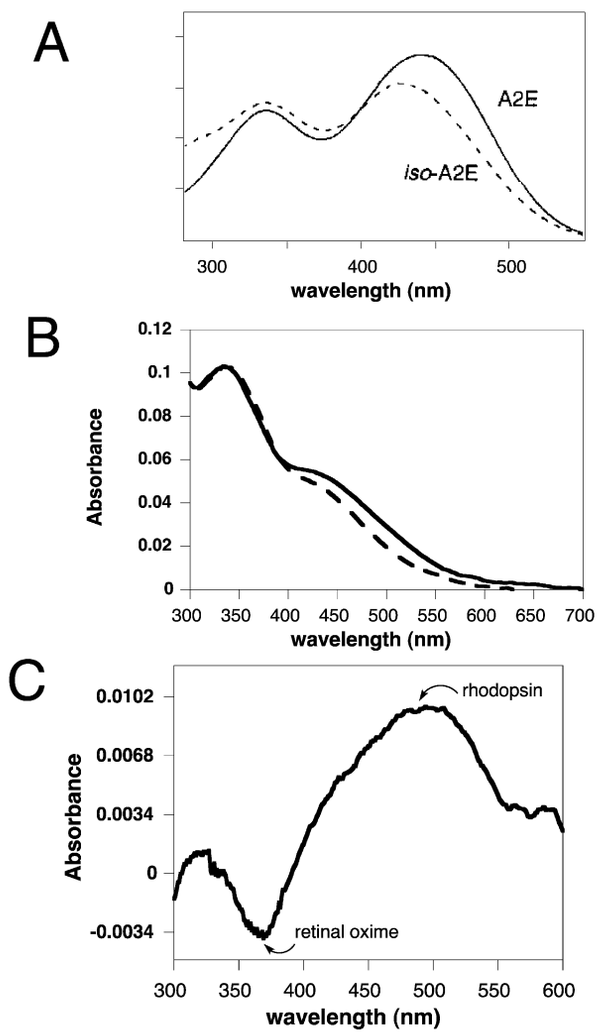

Fig. 4 A) UV–VIS spectra of A2E (solid line) and iso-A2E (dashed line) in methanol. A2E: λ_{max} 439 nm (ε_M 36 900), 336 (ε_M 25 600); iso-A2E: λ_{max} 426 nm (ε_M 31 000), 335 (ε_M 27 000) B) UV–VIS spectra of cellulose filtered A2-Rh before (solid line) and after (dashed line) bleaching for 2 min at 25 °C with >500 nm light in the presence of 50 mM hydroxylamine. C) Difference spectrum (dark–bleached) showing the loss of 500 nm pigment and generation of all-*trans* retinal oxime at 365 nm.

rhodopsin with higher concentrations of retinal did not produce a higher fluorescent intensity than that observed in lane 4 (data not shown). These data suggest that A2-adduct formation saturates at around 8 equivalents of retinal, and that the species likely generated at these higher retinal concentrations is a rhodopsin adduct burdened with pairs of retinals which combine to yield a fluorescent moiety at three sites on the protein. However, under conditions of endogenous release of retinal from isolated bovine rod outer segments, we were unable to detect any fluorescence associated with the resulting opsin when bleaching experiments were carried out similar to those described in whole rat retina.[2]

Encouraged by the formation of an A2-rhodopsin adduct in rod outer segments with exogenous retinal, we proceeded to cleave this protein adduct with cathepsin D, the major proteolytic enzyme found in RPE lysosomes.[28] Our objective was to generate small peptide fragments containing an A2-moiety which may be deposited in RPE cells and represent yet uncharacterized components of lipofuscin. After overnight cleavage under acidic conditions that mimicked lysosomes (pH 3.5), the resulting peptides exhibited little fluorescence when compared to the original intact sample imaged on a 10% tricine gel. Loss of fluorescence did not appear to be due to acid degradation of the A2 moiety with time, since prolonged

exposure of A2-Rh to acid conditions (pH 3.5) without enzyme caused no significant changes in the UV spectrum of A2-Rh (data not shown). Cleavage for 1.5 h did produce a peptide (~30 kDa) with high A2-fluorescence, suggesting that cathepsin D initially cleaves rhodopsin at the outermost periphery.

Discussion

Fluorescent and UV profiles of this new rhodopsin adduct strongly suggest that at saturating levels of retinal (~8 equivalents), three A2 moieties are formed on rhodopsin in native outer segment membranes. Given that there are five solvent accessible lysines on the thermolysin fragment comprising helices I–V, and, at most, only four such lysines in the lower molecular weight fragment (helices VI–VII), two of the three A2-adducts form on what must be the third cytoplasmic loop and/or the 8th helix[29] of rhodopsin (Fig. 2A), since all of the solvent exposed lysine residues in this fragment (245, 248, 311, and 325) reside in this region. It has been shown that there is a non-covalent partial-agonist binding site for all-*trans*-retinal on opsin and it is postulated to involve the region surrounding cysteines 322 and 323, as depalmitoylation of these residues dramatically decreases the binding affinity of retinal.[30] Thus, it seems that the high affinity of retinal to rhodopsin in the vicinity of cysteine 323 and 323 could be partially responsible for preferred formation of A2-adducts in this region compared to the rest of the molecule where no pre-binding occurs.

Of particular interest with this newly discovered A2-rhodopsin is its potential function in aged rod outer segments, as the covalently bound A2 groups may significantly affect binding of the G-protein transducin on the cytoplasmic side of rhodopsin. Given that A2E absorbs light in roughly the same region as dark-adapted rhodopsin, an A2-moiety directly attached to the protein may trap incoming photons, thereby lowering the sensitivity of photoisomerization of the rhodopsin chromophore. It remains to be seen whether ABCR knock-out mice, which are appropriate models for high all-*trans*-retinal concentrations, contain A2-rhodopsin in their outer segments.

From a chemical reactivity stand-point, it is interesting that under the tested conditions, A2-PE forms preferentially over A2-Rh. But it seems that A2-PE formation saturates at only around three equivalents of retinal, after which, A2-Rh begins to form. This data suggests that there may be only one phosphatidylethanolamine molecule tightly associated with each rhodopsin molecule and thus only one lipid available to bind retinal and ultimately form A2-PE. Indeed, difference FT-IR measurements on membrane bound rhodopsin show the release of a single lipid group bound to rhodopsin upon reaching the active MII state.[31] Furthermore, reaction of dipalmitoyl phosphatidylethanolamine (DP-PE) with retinal was previously shown to give a very low yield of A2-PE when compared to reaction in isolated rod outer segments,[15] suggesting that A2-PE formation may not be particularly favorable with bulk phospholipid.

We are also interested in the possible existence of A2-peptide species in the lysosomal compartments of RPE cells. Thus A2-rhodopsin was subjected to cleavage by cathepsin D, the major proteolytic enzyme in lysosomes. After HPLC identification of peptide fragments containing an A2 group, we will begin to search for these protein fragments in RPE cells, as rhodopsin, which comprises more than 90% of ROS membrane proteins, would be a likely source for A2-peptide formation.

Experimental

Preparation of rod outer segments (ROS)

ROS were isolated from frozen bovine retina (W.L. Lawson Co., Lincoln, Nebraska) by sucrose flotation {35% (w/v) in 10 mM Tris, 60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol (DTT) (pH 8.0)} followed by a sucrose gradient[32]{26% to 35% (w/v)}. ROS were washed and resuspended in a HEPES buffer {10 mM HEPES, 50 μM diethylenetriaminepentaacetic acid, 100 μM toluenesulfonyl fluoride, and 1 mM DTT (pH 7.0)}. Some samples were washed with 5.0 M urea {10 mM Tris buffer (pH 7.5), 60 min incubation at 4 °C} to remove membrane associated proteins. ROS membranes were then resuspended in HEPES buffer.

Retinal incubations

All-*trans*-retinal was synthesized in two steps starting from retinyl acetate. Briefly, retinyl acetate was treated with K_2CO_3 in MeOH–THF (6 : 1) to produce all-*trans*-retinol. Oxidation to retinal was then achieved using excess MnO_2 in hexane. Aliquots of a 25 mM stock solution of retinal in Me_2SO were added to ROS samples for incubation.

Purification of A2-rhodopsin

The crude A2-protein adduct (0.5 mg rhodopsin in 100 μl) was solubilized with 10 μl of a 7.5% CHAPS buffer. The mixture was pelleted on a tabletop centrifuge (15000 rpm, 10 min) to remove insoluble components, and the supernatant was then applied to a concanavalin A-Sepharose suspension (~1 ml) equilibrated with 0.75% CHAPS and 10 mM Tris-HCl (pH 7.4). After 2 hours incubation, the column was washed with three bed volumes of CHAPS buffer, followed by five bed volumes of 300 mM methyl α-D-mannopyranoside to elute the rhodopsin. All manipulations were carried out at 4 °C. Rhodopsin concentrations were determined by measuring a difference optical absorption spectrum before and after bleaching with white light in the presence of 50 mM hydroxylamine (pH 7.0). An extinction coefficient of 40600 M^{-1} cm^{-1} for rhodopsin absorbance at 500 nm was used.[27]

A2-rhodopsin in detergent was further purified using a Millipore Ultrafree®-MC microcentrifuge filter (Sigma) with a 30000 MW cut-off. 20 μl of 1 M hydroxylamine (pH 7.0) and 2 drops of methanol were added to 400 μl of sample and then spun at 15000 rpm for 45 min at 4 °C in the dark. The procedure was repeated once before the protein was used in further experiments.

Enzymatic degradation

Thermolysin cleavage: A2-rhodopsin adduct in ROS suspension (typically 200–400 μg) was suspended in 10 mM Tris-HCl (pH 7.4) with 5 mM $CaCl_2$ and 0.1 mg ml^{-1} thermolysin.[24] After 3 h incubation at 37 °C, the reaction was quenched with 50 mM EDTA and immediately frozen (−80 °C) or used for further analysis.

Cathepsin D cleavage[28]

A2-rhodopsin (100–200 μg) in disk membrane was added to a 0.2 M sodium acetate buffer (pH 3.5) and cathepsin D (purified from bovine kidney, CalBioChem) was added in 20 mM sodium phosphate buffer (pH 7.2) to achieve a rhodopsin : protease ratio of 10 : 1. The reaction was held at 37 °C for various time periods and then stopped with pepstatin (5 μg/ml) and the pH neutralized with 0.5 M sodium phosphate (pH 7.2). Aliquots were sedimented and used for further analysis.

Stoichiometry by mass spectrometry

Urea-washed ROS and concanavalin A purified A2-rhodopsin (40 μg in 10 μl) were solubilized with 30 μl 0.1% dodecyl maltoside and then subjected to N-terminal glycolysis using one unit of Glycopeptidase F (Sigma). After 1 h incubation at 4 °C in the dark, the samples were diluted with glycerol to make a 33% w/v solution. The samples were mixed in a 5 : 1 ratio with

saturated MALDI matrix (sinapinic acid, Aldrich). 1 μl was spotted per well. MALDI TOF spectra were performed on the Voyager DE (Perceptive Biosystems, MA) with an accelerating voltage of 25 kV. Spectra were averaged over 100 laser shots.

Protein separation and fluorescent imaging

Protein samples (5–25 μg) were loaded onto SDS tris-tricine gels (10% acrylamide) for electrophoresis. The samples were dissolved in an equal volume of tricine sample buffer (10 mM Tris pH 6.8, 24% v/v glycerol, 0.8% SDS, 1 mM DTT, and Coomassie blue G-250), and disulfide bonds were reduced with β-mercaptoethanol. Run gels were first imaged by the Image Station 440CF fluorometer (Kodak, Rochester, NY) under 300–400 nm excitation with 10 μW cm^{-2} illumination energy and then stained with Coomassie Brilliant Blue. The Kodak 1D v3.5 software package (Kodak, Rochester, NY) was used to determine the relative band intensities.

Abbreviations

A2E, pyridinium bisretinoid; A2-PE, phosphatidylpyridinium bisretinoid; A2, bisretinoid; ABCR, retina-specific ATP-binding cassette transporter; AMD, age-related macular degeneration; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane sulfonate; DP-PE, dipalmitoyl phosphatidylethanolamine; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethane sulfonic acid]; HPLC, high performance liquid chromatography; MALDI-TOF-MS, matrix assisted laser desorption ionization–time of flight mass spectrometry; NRPE, *N*-retinylidenephosphatidylethanolamine; Rh, rhodopsin; RmP, rim protein; RPE, retinal pigment epithelium; ROS, rod outer segments; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Acknowledgements

This research was supported by NIH grants GM 36564, GM 34509 and EY 12951. We would like to thank the lab of Professor Dalibor Sames (Columbia University) for the use of their fluorometer, Gong Chen, Doris Rieter, Alexander Heim, and Heidi Vollmer for invaluable discussions, and Jacqueline Cockrell for experimental assistance. We are grateful for the support provided to N.F. by an NIH Vision training grant (EY 139933–01).

References

1 C. A. Parish, M. Hashimoto, K. Nakanishi, J. Dillon and J. Sparrow, *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 14609–14613.
2 S. Ben-Shabat, C. A. Parish, H. R. Vollmer, Y. Itagaki, N. Fishkin, K. Nakanishi and J. R. Sparrow, *J. Biol. Chem.*, 2002, **277**, 7183–7190.
3 C. J. Kennedy, P. E. Rakoczy and I. J. Constable, *Eye*, 1995, **9**, 763–771.
4 C. K. Dorey, G. Wu, D. Ebenstein, A. Garsd and J. J. Weiter, *Invest. Ophthalmol. Vis. Sci.*, 1989, **30**, 1691–1699.
5 L. Feeney-Burns, E. S. Hilderbrand and S. Eldridge, *Invest. Ophthalmol. Vis. Sci.*, 1984, **25**, 195–200.
6 M. F. Rabb, M. O. Tso and G. A. Fishman, *Ophthalmology*, 1986, **93**, 1443–1451.
7 S. S. Schochet, Jr., R. L. Font and H. H. Morris, *Arch. Ophthalmol.*, 1980, **98**, 1083–1088.
8 F. C. Delori, D. G. Goger and C. K. Dorey, *Invest. Ophthalmol. Vis. Sci.*, 2001, **42**, 1855–1866.
9 F. G. Holz, C. Bellman, S. Staudt, F. Schutt and H. E. Volcker, *Invest. Ophthalmol. Vis. Sci.*, 2001, **42**, 1051–1056.
10 F. G. Holz, C. Bellmann, M. Margaritidis, F. Schutt, T. P. Otto and H. E. Volcker, *Graefe's Arch. Clin. Exp. Ophthalmol.*, 1999, **237**, 145–152.
11 N. Sakai, J. Decatur, K. Nakanishi and G. E. Eldred, *J. Am. Chem. Soc.*, 1996, **118**, 1559–1560.
12 R. X.-F. Ren, N. Sakai and K. Nakanishi, *J. Am. Chem. Soc.*, 1997, **119**, 3619–3620.
13 G. E. Eldred and M. R. Lasky, *Nature*, 1993, **361**, 724–726.
14 G. E. Eldred and M. L. Katz, *Exp. Eye Res.*, 1988, **47**, 71–86.
15 J. Liu, Y. Itagaki, S. Ben-Shabat, K. Nakanishi and J. R. Sparrow, *J. Biol. Chem.*, 2000, **275**, 29354–29360.
16 R. Allikmets, N. F. Shroyer, N. Singh, J. M. Seddon, R. A. Lewis, P. S. Bernstein, A. Peiffer, N. A. Zabriskie, Y. Li, A. Hutchinson, M. Dean, J. R. Lupski and M. Leppert, *Science*, 1997, **277**, 1805–1807.
17 R. Allikmets, N. Singh, H. Sun, N. F. Shroyer, A. Hutchinson, A. Chidambaram, B. Gerrard, L. Baird, D. Stauffer, A. Peiffer, A. Rattner, P. Smallwood, Y. Li, K. L. Anderson, R. A. Lewis, J. Nathans, M. Leppert, M. Dean and J. R. Lupski, *Nat. Genet.*, 1997, **15**, 236–246.
18 N. F. Shroyer, R. A. Lewis, R. Allikmets, N. Singh, M. Dean, M. Leppert and J. R. Lupski, *Vision Res.*, 1999, **39**, 2537–2544.
19 J. Ahn, J. T. Wong and R. S. Molday, *J. Biol. Chem.*, 2000, **275**, 20399–20405.
20 H. Sun, R. S. Molday and J. Nathans, *J. Biol. Chem.*, 1999, **274**, 8269–8281.
21 J. Weng, N. L. Mata, S. M. Azarian, R. T. Tzekov, D. G. Birch and G. H. Travis, *Cell*, 1999, **98**, 13–23.
22 R. R. Rando, *Chem. Rev.*, 2001, **101**, 1881–1896.
23 K. Tanaka, M. Kamatani, H. Mori, S. Fujii, K. Ikeda, M. Hisada, Y. Itagaki and S. Katsumura, *Tetrahedron*, 1999, **55**, 1657.
24 Y. Imamoto, M. Kataoka, F. Tokunaga and K. Palczewski, *Biochemistry*, 2000, **39**, 15225–15233.
25 Z. Ablonczy, D. R. Knapp, R. Darrow, D. T. Organisciak and R. K. Crouch, *Mol. Vision*, 2000, **6**, 109–115.
26 S. Ben-Shabat, Y. Itagaki, S. Jockusch, J. R. Sparrow, N. J. Turro and K. Nakanishi, *Angew. Chem., Int. Ed.*, 2002, **41**, 814–817.
27 G. Wald and P. K. Brown, *J. Gen. Physiol.*, 1952, **35**, 797–821.
28 C. Regan, W. de Grip, F. Daemen and S. Bonting, *Exp. Eye Res.*, 1980, **30**, 183–191.
29 K. Palczewski, T. Kumasaka, T. Hori, C. A. Behnke, H. Motoshima, B. A. Fox, I. Le Trong, D. C. Teller, T. Okada, R. E. Stenkamp, M. Yamamoto and M. Miyano, *Science*, 2000, **289**, 739–745.
30 K. Sachs, D. Maretzki, C. K. Meyer and K. P. Hofmann, *J. Biol. Chem.*, 2000, **275**, 6189–6194.
31 M. Beck, F. Siebert and T. P. Sakmar, *FEBS Lett.*, 1998, **436**, 304–308.
32 Y. Fukada, T. Okano, Y. Shichida, T. Yoshizawa, A. Trehan, D. Mead, M. Denny, A. E. Asato and R. S. H. Liu, *Biochemistry*, 1990, **29**, 3133–3140.
33 J. P. Sparrow, K. Nakanishi and C. A. Parish, *Invest. Ophthalmol. Vis. Sci.*, 2000, **41**, 1981–9.
34 J. R. Sparrow and B. Cai, *Invest. Ophthalmol. Vis. Sci.*, 2001, **42**, 1356–62.