ELUCIDATING THE MECHANISM BEHIND ALTERED RETINAL DYNAMICS IN AGE-RELATED MACULAR DEGENERATION

by

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Elucidating the Mechanism Behind Altered Retinal Dynamics in Age-Related Macular Degeneration

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Abstract: Age-related macular degeneration (AMD) is among the leading causes of blindness in the western hemisphere, with approximately 30% of people over 60 years old showing symptoms. AMD is characterized by a significant lowering in the zinc concentration in the eye, the formation of dense pockets of highly oxidized lipid particles called drusen in the retinal pigment epithelial (RPE) cells, and altered capability of the RPE cells to regenerate retinal. However, it is unclear how changes in the oxidative state of the lipids and lower zinc concentrations alter rhodopsin photodynamics. The activity of rhodopsin is dependent on the receptor's lipid environment, with changes in activity occurring with alterations in membrane curvature, thickness, and fluidity. Malfunction of rhodopsin has been linked to multiple eye disorders that cause visual impairment and blindness, including AMD. Much remains unknown about the effect of specific rhodopsin-lipid interactions and how zinc works to stabilize the receptor. To elucidate these effects on rhodopsin stability, we will perform trials to investigate lipid binding and the effects of bulk layer properties on rhodopsin activation. Furthermore, zinc titrations will be performed at varying concentrations to determine how zinc works to stabilize the receptor.

A. SPECIFIC AIMS

The leading cause of blindness in people over the age of 65 is age-related macular degeneration (AMD).[1] In the general population, AMD is the third leading cause of blindness after cataracts and glaucoma.[2,3] Symptoms of AMD include loss of central vision and a decrease in low light level sensitivity. As the disease progresses, pockets of highly oxidized lipids called drusen form in the retinal pigment epithelial (RPE) layer as a result of oxidative stress in the eye.[3] Drusen formation leads to eventual apoptosis of the RPE. This results in a decreased rate of retinal regeneration and a lower concentration of rhodopsin in the eye, decreasing light sensitivity. These drusen are formed from highly oxidized lipids that form during ageing. The effect of these oxidized lipids on rhodopsin stability remain unclear.

Currently there are no cures for AMD, but some treatments have been shown to delay the onset and decrease the severity of AMD. Current treatments for AMD consist of medications containing zinc. Little is known about why AMD pathology leads to decreased levels of zinc. It has previously been shown that rhodopsin binds zinc, which leads to a stabilization of rhodopsin. *The research proposed here will investigate how oxidized lipids and zinc alter rhodopsin photoactivation, leading to the decreased light sensitivity.*[4]

Aim 1. Determine the effects of oxidized lipids on rhodopsin photoactivation. As previously stated much is still unknown about how specific lipid binding events can allosterically modulate the activity or stability of rhodopsin.[4] Determining the effect of individual lipids on rhodopsin stabilization can provide key insight into how diseased pathology's destabilize rhodopsin. To probe the effect of lipids on rhodopsin stability a titration of different lipids at varying ratios will be used. In addition, lipid membrane mimetics called nanodisc, will be used to better mimic the bilayer environment of rhodopsin.[5,6] Nanodiscs will be made with increasing concentrations of oxidized lipids to analyze how oxidative stress alters rhodopsin photoactivation. We hypothesize that oxidation of lipids will decrease the activity of rhodopsin and favor the inactive MI state of the receptor. The completion of Aim 1 will provide key insights into AMD pathology and provide new avenues for therapeutic targets.

Aim 2. Determine the effects of zinc concentration on rhodopsin stability. The eye has one of the highest zinc concentrations found in the body, and low zinc levels in the eye are correlated with the pathology of AMD. Much is still not known about the mechanism through which low zinc concentrations lead to an increase in the onset and severity of AMD. We hypothesize that zinc stabilizes dark-state rhodopsin, preventing its activation to the MII product. Titrations with varying concentration of zinc, from abnormally low to high levels, will be used in conjunction with native MS to elucidate the mechanism through which lower zinc concentrations can lead to a diseased state.

This proposal seeks to understand how alterations in lipid composition and zinc concentration throughout the progression of AMD affect rhodopsin stability. The completion of this proposal will provide key insights into the pathology of AMD, leading to the identification of new therapeutic approaches for AMD. Rhodopsin is a prototypical GPCR that is commonly used as a model for other GPCRs. As a result, this proposal will provide new avenues for therapeutic research for AMD patients.

B. Background and Significance

Age-related Macular Degeneration. AMD is among the leading cause of blindness for elderly patients, accounting for 54% of patients with legal blindness over the age of 65.[3] The pathology of AMD largely affects the macula of the eye, which is a highly pigmented region of the retina responsible for high-resolution vision. The macula consists of the retinal pigment epithelial cells (RPE), the Bruch membrane, and the choroid. The choroid region is responsible for delivering nutrients to the eye, and the RPE cells are responsible for phagocytosis of waste material and regeneration of free retinal.[7] The RPE cells are highly important to the rhodopsin photocycle because they regenerate retinal for opsin to bind to and re-enter the cycle.[7] As the severity of AMD progresses, highly dense particles of oxidized lipids called drusen form in both the RPE layer of the eye and the Bruch membrane, which provides support to the retina layer (Figure 1).[8] The formation of drusen and alterations in the lipid environment of the macula alter the capability of the RPE to regenerate free retinal through inhibition of an ABC transporter, ABCA1.[9] This lowers the concentration of rhodopsin, lowering light sensitivity and leading to eventual loss of central vision. As the disease progresses, angiogenesis from the choroid into the RPE cells causes leakage of cell material and eventual apoptosis of the RPE layer.[10] Although the causes, risk factors, and the pathology of AMD are well understood, the underlying molecular causes for AMD remain unclear.

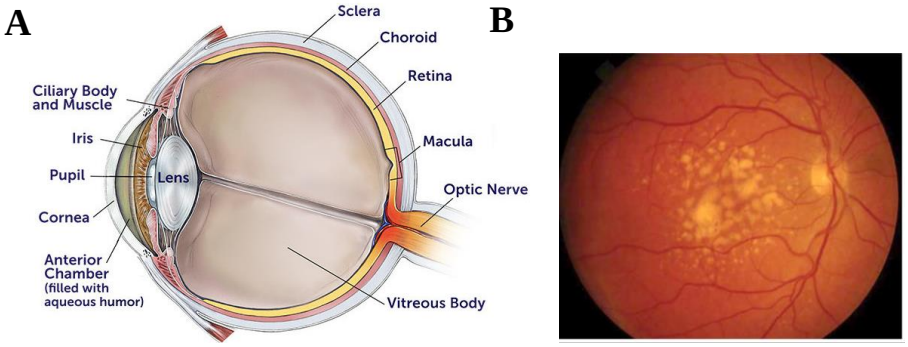

Figure 1. (A) The anatomy of the eye. (B) Image of a patient diagnosed with AMD showing the formation of drusen (yellow regions) in the eye.

Rhodopsin Mechanisms. Rhodopsin is found in the disc membrane of the rod outer spectrum (ROS) of the eye. Rhodopsin is a photopigment that allows for vision in low-light level or scoptic conditions. As a GPCR, rhodopsin contains a seven transmembrane alpha helical core with three extracellular (ECL) and three intracellular (ICL) loop regions (Figure 2).[11,12] Rhodopsin differs from other GPCRs because it has no basal activity in the dark state as a result of the Schiff base linkage between the chromophore and Lys^{296}.[13] When the protein photoactivates, the chromophore undergoes photoisomerization from 11-*cis* retinal to all-*trans* retinal.[14] After rhodopsin has been exposed to as few as one photon, it undergoes conformational changes that result in short-lived intermediate products. The photocycle will reach an equilibrium between the inactive and stable Meta I (MI) and the active but unstable Meta II state (MII) before decaying into free retinal and opsin (Figure 2).[14,15,16]

Rhodopsin-Lipid Interactions. The activity of membrane receptors has been shown to be highly dependent on their lipid environment. These lipids can affect receptor activity through allosteric modulation or through alterations in the membrane's curvature, fluidity, and thickness.[17,18] For example, it has been determined that rhodopsin is highly dependent upon its lipid environment and that alterations in the lipid content can directly affect the phototransduction cascade and transducin binding.[19] Changes in the composition of lipids alters rhodopsin activity through changes in bulk layer properties, such as membrane curvature forces.[20] The MII state is favored by a negative curvature, and the MI state is favored by zero curvature force.[21] Furthermore, the membrane fluidity is important to rhodopsin activity. Recent research provides support for the fact that direct binding of lipids can allosterically regulate other Class-A GPCRs, such as the β_2AR, through specific electrostatic interactions with the transmembrane helices.[22] To preserve a more natural environment for rhodopsin, nanodiscs will be used to mimic the lipid bilayer of the membrane. Nanodiscs are self-assembling bilayers of lipids that are encapsulated by two amphipathic membrane scaffold proteins (MSP).[6] This proposal will study the effects of direct lipid binding and bulk layer properties on the alteration in rhodopsin photoactivation seen in patients with AMD.

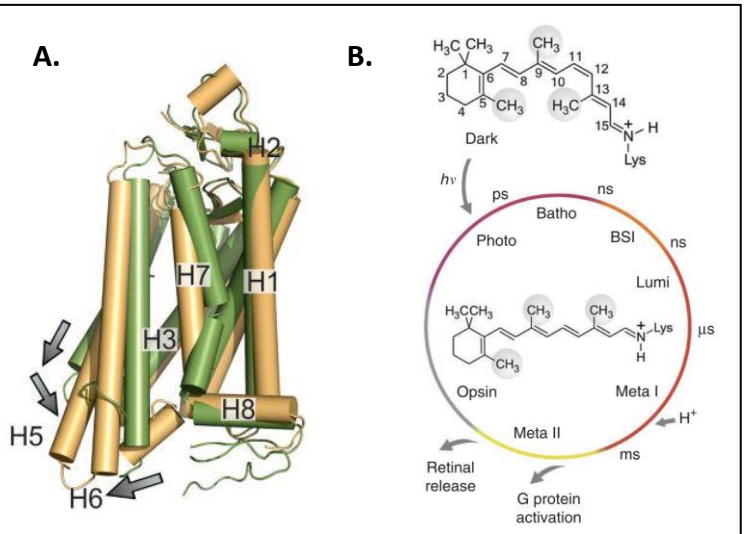

Figure 2. (A) Helical rearrangements occur upon the shift from the inactive MI (*green*) to the active MII (*yellow*) state of Rho.[18] (B) Photoactivation of Rhodopsin converts the retinal chromophore from 11-*cis* to all *trans*, yielding multiple photointermediates.[15]

Protein metal binding. Malfunction and mutations of metalloenzymes can cause a variety of diseases, including AMD.[23] Rhodopsin has been shown to bind zinc, which affects its photoactivity. AMD patients show low levels of zinc in comparison to healthy patients. Current treatments for AMD increase the cellular concentration of zinc, which delays progression of the disease. Those who do not receive treatment progress to a severe form of AMD, called wet-AMD, which is characterized by angiogenesis into the retina layer from the choroid.[8] It has previously been determined that rhodopsin contains at least two binding sites for zinc. These binding sites are known to have different binding affinities and have shown different results in how they interact with rhodopsin. As a result, it remains unclear how the lowering of zinc levels directly affects rhodopsin stability.

Native Mass Spectrometry. Historically, GPCRs have been studied using X-ray crystallography, cryo-EM, and NMR.[24,25,26] The highly dynamic nature of GPCRs along with their insolubility outside their host environment has made them difficult to characterize by conventional techniques. One technique that has gained popularity in recent years for the study of membrane proteins is native mass spectrometry (MS). Native MS allows for the use of less sample volume and lower concentrations. Native electrospray ionization MS (ESI-MS) is able to preserve the non-

covalent interactions of proteins through gentler ionization conditions, including lower spray voltage, lower source temperatures, neutral pH buffers, and volatile aqueous buffers.[27] The preservation of non-covalent interactions allows for oligomerization, stoichiometry, and binding events to be measured.[28,29] This proposal will use nanodiscs composed of varying lipid compositions and analyzed using native MS. This will allow for the determination of how different lipids can change the activity of rhodopsin. Obtaining a better understanding of how lipids and metal binding affect rhodopsin activity will provide key insight into the progression of AMD

C. Research Design

Aim 1. Analyze the effects of lipid oxidation on rhodopsin photoactivation

1.1 Introduction. The goal of this aim is to analyze how oxidized lipids lead to a diseased pathology in patients with AMD by causing differences in the stability and photoactivity of rhodopsin and its capability to become photoactivated. Patients with AMD have a higher concentration of oxidized lipids in the eye.[8] Alterations in the lipid content alters the activity of an ATP-binding cassette (ABC) transporter, ABCA1 that aids in the regeneration of free retinal through its flippase activity.[30] The increase in oxidized lipids decreases ABCA1's flippase capability, lowering the concentration of retinal the eye.[9] Research has shown the different lipids in recombinant membranes cause alterations in rhodopsin's functionality.[31] It is still not known how oxidized lipids affect receptor activity through direct binding and bulk bilayer effects. One hypothesis is that oxidized lipids alter the stability of rhodopsin by direct binding to rhodopsin or modulating the membrane properties.[4] The goal of this aim is to study how the binding of lipids to rhodopsin can allosterically modulate the photoactivity of the receptor.

1.2 Research Design. We will first purify rhodopsin from the rod outer segment (ROS) of bovine retinas (**1.2.1**). Following purification, rhodopsin will be analyzed using native MS in detergent to obtain basal level activity information. Next, direct lipid binding experiments will be performed and analyzed using native MS to determine extent of lipid binding and the effects of binding on activity (**1.2.2-1.2.3**). Rhodopsin will be incorporated into nanodiscs with varying degrees of oxidized lipids. (**1.2.4**). Nanodisc will be introduced into the MS in the dark state followed by online photobleaching. Analysis of the changes in rhodopsin activation with different membrane compositions will provide evidence for how oxidized lipids directly alter rhodopsin activation.

1.2.1 Protein Purification. Bovine rhodopsin will be extracted from ROS membranes using density ultracentrifugation. Rhodopsin will then be purified using a hydroxyapatite column according to previously established protocols.[32] The protein will then be exchanged into a 200 mM ammonium acetate buffer containing a detergent favorable for native MS analysis, such as tetraethylene glycol monooctyl ether (C8E4).[33,34] MSP1D1 will be expressed in *E. coli* and purified according to established protocols.[35]

1.2.2 Lipid Binding in Detergent. Preferential lipid binding studies to rhodopsin will use oxidized versions of POPC (PAZE-PC), cholesterol (hydroxy cholesterol), and POPS (1,2-dihexanoyl-sn-glycero-3-phospho-L-serine) (Figure 3). These lipids were chosen due to PC, PE, and PS being the most abundant lipid headgroup categories found in the eye. While, the most common types of lipids in the eye are polyunsaturated fatty acids the lipids chosen here have been commonly used in studies for protein-lipid binding and serve as a starting point for further studies

into the effect of chain length and degrees of unsaturation on rhodopsin activity. The exact kinds of lipids that are oxidized as a result of AMD pathology are not known, so we chose commercially available lipids that are of prevalence in the eye and have been shown to be important for rhodopsin activation. This will enable us to determine which lipids bind at a higher affinity to rhodopsin and at what concentration we see maximum levels of binding. The molar ratios of lipid:rhodopsin that will be used in these studies range from 0.1-100. This creates a titration with up to 100 lipids bound. The lipids will be titrated into rhodopsin that has been purified in C8E4. We will collect the native mass spectrum of dark state rhodopsin at the varying lipid concentrations. The binding of lipids to rhodopsin will create additional peaks in the spectrum that correspond to the mass of various protein-lipid complexes. We will measure lipid binding by quantifying the relative intensities of peaks in the mass spectrum for rhodopsin and rhodopsin-lipid complexes.

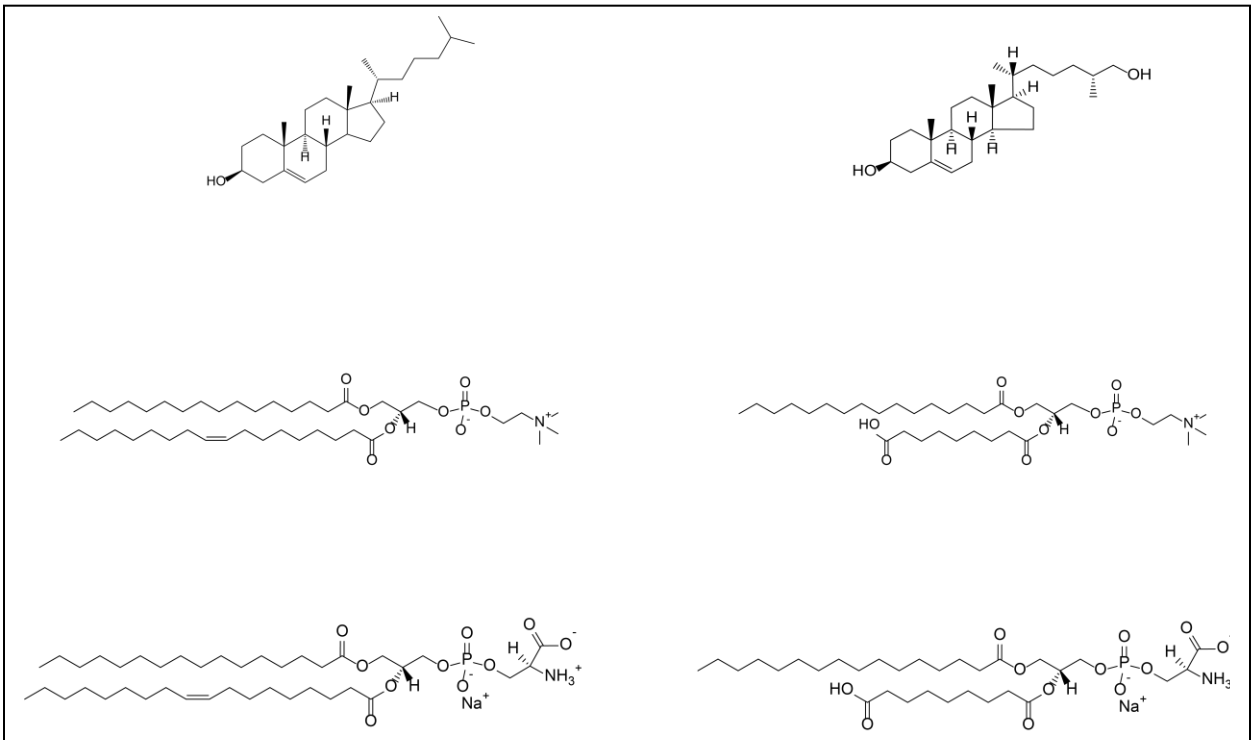

Figure 3. Figures of the non-oxidized lipids (cholesterol, POPC, and POPS) on the left and the oxidized versions (hydroxy cholesterol, PAZE-PC and oxPOPS) on the right.

1.2.3 Activity of Rhodopsin as a Function of Lipid Binding. After measuring the extent of lipid binding, (**1.2.2**) we will measure how lipid binding affects rhodopsin activity. As described above, we will collect mass spectra for three minutes to establish a baseline before light activation occurs. We hypothesize that photoactivation converts rhodopsin into the unstable MII product. This conversion creates an opening within the transmembrane regions exposing hydrophobic residues, causing rhodopsin to aggregate out of solution. This aggregation lowers the intensity in the signal that is measured by MS. Measuring the intensity as a function of time thus provides a direct readout of aggregation kinetics and thus the activity. The timescale of the conversion from the MII to opsin and free retinal is on the minute timescale. The temporal resolution of our experiments is on the minute to second timescale allowing the measurement of this kinetic process.

In addition, research has shown that using time-resolved mass spectrometry (TRMS) can achieve sub-millisecond temporal resolution. By having a constant stream of analyte flow to the MS the millisecond temporal resolution can occur. To confirm that kinetics obtained from the MS our applicable we will verify using more conventional spectrophotometric techniques, such as UV/vis.[36,37]

1.2.4 Nanodisc Assembly. While rhodopsin is stable in the detergent micelle environment, the micelle does not account for the interactions that occur within the lipid membrane of a cell. Thus, additional experiments will be performed with rhodopsin in varying types of nanodiscs to provide further evidence on how these oxidized lipids affect rhodopsin stability. Rhodopsin has previously been used in nanodiscs and has been shown to retain natural fold and function.[38,6] To create nanodiscs with monomeric rhodopsin embedded, detergent solubilized rhodopsin will be incubated with lipids and membrane scaffold protein (MSP) at previously determined ratios. [32]. Rhodopsin will be incorporated into nanodiscs as previously described.[32] The reconstituted mixture will be allowed to incubate in the dark and on ice for one hour before removal of the detergent with hydrophobic beads, which induces self-assembly of the nanodiscs.[6] Nanodiscs will be allowed to form overnight at 4°C. Following the nanodisc assembly, density ultracentrifugation will separate empty nanodiscs from nanodiscs containing rhodopsin.[39]

Historically, nanodiscs have been made up of one lipid or two lipid systems.[40,41] Control experiments will use nanodiscs composed of pure POPC will establish baseline values for rhodopsin photoactivation in nanodisc (**1.2.5**). Next, nanodiscs made up of increasing concentrations of PAZE-PC, ranging from 0-10% oxidized lipids, will be introduced into the mass spectrometer following the protocol outlined in section **1.2.3**. Subsequently, nanodiscs will be assembled using both a mixture of POPC and cholesterol and a mixture of POPC and POPS. Each nanodisc will first be made with 0% oxidized lipids and increased to a final concentration of 40% oxidized lipids. In addition to the two-component lipid nanodiscs, nanodiscs will be made containing POPC, POPS, and cholesterol with the same amounts of oxidation as described above.

1.2.5 Nanodisc Native MS Analysis. Purified nanodiscs containing rhodopsin will be analyzed on an Ultra-High Mass Resolution (UHMR) Q-Exactive mass spectrometer. To preserve the intact nanodisc assembly, low-collision voltage and charge manipulation reagents, such as imidazole and various carbonates will be used.[42] Photolysis of rhodopsin will be induced as described previously, through the use of a green LED.[43] Upon photoactivation, the active MII state of rhodopsin decays into opsin through the loss of retinal. Retinal loss of approximately 280 Da from rhodopsin will be measured as a function of time and used to quantify the rate of photoactivation. We hypothesize that the increase in the concentration of oxidized lipids inside the nanodiscs will decrease activity and slow retinal loss.

The mass spectra of nanodiscs are highly complex due to the heterogeneity of the nanodisc.[42] MSP1D1 makes nanodiscs with approximately 150 lipids, but this can vary by +/- 7 lipids.[44,45] As more components are added, such as the different types of lipids, the heterogeneity and thus the complexity increases. The raw mass spectra will be deconvolved using UniDec software to convert from *m/z* to mass.[46] As rhodopsin is photobleached we will measure the intensity of the peak corresponding to holo-rhodopsin, which is shifted to a lower mass by a loss of 280 Da.[47,48] The intensity of the bleached rhodopsin peak will be measured as a function of time to determine the rate at which rhodopsin is becoming photoactivated in these different nanodiscs.

1.3 Expected Outcomes. The overall goal of this aim is to provide evidence towards how binding of oxidized lipids to rhodopsin affects the stability of the receptor and ultimately leads to disease pathology. The completion of Aim 1 will provide key insights into how oxidized lipids bind rhodopsin and affect the rate at which is becomes photobleached. Furthermore, we expect to determine how changes in the oxidative state of the surrounding lipid bilayer will affect rhodopsin's capability to photoactivate in nanodiscs.

1.4 Impacts. AMD is a major cause of blindness worldwide, affecting over a million people worldwide. However, little is known about the effect that oxidation of lipids in the eye has on the activation of rhodopsin. Currently, there are only two treatment options for AMD that delay the onset of the disease. By better understanding how these oxidized lipids affect rhodopsin, better therapeutic targets can be developed for treatment of patients. Furthermore, the results from the above experiments can be used in additional experiments to determine how oxidative stress can affect other GPCRs and membrane proteins as a whole.

1.5 Potential Pitfalls and Solutions. The proposed analysis relies on the capability to measure the loss of retinal from nanodiscs upon photoactivation. However, the complex nature of nanodiscs, multiple post-translational modifications (PTMs) on rhodopsin, and the overlapping lipid signals could cause spectra to be unresolvable. If this occurs, UV/Vis spectroscopy will be used to measure the changes in the kinetics of rhodopsin photoactivation with the different lipids and nanodisc compositions. This method enables the completion of this aim; however, UV/Vis does not provide results at the same level of detail as MS experiments.

The oxidized lipids used in this study have shortened alkyl chain lengths in comparison to the non-oxidized version. Alterations in membrane thickness have been shown to cause hydrophobic mismatch, which leads to changes in receptor activity and oligomerization.[17] Thus, the changes in rhodopsin activity could be a result of the difference in membrane thickness and not a result of the binding of the oxidized lipids. To determine if the result is due to lipid binding, rhodopsin can be ejected from the nanodisc to determine which lipids are bound to compare to results from **1.2.3**.

Aim 2. Determine the effects of zinc concentration on rhodopsin activity

2.1 Introduction. Zinc is an essential mineral for the body, playing important roles in enzymatic activity of many proteins, immune health, protein synthesis, and cell division. The highest concentration of zinc is found in bones, muscles, kidneys, and eyes. X-ray crystal structures have shown that rhodopsin binds zinc and has up to four zinc binding sites.[49,50] Zinc binding to histidine residues in the transmembrane region have been shown to cause stabilization of rhodopsin, causing a decrease in the rate of rhodopsin photoactivation. When the binding of zinc occurs on the intradiscal side, it destabilizes rhodopsin by decreasing its thermostability. At low concentrations, zinc binding stabilizes rhodopsin through binding to the intramembrane site. Higher concentrations of zinc cause thermal destabilization by binding to the intradiscal side.[51] Changes in rhodopsin stability through zinc binding can be observed through shifts in the equilibrium. These shifts can be seen in a change in the ratio of the MI/MII upon addition of higher zinc concentrations.[52] This aim will seek to determine the residues important for zinc binding and how different zinc binding sites lead to differences in rhodopsin stability.

2.1.1 Preliminary Data. Preliminary data shows that by increasing the concentration of zinc, the intensity of the unbound rhodopsin peak decreases as up to two zinc ions bind (Figure 4). Furthermore, the kinetics of the MS signal decay caused by photobleaching was compared between

0, 100, 500, 750, and 1000 ppm of zinc. Addition of zinc at low levels stabilizes the receptor and reaches the highest stability at 500, which is the approximate concentration of zinc in the retina.

2.2 Research Design. Varying levels of zinc will be titrated into rhodopsin that has been purified and buffer exchanged for MS analysis. The concentrations of zinc that will be used vary from low levels to high levels of zinc when compared to the previously determined zinc concentration in the eye. After the full titration has been performed, mutagenesis studies will be used to identify key residues for zinc binding. In addition, a titration of zinc with lipids (POPC, cholesterol, and POPS) will be performed to understand how the binding of different lipids affects zinc binding and the stability of rhodopsin.

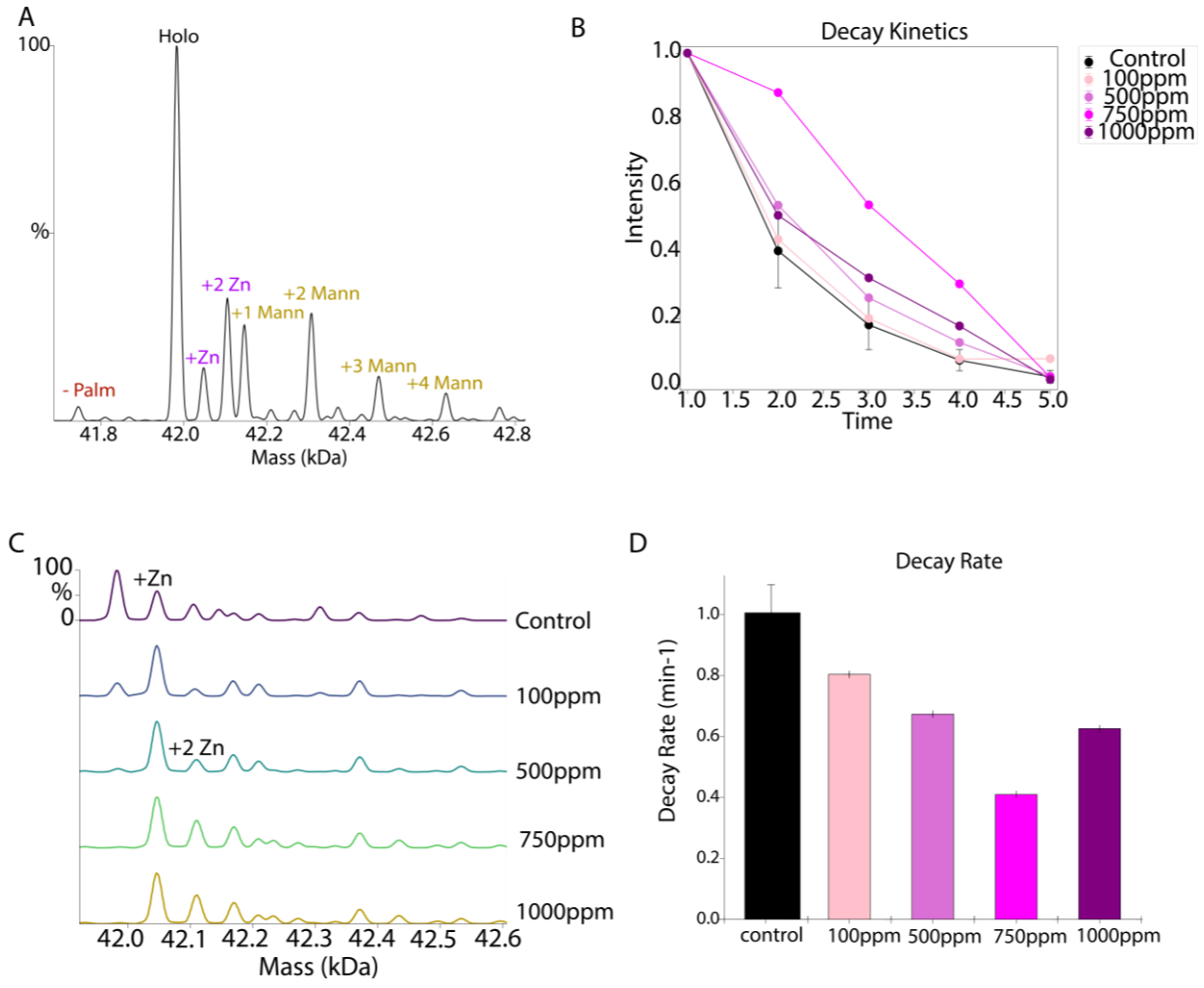

Figure 4. (A) Deconvolved mass spectrum of rhodopsin with the common PTMs assigned. (B) Decay and (D) decay rates for rhodopsin signal during photoactivation with different concentration of zinc added. (C) Deconvolved spectra show the increase in one or two zinc bound as the concentration of zinc increases.

2.2.1 Zinc Titrations. Preliminary data has shown that zinc influences the rate of rhodopsin photobleaching (Figure 4). However, the preliminary data lacks sufficient data points to determine

the different zinc binding regimes that we expect to see.[50] Therefore, this aim will use a full titration to measure the binding affinities and the optimal zinc concentration for rhodopsin stabilization. Rhodopsin will be purified according to the protocol described above. Zinc acetate will be used for the titrations as the acetate counterion is compatible with MS. For these experiments, a stock of zinc acetate at 2000 ppm in water will be mixed with rhodopsin to obtain final concentrations ranging from 50-1500 ppm. Each sample will be allowed to incubate for 10 minutes before introduction to the MS. After 10 minutes, of incubation a dark state spectrum will be collected providing information about how much zinc binding occurs. Following dark state analysis online photobleaching using a green LED will occur. By measuring the intensity of the signal as a function of time, we will measure how quickly rhodopsin decays into the unstable MII state. Zinc has previously been shown to stabilize rhodopsin through ionic interactions.[33] Therefore upon the addition of zinc to the sample we would expect to see an increase in the time that it takes for the signal to completely decay. Data will be analyzed according to the procedure outlined in **1.2.2** where we will fit the decay to an exponential equation to obtain a rate constant for the different solutions. These rates will then be compared as a function of zinc concentration to analyze whether zinc is stabilizing the receptor. UV/Vis spectroscopy has largely been used to characterize rhodopsin and analyze changes in the activation capability of the receptor. Therefore, UV/Vis will also be used to validate the photobleaching kinetic results obtained by MS. [53]

2.2.2 Binding Site Mutagenesis. To probe the effects of the specific ionic interactions between the residues in the core of rhodopsin and the zinc ions, we will mutate residues that have been shown to be important for zinc binding.[50] These mutation experiments will provide key evidence for which specific residues are important in zinc binding and the activity of rhodopsin. Glu^{122} and His^{211} are residues that have been shown to bind zinc through X-ray crystallography studies.[49] We will make single and double mutants of Glu^{122} and His^{211} to Ala. To perform these experiments, a plasmid containing the mutations will be purchased from Addgene. The plasmids will be transfected into cells and expressed following a previous protocol.[54] Briefly, expression of non-mutated and the mutated opsin genes will be performed in HEK293 cells. Cells will be harvested 40 hours after transfection and treated with 50 μM 11-cis retinal in the dark for 3 hours. Following treatment with 11-cis retinal, the membrane pellet will be collected through ultracentrifugation followed by protein extraction in a buffer containing DDM. The supernatant will be collected and further purified using a Rho-1D4-Sepharose column, which uses an antibody specific for the C-terminal tail of rhodopsin for affinity purification. Rhodopsin will be eluted using an excess of the 1D4 peptide to compete with the rhodopsin binding. Similar to the above experiments, both the control and mutants will be analyzed using native MS in the dark state to measure zinc binding. The extent of zinc binding of the mutants will be compared to between the wild type and both the single and double mutants. We expect that minimal zinc binding will occur in the double mutants and intermediate binding in the single mutants. Online photoactivation of rhodopsin will be used to measure how the activity changes when the residues listed above are changed. Differences in both the activity and stability of the receptor in conjunction with the binding data will provide insight into how zinc binding at each site influences the activity.

2.2.5 Zinc and Lipid Cooperativity Titrations. Preliminary data from our lab has shown interesting trends in the amount of bound zinc to rhodopsin with different lipids present. To understand this cooperativity, we will titrate varying zinc concentrations in the presence of different lipids and measure binding of both zinc and lipids. The lipid concentration in these

experiments will be fixed at the optimal ratio determined in section **1.2.3,** and zinc will be titrated in at concentrations ranging from 0–1000 ppm, increasing in increments of 200 ppm. The ratio of the intensities of the zero, one, and two zinc bound peaks will be plotted as a function of the zinc concentration. Using these specific points, we will determine the effect that lipid binding has on the number of zinc ions bound to rhodopsin. Additionally, online photobleaching will be used to determine how zinc and lipid binding effect activity.

2.3 Expected Outcomes. The goal of Aim 2 is to determine the effects that zinc binding has on rhodopsin stability. By performing a complete titration of zinc, the observation of the different binding sites can be determined as well as the binding affinities for each site. We expect that zinc will stabilize rhodopsin by binding to the high affinity binding site. Once the concentration of zinc is higher, the second binding site will cause destabilization of the receptor. Furthermore, mutagenesis experiments will determine important residues for zinc binding and how these residues affect rhodopsin stability. In addition, by performing the experiments in **2.2.5** we will gain knowledge of how the lipids themselves affect the zinc binding and in turn affect rhodopsin photoactivation.

2.4 Impacts. The eye contains among the highest levels of zinc found in the body. Furthermore, the highest levels of zinc are found in the RPE cells and the choroid, suggesting it is important for eye health. Zinc concentrations in the eye can reach levels of 500 to 600 ppm. Low levels of zinc have been linked to many diseases, including AMD. The current treatment methods for AMD consist of introducing more zinc into the patient's diet, which has been shown to delay onset of the disease. The completion of this aim will provide key mechanistic insight in the role that zinc plays in the disease pathology. Furthermore, by performing the mutagenesis studies, the key residues for the binding will be determined, which will identify mutations that lead to rhodopsin destabilization. A better understanding of zinc's role in this disease will lead to an advancement in how zinc can be used to prevent onset and decrease severity of AMD.

2.5 Potential Pitfalls and Solutions Non-specific binding of zinc to rhodopsin may occur as a result of the ESI process. This happens when residual zinc is left behind when the solvent evaporates from the droplet. To determine, if the zinc biding seen is specific a counterion of similar mass and charge, such as magnesium, will be used. These experiments will use magnesium acetate as the counterion and will be titrated into the zinc samples at similar concentrations. If the zinc binding is a result of non-specific binding, then the magnesium ion will displace the zinc. The amount of magnesium ion bound at the same concentration will indicate the nonspecific binding level and allow the zinc binding to be corrected.

Conclusion and Future directions. The overall goal of this proposal is to characterize how changes in the membrane and cellular environment of rhodopsin can affect its photoactivation. **Aim 1** will characterize how oxidative damage to lipids in AMD causes alterations in rhodopsin photodynamics, leading to a decrease in low-light level sensitivity. Completion of **Aim 1** will provide novel insight into how oxidative stress leads to a decrease in the light sensitivity of rhodopsin due to direct lipid binding events and changes to bulk layer properties. **Aim 2** will determine the effect of zinc on rhodopsin activity. Furthermore **Aim 2** provides a novel approach to measuring zinc binding effects on receptor stability. This will reveal how this important metal affects receptor activity and may aid in disease treatment. The completion of both aims in this proposal will give new insight into how the progression of disease pathology occurs and may

provide new avenues for therapeutic development. Overall, the success of the proposal aims to provide key insight into how rhodopsin malfunction contributes to the pathology of AMD**.**

As previously stated, the type of lipids that are oxidized in AMD are unknown and the lipids chosen here were based on their overall prevalence in the eye. Future directions will aim to determine pertinent information about which lipids are oxidized in patients suffering from AMD. Lipid extraction will be performed on eyes that are obtained from healthy patients and those suffering with different stages of AMD. Lipidomics will be performed on the samples to determine what kinds of oxidized lipids are present in the patient samples. In addition, rhodopsin will be extracted from the membranes with a gentle detergent and gradual delipidation will be used to determine the lipids that are bound tightly to rhodopsin versus those that are loosely bound.[55] Determining the lipids that are more tightly bound to rhodopsin as the disease progresses, along with the above-mentioned studies, will provide key insight into the pathology of the disease.

Although AMD is one of the leading causes of blindness in the elderly, there are many other eye diseases that are linked to rhodopsin. For example, autosomal dominate retinitis pigmentosa (adRP) causes complete night blindness in patients. There have been many studies on what leads to a disease and many mutations have been discovered. Using MS, we will determine how these residues lead to destabilization, whether through changes in types or amount of lipid binding or alterations to amount of zinc bound. Specific resides of interest include Lys^{296}, Pro^{267}, Glu^{113}, and residues in the conserved D(E)RY and NPXXY motifs.[56,57,58] These mutations will be analyzed within nanodisc and activity will be measured using the same protocol outlined in section **1.2.5** to determine how these mutations alter rhodopsin photoactivation. These future directions will further seek to understand how changes in the environment of rhodopsin lead to a disease pathology in hopes of finding better therapeutic targets.

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