CIRCADIAN PROTEOME CHANGES IN PHOTORECEPTOR OUTER SEGMENTS

by

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(date) __________________
December 7, 2009

*We also certify that written approval has been obtained for any proprietary material contained therein.
DEDICATION

I dedicate this thesis to my Grandmother, Jaroslava, who has always been my role model and is missed greatly, my Mom, Saša, and my brother, Tomáš, who have always encouraged me and supported my crazy ideas without questions, and my husband, Pete, who has been extremely supportive and always willing to help whether it was with my midnight experiments, taking pictures or correcting my English. I wouldn’t have been able to finish without you!
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### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMD</td>
<td>age related macular degeneration</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriamine pentaacetic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GBB5</td>
<td>Guanine nucleotide binding protein beta 5</td>
</tr>
<tr>
<td>GuHCl</td>
<td>guanidine hydrochloride</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IA</td>
<td>iodoacetamide</td>
</tr>
<tr>
<td>INL</td>
<td>inner nuclear layer</td>
</tr>
<tr>
<td>IS</td>
<td>inner segments</td>
</tr>
<tr>
<td>LCM</td>
<td>laser capture microscopy</td>
</tr>
<tr>
<td>LC-MRM</td>
<td>liquid chromatography multiple reaction monitoring</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>LEDGF</td>
<td>lens epithelium derived growth factor</td>
</tr>
<tr>
<td>Lys C</td>
<td>lysyl endopeptidase</td>
</tr>
<tr>
<td>Lys N</td>
<td>peptidyl lys metaloprotease</td>
</tr>
<tr>
<td>OCT</td>
<td>optimum cutting temperature</td>
</tr>
<tr>
<td>ONL</td>
<td>outer nuclear layer</td>
</tr>
<tr>
<td>OS</td>
<td>outer segments</td>
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</table>
PBS  - phosphate buffered saline
PBST  - phosphate buffered saline with triton
PE    - pigment epithelium
PMSF  - phenylmethysulphonyl fluoride
R9AP  - regulator of signaling 9 anchor protein
RCS   - Royal College of Surgeons
RGS9  - regulator of signaling 9
RK    - rhodopsin kinase
RP    - retinitis pigmentosa
RPE   - retinal pigment epithelium
SDS   - sodium dodecyl sulfate
TFA   - trifluoroacetic acid
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Circadian Proteome Changes in Photoreceptor Outer Segments

ABSTRACT

by

DAGMAR HÁJKOVÁ LEARY

The goal of this study was to better understand the molecular mechanisms of light-induced photoreceptor cell death. Apoptotic photoreceptor cell death is the cause of several common retinal diseases (e.g. age related macular degeneration, retinitis pigmentosa). Light-induced photoreceptor cell degeneration in laboratory animals is one of the experimental models for studying such human retinal diseases. It begins with the absorption of light by rhodopsin in the photoreceptor outer segments (OS) which eventually leads to apoptotic photoreceptor cell death but the exact molecular mechanisms are yet to be elucidated. To gain insight into these mechanisms we investigated the light-induced and circadian changes in the OS proteome using a quantitative proteomic method called proteolytic $^{18}$O labeling. The first study investigated the changes of OS proteome upon intense light exposure. Rapigest, acid cleavable detergent, was used to solubilize OS proteins for tryptic digestion and $^{18}$O
labeling. The quantities of 11 proteins were found to differ by more than 2-fold upon light exposure. Eight proteins were phototransduction proteins and seven of these were altered to reduce efficiency or quench phototransduction during light exposure. In contrast, the amount of rhodopsin kinase was reduced by 2-fold after light exposure, suggesting attenuation in the mechanism of quenching phototransduction. Our findings suggest that this reduction may be a contributing factor to light-induced photoreceptor cell death. The second study investigated OS proteome differences between midnight and noon. This study was designed to elucidate the mechanisms of circadian dependent susceptibility to light induced damage, since the susceptibility is greater at night than daytime. OS proteins were fractionated by 1D-SDS-PAGE prior to $^{18}$O labeling. Most OS protein amounts remained unchanged at night versus daytime. The most notable difference was the lower amount of anti-stress proteins (crystallin αB, and peroxiredoxin 1) at midnight, suggesting the link with the greater nighttime OS susceptibility to light. Our studies create a foundation for understanding the mechanisms of light-induced photoreceptor cell death.
CHAPTER 1: BACKGROUND

The mechanism of degenerative retinal diseases are difficult to study in humans, therefore the use of animal models is essential. Light-induced photoreceptor cell degeneration in laboratory animals is a model for many human retinal diseases in which the photoreceptors die by apoptosis, resulting in loss of vision in the individuals. However, the molecular mechanisms of this cell death are poorly understood. Intriguingly, susceptibility of photoreceptors to light is greater at night than daytime. The mechanisms underlying this circadian dependent susceptibility to light damage are not well understood. The studies described herein were performed to identify light-induced and circadian changes occurring in the photoreceptor OS, the compartments where photons are absorbed and changed into neuronal impulses. The results are expected to help to better understand the mechanisms of light-induced photoreceptor degeneration and have potential to lead to better clinical strategies to prevent and treat retinal degenerative diseases.

Vision

Vision is the ability of brain and eye to detect light. Light reflects from an object and enters the eye through transparent tissue called the cornea (Figure 1.1). It passes through a narrow opening – the pupil, and reaches the lens. The lens focuses the reflected light onto the retina which is a thin tissue present in the back of the eye. It consists of different neuronal cells organized into layers (Figure 1.2). This layered organization is suitable for signal transduction. The inner most layer consists of the photoreceptor cells. This is
where light is absorbed and changed into a neuronal signal. The impulse is then conveyed to the outer retinal layers to non-photoreceptor neurons. Then it travels via the optic nerve to the brain and where an image of the object is created. Vision is a very important aspect of life for every individual and vision impairment is a major handicap making every day life more difficult. There are no effective therapies for many retinal diseases that cause a loss of vision, therefore it is very important to study the mechanisms of these diseases.

**Photoreceptor Cells**

Photoreceptors are the essential components of the visual system. There are two types of photoreceptor cells: rods and cones. Rods can detect low intensity light and enable night vision. Cones detect higher intensity light, allow the distinguishing small details, and enable color vision. The central portion of the retina is called the macula and consists of rods and cones. This area is responsible for central vision. In humans, cones are concentrated in an area called the fovea which is in the middle of the macula (Figure 1.1), however rodents do not have a fovea and their cones are evenly distributed throughout the retina. Rod and cone photoreceptor cells are examples of cells polarized by structure and function. These cells consist of outer segments (OS) and inner segments (IS) attached to each other by narrow connecting cilium, and the synaptic region where information from photoreceptors is transmitted to other neuronal cells in the retina (Figure 1.3). The OS is a highly specialized part of the cell where photons are absorbed, initiating the phototransduction cascade. It has been estimated that the visual pigment rhodopsin accounts for more than 80% of the protein (w/w) in the OS [1]. Oxidative damage to OS could be created by excess light absorption. This is why parts of the OS are phagocytosed.
by retinal pigment epithelium (RPE) every morning as new disks are created at the bottom of the OS. It takes approximately 10 days to completely renew the OS. The IS contains major cellular organelles such as the nucleus and mitochondria. Since the OS does not contain protein synthesis machinery, all the proteins in the OS must be synthesized in the IS and transported to the OS.

**Phototransduction Cascade**

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contains major cellular organelles such as the nucleus and mitochondria. Since the OS does not contain protein synthesis machinery, all the proteins in the OS must be synthesized in the IS and transported to the OS.

**Phototransduction Cascade**

Phototransduction originates in OS. The visual pigment of rod photoreceptor, rhodopsin, consists of protein moiety opsin and chromophore 11-cis-retinal. Cone cells contain the cone opsin and 11-cis-retinal. Photoreceptors belong to the G-protein coupled receptor family. Upon absorption of light the chromophore, 11-cis-retinal, is isomerized to all-trans retinal that causes conformational changes in opsin and that facilitates the binding and activation of a G-protein called transducin. One subunit of transducin then separates from the others and binds to the inhibitory subunit of phosphodiesterase and subsequently activates this enzyme which converts guanosine 3’,5’-cyclic-monophosphate (cGMP) to guanosine monophosphate (GMP). The decreasing concentration of cGMP causes cGMP gated cation channel to close, resulting in hyperpolarization of the cell. Once rhodopsin is activated it is necessary to turn it off because spontaneous decay would be too slow. A protein called rhodopsin kinase (RK) initiate the deactivation cascade by phosphorylating the activated rhodopsin that creates a binding site for arrestin which stearically interferes with transducin’s binding [2]. In addition to deactivating rhodopsin, transducin mediated signaling could be quenched. The complex of G-protein beta 5 (GBB5), regulator of signaling 9 (RGS9) and regulator of signaling 9 anchor protein (R9AP) binds to the transducin α subunit-phosphodiesterase complex. This stimulates the GTPase activity of transducin α subunit and results in dissociation of the transducin α subunit from
phosphodiesterase, therefore slowing down the hydrolysis of cGMP, allowing the cGMP channels to open (Figure 1.4). New cycle of signaling can then begin.

**Photoreceptor Degenerative Diseases**

A large variety of diseases affect the retina. A subset of these diseases is caused by photoreceptor degeneration. One group of such diseases is called retinitis pigmentosa (RP) and is represented by heterogeneous familiar disorders in which photoreceptors and then RPE progressively degenerate. The most common cause of these diseases are gene mutations inherited from one or both parents. Many genes involved in this disease have already been identified [3]. The age of RP onset differs between individuals but is typically in adolescence [4]. As rods are the first photoreceptors affected, an early symptom of RP is night blindness. Loss of vision progresses from the periphery to the center. It is the most common type of retinal degeneration affecting about 1.5 million people in the world, 1 in every 3700 (~0.03%) individuals is affected by this disease [5]. Some world regions e.g. South India have a much higher prevalence of this disease (as much as 1 in 400 for rural populations) [6]. Unfortunately there is no cure or treatment to reverse the progress of this disease. Therapy is mainly focused on slowing down the photoreceptor degeneration. Detailed RP description and current treatment trends can be found in a review by Shintani et al[7].

One other group of retinal degenerative diseases is called age related macular degeneration (AMD) and is characterized by slow and at first asymptomatic development of drusen – whitish yellow deposits under the sensory retina and RPE. Photoreceptor degeneration usually starts in the macula and leads to the loss of central vision. The exact
cause of AMD is not known. Oxidative stress, mitochondrial dysfunction, genetics, and inflammatory processes may play a role [8-13]. Smoking, high blood pressure, and high body mass index are risk factors for AMD and if controlled, they could reduce the risk of developing AMD by half [14]. There is no cure for AMD, even though several treatments can slow its progression (treatments are reviewed by Coleman et al.[15]). AMD is the most common cause of blindness in elderly Americans of European descent as well as in many Europeans [16]. The prevalence of this disease is 0.2% but as our population lives longer its prevalence will be getting larger in the near future.

Animal Models for Retinal Diseases

It is extremely difficult to study degenerative retinal diseases in human patients. It would be impossible to study the mechanism of these diseases without animal models. There are several genetic mutants available to study retinal degeneration in mice (reviewed by Rakoczy et al.[17], and Dalke et al.[18]). Many of these mutations are in genes coding for proteins involved in phototransduction (i.e. subunits of phosphodiesterase, rhodopsin) or proteins involved in structural organization of the OS (i.e. peripherin).

Light-induced photoreceptor cell degeneration in laboratory animals is an example of a model for degenerative diseases as well. In this model, photoreceptors die by apoptosis caused by excessive illumination. As mentioned above, apoptosis is also the putative cell death mechanism in many retinal degeneration diseases. Light damage to photoreceptors was first reported by Noell et al. [19] in rats in 1966 and is still widely studied. Studies on several transgenic mice with visual cycle gene knock-outs (RPE65 [20], cellular retinaldehyde-binding protein [21], transducin alpha[22]) and animals that were
administered inhibitors of the visual cycle (retinylamine – [23], halothane [24]), revealed that these animals were protected from light-induced photoreceptor damage. Antioxidants given to laboratory animals prior to light exposure prevented light damage as well [25]. This suggests that oxidative stress plays a role in light damage [26].

Adaptation to bright cyclic light in wild type albino rats alleviated photoreceptor light damage [27]. On the other hand, excess continuous light stimulation of rhodopsin caused rods to be more sensitive to light damage in mouse knock outs for genes participating in signal quenching. It has been reported that arrestin [28, 29] and RK [30] knock out mice are much more susceptible to light damage. This suggests that prolonged activation of phototransduction signaling is responsible at least in part for the susceptibility to light damage. All of these reports support the notion that photoreceptor cell death begins in the OS by rhodopsin activation and that an active visual cycle to regenerate 11-cis retinal is required for cell death to occur, and properly quenching the activated rhodopsin is important to protect the cells from damaging light. Figure 1.5 summarizes the current knowledge of photoreceptor cell death in this animal model.

Several studies [31, 32] have demonstrated that the damage to photoreceptor cells by acute intense light was far more severe when the light exposure occurred during nighttime versus daytime. Figures 1.6 and 1.7 show the histology of retinas immediately and 14 days after light exposure at different times of day. Retinas from cyclic light reared rats exposed at 1 am completely lost the layered structure and most photoreceptor cells. On the other hand, retinas from rats raised in the same cyclic light environment and exposed at 9 am or at 5 pm had comparable structural morphology to the unexposed
control rats. The authors suggested that circadian rhythm may be responsible for the differences in light susceptibility.

**Circadian Rhythm**

Many biological activities of living organisms are organized in cycles of different length. The cycle lasting approximately 24 h is called circadian. In mammals, the main clock generating these rhythms is in suprachiasmatic nucleus in hypothalamus. It controls organization of different processes in the organism e.g. food intake, hormone release, body temperature, metabolism etc. On a molecular basis, a group of clock genes is rhythmically (cycle is approximately 24h) expressed to positively and negatively regulate the transcription of clock controlled genes. Zeitbergers – “time givers” are external factors which indicate the astronomical time. Light is the major zeitberger and it resets the clock daily in the suprachiasmatic nucleus, however circadian rhythms persist in complete darkness if other zeitbergers (food intake, social or physical activities) are present. In the absence of zeitbergers the circadian cycle is free-running and its period differs from 24 hours [33].

Pacemakers outside the suprachiasmatic nuclei were discovered in peripheral tissues. One peripheral pacemaker was thought to be in the retina [34] and in early 1990’s experiments in rats with lesions in suprachiasmatic nuclei provided more experimental evidence of its existence [35]. Other experimental results suggested that it controls the circadian processes in the eye [36]. In 1996 it was discovered that melatonin synthesis follows a circadian cycle in cultured retina [37], giving direct evidence of a suprachiasmatic nuclei independent circadian clock in the retina. This clock also controls dopamine release,
phototransduction proteins, and metabolism in the retina (summarized by Grewal et al. [38]).

**Proteomic Analysis of Photoreceptor Outer Segments**

As mentioned above, it has been estimated that the visual pigment rhodopsin accounts for more than 80% of the protein in the OS [1]. The OS does not contain protein synthesis machinery, therefore all the proteins in the OS must be synthesized in the IS and transported into OS. Therefore, quantification of mRNA cannot be used to estimate the protein expression profile.

It has been reported that light exposure causes the amounts of several OS proteins to change. The proteins that have been shown to translocate between OS and IS are transducins, arrestin and recoverin. Western Blot analysis or immunohistochemistry have been used to identify the translocating proteins. These techniques are well established; however, they require foreknowledge of the proteins that may change their location and they are not high throughput. On the other hand, comparative proteomic techniques are capable of identifying and quantifying these without a priori knowledge, are relatively high throughput, but they are not yet well established techniques and results require validation.

Most proteomic studies analyzing biological samples to identify up- or down-regulated proteins upon genetic or environmental change have been based on two dimensional gel electrophoresis (2D-PAGE) despite the fact that this method has poor dynamic range and is biased toward abundant and soluble proteins. An alternative proteomic method to 2D-PAGE is mass spectrometry based in vitro stable isotopic labeling (for workflow see Figure 1.8). In this comparative proteomic method, proteins from control and
experimental samples are digested by specific protease (e.g. trypsin). The peptides generated from the control sample are then labeled with naturally abundant isotope (light) and peptides from the experimental sample are labeled with its heavier isotope or vice versa. The samples are then mixed in 1:1 ratio and analyzed by mass spectrometry. The relative content of each peptide is then calculated by comparing the peak intensities of the heavy and light peptides in the mass spectrum. The relative abundance of the peptide corresponds to the abundance of the parental protein in the original samples.
Figure 1.1 – Light Path through the Eye

Light reflects from an object and enters the eye through transparent hard tissue called the cornea, into the anterior chamber. It passes through a narrow opening – the pupil, to reach the lens. Its function is to focus light onto the retina. It is here where photoreceptors turn light into a neuronal impulse which is then conveyed through the optic nerve to the brain, where an image of the object is created. Humans have two kinds of photoreceptors – rods and cones. Cones are concentrated in an area called the macula. A purple arrow represents the light reflected from an object.

Figure 1.2 - Layered Structure of the Retina

A 10 μm cryosection of rat eyeball stained with cresyl violet to create a contrast image of different layers in the retina is shown above. An arrow represents direction of light from the environment. Distinguishable layers: RPE - Retinal Pigment Epithelium, OS – Photoreceptor Outer Segments, IS – Photoreceptor Inner Segments, ONL – Outer Nuclear Layer, representing nuclei of non photoreceptor neurons in the retina.
Figure 1.3 – Structure of Rod and Cone Photoreceptors

Photoreceptors are polarized sensory neurons. Rods (left) and cones (right) have three cellular compartments. Outer segments (OS) are stacks of membrane disks rich in the visual pigment rhodopsin. It is here where phototransduction originates. Interestingly, this cellular part doesn’t contain any protein synthesis machinery. All OS proteins are synthesized in the inner segments (IS), and then transported to here. IS also contain other vital organelles i.e. mitochondria, and the nucleus. Neuronal impulses created in the OS pass through the IS till they reach the synapse where they are transmitted to other retinal neurons.
Figure 1.4 – Phototransduction cascade


Under dark conditions, cGMP gated channels are opened and phototransduction proteins - rhodopsin, transducin and phosphodiesterase are inactive (The first disk). Upon light absorption by rhodopsin, transducin is activated and the α subunit to dissociates from subunits β and γ, and binds to the inhibitory subunit (γ) of phosphodiesterase and activates the enzyme which hydrolyzes cGMP. cGMP gated channels close, and photoreceptor cell is hyperpolarized. (The second disk). Activated rhodopsin is then phosphorylated by RK to create a binding site for arrestin which stearically interferes with binding of a new subunit of transducin alpha. Complex of G-protein beta 5 (GBB5),
Regulator of signaling 9, Regulator of signaling 9 anchor protein (R9AP) binds to transducin alpha-phosphodiesterase complex. Transducin alpha hydrolyzes GTP and dissociates from phosphodiesterase, slowing down the hydrolysis of cGMP, allowing the cGMP channels to open (The third disk). All phototransduction proteins are then ready for a new signaling cycle.
Figure 1.5 – Summary of Studies about Light Induced Photoreceptor Cell Death

1. Photoreceptor light damage starts in the OS by rhodopsin activation. Green intense light ($\lambda=480-590$ nm) caused the highest damage to photoreceptors (ref), interestingly, this wavelength corresponds to rhodopsin absorption maximum [19].

2. Animals with knock out genes from chromophore regeneration cycle are protected from light induced cell death. This suggests that this cycle has to be intact for light damage to occur [20, 21].

3. Administration of antioxidants prior to light exposure prevents light damage. This suggests that oxidative stress may be involved [25].
4. Animal knock outs of apoptotic genes (bax and bak) were protected from light damage. This suggests apoptosis is the putative cell death mechanism in this animal model however the trigger of the apoptotic cascade is still not understood.
Figure 1.6 – Histology of Retinal Tissue Immediately after Light Exposure

Top row shows retinas from cyclic light reared rats; bottom row shows retinas from dark reared rats. A,E were exposed at 9:00 am. B,F were exposed at 5:00 pm. C,G were exposed at 01:00 am. D,H represent unexposed controls.

Abbreviations: PE, pigment epithelium; OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; Representative cone nuclei are indicated by arrows. Scale bar 20 µm.

Figure was originally printed in Photochemistry and Photobiology, 75 (5) Pgs. 547-553, Evidence for circadian rhythm of susceptibility to retinal light damage by Vaughan DK, Nemke JL, Fliesler SJ, Darrow RM, Organisciak DT, Figure 3 on page 550. Figure reprinted with permission from Wiley-Blackwell.
Figure 1.7 – Histology of Retinal Tissue 14 Days after Light Exposure

Top row shows retinas from cyclic light reared rats; bottom row shows retinas from dark reared rats. A,E were exposed at 9:00 am. B,F were exposed at 5:00 pm. C,G were exposed at 01:00 am. D,H represent unexposed controls.

Abbreviations: PE, pigment epithelium; OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; Representative cone nuclei are indicated by arrows. Scale bar 20 µm.

Figure was originally printed in Photochemistry and Photobiology, 75 (5) Pgs. 547-553, Evidence for circadian rhythm of susceptibility to retinal light damage by Vaughan DK, Nemke JL, Fliesler SJ, Darrow RM, Organisciak DT, Figure 4 on page 550. Figure reprinted with permission from Wiley-Blackwell.
Figure 1.8 – Isotopic Labeling Workflow for Forward Experiment

- **Control sample**
  - Digest by protease
  - Label with *light* isotope
    - Mix in 1:1 ratio
      - LC-MS/MS analysis
        - Calculate ratio of *heavy* to *light* peptides
          - Determine ratios of proteins in control to experimental sample
Our laboratory has been developing a stable isotope labeling technique called $^{18}$O labeling. This technique utilizes a protease to incorporate either light ($^{16}$O) or heavy ($^{18}$O) isotopes of oxygen into carboxyl groups at the C-terminus of peptides. The labeling is very specific to the residues which are recognized by the protease (Figure 2.1). Analysis of samples labeled by heavy and light isotopes is described in Figure 2.2. After mixing control and experimental samples, peptides are injected onto reverse phase chromatographic columns. Heavy and light peptides with the same sequence coelute from the chromatographic column and are analyzed by LC-MS. Peptide ratio calculation from LC-MS spectrum is explained in Figure 2.3. MS/MS spectra are used for peptide identification. It is critical to achieve either only single $^{18}$O or complete two $^{18}$O atom incorporation into peptides to achieve accurate quantification results. When more isotopic species are present the data interpretation becomes very complicated and inaccurate. Our laboratory has already determined the conditions of labeling under which only one $^{18}$O is incorporated into C-terminus of peptides by the protease, Lys N [39, 40]. This chapter summarizes our findings of the conditions under which both oxygen atoms in peptide carboxy termini are exchanged by Lys-C or trypsin.
Figure 2.1 – Amide Bond Hydrolysis and Carboxy Oxygen Exchange Reaction Catalyzed by Lys-C and Trypsin

The proteases recognize specific residues (Lys by Lys-C and Lys and Arg by trypsin) in protein and hydrolyze the C-terminus of these residues (a). The optimum pH for this amide bond hydrolysis reaction is around 9.0. These proteases also interact with the products of the amide bond hydrolysis reaction and exchange the C-terminal carboxyl oxygen with the oxygen of water. This reaction can be detected when the solvent contains heavy isotope of oxygen, $^{18}$O (b). If the reaction continues both oxygen atoms in the C-terminal carboxyl group eventually exchanged with the oxygen of $\text{H}_2^{18}$O. The optimum pH of this reaction was determined in the study described later.
Figure 2.2 – Scheme of LC-MS/MS analysis of $^{18}$O labeled samples.

Heavy and light peptides are injected onto reverse phase HPLC column A) and chromatographically separated from other tryptic peptides. B) Heavy and light peptides of the same sequence co-elute from reverse phase column. C) Eluted peptides are ionized and analyzed by high resolution mass spectrometer. The precursor ion masses of heavy and light peptides are different therefore can be resolved by this method. The intensity of each peptide is used for relative ratio calculation. D) MS/MS analysis of these peptides are used to identify the proteins from which these peptides were derived. Figure is adapted from Miyagi and Rao, Proteolytic $^{18}$O Labeling Strategies for Quantitative Proteomics, Mass Spectrometry Reviews, 2007, 26, 121–136.
Figure 2.3 – Calculation of $^{18}$O Ratio from LC-MS/MS Spectra

The corrected (cor) $^{16}$O is calculated from the observed (obs) intensity of the $^{16}$O labeled peptide and the monoisotopic peak intensities of the $^{18}$O-labeled species according to the equations above, where X is the theoretical fractional intensity of the M+4 isotopic peak contributed by the $^{16}$O labeled peptide. Relative abundances of each ion species in the sample are $^{16}$O$_2$ 0.25 %, $^{16}$O$_1$$^{18}$O$_1$ 9.5%, and $^{18}$O$_2$ 90.25%. These abundances are only valid when 95% H$_2$$^{18}$O is used. The cor $^{18}$O/cor $^{16}$O then corresponds to the ratio of the protein from which the peptide was created.
The pH Dependency of the Carboxyl Oxygen Exchange Reaction Catalyzed by Lysyl Endopeptidase and Trypsin

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ABSTRACT

The pH dependency of the carboxyl oxygen exchange reaction catalyzed by lysyl endopeptidase (Lys-C) and trypsin has been studied. The reaction was quantitatively monitored by measuring the incorporation of $^{18}$O atom into the $\alpha$-carboxyl group of $N^\alpha$-acetyl-L-lysine from H$_2$ $^{18}$O solvent. The optimum pHs of the carboxyl oxygen exchange reaction catalyzed by Lys-C and trypsin were found to be pH 5.0 and 6.0, respectively, which were significantly shifted toward acidic pHs compared to the most favorable pHs of their amidase activities for $N^\alpha$-acetyl-L-lysine amide in the pHs examined. Steady state kinetics parameters were also determined for both enzymes at two different pHs, one at the pH optimum for their carboxyl oxygen exchange activity (pH 5 - 6) and the other at the favorable pH for their amidase activity (pH 8 – 9). Significantly lower $K_m$ (2-fold lower for Lys-C, 3-fold lower for trypsin), and higher $k_{cat}$ values (1.5-fold higher for Lys-C, 5-fold higher for trypsin) were obtained at the acidic pHs compared to the alkaline pHs, suggesting that Lys-C and trypsin have higher substrate binding affinities and higher catalytic rates at the acidic pHs than at the alkaline pHs. The higher carboxyl oxygen exchange activities at the acidic pHs also were confirmed with peptide substrates derived from apomyoglobin. These findings are significant towards the goal of improving the efficiency of the Lys-C and trypsin catalyzed $^{18}$O labeling reactions and are thus pertinent to improving the accuracy and reliability of quantitative proteomic experiments utilizing $^{18}$O labeling.
INTRODUCTION

Hydrolysis of a protein in H$_2^{18}$O solvent by serine proteases results in incorporation of two $^{18}$O atoms into the carboxyl terminus of each proteolytically generated peptide, if sufficient time was given for the reaction to reach equilibrium[41-44]. Studies done by Yao and coworkers clearly demonstrated that the two $^{18}$O atoms are incorporated by trypsin at equilibrium via the following two step mechanism: [44]

$$RC^{16}ONHR' + H_2^{18}O \rightarrow RC^{16}O^{18}O^- + ^{18}H_3NR'$$

$$RC^{16}O^{18}O^- + H_2^{18}O \rightarrow RC^{18}O^{18}O^- + H_2^{16}O$$

The first $^{18}$O atom is incorporated from the H$_2^{18}$O solvent upon proteolytic cleavage of a peptide bond, as shown in reaction 1. The second $^{18}$O atom incorporation is essentially a carboxyl oxygen exchange reaction as shown in the reaction 2, which occurs following the proteolytic cleavage. The protease continues to interact with the peptide product and exchange the carboxyl oxygen, which results in two $^{18}$O atoms incorporation, if the first $^{18}$O atom retains (if not, which incorporates another $^{18}$O atom, but concomitantly loose the first $^{18}$O atom as a leaving $^{18}$OH$^-$, results in a net single $^{18}$O atom incorporation). Thus, the reaction 2 is required to occur multiple times on the peptide to achieve complete incorporation of two $^{18}$O atoms. If the reaction continues, both the oxygens in the C-terminal carboxyl group of the peptide should eventually come to equilibrium with oxygen from the H$_2^{18}$O solvent.

There has been an increasing interest in the use of protease catalyzed $^{18}$O labeling for quantitative proteomics[45, 46]. However, this method often suffers because of the generation of a mixture of isotopic isoforms resulting from the variable incorporation of
either one or two $^{18}$O atoms ($^{18}$O$_1/^{18}$O$_2$) into each peptide species [47], presumably because the second $^{18}$O atom incorporation reaction (carboxyl oxygen exchange reaction) is extremely slow under the conditions commonly used for protein digestion, leading to incomplete and variable exchange in the time frame of the proteolytic reaction. This complicates the quantification of the peptides and increases the error in the calculations of $^{16}$O- and $^{18}$O-labeled peptide ratios [39]. Complete incorporation of two $^{18}$O atoms would obviate this difficulty. Finding reaction conditions to achieve this would be a significant step towards improving quantitative proteomics methods utilizing Lys-C and trypsin catalyzed $^{18}$O labeling.

The carboxyl oxygen exchange reaction was found by Rittenberg and Sprinson for chymotrypsin fifty years ago [48]. Shortly after the finding, Doherty and Vaslow demonstrated that the binding of acetyl-3,5-dibromo-L-tyrosine to chymotrypsin is tighter at acidic pH than alkaline pH by an enzyme-substrate equilibrium experiment [49]. The kinetic parameters of chymotrypsin-catalyzed carboxyl oxygen exchange reaction have also been reported by Vaslow [50] for acetyl-3,5-dibromo-L-tyrosine at pH 7.2 and by Bender and Kemp [51] for benzoyl-L-phenylalanine and acetyl-L-tryptophane at pH 7.8. Recently, Yao and coworkers have reported kinetic parameters of trypsin-catalyzed carboxyl oxygen exchange reaction at pH 8.0 for short peptide substrates [44]. However, pH studies were not performed in these kinetic studies. A recent study done by Zang and coworkers showed that trypsin-catalyzed carboxyl oxygen exchange reaction at pH 6.75 is more efficient than at pH 8.50 based on an experiment that measured the changes of isotopic peaks of the labeled peptides after 20 h of labeling reactions [45]. Also, a study
performed by Staes and coworkers showed that complete two $^{18}$O atoms incorporation can be achieved after overnight reaction at pH 4.5 [52]. These two earlier works suggested that the optimum pH of trypsin catalyzed carboxyl oxygen exchange reaction is shifted toward acidic pH compared to the favorable pH for its amidase activity. However, these earlier works did not measure the initial rate of the reaction, therefore no quantitative information on the rate of the reaction were obtained. As far as we know the effect of pH on the kinetic parameters has not been studied before.

The purpose of this study is to better understand the catalytic mechanism of carboxyl oxygen exchange reaction promoted by serine proteases. Lys-C and trypsin were chosen, because they have been the most utilized proteases in $^{18}$O labeling experiment. A better understanding of the mechanism will lead to the development of a more reliable quantitative proteomic method. Since the substrate for carboxyl oxygen exchange reaction (RCOO$^-$) has a negative charge at the C-terminus at physiological pH, while the corresponding substrate for amide hydrolysis reaction (RCONH$_2$) does not, it seems reasonable to assume that the mechanism of enzyme-substrate complex formation in the carboxyl oxygen exchange reaction is somewhat different from the mechanism in the amide bond hydrolysis reaction. For this reason, we examined the pH dependency of the carboxyl oxygen exchange reaction for $N^\alpha$-acetyl-L-lysine (Ac-Lys-OH) catalyzed by Lys-C and trypsin, utilizing H$_2$ $^{18}$O as a carboxyl oxygen labeling agent. The single $^{18}$O atom incorporated reaction product, Ac-Lys-$^{18}$OH, was quantified by stable isotope dilution tandem mass spectrometry. Steady state kinetics parameters for the carboxyl oxygen exchange reaction were also obtained. Based on these results conditions for use
of Lys-C and trypsin to efficiently incorporate two $^{18}$O atoms into the carboxyl termini of peptides are suggested.

MATERIALS AND METHODS

Materials

$N^\alpha$-acetyl-L-lysine (Ac-Lys-OH), $N^\alpha$-acetyl-L-lysine amide (Ac-Lys-NH$_2$) and $N^\varepsilon$-tert-butyloxycarbonyl-L-lysine (H-Lys(Boc)-OH) were purchased from Bachem AG (Torrance, CA). Horse apomyoglobin and $[^{13}C_4]$acetic anhydride were obtained from Sigma-Aldrich (St. Louis, MO). Oxygen-18 enriched water (>95%) was obtained from Cambridge Isotope Laboratories (Andover, MA). Lys-C from Achromobacter lyticus was purchased from Wako Chemicals USA (Richmond, VA). Sequencing grade modified porcine trypsin was obtained from Promega (Madison, WI). All other chemicals and materials were either reagent grade or were of the highest quality that was commercially available.

$N^\alpha$-$[^{13}C_2]$acetyl-lysine ($[^{13}C_2]$Ac-Lys-OH) was synthesized by acetylating H-Lys(Boc)-OH (50mM) by $[^{13}C_4]$acetic anhydride (2mM) for 16 h at 25 °C in 0.8 mM N-ethylmorpholine, 50% acetonitrile in water. After the reaction, the reaction mixture was dried in a speed-vac, and then the Boc group was cleaved by 3 M HCl in ethyl acetate for 30 min at 25°C. The product, $[^{13}C_2]$Ac-Lys-OH, was extracted in water and purified by reverse phase HPLC. The molecular weight of the purified $[^{13}C_2]$Ac-Lys-OH was determined by high resolution mass spectrometry [$^{13}C_2C_6H_{17}N_2O_3$ (M + H$^+$): calculated, 191.1306; found, 191.1261]. $[^{13}C_2]$Ac-Lys-OH in water was quantified by
spectrophotometry at 215 nm using a molar absorption coefficient for Ac-Lys-OH of 877 M\(^{-1}\)•cm\(^{-1}\) and used for the following experiments.

Measurement of the pH dependency of the carboxyl oxygen exchange reaction for Ac-Lys-OH and the amidase activity for Ac-Lys-NH\(_2\)

The initial rate of carboxyl oxygen exchange reaction catalyzed by Lys-C and trypsin was determined by monitoring the rate of \(^{18}\)O atom incorporation into the carboxyl terminus of Ac-Lys-OH. For the pH studies, a 10 mM solution of Ac-Lys-OH in aqueous buffers at different pHs (specified below) was incubated with 1 \(\mu\)M Lys-C or trypsin at 25 °C in 95% H\(_2\)^\(^{18}\)O buffered at various pHs. The buffer solutions used were 50 mM citrate at pH 4.0, 5.0 and 6.0 (pH adjusted by addition of NaOH), 50 mM phosphate at pH 7.0 and 8.0 (pH adjusted by addition of NaOH) and 50 mM Tris at pH 9.0 (pH adjusted by addition of HCl). The duration of the reaction was 5 min for Lys-C and 20 min for trypsin. All the reactions were confirmed to be linear within the time range. The total volume of the reaction mixture was 50 \(\mu\)L. The reaction was stopped by adding 4-times the volume of 80% acetonitrile/1% formic acid (v/v) in water containing a constant amount of internal standard, \(\left[^{13}\text{C}_2\right]\)Ac-Lys-OH (0.75 \(\mu\)g), dried in speed-vac concentrator, and redissolved in 50 \(\mu\)L of 5% acetonitrile/0.1% heptafluorobutyric acid (HFBA) (v/v) in water. The single \(^{18}\)O atom incorporated reaction product, Ac-Lys-\(^{18}\)OH, in the reconstituted reaction solution was then quantified by stable isotope dilution tandem mass spectrometry, as described below. All reactions were carried out in triplicate.
The amidase activity of Lys-C and trypsin was measured by monitoring the initial rate of hydrolysis of Ac-Lys-NH$_2$ in H$_2^{18}$O. Ac-Lys-NH$_2$ (10 mM) was incubated with 0.2 $\mu$M Lys-C or 1 $\mu$M trypsin at 25 °C in 95% H$_2^{18}$O at various pHs. The buffer solutions used were the same as described above for the carboxyl oxygen exchange reactions. The duration of the reaction was 5 min for Lys-C and 20 min for trypsin. The single $^{18}$O atom incorporated reaction product, Ac-Lys-$^{18}$OH, was quantified after addition of a $[^{13}$C$_2]$Ac-Lys-OH internal standard (0.75 $\mu$g) by stable isotope dilution tandem mass spectrometry, as described below. All reactions were carried out in triplicate.

**Measurement of the steady-state kinetic parameters for the carboxyl oxygen exchange reaction**

The steady-state kinetic parameters for the carboxyl oxygen exchange reaction by Lys-C and trypsin were obtained at two different pHs (pH 9.0 and 5.0 for Lys-C and pH 8.0 and 6.0 for trypsin). Ac-Lys-OH (0.0625 – 10 mM) was incubated with 1 $\mu$M Lys-C or trypsin at 30 °C in 95% H$_2^{18}$O buffered at various pHs (described above). The total volume of the reaction was 20 $\mu$L. The duration of the reaction was 10 min for Lys-C and 20 min for trypsin. The single $^{18}$O atom incorporated reaction product, Ac-Lys-$^{18}$OH, was quantified after addition of a $[^{13}$C$_2]$Ac-Lys-OH internal standard (0.75 $\mu$g) by stable isotope dilution tandem mass spectrometry, as described below. All reactions were carried out in triplicate. Km and Vmax values were calculated by fitting the Michaelis-Menten equation to the experimental data using SigmaPlot 9.01 (Systat Software, Richmond, California). $k_{cat}$ values were calculated by dividing the obtained Vmax values by the total enzyme amount added to the system.
Quantification of Ac-Lys-\(^{18}\)OH by stable isotope dilution tandem mass spectrometry

An Agilent 1100 HPLC system (Palo Alto, California) coupled to an API 3000 triple quadrupole mass spectrometer equipped with a TurboIonSpray™ ion source (Applied Biosystems-MDS-Sciex, Foster City, CA) was used to quantify Ac-Lys-\(^{18}\)OH. The reconstituted reaction mixtures were injected (typically 5 \(\mu\)L) onto a reversed-phase C18 column (2.1 \(\times\) 150 mm, 5 \(\mu\)m, 300 Å, Vydac) equilibrated with 5% acetonitrile/0.1% HFBA (v/v) in water and then eluted with the same solvent at a flow rate of 200 \(\mu\)L/min. A switching valve (Valco Instrument, Houston, TX) was placed between the HPLC system and the mass spectrometer to divert non-volatile salt fractions from the mass spectrometer at the beginning of the run. The switching valve was switched in-line with the mass spectrometer at 3 min after the injection. The elution time of Ac-Lys-\(^{18}\)OH was 3.8 min.

The ion spray voltage and the ion source probe temperature were set at 5 kV and 400 \(^{\circ}\)C, respectively. Nitrogen was used both as a nebulizer and as an auxiliary gas, at flow rates of 3 and 8 L/min, respectively. A multiple reaction monitoring mode was used for monitoring Ac-Lys-\(^{18}\)OH and [\(^{13}\)C\(_2\)]Ac-Lys-OH. The precursor and fragment ions monitored were m/z 191 (Ac-Lys-\(^{18}\)OH + H) and 149 (H-Lys-\(^{18}\)OH + H) for Ac-Lys-\(^{18}\)OH and m/z 191 ([\(^{13}\)C\(_2\)]Ac-Lys-OH + H) and 147 (H-Lys-\(^{16}\)OH + H) for [\(^{13}\)C\(_2\)]Ac-Lys-OH, respectively. The collision energy was 20 eV for both the analyte and internal standard. Nitrogen was used as the collision gas.
Amounts of Ac-Lys-\(^{18}\text{O}\) in the reaction mixture were determined from the chromatographic peak area ratios of the analyte over the internal standard, assuming identical ionization efficiencies and identical yields of fragment ions for Ac-Lys-\(^{18}\text{O}\) and \([^{13}\text{C}_2]\text{Ac-Lys-OH}\). The experimentally determined amounts of Ac-Lys-\(^{18}\text{O}\) were corrected upward 5\%, accounting for \(^{16}\text{O}\) incorporation resulting from the \(\text{H}_2^{18}\text{O}\) solvent having a 5\% \(\text{H}_2^{16}\text{O}\) content.\(^8\) The assay method showed good linearity over the concentration range of 1.95 – 1,000 \(\mu\text{M}\) (\(r^2>0.999\)). A S/N=20 was obtained for 1.95 \(\mu\text{M}\) Ac-Lys-OH.

Measurement of the carboxyl oxygen exchange reaction for Lys-C and tryptic peptides of apomyoglobin

Apomyoglobin (34 \(\mu\text{g}\)) was digested for 16 h at 25 °C with either 1\(\mu\text{g}\) of Lys-C or trypsin in 100 mM ammonium bicarbonate in \(\text{H}_2^{16}\text{O}\) solvent. The digestion was stopped by adding formic acid to a final concentration of 1% and by boiling the reaction mixture for 2 min. The resulting Lys-C and tryptic peptides were lyophilized and used in the following experiments.

The rate of the carboxyl oxygen exchange reaction into the Lys-C and tryptic peptides by Lys-C and trypsin, respectively, were semi-quantitatively measured by looking at the rate of \(^{18}\text{O}\) atom incorporation into the carboxyl termini of the peptides by LC/MS (described below). The Lys-C and tryptic digestes (both 1.5 \(\mu\text{g}\)) were incubated with Lys-C and trypsin (both 0.03 \(\mu\text{g}\)), respectively, in \(\text{H}_2^{18}\text{O}\) at 30°C buffered (see above) at pH 5.0 or 9.0 for Lys-C and pH 6.0 or 8.0 for trypsin for various reaction times (1 min-60 min).
The total reaction volume was 50 μL. Aliquots (5 μL) of the reaction mixtures were taken after 1, 5, 10, and 60 min. The reactions were stopped by mixing with 40 μL of 10% formic acid and by heating at 95 °C for 2 min. After the heat treatment, the solvents were evaporated in the speed-vac concentrator and the samples were stored at -20 °C until use. The stored ¹⁸O labeled peptide mixtures were reconstituted in 20 μl of aqueous 0.1% trifluoroacetic acid (TFA), desalted by a C18 ZipTip as per the manufacturer’s instructions (Millipore, Bedford, MA), and subjected to LC/MS analysis using an UltiMate nano HPLC system (Dionex, San Francisco, CA) interfaced to a QStar quadrupole/time of flight mass spectrometer (Applied Biosystems-MDS Sciex, Foster City, CA), as described previously.² Briefly, the peptide mixture (5 μl) was injected directly into a reverse-phase analytical column (0.075 × 60 mm, New Objective Inc., Woburn, MA) packed with Jupiter C18 media (Phenomenex, Torrance, CA), washed with 2% acetonitrile/0.1% formic acid (v/v) in water for 15 min at flow rate of 2 μL/min. The peptides were then eluted with a 40 min linear gradient of 2% acetonitrile/0.1% formic acid (v/v) in water to 40% acetonitrile/0.1% formic acid (v/v) in water at a flow rate of 200 nL/min. The column effluent was directed on-line to a nano-electrospray ion source. The total ion current was obtained in the mass range of m/z 400-1600 in the positive ion mode. The electrospray and orifice voltages were 2050V and 65V, respectively.
RESULTS AND DISCUSSION

pH dependency of the carboxyl oxygen exchange reaction for Ac-Lys-OH

The pH dependency of the carboxyl oxygen exchange reaction measured by the incorporation of an $^{18}$O atom into Ac-Lys-OH catalyzed by Lys-C and trypsin is shown in Figure 2.4 a) and b), respectively. The pH dependency of their amidase activities obtained for the hydrolysis of Ac-Lys-NH$_2$ are also shown in the figure. The optimum pHs of the carboxyl oxygen exchange reaction catalyzed by Lys-C and trypsin were found to be pH 5.0 and 6.0, respectively, which were 3 - 4 pH units lower than the most favorable pHs of their amidase activities (pH 9) within the pH range examined.

Steady-state kinetic parameters for the carboxyl oxygen exchange reaction

To better understand the catalytic mechanism of carboxyl oxygen exchange reaction catalyzed by Lys-C and trypsin, we obtained steady-state kinetic parameters for the carboxyl oxygen exchange reaction of Ac-Lys-OH in H$_2^{18}$O solvent, assuming negligible kinetic isotope effect of H$_2^{18}$O solvent. The reactions were performed at the acidic and alkaline pH optimums of the carboxyl oxygen exchange and amidase reactions, respectively. Figure 2.5 a) shows the effects of substrate concentration on the initial velocity of the carboxyl oxygen exchange reaction catalyzed by Lys-C at pH 5.0 and 9.0. Similarly, Figure 2.5 b) shows the effects of substrate concentration on the initial velocity of the carboxyl oxygen exchange reaction catalyzed by trypsin at pH 6.0 and 8.0. From the plots in Figure 2.5 a) and b), it is noticeable that the $K_m$ values decrease and the $V_{max}$ values increase with a decrease in pH. Note that the high pH trypsin catalyzed reaction
was done at pH 8.0, because the rate of the carboxyl oxygen exchange was too low at pH 9.0 (most favorable pH for the amidase activity in the pHs examined) to accurately measure the kinetic parameters.

Table 2.1 summarizes the $K_m$ and $k_{cat}$ values calculated by fitting the Michaelis-Menten equation to the experimental data for the two enzymes at acidic and alkaline pHs. For Lys-C at pH 5.0, the $K_m$ was about 2-fold smaller, the $k_{cat}$ was about 1.5-fold higher, and the $k_{cat}/K_m$ was about 2.5-fold higher than the $K_m$, $k_{cat}$, and $k_{cat}/K_m$, respectively, at pH 9.0. For trypsin at pH 6.0, the $K_m$ was about 3-fold smaller, the $k_{cat}$ was about 5-fold higher, and the $k_{cat}/K_m$ was about 16-fold higher than the $K_m$, $k_{cat}$, and $k_{cat}/K_m$, respectively, at pH 8.0. These results show that Lys-C and trypsin have higher substrate binding affinities and higher catalytic rates at the acidic pHs. The higher substrate binding affinities of Lys-C and trypsin to the substrate for carboxyl oxygen exchange reaction at the acidic pHs are consistent with the result obtained for chymotrypsin by Doherty and Vaslow [49], therefore, this enzymatic property may be a common feature of serine proteases.

The experimentally determined $k_{cat}/K_m$ value for carboxyl oxygen exchange reaction by Lys-C at pH 5.0 was 1.42 mM$^{-1}$•s$^{-1}$ (Table 2.1), which is only 3.4-fold lower than the reported $k_{cat}/K_m$ value for amide hydrolysis of $N^\alpha$-benzoyl-L-lysine amide at pH 8.5 (4.81 mM$^{-1}$•s$^{-1}$)[53], suggesting that the carboxyl oxygen exchange catalytic activity of Lys-C at the acidic pH is almost equivalent with its amidase activity at alkaline pH. Similarly, for trypsin at pH 6.0, the experimentally determined $k_{cat}/K_m$ value for
The higher value of $k_{cat}/K_m$ for Lys-C at pH 5.0 compared to the value at pH 9.0 is due mainly to the effect of the decreased value of the $K_m$ component on the ratio, while both the $K_m$ and $k_{cat}$ significantly contribute to the higher $k_{cat}/K_m$ of trypsin at the acidic pH. The results suggest that Lys-C catalyzes the reaction more efficiently than trypsin at alkaline pH by as yet unknown mechanism.

It was also found that $k_{cat}/K_m$ of Lys-C at pH 5.0 was about 17-fold higher than that of trypsin at pH 6.0. This is attributed to the approximate 2-fold lower $K_m$ and 10-fold higher $k_{cat}$ of Lys-C for Ac-Lys-OH. This indicates that Lys-C has higher carboxyl oxygen exchange activity than trypsin.

**Rate of the carboxyl oxygen exchange reaction for peptide substrates**

We semi-quantitatively measured the rate of $^{18}$O atom incorporation into Lys-C and tryptic peptides derived from apomyoglobin. This was done to test whether the increased rate of carboxyl oxygen exchange noted for the reaction of Ac-Lys-OH at acidic pH could be generalized to peptide substrates. Lys-C and tryptic digest of apomyoglobin prepared in H$_2$ $^{16}$O solvent were incubated with Lys-C and trypsin in H$_2$ $^{18}$O solvent at the favorable pHs of the carboxyl oxygen exchange and amidase reactions for various
reaction times. The resulting $^{18}$O labeled peptides were analyzed by LC/MS. Figure 2.6 shows the mass spectra of a representative $^{18}$O-labelled apomyoglobin peptide that has a lysine residue at the C-terminus (Lys-peptide). The Lys-C results clearly show that there was complete incorporation of two $^{18}$O atoms (m/z 502.9) within 1 min of incubation at pH 5.0 (Fig. 2.6a), while the dominant result at pH 9.0 was the incorporation of only one $^{18}$O atom (m/z 502.3) (Fig. 2.6b). In the case of trypsin, complete incorporation of two $^{18}$O atoms was achieved within 10 min of incubation at pH 6.0 (Fig. 2.6c), while the dominant result at pH 8.0 was the incorporation of only one $^{18}$O atom, and complete incorporation of two $^{18}$O atoms was not achieved even after 60 min of incubation (Fig. 2.6d). All the other Lys-peptides showed similar results (data not shown). Note that a small fraction of one $^{18}$O atom incorporated peak (m/z 502.3) exists even after achieving complete incorporation of two $^{18}$O atoms because of the purity of H$_2$ $^{18}$O solvent (95% H$_2$ $^{18}$O and 5% H$_2$ $^{16}$O) used in the experiments.

These results demonstrate that the enhanced rate of carboxyl oxygen exchange reaction by Lys-C and trypsin at acidic pH for Ac-Lys-OH can be generalized to peptide substrates. They also show that the carboxyl oxygen exchange activity of Lys-C for peptide substrates is significantly higher than that of trypsin, consistent with the results obtained in the Ac-Lys-OH experiments.

Figure 2.7 shows the mass spectra of a representative $^{18}$O-labelled peptide that has an arginine residue at the C-terminus (Arg-peptide), which was also labeled by trypsin as described above. Complete incorporation of two $^{18}$O atoms (m/z 537.6) was achieved
within 10 min of incubation at pH 6.0 (Fig. 2.7a), while it took 60 min at pH 8.0 (Fig. 2.7b), showing that the higher carboxyl oxygen exchange rate at the acidic pH found for Lys-peptides can be expanded to Arg-peptides.

It is noticeable that the rate of $^{18}$O atoms incorporation into the Arg-peptide by trypsin is obviously faster than the rate into the Lys-peptide (Fig. 2.7a and b for Arg-peptide and Fig. 2.6c and d for Lys-peptide). After 1 min of incubation, dominant peak is two $^{18}$O atoms (m/z 537.6) incorporated peak at both pH 6.0 and 8.0 for the Arg-peptide (Fig. 2.7a and b), while the dominant result for Lys-peptide was the incorporation of only one $^{18}$O atom (m/z 502.3) (Fig. 2.6c and d). All the other Arg-peptides derived from apomyoglobin showed faster $^{18}$O atom incorporation rates than Lys-peptides (data not shown). The faster oxygen exchange reaction by trypsin for Arg-peptide has also been demonstrated by Yao and coworkers for peptide substrates, YGGFMK and YGGFMR, and for tryptic peptides derived from apomyoglobin [44].

CONCLUDING REMARKS

Our study demonstrated that the rate of carboxyl oxygen exchange reaction by Lys-C and trypsin can be accelerated significantly by carrying out the labeling reaction at the acidic pH conditions used in the present work. The catalytic activities ($k_{cat}/K_m$) of Lys-C and trypsin at the acidic pHs were 2.5-fold and 17-fold higher than those at the alkaline pHs, respectively. This finding is significant towards improving the accuracy of $^{18}$O labeling in quantitative proteomic experiments; by identifying the conditions that lead to the complete incorporation of two $^{18}$O atoms into peptides. We recommend carrying out $^{18}$O
labeling by Lys-C and trypsin under the acidic pH conditions specified in the present work. This study strongly supports the use of the separate experiments for digestion of proteins and $^{18}$O labeling of the generated peptides [44], each can be optimized separately (the digestion at pH 8, and the labeling at pH 5-6). Endoproteinase Glu-C and chymotrypsin also have been shown to be effective catalysts to incorporate two $^{18}$O atoms into peptides [55]. Based on the studies obtained with Lys-C and trypsin, the optimum pHs of the carboxyl oxygen exchange reaction catalyzed by these two proteases is assumed to be acidic. However, the optimum pHs need to be confirmed.

It was found that the activity of carboxyl oxygen exchange reaction of Lys-C ($k_{\text{cat}}/K_m$) at pH 5.0 was 17-fold higher than that of trypsin at pH 6.0 when Ac-Lys-OH was used as a substrate. The amidase activities of Lys-C for $N^\alpha$-benzoyl-L-lysine amide and $N^\alpha$-benzoyl-lysine-$p$-nitroanilide have been reported to be an order of magnitude higher than that of trypsin [53, 56]. Thus, it is apparent that the use of Lys-C as a catalyst in $^{18}$O labeling experiment has an advantage over trypsin in terms of efficiency of $^{18}$O labeling in both the first and the second $^{18}$O atom incorporation (the reaction 1 and 2, respectively, in Introduction). However, Lys-C produces peptides that are larger as compared with peptides produced by trypsin, therefore a high resolution mass spectrometer may be required for the analysis of the peptides because of the tendency of larger peptides to produce higher charge states than smaller peptides when electrospray is used.

A number of kinetic studies of serine proteases have shown that the unprotonated state of the active site histidine residue is required to express the amide hydrolysis catalytic
activity[57, 58]. This unprotonated histidine state promotes the nucleophilic attack by an active site serine hydroxyl group on the carbonyl carbon of the substrate, leading to amide bond hydrolysis. The p\(\text{Ka}\) of the active site histidine residues of Lys-C and trypsin have been reported to be 6.0 and 6.6 [59, 60], respectively. Therefore, a significant portion of the active site histidine residues of both proteases are expected to be in the protonated state at the optimum pHs of the carboxyl oxygen exchange reaction (pH 5.0 for Lys-C and pH 6.0 for trypsin). Thus, a different mechanism is implied for the carboxyl oxygen exchange reaction at acidic pH compared to the amide bond hydrolysis reaction. Further work is necessary to elucidate the detailed catalytic mechanism of carboxyl oxygen exchange reaction.

ACKNOWLEDGMENTS

The authors thank Drs. David J. Anderson (Cleveland State University, Cleveland, OH, USA) and Takashi Nakazawa (Nara Women’s University, Nara, Japan) for helpful discussions and editorial advice. This work was supported in part by NIH grant number RO3 EY014020 (to M. Miyagi) from the National Eye Institute, P20 RR016741 from the INBRE program of the National Center for Research Resources, and P20RR017699 from the COBRE program of the National Center for Research Resources.
Figure 2.4 - Effects of pH on the Initial Velocities of the Carboxyl Oxygen Exchange and Amidase Activities of Lys-C and trypsin.

For carboxyl oxygen exchange activity, 10 mM solution of Ac-Lys-OH in aqueous buffers at different pHs was incubated with 1 μM Lys-C or trypsin at 25 °C in 95% H₂¹⁸O buffered at various pHs. For amidase activity, Ac-Lys-NH₂ (10 mM) was incubated with 0.2 μM Lys-C or 1 μM trypsin at 25 °C in 95% H₂¹⁸O at various pHs. The single ¹⁸O atom incorporated reaction product, Ac-Lys-¹⁸OH, was quantified by stable isotope dilution tandem mass spectrometry as described in Materials and Methods. (a) pH dependency of the carboxyl oxygen exchange and amidase activities of Lys-C. (b) pH dependency of the carboxyl oxygen exchange and amidase activities of trypsin. -○-: carboxyl oxygen exchange activity, -●-: amidase activity. All reactions were carried out in triplicate.
Figure 2.5 - Effects of substrate concentration on the initial velocity of the carboxyl oxygen exchange reaction.

Various concentrations of Ac-Lys-OH were incubated with Lys-C or trypsin, both 1 μM, in 95% H2 18O solvent at 30 °C at pH 5.0 and 9.0 for Lys-C reaction and at pH 6.0 and 8.0 for trypsin reaction. The single 18O atom incorporated reaction product, Ac-Lys-18OH, was quantified by stable isotope dilution tandem mass spectrometry as described in Materials and Methods. (a) Effect of the substrate concentration of the carboxyl oxygen exchange reaction catalyzed by Lys-C. (b) Effect of the substrate concentration on the carboxyl oxygen exchange reaction catalyzed by trypsin. -○-: acidic pH, -●-: alkaline pH. All reactions were carried out in triplicate.
Table 2.1 - Kinetic parameters for carboxyl oxygen exchange reaction

Average values calculated from 3 independent experiments are shown ± standard deviations.

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<td>1.48 (± 0.21)</td>
<td>0.123 (± 0.007)</td>
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<td>4.89 (± 0.91)</td>
<td>0.025 (± 0.003)</td>
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$^a$ Numbers in the parentheses are standard deviations of the mean values.
Figure 2.6 - Mass Spectra of a Representative $^{18}$O labeled Lys-peptide Catalyzed by Lys-C and Trypsin.

Apomyoglobin Lys-C and trypsic digest (both 1.5 µg) prepared in H$_2^{16}$O solvent were incubated with Lys-C and trypsin (both 0.03 µg), respectively, in 95% H$_2^{18}$O solvent at 30°C for various reaction times (1 min-60 min). After the reaction, the resulting $^{18}$O labeled peptides were analyzed by LC/MS as described in Materials and Methods. (a) and
(b): (M + 3H)$^{3+}$ ions of peptide HPGDFGADAGMAMTK labeled with $^{18}$O by Lys-C at pH 5.0 and 9.0, respectively, for different reaction times. (c) and (d): (M + 3H)$^{3+}$ ions of the same peptide labeled with $^{18}$O by trypsin at pH 6.0 and 8.0, respectively, for different reaction times. m/z 501.6 is a peptide peak incorporated no $^{18}$O atom, m/z 502.3 is a peptide peak incorporated only one $^{18}$O atom, and m/z 502.9 is a peptide peak incorporated two $^{18}$O atoms.
Figure 2.7- Mass spectra of a representative $^{18}$O labeled Arg-peptide catalyzed by trypsin. Apomyoglobin tryptic digest (1.5 μg) prepared in H$_2^{16}$O solvent were incubated with trypsin (0.03 μg) in 95% H$_2^{18}$O solvent at 30°C for various reaction times (1 min-60 min). After the reaction, the resulting $^{18}$O labeled peptides were analyzed by LC/MS as described in Materials and Methods. (a) and (b): (M + 3H)$^3+$ ions of peptide
VEADIAGHQEVLIR labeled with $^{18}\text{O}$ by trypsin at pH 6.0 and 8.0, respectively, for different reaction times. m/z 536.3 is a peptide peak incorporated no $^{18}\text{O}$ atom, m/z 536.9 is a peptide peak incorporated only one $^{18}\text{O}$ atom, and m/z 537.6 is a peptide peak incorporated two $^{18}\text{O}$ atoms.
CHAPTER 3 – PROTEOMIC CHANGES IN THE PHOTORECEPTOR OUTER SEGMENT UPON INTENSE LIGHT EXPOSURE

This chapter will summarize information about developing an efficient $^{18}$O labeling method and $^{18}$O labeling workflow suitable for analyzing photoreceptor OS. This workflow was then applied to identify proteome differences between unexposed and 8h light exposed OS.

Validation of $^{18}$O Labeling using Bovine OS Proteins

Experiments from the previous section suggested that it is advantageous to carry out protease digestion at a basic pH and $^{18}$O labeling at an acidic pH in order to achieve complete two $^{18}$O atom incorporation into the C-terminal carboxyl groups of peptides. To test this hypothesis, we designed a series of validation experiments using bovine serum albumin (BSA) as a model protein and bovine OS.

MATERIALS AND METHODS

Materials

BSA was purchased from Sigma (St. Luis, MO), bovine retinas were from JA&WL Lawson Company (Lincoln, NE). Oxygen-18 enriched water (95%) was obtained from Isotec (Miamisburgh, OH). Sequencing grade modified porcine trypsin was purchased from Promega (Madison, WI). Acid cleavable detergent Rapigest was acquired from
Waters (Milford, MA). All other materials used for this experiment were either reagent grade or were of the highest quality that was commercially available.

Digestion and Labeling of Bovine Serum Albumin

Figure 3.1 shows a simplified workflow for this experiment. BSA (300 µg) in 450 µL of 100 mM ammonium bicarbonate was reduced by 10mM dithiothreitol (DTT) and alkylated by 25 mM iodoacetamide (IA). Modified porcine trypsin (9 µg) was added and samples were incubated overnight. Six equal aliquots of the digest containing 40 µg of BSA (60 µL) were completely dried using a speed vac. Three aliquots were re-dissolved in 50 µL of citrate buffer (pH6) made with 95% 18O water, the other three aliquots, in 50 µL citrate buffer (pH6) made with 16O water. Modified porcine trypsin was added (1 µg) to each aliquot and samples were labeled overnight. To prevent protease catalyzed back exchange of oxygen atoms upon mixing, trypsin has to be deactivated. The most efficient way of trypsin deactivation is reducing the three disulfide bonds in trypsin and alkylating the generated cysteine residues, which irreversibly inactivate trypsin. Trypsin was denatured in 4M guanidine hydrochloride (GuHCl) (adding 50µL of 8M GuHCl in either water) and the pH of the solution was raised to about 8 (adding 1µL of 3M Tris HCl buffer of pH 8.8, checking pH of the solution with pH paper to ensure that it was raised to 8.0). Trypsin was then reduced (1mM DTT in acetonitrile) and alkylated (2.5mM IA in acetonitrile). Samples labeled with 18O and samples labeled with 16O were mixed in different ratios based on protein amount (0.4:1, 1:1, 2:1). All the samples were then desalted by C18 column (The Nest Group, Inc., Southborough, MA), dried by speed vac,
and stored at -20 °C until analyzed. To ensure that digestion and labeling were successful, an aliquot of 16O labeled sample and an aliquot of 18O sample were also analyzed separately.

**Preparation of Bovine Outer Segments**

OS were prepared from bovine retina as described previously [1] by sucrose gradient ultracentrifugation. All steps were carried out under dim red light illumination. The rhodopsin rich fraction, also known as Band I, was used in this study.

**Protein Extraction from Bovine Outer Segments**

In total, 9 aliquots of bovine OS prepared from the same retinal homogenate were used in this study. In all aliquots, proteins were precipitated by mixing photoreceptor OS with an equal volume of PBS containing 20 % sucrose followed by centrifugation at 10,000 g for 5 min using a refrigerated tabletop centrifuge (Kendro Laboratory Products, Newtown, CT) at 4 °C. Proteins were then dissolved in 2 % sodium dodecyl sulfate (SDS) in 50 mM ammonium bicarbonate. Proteins were reduced by 10 mM DTT and S-alkylated by 25 mM iodoacetamide. The reduced and S-alkylated proteins were precipitated by mixing them with an excess of ice cold acetone (1:6) and leaving for at least 2 h at -20°C. The supernatant was removed following centrifugation (~ 1000 g for 15 s) in a tabletop centrifuge and pellet was washed twice with ice cold acetone to remove excess SDS from the pellet. Protein precipitate was redissolved in either 0.2% SDS, 0.1 % Rapigest (w / v) (Waters Corporation, Milford, MA) [61, 62] or in 8M urea prepared in 50 mM ammonium bicarbonate buffer. The amount of reduced and S-carbamidomethylated
protein dissolved by each reagent was estimated using DC Protein assay kit (Bio-Rad, Hercules, CA).

**Digestion and Labeling of Bovine Outer Segments**

A similar workflow to the one described in the BSA experiment was employed. In total, 6 aliquots of bovine OS prepared from the same retinal homogenate were used in this study. Proteins were extracted from the bovine OS by 0.1% Rapigest as described above. The amount of reduced and S-carbamidomethylated protein dissolved in 0.1% Rapigest solution in each aliquot was estimated using DC Protein assay kit (Bio-Rad, Hercules, CA). Each aliquot contained approximately 30 μg of protein. All aliquots were digested separately in H$_2^{16}$O by trypsin (1:100 enzyme to substrate ratio) at 25 °C for 18 h. Following digestion, Rapigest was cleaved by adding formic acid (final concentration 0.1% (v/v)) to the digest and incubating at 40 °C for 2 h. The resulting turbid solution containing, tryptic peptides and cleaved Rapigest, was loaded onto a Vydac C18 column (The Nest Group, Inc., Southborough, MA) and desalted as per manufacturer’s directions. The sample was eluted from the column using 60% (v/v) acetonitrile containing 0.1% formic acid and dried on a speed-vac (Thermo Fisher Scientific, Waltham, MA). The dried elute was reconstituted with 100 mM citrate buffer (pH 6). The samples were dried by a speed-vac, and then reconstituted in either H$_2^{16}$O or H$_2^{18}$O. Trypsin was added to the solutions to incorporate either $^{16}$O or $^{18}$O into the carboxyl termini of the peptides from the solvent water. The reaction was carried out at 25 °C for 18 h at a trypsin to protein ratio of 1:25 (w/w). After the reaction, 8M GuHCl solution in either $^{16}$O or $^{18}$O water was added to the reaction mixture obtaining final concentration of 4M GuHCl, the pH of
the solutions was increased by addition of Tris base. Trypsin was inactivated by reduction with 1 mM DTT, followed by alkylation with 2.5 mM IA. The resulting $^{16}$O- and $^{18}$O-labeled peptide solutions were mixed together in equal proportions and desalted using a Vydac C18 reverse phase column. The elute was dried by a speed-vac, reconstituted with 0.1% formic acid, and analyzed by LC-MS/MS.

**LC-MS/MS analysis**

Prior to LC-MS/MS analysis, samples were dissolved in 0.1% formic acid (FA) and then injected into the nano-liquid chromatography (LC) system (Ultimate 3000, Dionex, Sunnyvale, CA) onto a trapping pre-column (C18, PepMap100, 300 μm ×5 mm, 5 μm particle size, 100 Å; Dionex, Germering, Germany) followed by a reversed phase column (C18, 75μm×150 mm, 3 μm, 100 Å; Dionex). Peptides were eluted using mobile phases A (0.1 % formic acid in water) and B (80 % acetonitrile, 0.1 % formic acid in water). Eluting gradient was 0.8% of increasing mobile phase B per minute. The LC was coupled to a high resolution mass spectrometer (LTQ-Orbitrap, Thermo-Fisher, Waltham, MA) equipped with a Packed-Tip nanospray ionization Probe (Thermo Eectron Corp., Bremen, Germany) and operated in a positive ion mode. The peptides were infused at a flow rate of 300 nL/min via the silica non-coated PicoTip emitter (FS360-20-10-C12, New Objective Inc., Woburn, MA) at a voltage of 2.4 kV. The capillary temperature was maintained at 200 °C. Full MS spectra were recorded in the Orbitrap, and the tandem mass spectra of the 5 most intense ions were recorded by the LTQ ion trap at normalized collision energy of 35 eV, isolation width 2.5 Da and activation Q at 0.250 and an injection time of 300 ms.
Peptide and Protein Identification

Mass spectrometry data was acquired using Xcalibur software (Thermo Electron Corp.) and submitted for database search to the Mascot server using a client application, Mascot Daemon version 2.2.0 (Matrix Science, London, UK). Peptides were identified by comparing all of the experimental product ion spectra of the peptides to the Swiss-Prot v56 (392667 sequences; 141217034 residues) Mammalia database using the Mascot database search engine (version 2.2, Matrix Science, London, UK). S-carbamidomethylation of cysteine was set as a fixed modification. Oxidation of methionine (methionine sulfoxide), one or two C-terminal $^{18}$O labels, and acetylation at the protein N-terminus were set as variable modifications. Mass tolerance for protein identification precursor ion was set at 20 ppm and for product ion was set to 1 Da.

Peptide and Protein Quantification

Custom built software Relative Quantification O18.1.3.1 (developed by our collaborators Quanhu Sheng and Haixu Tang, PhD., University of Indiana, Bloomington, IN) was used to calculate $^{18}$O/$^{16}$O labeled peptide ratios. Mascot search result file from each LC-MS/MS sample in peptide format was converted to plain text file and served as input data. Proteins identified by at least 2 peptides with ion score of 20 were considered for quantification. This software uses algorithms previously described by Eckel-Passow et al. [63] The same software was utilized to calculate overall peptide ratios by linear regression from all individual peptide ratios.
RESULTS AND DISCUSSION

Digestion and labeling of Bovine Serum Albumin

This experiment was designed to investigate whether separating digestion (pH 8) and labeling (pH 5) can improve $^{18}$O atom incorporation into the C-terminus carboxyl groups in peptides derived from BSA and to test the accuracy of the $^{18}$O labeling method. We first analyzed $^{18}$O and $^{16}$O labeled digests prior to mixing to ensure that digestion and labeling worked properly. We observed 4 Da mass shift in LC-MS spectrum for most peptides in the $^{18}$O labeled sample, suggesting complete carboxyl oxygen exchange of both oxygens in the C-terminus (Figure 3.2). We then went on to test the accuracy of our method, therefore the $^{18}$O and $^{16}$O digests were mixed in different ratios ($^{16}$O:$^{18}$O ratios - 1:0.4, 1:1, 1:1.5 and 1:2). Peptide ratios were calculated from their mass spectra. Results showed that the calculated $^{18}$O/$^{16}$O BSA peptide ratios were very similar to the theoretical ratios for most peptides. Figure 3.3 shows representative mass spectra of BSA peptide (YICDNQDTISSK) from different samples. The experimentally determined ratios (1:0.38, 1:1.13, 1:2.18) were very close to the theoretical $^{16}$O:$^{18}$O ratios (1:0.4, 1:1, 1:2).

The experimentally determined ratios were then plotted against the theoretical ratios (Figure 3.4) The equation of the curve for this particular peptide was $y = 1.101x + 0.005$ and gave $R^2 = 0.998$. Thus, the data demonstrates that this method can provide accurate ratios.
Protein Extraction from Bovine Outer Segments

In order to establish a highly efficient protein extraction procedure for the OS proteins, three different methods of extraction were tested. In the first method SDS was used to extract proteins. SDS is a very powerful detergent and is known to dissolve membrane proteins well. However, SDS is not compatible with mass spectrometry therefore it has to be removed from the sample prior to analysis. In the second extraction method an acid cleavable detergent Rapigest™ (Waters, Milford, MA) was used. The hydrophobic and hydrophilic part of this detergent can be removed prior to analysis thus making it compatible with mass spectroscopy. 8M urea also was used in the extraction method for comparison because this has been the most commonly used agent to solubilize proteins in shotgun proteomics. Urea can be removed by desalting prior to mass spectrometry analysis. The results summarized in Figure 3.5 show that the protein extraction efficiency of Rapigest is comparable to SDS, while 8M urea is not effective in solubilizing the OS proteins.

Digestion and Labeling of Bovine Outer Segments

This experiment was designed to test the developed protein extraction and labeling method on biological sample which is similar to rat OS. The theoretical ratio of all proteins in the analyzed sample was 1:1. Individual peptide ratios assigned to OS proteins were approximately 1 (Table 3.1). The overall calculated ratios of all peptides were 1.01, 1.15, and 0.94 in the three independent labeling experiments (Figure 3.6). This result proved that our extraction and labeling method was successfully validated on biological sample mimicking the rat OS.
CONCLUDING REMARKS

This section focused on validating our labeling method and developing a suitable workflow for analyzing rat OS. The results demonstrate that this workflow can extract OS proteins efficiently and provide accurate quantification results.
Figure 3.1 – Workflow for BSA labeling experiment
Figure 3.2 – Mass Spectra of YICDNQDTISSK Peptide from BSA Digest

Sample spectrum of YICDNQDTISSK peptide from $^{16}$O (m/z=722.3, z=2 ) (a) and $^{18}$O labeled sample (m/z=724.3, z=2) (b). The mass difference of 4 Daltons is observed in sample labeled by $^{18}$O under our experimental conditions, proving complete labeling of both of the c-terminal carboxyl oxygens.
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Table 3.1– Example of Peptide Ratios from Bovine Outer Segment Labeling Experiment
Figure 3.3 – Mass Spectra of YICDNQDTISSK Peptide in Different Samples

LC-MS spectra of YICDNQDTISSK peptide ($^{16}$O labeled -m/z = 722.3, z = 2, $^{18}$O labeled m/z = 724.3, z = 2) from individual samples are shown above. Numbers on the left represent the ratio in which the samples were mixed. This is the theoretical $^{18}$O/$^{16}$O ratio of all peptides in the sample. Numbers on the right represent the experimentally determined $^{18}$O/$^{16}$O ratio of YICDNQDTISSK peptide from our software.
Figure 3.4 – Plot of Experimentally Determined versus Theoretical YICDNQDTISSK Peptide Ratios

Experimentally determined ratios were plotted against the theoretical ratios. Least square method was used to fit line through the data points. The equation of the line and its $R^2$ value are shown in left top corner of the graph. The slope of the fitted line is 1.101, this suggests that our experimentally determined ratios were very similar to the theoretical ratios for the YICDNQDTISSK peptide.
Figure 3.5 – Protein Extraction from Bovine Outer Segments

Protein amounts extracted with different agents are shown. The results are from experiments that were run in triplicate.
Figure 3.6 – $^{18}$O/$^{16}$O Ratio of All Peptides Present in Bovine OS Samples

The intensity of each $^{18}$O peptide is plotted against the intensity of its $^{16}$O peptide equivalent. Each point (blue diamond) represents an individual peptide ratio. The overall peptide ratio in the bovine OS sample is given by the slope (shown in left top corner) of the fitted line (red). $R^2$ value and F probability of the line fit are shown in the left top corner. The inset represents the enlargement of the area in the grey square at the origin. Three independent labeling experiments were carried out and each is represented by a plot.
Proteomic Changes in the Photoreceptor Outer Segment Upon Intense Light Exposure

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ABSTRACT

Acute light-induced photoreceptor degeneration has been studied in experimental animals as a model for photoreceptor cell loss in human retinal degenerative diseases. Light absorption by rhodopsin in rod photoreceptor outer segments (OS) induces oxidative stress and initiates apoptotic cell death. However, the molecular events that induce oxidative stress and initiate the apoptotic cascade remain poorly understood. To better understand the molecular mechanisms of light-induced photoreceptor cell death, we studied the proteomic changes in OS upon intense light exposure by using a proteolytic $^{18}$O labeling method. Of 171 proteins identified, the relative abundance of 98 proteins in light-exposed and unexposed OS was determined. The quantities of 11 proteins were found to differ by more than 2-fold between light-exposed OS and those remaining in darkness. Among the 11 proteins, 8 were phototransduction proteins and 7 of these were altered such that the efficiency of phototransduction would be reduced or quenched during light exposure. In contrast, the amount of OS rhodopsin kinase was reduced by 2-fold after light exposure, suggesting attenuation in the mechanism of quenching phototransduction. Liquid chromatography multiple reaction monitoring (LC-MRM) was performed to confirm this reduction in the quantity of rhodopsin kinase. As revealed by immunofluorescence microscopy, this reduction of rhodopsin kinase is not a result of protein translocation from the outer to the inner segment. Collectively, our findings suggest that the absolute quantity of rhodopsin kinase in rod photoreceptors is reduced upon light stimulation and that this reduction may be a contributing factor to light-induced photoreceptor cell death. This report provides new insights into the proteomic
changes in the OS upon intense light exposure and creates a foundation for understanding the mechanisms of light-induced photoreceptor cell death.

INTRODUCTION

Acute light-induced photoreceptor cell degeneration has been studied in experimental animals for over 40 years as a model for retinal cell loss arising from human retinal degenerative diseases [64]. A number of experiments have suggested that light-induced photoreceptor degeneration begins with absorption of light by rhodopsin in the photoreceptor outer segments (OS)[21, 23, 65, 66]. While this rhodopsin “bleaching” activates transducin as a part of normal vision, excessive activation may trigger photoreceptor degeneration [67]. In addition to extensive transducin activation, photo-oxidative stress is another potential mechanism to induce photoreceptor degeneration. Administration of antioxidants reduces the extent of rhodopsin mediated photo-oxidative stress and prevents photoreceptor cell death [68-72]. Although the light absorption step in photo-oxidative stress is reasonably well characterized, the molecular events that mediate or regulate the subsequent apoptotic cascade remain poorly understood.

Photoreceptor cells have evolved to maximally absorb visible light, which occurs in the OS, a photoreceptor cell sub-compartment. OS is filled with stacks of photosensitive membranes (disks) that contain the visual pigment rhodopsin and much of the phototransduction machinery embedded within a highly unsaturated phospholipid bilayer. In the dark adapted state, photoreceptor opsin apoprotein is bound with 11-cis-retinal to form rhodopsin. Rhodopsin comprises more than 80% of total OS membrane protein [1], and forms paracrystalline arrays in disk membranes of rodent photoreceptors [73].
Rhodopsin concentration can reach 4.6 mM in disk membranes [74], which may contribute to the retina’s susceptibility to light damage. The availability of proteins involved in quenching the phototransduction cascade [75], such as arrestin[28, 76] and rhodopsin kinase [28], has been shown to be important for protection from light-induced damage. Deactivation of rhodopsin, mediated by rhodopsin kinase and arrestin, is one of the rate limiting steps in the phototransduction cascade [77]. To understand how the photo-susceptibility of photoreceptors is modulated during and after induction of light damage, it is essential to quantitatively characterize these proteins involved in quenching phototransduction.

Light exposure causes certain proteins to translocate between the OS and other photoreceptor compartments [78]. Given the dynamic subcellular re-localization of these proteins, examination of retinal mRNA levels provides only limited information about their quantities in OS. While Western blot analysis and immunohistochemistry can be used to quantify OS proteins, these antibody-based techniques are low-throughput and require foreknowledge of the proteins being analyzed. Among the methods currently available, the proteomic approach is the only high-throughput method that does not require foreknowledge of proteins expressed in biological systems. However, proteomic analysis of OS proteins is challenging because many proteins involved in phototransduction are either integral membrane proteins or proteins modified by lipids. Since those membrane associated proteins are hard to solubilize, 2D-gel electrophoresis-based methods are not compatible with OS samples. Indeed, only a few proteomic studies examining this cell compartment have been reported so far [79-81], with none taking a quantitative approach.
To gain insight into the mechanisms that may contribute to light-induced photoreceptor degeneration, we compared OS proteins from light-exposed and unexposed rats with a proteolytic $^{18}$O labeling method [82]. Of 98 proteins that were quantitatively analyzed, 11 proteins showed significant changes upon light exposure. Among the significantly changed proteins, rhodopsin kinase was further analyzed by liquid chromatography multiple reaction monitoring (LC-MRM) and immunofluorescence microscopy.

MATERIALS AND METHODS

Materials
Oxygen-18 enriched water was obtained from Cambridge Isotope Laboratories (Andover, MA) or from Isotec (Miamisburg, OH). Sequencing grade modified porcine trypsin was purchased from Promega (Madison, WI). All other chemicals and materials were either reagent grade or were of the highest quality that was commercially available. All carbon-13 ($^{13}$C), nitrogen 15 ($^{15}$N) labeled peptides were synthesized by Sigma Genosys (The Woodlands, TX) or AnaSpec (San Jose, CA).

Light exposure and harvesting of rat retinal tissue
Male Sprague-Dawley weanling rats (Harlan Inc., Indianapolis, IN) were reared in a dim (20-40 lux) cyclic light environment (lights were on at 8 AM and off at 8 PM) for at least 40 days. The rats were fed standard rat chow (Teklad, Madison, WI) and given water *ad libitum*. After the 40 day cyclic light rearing period, randomly selected animals were assigned to control (4 rats x 4 replicates) or experimental (4 rats x 4 replicates) groups.
and were dark adapted for 16 h. After dark adaptation, the experimental group was exposed to bright green light (wavelength range 490-580 nm, light intensity 1200 Lux) for 8 h starting at 1 AM in animal treatment chambers (Figure 3.7 a-c) made from cylindrical green plexiglas (Cat # 2092, Dayton Plastic, Dayton, Ohio) [83]. This duration and intensity of light has been shown to damage photoreceptors such that it results in approximately 50% photoreceptor loss when measured 2 weeks later [31]. The control group remained in darkness for the same 8 h. The rats were terminated in a chamber with a CO2-saturated atmosphere (Figure 3.8) under dim red illumination immediately after the 8 h light or dark period. Retinas were excised under dim red illumination within two minutes of death and rinsed with phosphate buffered saline (PBS). Detailed description of retina dissection can be found in Figure 3.9. It should be noted that because the process of light-induced photoreceptor cell death occurs over several days to 2 weeks, photoreceptor cell loss immediately after light exposure is negligible [70].

Preparation of photoreceptor outer segments

Retinas from four rats were combined and used to prepare and isolate photoreceptor OS by sucrose density ultracentrifugation [83]. All solvents used for rat OS preparations contained protease inhibitors (1 mM EDTA, 0.2 mM PMSF, 0.7 μg/μl leupeptin, and 0.5 μg/μl pepstatin A) and 100 μM diethylenetriamine pentaacetic acid (DTPA) to inhibit protein degradation and oxidation, respectively. Cryotubes with rat retinas were taken out of liquid nitrogen storage tank and placed overnight to -20°C. The next day, retinas were completely thawed on ice. 8 retinas from same group were placed into glass tube
containing 1mL of ice cold 47% sucrose solution in phosphate buffer. Potter-Elvehjem homogenizer (Teflon pestle with glass tube and loose fit on the bottom - Figure 3.10) was used to gently grind retinas on ice. The pestle was turned for 15s clockwise rubbing against the bottom of the glass tube and then lifted up. Solution was allowed to drop to the bottom of the tube. This procedure was repeated one more time. Resulting homogenate was poured into filtering apparatus (Figure 3.11) which consisted of glass syringe equipped with two metal mesh filters (top mesh size 50, bottom mesh size 400). The filtrate was collected into a 5 mL plastic centrifuge tube (Beckmen, cat. no. 344057). The filtering apparatus was rinsed with 1mL of ice cold 47% sucrose solution which was collected into the same tube as the homogenate. Retinal homogenate was overlaid with the remaining ice cold sucrose solutions in phosphate buffer (42%, 37%, and 32%). Centrifuge tube was then placed into a prechilled swinging bucket for the Ti-55 rotor (Beckman). Swinging buckets were carefully balanced one against an other and placed on Ti-55 rotor. Extreme caution was taken not to disturb the individual sucrose layers when placing the rotor into Beckmen ultracentrifuge (S-class centrifuge, Beckmen-Coulter, Chino, CA). Samples were centrifuged at 35000 RPM for 1 h. After the spin cycle, OS band 1 and band 2 were collected (Figure 3.12) and stored at -80°C until use. Only band 1 fractions, which represent the purest OS preparation [84], were used in this study. To prevent possible light-induced protein migration in vitro, retinal dissections and OS preparations were done under dim red light.

An aliquot of OS sample not exposed to light was analyzed by electron microscopy by Hisashi Fujioka, PhD. (director of the Electron Microscopy Facility at Case Western Reserve University) to ensure intact disks were prepared. OS pellet was resuspended in
0.1% glutaraldehyde in a 0.1 M phosphate buffer, pH 5.7.4 for this purpose. OS were allowed to fix for 2 hours, washed and embedded in LR White resin (Polysciences, Inc.). Electron micrograph of unexposed OS preparation shows that intact disk membranes were isolated (Figure 3.13).

**Proteolytic \(^{18}\)O labeling**

The isolated OS material, which was stored in a 37% sucrose solution (300 μL), was mixed with an equal volume of PBS containing 20% sucrose, followed by centrifugation at 10,000 g for 5 min using a refrigerated tabletop centrifuge at 4°C. Workflow for proteolytic \(^{18}\)O labeling is shown in Figure 3.14. The precipitated OS were dissolved in 30 μL of 2% sodium dodecyl sulfate (SDS) in 50 mM ammonium bicarbonate, and the extracted proteins were reduced by 10 mM DTT and \(S\)-alkylated by 25 mM iodoacetamide [40]. The reduced and \(S\)-alkylated proteins were then precipitated by mixing with a 6-fold excess volume of ice-cold acetone and by incubation for at least 2 h at -20°C. The precipitated protein was then centrifuged at ~1,000 g for 15 s in a tabletop centrifuge and washed twice with ice-cold acetone to remove excess SDS. The protein pellet was redissolved in 0.1% acid cleavable surfactant, Rapigest [85] (w/ v) (Waters Corporation, Milford, MA), in 50 mM ammonium bicarbonate. The amount of protein dissolved in 0.1% Rapigest solution was determined by using the DC Protein assay kit (Bio-Rad, Hercules, CA). Each OS sample contained approximately 25 μg of protein.

The protein concentrations in control and light treated samples were adjusted with 0.1% Rapigest solution in 50 mM ammonium bicarbonate to be equal to one another before digestion. The light exposed and unexposed OS sample proteins were digested separately.
in H$_2^{16}$O by trypsin (1:100 substrate to protein ratio, w/w) at 25°C for 18 h. Following digestion, Rapigest was cleaved by adding formic acid (final concentration 0.1% (v/v)) and incubating at 40°C for 2 h. The resulting turbid solution containing tryptic peptides was loaded onto a Vydac C18 column (The Nest Group, Inc., Southborough, MA) and desalted according to the manufacturer’s directions. The peptides were eluted from the column using 60% (v/v) acetonitrile containing 0.1% formic acid and dried in a Speed-vac concentrator. The dried peptides from light-exposed and unexposed OS were dissolved in 100 mM citrate buffer pH 6 (50 μL) made with H$_2^{16}$O and H$_2^{18}$O, respectively. The peptides were then incubated with trypsin (1:25 enzyme to substrate ratio, w/w) at 25°C for 18 h to incorporate $^{16}$O and $^{18}$O, respectively, into the carboxyl termini of the peptides. This acidic pH condition has been demonstrated to provide higher $^{18}$O labeling efficiency compared to the typical alkaline pH condition (pH 8) [86]. After the reaction, solid guanidine HCl was added to a final concentration of 4 M, and the pH adjusted to approximately pH 8 by adding Tris base. Trypsin was then inactivated by reduction with 1 mM DTT at room temperature for 1 h, followed by alkylation with 2.5 mM iodoacetamide at room temperature for 30 min. The resulting $^{16}$O- and $^{18}$O-labeled peptide solutions were mixed together in equal proportions and desalted using a Vydac C18 reverse phase column. The eluate was dried in a Speed-vac, reconstituted with 0.1% formic acid, and analyzed by LC-MS/MS. In addition to this forward labeling experiment (light exposed OS labeled with $^{18}$O, unexposed OS labeled with $^{16}$O) we also carried out a reverse labeling experiment (unexposed OS labeled with $^{18}$O, light exposed OS labeled with $^{16}$O).
**LC-MS/MS analysis**

Chromatographic separation of the protein digest was performed by an Ultimate 3000 nano-HPLC (Dionex, Germering, Germany) with a trapping pre-column (C18, PepMap100, 300 μm × 5 mm, 5 μm, 100 Å; Dionex, Germering, Germany) followed by a reverse phase column (C18, 75 μm × 150 mm, 3 μm, 100 Å; Dionex). Peptides were injected onto the trapping column, which was equilibrated with 0.1% formic acid in water and washed for 5 min with the same solvent at a flow rate of 10 μL/min. After washing, the trapping column was switched in-line with the reverse-phase analytical column and bound peptides eluted using solvents A (0.1% formic acid in water) and B (0.04% formic acid in 80% acetonitrile, 20% water) with a linear gradient of 2% per min, starting with 100% of solvent A at a flow rate of 300 nL/min. The eluted peptides were introduced into a Finnigan linear ion trap Fourier transform (LTQ FT ICR) hybrid mass spectrometer (Thermo Electron Corp., Bremen, Germany) equipped with a 7 T superconducting electromagnet (biological replicates 1 and 2) or LTQ-Orbitrap (Thermo Electron Corp., Bremen, Germany) (biological replicates 3 and 4) mass spectrometer via a silica non-coated PicoTip emitter (FS360-20-10-C12, New Objective Inc., Woburn, MA) at a voltage of 2.2 kV. The capillary temperature was maintained at 200°C. Full MS spectra were recorded in the FT ICR cell or Orbitrap, and then the tandem mass spectra of the six most intense ions were recorded by the LTQ ion trap at a collision energy of 35 eV, isolation width 2.5 Da, and activation Q at 0.250.
Protein identification

Proteins were identified by comparing all of the experimental peptide MS/MS spectra to the Swiss-Prot (version 57) Rodentia database (25165 proteins) using Mascot database search software (version 2.1.04, Matrix Science, London, UK). S-carbamidomethylation of cysteine was set as a fixed modification while oxidation of methionine (methionine sulfoxide) and C-terminal $^{18}$O modification were variable modifications. The mass tolerance for the precursor ion was set to 10 ppm, and for the product ion it was set to 1 Da. Strict trypsin specificity was applied, allowing for one missed cleavage. Only peptides with a minimum score of 20 were considered significant. Scaffold software (Version Scaffold-2_06_00, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identification. Peptide identifications were accepted if they could be established at an ion score greater than 20, as specified by the Peptide Prophet algorithm [24]. Protein identifications were accepted if they could be established at greater than 95% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [87]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Calculation of $16$O/$18$O-peptide ratio

In-house software (Relative Quantification O18.1.2.2) employing a least squares regression algorithm [88] was used for the calculation of $^{16}$O/$^{18}$O peptide ratios. This software plots $^{16}$O/$^{18}$O-peptide intensities of all peptides identified from the same protein, and the slope of the linear regression fit is used as a $^{16}$O/$^{18}$O peptide ratio for that protein.
Only proteins with $R^2 \geq 0.85$ and a linear regression F-probability greater than 0.85 in at least 1 LC-MS/MS analysis are reported as quantified proteins. Proteins with $R^2$ values or F-probabilities out of our range were manually investigated for containing possible peptide outliers. An obvious outlier was defined as a peptide whose removal changed the protein $R^2$ value by more than 0.2 or increased the F-probability to >0.85. If an obvious outlier was detected, it was removed from the peptide list.

The slope of the linear regression fit from all the peptide ratios from all the proteins in the particular sample was also obtained after removing peptides from the following proteins, whose amounts are known to change upon light exposure: arrestin, transducin (Gt$\alpha$, Gt$\beta$ and Gt$\gamma$), and recoverin (Figure 3.15). The slope value was then used to normalize the individual protein ratios. This is expected to decrease the influence of experimental error (e.g. pipetting error during sample mixing) on the calculated ratios.

**LC-MRM**

Isotope dilution tandem mass spectrometry using liquid chromatography multiple reaction monitoring (LC-MRM) was used to verify the results of our proteomic study for rhodopsin and RK. Reference peptides incorporating stable isotopes at the C-terminal lysine residue were synthesized for rhodopsin (EAAQQQESATTQ$^{[13C_6,15N_2]}$K), and RK (GITVEEAAPTQ$^{[13C_6,15N_2]}$K). These tryptic peptides were chosen from among the peptides quantified in our proteomic study and did not contain any known modification sites. Known amounts of these reference peptides were introduced into the tryptic digest of light-exposed and unexposed OS proteins (25 $\mu$g) and were used to quantify native peptides derived from rhodopsin and RK. The digests containing known amounts of the
reference peptides were injected onto a C18 reverse phase column (Alltech Altima HP C18, 3um, 1 x 150 mm) equilibrated with 0.02% trifluoroacetic acid (TFA), and then peptides were eluted with a linear gradient of acetonitrile (2%/min) in the presence of 0.1% TFA at a flow rate of 100 μL/min. LC Packings Ultimate (Dionex, Germering, Germany) liquid chromatographic system was used for the chromatography. The peptides eluted from the column were directly introduced into a API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a Turbo IonSpray ion source and monitored by MRM. The following are the native and reference peptide sequences and the precursor/fragment ions monitored for rhodopsin: EAAAQQQESATTQK [m/z 745.8 (z = 2)/1020.2 (z = 1)] and EAAAQQQESATTQ[13C6,15N2]K [m/z 750.0 (z = 2)/1028.5 (z = 1), and for RK: GITVEEAAPTAK [m/z 593.8 (z = 2)/816.4 (z = 1)] and GITVEEAAPTA[13C6,15N2]K [m/z 597.8 (z = 2)/824.3 (z = 1)]. Chromatographic peak areas of the native and of the reference peptides were calculated using Analyst Software 1.4.1 (Applied Biosystems), and the area ratios of native/reference peptide were obtained. Then, the ratio for the light-exposed sample was divided by the ratio for the unexposed sample. Two OS preparations were used for the analysis.

Immunocytochemistry

After termination, eyes were removed from animals. Eyecups were prepared by carefully removing the cornea and lens. Eyes were dissected under light for light-exposed samples and under dim red light illumination for unexposed control samples. The eyecups were fixed with 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.4) for at least 6
hours. Then, the paraformaldehyde solution was exchanged in three concentration steps (5%, 10%, 15% sucrose) for 20% sucrose in 100 mM phosphate buffer (pH 7.4). Eyecups were then incubated overnight in a mixture of 20% sucrose phosphate buffer and Optimal Cutting Temperature (OCT) compound at a 2:1 ratio. Eyecups were frozen in isopentane cooled by liquid nitrogen and sectioned at 12 µm using a Leica CM 1850 Cryostat (Leica Microsystems Inc., Bannockburn, IL). For immunofluorescence, eyecup sections were first blocked for nonspecific labeling by incubating in 1.5% normal goat serum in PBST buffer (136 mM NaCl, 11.4 mM sodium phosphate, 0.1% Triton X-100, pH 7.4) for 15 min at room temperature. Sections were then incubated with antirhodopsin kinase antibody [89] in PBST overnight at 4°C. Sections were rinsed in PBST and incubated with indocarbocyanine (Cy3)-conjugated secondary antibodies. Sections were then rinsed in PBST and mounted in 50 µl of 2% 1,4-diazabicyclo-2,2,2-octane in 90% glycerol to slow photobleaching. Sections were analyzed under a Leica DM6000 B microscope (Leica Microsystems Inc.) equipped with a RETIGA EXi CCD camera (QImaging, Burnaby, BC, Canada).

RESULTS

Comparative proteomic study on intense light exposed OS

Proteins from four biological replicates of light-exposed and unexposed OS were subjected to proteolytic $^{18}$O labeling and analyzed by LC-MS/MS. All replicates were subjected to forward (F1-F4 experiment) and reverse (R1-R4 experiment) $^{18}$O labeling. A total of 171 proteins were identified of which 58% (100 proteins) were classified as
membrane proteins (Supplemental Table 1), demonstrating that the method we used was effective at identifying membrane proteins. Out of 171 proteins identified, 98 passed our statistical criteria ($R^2>0.85$ and F-probability$>0.85$) for quantification (Supplemental Table 2). Figure 3.15 shows the plots of the protein ratios obtained in the forward experiment against those for the same proteins in the reverse experiment (Fig. 3.16a: F1 vs. R1 experiment, Fig 3.16b: F2 vs. R2 experiment, Fig 3.16c: F3 vs. R3, and Fig 3.16d F4 vs R4). Linear regression analyses on the plots were performed and the regression line (bold line) as well as the confidence interval lines with $\alpha = 0.01$ (dotted lines) are shown in the figure. The theoretical line $y=1$ (represented by the diagonal of each panel) is within the confidence interval lines for all replicates, demonstrating that the results between the forward and reverse labeling experiment with the same biological sample were consistent.

The quantities of 11 proteins differed by at least 2-fold between light-exposed and unexposed OS samples in at least three biological replicates. Proteins altered in only one or two replicates were eliminated from consideration. Among the 11 proteins, 8 were proteins involved in phototransduction: arrestin, guanine nucleotide-binding protein Gt$\alpha$, Gt$\beta$ and Gt$\gamma$, recoverin, rhodopsin kinase, and rod cGMP-specific 3',5'-cyclic phosphodiesterase $\alpha$ and $\beta$ (Table 3.2). The quantities of several other phototransduction proteins were not altered by light exposure (Table 3.2). Those proteins were cGMP-gated cation channel $\alpha$, guanine nucleotide-binding protein $\beta$-5, guanylyl cyclase GC-E, regulator of G-protein signaling 9 and rhodopsin. Besides phototransduction proteins, we found three non-phototransduction proteins whose amounts increased more than 2-fold in
light-exposed samples (Table 3.3). They include ubiquitin, heat shock protein HSP 90-alpha and heat shock cognate 71 kDa protein.

A total of 8 glycolytic enzymes were quantified and found to be unchanged by light (Table 3.4). Major OS integral membrane proteins, such as rod outer segment membrane protein 1 (ROM1) and retinal-specific ATP-binding cassette transporter, were also found to be unchanged (Supplemental Table 2).

**LC-MRM analysis**

Rhodopsin kinase and rhodopsin were quantified by LC-MRM in two biological replicates to verify our proteomic results. Rhodopsin was selected to ensure that no significant photoreceptor degeneration was induced during light exposure. Rhodopsin kinase was selected because the decrease in protein amount upon light exposure did not appear to be a common adaptive change to light (see the Discussion), and therefore, was unique among the identified changes. The first LC-MRM verification experiment was carried out with the same sample (biological replicate 1) that was used for the proteomic study described above. In this sample, rhodopsin levels did not change upon light exposure, while the amount of rhodopsin kinase decreased 2-fold after light exposure (Table 3.5). In our proteomics study the light exposed/unexposed ratios of the rhodopsin peptide, EAAQQQESATTQK, and the rhodopsin kinase peptide, GITVEEAAAPTAK, between F1 and R1 was 1.26 and 0.42, respectively. Thus, the results were consistent with the proteomic results. In the second LC-MRM experiment, a new preparation of rat OS was analyzed. The light exposed/unexposed ratios of rhodopsin and rhodopsin kinase in the newly prepared OS were 0.75 and 0.26, respectively. Thus, the LC-MRM results
confirm that the amount of rhodopsin was not altered by 8 hr light exposure, while rhodopsin kinase in the OS decreased more than 2-fold.

**Immunocytochemistry**

We asked whether the reduction in the RK quantified by MS analysis was due to its translocation from OS to IS, or due to a reduction of the absolute level in photoreceptors. To discriminate between those possibilities, localization of RK was examined by immunofluorescence microscopy. Cryosections of eye cups were prepared from the rats exposed (Figure 3.17 a) and unexposed (Figure 3.17 b) to light, under identical conditions used for OS sample preparation. RK was localized exclusively in the rod and cone photoreceptor OS, regardless of light conditions. This indicates that the absolute quantity of RK decreased in the photoreceptors of light exposed rats.

**DISCUSSION**

Among the 8 phototransduction proteins found to be altered by light, 7 proteins reduce or quench phototransduction signaling upon intense light exposure, and therefore their changes appear to be adaptive changes to light. For instance, the amount of proteins involved in the activation steps of the phototransduction cascade (guanine nucleotide-binding protein Gtα, Gtβ and Gtγ, and rod cGMP-specific 3',5'-cyclic phosphodiesterase α and β) decreased 3.2- to 6.7-fold. In contrast, the level of arrestin, which is involved in quenching the signaling cascade, increased greater than 10-fold. The decrease in recoverin (5.6 fold) also can be considered adaptive, because it reduces the inhibitory constraint that recoverin imposes on rhodopsin kinase [90]. Proteins involved in the
activation and deactivation of phototransduction cascade are depicted in Figure 3.18 a and b, respectively.

One unusual protein was rhodopsin kinase, whose amount decreased 2-fold after light exposure. This result was verified by LC-MRM analysis on two OS preparations, which showed a 2.0- and 3.8-fold reduction of this protein after light exposure (Table 3.5). Rhodopsin kinase helps to quench the light-induced transduction signaling cascade by phosphorylating rhodopsin; therefore, an increased amount of this protein would be expected to deactivate the cascade. Indeed, mice lacking rhodopsin kinase are much more susceptible to photoreceptor cell damage from light compared to wild-type animals, which is due to prolonged activation of rhodopsin [28, 67]. Furthermore, hemizygous rhodopsin kinase knockout mice express ~ 2 fold less enzyme than wild type, and have a slower dark recovery rate to dim light flashes [28]. Therefore, it is reasonable to believe that a 2-fold reduction of rhodopsin kinase can provide a physiologically relevant change in the rod photoreponse. Our finding suggests that the loss of rhodopsin kinase might be a contributing factor to light-induced photoreceptor degeneration by prolonging the lifetime of activated rhodopsin.

Since the total rhodopsin kinase amount in rod OS appears to be reduced by light, we propose two alternate possibilities: 1) the degradation of rhodopsin kinase was accelerated upon light exposure, or 2) rhodopsin kinase is continuously degraded, and its synthesis was slowed by light exposure. Previous studies suggested that rhodopsin kinase does not change its distribution upon light exposure [90], a conclusion mainly drawn from Western blotting analyses. Although Western blotting is useful to study relative protein quantities among different subcellular compartments in the
photoreceptors, it does not quantify proteins at the same accuracy as LC-MRM. In contrast to the previous reports, our LC-MRM quantification clearly indicates that there is a mechanism to reduce rhodopsin kinase amount in OS upon light exposure, without changing the overall distribution in the photoreceptor cells. Among the eight phototransduction proteins whose amounts were altered by light, four proteins, guanine nucleotide-binding protein Gtα, Gtβ and Gtγ [91, 92], and recoverin [90] have been reported to translocate from OS to other compartments in photoreceptor cells upon light exposure, while arrestin is known to translocate in the opposite direction [92, 93]. In our proteomic study, the amounts of guanine nucleotide-binding protein Gtα, Gtβ and Gtγ, and recoverin were reduced 5.3-, 5.9-, 6.7-, and 5.6-fold, respectively, while arrestin increased more than 10-fold after light exposure (Table 3.2), consistent with previous findings. We also found that the amounts of rod cGMP-specific 3',5'-cyclic phosphodiesterase α and β were reduced 3.2- and 3.4-fold, respectively, after light exposure (Table 3.2). However, a previous study did not observe light-driven translocation of these proteins [92]. This discrepancy may be due to the differences in light treatment conditions. Whereas we used intense (1200 lux) and prolonged (8 h) light treatments, mild (250 lux) and short (1 h) light treatments were used in the previous report. Further studies are necessary to clarify whether the reduced amounts of these proteins in the OS after light exposure are due to translocation of these proteins out of the OS or are due to other mechanisms.

We found that the levels of heat shock protein HSP 90-alpha, heat shock cognate 71 kDa protein and ubiquitin are elevated more than 2-fold after light exposure (Table 3.3). Heat shock proteins are molecular chaperones and known to be induced under various stress
conditions [94]. Their major function is to assist in the folding of nascent polypeptides or denatured proteins that result from various cellular stresses [95]. Thus, our results imply that misfolded proteins accumulate in the OS upon intense light exposure and that the induction of heat shock proteins is to protect cells against light-induced oxidative stress. Ubiquitin is a protein modifier which can be covalently attached to proteins that marks the proteins for degradation by the proteasome [96]. The existence of ubiquitin-dependent proteolysis pathways in OS have been reported [97]. Therefore, the elevated ubiquitin level may be suggesting a higher activity of ubiquitin-proteasome pathways in intense light exposed OS.

A total of 8 glycolytic enzymes were quantified and found to be unchanged by light (Table 3.4), suggesting that no significant change occurred in glycolysis. No significant changes in major OS integral membrane proteins, including rhodopsin, rod outer segment membrane protein 1 (ROM1), cGMP-gated cation channel alpha-1 and retinal-specific ATP-binding cassette transporter, were found (Supplemental Table 2). The fact that the levels of soluble glycolytic enzymes and major OS integral membrane proteins were unchanged after light indicates that light-induced membrane damage, that may have caused a leaky OS, was not significant.

This is the first comparative proteomic study of intense light-induced changes in OS proteins and provides a foundation for further proteomic research. Our study led to the unexpected discovery of changes in the quantity of rhodopsin kinase during intense light exposure, a finding that could improve our understanding of light-induced photoreceptor apoptosis. It is currently unknown how light regulates the quantities of specific proteins in photoreceptor cells. In our analysis, another isoprenylated protein, rod cGMP-specific
3',5'-cyclic phosphodiesterase α and β were down-regulated similar to rhodopsin kinase (Table 3.2). An intriguing hypothesis is that rod cGMP-specific 3',5'-cyclic phosphodiesterase α and β and rhodopsin kinase are degraded specifically by a common pathway. Recently, a prenyl binding protein, PrBP/delta was demonstrated to be involved in specific trafficking of rhodopsin kinase and PDE6 to the photoreceptor outer segment [98]. Similarly, there could be a specific mechanism to traffic the isoprenylated proteins to the proteolytic machinery in photoreceptors. This novel protein quality/quantity control pathway warrants further studies.

ACKNOWLEDGMENTS

The authors thank Gaurav S. J. B. Rana for assistance in statistical analysis and Kerrie Lodowski for helpful discussions and editorial advice. The authors are grateful to Krzysztof Palczewski for generously providing us with anti-rhodopsin kinase monoclonal antibody G8. This study was supported by an NIH grant P30EY-11373 (Visual Sciences Research Center of Case Western Reserve University) and funds from Case Western Reserve University and the Cleveland Foundation (MM), and by the Ohio Lions Eye Research Foundation and M. Petticrew of Springfield OH (DTO).
### Table 3.2 – Proteins Involved in Phototransduction

<table>
<thead>
<tr>
<th>SwissProt Accession #</th>
<th>SwissProt ID</th>
<th>Protein Name</th>
<th>MW (Da)</th>
<th>Average protein ratio</th>
<th>Standard deviation</th>
<th>P-value</th>
<th># of unique peptides quantified</th>
</tr>
</thead>
<tbody>
<tr>
<td>P15887</td>
<td>ARRS_RAT</td>
<td>Arrestin</td>
<td>45206</td>
<td>&gt;10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01</td>
<td>&lt;0.001</td>
<td>22</td>
</tr>
</tbody>
</table>

#### Proteins found in greater amount in light exposed OS

| P20612                | GNAT1_MOUSE<sup>5</sup> | Guanine nucleotide-binding protein G(t) alpha-1 | 40397   | 0.19 | 0.01 | <0.001 | 16 |
| P64311                | GNB1_RAT        | Guanine nucleotide-binding protein G(I)/G(S)/G(T)beta-1 | 38151   | 0.17 | 0.01 | <0.001 | 17 |
| Q651O2                | GBG1_MOUSE      | Guanine nucleotide-binding protein G(T) gamma-T1 | 8593    | 0.15 | 0.02 | <0.001 | 9  |
| P34057                | RECO_MOUSE      | Recoverin   | 23449   | 0.18 | 0.11 | 0.006  | 6  |
| Q63051                | RK_RAT          | Rhodopsin kinase | 64298   | 0.51 | 0.10 | 0.002  | 15 |
| P27664                | PDE6A_MOUSE     | Rod cGMP-specific 3',5'-cyclic phosphodiesterase alpha | 100437  | 0.31 | 0.06 | <0.001 | 18 |
| P23440                | PDE6B_MOUSE     | Rod cGMP-specific 3',5'-cyclic phosphodiesterase beta | 99693   | 0.29 | 0.09 | 0.001  | 8  |

#### Proteins found in lesser amount in light exposed OS

| Q62927                | CNGA1_RAT      | cGMP-gated cation channel alpha-1 | 79577   | 0.99 | 0.06 | 0.654  | 8  |
| P62682                | GBBS_RAT       | Guanine nucleotide-binding protein beta-5 | 38505   | 0.98 | 0.12 | 0.533  | 5  |
| P51840                | GUC2E_RAT      | Guanylyl cyclase GC-E | 121750  | 0.98 | 0.17 | 0.834  | 18 |
| P49605                | RGSS_RAT       | Regulator of G-protein signaling 9 | 77653   | 0.94 | 0.23 | 0.529  | 6  |
| P54189                | OPSS_RAT       | Rhodopsin | 39586   | 1.4  | 0.5  | 0.229  | 6  |

<sup>a</sup> Calculated using protein ratios obtained from four biological replicates. The ratios are light exposed/unexposed.

<sup>b</sup> Exact value is not shown, because this proteolytic method cannot provide accurate value when the protein ratio exceeds greater than 10. Exact value can be seen in Supplemental Table 2.

<sup>c</sup> Mouse SwissProt accession numbers and IDs are used for proteins for which rat sequences have not been reviewed in SwissProt database version 57.0.
Table 3.3 – Other proteins altered by light exposure

<table>
<thead>
<tr>
<th>SwissProt Accession #</th>
<th>SwissProt ID</th>
<th>Protein Name</th>
<th>MW (Da)</th>
<th>Average protein ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Standard deviation</th>
<th>P-value</th>
<th># of unique peptides quantified</th>
</tr>
</thead>
<tbody>
<tr>
<td>P62989</td>
<td>UBIQ_RAT</td>
<td>Ubiquitin</td>
<td>8560</td>
<td>3.97</td>
<td>1.04</td>
<td>0.011</td>
<td>4</td>
</tr>
<tr>
<td>P82995</td>
<td>HS90A_RAT</td>
<td>Heat shock protein HSP 90-alpha</td>
<td>85134</td>
<td>4.13</td>
<td>1.13</td>
<td>0.041</td>
<td>11</td>
</tr>
<tr>
<td>P63018</td>
<td>HSP7C_RAT</td>
<td>Heat shock cognate 71 kDa protein</td>
<td>70989</td>
<td>2.50</td>
<td>0.90</td>
<td>0.045</td>
<td>14</td>
</tr>
</tbody>
</table>

<sup>a</sup>Calculated using protein ratios obtained from the eight proteomic experiments on four biological replicates. The ratios are light exposed/unexposed.
<table>
<thead>
<tr>
<th>SwissProt Accession #</th>
<th>SwissProt ID</th>
<th>Protein Name</th>
<th>MW (Da)</th>
<th>Average protein ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Standard deviation</th>
<th>P-value</th>
<th># of unique peptides quantified</th>
</tr>
</thead>
<tbody>
<tr>
<td>P05065</td>
<td>ALDOA_RAT</td>
<td>Fructose biphosphat aldolase alpha</td>
<td>39763</td>
<td>0.78</td>
<td>0.11</td>
<td>0.028</td>
<td>13</td>
</tr>
<tr>
<td>P09117</td>
<td>ALDOC_RAT</td>
<td>Fructose biphosphat aldolase gamma</td>
<td>39658</td>
<td>1.05</td>
<td>0.48</td>
<td>0.837</td>
<td>12</td>
</tr>
<tr>
<td>P04764</td>
<td>ENOA_RAT</td>
<td>Alpha enolase</td>
<td>47440</td>
<td>0.71</td>
<td>0.15</td>
<td>0.030</td>
<td>15</td>
</tr>
<tr>
<td>P07323</td>
<td>ENOG_RAT</td>
<td>Gamma enolase</td>
<td>47510</td>
<td>1.00</td>
<td>0.27</td>
<td>0.980</td>
<td>4</td>
</tr>
<tr>
<td>P04797</td>
<td>G3P_RAT</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>36090</td>
<td>1.06</td>
<td>0.12</td>
<td>0.351</td>
<td>10</td>
</tr>
<tr>
<td>P11980</td>
<td>KPYM_RAT</td>
<td>Pyruvate kinase isozymes M1/M2</td>
<td>58294</td>
<td>0.82</td>
<td>0.14</td>
<td>0.085</td>
<td>16</td>
</tr>
<tr>
<td>P25113</td>
<td>PGAM1_RAT</td>
<td>Phosphoglycerate mutase 1</td>
<td>28928</td>
<td>0.65</td>
<td>0.34</td>
<td>0.21</td>
<td>6</td>
</tr>
<tr>
<td>P16617</td>
<td>PGK1_RAT</td>
<td>Phosphoglycerate kinase</td>
<td>44809</td>
<td>0.81</td>
<td>0.11</td>
<td>0.106</td>
<td>9</td>
</tr>
<tr>
<td>P48500</td>
<td>TPIS_RAT</td>
<td>Triose phosphate isomerase</td>
<td>27345</td>
<td>1.00</td>
<td>0.42</td>
<td>0.986</td>
<td>7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Calculated using protein ratios obtained from four biological replicates. The ratios are light exposed/unexposed.

Table 3.4 – Ratios of Glycolytic Enzymes
<table>
<thead>
<tr>
<th>Protein</th>
<th>1st replicate</th>
<th>2nd replicate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>aRatio</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>rhodopsin</td>
<td>1.09</td>
<td>0.10</td>
</tr>
<tr>
<td>rhodopsin kinase</td>
<td>0.52</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*a(light exposed/unexposed)

Table 3.5 - Rhodopsin and rhodopsin kinase ratios analyzed by LC-MRM.
Figure 3.7 – Animal Light Exposure

Animals (2 male Sprague-Dawley rats) were placed in a light chamber for 8h starting in the middle of their dark cycle. a) Side view of the experimental light chamber and b) Front view of the light chamber. Animals were in a calm state and kept their eyes opened for most of the time in the light chamber (Figure 3.7 c).
Figure 3.8 – CO₂ Chamber Setup

Animals in a cage were placed inside this CO₂ chamber. This was very convenient because the animals were not needlessly stressed by handling.
a) cornea

b) retina

c) retina
Figure 3.9 – Detailed Description of Rat Retina Dissection

Rat was placed on its side and curved forceps were used to take a hold of the rat eyeball, forceps were in right hand. The forceps were turned counter-clockwise, and hands were switched – ended with the forceps in the left hand. This released the eyeball from the eye socket and ensured the right grip. Caution was taken not to squeeze the forceps too hard (a). A large slit was cut into cornea with a razor blade and the lens was removed (b). By slowly pulling the forceps up, vitreous humor was removed while the retina stayed in the curve of the forceps. The retina was then placed in cold wash buffer, and kept on ice (c) until it was placed into appropriately labeled cryotube and snap frozen in liquid nitrogen.
Figure 3.10 – Potter-Elvejem Type Homogenizer with a Glass Tube
Figure 3.11 – Filtering Apparatus for Rat OS Preparation

Top filter (mesh 50)

Bottom filter (mesh 400)

Centrifuge tube
Figure 3.12 – Rat OS Preparation by Sucrose Gradient

1. Slowly thawed retinas
2. Placed retinas into 47% sucrose in phosphate buffer
3. Ground retinas with teflon loose fitting homogenizer
4. Filtered homogenate
5. Overlayed homogenate with other sucrose solutions
6. Centrifuged
7. Collected OS bands

- Band 1: 32%
- Band 2: 37%
- 42%
- 47%
Figure 3.13 – Electron Micrograph of Photoreceptor Outer Segments

The OS aliquot was analyzed by electron microscopy to ensure that disk membranes were intact and were not disrupted during our preparation.
Control sample

OS from rats not exposed to light

Extract proteins with 2% SDS

Reduce and alkylate proteins

Acetone precipitate proteins

Solubilize proteins in 0.1% Rapigest

Digest at basic pH
label with $^{16}$O at acidic pH

Mix in 1:1 ratio

LC-MS/MS analysis

Database search to assign proteins

Calculate ratio of $^{18}$O to $^{16}$O peptides

Calculate ratio of proteins

Experimental sample

OS from rats exposed to 8h light

Extract proteins with 2% SDS

Reduce and alkylate proteins

Acetone precipitate proteins

Solubilize proteins in 0.1% Rapigest

Digest at basic pH
label with $^{18}$O at acidic pH

Figure 3.14 – Workflow for Forward Labeling of Light Exposed OS

This figure shows the experimental steps in the forward labeling experiment. The reverse labeling experiment followed the same workflow except the light exposed OS was labeled with $^{16}$O and the unexposed OS was labeled with $^{18}$O. Four biological replicates of pooled OS preparations (8 retinas/sample) were examined. All replicates were subjected to forward and reverse labeling.
All peptide regression plot is shown in a), each blue square represents individual peptide ratio in sample F1. Peptides belonging to proteins with extreme changes (arrestin, transducins α, β, γ, and recoverin) were removed from the plot (b) to determine the overall peptide ratio (in red circle) for each sample. This overall peptide ratio was used to normalize ratios of all proteins in this sample. E.g. if protein in sample F1 had a ratio of 1.00 it was then corrected to 1.24.
Linear regression analysis of protein ratios in forward and reverse $^{18}$O labeling experiments. A simple linear regression analysis of individual protein ratios in the R1 experiment as a function of those in the F1 experiment (a), R2 as a function of F2 (b), R3 as a function of F3 (c), and R4 as a function of F4 (d) were conducted. The regression line (bold line) and the confidence interval lines with $\alpha = 0.01$ (dotted lines) are shown. The equations and $R^2$ values for the regression lines are shown in left top corner of each panel. Ratios greater than 3.0 were excluded from the analysis.
Figure 3.17 - Immunocytochemical Analysis of Rhodopsin Kinase.

Immunocytochemical analysis of rhodopsin kinase. Representative immunofluorescence images from rats unexposed to light (a) and exposed to light for 8 hr (b) are shown. Rhodopsin kinase (red) was detectable only in the OS. Nuclei were stained with 4',6-diamidino-2-phenylindole (blue) and cone photoreceptors with fluorescein-conjugated peanut agglutinin (green). OS, photoreceptor outer segments; IS, photoreceptor inner segments; ONL, outer nuclear layer; INL, inner nuclear layer. Three light exposed and three unexposed rats were analyzed.
Molecular steps in activation (a) and deactivation (b) of the phototransduction cascade.

The activation cascade is initiated upon photon absorption by rhodopsin. Absorption of a photon by rhodopsin causes a series of conformational changes in the protein leading to an activated state of rhodopsin. The activated rhodopsin binds a heterotrimeric protein transducin and allows for exchange of GDP nucleotide for GTP in the $\alpha$ subunit of transducin. The GTP bound $\alpha$ subunit dissociates from the $\beta$ and $\gamma$ subunits of transducin and activates phosphodiesterase that catalyzes hydrolysis of cGMP. In response to a decreased concentration of cGMP, cGMP gated cation channels in the plasma membrane.
close, resulting in decreased calcium levels which cause the photoreceptor cell to become hyperpolarized (a). Recoverin is normally bound to rhodopsin kinase when the calcium concentration is high. However, during phototransduction the calcium levels fall, resulting in the release of rhodopsin kinase that leads to initiation of the deactivation cascade of rhodopsin. The activated rhodopsin is first phosphorylated by rhodopsin kinase. The phosphorylated rhodopsin allows arrestin to bind, which stearically interferes with the binding of the α subunit of transducin. In addition to deactivating rhodopsin, transducin mediated signaling could be quenched. The complex of G-protein beta 5 (GBB5), regulator of signaling 9 (RGS9) and regulator of signaling 9 anchor protein (R9AP) binds to the transducin α subunit-phosphodiesterase complex. This stimulates the GTPase activity of transducin α subunit and results in dissociation of the transducin α subunit from phosphodiesterase, therefore slowing down the hydrolysis of cGMP, allowing the cGMP channels to open (b). Adapted from Burns and Arshavsky [99].
CHAPTER 4 – CIRCADIAN PROTEOME CHANGES IN PHOTORECEPTOR OUTER SEGMENTS

The third chapter will describe further improvement and application of the final $^{18}$O labeling workflow. It summarizes information about circadian proteome differences in photoreceptor OS. These differences may explain the circadian dependence of photoreceptor susceptibility to light damage.

**Final Improvement of $^{18}$O labeling workflow**

After applying $^{18}$O labeling to identify light induced changes in the photoreceptor OS, it became evident that further improvement of our workflow was necessary to quantitatively analyze more OS proteins. It is likely that we are missing quantitative information of low abundant OS proteins due to the complexity of our samples. It is a common practice in proteomics to fractionate complex samples to increase protein sequence coverage as well as numbers of identified proteins. There are many different fractionation techniques at different level of the proteomic experiment. We have tested fractionation of OS samples at the peptide level using strong cation exchange columns. Unfortunately this separation technique was not fractionating the OS samples efficiently as most of the OS peptides eluted in the same fraction and many peptides were present in multiple fractions. This is why we decided to proceed with fractionation at the protein level using 1D-SDS-PAGE. This approach was tested on bovine OS to test the fractionation efficiency of OS samples.
MATERIALS AND METHODS

Materials

Bovine retinas were purchased from JA&WL Lawson Company (Lincoln, NE). Oxygen-18 enriched water (95%) was obtained from Isotec (Miamisburgh, OH). Sequencing grade modified porcine trypsin was purchased from Promega. Precast 1D-SDS-PAGE gradient gels were obtained from Invitrogen.

METHODS

Preparation of Bovine Outer Segments

OS were prepared from bovine retina as described previously [1] by sucrose gradient ultracentrifugation. All steps were carried out under dim red light illumination. Rhodopsin rich fraction also known as Band I was used in this study.

1D-SDS-PAGE Separation of Bovine Outer Segments

Proteins in two aliquots of bovine OS (100 µL) were precipitated by 20% sucrose solution in 100 mM phosphate buffer. Pellet was redissolved in 200mM Tris HCl containing 5% SDS to ensure proper solubilization of all OS proteins prior to reduction by DTT and alkylation by iodoacetamide. Samples were then subjected to gel electrophoresis (Lane 1-2) using 4-12% gradient gel.
**In Gel Digestion and in Solution $^{18}$O labeling**

Lane 1 and lane 2 were cut into 7 bands as depicted in Figure 4.1. Special care was taken to cut both lanes at same molecular weight markers. Gel bands were crushed and proteins in each band were digested separately in $H_2^{16}O$ by trypsin (1:100 enzyme:substrate ratio) at 25 °C for 18 h. Resulting peptides were extracted from gel by repeated sonication in 0.1% formic acid in 50% acetonitrile then dried. The dried peptide extracts were reconstituted with 100 mM citrate buffer pH 6. The samples were dried in a speed-vac, and then reconstituted in either $H_2^{16}O$ or $H_2^{18}O$. Trypsin was added to the solutions to incorporate either $^{16}O$ or $^{18}O$ into the carboxyl termini of the peptides from the solvent water. The reaction was carried out at 25 °C for 18 h at a trypsin to protein ratio of 1:25 (w/w). After the reaction, equal volumes of 8 M guanidine HCl solution in either $^{16}O$ or $^{18}O$ water were added, pH of solutions was increased by addition of Tris base, and trypsin was inactivated by reduction with 1 mM DTT, followed by alkylation with 2.5 mM iodoacetamide. The resulting $^{16}O$- and $^{18}O$-labeled peptide solutions were mixed together in equal proportions and desalted using a Vydac C18 reverse phase column. The eluted were dried in a speed-vac, reconstituted with 0.1% formic acid, and analyzed by LC-MS/MS. LC-MS/MS method, protein identification and quantification are described above under Validation of $^{18}$O Labeling on Model Protein and Bovine OS.

**RESULTS**

1D-SDS-PAGE separation of bovine OS

Image of 1D-SDS-PAGE is shown in Figure 4.1. Both lanes show similar separation profile and intensities suggesting similar protein amount. Each lane was carefully cut into
7 bands according to the black lines in this figure. This step was added into our workflow because it allowed the use of SDS to solubilize OS proteins. It also fractionated OS proteins into less complex samples and increased the coverage of identified proteins.

**In Gel Digestion and in Solution $^{18}$O labeling**

The total number of quantified peptides increased by more than 5 fold (compare Figure 3.6 to Figure 4.2 a-g). Most proteins were quantified in only one fraction, suggesting that the separation technique was suitable for OS sample, however several high abundant proteins (i.e. rhodopsin, transducin $\alpha$) were quantified in multiple bands. Overall $^{18}$O/$^{16}$O peptide ratios in individual bands varied from 0.71-1.10 (Figure 4.2).

**CONCLUDING REMARKS**

Fractionating OS samples by 1D-SDS-PAGE can be used in our labeling workflow. It increased the coverage of identified proteins as well as the number of quantified proteins.
Two aliquots of the same bovine OS preparation were reduced, alkylated then loaded onto 1D-SDS-PAGE and separated by electrophoresis. Lane 1 and 2 were cut into 7 bands as depicted above. Each band was digested by trypsin. The resulting peptides were extracted and labeled in solution with either $^{16}$O (lane 1) or $^{18}$O (lane 3). Prior to LC-
MS/MS analysis samples corresponding to the same band in lane 1 and 3 were mixed in 1:1 ratios.
Figure 4.2 – $^{18}$O vs $^{16}$O Peptide Intensity for Each OS Band

The intensities of $^{18}$O peptides were plotted against the intensities of their $^{16}$O peptide equivalents. Each point (blue diamond) represents individual peptide ratio. The overall peptide ratio in band 1 of the bovine OS sample, $R^2$ value and F probability of the line fit is shown in left top corner of Figure 4.2a. Figures 4.2 b-g correspond to band 2-7, respectively. The experimental overall ratios varied from 0.71-1.10.
Circadian Proteome Changes in Photoreceptor Outer Segments

ABSTRACT

Intriguingly, susceptibility of photoreceptors to light is greater at nighttime than at daytime. However, molecular mechanisms behind this phenomenon are not known. To better understand the mechanisms of circadian dependent susceptibility of photoreceptors to light, we studied the proteome differences in photoreceptor OS between midnight and noon by 1D-SDS PAGE gel followed with proteolytic $^{18}$O labeling. In total, 4 biological replicates were analyzed. Several protein ratios from this proteomic approach were verified by liquid chromatography multiple reaction monitoring (LC-MRM), and their cellular localizations were studied by immunofluorescence. Most OS proteins remained unchanged between daytime and nighttime (177 out of 185). The notable difference was the lower amount of anti-stress proteins (crystallin αB-chain, and peroxiredoxin 1) at midnight compared to noon, suggesting that nighttime OS is more susceptible to light-induced stress. LC-MRM confirmed the reduction in crystallin αB amount. Our data suggests that the lower amount of anti-stress proteins may be one of the contributing factors to the nighttime light susceptibility of photoreceptors. It is consistent with previous reports documenting protection of photoreceptors by overexpression of survival factor Lens Epithelium Derived Growth Factor (LEDGF) which then stimulates the expression of stress related genes including crystallin αB. Light exposure for 1 h to strong green light completely abolished the circadian changes suggesting that light is much stronger stimulus than the circadian rhythm.
INTRODUCTION

To identify circadian differences, comparative proteomics utilizing $^{18}$O labeling by trypsin was used. Previous quantitative proteomic study focused on identifying circadian differences in mouse retina by 2D gel electrophoresis identified changes in proteins involved in vesicular trafficking, metabolism, calcium binding, RNA binding and protein folding [100].

Our goal was to identify and quantify proteins in the OS at nighttime versus daytime. According to our results the differences in the OS proteome between daytime and nighttime are small (the amount of only 13 out of 185 proteins changed in the OS). Even though the changes in protein amount reported here were statistically significant, in many cases the differences were less than two fold. We further focused on validating the amount of small heat shock protein crystallin $\alpha$B which was found in lesser amount at midnight than at noon. This protein has been previously reported to have antiapoptotic effect therefore could play a role in the photoreceptor protection against light induced apoptosis during daytime when its amount is higher. The differentially expressed proteins found in this study may play a protective role against light damage in the retina and could lead us to uncover possible mechanisms of photoreceptor light damage.

MATERIALS AND METHODS

All procedures involving rats followed the protocols outlined in the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in
Ophthalmic and Vision Research, and animal protocol approved by Case Western Reserve University. Experimental workflow for entire study is shown in Figure 4.3.

**Animal treatment**

Male Sprague-Dawley rats (Harlan Inc., Indianapolis, IN) postnatal age 21 days were reared in a cyclic light environment 12 hours dark/light cycle each day for at least 40 days; lights were on at 6 AM and off at 6 PM. The rats were fed a standard rat chow (Teklad, Madison, WI) and given water ad libitum. Rats were randomly assigned to four groups (number of animals per group = 4). Four independent experiments were carried out. Group 1 and 3 were dark adapted from 6 pm, Group 2 and 4 were dark adapted from 7am. Groups 3 and 4 were placed into light chamber one hour prior to sacrifice and were exposed to green intense light (1200 Lux). Groups 1 and 3 were killed at noon, Groups 2 and 4 were killed at midnight in the atmosphere saturated by carbon dioxide. Immediately after death, retinas were excised and rinsed in phosphate buffer (PB) containing 32% sucrose, phosphatase and protease inhibitors (Sigma-Aldrich, Saint Louis, MO). Retinas were then frozen and stored in liquid nitrogen until further use. All mentioned steps were carried out under dim red light illumination for Group 1 and 3.

**Preparation of outer segments**

Retinas were kept in liquid nitrogen until OS were prepared by sucrose density gradient ultracentrifugation [84] and as described above. All solvents used to prepare OS contained protease and phosphatase inhibitors to prevent protein degradation and dephosphorylation. Only band 1 fractions were used in our study. OS were pelleted by
centrifugation (15000 g, 5 min, 4°C) with equal volumes of 20% sucrose in PB. OS pellets were stored at -80°C until further use.

**1D-SDS-PAGE protein separation**

OS from retinas prepared at noon or midnight were thawed on ice and dissolved in 200 mM Tris HCl buffer containing 5% sodium dodecyl sulfate (SDS). Protein concentration was estimated using RC DC Protein assay from BioRad. This method is based on modified Lowry method. Protein amount was adjusted to 50 μg in each sample. Proteins were reduced by dithiothreitol, alkylated by iodoacetamide, then loaded on 1D-SDS-PAGE precast gel (Invitrogen, Carlsbad, CA). Samples from noon and midnight were loaded on the same gel into adjacent lanes to enable reproducible fractionation of both samples.

**In-gel digestion and proteolytic $^{18}$O labeling**

Each sample gel lane was cut into 6 bands. Gel bands were washed by 200 mM ammonium bicarbonate in 50% acetonitrile, dried by 100% acetonitrile, and rehydrated by 100 mM ammonium bicarbonate. Bands were crushed and digested by porcine trypsin overnight. Peptides were extracted from gel, dried on the speed-vac (Thermo Savant, Asheville, NC), and redissolved in 100 mM citrate buffer pH 6. Peptide solutions were split into two fractions. Both fractions were dried on the speed vac. One fraction was then dissolved in $H_2^{16}$O the other fraction was dissolved in $H_2^{18}$O. Porcine trypsin in either water was added to the respective fractions. Peptides were labeled overnight at room temperature on a horizontal shaker. After labeling, porcine trypsin was deactivated at pH 8 by reduction and alkylation. Noon samples labeled by $^{16}$O and midnight samples
labeled by $^{18}$O were mixed for forward labeling experiment. Mixing noon samples
labeled by $^{18}$O and midnight samples labeled by $^{16}$O presented a reverse labeling
experiment. All samples were desalted on C18 silica column (Vydac, The Nest Group,
Southborough, MA) as described in Appendix 2 under step 20, dried on the speed vac
and stored at -20°C until further use.

**LC-MS/MS analysis**

All samples were dissolved in 0.1% formic acid and then injected into nano-liquid
chromatography (LC) system (Ultimate 3000, Dionex, Sunnyvale, CA) onto trapping pre-
column (C18, PepMap100, 300 μm ×5 mm, 5 μm particle size, 100 Å; Dionex,
Germering, Germany) followed by a reversed phase column (C18, 75μm×150 mm, 3 μm,
100 Å; Dionex), using a mobile phase A (0.1 % formic acid in water) and B (0.1 %
formic acid in 80 % acetonitrile). Eluting gradient was 0.8% of increasing mobile phase
B per minute. LC was coupled to high resolution mass spectrometer (LTQ-Orbitrap,
Thermo-Fisher, Waltham, MA) equipped with Packed-Tip nanospray ionization Probe
(Thermo Electron Corp., Bremen, Germany) operated in a positive ion mode. The
peptides were infused at a flow rate of 300 nL/min via the silica non-coated PicoTip
emitter (FS360-20-10-C12, New Objective Inc., Woburn, MA) at a voltage of 2.4 kV.
The capillary temperature was maintained at 200 °C. Full MS spectra were recorded in
the Orbitrap, and then the tandem mass spectra of 5 most intense ions were recorded by
the LTQ ion trap at normalized collision energy of 35 eV, isolation width 2.5 Da and
activation Q at 0.250 and injection time of 300 ms.
Protein identification

Data was acquired using Xcalibur software (Thermo Electron Corp.) and submitted for database search to the Mascot server using a client application, Mascot Daemon version 2.2.0 (Matrix Science, London, UK). Proteins were identified by comparing all of the experimental product ion spectra of the peptides to the Swiss-Prot v56 (392667 sequences; 141217034 residues) Rodentia (24760 sequences) database using the Mascot database search engine (version 2.2, Matrix Science, London, UK). S-carbamidomethylation of cysteine was set as a fixed modification. Oxidation of methionine (methionine sulfoxide), one or two C-terminal $^{18}$O labels, and acetylation at the protein N-terminus were set as variable modifications. Mass tolerance for protein identification precursor ion was set at 20 ppm and for product ion was set to 1 Da. Strict trypsin specificity allowed for only one missed cleavage site. Mascot results were further analyzed by Scaffold Proteome Software (version 2_05_01 by Proteome Software Inc., Portland, OR). All mascot search results were loaded (total 48) as separate biological samples. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Mascot identifications required at least ion scores to be greater than both the associated identity scores and 20. Protein identifications were accepted if they contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.
Protein quantification

Custom built software Relative Quantification O18.1.3.1 (developed by our colaborators Quanhu Sheng and Haixu Tang, PhD., University of Indiana, Bloomington, IN) was used to calculate $^{18}$O/$^{16}$O labeled peptide ratios. Mascot search result file from each LC-MS/MS sample in peptide format was converted to plain text file and served as input data. Proteins identified by at least 2 peptides with ion score of 20 were considered for quantification. The same software was utilized to calculate raw protein ratios by linear regression from individual peptide ratios assigned to the particular protein. The fact that only identified proteins are submitted for quantification creates a limitation of this technique. If protein was not submitted for MS/MS analysis there is no way a peptide sequence would be assigned. Proteins quantified by 2 peptides with $R^2$ value of more than 0.8 and F-probability value for predicted ratio greater than 0.9 in at least two biological samples were then submitted for further statistical analysis. We are aware of a limitation of our method in quantifying only identified peptides and proteins as this creates a potential problem of missing peptide ratios due to the missing MS/MS spectrum in the particular LC-MS/MS sample. In this case, missing ratio doesn’t necessarily mean that the protein was present in undetectable amount. It can simply mean that the particular peptide wasn’t submitted to analysis by MS/MS and therefore wasn’t identified. This is the reason that some proteins have mean midnight/noon ratios calculated from N<4. Further improvement of this quantification method is a subject of ongoing research in our laboratory and will not require peptide identification from the particular LC-MS/MS sample for quantification.
Statistical analysis

Scaffold Proteome Software (version 2_05_01 by Proteome Software Inc., Portland, OR) was used to filter our data prior to statistical analysis. Spectrum count of proteins quantified in multiple gel bands was investigated and only ratios from sample with the highest spectrum count were used, in this way the maximum number of ratios used per protein was 8 – (4 biological replicates analyzed by forward and reverse labeling analysis). Means of forward and reverse ratios for the same biological experiment were calculated and distribution plots of mean ratios from all biological replicates were created in JMP8 Software (SAS for Windows). Mean, standard deviation, and 95% confidence intervals were calculated for all proteins. Statistically significant changes in protein ratios (p<0.05) were identified by 1-sample t-test, testing the mean of the ratios to be different from 1 (=no change between midnight and noon).

LC-MRM validation of proteomic results

This experiment was designed to validate our proteomic results. Two biological replicates of noon and midnight OS were prepared as described above, dissolved by 0.1% Rapigest (Waters, Milford, MA) in 50 mM ammonium bicarbonate, reduced and alkylated, and digested by modified trypsin. Rapigest was cleaved by 1% formic acid, all samples were then desalted on Vyda C18 microcolumns (Nest Group Inc., Southborough, MA) and completely dried on speed-vac. Prior to analysis by liquid chromatography multiple reaction monitoring (LC-MRM) samples were dissolved in 0.1% trifluoroacetic acid (TFA) in water and spiked with AQUA-peptides (Sigma Genosys, The Woodlands,TX) - heavy labeled tryptic peptides (13C and 15N labeled C-terminal arginine or lysine) unique
to 9 proteins (7 involved in phototransduction, 2 anti-stress proteins). These peptides were used as internal standards and their amount ranged from 1 pmol (low abundant proteins i.e. Crystallin α-B-chain, Peroxiredoxin 1) – 3.9 nmol (rhodopsin). Samples were injected onto reverse phase column (Alltech Altima HP C18, ID 1mm, length 150mm, particle size 3µm) by LC Packings Ultimate 3000 (Sunnyvale, CA, USA) at 100 uL/min flow rate. 0.02% TFA in water or acetonitrile was used as mobile phase A or B, respectively. Peptides were eluted by LC gradient from 5 - 25% of B, starting after 10 min of column equilibration with mobile phase A, then they were introduced to API 4000 Q-trap mass analyzer (Sciex, Toronto, ON) equipped with TurboIon spray ion source (Sciex, Toronto, ON). Table 2.7 lists sequences unique to the proteins of interest and the transitions monitored by mass spectrometer. Analyst 1.4.2 software (Sciex, Toronto, ON) was used to acquire and analyze LC-MRM data.

**Immunofluorescence**

Animals for this experiment were raised under same light conditions as for the proteomic and LC-MRM studies, and killed at noon or midnight as described above. 4 biological replicates were analyzed by this method. Eyes were then removed and dissected under dim red light. The eyecups were fixed in 4% paraformaldehyde in 100 mM phosphate buffer for at least 6 hours. In three concentration steps (5%, 10%, and 15% sucrose), paraformaldehyde was replaced by 20% sucrose in 100 mM phosphate buffer. Finally, eyecups were incubated in a 2:1 mixture of 20% sucrose in 100 mM phosphate buffer and Optimal Cutting Temperature (OCT) compound. Eyecups were frozen in isopentane cooled by liquid nitrogen, and cut into 12µm sections on Leica CM 1850 Cryostat (Leica
Microsystems Inc., Bannockburn, IL). For immunofluorescence labeling, sections were first blocked for nonspecific labeling by incubating in 1.5% normal goat serum in PBST buffer (136mM NaCl, 11.4 mM sodium phosphate, 0.1% Triton X-100, pH 7.4) for 15 min at room temperature and then incubated overnight with primary polyclonal antibody against Crystallin α-B-chain (Stressgen, Ann Arbor, MI, product no.: SPA-223), diluted in PBST overnight at 4 °C. Sections were rinsed by PBST and incubated with indocarbocynine (Cy3 or Cy5)-conjugated secondary antirabbit antibodies. Sections were then rinsed and mounted in 50 μl of 2% 1,4-diazabicyclo-2,2,2–octane in 90% glycerol to slow photobleaching. Sections were analyzed under a Leica DM6000 B microscope (Leica Microsystems Inc.) equipped with a RETIGA EXi CCD camera (QImaging, Burnaby, BC).

Pathway analysis

Metacore software was used to find proteins which directly interact with Crystallin αB or control its expression.

RESULTS

A representative gel image is shown in Figure 4.4. Noon and midnight samples from all biological replicates showed a similar separation profile on 1D SDS PAGE gel. This suggests no significant changes in abundant OS proteome profiles between noon and midnight. Proteome changes can be observed between light exposed and unexposed samples especially in band 4 where arrestin (M=48kDa) is expected to migrate. This protein has been previously reported to translocate into OS upon light exposure. Each lane in Figure 4.4 was fractionated into 6 bands. Special care was taken to cut at the same
molecular weights and in between visible protein bands. After fractionation, all bands were subjected to digestion followed by either a forward or reverse $^{18}$O labeling. Thus each biological replicate yielded 12 different LC-MS/MS experiments. Four biological replicates were analyzed yielding 48 different raw data files for no light exposure and 48 files for 1h light exposure experiment. All these files were submitted to database search by Mascot for protein identification. All proteins identified by more than 2 peptides with ion score higher than 20 were submitted for quantification.

In no light exposure experiment, we identified 388 proteins (Supplementary Table 3) from which about 180 were quantified in at least 3 biological replicates and further statistically analyzed (Supplementary Table 4). We identified only 8 proteins which were changing significantly, p-value <0.05 (Table 4.2a and b). Representative distribution of ratios and p-value plots for protein increasing (RGS9) and decreasing (crystallin $\alpha$B-chain) amounts are shown in Figure 4.5.

Proteins involved in response to oxidative stress crystallin $\alpha$-B-chain and peroxiredoxin-1 were found in lower amounts at midnight. A protein involved in phototransduction (RGS9) was present in higher amount at midnight. These three proteins were selected for validation by LC-MRM because the proteomic results identified the greatest differences in these proteins between noon and midnight. In addition to these proteins, we targeted 3 phototransduction proteins (rhodopsin, arrestin, and rod specific phosphodiesterase 6$\alpha$) because these proteins didn’t show changes in our proteomic study and would serve as controls for loading and sample processing as well as comparison of the two techniques.

Protein ratios of crystallin $\alpha$B and RGS9 were successfully validated by LC-MRM (Table 4.3). Ratio of peroxiredoxin 1 was not successfully validated by this method and
further experiments are necessary to investigate the real ratio of this protein between midnight and noon. Rhodopsin, arrestin, and rod specific phosphodiesterase 6α had average ratio ~1 by LC-MRM method suggesting equal loading of noon and midnight samples and validating the result of proteomic study where these proteins did not change.

In 1h light exposure experiment, we identified about 370 proteins (Supplementary table 5) from which about 180 proteins were further statistically analyzed (Supplementary table 6) because they were quantified in 3 or more biological replicates. Nine proteins were significantly changing in this experiment (Table 4.4 a and b). Even though these proteins were changing significantly their fold change was small. No proteins were selected for LC-MRM validation from this experiment.

DISCUSSION

Our results suggest that the circadian proteome changes in retina are small. Even though the numbers of identified and quantified proteins were very similar in the no light and 1h light exposure experiment, there was no overlap in the significantly changing proteins. This suggests that light is a much stronger stimulus than circadian rhythm to change the proteome profiles. In both experiments, all the proteins changed their amount by less than two fold between day and night.

In no light exposure experiment, stress response proteins (crystallin αB, peroxiredoxin 1) were present in lower amounts at midnight. It is known that excess light causes oxidative stress. Peroxiredoxin 1 is one of the enzymes which can prevent oxidative stress damage. If it is present in lower amounts at night it can cause lower ability of photoreceptor to cope with the oxidative stress. The ratio obtained by LC-MRM method, which is
considered to be more sensitive, did not show any changes of this protein. Since this ratio was not successfully validated, further experiments are necessary to clarify whether the amount of this protein changes in a circadian manner.

Crystallin αB is a small heat shock protein and was present in lower amount at midnight. Its mean midnight/noon protein ratio and standard deviation by both $^{18}$O labeling and LC-MRM technique were very similar (0.45±0.22 – proteomics and 0.49±0.27 – LC-MRM). The differences of crystallin expression in retina between individuals have been reported previously [101]. Previous reports have suggested that crystallin αB has an important role in retinal degeneration caused by light or genetical factors. This protein increases its amount when cells are exposed to different challenges. Several models of retinal degeneration e.g. Royal College of Surgeons (RCS), and P23H rhodopsin transgenic rats had reportedly larger amounts of retinal crystallin proteins after the onset of degeneration than normal rats [102]. Crystallin αB was reported to increase the expression in photoreceptors overlaying drusen in AMD affected human retinas [103]. Pathway analysis (Figure 4.7) identified LEDGF (lens epithelium derived growth factor) as a growth factor directly controlling expression of crystallin αB. Machida et al.[104] reported that treating light damaged normal rats with this survival factor (LEDGF prosurvival function reviewed by Shinohara et al.[105]) had a protective effect against retinal degeneration. The retinas from eyes injected in vitrous with LEDGF-GST fusion protein had 2-3 times thicker outer nuclear layer (corresponding to photoreceptor cells) than retinas from eyes treated only with vehicle (Figure 4.8) [104]. They observed higher expression of heat shock protein 25 and crystallin αB in treated retinas, suggesting that
these two proteins may have a protective role against photoreceptor degeneration. In an
other study, light induced increase of crystallin αB in the OS was reported [106]. All
these reports directly support our finding that lower amount of crystallin αB at midnight
can be responsible for the higher susceptibility of photoreceptors to light damage at night.
Our results and all these reports directly support our hypothesis that lower amount of
crystallin αB at midnight is responsible for the higher susceptibility of photoreceptors to
light damage at night. We found that the amount of RGS 9 was higher at midnight.
However, the role of RGS 9 in the circadian susceptibility of photoreceptors to the light
damage is not clear.

This study represents the first circadian study of proteome changes in the photoreceptor
OS. Shot gun quantitative proteomic approach followed by the targeted quantitative LC-
MRM technique was employed. Higher amount of Regulator of signaling 9 and lower
amounts of crystallin αB were found at midnight than at noon. Based on the results of our
study, further experiments can be designed to investigate the exact mechanisms of light
induced photoreceptor damage and the role of these two proteins in the circadian
dependent susceptibility to light damage.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide sequence</th>
<th>Native protein Q1/Q3 transition</th>
<th>Internal standard Q1/Q3 transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodopsin</td>
<td>EAAAQQQGSATTQK</td>
<td>745.0(z=2)/1020.2(z=1)</td>
<td>750.0(z=2)/1028.5(z=1)</td>
</tr>
<tr>
<td>Arrestin</td>
<td>TLVLVPLLNNR</td>
<td>661.6(z=2)/797.4(z=1)</td>
<td>666.6(z=2)/807.4(z=1)</td>
</tr>
<tr>
<td>Rod phosphodiesterase 6 α</td>
<td>FHIPQEALVR</td>
<td>605.3(z=2)/812.8(z=1)</td>
<td>610.5(z=2)/822.7(z=1)</td>
</tr>
<tr>
<td>Regulator of signaling 9</td>
<td>LVEVPTK</td>
<td>393.0(z=2)/573.5(z=1)</td>
<td>397.0(z=2)/581.5(z=1)</td>
</tr>
<tr>
<td>Crystallin α-B chain</td>
<td>VLGDVIEVHGK</td>
<td>583.3(z=2)/953.7(z=1)</td>
<td>587.3(z=2)/961.7(z=1)</td>
</tr>
<tr>
<td>Peroxiredoxin 1</td>
<td>IGHPAPSFK</td>
<td>477.6(z=2)/646.5(z=1)</td>
<td>481.6(z=2)/654.5(z=1)</td>
</tr>
</tbody>
</table>

Table 4.1 – Peptide sequences used in LC-MRM experiment
Table 4.2a - Proteins in lower amounts at midnight without light exposure

<table>
<thead>
<tr>
<th>Swiss Prot Accession</th>
<th>Protein Name</th>
<th>Swiss Prot ID</th>
<th>N</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>95% confidence interval</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P05708</td>
<td>Alpha-crystallin B chain</td>
<td>CRYAB_RAT</td>
<td>3</td>
<td>0.45</td>
<td>0.22</td>
<td>(-0.091, 0.996)</td>
<td>-4.33</td>
<td>0.049</td>
</tr>
<tr>
<td>P09606</td>
<td>Tubulin beta-2A chain</td>
<td>TBB2A_RAT</td>
<td>4</td>
<td>0.65</td>
<td>0.21</td>
<td>(0.311, 0.989)</td>
<td>-3.28</td>
<td>0.046</td>
</tr>
<tr>
<td>P10111</td>
<td>Peroxiredoxin-1</td>
<td>PRDX1_RAT</td>
<td>4</td>
<td>0.70</td>
<td>0.18</td>
<td>(0.420, 0.983)</td>
<td>-3.38</td>
<td>0.043</td>
</tr>
<tr>
<td>P23928</td>
<td>Brain acid soluble protein 1</td>
<td>BASP_RAT</td>
<td>4</td>
<td>0.77</td>
<td>0.09</td>
<td>(0.617, 0.916)</td>
<td>-4.98</td>
<td>0.016</td>
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<tr>
<td>P30009</td>
<td>Peptidyl-prolyl cis-trans isomerase A</td>
<td>PPIA_RAT</td>
<td>4</td>
<td>0.77</td>
<td>0.13</td>
<td>(0.566, 0.979)</td>
<td>-3.51</td>
<td>0.039</td>
</tr>
<tr>
<td>P37377</td>
<td>Hexokinase-1</td>
<td>HXK1_RAT</td>
<td>4</td>
<td>0.77</td>
<td>0.09</td>
<td>(0.633, 0.915)</td>
<td>-5.11</td>
<td>0.015</td>
</tr>
<tr>
<td>P49805</td>
<td>Myristoylated alanine-rich C-kinase substrate</td>
<td>MARCS_RAT</td>
<td>4</td>
<td>0.80</td>
<td>0.08</td>
<td>(0.667, 0.933)</td>
<td>-4.8</td>
<td>0.017</td>
</tr>
<tr>
<td>P61027</td>
<td>Glutamine synthetase</td>
<td>GLNA_RAT</td>
<td>4</td>
<td>0.81</td>
<td>0.09</td>
<td>(0.670, 0.943)</td>
<td>-4.51</td>
<td>0.020</td>
</tr>
<tr>
<td>P84817</td>
<td>Retinaldehyde-binding protein 1</td>
<td>RLBP1_MOUSE</td>
<td>3</td>
<td>0.83</td>
<td>0.06</td>
<td>(0.684, 0.983)</td>
<td>-4.79</td>
<td>0.041</td>
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Table 4.2b – Proteins in higher amounts at midnight without light exposure

<table>
<thead>
<tr>
<th>Swiss Prot Accession</th>
<th>Protein Name</th>
<th>Swiss Prot ID</th>
<th>N</th>
<th>Mean</th>
<th>Standard deviation</th>
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<th>t-value</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>P85108</td>
<td>Mitochondrial fission 1 protein</td>
<td>FIS1_RAT</td>
<td>4</td>
<td>1.07</td>
<td>0.04</td>
<td>(1.009, 1.131)</td>
<td>3.66</td>
<td>0.035</td>
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<tr>
<td>Q05175</td>
<td>Ras-related protein Rab-10</td>
<td>RAB10_MOUSE</td>
<td>3</td>
<td>1.15</td>
<td>0.05</td>
<td>(1.022, 1.275)</td>
<td>5.03</td>
<td>0.037</td>
</tr>
<tr>
<td>Q63716</td>
<td>Alpha-synuclein</td>
<td>SYUA_MOUSE</td>
<td>3</td>
<td>1.18</td>
<td>0.07</td>
<td>(1.013, 1.350)</td>
<td>4.64</td>
<td>0.044</td>
</tr>
<tr>
<td>Q9Z275</td>
<td>Regulator of G-protein signaling 9</td>
<td>RGS9_RAT</td>
<td>4</td>
<td>1.48</td>
<td>0.30</td>
<td>(1.010, 1.958)</td>
<td>3.25</td>
<td>0.048</td>
</tr>
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</table>

*a* Number of biological replicates,  
*b* Mean midnight/noon ratio for all biological replicates,  
*c* Standard deviation of the mean midnight/noon ratio,  
*d* 95% confidence interval for the mean midnight/noon ratios (min, max)
<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Average&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio</th>
<th>Stdev&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CV(%)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodopsin</td>
<td>1.00</td>
<td>0.11</td>
<td>11.20</td>
<td></td>
</tr>
<tr>
<td>Arrestin</td>
<td>1.01</td>
<td>0.04</td>
<td>3.71</td>
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<tr>
<td>Rod specific phosphodiesterase 6 α</td>
<td>1.01</td>
<td>0.04</td>
<td>3.55</td>
<td></td>
</tr>
<tr>
<td>Regulator of signaling 9</td>
<td>1.19</td>
<td>0.05</td>
<td>3.92</td>
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<tr>
<td>Crystallin α-B chain</td>
<td>0.49</td>
<td>0.27</td>
<td>56.01</td>
<td></td>
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<tr>
<td>Peroxiredoxin 1</td>
<td>1.06</td>
<td>0.22</td>
<td>20.90</td>
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</tbody>
</table>

Table 4.3 – Results of LC-MRM validation
### Table 4.4a – Proteins in lower amounts at midnight after 1h light exposure

<table>
<thead>
<tr>
<th>Swiss Prot Accession</th>
<th>Protein Name</th>
<th>SwissProt ID</th>
<th>N</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>95% Confidence Interval</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P28663</td>
<td>Beta-soluble NSF attachment protein</td>
<td>SNAB_MOUSE</td>
<td>3</td>
<td>0.86</td>
<td>0.05</td>
<td>(0.732, 0.988)</td>
<td>-4.70</td>
<td>0.042</td>
</tr>
<tr>
<td>P70566</td>
<td>Tropomodulin-2</td>
<td>TMOD2_RAT</td>
<td>3</td>
<td>0.86</td>
<td>0.01</td>
<td>(0.831, 0.891)</td>
<td>-19.97</td>
<td>0.002</td>
</tr>
<tr>
<td>P60881</td>
<td>Synaptosomal-associated protein 25</td>
<td>SNP25_RAT</td>
<td>4</td>
<td>0.84</td>
<td>0.04</td>
<td>(0.776, 0.912)</td>
<td>-7.27</td>
<td>0.005</td>
</tr>
<tr>
<td>P60711</td>
<td>Actin, cytoplasmic 1</td>
<td>ACTB_RAT</td>
<td>4</td>
<td>0.89</td>
<td>0.05</td>
<td>(0.803, 0.975)</td>
<td>-4.10</td>
<td>0.026</td>
</tr>
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</table>

### Table 4.4b – Proteins in higher amounts at midnight after 1h light exposure

<table>
<thead>
<tr>
<th>Swiss Prot Accession</th>
<th>Protein Name</th>
<th>SwissProt ID</th>
<th>N</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>95% Confidence Interval</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q6MG60</td>
<td>N(G),N(G)-dimethylarginine dimethylaminohydrolase 2</td>
<td>DDAH2_RAT</td>
<td>3</td>
<td>1.13</td>
<td>0.02</td>
<td>(1.0825, 1.1766)</td>
<td>11.85</td>
<td>0.007</td>
</tr>
<tr>
<td>P11951</td>
<td>Cytochrome c oxidase polypeptide Vlc-2</td>
<td>CX6C2_RAT</td>
<td>3</td>
<td>1.18</td>
<td>0.06</td>
<td>(1.0272, 1.3397)</td>
<td>5.05</td>
<td>0.037</td>
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<tr>
<td>P31044</td>
<td>Phosphatidylethanolamine-binding protein 1</td>
<td>PEBP1_RAT</td>
<td>4</td>
<td>1.20</td>
<td>0.10</td>
<td>(1.0407, 1.3522)</td>
<td>4.02</td>
<td>0.028</td>
</tr>
<tr>
<td>P05065</td>
<td>Fructose-bisphosphate aldolase A</td>
<td>ALDOA_RAT</td>
<td>4</td>
<td>1.38</td>
<td>0.08</td>
<td>(1.2573, 1.500)</td>
<td>9.93</td>
<td>0.002</td>
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<tr>
<td>Q99MH9</td>
<td>Bardet-Biedl syndrome 2 protein homolog</td>
<td>BBS2_RAT</td>
<td>3</td>
<td>1.79</td>
<td>0.29</td>
<td>(1.078, 2.509)</td>
<td>4.77</td>
<td>0.041</td>
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Figure 4.3 – Workflow for Forward Labeling Circadian Experiment

All 4 biological replicates were subjected to the same workflow. The scheme for the forward labeling experiment is shown here. The reverse labeling experiment followed the same scheme but noon OS were labeled with $^{18}$O water and midnight OS were labeled with $^{16}$O water. See Materials and Methods for detailed description.
Figure 4.4 – Representative 1D-SDS Gel Image
Figure 4.5 - Statistical Analysis of Crystallin αB and Regulator of Signaling 9 Midnight/noon Protein Ratios

Distribution analysis of midnight/noon ratios and the probability box plot is shown for regulator of signaling 9 (N=4) (a) and crystallin αB (N=3) (b). The diamond represents the 95% confidence interval of each protein. The square box is 25%-75% percentile. The vertical line represents the mean midnight/noon ratio value. P-value animations from two
sided t-test of the mean noon/midnight ratio against the hypothesized mean = 1 (no change) for regulator of signaling 9 (N=4) and (c) crystallin αB (N=3) (d) are shown.

Figure 4.6 - Representative Image of Immunofluorescence with Crystallin α-B-chain Antibody.

20 um sections were incubated overnight with primary polyclonal antibody against crystallin α-B-chain (Stressgen, Ann Arbor, MI, product no.: SPA-223), diluted in PBST overnight at 4 °C. Sections were rinsed by PBST and incubated with indocarbocynine (Cy3 or Cy5)-conjugated secondary anti-rabbit antibodies. Sections were then rinsed and mounted in 50 μl of 2% 1,4-diazabicyclo-2,2,2–octane in 90% glycerol to slow photobleaching. Faint staining can be observed in the photoreceptor inner (IS) and outer (OS) segments.
Figure 4.7 – Network analysis of crystallin αB
Figure 4.8 – LEDGF (left) and vehicle treated eye after in a light exposed rat

Figure reprinted with permission from Association for Research in Vision and Ophthalmology (all rights reserved). This figure was originally printed as Figure 1a in Machida S, Chaudhry P, Shinohara T, et al. Lens epithelium-derived growth factor promotes photoreceptor survival in light-damaged and RCS rats. Invest Ophthalmol Vis Sci. 2001;42:1087-1095.
CHAPTER 5 – FUTURE DIRECTIONS AND SUMMARY

Future Directions

Many questions still remain unanswered and the search for answers to these will be the focus of future studies. Some of these questions are discussed below.

FUTURE METHOD IMPROVEMENT

Is it possible to improve the developed quantitative workflow?

The developed workflow is mature and can be applied to analyze other samples; however there is always space for improvement. Only the proteins identified by 2 or more peptides with Mascot score of at least 20 are submitted for quantification. This limits the number of proteins that can be quantified by $^{18}$O labeling. Peptide identification, unlike quantification, depends on the MS/MS spectra. Since our shot-gun proteomic analysis was carried out in a data dependent manner, only the 5 most intense peptides were submitted for MS/MS analysis at a given time. Therefore many peptides were not submitted for MS/MS analysis and thus were not identified. This means that the requirement of 2 peptides identified from 1 protein prior to quantification causes many proteins to have no quantitative information or have this information from only 1 biological replicate. No further statistical analysis can be carried out on such data and therefore it is a high priority to reduce the number of missing values.

A possible solution to increase the number of identified peptides is to create a library of all identified peptides for particular biological sample. Since the same biological sample is analyzed by LC-MS/MS more than once (in our case it was 48 times), multiple raw
files are available. Each raw file contains different MS/MS spectra. This means different peptides will be identified for each raw file. The mentioned library would contain the sequence information for precursors which were submitted for MS/MS analysis in any of the LC-MS/MS runs. In case of missing MS/MS information in one raw file, the library could be used to retrieve sequence information for precursors which were submitted to MS/MS in the past. This workflow would contain the following steps:

1. Each LC-MS/MS raw file would be searched by Mascot - as it is in the current workflow.

2. All search results would be combined and a peptide sequence library would be created for the particular sample e.g. for the rat OS. In other words, this library would contain all precursor ion masses and sequences which were ever identified by Mascot in rat OS. If the sample is different than rat OS, a new library is to be created.

3. Precursor ions which were missing sequences due to missing MS/MS spectrum would be extracted from an LC-MS/MS raw file and compared to the library of identified peptides.

4. Sequences for precursor ion masses would be retrieved from the library. In the case of retrieving multiple sequences for one precursor ion mass, LC retention time would be used to distinguish the hydrophobicity of the sequence.

5. The newly identified peptides would be quantified.

This approach is expected to eliminate missing data points and thus increase the number of proteins quantified in more than 1 biological replicate.
FUTURE BIOLOGICAL STUDIES

Is crystallin αB a protective protein?

To investigate whether crystallin αB is a protective protein, crystallin αB knock out mice can be used. An experiment similar to the one described by Organisciak et al.[70] will be carried out. The knock out and control mice will be reared in cyclic light for at least 40 days. All mice will be dark adapted for at least 16 h and then randomly assigned to two groups. One group of control and knock out mice will be exposed to strong green light (2000 Lux, wavelength range 490-580 nm) for 8h starting at 9 am, then placed into dark for 14 days. The other group will be kept in dark for the whole experimental period. Eyes will be dissected and examined for morphology changes. We expect to see differences in morphology between the knock out and the control mice after light exposure. If crystallin αB is indeed a protective protein, the photoreceptors in knock out mice after light exposure should undergo apoptosis while the photoreceptors in control mice are expected to be protected.

Are there circadian differences in post-translational modifications of OS proteins?

The identified circadian differences in protein amounts are strikingly small. In this study, we focused only on proteins that changed amounts between noon and midnight, however proteins also may change their post-translational modifications in circadian manner. These modifications can change or regulate protein function. In order to study these modifications, OS samples would have to be enriched for the particular modification (e.g. phosphorylation, glycosylation, etc.) and then analyzed by the above described $^{18}$O
labeling workflow. The identified modification differences can provide valuable information about the protective mechanism of photoreceptor cells.

Are there circadian differences in photoreceptor transcriptome?

The pathway analysis identified several transcription factors which control expression of crystallin αB – LEDGF, SP1, c-Fos, p52 (Figure 4.7). It is in our interest to investigate whether the amount of mRNA corresponding to these transcription factors change their amount or to identify other transcription factors which would change between nighttime and daytime. This study would uncover upstream circadian changes suggested by our proteomic study. In order to study the transcriptome, photoreceptor nuclei would have to be separated from other nuclei in the retina and this is still a very challenging task. At the present time, the only technique which can isolate photoreceptor nuclei is laser capture microscopy (LCM). In this technique, cryosections (10µm thick) of frozen eyes are placed onto a special membrane coated glass slide and stained with cresyl violet to increase contrast of individual retinal layers. This slide is then inserted into the LCM machine and photoreceptor nuclei are cut out with a laser and collected on a membrane coated cap. Examples of retinal section before and after cutting as well as the cut out photoreceptor nuclei are shown in Figure 5.1. After collecting sufficient amounts of photoreceptor nuclei, caps are retrieved from the LCM machine and placed on top of a small tube with lysis buffer. Tube is inverted several times to ensure that all acquired tissue is released from the cap and cells are properly lysed. The next day, mRNA is extracted from the tissue and analyzed by either RT-PCR or microarray. Experiments to test this technique on rat eyes have already carried out. Special care had to be taken in
sample preparation not to contaminate the biological sample with RNAases. Dissected eyeballs in the OCT were immediately frozen in liquid nitrogen. Slides were only handled with gloves and cryostat was always properly cleaned to prevent contamination. We tested several thicknesses (10, 20, 30µm) of frozen sections and 10 µm sections were the most suitable. Thicker sections were difficult to cut evenly on cryostat because retinas would tear, making it impossible to collect intact photoreceptor nuclei.

Figure 5.1 – LCM of Photoreceptor Nuclei

A frozen section of retina is stained with cresyl violet to increase contrast of retinal layers. The section is then inserted into LCM and the photoreceptor nuclei are dissected by laser. From left to right: retina before dissection, retina after dissection, dissected nuclei
Summary

The above studies describe proteomic method development and optimization for studying membrane rich samples – OS. The final workflow was used to study light induced and circadian changes in the OS proteome. Our results revealed the previously unpublished reduction of RK upon light exposure. This protein is responsible for rhodopsin deactivation and its reduction can cause prolonged rhodopsin signaling which was reported to be important in light induced apoptosis [67]. Therefore, the reduction of RK may be a contributing factor of light induced cell death. Elevation of heat shock proteins and ubiquitin in the OS upon light exposure was also discovered, suggesting that excessive light exposure may cause accumulation of misfolded proteins and activates the ubiquitin-proteasome protein degradation system. Another small heat shock protein, crystallin αB was found to be expressed lesser amount in OS at midnight than at noon, suggesting that this protein may be responsible for the higher susceptibility of the photoreceptor to light induced apoptosis at night. Crystallin αB has previously been reported to be elevated in light exposed OS [106], suggesting a protective role of this protein against light-induced photoreceptor cell death.

These studies represent the first reports of quantitative proteomics in OS. They provide information about proteins which can possibly protect photoreceptor cells from light induced apoptosis, the putative cell death mechanism in many retinal degenerative diseases.
APPENDIX 1 – PROTOCOL FOR EYEBALL AND RETINA DISSECTION AND OUTER SEGMENT PREPARATION

Gather the following:

Stand
Paper towels
Box of razor blades
Tweezers
Ice buckets
Liquid nitrogen
Buffer solutions – test tubes in ice bucket
Glass test tubes – 2 15x 85, 8 tubes 12x85
Glass syringes with mesh filters
Homogenizer
Beckmen centrifuge tubes 5 mL – catalog no. 344057
Swinging bucket rotor Ti-55, 50.1 was used previously
Beckmen Coulter Class S ultracentrifuge
Single use surgical blade (no. 11, Feather Safety Razor Co. Ltd., Japan)
Small spring scissors (Castroviejo Spring Scissors cat. no. 15017-10, Fine Science Tools Inc., San Francisco)
Small Petri dishes

Preparation of the dark room

- Place paper towels on the benches
- Put the syringes on stands
• Find a container to sacrifice the rats in
• Ensure CO₂ tank is full

Solution preparation

Phosphate buffered saline (PBS) – NaCl 8g/L, NaH₂PO₄ 0.148 g/L

Phosphate buffer (PB) – 2.022 g Na₂HPO₄ g and 0.588 g NaH₂PO₄ add water to 100mL

100 x Phosphatase inhibitors and 10 x Protease inhibitors – 1 vial 10 mL (Sigma Aldrich, Saint Louis, catalog number P5726 and P2714)

<table>
<thead>
<tr>
<th>Solution</th>
<th>PB (mL)</th>
<th>Sucrose (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32% sucrose PB</td>
<td>3.75</td>
<td>4.80</td>
</tr>
<tr>
<td>37% sucrose PB</td>
<td>3.75</td>
<td>5.55</td>
</tr>
<tr>
<td>42% sucrose PB</td>
<td>3.75</td>
<td>6.30</td>
</tr>
<tr>
<td>47% sucrose PB</td>
<td>3.75</td>
<td>7.05</td>
</tr>
</tbody>
</table>

Add phosphatase (150 µL) and protease (1.5 mL) inhibitors then add water to 15 mL

Aliquote 2 mL of 47% of sucrose PB, 1.1 mL of 42% sucrose PB, 1.5ml of 37% sucrose PB, 1 mL of 32% sucrose PB – keep solutions on ice at all times

Centrifuge preparation

• Place Ti-55 or 50.1 rotor into ultracentrifuge
• Turn centrifuge on to cool to 4 deg C

Kill at night

• Move rats from the animal room at 7 am and placed into dark room
• At **midnight** sacrifice the rats with \( CO_2 \)

Retina preparation

• Dissect the eye – grab eye with round tweezers, cut cornea with razor blade, remove the lens, and squeeze retina out
• Rinse retina into phosphate buffered saline solution with protease and phosphatase inhibitors in Petri dish on ice
• Place retina into labeled cryotubes and snap freeze in liquid nitrogen. Tubes must be labeled before they are placed into liquid nitrogen because Sharpie does not write on cold tubes!
• Wrap cryotubes with correctly labeled aluminum foil. Place back into liquid nitrogen. When all retinas are dissected, transfer wrapped tubes into nitrogen dewer for storage.

**Kill during the day**

• Move rats from the animal room **after** their night cycle begins - **after 6pm and before 6 am**
• Place rats into the dark room and sacrificed at **noon**

Other procedures are the same as for night sacrificing

**Preparation of outer segments pellet**

• Place retinas into -20 °C room overnight
• Next day, slowly thaw retinas on ice
• Place completely thawed retina into glass tube (which belongs to the Teflon homogenizer) with 1mL of 47% sucrose PB
• Homogenize for 5 s, again homogenize for 15 s
• Pour homogenate into glass syringe, let drip into the 5mL Beckmen centrifuge tube
• Wash homogenizer with an other 1 mL of 47% sucrose PB
• Overlay with 1.1 mL of 42% of sucrose PB, 1.5 mL or 37% sucrose PB and fill with 32% sucrose PB to 2-3mm from the top of the tube
• Gently place tube into swinging bucket and screw the top on.
• Balance two tubes against each other for the centrifuge
• Spin at 4 °C at 35000 rpm (50.1 rotor or Ti-55) for 1 hour
• Collect band 1 and band 2 – 500 mL per one tube of each fraction
• Add equal volume of 20% sucrose PB to the collected fractions (1:1 ratio of ROS and 20% sucrose PB)
• Spin at 4 °C at the bench top centrifuge for 5 min save the pellet at -80 °C
Preparing sample for immunohistochemistry

Prior to killing, animals are treated the same way as in the retina dissection experiment.

Buffers:

10% paraformaldehyde solution in 100 mM phosphate buffer – MUST BE DONE in the hood!!! Put paraformaldehyde into a special glass bottle with magnetic stirrer. While gently heating and stirring (using hot plate) dissolve paraformaldehyde in 100mM phosphate buffer. This may take up to 1 hour. At first, solution is very cloudy and milky, after reaching 60°C it clears up. Cool down solution and store at – 20°C until use. Dilute this solution to 4% paraformaldehyde right before fixation.

Phosphate buffered saline (PBS) – see above

2:1 mixture of 20% sucrose:Optimum Cutting Temperature (OCT) compound

- With a permanent marker mark the bottom of the eye
- Cut out the whole eyecup and place into 50 mL conical tube with 4% paraformaldehyde in 0.1M phosphate buffer pH 7.3 to fix the tissue
- Allow several minutes (up to 5 min) to let fixative enter the eye
- Transfer eyeball from fixative into PBS in Petri dish. Poke hole into cornea with stainless steel surgical blade.
- Insert small spring scissors (Castroviejo Spring Scissors cat. no. 15017-10, Fine Science Tools Inc., San Francisco) into the hole and cut out cornea completely – cut close but away from the edge of sclera.
- Take lens out carefully, taking extra care not to change shape of the eyeball, eyecup can easily collapse in when lens is taken out!
- Place the eyecup into 50 mL tubes with ~7.5mL of 4% paraformaldehyde solution to fix for 6 more hours at 4 °C on a rocker. Wrap tubes with aluminum foil to prevent bleaching before freezing.
- After fixing replace paraformaldehyde solution with 5% (w/v) sucrose in phosphate buffer. Incubate 5 min at 4 °C on a rocker.
- Carefully pour out solution and replace with 5% (w/v) sucrose in phosphate buffer. Incubate 30 min at 4°C on a rocker.
• Carefully pour out solution and replace with 10% (w/v) sucrose in phosphate buffer. Incubate 5 min at 4°C on a rocker.
• Carefully pour out solution and replace with 10% (w/v) sucrose in phosphate buffer. Incubate 30 min at 4°C on a rocker.
• Carefully pour out solution and replace with 15% (w/v) sucrose in phosphate buffer. Incubate 5 min at 4°C on a rocker.
• Carefully pour out solution and replace with 15% (w/v) sucrose in phosphate buffer. Incubate 30 min at 4°C on a rocker.
• Carefully pour out solution and replace with 20% (w/v) sucrose in phosphate buffer. Incubate 5 min at 4°C on a rocker.
• Carefully pour out solution and replace with 20% (w/v) sucrose in phosphate buffer. Incubate 30 min at 4°C on a rocker.
• Carefully pour out solution and replace with 2:1 mixture of 20% (w/v) sucrose in phosphate buffer: OCT. Incubate overnight at 4°C on a rocker.
• Transfer specimen into cryo-molds with 2:1 mixture of 20% (w/v) sucrose
• Orient eyeballs according to the picture below.
• Freeze specimens by isopentane chilled with liquid nitrogen
• Preserve frozen samples in -80°C. To prevent drying of the tissue store it with ice in a sealed plastic bag.
APPENDIX 2 – PROTOCOL FOR IN GEL DIGESTION FOLLOWED BY ¹⁸O LABELING

1. Dissolve samples in 5% SDS 200mM Tris buffer
2. Reduce and alkylate proteins in solution by 10 mM DTT and 25 mM iodoacetamide
3. Separate samples on 1 D PAGE bis-tris system from Invitrogen (10mm gels, 4-12% gradient, 90 min, 160V)
4. Obtain image of the gel.
5. Cut each sample lane into 6 bands, ensure that cutting is consistent between lanes and make note of cutlines.
6. Wash all bands with 200mM ammonium bicarbonate in 50% acetonitrile (three times) and 100% acetonitrile then dry on the speed vac(once).
7. Add trypsin (~2 ug/sample) and 100 μL of 100mM ammonium bicarbonate
8. Crush bands into small pieces.
9. Incubate overnight
10. Extract peptides by sonication, and washing with 0.1% formic acid in 60 % acetonitrile (twice) and 100% acetonitrile (once)
11. Dry digests completely
12. Add 200 mM citrate buffer pH 6 and dry
13. Add 16 and 18 O water to all samples accordingly to dilute citrate buffer to 100 mM
14. Add trypsin to the samples in either 16 or 18 O water (1μg/sample)
15. Incubate overnight.
16. Denature trypsin by adding 8M Guanidine HCl to samples in equal volume as the reaction volume – must be either in 16 or 18O water!!
17. Add 1 M Tris pH 8 to raise pH to ~8 – usually 2-3 μL
18. Reduce and alkylate trypsin by 10 mM DTT (in acetonitrile) and 25 mM iodoacacetamide (in acetonitrile)
19. Mix samples in 1:1 ratio of 16 and 18O reactions – pipet the certain volume from 16O reaction and place into new tube, then pipet the same volume from 18O reaction and put in the tube where 16O aliquot is. Mix right before desalting to prevent 18O exchange if trypsin was not properly deactivated!
20. Desalt samples on C18 column from Vydac
   (1. wet column with 200 μL of 100% methanol, 2. wash column with 200 uL of 0.1% formic acid in 60% acetonitrile 3. equilibrate column with 200 uL of 0.1% formic acid in HPLC water (repeat 3 times) 4. load sample onto column from top 5. wash column with 200 uL of 0.1% formic acid in water 6. elute sample with 200 uL of 0.1% formic acid in 60% acetonitrile)
21. Dry samples.
22. Reconstitute samples in 0.1% formic acid
23. Inject samples into LC-MS/MS
REFERENCES


