STRUCTURAL ENDEAVORS IN THE RETINOID (VISUAL) CYCLE

by

LUKAS HOFMANN

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Pharmacology

CASE WESTERN RESERVE UNIVERSITY

August, 2017
CASE WESTERN RESERVE UNIVERSITY
SCHOOL OF GRADUATE STUDIES

We hereby approve the thesis/dissertation of

Lukas Hofmann

candidate for the degree of Doctor of Philosophy*

Committee Chair

Jason Mears, Ph.D.

Committee Member

Krzysztof Palczewski, Ph.D.

Committee Member

Marvin Nieman, Ph.D.

Committee Member

Focco van den Akker, Ph.D.

Committee Member

Marcin Golczak, Ph.D.

Date of Defense

05.19.2017

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

First, I would like to thank my advisor, Dr. Krzysztof Palczewski for giving me such interesting and challenging projects, dedicating his valuable time to help me through my experimental work and all aspects of my training, and for also taking time to mentor me during my development as a scientist and beyond.

Thank you to all my colleagues in the Palczewski laboratory, especially Drs. Tsybovsky and Alexander for all their motivational support and for their guidance in our experimental endeavors, thank you. Dr. Webster provided tremendous support for all manuscripts. David Peck and Tivi were essential in all ways providing solutions to my experimental necessities. Dr. Sun, thank you for providing me the essential training that I was able to use throughout this work.

I particularly wish to thank Drs. Marcin Golczak and Ardeschir Vahedi-Faridi for their help and support with my projects. Marcin and Ardi have been mentors and friends throughout my tenure as a Ph.D. student. Thank you for your time and friendship.

I owe a special thanks to our collaborators, Dr. von Lintig and his group members for their support of my research. Thanks to Dr. Darwin O. Babino for his generous support with retinoids.

I owe special thanks to the members of my Ph.D. thesis committee, Drs. Jason Mears, Marvin Nieman, Focco van den Akker, and Marcin Golczak for all their insightful and constructive analysis of my work.
During my graduate studies, I was supported by a Swiss National Science Foundation (SNSF) Doc.Mobility fellowship. I would like to thank the research committee for financial support.
Abbreviations

ANS, 1-anilinonaphthalene-8-sulfonate; BFC, BODIPY FL L-cystine; BCM, blue cone monochromacy; CB1, cannabinoid receptor 1; CR, cone–rod homeobox; CPM, N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl] maleimide; DDM, n-dodecyl β-D-maltopyranoside; DHRS, dehydrogenase/reductase SDR family member; DSF, differential scanning fluorimetry; EPPIC, Evolutionary Protein-Protein Interface Classifier; EL, extracellular loop; $E_{\alpha}^{I}$, isomerization energy; $E_{\alpha}^{T}$, thermal activation energy; $E_{\alpha}^{P}$, photochemical activation energy; EM, electron microscopy; GPCR, G protein–coupled receptor; H/D, hydrogen deuterium; HRM, high resolution melt analysis; IL, intracellular loop; IR, infrared; Kd, dissociation constant; LCA, Leber congenital amaurosis; LCR, locus control region; L/LWS, human red long wavelength sensitive; LWS, long wavelength sensitive; MD, molecular dynamics; MS, mass spectrometry; M/LWS, human green long wavelength sensitive; MWS or Rh2, medium wavelength sensitive; NMR, Nuclear magnetic resonance spectroscopy; MW, molecular weight; NRL, neural retina leucine zipper; NR2E3: nuclear receptor subfamily 2, group E, member 3; PBS, phosphate buffered saline; PDH, photoreceptor dehydrogenase; PISA, Proteins, Interfaces, Structures and Assemblies; PR, promoter region; QM/MM, quantum mechanics/molecular mechanics; RER, rhodopsin enhancer region; Rh1, rhodopsin; RDH12, retinol dehydrogenase 12; RMSD, root-mean-square deviation; ROS, rod outer segments; RT-PCR, reverse transcription polymerase chain reaction; SDR, short-chain dehydrogenase;
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SWS1, short wavelength sensitive type 1; (S1), first electronically excited singlet state; (S0), ground electronic state; TM, transmembrane helix; UV, ultraviolet; VIS, visible; 5-HT\textsubscript{1B}, 5-hydroxytryptamine receptor 1B; 5-HT\textsubscript{2B}, 5-hydroxytryptamine receptor 2B.
“Vitamin A is the precursor of visual purple as well as the product of its decomposition. The visual processes therefore constitute a cycle.” This statement by George Wald in 1935 described in astounding simplicity the visual cycle and the recycling of the universal chromophore 11–cis retinal. Here, we present recent findings derived from genetic, biochemical and physiological studies that provide a more advanced understanding of the retinoid cycle and color perception. The energetics of cis–trans isomerization of 11–cis–retinal accounts for color perception in the wide range of our visible spectrum and explains how human photoreceptors can absorb light in the near infrared (IR). Structural homology models of visual pigments reveal complex interactions of the opsin protein moieties with 11–cis–retinal and how certain color blinding mutations can impair secondary structural elements of these G protein–coupled receptors (GPCRs). Studies of hydrogen/deuterium exchange also confirmed the dimeric state of human green cone opsin, and revealed the potential role of intracellular loop 2 at the dimer interface in opsin activity. Additionally, these
studies demonstrated an alternative chromophore exit site involving a Pro-Pro motif at transmembrane helix 4. Once all-trans-retinal is released from opsin, it is reduced by a retinol dehydrogenase in concert with its co-factor NADPH. This first reaction of the retinoid cycle results in reduced all-trans-retinol, which is then recycled back to 11-cis-retinal which serves as the light-sensitive chromophore for visual pigment molecules. Finally, we solved the structure of the Drosophila melanogaster photoreceptor dehydrogenase. The crystal structure of this enzyme provided insight into the first reaction step of the visual cycle and revealed the likely location of disease related mutations in the homologous human retinol dehydrogenase 12.
Chapter 1

Advances in understanding the molecular basis of the first steps in color vision

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1.1 Introduction

Color vision provides the primary human sensory perception of our environment and its understanding requires input from a variety of scientific fields (Bowmaker, 2008). This is initiated when photons of light penetrate the eye to reach the retina causing ‘excitation and photoisomerization’ of the 11-cis-retinylidene moieties bound to their protein opsins. Photoactivation of these visual GPCRs starts a series of enzymatic reactions collectively termed as phototransduction (Polans et al., 1996; Ridge et al., 2003; Yau and Hardie, 2009). Photopigments represent an exclusive class of receptor proteins that cannot be activated by a classic molecular ligand (Kefalov, 2012b; Orban et al., 2014; Palczewski, 2006a; Rieke and Baylor, 1998; Salon et al., 2011). Instead, photopigments contain a prosthetic group, 11-cis-retinal, that once photoisomerized must be continuously regenerated to maintain vision via the retinoid cycle (termed in the past as the visual cycle) (Kiser et al., 2014; Palczewski, 2012; von Lintig et al., 2010). Thus, understanding how light interacts with nature’s most sophisticated receptors – GPCRs – represents a unique scientific challenge. The phototransduction pathways within rods and cones are quite similar. In principle these pathways are designed on a common strategy involving homologous gene products with similar functions that differ significantly in some properties such as visual pigments sensitivities to light and their regeneration, kinetics and regulation of the reactions they initiate (reviewed in (Imai et al., 2005; Imamoto and Shichida, 2014a; Kefalov, 2012b; Korenbrot, 2012; Luo et al., 2008; Wang and Kefalov,
Moreover, vision is the major sensory perception system in various species, which makes it a favored target for comparative anatomy. In this review we address several topics of current interest in the first steps in color vision. These include the energetic aspects, disease phenotypes of color vision, and a comparative analysis of the organization of visual color receptors in different species.

1.2 Terminology and phylogeny of human cone visual pigments

1.2.1 Background

Visual pigments consist of different protein moieties and universal chromophore 11-cis-retinal (Nathans, 1999; Stenkamp et al., 2002). The visual system of vertebrates encompasses five evolutionarily distinct classes of visual pigments: rhodopsin (Rh1), LWS, MWS (or Rh2), SWS1 and SWS2. Whether and how melanopsin contributes to vision is a topic of current research (Barrionuevo and Cao, 2014; Horiguchi et al., 2013; Schmidt et al., 2014b). All bind to the universal chromophore, 11–cis–retinal via a Schiff base, thus the difference in absorption originates from the different opsin protein moiety. Vertebrate photopigments contain glutamate as a primary counter anion in their third transmembrane sequence at position 113 to stabilize the protonated Schiff base (Collin, 2004; Jacobs, 2009; Nathans, 1999). Invertebrate visual pigments contain Y113 or F113 and a primary counter ion at position E181 in the second extracellular loop.
(EL2). (Ramos et al., 2007; Terakita et al., 2004; Terakita et al., 2000). The Rh1 class consists of rhodopsins responsible for scotopic vision. Phylogenetic analyses based on amino acid sequences revealed that the Rh1 class evolved from an ancestral short wavelength sensitive cone pigment about 540 million years ago (Bowmaker, 2008; Collin et al., 2003; Okano et al., 1992; Peichl, 2005). The other four classes known as cone pigments are responsible for photopic vision. Cone visual pigments are categorized according to both their maximum wavelengths of light absorption ($\lambda_{max}$) with bound chromophore and amino acid sequences: long wavelength sensitive (LWS) pigments absorb at a $\lambda_{max}$ of $500 - 570$ nm, medium wavelength sensitive (MWS or Rh2) pigments absorb at a $\lambda_{max}$ of $480 - 530$ nm and two short wavelength sensitive (SWS1, SWS2) pigments absorb at a $\lambda_{max}$ of $354 - 445$ nm and a $\lambda_{max}$ of $400 - 470$ nm, respectively (Hunt et al., 2009; Jacobs, 2008; Yokoyama, 2008).

1.2.2 Unique photoreceptor physiology improves color perception

Rod and cone photoreceptors form a unique blend of these cells in the retina. Comparison of retinas among different species revealed that the organization of the retina and photoreceptor cells varies as much as the species themselves (Walls, 1942). For instance, the ratio between rod and cone cells in nocturnal species is about 200:1 whereas it is about 20:1 in diurnal species (Peichl, 2005). Furthermore, expression of two different classes of opsins, SWS and either MWS or LWS in one photoreceptor cell is an exclusive feature found in the house mouse, guinea pig and rabbit (Rohlich et al., 1994). SWS photopigments are
unique cone pigments localized at the autosomal loci of their genes and they display the highest variability among species with respect to both their organization within the retina and their absorption maxima ranging from 360 nm for the SWS type 1 (SWS1) to 420 nm for the SWS type 2 (SWS2) pigment (Hunt and Peichl, 2014). The ability to tune 11−cis−retinal over such a broad range is due to several factors including the variable protonation state of the retinylidene moiety (Lewis et al., 2000; Nathans, 1990; Terakita et al., 2004). Furthermore, oil droplets, a special feature, are found in photoreceptor cells of birds, turtles, lizards, some fish and Marsupialia (Ahnelt et al., 1996; Arrese et al., 1999; Hannover, 1840). Oil droplets supposedly improve the quality of vision by increasing the signal to noise ratio, color contrast and hue discrimination (Vorobyev, 2003). This improved sensitivity is also maintained by a number of carotenoids located in oil droplets that absorb light at specific wavelengths (Goldsmith et al., 1984; Hart et al., 2006; Ives et al., 1983; Knott et al., 2010; L. Fischer 1984).

1.2.3 Properties of trichromacy

Trichromatic vision implies the ability to detect light with three independent receptors differing in their wavelength sensitivities. Differences in \( \lambda_{max} \) between human long wavelength sensitive (L/LWS) and human medium wavelength sensitive (M/LWS) cone pigments improve distance−dependent discrimination between young and mature leaves, leaves and fruits, or ripe and unripe fruits (Bompas et al., 2013; de Lima et al., 2015; Dominy and Lucas, 2001; Matsumoto
et al., 2014; Melin et al., 2014; Regan et al., 2001). Improved detection and selection of nutrients contributes further to an evolutionary advantage. Others have proposed an advantage of color vision can be found in the faster recognition and better memorization of colored things (Bredart et al., 2014; Gegenfurtner and Rieger, 2000; Wichmann et al., 2002). Environmental factors and nocturnal living habits have fostered a reduction from four to three classes of cone photopigments in mammals including humans (Bowmaker, 2008). Humans carry the Rh1 rhodopsin gene, as do all mammals, along with two variants of LWS cone opsin genes: the L/LWS and M/LWS cone opsin genes responsible for long wavelength and medium wavelength absorption. A third opsin encoded by the SWS1 gene is needed to attain trichromacy (Hunt et al., 2009; Nathans, 1999). Further, the three cone photopigments with their distinct sensitivities can match the visible spectrum by combination of three colors, namely red, green and blue (Figure 1).
Figure 1: Shown are the visible and IR spectra with normalized cone and rod spectral sensitivities according to the 10–deg fundamentals based on the Stiles and Burch 10–deg color matching functions (CMFs) (Stockman and Sharpe, 2000). The normalized sensitivity of the visual spectrum is combined with the corresponding IR spectrum determined by the two–photon effect. The corresponding IR spectrum shows a sensitivity maximum of 50% due to the two–photon effect. The white vertical line separating the classical visible from the near IR spectra indicates the boundary between these two spectra.

An opposing processing theory of color vision is another physiological model which takes into account that certain pairs of colors cannot be seen together, e.g. red and green or blue and yellow (Figure 2) (Dacey, 1996; Lee, 2014; Rowe, 2002). Electrophysiological recordings support the idea that responding neurons of the lateral geniculate nucleus can be divided into four wavelength–dependent groups. These groups are characterized according to the opposing system: +Red –Green, +Green –Red, +Blue –Yellow and +Yellow –Blue (Figure 2) (De Valois et al., 1966).
Figure 2: A simplified model of light absorption and neuronal processing according to the color opponent theory modified by (Schmidt et al., 2014a). Representation of blue, green, yellow and red monochromatic light form short to long wavelength are represented with increasing wavelength. The corresponding qualitative and normalized cone spectral sensitivities are illustrated with the size of each cone. The cones are colored according to their spectral sensitivity, red for L/LWS, green for M/LWS and blue for SWS1 cone photoreceptor cells. A simplified processing of the cone signals through the midget ganglion cells is represented by grey lines which finally results in the color perception. Nevertheless, contributions of rod photoreceptor cells and melanopsin containing retinal ganglion cells are still missing (Barrionuevo and Cao, 2014).

Besides these two theories of trichromacy and opponent colors, recent findings suggest the contribution of a fourth pigment – melanopsin – at high photopic levels to peripheral vision (Horiguchi et al., 2013; Schmidt et al., 2014b), and perhaps fifth, rod photoreceptor cells contribute to color vision (Barrionuevo and
Cao, 2014). Current issues relating to color vision with an emphasis of neuronal processing is reviewed by (Lee, 2014).

Thus, colors play a major role in communication within and between species and can be considered a first primitive universal language. Communicating through colors can be a rapid, beautiful, meaningful, and subtle way of interaction. For instance, colors and color perception are crucial in avoidance of predators (camouflage), mimicry, warning or improving the chances of mating to assure successful reproduction and survival (Bloch, 2015; Jacobs and Nathans, 2009; Sandkam et al., 2015; Yokoyama, 2000).

1.3 Interplay of photoreceptors and neurons in color vision

Visual perception begins when photons of different energies interact with chromophores in distinct types of photoreceptor cells (Kefalov, 2012b; Rieke and Baylor, 1998). These interactions transform the electromagnetic information into an electrochemical potential which then is modified, conducted and analyzed via different types of neurons (De Valois et al., 1966; Luo et al., 2008; Schmidt et al., 2014a). Color vision per se is processed by the interconnected neurons, because the cone photoreceptor cells themselves are ‘colorblind’. A simple fact is that in scotopic vision the environment appears as black and white, although rod photoreceptor cells have their maximal sensitivities in the green spectral region (Figure 1) (Lee, 2014; Schmidt et al., 2014a; Stockman, 2010). In the following
paragraphs, we focus on the interplay of light with pigments and how neurons shape our color perception.

1.3.1 Multichromacy and its dimensions

Three cone photoreceptor types, each designated according to the opsin gene expressed, mediate human color vision. Thus, short wavelength sensitive cones contain SWS1 pigment, medium wavelength sensitive cones contain M/LWS pigment, and long wavelength sensitive cones contain L/LWS pigment. The wavelength of maximum absorption is around 420 nm for human SWS1 cones, near 530 nm for human M/LWS cones, and about 557 nm for human L/LWS cones (Dartnall et al., 1983; Merbs and Nathans, 1992; Neitz and Neitz, 2011; Stockman et al., 1993; Yoshizawa, 1994). Due to the spectral sensitivity of these three pigments and the neuronal processing, Humans can discriminate colors in the visible range of light from about 420 nm to 680 nm (Figure 1 and Figure 2, Table 1 and Table 2) (Jacobs and Nathans, 2009). Aside from trichromatic vision, there also is di- or tetrachromatic vision. Tetrachromatic vision, for example, is found in chickens and pigeons that possess an additional UV-sensitive photoreceptor besides the three usual photoreceptor types (Bowmaker, 1984; Vorobyev et al., 1998; Yoshizawa, 1994). The dimensionality of chromatic vision is not directly correlated with the number of pigment classes. Indeed, the presence of four cone classes does not implement tetrachromacy per se; only behavioral studies could reveal this correlation (Bowmaker, 2008). Albeit that the mantis shrimp has 12 types of photoreceptor cells ranging from ultraviolet (UV) to
deep red, these 12 photoreceptor cell types do not hint at dodecachromacy. However, this shrimp certainly can absorb a broader spectrum of light than other species with less photoreceptors (Marshall and Oberwinkler, 1999). Recent, behavioral studies on the *mantis shrimp* showed that the color perception is different from the known human color opponent theory. Further, the 12 photoreceptors allow the *mantis shrimp* to detect colors more quickly. Thus the advantage lies in the color detection rather than the color discrimination (Land and Osorio, 2014; Thoen et al., 2014).
Table 1: A summary of published human opsin absorption maxima ($\lambda_{max}$) from 1964 to 2011. Data here show that the absorption maxima are not single values but instead represent an absorption range. This absorption range is due not only to different experimental methods but also to polymorphisms within the opsins. For simplicity, we have used values published previously (Neitz and Neitz, 2011).

<table>
<thead>
<tr>
<th>Human Rhodopsin $\lambda_{max}$</th>
<th>496 nm (Dartnall et al., 1983), 497 nm (Bowmaker and Dartnall, 1980), 505 nm (Brown and Wald, 1964), 498 nm (Nathans, 1990).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human SWS1: $\lambda_{max}$</td>
<td>426 nm (Merbs and Nathans, 1992), 424 nm (Oprian et al., 1991), 419 nm (Dartnall et al., 1983), 410 nm (Asenjo et al., 1994), 420 nm (Bowmaker and Dartnall, 1980), 450 nm (Brown and Wald, 1964), 420 nm (Neitz and Neitz, 2011).</td>
</tr>
<tr>
<td>Human M/LWS: $\lambda_{max}$</td>
<td>530 nm (Merbs and Nathans, 1992), 530 nm (Oprian et al., 1991), 530 nm (Dartnall et al., 1983), 532 nm (Asenjo et al., 1994), 533 nm (Bowmaker and Dartnall, 1980), 525 nm (Brown and Wald, 1964), 530 nm (Neitz and Neitz, 2011).</td>
</tr>
<tr>
<td>Human L/LWS: $\lambda_{max}$</td>
<td>552 nm (Merbs and Nathans, 1992), 560 nm (Oprian et al., 1991), 558 nm (Dartnall et al., 1983), 563 nm (Asenjo et al., 1994), 562 nm (Bowmaker and Dartnall, 1980), 555 nm (Brown and Wald, 1964), 557.5 nm (Neitz and Neitz, 2011).</td>
</tr>
<tr>
<td>Human melanopsin $\lambda_{max}$</td>
<td>479 nm (Bailes and Lucas, 2013)</td>
</tr>
</tbody>
</table>
Table 2: Conversion of specific absorption maxima ($\lambda_{max}$) into corresponding energy units (Neitz and Neitz, 2011).

\[ E = \frac{hc}{\lambda_{max}} \]  

\[ c = 299792458 \frac{m}{s} \]  

\[ h = 6.62606957(29) \cdot 10^{-34} Js \]

<table>
<thead>
<tr>
<th>Wavelength ($\lambda_{max}$)</th>
<th>Energy (E)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[nm]</td>
<td>[kJ mol$^{-1}$]</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Rhodopsin 498 nm</td>
<td>240.21</td>
</tr>
<tr>
<td>SWS1 420 nm</td>
<td>284.83</td>
</tr>
<tr>
<td>M/LWS 530 nm</td>
<td>225.71</td>
</tr>
<tr>
<td>L/LWS 557.5 nm</td>
<td>214.58</td>
</tr>
<tr>
<td>Melanopsin 479 nm</td>
<td>249.74</td>
</tr>
</tbody>
</table>

$^a$ Calculated from

1.3.2 Neuronal contribution to color perception

Color vision and all other sensory perceptions are processed and evaluated by numerous neurons (Bowmaker, 2008). About 37% (or up to 60% depending on the literature source) of the human striate cortex is involved in the evaluation of only 3% of the visual field (Horton and Hoyt, 1991; Pahlberg and Sampath, 2011; Tolhurst and Ling, 1988; Wong and Sharpe, 1999). By comparing the number of
involved neurons per sensory process, it becomes obvious that visual stimuli provide the most information about our environment. Post-photoreceptor signaling plays a major role in the discrimination and processing of visual stimuli (Bitensky et al., 1985; Conway et al., 2010; Dacey et al., 1996; De Valois and De Valois, 1993; Lee, 2014; Mancuso et al., 2010; Neitz and Neitz, 2011; Schmidt et al., 2014a; Stockman, 2010). The three–stage Müller zone model or the multi–stage color model by DeValois and DeValois provide a simplified and still controversial hypothesis of signal processing initiated by photoreceptors (De Valois and De Valois, 1993; Mancuso et al., 2010; Müller, 1930). Current models of color vision emphasize inputs from three types of cone photoreceptor cells and rod photoreceptor cells, which also contribute to daylight vision in the retina (Barrionuevo and Cao, 2014; Barrionuevo et al., 2014). Here rod photoreceptor cells elicit a light intensity–dependent signal modulated by horizontal cells (Szikra et al., 2014). Positing that stimuli elicited by rods and cones are modified by each other along neuronal pathways suggests that color vision results from more than a complex interplay between cone photoreceptor cells and other neurons (Barrionuevo and Cao, 2014; Barrionuevo et al., 2014; Conway et al., 2010; De Valois and De Valois, 1993; Dunn et al., 2007; Szikra et al., 2014). A revised model (Figure 2) of the opponent color theory by De Valois and De Valois is provided by (Schmidt et al., 2014a). It states that cone opponent signals are processed through midget ganglion cells, where signals from cones are combined with those from horizontal cells. Thereby the ON/OFF state of bipolar cells and the SWS1 cone input plays a major role in the transmission of the hue
sensations yellow, blue, green and red. Based on this model, four hue mechanism are proposed: yellow, $L - (S + M)$; blue, $(S + M) - L$; green, $M - (S + L)$; and red, $(S + L) - M$ (Figure 2) (Schmidt et al., 2014a). Melanopsin signaling can contribute to the contrast sensitivity in vision (Schmidt et al., 2014b). A model based on principal component analysis suggests how rod photoreceptor cells and melanopsin contributions could be achieved. In this principal component analysis, the most important chromatic contribution is also the blue–yellow input (Barrionuevo and Cao, 2014). How these signals from cone, rod photoreceptor cells and melanopsin-containing ganglion cell are processed and if the current models of color perception need to be expanded remains topics of ongoing research.

1.4 Energetics of cis–trans isomerization of 11–cis–retinal

In the following paragraphs, we focus on the energetics of cis–trans isomerization of 11–cis–retinal triggered by interaction with photons of specific energy (Barlow et al., 1993; Frutos et al., 2007; Gozem et al., 2012; Luo, 2000; McBee et al., 2001; Polli et al., 2010). Moreover, we discuss which physicochemical properties limit the visible spectrum and how these can be overcome.

To comprehend the principles of spectral tuning it is crucial to consider the mechanism of photoactivation from an energetic perspective. Photoactivation by a single photon generally leads to the same outcome, namely excitation of the
chromophore and subsequent photoisomerization of retinal from 11-cis-retinal to the all-trans-configuration (Frutos et al., 2007; Gozem et al., 2012; Luo et al., 2008; Polli et al., 2010). To understand the activation by photons of different wavelengths or energies, it is critical to determine how the energetics vary with different interactions of the retinoid chromophore with its local environment. Such interactions form the basis of spectral tuning. Hence, characterizing the energetics of these interactions provides insights into how visual pigments accomplish the absorption of a wide UV-visible-IR spectrum of light with the same retinal chromophore, which by itself can only absorb light in the UV range (Fujimoto et al., 2009; Okada et al., 2002a; Rajamani et al., 2011). Considering the process of photoactivation from an interdisciplinary perspective reveals the limitations that energetics and physiology impose on color perception at an early stage, where light passes through the cornea, lens, vitreous and ultimately interacts with the chromophore in the retina. Thus activation of visual pigment proteins requires breakage of a π bond. A minimal energy of $E_a \sim 156.4 \frac{kJ}{mol}$ (37.4 kcal/mol) in rhodopsin or $E_a' \sim 61.8 \frac{kJ}{mol}$ (14.8 kcal/mol) in solution must be provided to break the π bond and cause isomerization (Dilger et al., 2015a; Luo, 2000). This energy level limits the visual spectrum on the IR side. Furthermore, the reduced transmission of UV-light by predominantly lens and cornea and to a lesser extend vitreous limits our light absorption on the short wavelength side of the spectrum and also protects the retina from radiation damage (Ambach et al., 1994; Norren and Vos, 1974; Walls, 1942). The human lens starts transmitting
50 % of light at a wavelength range of 425 – 475 nm (Kessel et al., 2010; Lei and Yao, 2006). This 50 % light transmission decreases with age and shifts towards 575 nm (Artigas et al., 2012; Dillon et al., 1999; Kessel et al., 2010). Further, cornea and aqueous humor show a more complex light transmission after 1300 nm with two declines at ~1400 nm and ~2000 nm. Moreover, the vitreous humor transmission shows two declines at ~1000 nm and ~1200 nm (Boettner and Wolter, 1962; van den Berg and Spekreijse, 1997). Thus the combined absorption of cornea, aqueous humor, lens and vitreous humor shapes the range of electromagnetic spectra of visual perception in humans.

1.4.1 Molecular insights into spectral tuning

11-cis-Retinal is the universal chromophore responsible for absorbing light in all four types of photoreceptors and melanopsin with the opsin and bound chromophore specifying the wavelength of absorbed light (Kiser et al., 2014). This unprotonated chromophore has an absorption maximum at about 380 nm in methanol solution and undergoes a bathochromic shift (towards longer wavelength) upon protonation or covalent binding (Schiff base formation) (Blatz and Liebman, 1973; Fujimoto et al., 2005; Sakmar, 2012). Moreover, electrostatic interactions between the retinylidene moiety and the opsin protein environment cause additional tuning of wavelength absorption (Coto et al., 2006a; Coto et al., 2006b; Zhou et al., 2014). These interactions include the pigments’ different amino acid residues and also their spatial orientation, internal water molecules and counter ions. Overall covalent and non–covalent interactions of the bound
retinal are responsible for the bond length alterations and conformations of the chromophore crucial for fine-tuning spectral absorption (Coto et al., 2006b; Fujimoto et al., 2009; Okada et al., 2002a; Rajamani et al., 2011). Thus, binding to amino acid residues, waters or counter ions that increase electron delocalization and decrease bond length alternation induce a bathochromic shift, whereas decreased electron delocalization and increased bond length alternation cause a hypsochromic shift (towards shorter) wavelength of the chromophore (Fujimoto et al., 2006; Sekharan et al., 2012). From a quantum chemical perspective a bathochromic or hypsochromic shift results from the relative stabilization of the excited electronic state ($S_1$) of the retinal chromophore with respect to the ground state ($S_0$). The electronic structure (charge distribution) of the excited state and ground state are different. Therefore, the interactions that stabilize the charge distribution of the $S_1$ with respect to the charge distribution of the $S_0$ will cause a bathochromic shift. The same is true for a stabilization or destabilization of the $S_0$ with respect to the $S_1$ (Melaccio et al., 2012). Because no cone pigment structure is yet available, it is difficult to predict the coordinates of protein residues, counter anion hydrogen bond networks or waters to determine exactly how nature achieves specific spectral tuning for color vision. All our structural information about cone pigments (Stenkamp et al., 2002; Teller et al., 2003) are derived from modeling studies based on rhodopsin structure (Palczewski et al., 2000).

All five photopigments including melanopsin have their maximal absorption ($\lambda_{max}$) at a unique wavelength. These maxima were measured and determined
over decades by several groups using different methodologies (Asenjo et al., 1994; Bailes and Lucas, 2013; Bowmaker and Dartnall, 1980; Brown and Wald, 1964; Carroll et al., 2002; Dartnall et al., 1983; Merbs and Nathans, 1992; Oprian et al., 1991). The data reveal that there is a natural variation in $\lambda_{\text{max}}$ caused predominantly by mutations in the 2, 3 and 4 exon of L/LWS and M/LWS genes in normal color vision. Here we report a summary of current available absorption maxima and calculate the energies of the specific wavelengths shown (Table 1 and Table 2, and Figure 1). Throughout this review we will refer to the $\lambda_{\text{max}}$ values published by (Neitz and Neitz, 2011).

1.4.2 Physicochemical limits of human visible spectra

A photopigment is activated with a quantum yield of 0.65 when a photon of an electromagnetic wave with energy close to the absorption maxima strikes the chromophore (Kefalov et al., 2003; Kim et al., 2001, 2003; Liu and Colmenares, 2003; Rieke and Baylor, 1998). Measurements of absolute thresholds in human vision revealed that a single photon cannot provoke a physiological response. Absolute thresholds for humans to detect a signal through cones and rods are about 200 and 50 photons, respectively (Koenig and Hofer, 2011). A visual stimulus is elicited when the energy of a photon meets the energy required to promote the chromophore from an electronic ground state ($S_0$) to the first electronically excited singlet state ($S_1$) where it undergoes cis–trans isomerization to the photoproduct begins. Such photoisomerization involves the passage through a $S_0/S_1$ conical intersection that can be found along the plot of
the energetics of the reaction (Barlow et al., 1993; Frutos et al., 2007; Gozem et al., 2012; Polli et al., 2010). The body temperature contributes continuously to the vibrational mode of the electronic ground state of the chromophore and thus destabilizes the ground state (Figure 3) (Ala-Laurila et al., 2004; Luo et al., 2011). QM/MM calculations of the excitation energies on free retinal in the gas phase range from \( \Delta E_{S_0\rightarrow S_1} = 197.8 \text{ kJ mol}^{-1} (47.3 \text{ kcal mol}^{-1}) \) to 232.5 \( \text{ kJ mol}^{-1} (55.6 \text{ kcal mol}^{-1}) \). The calculations of the chromophore in solution resulted in higher values because solvents stabilize \( S_0 \) relative to \( S_1 \) \( \Delta E_{S_0\rightarrow S_1} = 205.5 \text{ kJ mol}^{-1} (49.1 \text{ kcal mol}^{-1}) \) to 284.6 \( \text{ kJ mol}^{-1} (68.0 \text{ kcal mol}^{-1}) \) (Bravaya et al., 2007). The energy barrier for isomerization of the free retinal protonated Schiff–base chromophore was determined to be: \( E_a^f = 61.8 \text{ kJ mol}^{-1} (14.8 \text{ kcal mol}^{-1}) \), and the corresponding wavelength was \( \lambda = 1937.3 \text{ nm} \) (Dilger et al., 2015b). Conversely, the energy barriers for chromophore isomerization in rhodopsin are higher compared to the free chromophore. Thus, they suggest that the pigment proteins restrict isomerization and thereby regulate sensitivity (Bravaya et al., 2007; Dilger et al., 2015a; Lorenz-Fonfria et al., 2010; Sugihara et al., 2002). The existence of an isomerization barrier stands in contrast to the barrier–free \( \text{cis–trans} \) isomerization as stated earlier by (Barlow et al., 1993; Frutos et al., 2007; Polli et al., 2010). Different activation energies are reported for the thermal activation path ranging from \( E_a^T \sim 91.63 \text{ kJ mol}^{-1} (21.9 \text{ kcal mol}^{-1}) \) to 200.96 \( \text{ kJ mol}^{-1} (48.03 \text{ kcal mol}^{-1}) \) (Baylor et al., 1980; Guo et al., 2014; Luo et al., 2011), and for the photochemical activation path from \( E_a^P \sim 188 \text{ kJ mol}^{-1} (45 \text{ kcal mol}^{-1}) \) to 240.21 \( \text{ kJ mol}^{-1} (62 \text{ kcal mol}^{-1}) \).
The range of activation energy can be explained by different calculation models or protonation states of the chromophore. Thereby, deprotonation leads to a destabilization of the ground state and thus lowers the energy barrier for isomerization (Barlow et al., 1993). Others suggest that the difference of activation energy is found in conformational fluctuations of the binding pocket rather than the chromophore (Lorenz-Fonfria et al., 2010). Conformational fluctuations would further support the model of a different thermal-dependent isomerization mechanism controlled by an energy barrier $E_a^T$ (Frutos et al., 2007; Gozem et al., 2012; Polli et al., 2010). An example of a deprotonated chromophore can be found in the UV pigment (SWS1) of the Siberian hamster. The thermal activation energy of UV pigment (SWS1) from a Siberian hamster is only half that of bovine rhodopsin (depending on the literature), about $96.3 \frac{kJ}{mol}$ ($23.0 \frac{kcal}{mol}$), which corresponds to $\lambda = 1243.1$ nm (Mooney et al., 2015). This result appears counter intuitive because the UV pigment (SWS1) is triggered by photons of higher energy than rhodopsin. Therefore, higher activation energy would be expected for UV pigments. The reason for the higher thermal activation energy of rhodopsin lies in its increased photosensitivity compared to that of cone pigments. Whether the different sensitivity between the SWS1 photopigment and bovine rhodopsin only depends on the protonated state or if there are other factors, is part of ongoing research. The interdependence of the activation energy and maximal absorption in different
photopigments is addressed by the Stiles–Lewis–Barlow hypothesis that includes the thermal activation energy \( E_a^T \) as a constant which depends on the photon energy and thus the maximal absorption such as \( E_a^T = \text{const.} \times \frac{1}{\lambda_{\text{max}}} \), or \( \text{const.} \times \lambda_{\text{max}} \) (Barlow, 1957; Lewis, 1955; Stiles, 1948). Spectroscopic and electrophysiological measurements combined with statistics indicate that \( E_a^T \) does not show a strict dependence on \( \lambda_{\text{max}} \) or on \( \frac{1}{\lambda_{\text{max}}} \) for all pigments (Ala-Laurila et al., 2003; Ala-Laurila et al., 2004; Koskelainen et al., 2000). Nevertheless, recent findings generated by quantum mechanical calculations showed that \( E_a^T \) of bovine rhodopsin indeed depends on \( \frac{1}{\lambda_{\text{max}}} \), which supports an “anti–Barlow” correlation (Dilger et al., 2015a; Gozem et al., 2012). Thus, the interplay between the activation energy and absorption maxima is still a topic of debate. Only a combined approach of structural biology, computational chemistry and photochemistry will provide us further insight into the energetics of nature’s photoreceptors.

From a pure energetic perspective based on bond dissociation, neglecting the photochemical reaction path as described above, the isomerization of 11–cis–retinal to all–trans–retinal requires the breakage of a \( \pi \) bond. This \( \text{C–C–}\pi \) bond dissociation energy at the 11 and 12 position of the rhodopsin complex was stated to be \( 156.4 \pm 6.3 \frac{\text{kJ}}{\text{mol}} \) \( (37.4 \pm 1.5 \frac{\text{kJ}}{\text{mol}}) \), with the corresponding wavelength \( \lambda = 764.5 \text{ nm} \) (Luo, 2000). The thermal isomerization of the chromophore in rhodopsin, which supposedly undergoes a different path as the photoactivation
path requires an activation energy of about $E_{a}^{T} \sim 146 \frac{kJ}{mol}$ (35 $\frac{cal}{mol}$), with the corresponding wavelength $\lambda = 819.0$ nm (Gozem et al., 2012). Both wavelengths also relate to the energetic minimum of the human visible spectrum. Thus, it remains to be answered if the cone photopigments also have the ability to undergo both activation pathways, one through a $\lambda_{\text{max}}$ specific photoactivation path and a photopigment universal thermal activation path. Moreover, the maximal energy limit of the human visible spectrum is about 380 nm which is $314.6 \frac{kJ}{mol}$ (75.2 $\frac{cal}{mol}$). With this amount of energy, it is possible to break weak C–H or C–C single bonds (Luo, 2000). Breakage of a C–C or C–H bond would lead to a decay of the chromophore or other macromolecules which would harm the retinoid cycle and thus impair vision (Kiser et al., 2014). However, the lens and vitreous show a higher absorption of short wavelength light that prevents damage to the retina by high energy irradiation (Norren and Vos, 1974). These data provide a physicochemical explanation for the natural limits of the human visual spectrum. However, a recent publication about IR vision indicates that these limits can be extended by two–photon isomerization with modern instruments. Thereby, the two-photon effect enables to detect IR light emitted by a laser (Palczewska et al., 2014).

1.4.3 The interplay of light and heat on the activation energy

Studies about the interplay of light and heat in bovine rhodopsin revealed that the thermal activation energy ($E_{a}^{T}$) showed an increased temperature dependence
$E_a^T = 202.8 \, \frac{kJ}{mol} \, (48.5 \, \frac{kcal}{mol})$ for wavelengths beyond 590 nm. Conversely, no thermal contribution was needed at wavelengths shorter than 590 nm (St George, 1952). The activation energy above 750 nm corresponds to the thermal activation energy of bovine rhodopsin mentioned above. Taken together, the thermal activation energy of bovine rhodopsin lies between $E_a^T \approx 91.63 \, \frac{kJ}{mol} \, (21.9 \, \frac{kcal}{mol})$ and $200.96 \, \frac{kJ}{mol} \, (48.03 \, \frac{kcal}{mol})$ (Baylor et al., 1980; Guo et al., 2014; Luo et al., 2011; Lythgoe and Quilliam, 1938) and the photochemical activation energy $E_a^P \approx 188 \, \frac{kJ}{mol} \, (45 \, \frac{kcal}{mol})$ to $240.21 \, \frac{kJ}{mol} \, (57.41 \, \frac{kcal}{mol})$ (Barlow et al., 1993; Cooper, 1979; Gozem et al., 2012; Lorenz-Fonfria et al., 2010; Luo et al., 2011; St George, 1952). A further indication of a higher $E_a^T$ is shown by two different toad rhodopsins, where different $E_a^T$ values of $185.4 \, \frac{kJ}{mol} \, (44.3 \, \frac{kcal}{mol})$ and $204.2 \, \frac{kJ}{mol} \, (48.8 \, \frac{kcal}{mol})$ were observed despite their similar absorption spectra (Ala-Laurila et al., 2002). A recent in vitro experiment revealed an unusual temperature dependence of $E_a^T$, for bovine rhodopsin based on a rearrangement of the hydrogen network within the retinal–binding pocket. At a temperature of 310.15 K (37˚ C), $E_a^T = 92.0 \, \frac{kJ}{mol} \, (22.0 \, \frac{kcal}{mol})$ and at 317.15 K (44˚ C), $E_a^T = 477.0 \, \frac{kJ}{mol} \, (114.0 \, \frac{kcal}{mol})$ (Guo et al., 2014). The reported differences in $E_a^T$ between (Ala-Laurila et al., 2002) and (Guo et al., 2014) are due to the different decay models used. The latter takes into account that thermal decay of rhodopsin measured at 500 nm consists of both the thermal isomerization and hydrolysis of the Schiff base (Liu et al., 2011; Liu et al., 2009). Furthermore, basic Boltzmann statistics were used to describe the thermal
activation of a complex molecule such as retinal. Basic Boltzmann statistics reduce a complex reaction to a simple process like the collision of gas molecules (Luo et al., 2011). Thus, the use of basic Boltzmann statistics and conventional Arrhenius analysis contribute further to the difference found between the above stated activation energies. Furthermore, the lipidic environment of rhodopsin changes at higher temperatures and thus should influence the decay of rhodopsin (Garavito and Ferguson-Miller, 2001; Paleologos et al., 2005; Stalikas, 2002; Vautier-Giongo and Bales, 2003). Recent findings report that the isomerization of the chromophore is an exclusive mechanism for thermal activation (Yanagawa et al., 2015). Further, they found a relationship between the thermal stability of rhodopsin, the low dark noise and the stability of the activated Meta II state (Yanagawa et al., 2015). Discrepancies between theoretical activation energies of $11\text{cis}$–retinal isomerization and minor deviance from experimental energies indicate that the mathematical models are improving; however, they are still a matter of controversy in the fields of computational chemistry, photochemistry and biochemistry, thus much remains to be answered.

1.4.4 Human vision beyond the visible spectra

Human rhodopsin sensitivity was calculated and measured at 1050 nm ($114.0 \text{kJ mol}^{-1}$, or 27.2 $\text{kcal mol}^{-1}$) (Griffin et al., 1947). These calculations based on classic Maxwell–Boltzmann distribution suggested that rhodopsin bleaches and absorbs light even at 1050 nm and with $10^{-12.4}$ times less sensitivity than at 510 nm
(Griffin et al., 1947; St George, 1952). Moreover, *in vitro* investigations revealed that the relative sensitivity at long wavelengths increases upon warming and also shifts $\lambda_{max}$ to shorter wavelengths (Ala-Laurila et al., 2003). The threshold of the activation energy $184.1 \frac{kJ}{mol}$ (44.0 $\frac{kcal}{mol}$) at 1050 nm (114.0 $\frac{kJ}{mol}$, or 27.2 $\frac{kcal}{mol}$) can be achieved with the addition of thermal energy provided from the human physiological temperature of 310.15 K (37˚ C) (72.3 $\frac{kJ}{mol}$, 16.8 $\frac{kcal}{mol}$) (Figure 3). However, this activation requires $10^{12.4}$ more photons due to the reduced sensitivity of bovine rhodopsin at 1050 nm (St George, 1952). Figure 3 shows the combinations of thermal energy (red bars) and quantum energy (blue bars) at various wavelengths. The thermal energy contribution facilitates activation from 675 nm to 1050.9 nm where the quantum energy of light would not suffice to achieve the threshold of activation energy (black line). Recent findings demonstrate that mammalian photoreceptors can elicit a signal if irradiated with IR light at wavelengths longer than 800 nm (Palczewska et al., 2014). Here excitation is triggered by a two–photon effect yielding a total energy corresponding to twice the single photon energy (Figure 1). Thus, doubling the energy leads to a color perception corresponding to one photon in the visual spectra. Although this multi–photon excitation is a remarkable observation, the extent to which this two–photon excitation shapes our visual perception remains to be determined.
Figure 3: Photon specific energy contribution to opsin activation throughout the visible and IR spectra. The wavelength–dependent energy of light is depicted in blue; red bars correspond to the thermal energy contribution of 310.15 K (37° C), 70.3 $\text{kJ mol}^{-1}$ (16.8 $\text{Kcal mol}^{-1}$). The black horizontal line corresponds to the thermal activation energy of bovine rhodopsin, $E^*_a = 184.1 \text{ kJ mol}^{-1}$ (44.0 $\text{Kcal mol}^{-1}$) (Lythgoe and Quilliam, 1938). At 1050.9 nm the combined thermal and radiation energy crosses the threshold which implies the theoretical limit of mammalian light perception.

1.4.5 Phototransduction noise of photoreceptor cells

Phototransduction noise of photoreceptor cells determines our visual sensitivity (Aho et al., 1988; Angueyra and Rieke, 2013; Bulmer et al., 1957). Phototransduction noise originates from two different sources; one is thermal noise of visual pigments, other results from phototransduction downstream events (Figure 4). Thus, the extent to which each source contributes to this noise
depends on both the photoreceptor cell and photopigment type (Angueyra and Rieke, 2013; Kefalov, 2012b; Korenbrot, 2012; Rieke and Baylor, 2000). In addition to significant structural differences between rods and cone (Mustafi et al., 2009), cone photoreceptor cells bear an increased noise compared to rod photoreceptor cells (Barlow, 1957; Rieke and Baylor, 2000; Yanagawa et al., 2015). A comparison of phototransduction noise events in salamander long wavelength sensitive (LWS) and short wavelength sensitive (SWS) cone photoreceptor cells revealed that in LWS cones the noise is mainly caused by spontaneous activation of the photopigment (thermal noise), whereas in SWS cones downstream events are responsible for the phototransduction noise (Rieke and Baylor, 2000). A pharmacological approach with β-ionone as substrate showed that the differences in activity between cone and rod opsins predominantly originate from the opsin properties (Isayama et al., 2006). In contrast, a comparison of primate cone photoreceptor cells suggests that phototransduction noise in cone photoreceptor cells is dominated by noise from downstream events such as channel noise and fluctuations in cGMP concentrations and not by thermal noise (Figure 4) (Angueyra and Rieke, 2013; Fu et al., 2008; Palczewski, 2014). The thermal noise of visual pigments is determined by the energetic states of the chromophore or structural fluctuations of the binding pocket and ultimately shapes the spectral sensitivity of each photoreceptor cell (Aho et al., 1988; Gozem et al., 2012; Lorenz-Fonfria et al., 2010; Rinaldi et al., 2014). Recent findings determined two key residues E122 and I189 in rhodopsin which contribute to the lower dark noise compared to cone
visual pigments (Yanagawa et al., 2015). The source of noise that contributes to the photoreceptor sensitivity is still topic of ongoing research.

Figure 4: Phototransduction and the visual (retinoid) cycle in a rod outer segment. Phototransduction can be described in three stages shown from top to bottom in this cartoon. When light strikes rhodopsin (red), it
causes isomerization of the 11-cis-retinylidene chromophore to an all-trans configuration and a conformational change in the opsin protein. This, in turn, leads to formation of a complex with the heterotrimeric G protein, transducin. Nucleotide exchange in the transducin a-subunit from guanosine diphosphate to GTP causes dissociation of transducin with formation of the transducin a-subunit. This subunit interacts with tetrameric cGMP–specific PDE6, whereas the transducin bc-subunit complexes with phosducin. One activated rhodopsin molecule can activate dozens of transducin molecules in this first amplification stage of phototransduction. Displacement of the inhibitory c-subunit activates PDE in the second amplification step of phototransduction. The resulting decrease in the concentration of cGMP is associated with a decrease in intradiscal Ca2þ concentration because cGMP is a ligand for cGMP-gated cation channels (shown in blue in the plasma membrane), nonselective channels that also allow passage of Ca2þ in their cGMP-bound state. The low Ca2þ-level is maintained by the light-insensitive Naþ/Ca2þ-Kþ exchanger, which extrudes Ca2þ ions out against a gradient in exchange for Naþ and Kþ ions. Each of the above-activated molecules needs to return to its inactive state before absorption of the next photon. Thus, rhodopsin is phosphorylated at its C-terminus by GRK1 (or rhodopsin kinase [RK]), followed by binding of arrestin, a capping protein. Guanosine triphosphate is hydrolyzed by the a-subunit of transducin with the help of a GTPase-activating protein. Guanylate cyclase 1 and GC2 (GC, light/dark-brown box) are activated by Ca2þ-binding proteins (GCAP1 and GCAP2, black ball) in their Ca2þ-free forms to restore cGMP levels and open the cyclic nucleotide–gated cation channels in the plasma membrane. Guanylate cyclase-activating proteins are inactivated and GC activities return to their dark condition. Once GTP is hydrolyzed by the a-subunit of transducin along with phosphorylation of phosducin, the heterotrimeric G protein is restored. Opsin recombines with 11-cis-retinal and the rhodopsin thus formed is ready to be photoactivated. Note that all these processes take place on the cytoplasmic surfaces of disc and plasma membranes. The metabolic renewal of 11-cis-retinal takes place in photoreceptor outer segments and the RPE. First, alltrans-retinylidene is hydrolyzed from opsin and all-trans-retinal diffuses to the cytoplasmic side where it is reduced to an alcohol by membrane associated all-trans-retinol RDH. Lack of adequate RDH activity can lead to LCA (or RP); all diseases are depicted in red letters. A fraction of all-transretinal is released into the intradiscal side. There all-trans-retinal and phosphatidylethanolamine form a Schiff base and together are transported into the cytoplasmic side via ABCA4. Lack of ABCA4 transport activity is associated with Stargardt disease, whereas polymorphisms in this gene are associated with AMD. Retinol diffuses from the cytoplasm to the RPE where it becomes esterified by LRAT to form fatty acid retinyl esters. Such esters have a propensity to coalescence, thermodynamically driving the transfer from photoreceptor to RPE cells. The esters then serve
as substrates for the isomerization reaction catalyzed by the 65-kDa protein, RPE65. The resulting product, 11-cis-retinol, is oxidized to 11-cis-retinal by the 11-cisretinol specific RDH5 and dual specificity (cis and trans-retinols) RDHs, including RDH10; 11-cis-retinal diffuses back into the photoreceptor outer segments, a process thermodynamically driven by the formation of stable visual pigments. Essential for transporting and protecting these retinoids are intracellular and extracellular retinoid-binding proteins such as CRALBP, CRBP1, IRBP and retinol-binding protein 4 (RBP4). Inactivating mutations in the LRAT and RPE65 genes are causes of childhood blindness because these genes are nonredundant, whereas mutations in RDHs and retinoid-binding proteins have less severe effects but can be associated with RP, cone-rod dystrophy, fundus albipunctatus, fundus albescens or Bothnia dystrophy. Retinoids are retained in the eye as a result of LRAT activity. In the bloodstream, retinoids are bound to RBP4 and then enter the eye by passive transport with the help of STRA6. Mutations in the STRA6 gene cause Matthew-Wood Syndrome, a severe disease that includes obesity and mental retardation, as well as faulty eye development and blindness, indicating the importance of STRA6 for retinol transport into the brain and eye (Palczewski, 2014).

1.5 Molecular insights into color tuning

1.5.1 Molecular insights into SWS1 color tuning

The human SWS1 cone pigment induces a hypsochromic shift in the absorption maximum of 11-cis-retinal, with a published peak absorption of human SWS1 cone pigment ranging from 410 to 450 nm (Table 1 and Table 2 and Figure 1) (Asenjo et al., 1994; Bowmaker and Dartnall, 1980; Brown and Wald, 1964; Dartnall et al., 1983; Merbs and Nathans, 1992; Neitz and Neitz, 2011; Oprian et al., 1991). Several investigations combining site-directed mutagenesis, spectroscopy, modeling, quantum mechanics/molecular mechanics (QM/MM)
estimates and combinations of these approaches have provided insights into the molecular mechanism of spectral tuning by SWS1 cone pigment.

The low 62% identity in protein sequence between rhodopsin and SWS1 opsin indicates a difference in intramolecular interactions despite a similar function. Highly conserved residues and domains within visual pigments suggest their crucial role in function including in color tuning. For instance, conserved in human cone pigments, the F207 residue plays an important role in photoactivation and a minor role in spectral tuning. Replacing F207 with a V, Y, T or S consistently leads to a reduction or absence of absorption without changing λ_{max} (Kuemmel et al., 2013). Moreover, a less conserved acidic residue in visual pigments in EL2 D181 or E181 plays a crucial role in GPCR activation as a counterion switch (Terakita et al., 2004; Terakita et al., 2000; Yan et al., 2003). Thereby, GPCR activation causes a proton transfer from E181 to E113 through hydrogen bond network resulting in the activated GPCR conformation (Martinez-Mayorga et al., 2006; Ramos et al., 2007; Yan et al., 2003). However, the M/LWS and L/LWS photopigment do not contain an acidic residue at position 181, but an H197 residue which serves as chloride-binding site (Wang et al., 1993; Yan et al., 2003). If and how the chloride ion contributes to the activation mechanism as the above mentioned acidic residues D181 and E181 remains to be answered. Furthermore, sequence alignments of the visual pigments showed seven conserved residues in EL2: R177, P180, G182, S186, C187, G188, and D190 (rhodopsin numbering). These residues, though preserved, can play different roles in SWS1 and other visual pigments. Mutational and molecular dynamic
(MD) studies of *Xenopus* violet cone pigment, a SWS1 class opsin, revealed a hydrogen bonding network between EL2, EL3 and the N–terminus. Despite the conserved residues in EL2, bovine rhodopsin’s EL2 shows extensive interactions between EL3 and the N–terminus via a hydrogen network without direct interactions with EL3 or the N–terminus. Finally, mutagenesis studies revealed that E176 and S181 participate in protein folding and chromophore binding in *Xenopus* violet cone pigment (Chen et al., 2011). The differences at the N-terminal (extracellular) domain between cone pigments and rhodopsin provides a possible explanation for the increased chromophore dissociation. Cone visual pigments show a dark dissociation comparable to 500 photoisomerizations per s (Kefalov et al., 2005). More open conformation of the extracellular domain allow small nucleophilic agents to penetrate of the binding site and cause hydrolysis of the chromophore (Wald et al., 1955). Moreover, the SWS1 pigment Meta II state decays 40 times faster than rhodopsin, despite their similar photoresponses this effect could also originate from reduced cone pigment stability (Kefalov et al., 2003; Shi et al., 2007). According to QM/MM calculations, the molecular basis of spectral tuning within the UV to violet wavelength range involves stabilization of either the deprotonated (UV) or protonated (violet) Schiff base. Deprotonation increases bond length alternation and thus causes a hypsochromic shift of 11–cis–retinal absorption (Altun et al., 2011; Altun et al., 2009; Zhou et al., 2014). Additional structural modeling, QM/MM calculations and mutagenesis studies indicated that a complex hydrogen network forms an inter–helical lock within TM2, TM3 and TM6. This lock contributes to stabilization of the deprotonated Schiff
base and thus enables the spectral tuning of Siberian hamster UV cones (Sekharan et al., 2013). Mutagenesis studies showed that a single amino acid substitution, S90C, is responsible for the major hypsochromic shift of 34 to 46 nm in avian SWS1 pigment and, further supporting this observation, the C90S reverse substitution, caused a bathochromic shift of 38 nm in zebra finch. However, similar mutagenesis studies of mouse and bovine SWS1 pigments indicate that the S90C substitution showed only a marginal effect, whereas a single substitution at position F86Y was responsible for their 70 nm difference in absorbance (Sekharan et al., 2013; Yokoyama et al., 2000). Despite difficulties with interpretation, mutagenesis studies of photopigments combined with evaluation of their changes in absorption is a powerful approach. We must remember that a mutation without an effect on absorption could still change the hydrogen bond network if, for example, waters are rearranged in the binding pocket and compensate for the changed residue. Thus it is difficult to draw conclusions about spectral tuning from mutagenesis studies alone.

These results indicate that spectral tuning in different cone pigments and species is maintained by complex interactions involving different amino acids. Therefore, each cone opsin class from different species must be examined individually. Also, spectral tuning results from a complex interplay between multiple amino acid residues, hydrogen bond networks and waters within the chromophore−binding site. A combined approach of mutagenesis studies and QM/MM modeling would shed a brighter light on the mechanism of spectral tuning.
Both M/LWS and L/LWS cone photopigment induce a bathochromic shift in the absorption maximum of $11\text{--}cis$-retinal in respect to free retinal. Published absorption peaks of these cone visual pigments range from 552 – 563 nm and 525 – 533 nm, respectively (Table 1 and Table 2 and Figure 1) (Asenjo et al., 1994; Bowmaker and Dartnall, 1980; Brown and Wald, 1964; Dartnall et al., 1983; Merbs and Nathans, 1992; Neitz and Neitz, 2011; Oprian et al., 1991). Several approaches including mutagenesis, spectroscopy, modeling studies, and their combinations have yielded insights into the molecular mechanisms of spectral tuning by M/LWS and L/LWS cone pigments.

Alignment of the human L/LWS and M/LWS protein sequences displays a difference in a total of fifteen amino acids. Of these fifteen, seven amino acids are responsible for the spectral difference between human L/LWS and M/LWS cone pigments as determined by Oprian’s group. Listed from L/LWS to M/LWS, these amino acids are: S116Y, S180A, I230T, A233S, Y277F, T285A and Y309F (Figure 5) (Asenjo et al., 1994).
Figure 5: A structural comparison between the L/LWS and M/LWS opsin model is shown. Wire representation of the L/LWS and M/LWS model is based on the rhodopsin crystal structure. Spheres represent differences in the amino acid sequence between L/LWS and M/LWS. Red spheres indicate differences responsible for spectral tuning between L/LWS and M/LWS, whereas grey spheres do not contribute to color tuning, according to (Asenjo et al., 1994). Numbers within the LWS model help to orient between the magnifications and the GPCR overview. Fifteen changes in amino acid residues are magnified on a grey background; amino acid residues contributing to color tuning are in their corresponding colors, namely red for L/LWS and green for M/LWS. Changed amino acid residues that do not affect spectral tuning are colored according their atoms.
Other groups using mutagenesis found that five amino acids and their interactions are responsible for the spectral tuning between vertebrate LWS and Rh2 pigments: S180A, H197Y, Y277F, T285A, and A308S (Wang et al., 1993; Yokoyama et al., 2008). Three of these five mutations are identical with the seven amino acids responsible for the spectral tuning in the L/LWS and M/LWS pigments. These data show that spectral tuning resulting in the same spectral shift can be accomplished through different combinations of amino acid substitutions. Fifteen implicated amino acids differ between L/LWS and M/LWS. Thus theoretically there are $15^{20}$ possible mathematical combinations that could achieve the spectral absorption of the human L/LWS and M/LWS by only considering the changes in standard amino acids. These results reinforce the conclusion that spectral tuning involves a set of highly complex interactions amongst different residues, chromophore and internal waters which cannot be derived accurately through mutagenesis alone. Furthermore, the LWS cone pigments harbor an additional feature for spectral tuning called the ‘chloride effect’ wherein a chloride−binding site located near His197 contributes to the bathochromic shift of retinal in both human L/LWS and M/LWS cone pigments (Wang et al., 1993; Yamashita et al., 2013). The same effect is found in iodopsin (chicken LWS pigment) wherein depletion of chloride leads to a hypsochromic shift of about 40 nm (Hirano et al., 2001). The ‘chloride effect’ has been reported in mouse visual pigments where it also accounts for the above mentioned hypsochromic shift (Sun et al., 1997). Moreover, this chloride effect causes a faster decay of the Meta I state and, thereby, a more rapid formation of the active
state (Morizumi et al., 2012). Results published to date are derived from
mutagenesis of different amino acids close to the chromophore and their impacts
upon spectral tuning. Absorption of naturally occurring visual pigments illustrate
the extensive repertoire of absorption spectra which nature maintains ranging
from 355 nm in *Danio rerio* (Zebrafish) to 625 nm *Anolis carolinensis* (American
Chameleon) (Amora et al., 2008; Chinen et al., 2003; Kawamura and Yokoyama,
1993). An extreme example of artificial tuning was performed with human cellular
retinol–binding protein II. Systematic mutagenesis of amino acids close to the
chromophore led to absorption spectra ranging from 425 nm up to 644 nm
(Sakmar, 2012; Wang et al., 2012a). The described artificial spectral tuning
through the cellular retinol–binding protein II gave insights into the correlation
between electrostatic effects, counter anion, structure of 11–cis–retinal and
absorbed wavelength (Huntress et al., 2013). An artificially engineered system
such as this experiment demonstrates the marked ability of 11–cis–retinal to
absorb light over a broad spectrum. Yet it fails to provide further insights into how
nature and evolution accomplish color tuning. Besides the role of amino acid
residues on spectral tuning, investigations of rhodopsin revealed that internal
waters play a crucial role in the hydrogen bonding network and thus in spectral
tuning (Okada et al., 2002a). Because cone photopigment crystal structures are
lacking, the role of waters and the precise location of the hydrogen network have
yet to be documented. This fact reinforces the need for crystallographic
structures for each human cone pigment to fully understand the molecular details
underlying spectral tuning in cone photopigments.
1.6 Deficiencies in color vision

Inherited color vision deficiencies of various types and degrees occur in humans (Neitz and Neitz, 2011; Roosing et al., 2014). Among others, these include different types of monochromacy, the common red–green color deficiencies and blue–yellow color deficiency. There are two forms of monochromacy: Form 1, rod monochromacy or achromatopsia; and Form 2, blue cone monochromacy or incomplete achromatopsia. Form 1 is caused by mutations in the CNGA3 gene, CNGB3 gene, GNAT2 gene, or PDE6C/H gene. Patients suffering from rod monochromacy caused by mutations in CNGA3 have normal rod and cone pigment levels and only the rod responsivity (Alpern, 1974; Kohl et al., 2000; Kohl et al., 2002; Kohl et al., 1998). Mutations in the CNGB3 gene and the PDE6C gene alter the function of cones and also decrease the number of cone photoreceptors in the retina (Grau et al., 2011; Thiadens et al., 2009; Varsanyi et al., 2007). Form 2 is caused by mutations in the locus control region for the human long wavelength sensitive (L/LWS) and human medium wavelength sensitive (M/LWS) pigment genes or harmful mutations in both L/LWS and M/LWS genes which cause the absence of functional LWS cone pigments (Gardner et al., 2014; Gardner et al., 2009; Gardner et al., 2010; Mizrahi-Meissonnier et al., 2010; Nathans et al., 1989).

Red–green color deficiencies are largely due to increased non–homologous recombination of nearly identical sequences of L/LWS and M/LWS opsin genes. Gene duplication of the L/LWS gene led to a head–to–tail array of numerous
L/LWS and M/LWS genes. Numerous copies of L/LWS and M/LWS opsin genes in this array increase the probability of unequal crossing-over where one or more of the six exons are exchanged (Crognale et al., 1998; Deeb et al., 1992; Hayashi et al., 2006; Jagla et al., 2002; Nathans et al., 1986a; Neitz et al., 1989; Neitz et al., 1996). Successful gene therapy in red-green color blind primates shows that common color vision deficiency can be rescued. Moreover, successful treatment of a non-human adult primate indicates that gene therapy in a neuronal context can be effectively applied after the developmental stage (Mancuso et al., 2009). Another proposed rescue of daylight vision has been achieved by cell transplantation in mice (Santos-Ferreira et al., 2015).

Blue-yellow color deficiency can be caused by one of the six known mutations or other unreported mutations within the human short wavelength sensitive (SWS1) opsin gene (Table 3) (Neitz and Neitz, 2011). Interestingly, none of the mutated amino acid residues interact directly with the chromophore or show a shift of the SWS1 pigment spectral sensitivity (Figure 7). Only seven amino acid residues are responsible for the spectral shift between L/LWS and M/LWS pigment of which only three interact directly with the chromophore. This fact leads us to conclude that the six mutations in the SWS1 opsin must perturb the folding, hydrogen bond network, stability, activation or transport instead. One reason for the minor number of known mutations which shift the spectral absorption in the SWS1 pigment is that mutations causing a minor shift of the SWS1 pigment will not overlap with the absorption spectra of other visual pigments. Thus a mutation
causing a spectral shift of the SWS1 pigment will not result in a severe
compromising color vision deficiency and thus is not reported.

Disease causing mutations in LWS and SWS1 opsin genes.

<table>
<thead>
<tr>
<th>opsin</th>
<th>mutation</th>
<th>phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>L/LWS and M/LWS</td>
<td>N94K (Ueyama et al., 2002)</td>
<td>deutan</td>
</tr>
<tr>
<td></td>
<td>C203R (Winderickx et al., 1992b), (Nathans et al., 1993)</td>
<td>deutan, or BCM if both L/LWS and M/LWS are affected</td>
</tr>
<tr>
<td></td>
<td>W177R (Gardner et al., 2010)</td>
<td>X-linked cone-rod dystrophy</td>
</tr>
<tr>
<td></td>
<td>R247Ter (Nathans et al., 1993)</td>
<td>BCM with single L/LWS opsin gene</td>
</tr>
<tr>
<td></td>
<td>P307L (Nathans et al., 1993)</td>
<td>BCM with a single L/LWS-M/LWS hybrid gene</td>
</tr>
<tr>
<td></td>
<td>R330N (Ueyama et al., 2002)</td>
<td>deutan</td>
</tr>
<tr>
<td></td>
<td>G338E (Ueyama et al., 2002)</td>
<td>protan, deutan</td>
</tr>
<tr>
<td></td>
<td>L/M153, I/V171, A174, V178 and A/S180 (LIAVA, LVAVA, LIAVS or MIAVA) (Carroll et al., 2004; Gardner et al., 2014; Mizrahi-Meissonnier et al., 2010; Neitz et al., 2004; Ueyama et al., 2012)</td>
<td>protan, deutan</td>
</tr>
<tr>
<td>SWS1</td>
<td>G79R (Weitz et al., 1992a)</td>
<td>tritan</td>
</tr>
<tr>
<td></td>
<td>L56P (Gunther et al., 2006)</td>
<td>tritan</td>
</tr>
<tr>
<td></td>
<td>T190I (Baraas et al., 2012)</td>
<td>mild tritan (at high luminance), severe tritan (at low luminance)</td>
</tr>
<tr>
<td></td>
<td>S214P (Weitz et al., 1992a)</td>
<td>tritan</td>
</tr>
<tr>
<td></td>
<td>P264S (Weitz et al., 1992a)</td>
<td>tritan</td>
</tr>
<tr>
<td></td>
<td>R283Q (Baraas et al., 2007)</td>
<td>tritan, SWS1 cone degeneration</td>
</tr>
</tbody>
</table>

Table 3: Disease causing mutations in LWS and SWS1 opsin genes. *Mutations in L/LWS and M/LWS could cause similar impairments because high homology between these two pigments and are group together.
1.6.1 Tritanopia

SWS1 cones comprising about 6% of all human cones, are distributed independently throughout the retina and show a hexagonal packing with the neighboring photoreceptor cells (Curcio et al., 1987; Hofer et al., 2005; Lombardo et al., 2013a, b). Tritanopia is a SWS1 cone–related disease. Humans suffering from tritanopia are unable to differentiate colors in the middle to short wavelength region of the visible spectrum (Figure 6). Such variations of spectral discrimination are due to amino acid substitutions in the SWS1 cone pigment. Compared to the 8% prevalence of red–green visual defects, the occurrence of tritanopia is much lower affecting only 0.001% – 0.2% of the human population (Gunther et al., 2006). Tritanopia also increases during aging as cones become less sensitive to light with a reduction affecting the sensitivity of SWS1 cones at least as much as that of L/LWS and M/LWS cones (Eisner et al., 1987; Johnson et al., 1988; Shinomori et al., 2001; Werner and Steele, 1988). The age–related loss of short wavelength sensitivity cannot or only partially be attributed to yellowing of the lens. A comparison study with younger tested persons showed that other factors such as pupil size, background illumination level, iris color and macular pigment density can account for the reduced perception (Beirne et al., 2008a; Beirne et al., 2008b). Another explanation is found by neurological studies on the SWS1 cone pathway showed that a neural loss in the retinal circuit and lateral geniculate nucleus causes a delayed SWS1 cone OFF response and thus an altered sensitivity (Shinomori and Werner, 2012).
Figure 6: Comparison of normal and deficient color vision. Visible spectra illustrated for five types of human color blindness are adapted from (Karl R. Gegenfurtner, 2001). The spectra represent only severe cases of color vision deficiencies and a common spectrum for normal color vision. Actually, there are milder degrees of color vision deficiencies such as deuteranomaly and protanomaly. These anomalous trichromats contain at least one normal cone opsin and one set, which evidence a minor spectral shift from each other (Bollinger et al., 2004; Yamaguchi et al., 1997). Furthermore, 38% of Caucasian males with normal trichromacy contain the variation in the red opsin that alters the normal spectrum.

Genealogical studies have revealed that tritanopia is an autosomal dominant trait with incomplete penetrance, whereas protanopia and deuteranopia involve the X−chromosome and thus exhibit a X−linked pattern of inheritance (Krill et al., 1971; Smith et al., 1973; Wright, 1952). Six disease−causing mutations are known to cause tritanopia: L56P, G79R, T190I, S214P, P264S and R283Q (Table 3) (Baraas et al., 2007; Baraas et al., 2012; Gunther et al., 2006; Weitz et
al., 1992a; Weitz et al., 1992b). As illustrated in Figure 7, these mutations do not interact with the chromophore itself. However, replacement of a rigid nonpolar Pro residue, with a polar or a charged residue or vice versa can distort the overall protein structure, impair GPCR activation or transport, all of which result in the loss of blue–yellow color perception (Weitz et al., 1992a; Weitz et al., 1992b; Zalewska et al., 2014). The point mutation R283Q in the SWS1 opsin was shown to be associated with progressive SWS1 cone degeneration, analogous to the rod degeneration seen in retinitis pigmentosa (Baraas et al., 2007).

Figure 7: A schematic view of the SWS1 opsin with the six disease–causing mutations is depicted in black. Each mutation is displayed as green sticks, whereas the wild–type is displayed according to element color. The protein is shown in a ribbon representation colored according to its secondary structure. Red represents α–helical structures, cyan indicates beta strands and loops are shown in green. The structure is displayed as a wire ribbon, where mutations have changed the secondary structure of the wild–type protein.
1.6.2 Red–green color deficiency

Individuals suffering from red–green color deficiencies possess only two fully functional cone pigments, namely SWS1 and either M/LWS or L/LWS. The loss of functional L/LWS photopigment results in protanopia, whereas a shifted absorption of L/LWS photopigment causes protanomaly. The loss of functional M/LWS pigment results in deuteranopia, whereas a shifted absorption of M/LWS pigment causes deuteranomaly (Figure 6). Non–homologous recombination or point mutations within the L/LWS or M/LWS opsin genes produce these deficiencies (Figure 6) (Deeb, 2004; Neitz and Neitz, 2011). The spectral separation between the impaired L/LWS and M/LWS pigments determines the severity of the color vision defect. The small difference ($\Delta_{M/LWS/LWS} = 27.5$ nm) in spectral absorption between human L/LWS and M/LWS pigments is crucial for correct red–green color vision. A spectral shift of about 5 nm caused by the S180A mutation accounts for 18% of the total wavelength difference between L/LWS and M/LWS. A shift of 5 nm in the absorption of the SWS1 cone pigment would only account for 4.5% or 3.6% of the total absorption difference between SWS1 and M/LWS or L/LWS, respectively. Thus mutations that cause a minor shift in the SWS1 pigment will cause a smaller change in color perception compared to shifts in M/LWS or L/LWS pigment absorption (Pokorny et al., 1981).
Figure 8: A combined figure of transcription regulation, organization and crossover events of LWS opsin genes. L/LWS, M/LWS head to tail gene arrays are shown with the LCR located about 3.5 kb upstream. L/LWS genes are depicted in red, M/LWS genes in green. LCR regulates the expression of the first two LWS gene copies. Obviously a deletion or other damage of the LCR leads to BCM. The left and right crosses indicate unequal or equal crossovers, respectively during meiosis resulting in either a hybrid gene or exchange of a whole gene. The upper strand depicts the binding of LCR to the promoter region of the first L/LWS gene, whereas the lower strand represents the binding of LCR to the first M/LWS gene promoter via its three transcription factors. Percentages on the left indicate the distance–dependent probability of gene transcription regulated by LCR in Caucasian males (Carroll et al., 2000; McMahon et al., 2008).

The L/LWS and M/LWS opsin genes are located in a head–to–tail tandem array on the human X–chromosome (Figure 8) (Nathans et al., 1986b). The amino acid sequence identity between L/LWS and M/LWS opsins is 96%, and they have only 42% and 43% identity with the SWS1 opsin and rhodopsin (Nathans et al., 1986b; Okano et al., 1992). The number of copies and sequences of both the
The L/LWS and M/LWS opsin genes vary among individuals with normal color vision (Figure 8) (Neitz and Neitz, 1995; Yamaguchi et al., 1997). For example, the L/LWS opsin exists as two prominent Ser/Ala variants at position 180 in humans with normal color vision which differ in $\lambda_{max}$ by about 4 nm (Neitz and Jacobs, 1986). The high degree of inherited red–green color vision deficiency (~8%) can be explained by a high frequency of unequal homologous recombination (McClements et al., 2013; Roosing et al., 2014). The high frequency of recombinations is promoted by the high homology of the L/LWS and M/LWS opsins. Non–homologous pairing, gene conversion and unequal cross–overs within this tandem array result in color vision deficiencies of different severity such as protanopia, protanomaly, deuteranopia, deuteranomaly or blue–cone monochromacy (Figure 6 and Figure 8) (Deeb, 2004; Neitz and Neitz, 2011).

Transcription of both the L/LWS and M/LWS opsins is regulated via a 3.5 kb upstream locus control region (LCR) (Nathans et al., 1989; Wang et al., 1992). Therefore, the 5’–first and the 5’–second of numerous LWS opsin genes are transcribed and regulated by this LCR (Figure 8) (Carroll et al., 2002; Deeb, 2005; McMahon et al., 2008; Winderickx et al., 1992a; Yamaguchi et al., 1997). Deletion or damage of the LCR results in blue–cone monochromacy (BCM) (Figure 6 and Figure 8) (Nathans et al., 1989).

1.6.3 L/LWS–M/LWS genetics

The L/LWS and M/LWS genes consist of six exons, one more (the first exon) than the rhodopsin and SWS1 genes that contain only 5 exons (Nathans et al.,
Exon five of the L/LWS and M/LWS opsins is primarily responsible for the spectral differences in light absorption (Neitz and Jacobs, 1986). Because L/LWS and M/LWS opsin genes are located on the X−chromosome, females are theoretically capable of tetrachromacy. Addition of one more dimension within the visible spectra to trichromacy fails to produce a significant phenotypic advantage for these carriers (Jordan et al., 2010).

To date, several point mutations are known which result in red−green color deficiencies (Table 3). Some of these are summarized in (Carleton et al., 2005). Three different mutational mechanisms were categorized into three classes. First, deletion of the LCR; second, missense mutation in an LWS hybrid gene; and third, an exon 3 single−nucleotide polymorphism (Gardner et al., 2014). Here we report a selection (N94K, R247Ter, C203R, P307L, R330Q and G338E) of known class two and three mutations in L/LWS and M/LWS opsins and their corresponding disease phenotypes. All of these missense mutations result in either the absence of expression or lack of function of the corresponding photopigment. Males who have an X−chromosome opsin gene array in which there is a single gene containing one of these missense mutations, will experience blue cone monochromacy. In contrast, a male with two or more opsin genes on the X−chromosome in which one of the first two genes contains one of these missense mutations will be a dichromat. The type of dichromacy, protan or deutan, depends on whether the mutation is in the L/LWS opsin gene (protan) or in M/LWS opsin gene (deutan). The point mutation G338E in L/LWS or M/LWS opsin results in a protan or deutan dichromacy, respectively, because of the
inactivated photopigment (Ueyama et al., 2002). The mutation N94K, C203R or P307L in either L/LWS or M/LWS resulted in a lack of light absorption and thus in dichromacy (Nathans et al., 1993; Reyniers et al., 1995; Ueyama et al., 2002). The mutation R330Q found in M/LWS causes deutan dichromacy because of reduced absorption at 530 nm (Ueyama et al., 2002). The mutation R247Ter in a male with only a single LWS gene leads to blue–cone monochromacy due to an early stop codon after the fifth transmembrane helix (Nathans et al., 1993; Reitner et al., 1991). Furthermore, a deleterious combination of polymorphisms involving amino acids L153, I171, A174, V178 and A180 (LIAVA) can result in protanopia, deuteranopia or even blue–cone monochromacy (Figure 6) if present in both L/LWS and M/LWS genes (Carroll et al., 2004; Crognale et al., 2004; Gardner et al., 2014; Mizrahi-Meissonnier et al., 2010; Ueyama et al., 2012). Besides LIAVA, the two single nucleotide polymorphisms leading to amino acid combinations of LVAVA and MIAVA were recently found to cause aberrant splicing (Gardner et al., 2014; Ueyama et al., 2012).

X–linked cone dystrophy is yet another and more severe color vision deficiency. Here opsin dysfunction causes degeneration primarily of cone photoreceptors and subsequently rod photoreceptors resulting in severely impaired vision. Cone dystrophy differs from cone–rod dystrophy in that intact peripheral vision is retained in the former. Point mutations W177R and C203R in the third and fourth exon of the L/LWS– and M/LWS–genes are known to cause progressive X–linked cone dystrophy. Both mutations cause aggregation and accumulation of opsin within the endoplasmic reticulum of human neuroblastoma cells, similar to
the rhodopsin retinitis pigmentosa–causing mutation P23H (Dryja et al., 1990; Gardner et al., 2010). Compared to P23H rhodopsin, the W177R mutation cannot be rescued with 9–cis–retinal (Gardner et al., 2010). Moreover, the linkage disequilibrium between LIAVA and W177R supports the concept of abundant gene conversions between the two homologous L/LWS and M/LWS opsin genes (Gardner et al., 2010).

1.6.4 Transcription regulation of L/LWS and M/LWS opsin genes

Epigenetic studies have shown that rod and cone opsin transcription is regulated by several mechanisms, including interactions of at least three photoreceptor–specific transcription factors at the promoter or coding regions and looping of the cis–regulatory region LCR or rhodopsin enhancer region (RER) (Figure 8). The latter interaction decreases as the distance increases between the LCR or RER and the promoter or coding region (Figure 8). The three transcription factors are the cone–rod homeobox (CRX), the neural retina leucine zipper (NRL), and nuclear receptor subfamily 2, group E, member 3 (NR2E3) (Peng and Chen, 2011). GTF2IRD1 is an additional transcription factor found in human and mouse retina that might regulate the level and topology of rod and cone photoreceptor gene expression as well as the three known transcription factors (Farkas et al., 2013; Masuda et al., 2014). Understanding transcription regulation mechanisms could increase the probability of transcribing fully functional copies of either a L/LWS or M/LWS gene and thereby restoring color vision in individuals with impaired color perception (Cideciyan et al., 2013).
Recent advances in somatic cell research revealed that fully functional photoreceptor cells derived from induced fibroblasts can further up-regulate the endogenous \textit{NRL} and \textit{NR2E3} genes required for photoreceptor cell specification (Seko et al., 2014). Furthermore, successful transplantation of cone–like photoreceptors into mouse retinas with cone degeneration demonstrates the therapeutic potential of stem cell and gene therapies to cure these visual diseases (Dalkara et al., 2015; Santos-Ferreira et al., 2015). Finally, restoration of color vision in a red–green color blind primate also emphasizes the potential of gene therapy (Mancuso et al., 2009). Furthermore, genetic deletion of both SWS gene alleles slowed down cone photoreceptor cell degeneration in a Leber congenital amaurosis mouse model. This finding implicated that a combinatorial approach of gene therapy and pharmacological chaperones which improve folding of cone visual pigments would slow down photoreceptor degeneration (Zhang et al., 2015a).

1.7 Color vision among species

The retina is a highly specialized light sensor that can detect chromatic, spatial and temporal variations in light. This nerve tissue has also evolved adaptively to the particular environment of an animal’s habitat (Ahnelt and Kolb, 2000; Bloch, 2015; Peichl, 2005; Szel et al., 1994a). Thus, an animal possesses color vision if it can discriminate between stimuli that differ in their distributions of spectral energy. Or more precisely, it needs to possess: a) photoreceptors with distinct
photopigments, and b) a neuronal circuit which connects the outputs of these receptors with the central nervous system (Jacobs, 1993). The most sophisticated visual sensory system is found in the cones of sauropsids. Aside from several differing visual pigments and types of cone cells, they possess double cones, cone photoreceptor cells containing two outer segments with two different visual pigments such as LWS or RH2, and pigmented oil droplets that serve as chromatic filters (Figure 9). These features permit tetrachromatic vision and further improve hue and contrast discrimination (Ahnelt and Kolb, 2000; Vorobyev, 2003; Vorobyev et al., 1998). Varying ratios of cone and rod cells between different species indicate an environmentally–related sculpting of the retina which influences the numerical ratio and also the structures of rod and cone photoreceptor cells. Adaptation of the first mammals to nocturnal activity led to a rod–dominated retina which affected photoreceptor cells and subsequently also the organization of second–order neurons (Ahnelt and Kolb, 2000; Hart et al., 2006; Peichl, 2005).

In 1840, Adolph Hannover discovered colored oil droplets in bird cone photoreceptor cells (Hannover, 1840). Almost 100 years later Gordon Lynn Walls reported the presence of an oil droplet located before the disc membranes of cones or rods in a variety of species including sturgeon, marsh hawk, leopard frog, snapping turtle, western painted turtle, and marbled lungfish (Figure 9) (Walls, 1942). The presence of these oil droplets in cone photoreceptor cells of all birds, and some reptiles, amphibians, mammals and fish was later confirmed by several other groups (L. Fischer 1984; Robinson, 1994; Vorobyev, 2003). The
presence of an oil droplet in just a minority of varied species suggests that this feature could be an ancient feature of visual cells. Genealogical studies dated the origin of colored droplets to about 400 million years ago. That pigmented oil droplets are found exclusively in tetra or higher chromatic species indicates that they provide an advantage only when many different photopigment types are present (Robinson, 1994). Walls and Hannover noted that some oil droplets in cones contain dissolved yellow, orange or red pigments (Table 4) (Hannover, 1840; Walls, 1942). Light absorption by these oil droplets varied from 375 to 477 nm depending on their pigments.

Figure 9: A schematic representation of a cone photoreceptor cell containing an oil droplet. Localization of an oil droplet between the inner and outer segment is identical throughout various species (Walls, 1942)
Analysis of extracted pigments showed that these were carotenoid derivatives, such as astaxanthin, zeaxanthin or $\varepsilon$-carotene (Goldsmith et al., 1984). Further information about carotenoids and a structural perspective about their enzymatic cleavage was reviewed by (Sui et al., 2013). Some droplets contained up to two carotenoid pigments. Avian oil droplets have been classified based on their spectrophotometric and morphological properties (Table 4) (Goldsmith et al., 1984). Because pigmented oil droplets absorb light at specific wavelengths, they could enhance color contrast, acting as filters to exclude certain wavelengths of light. An additional function could be protection against damage by light in the short wavelength range (Goldsmith et al., 1984; Hart et al., 2006; L. Fischer 1984). Calculations of energy distribution indicate that oil droplets enhance the absolute sensitivity at one wavelength and also the signal–to–noise ratio of photoreceptor cells through absorption of scattered photons in the retina (Ives et al., 1983). In contrast, absorption of any wavelength of light in a dim lit environment would be disadvantageous. A study of pigmented oil droplets in chicken retina showed that the carotenoid density was proportional to the light intensity of their environment. This finding indicates that the visual pigment density and therefore the absorption could be adapted on a seasonal timescale to optimize color perception (Hart et al., 2006). Furthermore, the dietary uptake of carotenoids can modify the composition and concentration of carotenoids found in avian oil droplets (Knott et al., 2010). Taken together, the features of colored or non–colored oil droplets probably improve both color–contrast and hue discrimination. This improvement could be highly beneficial, especially where
colors play a crucial role in natural selection (Vorobyev, 2003; Vorobyev et al., 1998).

<table>
<thead>
<tr>
<th>Type</th>
<th>Visual appearance</th>
<th>Always present</th>
<th>$\lambda_{\text{max}}$ [nm]</th>
<th>Number of carotenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>Red</td>
<td>+</td>
<td>477 ± 2.3</td>
<td>1</td>
</tr>
<tr>
<td>O</td>
<td>Orange</td>
<td>−</td>
<td>425–477</td>
<td>1–2</td>
</tr>
<tr>
<td>Y</td>
<td>Yellow</td>
<td>+</td>
<td>435–455</td>
<td>1–2</td>
</tr>
<tr>
<td>P</td>
<td>Pale yellow or greenish</td>
<td>+</td>
<td>375–455</td>
<td>2</td>
</tr>
<tr>
<td>$C_j$</td>
<td>Colorless (or pale yellow)</td>
<td>+</td>
<td>375–405</td>
<td>1</td>
</tr>
<tr>
<td>$C_s$</td>
<td>Colorless (or pale yellow)</td>
<td>−</td>
<td>375–405</td>
<td>1</td>
</tr>
<tr>
<td>$T_j$</td>
<td>Transparent</td>
<td>−</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>$T_s$</td>
<td>Transparent</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4: Classification of oil droplet types based on carotenoid content. Lower case ‘j’ and ‘s’ indicate the relative size of the droplet, with ‘j’ representing the small and ‘s’ the large size. Adapted from (Goldsmith et al., 1984).

Through all the species the light absorbing prosthetic group in visual pigments is a retinal analog or retinal (Urich, 2013). So far four different visual chromophores were characterized; Retinal (RAL₁) is the universal chromophore found in most vertebrates and invertebrates (Wald, 1939a, b). Porphyropsin, 3-dehydroretinal (RAL₂) is found in fishes, some amphibians and reptiles (Allison et al., 2004; Suzuki et al., 1984a; Toyama et al., 2008; Wald, 1939b). Those two
chromophores (RAL₁ and RAL₂) compete for the same binding site in opsin proteins if present in the photoreceptor cells (Allison et al., 2004; Suzuki et al., 1984b). Xanthopsin, 3-hydroxyretinal (RAL₃) was exclusively discovered in the visual system of insects and occurs in two enantiomeric forms (3R and 3S) (Kirschfeld et al., 1977; Seki and Vogt, 1998; Vogt and Kirschfeld, 1983). 4-hydroxyretinal (RAL₄) the forth chromophore was discovered in firefly squid (Matsui et al., 1988). A comparison of RAL₂ and RAL₁ in salamander red rods demonstrated that RAL₁ improves dark noise levels compared to RAL₂ (Ala-Laurila et al., 2007). The origin and further advantages besides the peak absorption of the different chromophores RAL₁-₄ in visual pigments is not fully understood.

1.7.1 Variations of cone photoreceptor organization throughout species

When immunohistochemistry is used to compare mammalian retinas, the largest diversity of cone abundance is found in rodents. This finding is not surprising, as rodents constitute the most species-rich order and occupy a large variety of habitats. Moreover, the distribution of cone photoreceptor cells is similar in the retinas of all mammalian species. The highest cone density is found in the dorsotemporal quadrant of the retina’s Area centralis with a centroperipheral density gradient. A search for SWS cones revealed either their absence in species such as whales, seals and some nocturnal species or a distribution similar to LWS or MWS cones. Notably, SWS cones account for only about 5–10% of total cones distributed into three regions: first, a centro–peripheral
density gradient of SWS cones with a peak density at the Area centralis in the dorso-temporal quadrant; second, a high SWS cone concentration in the ventral retina, and third, a reverse topography of SWS cones with a high density in the peripheral retina and a low density in the central retina (Lukats et al., 2005; Peichl, 2005; Szel et al., 2000). Typically, photoreceptor cells contain only a single visual pigment in most species. Immunohistochemical analyses of the retina have revealed; however, that in some species that there are regions with dually pigmented cones co-expressing two pigments (Lukats et al., 2005). ERG studies in mice and Siberian hamsters revealed that dual cones are sensitive to UV and long wavelength light (Calderone and Jacobs, 1999; Jacobs et al., 2004). How these signals are processed and contribute to color perception is still unknown. Expression of two LWS and SWS opsins within one photoreceptor cell is a rare phenomenon in mammalian species. To date, such dually pigmented cones have been detected in the house mouse, guinea pig and rabbit (Rohlich et al., 1994). Dual cones first express the SWS opsin and then later in development express MWS and LWS opsins (Lukats et al., 2005; Szel et al., 1994b). The distribution of such pigmented cones throughout the retina differs among species. An interesting pattern is found in mice where SWS and LWS pigments are distributed along a dorsoventral gradient. In dorsal cones MWS and LWS opsins are preferentially expressed but in ventral cones, SWS opsin expression dominates. A high abundance of SWS pigments in the ventral region is also found in insectivores (Lukats et al., 2005). The function of these dually pigmented
cells can serve more in broadband detection than in specific color contrast discrimination (Peichl, 2005).

1.7.2 SWS cones: a distinct member of the retina

The uneven distribution of SWS cones within the retina is not fully understood. However, the increased SWS cone density in the ventral retina region in species such as rodents implies an evolutionary adaption to blue skylight because that is the background from which their predators attack (Lukats et al., 2005; Peichl, 2005). Two–photon imaging, statistics and immunohistochemistry were used to investigate the response properties of MWS and SWS cones based on their loci in mouse retina. The results indicate that the improved detection of achromatic contrast in the sky region originates from a higher gain of SWS cones in the ventral retina, and not from greater spectral sensitivity (Baden et al., 2013). Further, lower phototransduction noise in photoreceptor cells leads to higher visual sensitivity (Aho et al., 1988; Angueyra and Rieke, 2013). SWS photopigments have reduced noise compared to most LWS photopigments, thus the improved detection could partially result from a lower phototransduction noise in SWS cone photoreceptor cells (Rieke and Baylor, 2000). An exceptional feature of SWS cones is their complete absence in the retinas of bottlenose dolphins. Also notable is a distribution of SWS cones found in the ground squirrel that differs from the pattern exhibited by LWS and MWS cones (Figure 10). Loss of SWS cones can occur in distinct mammalian species, and loss of SWS cones is common in marine mammals. This observation seems counterintuitive as short
wavelength light is the major source of light in open, clear water (Griebel, 2002). LWS and rod pigments of marine mammals also exhibit a hypsochromic shift which possibly could compensate for their loss of SWS cones (Peichl, 2005; Peichl et al., 2001).

Figure 10: Topography of the spectral cone types in mammalian species. The bottlenose dolphin represents marine mammals lacking SWS cone photoreceptor cells. The ground squirrel represents a cone dominant diurnal species with a spot of high density of SWS cones in the dorso–temporal region of the retina. The rabbit contains SWS cone photoreceptors in an increasing gradient from the area centralis to the ventral region of the retina (Ahnelt and Kolb, 2000).
Humans and most primates have a cone–rich region in the retina termed the fovea which can be compared with the *Area centralis* of other mammals. Consisting of tightly packed cones, the fovea is almost devoid of rods (Figure 10). Moreover, entering light can avoid crossing secondary neurons because these are bent away from the fovea (Provis et al., 2013). This morphology makes the fovea well suited for high resolution vision of focused objects. Photoreceptor cells are organized within a mosaic in the retina, their distribution varying from a completely random to an ordered hexagonal topography. A hexagonal organization represents the closest packing comparable to crystal packing, wherein one cone photoreceptor is surrounded by six photoreceptor cells. This highly ordered packing is prominently found with cones, whereas rods disrupt the hexagonal topography by having four to five directly neighboring photoreceptor cells (Ahnelt and Kolb, 2000; Lombardo et al., 2013a, b; Pum et al., 1990). Such an arrangement allows the highest density of light sensors and thus one of the highest possible optical resolutions in nature. Conversely, irregularities in cone photoreceptor organization found in their mosaics are mainly due to curvature, local noise, interference from other cellular subpopulations, and meridional changes of size and composition (Ahnelt, 1998; Hirsch J Fau - Miller and Miller, 1987). Such irregularities result in areas where one photoreceptor class is predominant or absent, and as a result, these regions will be more or less sensitive to one wavelength. In humans and some primates, the number of SWS cones are markedly reduced in the foveal pit. SWS cones have the highest density within the fovea around the foveal pit. Although the impact of the foveal
organization is still unknown, it should be noted that short wavelength light is dispersed to a higher degree than medium or long wavelength light, depending on the wavelength–specific refractive index. This fact would explain why SWS cones are mainly located in the foveal ring surrounding the foveal pit (Ahnelt, 1998; Curcio et al., 1987; Lukats et al., 2005; Walls, 1942). Such foveal organization also compensates for the greater dispersion of short wavelength light through the cornea, lens and vitreous (Figure 10).

Figure 11: Schematic optics of the eye provides an explanation of the SWS1 cone distribution around the central fovea. Polychromatic light indicated by an arrow (left) undergoes different aberrations through the lens and vitreous. This process causes the different focus points behind (red), on (green) and in front (blue) of the retina. Dimensions of fovea are from (Kolb, 2015).

Furthermore, transmission of short wavelength light is reduced more than that of medium and long wavelength light. Dispersion plus transmission properties of the cornea and lens contribute to the explanation of why no SWS1 cone pigments
are found in the center of the fovea and why SWS cones account for only 5–10% of all cone photoreceptor cells. The fovea contains increased concentrations of carotenoids that absorb short wavelength light (Hammond et al., 1997). Thus, the ability to absorb blue light indicates a scavenger effect similar to that found in oil droplets, which absorb scattered photons within the eye and thereby, improve the signal to noise ratio. Limited space in the retina has forced nature to build the most effective, efficient and sophisticated light sensors with minimal redundancy in the photoreceptor cell population (Figure 11). As do all optical systems, the eye also suffers from chromatic anomalies depending on the wavelength of light. For example, short wavelengths exhibit greater deviations than long wavelength light resulting in myopia (near sightedness) for short wavelengths and hyperopia (far sightedness) for long wavelengths of light (Charman and Jennings, 1976). These abnormalities cause three optical effects, namely alterations in focus, position and magnification. The difference in focus stems from a longitudinal chromatic aberration, whereas differences in position and magnification are based on transverse chromatic aberrations (TCA) (Figure 11) (Gupta et al., 2010; Rucker and Osorio, 2008; Rynders et al., 1995). Herein we propose that the organization and distribution of photoreceptor cells within and around the fovea partially compensate for these abnormalities. Furthermore, neurons in the brain process visual stimuli and match them together to form a meaningful image (Artal et al., 2004; Ohlendorf et al., 2011; Sabesan and Yoon, 2010). Thus, organization of the fovea and processing through neurons compensate for the disruptive effects of chromatic aberrations. Besides the fovea, there is a
cone–rich rim consisting of L/LWS and M/LWS cone photoreceptors found at the ora serrata of the human retina. This organization of cones could contribute to color perception by detecting dispersed light, although it is function has not yet been fully established (Curcio et al., 1987; Mollon et al., 1998). Others propose three advantages for the band of cones at the edge of the retina with respect to peripheral vision. First, is to detect objects crossing the visual field based on a change in contrast. Second, cones can evoke a signal in bright daylight because they are not saturated under bright light conditions. Third, the time response of cones is more rapid than that of rods, allowing a faster and steady perception (Lamb, 2013; Williams, 1991). Measurements of color perception in the intermediate periphery confirmed the ability of peripheral red–green color detection (Hansen et al., 2009). How the cone–rich rim contributes to the peripheral color perception remains to be answered. Müller glial cells can contribute to our color perception as shown using a combined experimental and computational experiments (Agte et al., 2011; Franze et al., 2007; Labin et al., 2014). Thus, these neurons act as wavelength–dependent wave guides to selectively direct incoming photons to corresponding cone– or rod–photoreceptor cells, a concept reminiscent of the passage of light through fiber optics. The idea of Müller glial cells as waveguides is controversial in the field of vision. Questions of how Müller glial cells might select specific wavelengths and thus improve light perception or if Müller glial cells simply degrade vision remain to be answered.
1.8 Conclusions

Here we provide an overview of the first steps in color vision by combining the most recent relevant information in the fields of physics, chemistry and biology. History has revealed that such an interdisciplinary combination can contribute substantially to our knowledge of complex biological processes. However future progress will require new approaches involving additional disciplines to answer more complex scientific questions about light perception and the spectral tuning of cone pigments.

Visual perception starts with the absorption of photons, excitation of the retinal chromophore and the \textit{cis–trans}–isomerization of 11–\textit{cis}–retinal and ends with signal processing in our central nervous system. Thus, vision and the perception of color is a highly complex process involving an interplay of photons, chromophore, opsin proteins, neurons and their crosstalk. Herein, we discuss energetic and chemical properties of the chromophore, that provide insights into the natural limits of our visible spectra. The physical properties of light and its dispersion through the cornea, lens, and vitreous limit our color perception. Moreover, the distribution of LWS, MWS, and SWS cone photoreceptors throughout the retina seems shaped by evolution to optimize visual perception in the particular habitat of a species. The hypothesis that organization of photoreceptor cells at the fovea compensates for wavelength–dependent aberrations caused by the lens also seems likely. An exotic but nonetheless effective device to optimize color perception is the oil droplet found in the retinas.
of birds, fish, reptiles and marsupials. Oil droplets supposedly improve contrast by filtering out unwanted wavelengths of light through absorption by carotenoids which further improves the signal–to–noise ratio. Similar carotenoids are found in the retinas of mammals, especially at high concentrations in and around the fovea. Whether these carotenoids act as scavengers for scattered photons and thus have the same function as an oil droplet remains unknown.

Color vision deficiencies can be inherited or caused by various mutations or unequal crossovers, resulting in a reduced, shifted or absent wavelength sensitivity in at least one cone photopigment. To date, up to six point mutations in the SWS1 opsin are known to cause tritanopia, a deficiency in blue spectral sensitivity. The more frequent red–green color deficiency can be caused by various point mutations similar to those in tritanopia. Moreover, a major motif is found in the increased number of recombinants between the highly homologous L/LWS and M/LWS opsin genes. Transcription of these opsin genes is regulated by the LCR, such that deletion or mutation of the LCR leads to BCM. Furthermore, transcription of one of the numerous L/LWS or M/LWS opsin gene copies depends on the distance between the LCR and the copy; thus, the shorter this distance, the greater the transcription. Gene therapy can be used to cure red–green color blindness in adult primates (Mancuso et al., 2009), but how these findings can be applied to humans is a topic for future research.

Structural studies, computational chemistry, mutagenesis and biochemistry have provided insights into the spectral tuning of the universal chromophore, 11–cis–retinal. Nevertheless, we stress here that mutagenesis of single amino
acids and spectral analyses do not take the complex interactions of internal visual pigment waters, hydrogen bond networks and their rearrangements entirely into account. Thus, experimental data at a molecular level are desperately needed to decipher the secrets of nature’s spectral tuning.

1.9 Future directions

The highest resolution structures of cone pigments are urgently required to identify their side–chain arrangements and also to reveal water molecules and hydrogen bond networks that reside within the transmembrane bundles of visual GPCR helices. Such structures will demonstrate the multiple components that contribute to the spectral properties of visual pigments including: a) the organization of internal waters; b) protonation states of other pigment residues along with the helical bundles; and c) conformations and twisting of the chromophore within the binding pocket. To supplement this structural data, radiolytic footprinting methods should provide additional information about water interactions and movements. Moreover, cations and anions can significantly change the spectral tuning of visual pigments. These charged molecules need to be identified in the pigment structures and correlated with the corresponding spectra.

Combined X–ray crystallography, electron crystallography and radiolytic footprinting studies will provide details for cone pigments unprecedented in
GPCR structural determination. This information will constitute a platform for initiating molecular dynamics and quantum mechanics computational studies. Quantum mechanical calculations of the chromophore in the binding pocket will reveal the mechanism of spectral tuning for each of the cone pigments. Although such mechanisms have long been studied, direct observation and calculation will only be enabled with high-resolution structural data made possible through the multidisciplinary protocols outlined above. Hybrid QM/MM molecular dynamics trajectories will reveal structural changes that take place in the chromophore, the protein, and bound waters during the photochemical and thermal chromophore isomerization reaction.
Chapter 2

An effective thiol-reactive probe for differential scanning fluorimetry with a standard real-time polymerase chain reaction device

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"Reprinted from Analytical Biochemistry, Vol 499, Lukas Hofmann, Sahil Gulati, Avery Sears, Phoebe L. Stewart, Krzysztof Palczewski, An effective thiol-reactive probe for differential scanning fluorimetry with a standard real-time polymerase chain reaction device, 63-65, Copyright (2016), with permission from Elsevier."

https://dx.doi.org/10.1016/j.ab.2016.01.016
2 Assessing the thermal stability of visual GPCRs

2.1 Abstract

Popular in different sub-disciplines of biochemistry, differential scanning fluorimetry (DSF) is used to assess protein stability, transition states, or the Kd’s of various ligands, drug molecules and antibodies. Furthermore, this technique is employed to monitor protein-protein interactions and investigate the effects of disease-causing mutations on proteins. The popularity of DSF originates from the affordable, simple, sensitive and high-throughput format of the assay. Nevertheless, all fluorescent probes published to date are either incompatible with hydrophobic proteins/ligands, which precludes analyses of transmembrane or membrane associated proteins, or have excitation and detection wavelengths outside the range of RT-PCR machines, necessitating the use of dedicated devices. Herein, we describe a thiol-reactive probe BODIPY FL L-cystine (BFC) to overcome both of these shortcomings, utilizing both membrane and soluble proteins as examples. The results show the probe supports an inexpensive application of DSF measurements suitable for detection with standard RT-PCR machines in a hydrophilic or hydrophobic environment (Figure 12).
Figure 12: Reaction mechanism of BFC with an exposed Cys residue. Upon thermal unfolding Cys residues of a protein are exposed to the solvent. The thiol reactive dimer of BFC dissociates and binds to the Cys sulphydryl group. In its dimeric state BFC is self-quenched and exhibits reduced fluorescence however, upon monomerization the dye is unquenched and its fluorescence emission at $\lambda_{em} = 511$ nm can be detected using a standard RT-PCR filter (Haugland, 2002).
2.2 Introduction

Differential scanning fluorimetry (DSF) is a potent, fast and affordable method to investigate protein stability. The method has versatile applications in protein biochemistry, (membrane-) protein crystallography, electron microscopy, as well as ligand, drug and antibody screening (Alexandrov et al., 2008; Chari et al., 2015; Crowther et al., 2009; Epps et al., 2001; Ericsson et al., 2006; He et al., 2011; Liu et al., 2010; Niesen et al., 2007; Pantoliano et al., 2001; Senisterra et al., 2012; Tol et al., 2013; Vedadi et al., 2006). Thermal denaturation of a protein can be monitored with different fluorescent probes that interact with exposed hydrophobic moieties or Cys residues. Interaction of the fluorescent probe with the protein moiety or a single amino acid residue causes quenching or un-quenching of the fluorescent probe. The change of fluorescence is monitored by a UV/ VIS- detector and is plotted as a function of temperature (Pantoliano et al., 2001). The response curves then can be used to determine the melting temperature of the protein calculated as the first derivative of the sigmoidal curve. In case of a two-state transition the melting point corresponds to the temperature where 50% of the protein molecules are unfolded (Pantoliano et al., 2001). Other ways of determining the melting temperature are by normalizing the curves and utilizing the temperature at half of the total fluorescence intensity or by fitting the curve to the following Boltzmann equation (Ericsson et al., 2006; Mezzasalma et al., 2007):

\[
I = A + \frac{(B-A)}{1 + e^{\frac{(T-T_0)}{D}}} \tag{ii}
\]
Where, $I$ represents the fluorescence intensity, $x$ is temperature, $A$ and $B$ are the initial minimal and final maximal fluorescence intensities, respectively, $C$ is the melting temperature and $D$ is the slope factor (Ericsson et al., 2006). More in depth thermodynamic analysis of data obtained with DSF are described elsewhere (Layton and Hellinga, 2010). A change in the melting temperature caused by binding ligands, mutations or other changes in the protein environment indicates either a stabilization or destabilization of the protein (Niesen et al., 2007). Moreover, sigmoidal melting curves with multiple transitions are used to verify the unfolding process of proteins containing multiple domains with different stabilities or protein complexes (Senisterra et al., 2006). Recent DSF experiments on the oligomeric state of different proteins and protein complexes showed that the transition state of the melting curve provides insights into the folding and dispersity of the protein sample. Furthermore, iterative optimization of the buffer system, pH and ligands by DSF can result in monodisperse species as confirmed by negative stain EM (Chari et al., 2015).

The three commonly used reporter dyes to monitor thermal unfolding are: Sypro-orange, 1-anilinonaphthalene-8-sulfonate (ANS), and N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl] maleimide (CPM) (Table 5) (Alexandrov et al., 2008; Epps et al., 2001; Niesen et al., 2007; Slavik et al., 1982). Both Sypro-orange and ANS interact with the exposed hydrophobic moieties of the protein and consequently change their spectral properties. Sypro-orange ($\lambda_{em} = 570$ nm) increases its fluorescence intensity 5 times, whereas ANS ($\lambda_{em} = 500$ nm) additionally undergoes a hypsochromic shift upon binding (Epps et al., 2001;
Niesen et al., 2007; Slavik et al., 1982). CPM (λ<sub>em</sub> = 470 nm) is a thiol-reactive probe with unquenched fluorescence after binding to an exposed Cys residue (Alexandrov et al., 2008).

<table>
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<th>Probe</th>
<th>λ&lt;sub&gt;ex&lt;/sub&gt;</th>
<th>λ&lt;sub&gt;em&lt;/sub&gt;</th>
<th>mechanism</th>
<th>RT-PCR channel</th>
<th>limitation</th>
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<td>511 nm</td>
<td>thiol-reactive probe</td>
<td>FAM&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>Cysteine containing protein pH dependent reaction prone to reducing agents</td>
</tr>
<tr>
<td>CPM</td>
<td>384 nm</td>
<td>470 nm</td>
<td>thiol-reactive probe</td>
<td>HRM channel (QIAGEN only)</td>
<td>Cysteine containing protein pH dependent reaction prone to reducing agents UV-excitation (specialized filter)</td>
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<td>Sypro-orange</td>
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<td>570 nm</td>
<td>hydrophobic sites</td>
<td>SYBR® ROX&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>Limited application to proteins with hydrophobic binding sites or membrane proteins</td>
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<td>500 nm</td>
<td>hydrophobic sites</td>
<td>n/a</td>
<td>Limited application to proteins with hydrophobic binding sites or membrane proteins</td>
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Table 5: Comparison of probes used for DSF measurements. The limitations and advantages of each probe are described. BFC is limited only by the general properties of all thiol-reactive probes.

Each fluorescent probe has at least one major drawback preventing its use for universal melting temperature detection. Sypro-orange and ANS both function by interacting with exposed hydrophobic moieties. This limits the application of Sypro-orange and ANS to soluble proteins, proteins without hydrophobic binding sites or DSF measurements with hydrophilic ligands (Yeh et al., 2006). This shortcoming leaves CPM as the only reporter dye for DSF measurements of hydrophobic proteins or membrane proteins such as GPCRs (Alexandrov et al., 2008). However, the major drawback of this probe is its excitation (λ<sub>ex</sub> = 384 nm)
in the near UV-range, thus only RT-PCR machines with specialized filters can excite CPM. RT-PCR machines equipped with a high resolution melt (HRM) analysis channel can detect CPM but only at a sub-optimal wavelength (Crichton et al., 2015).

Herein, we introduce a thiol-reactive probe with an excitation and emission in the visible range that can be universally applied to soluble and membrane proteins. We found that the boron-dipyrromethene (BODIPY) based probe BFC enabled DSF measurements of various proteins in a hydrophobic environment and detection of the fluorescent signal ($\lambda_{\text{ex}} = 504$ and $\lambda_{\text{em}} = 511$ nm) with a standard RT-PCR machine. The functionality of the BFC probe as a reporter dye was assessed with chicken lysozyme C (UNIPROT ID: P00698) and bovine rhodopsin (UNIPROT ID: P02699), as a representative membrane protein.

2.3 Results

Validation of BFC as a fluorescent probe suitable for DSF measurements was carried out by following the thermal denaturation of lysozyme C (UNIPROT ID: P00698) in PBS pH 7.4. The melting temperature of lysozyme (74 ± 0.5 °C) was previously determined using different methodology such as: absorbance at 292 nm as a function of temperature; differential scanning calorimetry; DSF with Sypro-orange; and circular dichroism (Ichimura, 1991; JASCO.INC, 2015; Shih et al., 1995; Yeh et al., 2006). The working concentration of lysozyme was verified via a series of two-fold dilutions each in duplicate. The starting and final concentrations were 9 mg/ml and 4.3 μg/ml, respectively. The lowest protein
concentration that resulted in a concentration independent melting temperature $(\pm 0.5 \, ^\circ C)$ was 2.25 mg/ml. Therefore, a comparison between Sypro-orange and BFC was made with lysozyme at 2.25 mg/ml (Figure 13 A and B). The fitting of the melting curves with the Boltzmann equation resulted in a melting temperature calculated at 68.6 °C with Sypro-orange (Figure 13B) and 74.4 °C with the BFC probe (Figure 13D). The melting temperature obtained with BFC coincides with label-free measurements as stated above. Conversely, measurements with the Sypro-orange probe differed by 5.8 °C. This difference originates from the pH dependence of the lysozyme DSF data measured with the Sypro-orange probe as reported previously (Yeh et al., 2006). Agreement of the melting temperature between label free measurements and BFC supports the usage of this probe for DSF.
Figure 13: DSF measurements of lysozyme. a) Fluorescence emitted by Sypro-orange and BFC in the presence and absence of lysozyme. Measurements in the absence of protein are shown in violet for BFC and blue for Sypro-orange. Fluorescence in the presence of lysozyme is presented in green for BFC and red for Sypro-orange. b) Normalized fluorescence of lysozyme measured with BFC in violet and Sypro-orange in blue. Error bars indicate the standard deviation of quadruplicate samples. c) Thermal unfolding of lysozyme measured with Sypro-orange. The first transition of the normalized curve was fitted to the Boltzmann equation and resulted in a melting temperature determined at 68.6 °C. d) Thermal unfolding of lysozyme measured with BFC. The first transition of the normalized curve was fitted to the Boltzmann equation and resulted in a melting temperature of 74.4 °C. Variables A –D correspond to the values described by the Boltzmann equation.

The application of DSF with BFC to hydrophobic proteins, proteins with a hydrophobic binding site, or membrane proteins was verified with bovine
rhodopsin. The melting temperatures of bovine rhodopsin in ROS disk membranes are 71.9 °C and 55.9 °C for the dark and bleached states, respectively (Khan et al., 1991; Landin et al., 2001). The working concentration of bovine rhodopsin was verified via a series of two-fold dilutions, each in duplicate. The starting rhodopsin concentration was 2.34 mg/ml, and the final concentration was 1.14 μg/ml. The measurements with Sypro-orange could not be analyzed because of its high initial fluorescence and the absence of a transition (Figure 14 A and B). Conversely, the DSF measurements with BFC resulted in sigmoidal curves at all concentrations. Figure 14 B reveals the averaged quadruplicate DSF measurements at 9.14 μg/ml sample concentration. Analysis of this sample revealed a melting temperature of 72.1 °C, which is consistent with the published value (Figure 14 C). Nevertheless, the dilution series of bovine rhodopsin did not result in a concentration dependent melting temperature as obtained with lysozyme. This inconsistency could result from a concentration-dependent oligomerization or from a detergent induced effect (Edrington et al., 2008; Fotiadis et al., 2006; Jastrzebska et al., 2015; Jastrzebska et al., 2006; Jastrzebska et al., 2004; Khan et al., 1991; Landin et al., 2001). Overall, the data show that BFC is an effective probe to obtain DSF measurements of membrane proteins, hydrophobic proteins or proteins with a hydrophobic binding site, as exemplified by rhodopsin.
Figure 14: DSF measurements of bovine rhodopsin. a) Fluorescence emitted by Sypro-orange and BFC in the presence and absence of rhodopsin. Measurements in the absence of protein are shown in violet for BFC and blue for Sypro-orange. Fluorescence in the presence of rhodopsin is presented in green for BFC and red for Sypro-orange. b) Normalized fluorescence of rhodopsin measured with BFC in violet and Sypro-orange in blue. Error bars indicate the standard deviations of quadruplicate samples. The blue curve shows a high initial fluorescence and no transition curve upon unfolding. The violet curve shows an initial fluorescence which is reduced and subsequently increases upon unfolding of the protein. c) The normalized fluorescence of rhodopsin is fitted to the Boltzmann equation and resulted in a melting temperature of 72.1 °C. Variables A –D correspond to the values described by the Boltzmann equation.
2.4 Discussion

Comparing DSF measurements between Sypro-orange and BFC demonstrated the superior data obtained with BFC especially with membrane proteins. Moreover, the melting temperature of lysozyme measured with BFC is identical with the results obtained with label-free methods. BFC as a probe for DSF measurements should be considered if measurements with Sypro-orange result in a high initial fluorescence, or if sigmoidal curves cannot be obtained due to the presence of hydrophobic patches or binding sites (Figure 14 A). Further, the concentration-dependent melting temperatures observed with both lysozyme and rhodopsin indicate that DSF measurements have to be conducted at a fixed protein concentration to allow accurate comparison.

BFC belongs to the class of thiol-reactive probes, and therefore it suffers from similar shortcomings as CPM (Alexandrov et al., 2008). The main limitation is the pH dependence of thiol formation. At a pH above 8.0 the reaction becomes less selective and at a lower pH the reaction rate decreases. Disulfide bonds are prone to react with reducing agents such as β-mercaptoethanol or dithiothreitol. Thus, the sample must be free of reducing agents. Furthermore, some salts such as chlorides, bromides, and iodides have the ability to quench fluorescent signals and can bias the transition curve. As stated by others, the use of a reporter dye can interfere with ligand binding or, in the case of an autofluorescent ligand, perturb the measured signal (Alexandrov et al., 2008; Layton and Hellinga, 2010; Pantoliano et al., 2001; Tyagarajan et al., 2003). Nevertheless, we have shown
these shortcomings can be overcome by careful sample preparation, initial evaluation of the protein, probe concentrations, and careful data analyses.

As initially stated, a thorough review of DSF applications to various fields of biochemistry has been published (Senisterra et al., 2012). One specific application of the DSF assay is the assessment of protein stabilization to facilitate crystallization. An iterative optimization approach of variables such as buffer systems, pH values, salts and additives can result in a highly stable and monodispersed sample (Chari et al., 2015). Afterwards, the obtained stabilizing conditions and identified additives can be applied to a focused crystallization screen. One such example, is the so-called “Pi sampling method” that consists of 36 stock solutions (12 buffer systems, 12 precipitants, and 12 additives) which are combined to sample 96 unique conditions (Gorrec et al., 2011). Overall, DSF measures with BFC can be utilized to quickly establish parameters for crystallization to yield reproducible, significant and fruitful data.
Chapter 3

Structural Insights into the Drosophila melanogaster Retinol Dehydrogenase, a Member of the Short-Chain Dehydrogenase/Reductase Family

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3 Structure of the Drosophila melanogaster retinol dehydrogenase

3.1 Abstract

The 11-cis-retinylidene chromophore of the visual pigments isomerizes upon interaction with a photon, initiating a downstream cascade of signaling events that ultimately lead to visual perception. The 11-cis-retinylidene is regenerated through the enzymatic transformations collectively called visual cycle. The first and rate-limiting enzymatic reaction within this cycle, the reduction of all-trans-retinal to all-trans-retinol, is catalyzed by retinol dehydrogenases (Figure 15). Here, we solved the structure of the Drosophila melanogaster photoreceptor retinol dehydrogenase (PDH) that belongs to the short-chain dehydrogenase/reductase (SDR) family. This is the first reported structure of SDR that possesses this biologically important activity. Two crystal structures of the same enzyme grown under different conditions revealed a novel conformational change of the NAD$^+$ co-factor, likely representing conformational changes during the catalysis. Amide hydrogen–deuterium exchange of PDH demonstrates changes in the structure of the enzyme upon di-nucleotide binding. In Drosophila melanogaster loss of the PDH activity leads to photoreceptor degeneration that can be partially rescued by transgenic expression of human RDH12. Based on the structure of PDH, we analyzed mutations causing Leber congenital amaurosis (LCA) 13 in a homology model of human RDH12 to obtain insight into the molecular basis of disease-causing mutations in RDH12.
Figure 15: A PDH dimer residing on a modeled membrane with the bound substrate all-trans-retinal. The structure of the PDH dimer is represented in tan, and the co-factor and the modeled retinal are shown as sticks with their corresponding mesh surfaces. All-trans-retinal molecules floating within the membrane are represented by space-filling models colored orange with the oxygen in red. Membrane lipids are represented as sticks and colored according to their elements. The model features the proposed ‘kiss-and-run’ redox reaction carried out by PDH.

### 3.2 Introduction

One of the key reactions involved in vitamin A metabolism and in recycling of the chromophore in vision is the NAD(P)-dependent reversible redox reaction of retinol/retinal catalyzed by alcohol dehydrogenases (Parker and Crouch, 2010; Saari et al., 1998; Wielgus et al., 2010). A sub-class of enzymes from the SDR...
superfamily appears to have evolved specifically to catalyze the retinol dehydrogenase reaction (De Pont et al., 1970b; Haeseleer et al., 1998; Haeseleer et al., 2002; Kiser et al., 2014; Wang et al., 2010). In invertebrate photoreceptors, all-trans-retinylidene remains bound to opsin in a stably photo-activated form and a second photon of light is required to convert it back to 11-cis-3-OH-retinal (Montell, 1999, 2012; Seki et al., 1998; von Lintig, 2012; Wang et al., 2010; Wang et al., 2012b). In flies, the chromophore is required for rhodopsin maturation, and free chromophore can induce photoreceptor degeneration when opsin is lacking (Voolstra et al., 2010). However, there is recent evidence for some enzymatic turnover of the chromophore. A mutation was discovered in a gene encoding a pigment-cell-enriched NAD-dependent SDR, namely Drosophila melanogaster photoreceptor cell dehydrogenase or PDH that leads to light-dependent degeneration of photoreceptor cells. Light responses and rhodopsin levels were normal in young pdh-deficient flies or in old mutant flies maintained in the dark (Wang et al., 2010). However, under a light/dark cycle, pdh-deficient flies underwent photoreceptor degeneration with a progressive loss of retinoids and a concomitant loss of rhodopsin. Notably, this phenotype was partially rescued by ectopic expression of human RDH12 (NP_689656.2). These findings suggest that an enzymatic chromophore regeneration pathway (visual cycle) is required in flies for maintaining rhodopsin levels and the health of photoreceptors (Montell, 2012; Wang et al., 2010; Wang et al., 2012b).
In vertebrate vision, these redox reactions are enzymatically facilitated by retinol dehydrogenases with geometric (cis/trans) isomeric mono or dual specificities, such as RDH5, RDH8, RDH10, RDH11 and RDH12 (Parker and Crouch, 2010). Mutations in these enzymes either lead to mild (RDH5, RDH11) or severe (RDH12) impairments of vision, or can even be lethal (RDH10) due to the requirement of retinoic acid during embryonic development (Benayoun et al., 2009; Fingert et al., 2008; Janecke et al., 2004; Pang et al., 2005; Perrault et al., 2004; Sun et al., 2007; Thompson et al., 2005). As an example, mutations of the \textit{RDH12} gene result in severe visual dystrophies, such as LCA13 or retinitis pigmentosa 53 (RP 53) (Sun et al., 2007). Unfortunately, no structural studies were carried on the vertebrate enzymes, because the inherited difficulty in the expression and purification of the enzymes. Therefore, we turn into an invertebrate retinol dehydrogenase, PDH.

Here, we present on the crystal structure of \textit{Drosophila melanogaster} PDH. To our knowledge it is a first structure of a member of the retinol dehydrogenases from the SDR family. The structural data obtained through crystallization in the presence of β-ionone suggest a novel binary conformation state of the co-factor NAD$^+$ prior to binding of the second substrate (Benach et al., 1999; Tanaka et al., 1996; Winberg et al., 1999). Our findings also support the proposal that enzymatic activity relies on the dimerization as observed for other SDRs (Kristan et al., 2005). The model of functionally related RDH12 allows prediction of the impact of disease causing mutations within the catalytic domain including the dinucleotide-binding site of the protein or the dimerization interface.
3.3 Results

As described in the introduction, retinol dehydrogenases are key enzymes involved in the production of retinal that, in turn, is used in support of invertebrate and vertebrate vision, or further oxidized to retinoic acid, an important transcriptional regulator. Many years of work on vertebrate enzymes in our laboratory led to only rudimentary expression and stereospecificity determination of the enzymatic activity, but did not produce material that could be used for approaches of structural biology (Haeseleer et al., 1998; Haeseleer et al., 2002; Maeda et al., 2009; Maeda et al., 2007). In fact, no retinol dehydrogenase of the SDR family has been reported so far. Because the pdh-c mutant can be rescued with the RDH12 knock in in Drosophila melanogaster, we focused on this invertebrate enzyme (Montell, 2012; Wang et al., 2010; Wang et al., 2012b).
Figure 16: Crystal structure of the PDH dimer with NAD$^+$ and phenol (IPA) in the active site. The left hand panels illustrate the PDH dimer colored blue to red from the N- to C-terminus. The surface of the binding site is colored according to its hydrophobicity from blue to brown, blue for hydrophilic and brown for hydrophobic. The top view (A) shows the hydrophilic entry surface of the co-factor, and the side view (B) indicates the hydrophobic entry to the active site where phenol is located. The bottom view (C) shows the overlapping C-termini and the entry for the substrate. Panel (D) displays a 2D interaction diagram of the co-factor NAD$^+$, generated with LigPlot$^+$ (Laskowski and Swindells, 2011). Phenol is abbreviated as IPA and 1,2-ethanediol as EDO. The ligands and protein side chains are represented in balls-and-sticks, with the ligand bonds colored purple. Hydrogen bonds (in Å) are displayed as green dotted lines. Protein residues making unbounded contacts with the ligand are depicted using spiked arcs.
The structure of PDH in the presence of NAD\(^+\) and phenol, was determined by molecular replacement using the PDB entry 1B2L. PDH crystallized in the dimeric state with a Rossmann fold typical of SDRs (Figure 16 A - C) (Benach et al., 1998, 1999; Jornvall et al., 1995; Persson et al., 1991; Rao and Rossmann, 1973). Furthermore, the three-dimensional structure (RMSD = 1.35 Å) closely resembled the alcohol dehydrogenase from \textit{D. lebanonensis} (PDB entry 1B2L). The surface of the co-factor binding site in the presence of phenol had a funnel-like shape (Figure 16). NAD\(^+\) entry at the top of the funnel was characterized by hydrophilic patches, whereas the bottom entry site for the substrate was entirely hydrophobic. The substrate entry site was identically located in each monomer (Figure 16 B and C). This arrangement allows a membrane-residing substrate, such as retinaldehyde, to have a ‘kiss-and-run’ interaction with the catalytically active site of the enzyme (Figure 16 B – C). The C-termini of both monomers overlapped and collapsed on each other (Figure 16 C). The increased B-factor noted for the C-terminus (Figure 31) and results of previous research regarding membrane association of SDRs C- and N- termini indicate that the C-terminal conformation reported here is a crystallographic artefact (Lhor et al., 2015; Liden et al., 2003; Wang et al., 2001; Zhang et al., 2004).

Kd for NAD\(^+\) binding using differential scanning fluorimetry yielded a value of 170 ± 30 μM (Figure 34) (Hofmann et al., 2016). This value agreed with published Kd values for other members of the SDR family (Gonnella et al., 2011; Moon et al., 2012). The ligand interaction diagram based on the structure is shown in Figure 16 D.
Dimerization of short- and medium-chain dehydrogenases is a prerequisite for their activity (Jornvall et al., 1995; Juan and Gonzalez-Duarte, 1980; Kristan et al., 2005; Ladenstein et al., 1995; Mayoral et al., 2013; Niesen et al., 2010; Winberg et al., 1986; Wuxiuer et al., 2012). Calculations of the crystallographic dimer with Proteins, Interfaces, Structures and Assemblies (PISA) indicated a biologic origin of the dimeric state (Table 8) (Krissinel and Henrick, 2007). Furthermore, evolutionary analysis of the protein-protein interface with Evolutionary Protein-Protein Interface Classifier (EPPIC) supported data calculated by PISA that classified the interface as of biological origin (Figure 17 and Table 9) (Duarte et al., 2012). Residues contributing to the dimer interface were depicted in a stick representation (Figure 17) and were evaluated with EPPIC (Duarte et al., 2012). Gel filtration analysis of the heterologous PDH in the presence or absence of dinucleotide revealed a monomeric mass under both conditions. The theoretical molecular weight (MW) determined with the ExPASy server was 29,453 Da (Wilkins et al., 1999). The MW of the apo-state PDH as determined by gel filtration was 30,299 Da. In the presence of NAD+, the MW was 27,083 Da, and in presence of NADH, the MW was 25,606 Da. These masses provided evidence for a monomeric form of PDH expressed in E. coli, as previously reported for other SDRs (Mayoral et al., 2013; Shafqat et al., 2003). Here, the presence of co-factor resulted in a lower molecular weight, which can be explained by a more compact structure and thus a reduced hydrodynamic radius that delayed elution from the gel filtration column. Additionally, the oligomeric state of PDH was analyzed by determining its MW upon gel filtration at four protein concentrations
ranging from 20 mg/ml to 0.5 mg/ml. The MW of PDH indicated a sole monomeric species at all protein concentrations (Figure 17).

Figure 17: Dimer interface of PDH with interacting residues and analysis of the oligomeric state upon gel filtration. The top panel illustrates the PDH dimer, wherein the monomers are colored in cyan for chain A and green for chain B. Co-factors and phenol are represented in balls-and-sticks according their element colors. Residues contributing to the interface are either colored in blue for chain A or orange for chain B; these residues were verified by EPPIC (Table 9) (Duarte et al., 2012). The gel filtration analysis (bottom panel) was carried out with the Gel Filtration Standard (#151-1901) from BioRad. Standards were thyroglobulin (bovine), 670 kDa; γ-globulin (bovine), 158 kDa; ovalbumin (chicken), 44 kDa; myoglobin (horse), 17 kDa; vitamin B12, 1.35 kDa. PDH was injected both without (red) and with (violet) the dinucleotide NAD⁺ or NADH (green). Concentration dependent-oligomerization was analyzed at PDH concentrations ranging from 20 mg/ml down to 0.5 mg/ml.
Purified and heterologous expressed PDH from *E.coli* or Sf9 insect cells at various concentrations did not result in enzymatic activity. Therefore, we assessed the activity of PDH in a crystalline state, where concentration of the protein is significantly higher. As reported above, PDH was found in a dimeric state within crystals but not in solution. Indeed, it has been reported that SDRs remain catalytically active in a crystalized form (Perez-Miller and Hurley, 2003; Tanaka et al., 1996). Enzymatic redox reactions were carried out in the crystallization drops along with the suitable co-factor. Figure 18 shows the analysis of the reaction products by HPLC. The reduction of all-trans-retinal to all-trans-retinol is shown in Figure 18 A with the corresponding spectra. Oxidation of all-trans-retinol to all-trans-retinal is depicted in Figure 18 B. The naturally occurring substrate of *Drosophila melanogaster* PDH is 3-OH-retinal. Therefore, the enzymatic activity of PDH crystals was verified in the presence of this compound (Figure 33). Accordingly, extraction and analysis of the retinoid composition from *Drosophila melanogaster* fly heads revealed a significant reduction of both 11-cis- and all-trans-3-OH-retinal in *pdh1*-mutants (Figure 33).
Figure 18: HPLC analysis of PDH reaction products extracted from crystal drops. (A) Reduction of all-trans-retinal (c) to all-trans-retinol (e) in the presence (blue) and absence (red) of crystals. (B) Oxidation of all-trans-retinol (e) to all-trans-retinal (c) in the presence (blue) and absence (red) of crystals. The corresponding spectra of the different retinoid isomers a - e are shown on the right. The asterisk * indicates an unidentified compound.

As reported previously, the Ser-Tyr-Lys catalytic triad of SDRs is extended by a proton relay system (Filling et al., 2002; Jornvall et al., 1995; Kallberg et al., 2002; Mayoral et al., 2013; Oppermann et al., 1997b; Persson et al., 1995). Site-directed mutagenesis showed the importance of this catalytic triad for SDR enzymatic activity (Oppermann et al., 1997a). Among these three residues, Tyr is
the most conserved among different family members. Figure 19 A and B show the catalytic triad Ser137-Tyr150-Lys154 of PDH in the presence of phenol (Figure 19 A) and PDH crystallized in the presence β-ionone (Figure 19 B). The distances between the co-factor and the catalytic triad are depicted with dashed lines consistent with hydrogen bonds. Also, a sigma-pi interaction between Tyr150 and Lys154 is highlighted by an orange dashed line. The reactive hydride atom (yellow) of NADH was modeled based on the NAD$^+$ structure used for crystallization. Co-factor isomerization as an integral characteristic of the aldehyde dehydrogenase family has been the topic of several investigations (Gonzalez-Segura et al., 2009; Perez-Miller and Hurley, 2003; Talfournier et al., 2009). From extensive fluorescence measurements, a Mg$^{2+}$- and substrate-dependent co-factor isomerization reaction mechanism was proposed (Gonnella et al., 2013; Gonnella et al., 2011). However, to date, there is no structural evidence of a co-factor isomerization in the same SDR enzyme. Here, we showed an alternative binary state of NAD$^+$ complexed in a SDR (Figure 19 B and 4D, PDB: 5ILO). The conformation of the co-factor in both structures was confirmed with omit maps (Figure 35). A comparison of Figure 19 C and D revealed a hydrogen bond between Ser137 and the amide group of NAD$^+$. This interaction was not seen in the structure when phenol was present (Figure 19 C, PDB: 5ILG). Instead, Ser137 was within a hydrogen bond distance of phenol (Figure 19 A). Similar to human aldehyde dehydrogenases, isomerization leads to the alternative configuration of the co-factor (Figure 19).
Figure 19: The catalytic triad of PDH with phenol and co-factor in space group P222₁ (A and C) and with co-factor only in space group C121 (B and D). This 2D ligand interaction diagram was generated with BIOVIA, Discovery Studio Visualizer (BIOVIA, 2015). Panels A and B show the catalytic triad with the distances in black dashed lines and the σ-π interactions depicted in orange. The reactive hydrogen atom of NADH was modeled based on the NAD⁺ structure and is indicated in yellow. Conformations in panels B and D reveal an NAD⁺ flip wherein the amide group interacts with Ser137 and Tyr150 whereas in panels A and C the phenol interacts with Ser137, Tyr150 and the reactive hydrogen of NADH in its accessible conformation.

Calculations indicated an energetically favored binary complex conformation of NAD(H) co-factor as found in PDB: 5ILO (Table 10). The binary complex conformation of NAD(H) found there is about 27.24 kJ/mol more favorable than
the ternary complex conformation in 5ILG. The energetics of NAD(H) in vacuum and water supported previous analyses. NAD(H) in 5ILO is about 49.23 kJ/mol and 51.63 kJ/mol more stable as compared to the conformation found in 5ILG in vacuum and water, respectively. Thus, an energetic favored binary state is accommodated by the co-factor prior to substrate binding. Once the substrate binds, the nicotinamide group undergoes a flip to its accessible conformation (Figure 19 and Figure 20). Moreover, we conducted the energies calculated for each conformation in the presence and absence of the protein. The data support the nicotinamide flip (5ILO → 5ILG) upon substrate binding (Figure 20).

![Figure 20: Binding and reaction energy diagram of the co-factor NAD(H) in the absence and presence of substrate. First, co-factor binds in its energetically favorable conformation, stabilized by a hydrogen bridge with Ser137 (light blue segment). Second, co-factor flips upon substrate binding (dark blue segment), the hydrogen (yellow) of the co-factor is now in its accessible conformation for the phenol substrate. Third, the catalysis of hydride transfer occurs via a transition state (rose segment). Finally, the product and co-factor are released. This figure was edited from (Shafee, 2015).](image-url)
Hydrogen/deuterium-exchange studies of PDH in the presence and absence of NAD\(^+\) provided insights into the stabilization of the protein upon co-factor binding (Figure 21). The \(\beta\)-sheets within the Rossmann fold were stabilized by co-factor binding as was the loop between \(\beta\)-sheets 5 and 6. However, the extensive loop which connects \(\beta\)-sheets 6 and 7 exhibited higher flexibility. The flexibility and dynamics of the loop region between the \(\beta\)-sheets of the Rossmann fold were in agreement with the B-factors calculated from the crystal structure (5ILG and 5ILO) (Figure 31 and Figure 32). Figure 31 represents the B-factors of 5ILG wherein the increased thermal motion of the loop between \(\beta\)-sheets 6 and 7 agreed with the increased flexibility shown with hydrogen/deuterium exchange. The largest RMSD values between 5ILG and 5ILO were found in the loop region and the \(\alpha\)-helical portion of PDH (Figure 32). Larger RMSD values between 5ILO and 5ILG were found where the B-factors of both structures also were elevated (Figure 32).
Figure 21: PDH becomes less flexible upon dinucleotide binding as measured by hydrogen/deuterium exchange. Hydrogen/deuterium exchange rates of PDH are shown without dinucleotide (left) and in the presence of dinucleotide (right). PDH is colored according to the H/D-exchange rates (see scale below). NAD⁺ is represented in sticks and element colors. The beta-sheets are numbered 1 - 7 from the N- to C-terminus. Regions that were not evaluated are shown in grey.

As indicated in the introduction RDH12 is the human functional orthologue of PDH that reduces all-trans-retinal to all-trans-retinol in a NADP(H) dependent manner (Haeseleer et al., 2002). The RDH12 model was generated via the SWISS-MODEL server based on the PDH (5ILG) crystal structure (Arnold et al., 2006; Benkert et al., 2011; Biasini et al., 2014b). The resulting RDH12 model is
depicted in Figure 22. The disease causing mutations derived from the OMIM database are shown in Table 11. Out of the 16 listed mutations, two cause retinitis pigmentosa 53 and fourteen induce LCA 13 (McKusick, 1998). RP 53 mutations (776delG, Ala126Val) result in a mild and late onset variant of retinitis pigmentosa (Fingert et al., 2008). These mutations are shown as ball and stick representations in Figure 22. The majority of the mutations underlying LCA were present at sites which interact with the co-factor or directly with the catalytic triad (Table 11). Two mutations, Tyr226Cys and Thr155Ile, are known to cause LCA 13 and were located near the dimer interface.

Figure 22: LCA13 disease-causing mutations in RDH12 based on a model of the Drosophila PDH structure. These mutations are listed in Table 11. Mutations distant from the co-factor binding site are highlighted with a yellow background (Tyr226Cys and Thr155Ile). Mutations interacting with the co-factor or substrate-binding site are highlighted with a blue background. RDH12 is colored according to its secondary structural elements; beta sheets in cyan, helices in red and loops in green.
Mutations in retinol dehydrogenases from SDR family are associated with a broad spectrum of ocular diseases. We provide structural insights into the biochemical functioning of this class of enzymes by solving the structure of a Drosophila melanogaster photoreceptor dehydrogenase, PDH. Comparisons of two structures with phenol and β-ionone suggest potential substrate-induced conformational change of the co-factor NAD(H) during the catalysis. In enzymatic analysis of PDH strongly favors a native dimeric state. It was suggested that the substrate binding in Drosophila melanogaster alcohol dehydrogenase occurs at virtually the same cytosolic site as co-factor binding (Benach et al., 1999). Although this could be true for soluble and hydrophilic substrates, we propose that binding of hydrophobic substrates such as retinoids occurs at the opposite site. Our finding is supported by data regarding the potential membrane spanning N-terminus in some SDRs (Liden and Eriksson, 2006; Wang et al., 2001; Zhang et al., 2004). Hence, in a dimeric state which is crucial for the activity of PDH, the substrate entry faces the membrane, as suggested in Figure 16 B. Therefore, we propose a ‘kiss-and-run’ mechanism for the redox reactions of hydrophobic substrates that eliminates thermodynamically unfavorable extraction of retinoid from the phospholipid fraction by allowing the substrate to partially reside within the membrane. Moreover, the molecular basis of disease-causing mutations associated with severe impairments in human vision also was revealed by comparing the Drosophila melanogaster dehydrogenase with the functionally-related human orthologue, RDH12.
3.4 Discussion

The crystal structure of PDH is the first structural study on retinol dehydrogenases from SDR family. For the first time, we provide also structural evidence of a co-factor binary state of PDH that differs from published structures of SDRs. Structural data combined with energy calculations indicated that the co-factor NAD(H) undergoes a conformational change upon substrate binding to these enzymes. Thus, isomerization of NAD(H) takes place prior to substrate binding and co-factor release. The isomerization of NAD(H) was reported previously in the context of co-factor release (Bennett et al., 1982; Blackwell et al., 1987; Gonnella et al., 2013; Gonnella et al., 2011; Hammen et al., 2002). Such data were derived from fluorescence lifetime measurements and NMR studies of aldehyde dehydrogenase 1 (ALDH1), a member of the medium-chain dehydrogenases/reductases (MDRs) (Pares et al., 2008). It was demonstrated with real-time measurements of NADH fluorescence that three different species of NADH exist, free NADH and two bound species of NADH with shorter and longer fluorescence lifetimes (Gonnella et al., 2013; Gonnella et al., 2011). The species with the longer fluorescence lifetime (accessible state, 5ILG) is produced through isomerization and is dependent on the substrate concentration (Bennett et al., 1982; Gonnella et al., 2011). Based on the energy calculations of 5ILG and 5ILO and the corresponding NAD(H) conformation, we concluded that the structure found in 5ILO represents the binary state in the absence of substrate. Thus, the amide group of the co-factor interacts via a hydrogen bridge with
Ser137. Once the substrate binds, the nicotinamide group undergoes a flip to its accessible conformation (5ILG) wherein the hydride is within a hydrogen bond distance to the substrate (Figure 19 and Figure 20). This mechanism is supported by enzymatic analysis of other dehydrogenases (Bennett et al., 1982; MacGibbon et al., 1977).

DHRS4 (NP_001269916.1) and DHRS10 (NP_057330.2) are human SDRs that are dependent on either NADP(H) or NAD(H), respectively. Both SDRs are known to reduce or oxidize 3-ketosteroids or 3β-hydroxysteroids (Endo et al., 2009; Lukacik et al., 2007; Matsunaga et al., 2008). Figure 36 presents an alignment of the crystal structures of DHRS4 (red), DHRS10 (green) and PDH (yellow). The co-factor binding-site common to all SDR family members is nearly identical (low illuminance). This finding is further supported by the low RMSD values between these structures (e.g. DHRS4: PDH = 1.58 Å; DHRS10 : PDH = 1.07 Å; DHRS4 : DHRS10 = 0.95 Å). Differences in the structures were only found within two loop regions (high illuminance). These major differences were located at the substrate-binding site. The loop between β-sheets 4 and 5 showed a shortened connection in the PDH crystal structure. Further, the loop between β-sheets 6 and 7 revealed a completely rearranged structure as compared to the DHRS structures. This loop region is also associated with the substrate-binding site (Benach et al., 1999). The substrate-binding loop region in PDH interacted with the opposite loop between β-sheets 5 and 6 via a hydrogen bond network and highly structured waters (S7, S17, and S20 in 5ILG). The interaction
between these two opposite site loops strongly favored a substrate-binding site distanced from the hydrophobic opening as depicted in Figure 16 A - C.

Enzymatic assays with PDH were conducted in the crystalline state wherein PDH existed in a dimeric conformation. However, in solution PDH was found exclusively in a monomeric conformation (Figure 17). The lack of activity in solution could thus be explained by the monomeric state of not only PDH but other SDRs as well. The dependence of activity on the oligomeric state of SDR was previously investigated by site-directed mutagenesis (Kristan et al., 2005). Findings from that study are in agreement with our data indicating that a monomeric SDR is inactive. Furthermore, this finding explains the underlying molecular mechanism of disease-causing mutations found near the dimer interface of RDH12 (Figure 22). The importance of dimerization for the activity of SDRs offers a specific target for drugs to regulate the activity of a given SDR (Kristan et al., 2005).

We made an interesting observation related to the enzymatic activity of PDH. First, the previously developed assay was validated by using bovine rod outer segment membranes that expressed high levels of retinol dehydrogenase activity (de Pont et al., 1970a). This assay relies on direct detection of the retinol or retinal product, but no activity was detected in the various experimental settings described in Materials and Methods, including insect cell expression systems. This lack of activity could not be attributed to a nonproductive conformation of protein but rather to a major conformational change because the polypeptide fold
is very similar to other known SDRs. When the protein was precipitated with PEG to form an amorphous material, no activity was detected either. Only when crystals were present were the enzymatic products observed. As demonstrated biochemically, PDH is monomeric even at a concentration of 20 mg/mL, but in a crystal, the dehydrogenase is present in the dimeric form where its concentration was calculated from the Matthews volume to be 826 mg/mL. For most SDRs, the dimer is obligatory for enzymatic activity, but to the best of our knowledge, neither the monomeric nor the dimeric structures of the same enzyme are known (Kristan et al., 2005). The most straightforward interpretation of these phenomena is that monomeric PDH is inactive and that dimerization brings together sites that then become active. Such catalytically competent dimers could have a slightly differently arranged side chain induced by dimerization. PDH crystallizes under a very narrow range of conditions, and we failed to obtain crystals in a different space group where PDH existed in a monomeric state. In insect eyes, expression of PDH could require an anchor protein that further stabilizes the dimeric form. Indeed, as demonstrated previously and here by retinoid analyses, PDH is biologically active in vivo.

SDRs in general have very similar folds. Now with the results of the retinol dehydrogenase structure, it is clear that there is nothing unusual about this subclass of enzymes. Thus, we can now generate models across different RDHs with greater confidence. Moreover, the interface of the PDH identified here provides additional insights. Of particular interest are those RDHs associated with human retinal diseases such as severe LCA caused by mutations in RDH12.
Of the 16 listed mutations for RDH12, two cause retinitis pigmentosa 53 and 14 induce LCA 13 (McKusick, 1998). RP 53 mutations (776delG and Ala126Val) result in a mild and late onset variant of retinitis pigmentosa (Fingert et al., 2008). These mutations are shown as balls and sticks in Figure 22. Most mutations underlying LCA were found at sites that interact with the cofactor or directly with the catalytic triad (Table 11). These results suggest that inactivation or decreased activity of the enzyme could cause this disease. Two mutations, Tyr226Cys and Thr155Ile located near the dimer interface, are known to cause LCA 13. Further progress in expressing RDH12 will be needed to validate this supposition for the last two mutations.
Chapter 4

Hydrogen/Deuterium Exchange Mass Spectrometry of Human Green Opsin Reveals a Conserved Pro-Pro Motif in Extracellular Loop 2 of Monostable Visual G Protein-Coupled Receptors

This chapter was adapted or reprinted in part or in full from the following sources:

https://doi.org/10.1021/acs.biochem.7b00165
4 Hydrogen/Deuterium Exchange of the Green Cone Pigment, a Member of the Class A GPCRs

4.1 Abstract

Opsins comprise the protein component of light sensitive G-protein coupled receptors (GPCRs) in the retina of the eye that are responsible for the transduction of light into a biochemical signal. In this study, we used hydrogen/deuterium (H/D) exchange coupled with mass spectrometry to map conformational changes in green cone opsin upon light activation. We compare these findings with those reported for rhodopsin. The extent of H/D exchange in green cone opsin is more substantial than in rhodopsin both in the dark and bleached states. This suggests a higher structural heterogeneity of green cone opsin as compared to the corresponding states of rhodopsin. Further analysis reveals that green cone opsin exists in a dimeric form in both dark (inactive) and bleached (active) states, and that the predicted glycosylation sites at N\textsuperscript{32} and N\textsuperscript{34} of the green cone opsin are indeed glycosylated. Comparison of deuterium uptake between the inactive and active states of green cone opsin also disclosed the reduced solvent accessibility of the extracellular N-terminal region and the increased accessibility of the chromophore binding site. Increased H/D exchange at the extracellular side of transmembrane helix four (TM4) combined with an analysis of sequence alignments revealed a conserved Pro-Pro motif in extracellular loop 2 (EL2) of this monostable visual GPCR (Figure 23). These data present a novel insight into the locus of chromophore release at the
extracellular side of TM4 and TM5 and provide a foundation for future studies detailing its mechanism.

![Diagram of M/LWS pigment with emphasis on the EL2. Computational modeling of the P186M mutation in M/LWS reveals increased flexibility compared to WT in the EL2 (highlighted with yellow oval). TM4, TM5 and IL2 reside at the dimer interface and display reduced H/D exchange compared to the rest of the receptor. IL2 is less solvent exposed upon bleaching and potentially contributes to the dimer interface and activation properties of M/LWS pigment. Positive differences in percent deuteration between states ($\Delta\%\text{Deuteration (Dark-Bleached)}$) were represented using the following color coding: 0-4%, blue; 5-9%, violet; 10-14%, pink. Negative differences of percent deuteration were color coded as follows: 0-4%, cyan; 5-9%, green; 10-14% yellow; 15-19%, orange; 20-24% red. Undefined regions are represented in grey.]

Figure 23: Dimer interface of M/LWS pigment with emphasis on the EL2. Computational modeling of the P186M mutation in M/LWS reveals increased flexibility compared to WT in the EL2 (highlighted with yellow oval). TM4, TM5 and IL2 reside at the dimer interface and display reduced H/D exchange compared to the rest of the receptor. IL2 is less solvent exposed upon bleaching and potentially contributes to the dimer interface and activation properties of M/LWS pigment. Positive differences in percent deuteration between states ($\Delta\%\text{Deuteration (Dark-Bleached)}$) were represented using the following color coding: 0-4%, blue; 5-9%, violet; 10-14%, pink. Negative differences of percent deuteration were color coded as follows: 0-4%, cyan; 5-9%, green; 10-14% yellow; 15-19%, orange; 20-24% red. Undefined regions are represented in grey.
4.2 Introduction

Opsins form an essential part of the visual sensory system that function as photon detectors in the retina (Bowmaker, 2008; Hofmann and Palczewski, 2015a). The four opsins in the human retina - rhodopsin, short wavelength sensitive 1 (SWS1) opsin, medium wavelength sensitive 1 (M/LWS) opsin, and long wavelength sensitive 1 (L/LWS) opsin - differ in their spectral absorbance, sensitivity, regeneration kinetics, and regulation of downstream signaling (Imai et al., 2005; Imamoto and Shichida, 2014b; Kefalov, 2012a; Korenbrot, 2012; Lamb, 2013; Wang et al., 2012a). Nonetheless, all four pigments are activated by the isomerization of the chromophore 11-cis-retinal to all-trans-retinal, which is then released and regenerated back to 11-cis-retinal through the retinoid cycle (Kiser et al., 2012; Kiser et al., 2014; Kiser and Palczewski, 2016). Chromophore isomerization induces conformational changes in the light-sensitive GPCRs and initiates the binding of a G-protein, transducin (Jastrzebska et al., 2011a; Jastrzebska et al., 2011c; Jastrzebska et al., 2010; Kobilka, 2007; Rosenbaum et al., 2009). The two different conformational states, active and inactive, are known to be very stable and discrete in rhodopsin (Blankenship et al., 2015; Rosenbaum et al., 2009). However, the vast majority of GPCRs display different and more heterogeneous states which permit biased signaling and different active states induced by different ligands (Heydenreich et al., 2015; Steyaert and Kobilka, 2011; Zhang et al., 2015b). Ongoing difficulties associated with GPCR crystallization have prevented the experimental determination of the atomic
structures for SWS1, M/LWS, and L/LWS opsins (Chun et al., 2012; Ghosh et al., 2015; Zhang et al., 2015b). Therefore, understanding the structural underpinnings of their functional differences remains a challenging issue.

GPCRs in general can exist as functional monomers, homo/hetero- dimers or even higher oligomeric species (Fotiadis et al., 2006; Fotiadis et al., 2003; Gunkel et al., 2015; Gurevich and Gurevich, 2008; Jastrzebska, 2013; Palczewski, 2010; Vischer et al., 2011). These different oligomeric organizations may affect the signaling and other physiological properties of the receptors and can, therefore, serve as different targets for drug screens (Chen and Palczewski, 2016; Ferre et al., 2014; Hill, 2006; Prazeres and Martins, 2015). Opsins are known to form homo-dimers and higher oligomeric structures, as shown by cryo-electron microscopy, atomic force microscopy and fluorescence assays (Comar et al., 2014; Fotiadis et al., 2003; Gunkel et al., 2015; Jastrzebska et al., 2017). Disruption of the oligomeric organization of bovine rhodopsin with synthetic peptides confirmed that the interface is located between TM4 and TM5 in visual GPCRs (Jastrzebska et al., 2015). Recent analyses of the human M/LWS, L/LWS, and SWS1 cone opsins by fluorescence correlation spectroscopy also revealed a dimer interface between TM4 and TM5 (Jastrzebska et al., 2017).

Extensive research on the role of the extracellular loops (ELs) in GPCRs has demonstrated that ELs are involved in ligand recognition and binding, and can play the role of gatekeepers in GPCR signaling (Peeters et al., 2011; Wacker et al., 2017; Wheatley et al., 2012). GPCR structural studies demonstrated a high
variability in the sequence and length of the ELs (Katritch et al., 2012; Peeters et al., 2011; Wheatley et al., 2012). The ELs of light-sensitive GPCRs play a unique role. Unlike other GPCRs, which are activated by molecules, the opsins are activated by light (Kefalov, 2012a; Luo et al., 2011; Orban et al., 2014; Palczewski, 2006b; Reingruber et al., 2015; Rieke and Baylor, 1998). Thus, attention has focused on the potential role of the ELs in rhodopsin, because of the absence of a direct ligand interaction and their role in chromophore release (Ahuja et al., 2009b; Ahuja and Smith, 2009). Nuclear magnetic resonance spectroscopy (NMR) studies combined with crystallographic data about rhodopsin offered valuable insights into the motion of EL2 following activation (Ahuja et al., 2009a; Ahuja et al., 2009b; Ahuja and Smith, 2009). EL2 forms a lid on the retinal binding site and interacts directly with the chromophore (Jastrzebska et al., 2011b; Okada et al., 2002b; Palczewski et al., 2000). Photo activation leads to the displacement of EL2 in rhodopsin and rearrangement of the hydrogen bond network (Struts et al., 2007). The displacement of EL2 and TM4, TM5, and TM6 involves a conserved hydrogen bond network stretching throughout rhodopsin (Ahuja et al., 2009b; Blankenship et al., 2015). Thus, interactions among EL2, TM4 to TM6, and the hydrogen bond network are critical for opsin activation and chromophore release. Comparable studies on cone opsins have not been reported to date.

Visual pigments can be divided in two types: monostable, which release the chromophore upon photo-activation, or bistable, which retain the chromophore within the binding pocket (Shichida and Matsuyama, 2009; Terakita and Nagata,
A subset of bistable opsins have the ability to re-isomerize the chromophore (Koyanagi and Terakita, 2014; Shichida and Matsuyama, 2009; Tsukamoto and Terakita, 2010). To re-isomerize the chromophore, it is essential to retain the chromophore within the binding pocket. Mutagenesis studies of chicken rhodopsin and chicken green cone opsin revealed that Pro189 in EL2 has a crucial impact on the meta-II and meta-III decay rates. Pro189 is conserved in cone opsins but not rhodopsins, which suggests that residues within EL2 likely influence the chromophore release in monostable opsins (Kuwayama et al., 2002; Kuwayama et al., 2005; Yanagawa et al., 2015). Additionally, evaluation of the backbone configuration based on 500 high resolution protein crystal structures has shown a restricted conformational freedom of Pro and residues preceding Pro (Bajaj et al., 2007; Lovell et al., 2003; Reimer et al., 1998). This geometrical restriction originates from the pyrrolidine ring of Pro. Moreover, Pro residues found in transmembrane domains are known to induce helix kinks which are indispensable for the integrity of the transmembrane helices (Schmidt et al., 2016). Additionally, molecular dynamics simulations of α-helical transmembrane proteins (e.g. GPCRs) showed that Pro-induced hinges have a crucial role in signal transduction (Brandts et al., 1975; Elling et al., 2006; George and Heringa, 2002; Reimer and Fischer, 2002; Sansom and Weinstein, 2000). Thus, the conformational restriction and impact on α-helical structure reinforce the selection of Pro residues as switches for binary signaling triggered by the cis-trans isomerization of retinal in rhodopsin and the cone opsins (Smith, 2010).
H/D exchange measurements are used to probe protein dynamics and protein-ligand interactions (Bruning et al., 2007; Chalmers et al., 2006; Chalmers et al., 2011a; Orban et al., 2012b; Zhang et al., 2010), providing information complementary to other structural techniques such as NMR, X-ray crystallography, cryo-electron microscopy or computational modeling (Brock, 2012; Englander, 2006; Jaswal, 2013; Lossl et al., 2016). H/D exchange reflects the exposure of residues to the surrounding environment and depends on several parameters such as temperature, pH, ionic strength, exposure time, and hydrogen bonding. With careful experimental control of these first four parameters, detection of changes in secondary and tertiary structure, and protein dynamics is possible (Chalmers et al., 2011a; Chalmers et al., 2011b; Jaswal, 2013; Katta and Chait, 1991). Due to solvent exchange, this method is especially suited for GPCR analysis, considering the conserved hydrogen bond network involving water molecules that exists throughout this group (Blankenship et al., 2015; Hofmann and Palczewski, 2015b; Jastrzebska et al., 2011b) Here, we looked specifically at H/D exchange in the dark and bleached states of M/LWS (green) cone opsin.

Post-translational modifications of GPCRs play critical roles in protein folding, transport, and signaling (Dong et al., 2007; Morello and Bouvier, 1996; Zheng et al., 2013). In bovine rhodopsin, N-terminal glycosylation has been studied extensively and its importance in trafficking and signaling has been demonstrated (Hargrave, 1977, 2001; Hofmann and Palczewski, 2015b; Kaushal et al., 1994; Murray et al., 2009; Murray et al., 2015; Zhu et al., 2004). It was previously
proposed that green cone opsin, expressed in Sf9 insect cells, is glycosylated at its N-terminal region like rhodopsin but confirmation has been lacking until now (Vissers and DeGrip, 1996).

In this study, we employed H/D exchange coupled with mass spectrometry (MS), differential scanning fluorometry, bioinformatics, and computational modelling to investigate dynamic structural changes in green opsin that occur upon photo-activation and chromophore release. Additionally, MS analysis of green opsin verified the proposed N-terminal glycosylation at asparagine residues 32 and 34. H/D exchange analysis also revealed a Pro-Pro motif with increased exchange upon photo-activation and chromophore release. Sequence alignment of monostable and bistable opsins revealed that the Pro-Pro motif is conserved in all monostable opsins but does not occur in bistable opsins suggesting a role of TM4 in chromophore release. In addition, computational analysis indicated that the substitution P186M, as seen in bistable proteins, permits greater conformational flexibility, further implicating TM4 and EL2 in the process of chromophore release in monostable visual opsins (Figure 23).

4.3 Results

Purification of the green photopigment. The purity of the green cone opsin after final size exclusion chromatography was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and spectrophotometry (Figure 24 A to D). The reported extinction coefficient for the green photopigment is $\varepsilon_{530} = 40,000 \, \text{M} \cdot \text{cm}^{-1}$.
and the calculated $\varepsilon_{280} = 99,975$ M · cm$^{-1}$ (Vissers et al., 1998; Vissers and DeGrip, 1996). Thus, a theoretical ratio ($A_{280/530}$) of 2.49 would indicate a pure sample. Here we obtained an absorbance ratio $A_{280/530}$ of 2.86, which together with the data obtained by SDS-PAGE indicate a purity close to 100%.

Figure 24: Purification of green opsin by size exclusion chromatography and analysis of glycosylation by SDS-PAGE and immunoblotting. Panel A aggregates and oligomers eluted at fraction 9, while monomeric opsin eluted in fractions 12 to 18. The 1D4-peptide used for affinity chromatography eluted at fraction 25. Panel B shows the absorbance measurements of fraction 15. The peak at 530 nm originates from the chromophore bound to green opsin, the absorbance at 363 nm is due to free retinal in the buffer, and the 280 nm absorbance is the result of aromatic amino acid residues Trp, Tyr, and Phe. Panel C shows the Coomassie stain of glycosylated (lane 2) and de-glycosylated (lane 3) opsin. Following PNGase F treatment,
the de-glycosylated receptor undergoes a mobility shift towards lower molecular weight compared to the glycosylated receptor. Additionally, lane 3 shows a thin band attributed to PNGase F at about 32 kDa. Panel D displays the Western blot analysis of the glycosylated (lane 2) and de-glycosylated (lane 3) opsin. The monomer (30 kDa), dimer (60 kDa), tetramer (120 kDa) and higher oligomer species can be detected in both SDS-PAGE and Western blot analyses. Lanes 1 and 4 in both panels contain protein markers. Panel E displays differential scanning fluorimetry measurements of green opsin and bovine rhodopsin. The normalized fluorescence of green opsin is displayed in green and was fitted to the Boltzmann equation which resulted in a melting temperature of 48˚ C. The normalized fluorescence of bovine rhodopsin (red) was fitted to the Boltzmann equation which shows a melting temperature of 72˚ C. The difference between the melting temperatures is 24˚ C.

Green opsin dynamics from H/D exchange. The MS/MS spectra of the green opsin digest were analyzed with MassMatrix software based on the primary sequence of the L/MWS construct (see Appendix) (Xu and Freitas, 2007, 2008; Xu et al., 2008a; Xu et al., 2008b). A sequence coverage of 94.7% was achieved for the deuterated green pigment protein (Figure 25) by rigorous screening and selection of only significant peptide matches (see Methods for details).
Figure 25: Sequence, glycosylation and peptide coverage of the green opsin construct by mass spectrometry. A) Glycosylation sites are indicated in orange. The extracellular side of the receptor is displayed in green, the cytosolic domain in red, and trans-membrane domains are displayed in blue. Black lines indicate the peptide coverage in H$_2$O and D$_2$O both in dark and bleached states. The green line indicates coverage only in the D$_2$O dark state and the violet line displays peptide coverage only found in the D$_2$O bleached state. B) MS/MS spectrum of the N-terminal peptide, residues 29–45. Residues N32 and N34 were detected as D32 and D34, respectively. The sequence is displayed above the spectrum, and the two glycosylated residues are highlighted in orange. The b and y ions are shown in red and blue, respectively. The M notation refers to the molecular ion.
Overall, there was an increase in the uptake of deuterium in the bleached state compared with the dark state of the green photopigment. The difference map of H/D exchange between dark and bleached states revealed several major changes upon light activation (Figure 26). First, the chromophore binding pocket became more solvent accessible during activation (Figure 26 A), indicating a potential exit site for all-trans-retinal (Figure 26 A, B and E). The increased deuterium uptake started at TM1 and extended over TM2 and TM3 (Figure 26 A and D). The largest increase in deuterium uptake within the region of the binding pocket was observed on the extracellular side of TM4 (Figure 26). In contrast, the proposed dimer interface consisting of TM4 and TM5 was characterized by an overall reduction in deuterium exchange compared to the rest of the pigment molecule (Figure 26 B and Figure 27) (Jastrzebska et al., 2017). The N-terminal region and intracellular loop two (IL2) also showed less deuterium exchange in the bleached state of the photopigment (Figure 26 A, B and F); the largest reduction in deuterium uptake was observed at the N-terminal region (Figure 26 A, C and E). Extracellular loops (ELs) 1-3 exhibited little difference in deuterium uptake.
Figure 26: H/D exchange difference map of green opsin in the dark and bleached states. The percent deuteration of the dark state was subtracted from the percent deuteration of the bleached state and displayed on the surface of the receptor. Positive differences in percent deuteration between states ($\Delta$%Deuteration (Dark-Bleached)) were represented using the following color coding: 0-4%, blue; 5-9%, violet; 10-14%, pink. Negative differences of percent deuteration were color coded as follows: 0-4%, cyan; 5-9%, green; 10-14% yellow; 15-19%, orange; 20-24% red. Undefined regions are represented in grey. Panels A to D represent side views of the green pigment, E and F show top and bottom view, respectively.

Only TM1 to TM4 of the green opsin underwent a significant increase in deuterium uptake upon activation (Figure 27). Again, we saw an increased H/D exchange along the chromophore binding pocket. In contrast, the H/D exchange
levels of TM5 remained constant upon activation. Moreover, the cytosolic part of TM6 and TM7 demonstrated a decrease in its H/D exchange rate in the active conformation (Figure 27). Overall, the highest but constant deuterium uptake was observed in the extracellular parts of TM6 and TM7, including EL3 (Figure 27 and 5 A). These high exchange rates were observed in both dark and bleached states (Figure 26 E and Figure 27).

Figure 27: Trans-membrane analysis of H/D exchange of dark and bleached states. A model of green opsin is represented in grey with the chromophore in magenta. Trans-membrane domains in pairs with the left trans-membrane helix showing H/D data for the dark state, and the right helix showing H/D data for the bleached state. The H/D exchange is represented using the following color coding 0-4%, dark blue; 5-9% light blue; 10-14%, cyan; 15-19%, cadet blue; 20-24%, green, 25-29%, light green; 30-34%, yellow; 35-39%, orange; 40-44%, red. Undefined regions are represented in grey.
As reported previously, the dimer interface of the green opsin was localized between TM4 and TM5, with residues T230, S233, and V236 of TM5 being crucial for dimerization (Jastrzebska et al., 2017). The dimer interface between two green opsin molecules revealed two major changes upon activation (Figure 28). First, there was an increase in H/D exchange at the tip of the chromophore binding pocket located at TM4 on the extracellular side (Figure 28 A and E). Second, a decrease in H/D exchange was observed in IL2 (Figure 28 B and D). Further, both IL2s were in proximity with each other between the dimer interface (Figure 28 D). The reduced H/D exchange of IL2 and the location at the dimer interface of the green pigment indicates a potential role of IL2 in green opsin signaling influenced by dimerization.
Figure 28: Dimer interface analysis of dark and bleached states. Panel A to C Side views of the green dimer in the dark and bleached states. Panel D to E Bottom and top views of the dimer in the dark and bleached states. In all panels the dimer in the dark state is shown on the left; the dimer in the bleached state is shown on the right, with one monomer is displayed in cartoon representation and the second monomer displayed as a surface. Both the surface and cartoon representations show the dimer interface colored according to the H/D exchange. The H/D exchange is represented using the following color coding: 0-4%, dark blue; 5-9%, light blue; 10-14%, cyan; 15-19%, cadet blue; 20-24%, green; 25-29%, light green; 30-34%, yellow; 35-39%, orange; 40-44%, red. Undefined regions are represented in grey.

Glycosylation of the green photopigment. Previous analysis of the green photopigment expressed in Sf9 insect cells showed evidence for N-linked
glycosylation, although the prediction was not confirmed experimentally (Vissers and DeGrip, 1996). Here the glycosylation was validated by SDS-PAGE and Western blot analysis. PNGase F-treated pigment displayed a migration shift (Figure 24 C and D), resulting from the de-glycosylation of the green opsin. Additionally, the sites of glycosylation were verified by MS. Glycosylation is lost during ionization, and therefore glycosylated asparagine residues are converted to aspartate residues. Consequently, asparagine-linked glycosylation can be identified by assuming N to D transformations during the MS/MS analysis (Figure 25 A and B). Potential glycosylation sites of the green cone pigment were predicted with the NetNGlyc 1.0 Server prior to mass spectrometry (Gupta and Brunak, 2002; Steentoft et al., 2013). The top three asparagine residues with a glycosylation prediction score higher than 0.71 out of 1 (i.e. N32, N34, and N94 (see Appendix)) were verified by MS/MS data. The predicted glycosylation sites, N32 and N34, were confirmed by the MS/MS analysis of peptide 29TYTDSDSTRGPGENGPNY46 (Figure 25 B). A third glycosylation site (N94) with a potential of 0.7776 was indicated by the server but could not be confirmed by mass spectrometry (see Appendix).

Computational modeling. The H/D exchange data indicate a rearrangement of EL2 between the dark and bleached states of green opsin, suggesting a possible role in chromophore exchange. EL2 contains a Pro-Pro motif conserved across all monostable but not bistable visual pigment proteins (see Appendix). To further characterize the structural effect of the motif, a model of green opsin containing a single substitution, P186M, was compared to the green pigment model.
Analyzing the ten most energetically favorable structures resulting from independent optimization trajectories for both the green pigment and P186M models demonstrated that the P186M model has increased flexibility at the end of TM4 as compared to the green pigment model (compare Figure 29 A, B and D; and Figure 37). The increased flexibility in TM4 induced by the P186M mutation agreed with the reduction of geometric constrains induced by the exchange of a Pro residue. The region of increased flexibility corresponds to the site of increased deuterium uptake after photon-activation of the green cone opsin (Figure 26 A, D and 4). To verify that our modeling can identify structural changes with functional consequences due to a single amino acid substitution, we also prepared a model with a P205I point mutation in green opsin. P205I has previously been shown to affect the meta-II and meta-III decay rates in chicken green cone opsin (Kuwayama et al., 2002; Kuwayama et al., 2005; Yanagawa et al., 2015). Comparison of the structure ensembles between the P205I and green pigment models demonstrated an increased flexibility at the C-terminal region of EL2 in the P205I model, consistent with changes in the decay rates of meta conformers (compare Figure 29 A, C and D; and Figure 37). These data demonstrate that the computational modeling and RMSD analysis results agreed with mutagenesis studies that influence chromophore release in visual opsins (Kuwayama et al., 2002; Kuwayama et al., 2005; Yanagawa et al., 2015).
Figure 29: Structural models and RMSD analysis of EL2 of WT and with P186M and P205I substitutions. The ten energetically favored structures were aligned and displayed as cartoons from residue 180 to 224. A) The Pro-Pro motif in the WT green opsin highlighted by a red oval; the black oval indicates the region of variation in the structure caused by P205I. B) Structures resulting from the P186M mutation; the red oval indicates major variation in structure induced by the mutation (compare red oval region in A). C) Major variations in structure are caused by P205I (black oval; compare to black oval region in A). D) Heatmap of RMSD analysis between the ten energetically favored structures shown in A - C. The red and black boxes correspond to the ovals displayed in A - C.
4.4 Discussion

We previously reported an H/D exchange analysis of bovine rhodopsin (Orban et al., 2012b). Herein, we provide the H/D exchange analysis of the inactive and active states of green cone pigment and compare those findings with rhodopsin (Orban et al., 2012b). Overall, the differences between the inactive and active states observed in the green visual pigment and rhodopsin ranged from -25% to +15% and -50% to +40%, respectively. These differences indicate that the two states are more distinct in rhodopsin as compared to green cone opsin. It should be noted, however, that the analysis of the N-terminal region, IL1, IL2, and TM5 are lacking in the rhodopsin model because of low coverage. The reduced deuterium uptake upon bleaching in TM1 is common to both rhodopsin and the green pigment. Further, the increased deuterium exchange along the chromophore binding pocket is found in both pigments. Also in rhodopsin, the H/D exchange rate in the chromophore binding pocket peaks at the extracellular side of TM4. In green cone opsin we measured a reduced H/D exchange in IL2 upon activation, and the same is true for rhodopsin. Rhodopsin shows an increased H/D exchange in TM3 and a decreased exchange in IL3. Both these differences were not detected in green opsin. In green opsin, we detected an overall decrease in the H/D exchange rate for TM6 and TM7 upon activation. Bovine rhodopsin displays reduced exchange in TM6 but not in TM7 (Orban et al., 2012b).
The extracellular N-terminal region of green opsin is 18 amino acid residues longer than the corresponding region of rhodopsin (see Appendix). Mutagenesis experiments and molecular dynamic studies of the Xenopus violet cone pigment, revealed a hydrogen bonding network between N-terminal region, EL2, and EL3. In rhodopsin EL3 builds up a hydrogen network with the N-terminal region but not with EL2. These differences in the hydrogen network at the extracellular site between cone opsins and rhodopsin provide a possible explanation for the decreased H/D exchange upon activation (Chen et al., 2011; Coleman et al., 2017).

Thermal unfolding of bovine rhodopsin and green opsin was monitored by differential scanning fluorimetry (Figure 24 E) (Hofmann et al., 2016). The melting temperatures were found to be 48˚C and 72˚C for green opsin and rhodopsin, respectively. The melting temperature difference of 24˚C indicates a reduced stability of green opsin, which is in agreement with other class A GPCRs such as the human CB1 cannabinoid receptor (Shao et al., 2016). Unlike rhodopsin, most GPCRs are inherently unstable and express multiple ligand-specific active states (Heydenreich et al., 2015; Steyaert and Kobilka, 2011; Zhang et al., 2015b). Successful crystallization of rhodopsin in its active and inactive states without stabilizing agents, signaling partners or antibodies suggests, that both states are discrete and do not suffer from inherent instability and multiple heterogeneous states (Choe et al., 2011; Okada et al., 2004; Palczewski et al., 2000; Salom et al., 2006). The reduced stability of green cone pigment indicates higher mobility of the protein domains and thus an increased overall solvent exposure.
Increased solvent exposure reduces the differences in H/D exchange between different states such as dark and bleached states of green cone opsin. This difference in stability of green opsin versus rhodopsin complicates a direct comparison by state between these two light sensitive pigments.

We provide experimental data for a potential exit site for the photo isomerized chromophore all-trans-retinal between TM4 and TM5 at the extracellular surface of green opsin (Figure 30). The increased level of H/D exchange upon bleaching at the chromophore binding pocket domain is most prominent at the extracellular side of TM4. Moreover, the exit site between TM4 and TM5 is favored in dark state rhodopsin, based on molecular dynamics simulations (Wang and Duan, 2007). However, similar calculations on the bleached state of bovine rhodopsin and mutagenesis studies provide evidence that the exit site resides between TM5 and TM6, a finding we were not able to recapitulate for the green opsin with our H/D exchange experiments (Morrow and Chang, 2015; Piechnick et al., 2012; Schadel et al., 2003; Standfuss et al., 2011; Wang and Duan, 2011).

Alignment analysis of monostable visual opsin protein sequences from different species revealed a conserved Pro186-Pro187 sequence at TM4 (see Appendix). This conserved Pro-Pro motif coincides with the site of largest difference in H/D exchange upon photo-activation of the green cone pigment. Additionally, it was previously proposed that the Pro170 and Pro171 residues in bovine rhodopsin are involved in accommodating the perturbation of the helix upon receptor activation (Riek et al., 2008; Riek et al., 2001; Stenkamp et al., 2002). Also,
single mutations P170H and P171S in rhodopsin have been shown to cause retinitis pigmentosa in humans (Shah et al., 2014; Vaithinathan et al., 1994). Interestingly, bistable pigments which do not release the chromophore (e.g. Human Opn5 and visual bistable opsins of Drosophila melanogaster and Todarodes pacificus) only contain one conserved single Pro residue at TM4 (see Appendix) (Hamdorf, 1979; Koyanagi and Terakita, 2014; Tsukamoto and Terakita, 2010). Taken together, these data show that the presence of the Pro-Pro motif is crucial for a fully functional monostable visual opsin.

Figure 30: All-trans-retinal egress pathway near the extracellular side of M/WLS opsin. The retinylidene Schiff base and K312 are represented in spheres. The H/D exchange of the dark state was subtracted from the bleached state and displayed on the trans-membrane helices represented in cartoon. The H/D exchange is represented using the following color coding 0-4%, dark blue; 5-9% light blue; 10-14%, cyan; 15-19%, cadet blue; 20-24%, green, 25-29%, light green; 30-34%, yellow; 35-39%, orange; 40-44%, red. Undefined regions are represented in grey. The possible exchange route for water or all-trans-retinal is indicated with a yellow arrow.
The 5-hydroxytryptamine receptor 1B (5-HT\textsubscript{1B}) is the only crystallized GPCRs (PDB ID: 4IAQ and 4IAR) which contains a Pro-Pro motif at TM4 besides rhodopsin. The 5-HT\textsubscript{1B} receptor has a sequence similarity of 65% with the green pigment at the Pro-Pro site of TM4 (Figure 38). Comparison of the green cone pigment model with the 5-HT\textsubscript{1B} structure, revealed an alternative and extended TM4 and EL2 of 5-HT\textsubscript{1B} (Riek et al., 2008; Wang et al., 2013). Furthermore, recent data regarding the 5-hydroxytryptamine receptor 2B receptor combined with molecular dynamics simulations highlight the crucial role of the EL2 site in ligand binding (Wacker et al., 2017). These data support that the conserved Pro-Pro motif in TM4 including the EL2 in monostable vertebrate visual opsins is involved in chromophore release.

It remains unclear whether all-trans-retinal is released through TM4 and TM5 or if the relaxation of TM4 serves as a water counterbalance valve, as is found in other hydrophobic ligand transport proteins (Christen et al., 2015; Silvaroli et al., 2016). The changes of solvent accessibility at TM4 could also alter the solvent network within GPCRs which impacts the continuous H-bond network contributing to G-protein activation and thus regulates the GPCR activity (Angel et al., 2009; Blankenship et al., 2015; Jastrzebska et al., 2011b; Okada et al., 2002a, b; Sun et al., 2014; Yuan et al., 2014).
Chapter 5

Conclusions and Future directions
5 Summary

5.1 Assessing thermal stability of membrane proteins with a PCR device

We demonstrate here that BFC is a versatile thiol-reactive compound that can be used for high-throughput DSF measurements of membrane proteins, proteins with hydrophobic binding sites or hydrophobic ligands. Unlike CPM, the absorption and fluorescent emission of BFC allows signal detection with a standard RT-PCR device without specialized filters. Moreover, the initial dimeric state of BFC enhances the intensity of the fluorescent signal, producing greater assay sensitivity, and permitting lower concentrations of probe, if desired. These properties render BFC a superior probe for high-throughput DSF measurements with standard RT-PCR equipment.

5.2 Conformational changes in green cone pigment upon light activation

We employed H/D exchange coupled with MS, differential scanning fluorimetry, bioinformatics, and computational modelling to investigate dynamic structural changes in green cone opsin that occur upon photo-activation and chromophore release. In addition to ligand binding, post-translational modifications of GPCRs, including the visual pigments, can contribute to the dynamics of their structure as well as play critical roles in protein folding, transport, and signaling (Dong et al., 2007; Morello and Bouvier, 1996; Zheng et al., 2013). In bovine rhodopsin, N-terminal glycosylation has been studied extensively and its importance in trafficking and signaling has been demonstrated (Hargrave, 1977, 2001;
Hofmann and Palczewski, 2015b; Kaushal et al., 1994; Murray et al., 2009; Murray et al., 2015; Zhu et al., 2004). It was previously proposed that green cone opsin, expressed in Sf9 insect cells, is glycosylated at its N-terminal region like rhodopsin but confirmation has been lacking (Vissers and DeGrip, 1996). MS/MS analysis of green opsin verified the proposed N-terminal glycosylation at asparagine residues 32 and 34. H/D exchange analysis also revealed a Pro-Pro motif with increased exchange upon photo-activation and chromophore release. Sequence alignment of monostable and bistable opsins revealed that the Pro-Pro motif is conserved in all monostable opsins but does not occur in bistable opsins suggesting a role of TM4 in chromophore release. In addition, computational analysis indicated that the substitution P186M, as seen in bistable proteins, permits greater conformational flexibility, further implicating TM4 and EL2 in the process of chromophore release in monostable visual opsins.

Here, we also provide further evidence in support of the proposed dimer interface of green opsin (Jastrzebska et al., 2017). The dimer interface between TM4 and TM5 exhibits reduced H/D exchange, compared to the other TM domains. We also observed a reduction of H/D exchange in IL2 upon receptor activation, whereas the remaining ILs did not exhibit significant changes. IL2 resides at the dimer interface between TM4 and TM5. IL2 directly interacts with Gα and β-arrestin and thus is crucial for G-protein signaling and desensitization (Han et al., 2001; Huang and Tao, 2014; Rosenbaum et al., 2009). Thus, stabilization of IL2 through dimerization could potentially affect the interaction with signaling proteins and lead to an altered activation state of the receptor (Fotiadis et al., 2006;
Rosenbaum et al., 2009). It remains to be answered whether stabilization of IL2 leads to prolonged activation through increased G-protein interaction and reduced affinity towards arrestin or faster desensitization through decreased G-protein interaction and increased affinity towards arrestin (Fotiadis et al., 2006).

5.3 The PDH crystal structure provides mechanistic insight into the initial reaction of the retinoid cycle

Mutations in retinol dehydrogenases from the SDR family are associated with a broad spectrum of ocular diseases. We provide structural insights into the biochemical functioning of this class of enzymes by solving the structure of a Drosophila melanogaster photoreceptor dehydrogenase, PDH. Comparisons of two structures with phenol and β-ionone suggest potential substrate-induced conformational change of the co-factor NAD(H) during catalysis. Enzymatic analysis of PDH also strongly supports a native dimeric state. It was suggested that the substrate binding in Drosophila melanogaster PDH occurs at virtually the same cytosolic site as co-factor binding (Benach et al., 1999). Although this could be true for soluble and hydrophilic substrates, we propose that binding of hydrophobic substrates such as retinoids occurs at the cytosolic distant site. Our finding is supported by data regarding the potential membrane spanning N-terminus in some SDRs (Liden and Eriksson, 2006; Wang et al., 2001; Zhang et al., 2004). Hence, in a dimeric state which is crucial for the activity of PDH, the substrate entry faces the membrane. Therefore, we propose a ‘kiss-and-run’ mechanism for the redox reactions of hydrophobic substrates that eliminates
thermodynamically unfavorable extraction of retinoid from the phospholipid fraction by allowing the substrate to partially reside within the membrane. Moreover, the molecular basis of disease-causing mutations associated with severe impairments in human vision also was revealed by comparing the *Drosophila melanogaster* dehydrogenase with the functionally-related human orthologue, RDH12.

5.4 Conclusion

The obtained results from these studies provided in depth structural insights into the first steps of the retinoid cycle. We were further able to improve and simplify the DSF measurements for membrane proteins with the use of a novel and effective thiol reactive probe. The application of H/D exchange measurements provided insights into the structural changes of the human green cone pigment upon activation. These conformational changes cannot be verified by classical structural studies. Thus, we demonstrated that only a combined approach of H/D exchange and structural studies reveals a comprehensive understanding of how pigment proteins work and interact on a molecular level. Still, experimental structure data of cone pigments are lacking and thus a primary focus for future studies. Further, the high-resolution structure of the *Drosophila melanogaster* PDH revealed unprecedented insight into the mechanism of hydride transfer in SDRs. The obtained knowledge provides an explanation for substrate specificity and demonstrates how dimerization affects the enzymatic activity of this class of
enzymes. These insights can provide the basis for future drug studies targeting the redox pathway or cycle.
6 Appendix

6.1 An effective thiol-reactive probe for differential scanning fluorimetry with a standard RT-PCR device; Materials and Methods

Preparation and storage of the BFC probe. One mg of BFC was purchased from Invitrogen (B-20340). The probe was diluted to a final concentration of 10 mM in DMSO and stored in 1 μl aliquots in the dark at -20 °C. The final working concentration of 2 μM BFC was used throughout the DSF experiments by diluting the DMSO stock with deionized water before adding it to a sample.

Preparation and storage of the Sypro-orange probe. The Sypro-orange 5000x concentrate was purchased from Invitrogen (S-6650), and stored in the dark at 4 °C. A final working concentration of 5x Sypro-orange was used throughout the DSF experiments by adding the DMSO stock solution to the sample (Niesen et al., 2007). If the required volume was less than 1 μl, 5000x Sypro-orange was diluted in deionized water before adding it to a sample.

Preparation of the lysozyme samples. Lysozyme C (UNIPROT ID: P00698) was purchased from Sigma Aldrich (L6876). Lyophilized lysozyme powder was solubilized in PBS buffer (NaCl, 137 mM; KCl 2.7 mM; Na₂HPO₄, 10 mM; and KH₂PO₄, 1.8 mM) pH 7.4 to a final concentration of 10 mg/ml. A series of two-fold dilutions in PBS was prepared in a MicroAmp Fast Optical 96-Well Reaction Plate (4346906) from Applied Biosystems.
Purification of bovine rhodopsin (UNIPROT ID: P02699). All manipulations were carried out in a darkroom under dim red illumination (>650 nm). Rod outer segments (ROS) were prepared as previously described (Baker et al., 2015; Okada et al., 1994). Briefly, crude ROS were isolated from dark adapted bovine retina (W. L. Lawson Co., Lincoln, NE) by a sucrose gradient extraction method. The ROS membranes were washed with isotonic and hypotonic buffer to remove both soluble and membrane associated ROS proteins. Purified ROS membranes (20 mg/mL rhodopsin) were solubilized by the zinc/alkyl-glucoside extraction method and centrifuged at 100,000g for 40 min to extract rhodopsin (Okada et al., 1998). The clear supernatant was further purified by gel filtration chromatography on a Superdex-200 column (GE-Healthcare) in 50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM MgCl2 and 0.02% n-dodecyl β-D-maltoside. Purified rhodopsin was concentrated to 2.6 mg/ml and used for DSF experiments.

Experimental sample preparation and fluorescence measurements. The dilution series were constructed in duplicate. Each well contained 9 μl of protein sample plus 1 μl of dye. The plate was sealed with ClearSeal film (HR4-521) from Hampton Research. The final probe concentration was 2 μM for BFC and 5x times for Sypro-orange. The plate was incubated for 10 min on ice prior to DSF measurements. The dilution series revealed the optimal working concentration for DSF measurements, which was then verified in quadruplicate.

All DSF measurements were performed with a StepOnePlus System (4376600) from Applied Biosystems. The melting curve experiments were conducted and
recorded using StepOne Software v2.3 from Applied Biosystems. The SYBR®, FAM™ and ROX™ channels were used simultaneously to record the change in fluorescence. The run was set to cool down to 4 °C within 10 s, kept at 4 °C for 1 min and then increased 1 °C per min in a step and hold manner up to 99.9 °C. The multicomponent data was exported on a Microsoft EXCEL sheet and analyzed with the XLfit package 5 (Ericsson et al., 2006).

**Data analysis.** Data were analyzed by averaging and normalizing the fluorescence intensities from the multicomponent experiments. Normalized curves were fitted to the Boltzmann equation (i) using the XLfit package 5.

6.2 **Structural insights into the Drosophila melanogaster retinol dehydrogenase, a member of the short-chain dehydrogenase/reductase family; Materials and Methods**

**Protein Expression and Purification.** *Drosophila melanogaster pdh-c* (NM_001144487.2) was cloned in a pet-45b(+) vector and expressed in BL21 *Escherichia coli*. The integrity of this expression plasmid was confirmed by sequencing. The plasmid was transformed into the BL21 (DE3) *Escherichia coli* strain (New England Biolabs, Ipswich, MA) for protein expression studies. One-liter LB media cultures containing 100 mg of ampicillin were grown at 37 °C to an OD₆₀₀ of 0.6–0.9 after which the temperature was lowered to 25 °C and cultures were induced with 0.5 mM IPTG. After 6 h of growth, cells were harvested by centrifugation and stored at −80 °C. Bacterial cells were lysed by four subsequent passages through a French press at 20,000 psi. The lysis buffer was
50 mM Tris, pH 8.0, 500 mM NaCl and 20% glycerol. The lysate was centrifuged for 30 min at 10,000 x g. The supernatant was filtered through a 0.22 μm Millex GS filter unit (Millipore, Carrigtwohill, Ireland). The filtrate was loaded onto a nickel column in the presence of 5 mM imidazole, followed by a washing step with 50 mM imidazole until no change in absorbance at 280 nm was detected. Protein then was eluted with 20 mM HEPES, pH 7.0, 500 mM NaCl and 300 mM imidazole. The eluent was loaded onto a Sephacryl S300 HR column (GE Healthcare) equilibrated with 20 mM HEPES, pH 7.0, 680 mM NaCl and 0.1 mM DDT. Peak fractions were combined and concentrated to 2.5 mg protein/ml. Protein samples (≥95% purity as judged by SDS-PAGE) were placed on ice for immediate use.

**Protein Crystallization, Structural Determination, and Analyses.** PDH-C (NP_001137959.1) was crystallized by the sitting-drop vapor-diffusion method at 4 °C in the presence of 1 mM NAD or NADH. One μl of purified enzyme at 2.5 mg/ml in 20 mM HEPES, pH 7.0, containing 10% (w/v) glycerol and 1 mM dinucleotide was mixed with a reservoir solution containing 0.1 M BisTris, pH 6.5, 25% (w/v) PEG3350, and 10 mM phenol or 10 mM β-ionone in a 1:1 ratio. Cubic- or triangular-shaped crystals typically appeared within 3 days in the presence of 10 mM phenol or 10 mM β-ionone. Mature crystals were harvested, swished through a reservoir mixture containing 15% glycerol, and flash-cooled in liquid nitrogen before x-ray exposure (Pflugrath, 2004). Diffraction data were collected at the NE-CAT 24-ID-E beamline of the Advanced Proton Source. These data
were indexed, integrated, and scaled with the XDS package (Kabsch, 2010). The structure was determined by molecular replacement in Phaser using PDB entry 1B2L as the starting model (Benach et al., 1999; Biasini et al., 2014a; McCoy et al., 2007). Manual adjustments to the structure were made with COOT, and restrained refinement was carried out in REFMAC5 and phenix.refine (Afonine et al., 2012; Echols et al., 2012; Emsley et al., 2010; Murshudov et al., 2011). Structures were validated with MOLPROBITY and the wwPDB structure validation server (Chen et al., 2010; Read et al., 2011). A summary of the x-ray data and refinement statistics is shown in Table 6. Structural figures were prepared with PyMOL or Discovery Studio Visualizer (BIOVIA, 2015; Schrodinger, 2015). The Omit maps for the ligands were calculated with phenix.maps in absence of the ligands in the model file and with the initial mtz reflection file (Adams et al., 2010).

Table 6: Data collection and refinement statistics.

<table>
<thead>
<tr>
<th></th>
<th>5ILG (PDH phenol)</th>
<th>5ILO (PDH β-ionone)</th>
</tr>
</thead>
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<tr>
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<td>0.9792</td>
</tr>
<tr>
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<td>113.4 - 2.71 (2.807 - 2.71)</td>
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<td>space group</td>
<td>P 2 2 1 2 1</td>
<td>C 1 2 1</td>
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<tr>
<td>unit cell</td>
<td>44.195 93.477 114.674 90 90 90</td>
<td>70.159 62.646 114.113 90 96.26 90</td>
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<td>total reflections</td>
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<td>26344 (2219)</td>
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<tr>
<td>unique reflections</td>
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<td>13342 (1145)</td>
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<td>Metric</td>
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<td>Value 2</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>completeness (%)</td>
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<td>98.56 (85.64)</td>
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<td>9.73 (2.30)</td>
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<td>37.68</td>
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<tr>
<td>R-merge</td>
<td>0.1033 (0.7215)</td>
<td>0.1004 (0.3859)</td>
</tr>
<tr>
<td>R-meas</td>
<td>0.1162</td>
<td>0.142</td>
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<td>0.992 (0.873)</td>
<td>0.976 (0.765)</td>
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<tr>
<td>CC*</td>
<td>0.998 (0.966)</td>
<td>0.994 (0.931)</td>
</tr>
<tr>
<td>reflections used for R-free</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-work</td>
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<td>0.2026 (0.3088)</td>
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<tr>
<td>R-free</td>
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<td>0.2644 (0.3482)</td>
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<td>RMS(angles)</td>
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<td>98</td>
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<td>average B-factor</td>
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<td>39.50</td>
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<tr>
<td>ligands</td>
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<td>31.10</td>
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<tr>
<td>solvent</td>
<td>41.10</td>
<td>33.50</td>
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</table>

\(^1\)Statistics for the highest-resolution shell are shown in parentheses.
Figure 31: B-factor analysis of PDH crystallized in the presence of phenol. Regions with high B-factors are represented in yellow, orange, and red with increased tubing size (Schrodinger, 2015). Aside from the C-terminus, the substrate-binding site shows an increased B-factor and thus increased flexibility.
Figure 32: B-factor analysis and RMSD between the two crystal structures in the presence of either phenol (blue) or β-ionone (green). The highest B-factors occur at the C-terminus and the substrate-binding loop (residues 200 - 220). The RMSD between the two structures was calculated with VMD and shows the largest differences between the substrate-binding site and the C-terminus (Humphrey et al., 1996).

Table 7: Sequences of peptide fragments from PDH’s primary sequence showing normalized hydrogen deuterium uptake for apo-PDH and PDH in the presence of NAD⁺. Column 1 shows the peptide sequence from the PDH primary sequence.
Column 2 reports the m/z of the ion used to identify the peptide based on its MS/MS spectrum. Column 3 indicates the charge of the ion from Column 2, and Column 4 shows the number of theoretically exchangeable sites of the peptide fragment. Columns 5 and 6 indicate the uptake of the exchangeable sites without NAD⁺ (column 5) and in the presence of NAD⁺ (column 6). Columns 7 and 8 indicate uptakes normalized to 75% of the theoretical maximum exchangeable sites. Column 9 indicates the difference of uptake between the apo- and NAD⁺-bound state.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>m/z</th>
<th>z</th>
<th>No. of exchangeable sites (75%)</th>
<th>PDH apo</th>
<th>PDH with NAD⁺</th>
<th>PDH apo %</th>
<th>PDH with NAD⁺ %</th>
<th>Δ in uptake %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A⁸⁰VVTGGAGGIGL</td>
<td>971.64</td>
<td>1</td>
<td>8.25</td>
<td>4.46</td>
<td>3.36</td>
<td>54</td>
<td>41</td>
<td>13</td>
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<tr>
<td>A⁸⁰VVTGGAGGIGLQVS KQLL</td>
<td>1768.16</td>
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<td>13.5</td>
<td>4.81</td>
<td>3.58</td>
<td>36</td>
<td>27</td>
<td>9</td>
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<tr>
<td>L⁹⁰QVSKQLL</td>
<td>928.68</td>
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<td>5.25</td>
<td>0.7</td>
<td>0.64</td>
<td>13</td>
<td>12</td>
<td>1</td>
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<tr>
<td>A²⁷AGAAKVAIDL</td>
<td>557.07</td>
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<td>8.25</td>
<td>1.31</td>
<td>1.33</td>
<td>16</td>
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<tr>
<td>F⁹⁰VKLRAAHPTQSM</td>
<td>793.32</td>
<td>2</td>
<td>9</td>
<td>3.08</td>
<td>3.2</td>
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<tr>
<td>I³⁹IKMDVANKKGVEAT</td>
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<td>Y⁷⁴EEIAKTFGNID</td>
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<tr>
<td>I⁷⁷AKTFGNID</td>
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<tr>
<td>I³⁷⁶VNVAGIFNDKDQVRRTLL</td>
<td>1057.96</td>
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<td>13.5</td>
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<td>3.42</td>
<td>48</td>
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<td>23</td>
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<td>V¹⁰⁵NLGGIINSTL</td>
<td>1100.64</td>
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<td>6.75</td>
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<td>36</td>
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<td>15</td>
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<tr>
<td>S¹⁷⁶ALPYMGKDGNGK GGIVVNM</td>
<td>1004.86</td>
<td>2</td>
<td>13.5</td>
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<td>2.37</td>
<td>19</td>
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<td>S¹³⁸SVVGLDPMF</td>
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<td>1</td>
<td>6</td>
<td>3.63</td>
<td>2.94</td>
<td>61</td>
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<tr>
<td>I¹⁴⁶IPVYGATKAGIINF</td>
<td>1576.99</td>
<td>1</td>
<td>9</td>
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<td>T¹⁶¹RCLANEKY</td>
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<td>2</td>
<td>5.25</td>
<td>2.67</td>
<td>2.6</td>
<td>51</td>
<td>50</td>
<td>1</td>
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</table>
Activity Assay of PDH Crystals. Oxidation of all-trans-retinol in PDH crystals was assayed by monitoring the production of all-trans-retinal in the presence of NAD$^+$. (Perez-Miller and Hurley, 2003; Tanaka et al., 1996). The oxidation mixture added to the crystallization drop contained 1 mM NAD$^+$, 0.1 M phosphate buffer pH 8.0, 680 mM NaCl, 10% PEG200, 20% PEG3350 and 244 μM all-trans-retinol. Reduction of all-trans-retinal in PDH crystals was assayed with 1 mM NADH, 0.1 M phosphate buffer, pH 5.0, 680 mM NaCl, 10% PEG200, 20% PEG3350 and 431 μM all-trans-retinal. For each redox reaction, 96 crystallization drops were incubated for 2 h at room temperature before they were harvested. The control was carried out in crystallization drops without protein. The reaction was quenched with 100 μl methanol, and retinoids were extracted twice with 200 μl hexane. Retinoids were separated by normal phase HPLC in 90% hexane, 10% ethyl acetate and analyzed at both 325 nm and 360 nm. The reduction of 3-OH-retinal followed the same procedure as described above for all-trans-retinal. 3-OH-retinoids were obtained by enzymatically converting zeaxanthin by NINAB.
Analysis and separation of 3-OH-retinoids was accomplished by normal phase HPLC in 70% hexane and 30% ethyl acetate and analyzed at 325 nm. All steps were conducted under dim red light. The enzymatic activity was also tested with PDH precipitated without formation of the crystalline state by 25% PEG3350, 0.1 M BTP, pH 6.5 in addition to the purified protein and the reaction solution of 10% PEG200 and 20% PEG3350 in 0.1M BTP pH 6.5. No activity was observed in the precipitated PDH in identical reaction conditions as observed red-ox reaction for PDH in its crystalline state.

Extensive experiments were performed in order to verify the catalytic activity of PDH in solution. We tried the following conditions: (all these conditions were analyzed with retinal, retinol and 3-OH-retinal): in presence of detergent micelles CHAPS or DDM; in presence of detergent micelles CHAPS or DDM with bovine albumin; in presence of bovine albumin alone; in presence of synthetic liposomes the lipid composition mimics the bovine photoreceptor disc membranes: (44% PE, 44% PC, 44% PS by mass); all four isoforms (isoform A, NP_524105.2 (or NM_079381.3); isoform B, NP_730153.1 (or NM_168659.2); isoform C, NP_001137959.1 (or NM_001144487.2), isoform D, NP_001137960.1 (or NM_001144488.2) of PDH expressed in Sf9 insect cells and purified in microsomes; all four isoforms (A-D) of PDH expressed in Sf9 insect cells and purified in microsomes in presence of detergent micelles CHAPS or DDM; all four isoforms (A-D) of PDH expressed in Sf9 insect cells and purified in microsomes in presence of detergent micelles CHAPS or DDM with albumin; and all four isoforms (A-D) of PDH expressed in Sf9 insect cells and purified in microsomes
in presence of albumin. In all of these conditions no enzymatic activity was observed.

Figure 33: HPLC analysis of the PDH reaction products extracted from crystal drops and analysis of retinoids extracted from fly heads. (A) Reduction of 3-hydroxy-retinal (a and b) to 3-hydroxy-retinol (d and e) in the presence (blue) and absence (red) of crystals. (B) HPLC analysis of retinoids extracted from 100 mg of fly heads. Data from the PDH knockout fly heads are depicted in blue and show a decreased amount of extracted retinoids compared to the wild type in red. The corresponding spectra of the different retinoid isomers and zeaxanthin (a – k) are shown on the right. The asterisk * indicates an unidentified compound.

Isogenized w^{1118} (control flies) and pdh^1 flies were raised at 25°C on standard cornmeal-yeast medium under a 12 h light/12 h dark cycle (Wang et al., 2010). Control and mutant flies were collected in 50 mL Falcon tubes (5 g and 2.5 g,
respectively). All flies were frozen in liquid nitrogen for 5 min followed by vigorous vortexing that was repeated 5 times. The flies were then transferred to standard testing sieves to collect fly heads: the top sieve (710 μM No. 25, Fisher) collected the bodies and the bottom sieve (425 μM No. 40, Fisher) collected the heads.

Retinoids from 100 mg *Drosophila melanogaster* fly heads were extracted and analyzed as described previously (Wang et al., 2010). Dissected fly heads were disrupted in a glass homogenizer in 400 μl 2 M NH$_2$OH (pH 6.8). Then 400 μl of methanol and 200 μl of acetone were added to further homogenize and wash the sample. Retinoids were extracted with 250 ml diethyl ether and 250 ml petroleum benzene. The extraction was repeated three times with 300 μl petroleum benzene. The combined organic phases then were dried in a Speedvac. Lipophilic compounds were dissolved in 300 μl hexane and subjected to quantitative HPLC analysis as described previously (Oberhauser et al., 2008). All steps were conducted under a dim red safety light.

NINAB (*Galleria mellonella*) was expressed and assayed enzymatically as described previously (Babino et al., 2016) and was used for the production of 3-OH-retinal. DMN micelles loaded with substrate (zeaxanthin) were prepared as follows: 33 μL of 3% DMN detergent solution was mixed with 10 μM (final concentration) of substrate, and dissolved in acetone in a 2-mL Eppendorf tube. This mixture was then dried in a Speedvac. Then 100 μL of cell lysate was added to substrate and vortexed vigorously for 20 s and placed on an Eppendorf Thermomixer set at 28 °C for 30 min at 300 rpm. Reactions were stopped by
adding 100 μL of water and 400 μL of acetone. Lipids were extracted by adding 400 μL of diethyl ether and 500 μL petroleum ether, then vortexed for 3 x 10 s periods, centrifuged at 15,000g for 1 min and the resulting organic phase was collected. This extraction was performed twice and the combined organic phases were dried in a Speedvac.

**Protein Interface Classification by Evolutionary Analysis.** The dimer interface of PDHc was analyzed with both PISA and EPPIC (Duarte et al., 2012; Krissinel, 2010; Krissinel and Henrick, 2007). EPPIC classifies protein-protein interactions in a crystal lattice by including multiple sequence alignments of homologs with ≥60% sequence identity. This classification is based on a geometric measure, namely the number of core residues and two evolutionary indicators derived from the sequence entropy of homolog sequences. Both aim at detecting differential selection pressure between the interface core and either the rim or the remainder of the surface (Duarte et al., 2012). The protein-protein interaction of heterologous PDHc was assessed with FPLC. PDHc was analyzed either in the presence of 1 mM NAD⁺, 1 mM NADH or without dinucleotide on a Superdex 200 increase column (GE Healthcare, Pittsburgh, PA) using the equilibration buffer described in the purification protocol.
Table 8: Dimer interface statistics generated by PISA, CCP4 (Krissinel and Henrick, 2007).

### Interface summary:

<table>
<thead>
<tr>
<th></th>
<th>Monomer 1</th>
<th>Monomer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ID</strong></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td><strong>Class</strong></td>
<td>Protein</td>
<td>Protein</td>
</tr>
<tr>
<td><strong>Symmetry operation</strong></td>
<td>X,Y,Z</td>
<td>X,Y,Z</td>
</tr>
<tr>
<td><strong>Symmetry ID</strong></td>
<td>1_555</td>
<td>1_555</td>
</tr>
<tr>
<td><strong>Interface atoms</strong></td>
<td>234 (12.1%)</td>
<td>238 (11.9%)</td>
</tr>
<tr>
<td><strong>Surface atoms</strong></td>
<td>1111 (57.5%)</td>
<td>1157 (57.9%)</td>
</tr>
<tr>
<td><strong>Total atoms</strong></td>
<td>1933 (100.0%)</td>
<td>2000 (100.0%)</td>
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<tr>
<td><strong>Interface residues</strong></td>
<td>67 (26.0%)</td>
<td>65 (24.5%)</td>
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<tr>
<td><strong>Surface residues</strong></td>
<td>235 (91.1%)</td>
<td>244 (92.1%)</td>
</tr>
<tr>
<td><strong>Total residues</strong></td>
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<td>265 (100.0%)</td>
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<tr>
<td><strong>Buried ASA, Å²</strong></td>
<td>2365.7 (18.7%)</td>
<td>2396.6 (18.4%)</td>
</tr>
<tr>
<td><strong>Total ASA, Å²</strong></td>
<td>12621.9 (100.0%)</td>
<td>13053.8 (100.0%)</td>
</tr>
<tr>
<td><strong>Solvation energy (kcal/mol)</strong></td>
<td>-237.5</td>
<td>-246.8</td>
</tr>
<tr>
<td><strong>SE gain (kcal/mol)</strong></td>
<td>-21.2</td>
<td>-19.7</td>
</tr>
</tbody>
</table>

### Interface parameters:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IA: Interface Area</strong></td>
<td>2381.1</td>
</tr>
<tr>
<td><strong>DG: ΔG</strong></td>
<td>-40.9</td>
</tr>
<tr>
<td><strong>BE: Binding Energy</strong></td>
<td>-49.7</td>
</tr>
<tr>
<td><strong>PV: P-value</strong></td>
<td>0.0042</td>
</tr>
<tr>
<td><strong>HB: Hydrogen bonds</strong></td>
<td>13</td>
</tr>
<tr>
<td><strong>SB: Salt bridges</strong></td>
<td>8</td>
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<tr>
<td><strong>DS: Disulphide bonds</strong></td>
<td>0</td>
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</tbody>
</table>
Table 9: Dimer interface statistics generated by EPPIC (Duarte et al., 2012). The interface between the two monomers in the asymmetric unit is classified as BIO, and thus is biologically relevant.

<table>
<thead>
<tr>
<th>ID</th>
<th>Chains</th>
<th>Area (Å)</th>
<th>Operator</th>
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<th>Core-Surface</th>
<th>Final</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>B+A</td>
<td>2385.15</td>
<td>I</td>
<td>11 + 9</td>
<td>bio</td>
<td>bio</td>
<td>bio</td>
<td>BIO</td>
</tr>
</tbody>
</table>

Differential Scanning Fluorimetry (DSF). Protein stability was assessed by differential scanning fluorimetry as previously reported (Hofmann et al., 2016). Briefly, the protein was diluted to a concentration of 6 μM and screened in the presence of 2 μM reporter dye, BODIPY FL L-cystine. Fifteen μl of sample plus dye were screened against 5 μl of Hampton additive screen HT (Hampton Research, Aliso Viejo, CA). The melting temperature was determined with a Boltzmann fit. The Kd for NAD⁺ was determined with DSF measurements (Hofmann et al., 2016). Briefly, the melting temperature of the protein ligand complex was determined in triplicate using a two-fold dilution series from 18.75 mM to 0.009 mM NAD⁺. Melting curves were fitted to the Boltzmann equation and the resulting melting temperatures were analyzed with SigmaPlot version 11.0 according to a standard ligand-binding curve (Software).
Figure 34: Kd measurements of NAD$^+$ and PDH with DSF (Hofmann et al., 2016). The difference in melting temperature plotted against increasing NAD$^+$ concentrations revealed a $K_d$ for NAD$^+$ of 170 ± 30 μM. Data were fit with $f = B_{max} \cdot \text{abs}(x)/(K_d + \text{abs}(x))$ with SigmaPlot version 11.0 (Software).

**Energy Calculations.** Guassian09 software was used for quantum chemical calculations (Frisch et al., 2009). A two layer ONIOM model (Dapprich et al., 1999) was employed for calculations of the protein-NAD complex, with the NAD molecule treated at the Hartree-Fock theory level with 3-21 g basis sets (Binkley et al., 1980; Dobbs and Hehre, 1986, 1987a, b; Gordon et al., 1982; Pietro et al., 1982). The AMBER molecular mechanics force field (Cornell et al., 1995) was used for the protein. The complex was geometrically optimized and the total ONIOM energy is reported, as well as the energy for just the NAD within the complex. To obtain the energy of the two conformations of NAD in solution, the molecule was extracted from the optimized complex structures. A single-point energy calculation was then performed at the Hartree-Fock theory level with 3-21...
g basis sets and water as the solvent using the polarizable continuum model (Barone et al., 1997, 1998; Cammi et al., 1999, 2000; Cossi and Barone, 2000, 2001; Cossi et al., 1996; Cossi et al., 1999; Cossi et al., 2001, 2003; Cossi et al., 2002; Miertuš et al., 1981; Miertuš and Tomasi, 1982; Pascual-ahuir et al., 1994; Tomasi et al., 2005; Tomasi et al., 1999).

Table 10: Calculated energies of NAD with its conformations in the presence of the protein moiety, in vacuum and in water.

<table>
<thead>
<tr>
<th></th>
<th>Energy with protein in (kcal/mol)</th>
<th>NAD(H) in vacuum (kcal/mol)</th>
<th>NAD(H) in water (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenol (5ILG)</td>
<td>-1,829,534.26</td>
<td>-1,821,020.38</td>
<td>-1,821,100.09</td>
</tr>
<tr>
<td>β-ionone (5ILO)</td>
<td>-1,829,561.51</td>
<td>-1,821,069.61</td>
<td>-1,821,151.72</td>
</tr>
</tbody>
</table>
Figure 35: Omit maps of the co-factor binding site calculated in the absence of the co-factor (Echols et al., 2012). Panels A and B represent the PDH binding site crystallized in the presence of phenol. Panels C and D represent the binding site crystallized in the presence of β-ionone. Panels A and C show 2Fo-Fc maps contoured at 1.5σ, whereas B and D are Fo-Fc maps contoured at 3.0σ. All maps are represented in blue mesh. The catalytic triad of the active site and NAD⁺ are shown in sticks colored according to their elements (Schrodinger, 2015).

**Amide Hydrogen–Deuterium Exchange of PDH.** Amide hydrogen–deuterium exchange was performed as follows. Approximately 0.8 μg of purified PDH was diluted in 45 μl D₂O containing 20 mM HEPES, pH 7.0, and kept on ice for 5 min, 10 min, 15 min, 20 min, or 25 min. After exposure to deuterated buffer, the exchange was terminated with ice cold quenching buffer composed of D₂O and
20 mM HEPES, pH 7.0. Ten μl of pepsin (8.0 mg/ml, Worthington, Lakewood, NJ) was immediately added to the solution and the sample was digested for 15 min on ice. Next, the sample (100 μl) was loaded onto a Luna 20 × 2.00 mm C18 column (Phenomenex, Torrance, CA) with a temperature–controlled Hewlett–Packard autosampler set to 4 °C. Peptides were eluted with the following gradient sequence: 0–4 min, 98% H2O with 0.1 % (v/v) formic acid (A) and 2% acetonitrile with 0.1 % (v/v) formic acid (B); 4–12 min, 98% to 2% A. Separation was performed with an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) at a flow rate of 0.2 ml/min. The eluent was injected into a Thermo Finnigan LXQ (Thermo Scientific, Waltham, MA) MS equipped with an electrospray ionization source operated in a positive ion mode with other parameters adjusted as follows: activation type was set to collision-induced dissociation, normalized collision energy to 35 kV, capillary temperature to 370 °C, source voltage to 5 kV, capillary voltage to 43 V, tube lens to 105 V, and MS spectra were collected over a 200–2,000 m/z range. To avoid sample propagation from one HPLC run to another, each production run was followed by a mock injection of 10 μl of solution A with a resulting HPLC elution profile identical to the one described above. This run was followed by a 20 min equilibration run with 98% A and 2% B for 20 min. Experiments were performed at least in triplicate for each time point.

Analysis of Hydrogen–Deuterium Exchange. Peptides resulting from the pepsin digestion were identified by MS/MS by employing the online version of
MassMatrix (Xu and Freitas, 2009). Search settings used to examine a peptic digest were as follows: precursor ion tolerance, 0.8 Da; the maximum number of variable modifications allowed for each peptide sequence, 2; the minimum peptide length, 4 amino acids; the minimum pp score, 5; the pp tag score, 1.3; the maximum number of combinations of different modification sites for a peptide match with modifications, 1; and the maximum number of candidate peptide matches for each spectrum output in the result, 1 (Xu and Freitas, 2007). Peptides reproducibly identified in (Orban et al., 2012b) were used to construct the progress plots. Raw data in the form of relative signal intensity (%) as a function of m/z were extracted with XCalibur 2.1.0. Qual Browser was used for a recently described semi–automated peak detection and deconvolution procedure performed with HDExpress software (Orban et al., 2010; Weis et al., 2006). Briefly, after deuterium uptake was evaluated from the raw data, the value for every peptide fragment was normalized to 75% of the theoretically maximum exchangeable sites to account for the 75% deuteration accomplished experimentally (Table 7, column 4). For calculations of the maximum exchangeable sites, only peptide bonds were used to account for the amide exchange; deuterium exchange from side chains was considered negligible and thus not included. The number of maximum exchangeable sites was decreased by one for each P residue in a peptide sequence. Hydrogen–deuterium exchange was color coded based on the total percent of the theoretical maximum deuterium uptake at 10 min as follows: blue, 10–19%; violet, 20–29%; cyan, 30–39%; green, 40–49%; yellow, 50–59%; and orange, 60–69%.
**RDH12 Modelling.** The RDH12 model was calculated based on the PDH crystal structure template from the SWISS-MODEL Workspace (Arnold et al., 2006; Benkert et al., 2011; Biasini et al., 2014b). This model (32.48 sequence identity) yielded a GMQE value of 0.6 and a QMEAN4 of -9.66.

Figure 36: Superimposition of human and *Drosophila* short-chain dehydrogenases reveals major RMSD differences in the substrate-binding region. Human DHRS4 (red) is superimposed on, DHRS10 (green) and *Drosophila* PDH (yellow). RMSD values were as follows: between DHRS4 and DHRS10, 0.951 Å; DHRS4 and PDH, 1.582 Å; DHRS10 and PDH, 1.066 Å. The Rossmann fold is conserved among the short-chain dehydrogenase family members and thus shows a low RMSD value, indicated by light coloring. The substrate-binding site shows a large RMSD value indicated with a higher illuminance.
Table 11: Allelic variants causing LCA13 or RP53 in RDH12 from OMIM (McKusick, 1998).

<table>
<thead>
<tr>
<th>Number</th>
<th>Phenotype</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LCA 13</td>
<td>RDH12, TYR226CYS</td>
</tr>
<tr>
<td>2</td>
<td>LCA 13</td>
<td>RDH12, 5-BP DEL, NT806</td>
</tr>
<tr>
<td>3</td>
<td>LCA 13</td>
<td>RDH12, GLN189TER</td>
</tr>
<tr>
<td>4</td>
<td>LCA 13</td>
<td>RDH12, THR49MET</td>
</tr>
<tr>
<td>5</td>
<td>LCA 13</td>
<td>RDH12, ARG62TER</td>
</tr>
<tr>
<td>6</td>
<td>LCA 13</td>
<td>RDH12, GLY127TER</td>
</tr>
<tr>
<td>7</td>
<td>LCA 13</td>
<td>RDH12, HIS151ASN</td>
</tr>
<tr>
<td>8</td>
<td>LCA 13</td>
<td>RDH12, PRO230ALA</td>
</tr>
<tr>
<td>9</td>
<td>LCA 13</td>
<td>RDH12, HIS151ASP</td>
</tr>
<tr>
<td>10</td>
<td>LCA 13</td>
<td>RDH12, LEU99ILE</td>
</tr>
<tr>
<td>11</td>
<td>LCA 13</td>
<td>RDH12, SER175PRO</td>
</tr>
<tr>
<td>12</td>
<td>LCA 13</td>
<td>RDH12, ILE51ASN</td>
</tr>
<tr>
<td>13</td>
<td>LCA 13</td>
<td>RDH12, 658G-A, +1</td>
</tr>
<tr>
<td>14</td>
<td>LCA 13</td>
<td>RDH12, THR155ILE</td>
</tr>
<tr>
<td>15</td>
<td>RP 53</td>
<td>RDH12, 1-BP DEL, 776G</td>
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<tr>
<td>16</td>
<td>RP 53</td>
<td>RDH12, ALA126VAL</td>
</tr>
</tbody>
</table>

6.3 Hydrogen/Deuterium Exchange Mass Spectrometry of Human Green Opsin Reveals a Conserved Pro-Pro Motif in Extracellular Loop 2 of Monostable Visual G-protein Coupled Receptors; Materials and Methods

Sequence alignment of the M/LWS receptor and M/LWS construct. Sequences were aligned with the MUSCLE server provided by EMBL-EBI. (Edgar, 2004a, b; Li et al., 2015b; McWilliam et al., 2013) MUSCLE stands for
MULTIPLE SEQUENCE COMPARISON BY LOG-EXPECTATION. The M/LWS construct starts with a haemagglutinin signal sequence, which is cleaved during protein maturation in Sf9 insect cells. The C-terminus of the M/LWS construct was replaced with the 1D4 sequence TETSQVAPA.

**Cloning and Expression** The human green opsin gene (Uniprot Entry: P04001) was modified with a 1D4-tag at the C-terminal region (Molday and Molday, 2014). This construct was cloned into a modified pFastBac (Invitrogen) baculovirus expression vector containing the haemagglutinin (HA) signal sequence.

The modified green opsin construct was transformed into DH10Bac to produce a recombinant baculovirus with the Bac-to-Bac system (Invitrogen). The recombinant baculovirus then was used to infect an Sf9 insect cell culture at a
cell density of $3 \times 10^6$ cells /ml. Infected cells were grown for 60 h at 27 °C before harvesting, and cell pellets were stored at -80 °C for future use.

**Regeneration, solubilization and purification of green cone opsin.** Sf9 cell membranes from 5 liters of cell culture were disrupted with a Dounce homogenizer in a hypotonic buffer containing 25 mM HEPES, pH 7.0, and EDTA-free complete protease inhibitor cocktail (Roche, Indianapolis, IN). Cell lysis and washing of the membranes were performed by repeated passes in the Dounce homogenizer and centrifugation at 96,000 g for 20 min at 4 °C in the hypotonic buffer for a total of three times and then in a high osmotic buffer containing 1.0 M NaCl, 25 mM HEPES, pH 7.0, and EDTA-free complete protease inhibitor cocktail for three additional cycles. Cell membranes were centrifuged at 96,000 g for 20 min at 4 °C and stored at -80 °C. Prior to use, membranes were homogenized in 200 ml of 25 mM HEPES, pH 7.0, 50 mM NaCl. The following steps were enacted under dim red light. Green opsin was regenerated with 40 μM 11-cis-retinal solubilized in ethanol for 1 h. Membrane pellets were solubilized by adding 1% (w/v) n-dodecyl β-D-maltopyranoside (DDM; Anatrace, Maumee, OH) to a final volume of 250 ml, for 3 h at 4 °C. The supernatant was collected after centrifugation for 45 min at 95,834 g and incubated with 7.5 ml of Rho1D4 monoclonal antibody linked to CNBr-activated Sepharose 4B (GE Healthcare, Waukesha, WI) in a batch for 3 h at 4 °C. After binding, the beads were collected by sedimentation and applied to a gravity flow column (BioRad). This step was followed by washing with 500 ml of buffer consisting of 25 mM
HEPES, pH 7.0, 50 mM NaCl, and 0.02% (w/v) DDM. The green pigment was incubated with 10 ml of buffer and 1 mg/ml 1D4 peptide overnight and then was eluted with an additional 15 ml of buffer and 1 mg/ml 1D4 peptide. The eluted protein was concentrated to 500 μl with an Amicon 50 kDa NMWL cutoff filter (EMD Millipore, Billerica, MA). Finally, the pigment was passed through a Superdex 200 size-exclusion column (GE Healthcare) with a final buffer containing 20 mM HEPES, pH 7.0, 50 mM NaCl, and 0.02% DDM. The purified protein was concentrated to 1 mg/ml, flash frozen in 50 μl aliquots and stored at -80 °C for future use.

The purity of the green cone opsin after size exclusion chromatography was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and spectrophotometry (Figure 24 A to D). The reported extinction coefficient for the green photopigment is \( \varepsilon_{530} = 40,000 \text{ M} \cdot \text{cm}^{-1} \), and the calculated \( \varepsilon_{280} = 99,975 \text{ M} \cdot \text{cm}^{-1} \) (Vissers et al., 1998; Vissers and DeGrip, 1996). Thus, a theoretical ratio \( (A_{280/530}) \) of 2.49 would indicate a pure sample. Here we obtained an absorbance ratio \( A_{280/530} \) of 2.86, which together with the data obtained by SDS-PAGE indicate a purity close to 100%.

**Immunoblotting of green cone pigment.** 1D4 antibody was purified from mouse ascites fluid using a Pierce™ (Rockford, IL) Thiophilic Adsorption kit and conjugated to an alkaline phosphatase with the Lightning-Link® Alkaline Phosphatase kit (Innova Bioscience, Cambridge, UK). The gel was transferred to an Immobilon®-P Transfer Membrane with the eBlot Protein Transfer Device
The membrane was blocked with 5% nonfat-dry milk in TBST buffer for one hour. The membrane was then incubated with conjugated 1D4 antibody for three hours. After three washing steps with TBST for five min each, the membrane was developed for five min with Western Blue® Stabilized Substrate for Alkaline Phosphatase.

**Differential Scanning Fluorimetry.** Protein stability was assessed by differential scanning fluorimetry as previously reported (Hofmann et al., 2016). Briefly, bovine rhodopsin (Uniprot Entry: P02699) and the green pigment were diluted in 20 mM HEPES, pH 7.0, 50 mM NaCl, and 0.02% n-dodecyl β-D-maltopyranoside (DDM) to a concentration of 6 μM and assayed in the presence of 2 μM reporter dye, BODIPY FL L-cystine (ThermoFisher). The melting temperatures were determined in triplicate using a Boltzmann fit performed with SigmaPlot software, ver. 11.0 (Software).

**Amide H/D Exchange of Green Opsin.** Amide H/D exchange was performed and analyzed as described previously (Li et al., 2015a; Orban and Tsybovsky, 2015; Zhang et al., 2010). Briefly, 10 μg of purified green pigment was diluted in 25 μl D₂O, resulting in a final D₂O dilution of 80%. The solution was kept on ice and incubated for 10 min. This D₂O exposure time (i.e., 10 min) was chosen to achieve steady-state exchange conditions so deuterium uptake in each peptide could be compared between states (dark vs bleached) without the need to evaluate back exchange and have only one parameter that changes between states, i.e. light. Incubation time in D₂O was tested and optimized using data from
hundreds of peptides following previous protocols from similar experiments wherein the dilution was kept at 80% (Orban et al., 2010; Orban et al., 2012a; Orban et al., 2012b; Rajavel et al., 2016; Tsybovsky et al., 2013). After exposure to D₂O, the exchange was quenched with ice cold buffer composed of D₂O and formic acid (Sigma Aldrich), pH 2.5. The quenching buffer used in the non-deuterated samples was prepared with H₂O. Pepsin (Uniprot Entry: P00791) (Worthington), 2.5 mg/ml, was freshly prepared in H₂O for both deuterated and non-deuterated samples. Five μl were added to the protein solution immediately after addition of the quenching buffer. The sample then was digested for 5 min on ice. Next, the sample (30 μl) was loaded onto a C8 trap (2.1 mm Thermo Scientific) and a C4 column (2.1 mm × 50 mm, Thermo Scientific) using a temperature–controlled Accela 600 autosampler and pump (Thermo Scientific) with the temperature set to 4 °C. Peptides were eluted by the following gradient program: 0–40 min, 98% H₂O with 0.1 % (v/v) formic acid (A) and 2% acetonitrile with 0.1 % (v/v) formic acid (B), to 2% A and 98% B. Separation was achieved at a flow rate of 0.1 ml/min. The eluent was injected into a LTQ Velos linear trap quadropole mass spectrometer (Thermo Scientific) equipped with an electrospray ionization source operated in a positive ion mode.

**Settings for peptide detection.** Mass spectrometer settings were set as follows: activation type, collision-induced dissociation; normalized collision energy, 35 kV; capillary temperature, 370 °C; source voltage, 5 kV; capillary voltage, 43 V; tube lens, 105 V, and then MS spectra were collected over a 200–2,000 m/z range. To
avoid sample propagation from one run to the next, each production run was followed with a mock injection of 10 μl of solution A and the same gradient program as described in Methods. This run then was followed by a 20-min equilibration run with 98% A and 2% B for 20 min. Experiments were performed at least in triplicate for each time point.

**De-glycosylation of Green Opsin by PNGase F Digestion.** Purified green opsin was incubated with PNGase F at 22 °C for 2 h prior to SDS-PAGE. The effect of PNGase F digestion was monitored by SDS-PAGE and Western blot analysis (see Appendix).

**Analysis of H/D Exchange.** Peptides resulting from the pepsin digestion were identified by MS/MS with an offline local version of MassMatrix (Xu and Freitas, 2009). Search settings used to examine the peptic digest against the primary sequence of human green opsin construct (see Appendix) were as follows: precursor ion tolerance, 0.8 Da; maximum number of variable modifications allowed for each peptide sequence, 2; minimum peptide length, 5 amino acids; minimum pp score, 5; ppgtag score, 1.3; maximum number of combinations of different modification sites for a peptide match with modifications, 1; and maximum number of candidate peptide matches for each spectrum output in the result, 1 (Xu and Freitas, 2007). Raw data in the form of relative signal intensity (%) as a function of m/z were extracted with Xcalibur 2.1.0. Qual Browser was used for a recently described semi–automated peak detection and a deconvolution procedure was performed with HX-Express² software (Guttman et
al., 2013; Orban et al., 2010; Weis et al., 2006). The extent of H/D exchange was color coded based on the total percent of the theoretical maximum deuterium uptake at 10 min as follows: 0-4%, dark blue; 5-9% light blue; 10-14%, cyan; 15-19%, cadet blue; 20-24%, green, 25-29%, light green; 30-34%, yellow; 35-39%, orange; 40-44%, red. Undefined regions are represented in grey.

**H/D exchange analysis and normalization.** Deuterium uptake was evaluated from the raw data, the value for every peptide fragment was normalized to 80% of the theoretically maximum exchangeable sites to account for the 80% deuteration accomplished experimentally (Table 12, column 7). For calculations of the maximum exchangeable sites, only peptide bonds were used to account for the amide exchange; deuterium exchange from side chains was considered negligible and thus not included. The number of maximum exchangeable sites was decreased by one for each Pro residue in a peptide sequence under analysis (Table 12, column 6).
Table 12: Sequences of peptide fragments from the M/LWS construct sequence showing normalized deuterium uptake for dark M/LWS and bleached M/LWS receptor in the presence of DDM. Column 1 shows the peptide sequence from the M/LWS construct’s primary sequence. Column 2 reveals the charge of the ion from Column 5. Column 3 reports the retention time (min) of the peptide. Column 4 shows the detected mass of the peptide (MW(obs)=m/z*charge-charge). Column 5 indicates the mass over charge (m/z) of the ion used to identify the peptide based on its MS/MS spectrum and Column 6 shows the maximum number of theoretically exchangeable sites of the peptide fragment (max = number of non-proline peptide bonds in the peptide fragment). Column 7 reveals the uptake normalized to 80% of the theoretical maximum exchangeable sites. The 80% normalization reflects the dilution percentage in D$_2$O (see Methods). Columns 8-9 and 10-11 indicate the uptake of the exchangeable sites in dark state and in the bleached state, respectively. Column 12 shows the difference of uptake between the dark and bleached states. Columns 9 and 11 are colored and shaded according to a red (1) – yellow (25) – green (50) scale based on the value. Column 12 is colored and shaded in a green-red scale based on the value (red: negative, green: positive).
<table>
<thead>
<tr>
<th>peptide</th>
<th>charge</th>
<th>RT</th>
<th>MW(obs)</th>
<th>m/z</th>
<th>max</th>
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**Green Opsin Modelling.** A model of the human green cone opsin (Uniprot Entry: P04001) was created with the 2.2 Å resolution crystal structure of rhodopsin (PDB ID 1U19; Uniprot Entry: P02699). The EMBOSS Needle Pairwise Sequence Alignment tool was used to align the two sequences (Rice et al., 2000). The MEDELLER server generated an initial structure for the green cone opsin based on the sequence alignment (Kelm et al., 2010). The resulting MEDELLER structure then was used as input into the ROSETTA protein structure prediction suite, where the conformation of the N-terminal 20 amino acids was sampled sixty times with the loop building protocol and the best model by energy was selected (Lee et al.).

**Single point mutations of Green Opsin: P186M and P205I Models.** Two single-point mutations in green opsin were created with ROSETTA. The mutations P186M and P205I were introduced into the green opsin model with the fixed backbone design module of ROSETTA (Kuhlman et al., 2003). Next, all models underwent 1000 independent full-structure optimization trajectories in ROSETTA employing the membrane fast relax protocol (Barth et al., 2007). The ten most energetically favorable structures for each of the three models (green pigment, P186M, and P205I) were then selected for further analysis. Ten energetically favored models of each WT and the two mutants were aligned with PyMOL (Schrodinger, 2015). Root-mean-square deviation (RMSD) analysis between these aligned structures was carried out with the VMD RMSD Visualizer Tool heatmapper extension (Humphrey et al., 1996).
### The Pro-Pro motif in human opsins.

Sequence alignment of the M/LWS construct, human long wavelength sensitive opsin (L/LWS), bovine rhodopsin, and human short wave sensitive opsin (SWS1). Conserved Pro residues are highlighted in green, the conserved Ala residues in cyan, and the GWSRY domain in yellow.

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<tr>
<td>rhodopsin</td>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>SWS1</td>
<td>----------------------------------------------------------</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M/LWS_construct</th>
<th>FVFGRTWVGLTGYSTVGSLAFSTFVFGYACFGVFGFTTVYKSESTVWIFVMQLC</th>
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<tr>
<td>L/LWS</td>
<td>FVFGRTWVGLTGYSTVGSLAFSTFVFGYACFGVFGFTTVYKSESTVWIFVMQLC</td>
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<tr>
<td>rhodopsin</td>
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<td>SWS1</td>
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<tr>
<th>M/LWS_construct</th>
<th>VFPLMAALPAYFAKSATIYNPVIYIMMNKQFRNCMTVEKCGA-MTDESOTCSQKTEV</th>
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<tbody>
<tr>
<td>L/LWS</td>
<td>VFPLMAALPAYFAKSATIYNPVIYIMMNKQFRNCMTVEKCGA-MTDESOTCSQKTEV</td>
</tr>
<tr>
<td>rhodopsin</td>
<td>----------------------------------------------------------</td>
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<tr>
<td>SWS1</td>
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<tr>
<th>M/LWS_construct</th>
<th>YLVQLWLAIRAVAKQKQKESSTQKAEEKVTRMVMVMLAFCFCCNGPYPFACFAAANPYP</th>
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<tbody>
<tr>
<td>L/LWS</td>
<td>YLVQLWLAIRAVAKQKQKESSTQKAEEKVTRMVMVMLAFCFCCNGPYPFACFAAANPYP</td>
</tr>
<tr>
<td>rhodopsin</td>
<td>-----------------------------------------------------------</td>
</tr>
<tr>
<td>SWS1</td>
<td>-----------------------------------------------------------</td>
</tr>
</tbody>
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<thead>
<tr>
<th>M/LWS_construct</th>
<th>FPHLMALPFAFFAKTAYVYFVMRFRCNQLGKF-GVDSGELSSASSTKETE</th>
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</thead>
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<tr>
<td>L/LWS</td>
<td>FPHLMALPFAFFAKTAYVYFVMRFRCNQLGKF-GVDSGELSSASSTKETE</td>
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<tr>
<td>rhodopsin</td>
<td>----------------------------------------------------</td>
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<tr>
<td>SWS1</td>
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<tr>
<th>M/LWS_construct</th>
<th>----SQVAPA</th>
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<td>SSV--SSVPA</td>
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<tr>
<td>rhodopsin</td>
<td>----SQVAPA</td>
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<tr>
<td>SWS1</td>
<td>STVSTQVGPN</td>
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Excerpt of the sequence alignment containing the Pro-Pro motif. Alignment between the Human M/LWS receptor, Human long wavelength sensitive opsin (L/LWS), Bovine rhodopsin, Human short wave sensitive opsin (SWS1), Human opsin-5 (Opn5), Danio rerio red sensitive opsin 1 (lw1_dr), Danio rerio short wavelength sensitive opsin 2 (SWS2_dr), Danio rerio medium wavelength sensitive opsin (MW1_dr), Todarodes pacificus rhodopsin (rhodopsin_squid), and Drosophila melanogaster opsins 1-6 (rh1-6_dm). The conserved Pro residues are highlighted in green, the conserved Ala residues in cyan, and the GWSRY domain in yellow.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Alignment</th>
<th>Conserved Pro</th>
<th>Conserved Ala</th>
<th>GWSRY Domain</th>
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<tr>
<td>LW1_dr</td>
<td>AKWASAGIIFSWVWAASMCPPIFG-WSRYMPHGKTSICPDVFSGSDEPGVQSYMVVML</td>
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<tr>
<td>M/LWS</td>
<td>AKLAIVGIAFSWIWAAVTAPPIFG-WSRYMPHGKTSICPDVFSGSDEPGVQSYMVVML</td>
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<tr>
<td>L/LWS</td>
<td>AKLAIVGIAFSWIWAVTAPPIFG-WSRYMPHGKTSICPDVFSGSDEPGVQSYMVVML</td>
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</tr>
<tr>
<td>SWS1</td>
<td>SKHALTVLATWITIGVSVQFFG-WSRYMPHGKTSICPDVFSGSDEPGVQSYMVVML</td>
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<tr>
<td>SWS2_dr</td>
<td>TPHAIAGCIIWMMCAAGLWVLLK-WSRYMPHLCSCPDWYTTMKFMNFGMFM</td>
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<tr>
<td>MW1_dr</td>
<td>AHAMIAGAFTWIFACEMACVPPIFG-WSRYMPHLCSCPDWYTYTNDNRSFGFVFVF</td>
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<tr>
<td>Opn5</td>
<td>RKBAYICALAAANAYASFHHILH-LSWYVPEFPGTSTLCWLAUMSAGVQQVFLINIL</td>
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<tr>
<td>rhodopsin_squid</td>
<td>HHARAVIFVWILVWALWAAGI-LSWYVPEFPGTSTLCWLAUMSAGVQQVFLINIL</td>
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<tr>
<td>rh6_dm</td>
<td>TAAYGRLMVVTWIFACEMACVPPIFG-WSRYMPHLCSCPDWYTYTNDNRSFGFVFVF</td>
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<tr>
<td>rh1_dm</td>
<td>IRLALGKMAYFWMMSSWCAAGI-LSWYVPEFPGTSTLCWLAUMSAGVQQVFLINIL</td>
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<td>rh2_dm</td>
<td>IKTSAIMKIFWMMSSWCAAGI-LSWYVPEFPGTSTLCWLAUMSAGVQQVFLINIL</td>
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<tr>
<td>rh3_dm</td>
<td>HGGKIAMIMITYHAYFVCAKTVYTGFPYEGYLTSCFDVL-NDNFDTRLFVACIF</td>
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<tr>
<td>rh4_dm</td>
<td>HGGKIAMIMITYHAYFVCAKTVYTGFPYEGYLTSCFDVL-NDNFDTRLFVACIF</td>
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</table>
Quantitative RMSD analysis. The RMSD of ten energetically favored models was averaged and plotted by residue.

Figure 37: RMSD analysis by residue of green opsin and its P205I and P186M mutants. The RMSD of a residue is calculated relative to one structure in the ensemble of structures for a given opsin model. The RMSDs of a residue for all structures in an ensemble are then averaged. The average RMSD values are plotted against the amino acid residues 180 to 225. The average of the green cone pigment models is shown in blue, the mutant P205I and P186M are depicted in black and red, respectively. The highlighted area in red and black correspond to the red and black oval in Figure 29.

Sequence alignment of M/LWS opsin, and the 5-HT$_{1B}$, and 5-HT$_{2B}$ receptors.
These sequences were aligned with the MUSCLE server provided by EMBL-EBI. (Edgar, 2004a, b; Li et al., 2015b; McWilliam et al., 2013) MUSCLE stands for MUltiple Sequence Comparison by Log-Expectation. Conserved Pro residues are highlighted in green, the conserved Ala residues in cyan, and the GWSRY domain in yellow.
...
Non-overlapping local alignments of M/LWS opsin and 5-HT₁B protein sequences. LALIGN finds internal duplications by calculating non-intersecting local alignments of protein sequences (Li et al., 2015b; McWilliam et al., 2013). The Pro residues 186-187 and 183-184, corresponding to the M/LWS opsin and 5HT₁B receptor respectively, are highlighted in green.

Figure 38: Non-overlapping local alignments of the green pigment and 5-HT₁B protein sequences. The amino acid residues of interest, P186 and P187, are found in the local alignment of green opsin 55-191 aa and 5-HT₁B 51-187 aa, highlighted in orange. The similarity score is 65.2% and the sequence shares 26.1% identity with an E(1) < 7.7e-13.
References


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Software, S., SigmaPlot, 11.0 ed. Systat Software, Inc, San Jose, CA.


