Shedding Light on the Photoactivation of Visual Pigments

A Study Using FTIR Spectroscopy

Frank de Lange
Shedding Light on the Photoactivation of Visual Pigments

A Study Using FTIR Spectroscopy

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Frank de Lange

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About the cover: Rain over the Atlantic Ocean, Funchal, Madeira, Portugal. Inset: Salamander rod and cone photoreceptor cells. Immunolabeling (double labeling) with 4D2 (anti-rhodopsin N-terminal, red) and CERN956 (anti-L-cone opsin, green). The rod outer segments appear red, while the conical outer segments of the L-cones appear green. Height of the rod outer segments is approximately 25 micron. Courtesy of Dr. D.M. Sherry, College of Optometry, University of Houston, Houston TX, USA.

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Chapter 1

Introduction

1.1 General Introduction

In need of accurate information about our immediate environment, five senses evolved, of which sight developed to be the one with the highest spatial and temporal resolution. The fascinating complexity of the mechanism underlying visual perception has been the subject of biochemical, biophysical and neurological research for many years.

Images projected onto the retina are processed into a nerve signal in the rod and cone photoreceptor cells. In these cells, the photoactivation of light-sensitive proteins, called visual pigments, triggers a cascade of events using electro-magnetic energy to modulate electro-chemical gradients. Obviously, to allow for the rapid succession of images, as well as the detection of visual stimuli showing an over $10^8$-fold variation in illumination intensity, the intracellular signal transduction mechanism had to meet stringent requirements regarding gain, dynamic range, noise-level and response rate.

Signal transduction mediated by visual pigments has been extensively studied during the last 40 years and has evolved into a model system for signaling mediated by G-protein coupled receptors (GPCRs). Thus, the visual pigments are the prototypical members of a family of receptors, which currently contains over 500 sequences, including receptors for neurotransmitters, sensory stimuli and hormones, as a few of the numerous examples (1).

Although a wealth of knowledge has accumulated on the excitation and desensitization pathways of these important signaling mechanisms, much less is known about the molecular details of signal generation by the respective receptor molecules. Knowledge of their structures will provide important clues regarding their function and may provide a starting point for rational drug design.

Structure-function relationships in the molecular mechanism of activation of visual pigments are the central theme of this thesis. A combination of UV/visible and infrared spectroscopic techniques is applied to study the various phases in receptor activation. In this way, information is obtained on essential questions regarding the remarkable efficiency
as well as the pathway of intra-molecular signal expression in the visual pigments. The following sections aim at providing a nutshell overview of, firstly, the present knowledge on the primary processes in vision and, secondly, the experimental techniques used to obtain this information. Finally, the aims of this investigation and an outline of this thesis will be presented.

### 1.2 The molecular mechanism of vision

#### 1.2.1 Rod and cone photoreceptor cells

The human retina contains approximately 120 million rod and 6 million cone photoreceptor cells, named after their morphology. The fovea, or yellow spot, is located around the optic axis of the eye and contains the vast majority of cones but no rods. Bright-light (photopic) vision is provided by the cones, offering the highest spatial and temporal resolution as well as color discrimination. Color vision is mediated by three types of cone photoreceptor cells, each containing a distinct visual pigment absorbing maximally at 420, 530 and 560 nm, respectively. This has been verified experimentally by psychophysical methods (2), microspectrophotometry of individual human cones (3, 4), electroretinography (5), and by functional expression of the genes encoding the pigments (6, 7). In rods only one type of pigment, rhodopsin [498 nm, (8)], is expressed. Therefore, rods cannot discriminate between different colors. On the other hand, rods are far more light-sensitive than cones and may be used under dim-light (scotopic) conditions, where foveal vision cannot function.

A schematic representation of a rod cell is given in Figure 1.1 (9). Photoreceptor cells, in general, are divided into three domains: the outer- and inner segment and a synaptic terminal. The inner segment contains the nucleus, mitochondria and the other organelles which take part in the cell’s metabolism. The synaptic region is connected via bipolar cells to the ganglion cells. The characteristically shaped outer segment, juxtapositioned to the microvilli of the retinal pigment epithelium, contains the components dedicated to light-capture. The rod outer segment is packed with numerous flattened membrane discs which are very rich in rhodopsin. Cone outer segments, on the other hand, are generally shorter than those of the rods, and have infoldings in their plasma membrane, called sacs, containing the photoreceptor proteins. On a daily basis, new discs and sacs are formed in the ciliary region of the photoreceptor cells. The reason for the striking difference in outer segment morphology is still unclear. However, despite their morphological and functional differences, the general mechanism underlying the response to light in both cell types is believed to be similar (10–12).
1.2 The molecular mechanism of vision

Figure 1.1: Schematic drawing of a rod photoreceptor cell. Rhodopsin, consisting of the apoprotein opsin and 11-cis-retinal is shown on an expanded scale on the right hand side [adapted from (9)].

1.2.2 Phototransduction in rods and cones

The photoreceptor membrane

The photoreceptor membranes probably represent the most highly specialized biological membrane system. It essentially is a two-phase system in which the visual pigments comprise over 95% of total membrane protein content. Visual pigments are integral membrane proteins of ca. 40 kD molecular weight, called opsins, and contain a light sensitive ligand: 11-cis-retinal, an aldehyde derivative of vitamin A. The protein moiety is folded into seven largely alpha-helical transmembrane segments, forming a well defined binding pocket for the chromophore (Figure 1.1). The retinylidene ligand is covalently linked to a
lysine residue in the protein through a protonated Schiff base linkage (8). The UV/visible absorbance spectrum of detergent solubilized bovine rhodopsin is shown in Figure 1.2. Recent studies indicate that in cone opsins only a few amino acid replacements, involving

![Diagram of rhodopsin photocascade](image)

Figure 1.2: (a), The photocascade of rhodopsin. Time constants are given for room temperature; absorbance maxima (visible region) of the photointermediate conformations are indicated in parentheses. The cascade can be arrested at a specific intermediate by lowering the temperature to a specific range (see e.g. Figure 1.5). The active, G-protein binding Meta II intermediate (R*) is formed within milliseconds after illumination under physiological conditions. Meta II absorbs maximally at 380 nm; this is due to the deprotonation of the Schiff base linkage between all-trans-retinal and opsin during its formation. (b) Absorbance spectrum of rhodopsin in micellar solution. This spectrum consists primarily of three bands: the $\alpha$ band at 498 nm (chromophore), the $\beta$ or cis- band near 350 nm (chromophore), and the $\gamma$ band near 280 nm (protein).

hydroxyl bearing residues in the vicinity of the chromophore, are sufficient to modulate the electronic transition energy of the chromophore, and consequently, the spectral properties of the pigments (13–15). The mechanism of wavelength regulation in the visual pigments will be discussed in more detail in section 1.3.2 and in chapter 6.
1.2 The molecular mechanism of vision

In bovine rods, the main components of the lipid matrix of the disk membrane are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS), in a ratio of about 45/40/10 mole-percent of total lipid content (16, 17). The acyl chains were found to be mainly 22 in length, and polyunsaturated to a high percentage, 22:6ω3 being the predominant species (17). The role of this unusual composition is still largely unclear. Although both the phospholipid head-group as well as the acyl chain composition have been shown to influence the photoactivation of rhodopsin, there is good evidence that overall membrane properties, rather than specific chemical properties of the lipids, are involved in modulating pigment activation. More specifically, it has been shown that rhodopsin activation is promoted by the presence of lipids close to a $L_α$–$H_{II}$ phase boundary, suggesting that the membrane lipid bilayer has a direct influence on the energetics of the activation process [for a recent review see (18)].

Intracellular signaling in rods

Phototransduction is triggered by the photoinduced isomerization of the chromophore to the all-trans conformation. Under physiological conditions, the protein moiety very rapidly responds to the altered conformation of the ligand and adopts its active conformation, metarhodopsin II or $R^*$ within 1 ms (Figure 1.2a) [(8, 19, 20) and section 1.3.2]. In this state, rhodopsin is able to bind and activate the G-protein transducin (21, 22). The latter is a membrane associated hetero-trimer ($T\alpha$, 40 kD; $T\beta$, 38 kD; $T\gamma$, 8 kD). The N-terminal region of both the $T\alpha$ and $T\gamma$ subunit carry lipid modifications, supposedly functioning as sites of membrane attachment. Binding of transducin to metarhodopsin II is the result of a collisional coupling process, and is favored by the rapid lateral diffusion of rhodopsin through the membrane (23). Activation occurs through receptor-catalyzed GDP-GTP exchange in the $T\alpha$-subunit, eventually leading to the dissociation of the $T\alpha$-GTP subunit from $T\beta\gamma$. Then, $T\alpha$-GTP diffuses into the cytosol where it activates a cGMP phosphodiesterase, PDE, which subsequently catalyzes the hydrolysis of cGMP to GMP. The drop in cGMP effectuates the closure of cGMP-gated cation channels in the plasma membrane of the outer segment. This results in hyper-polarization of the plasma membrane and subsequent down-regulation of the release of neurotransmitter at the synaptic cleft. In this way, the light stimulus is finally transmitted to the adjacent neurons. [For recent reviews, see refs. (24, 25)].

The gain of the phototransduction process is very impressive: one photon, activating a single rhodopsin, can lead to the activation of in the order of $10^3$ transducin molecules, causing a decrease of up to $10^6$ molecules of cGMP, enough to produce a detectable change in the cGMP gated current within 0.5 sec (26). Of course, to obtain temporal resolution, efficient desensitization is equally important. However, the lifetime of the metarhodopsin II intermediate [which decays to opsin and all-trans retinal (Figure 1.2a)], as well as that of the $T\alpha$-GTP subunit (limited by its internal GTP-ase activity) both lie in the order of minutes at physiological temperature. These processes are obviously too slow, and parallel pathways have evolved. Metarhodopsin II becomes phosphorylated by rhodopsin kinase on multiple serine and threonine residues in the C-terminal region (27–29). Subsequently,
arrestin binds to the phosphorylated receptor and effectively inhibits further activation of transducin (30). This process is complete within one second. In addition, GTP-ase accelerating proteins may play a role in the targeted inactivation of the Tα-GTP complex [see e.g. (25)].

For the detection of another photon by the same photoreceptor protein, it needs to be released from arrestin, to become de-phosphorylated and to be regenerated with 11-cis-retinal. For this purpose, the all-trans retinal moiety is reduced to all-trans retinol, and subsequently transported to the retinal pigment epithelium (RPE) by a retinol binding protein. In the RPE, the retinol is esterified to a fatty acid, enzymatically isomerized to 11-cis-retinol, and subsequently oxidized to 11-cis-retinal, by an oxidoreductase. Next, the retinal is transported back to the photoreceptor cell and rebinds to opsin to form rhodopsin (31). The process of regeneration is rather slow, taking approximately half an hour to regain the full complement of dark adapted pigment [(32), Figure 1.2a].

To extend the range of light intensities over which they function, photoreceptor cells have to be able to i) quickly recover after intense bleaches, ii) adapt to background illumination, and iii) to show a very low dark-activity. Under scotopic (dim-light) conditions, the photodetection limit is determined by the dark-activity, or dark-noise, of the rod cell. The electrical signals corresponding to this dark-noise, as obtained from electro-physiological experiments, cannot be distinguished from light induced cellular responses and occur at a rate of about 0.02 events per cell per second. Recently, dark-noise has been mechanismically linked to the thermal activation of individual rhodopsin molecules (33). Thus, the noise can be related to the rhodopsin present in the rod cell and amounts to $10^{-11}$ events per rhodopsin per second. It was suggested that this extremely low noise rate is due to the unusually high $pK_a$ of over 16 of the Schiff base (34), inhibiting thermal excitation. While dark-noise has been related to intramolecular properties of rhodopsin, complex dynamical processes within the cell are responsible for the adaptation and recovery characteristics of the cell in bright light. In this respect, calcium was found to play a prominent role.

Photoactivation also effectuates a drop in intracellular calcium. This is due to the light induced closure of the cGMP gated cation channels, which reduces the influx of Ca$^{2+}$, while the efflux through the cell’s Na$^+$/Ca$^{2+}$-K$^+$ exchanger continues. In the process of background adaptation, three negative feedback mechanisms have so far been identified. First, the decrease in intracellular calcium increases the activity of a retinal specific guanylate cyclase [reviewed in (35)], an enzyme which catalyzes the formation of cGMP from GTP. Thus, the light induced hydrolysis of cGMP is counteracted. Second, the drop in free calcium increases the phosphorylation rate of metarhodopsin II, through the action of a protein called recoverin. Third, it has been shown that calcium mediates the apparent affinity for cGMP of the cGMP gated channels, so that when the calcium concentration drops, some of the channels reopen despite the lower cGMP-level. Indeed, the above mentioned feedback mechanisms both speed up the recovery of the cell’s reaction to intense stimuli and enable the cell to adapt to background illumination. See for recent reviews refs. (36, 37).

The discovery of new players in phototransduction is an ongoing process (38–40). So
far, several of the actors have been identified to play a role in various forms of night blindness (arrestin, rhodopsin kinase, Tα), and retinopathies (guanylate cyclase). Other hereditary malfunctions of the visual system, like color-blindness (41, 42) and Retinitis Pigmentosa (43, 44), were found to be linked to the visual pigments themselves.

The vast majority of data regarding phototransduction stems from experiments on rods. Although phototransduction in cones is accepted to be based on a similar mechanism, the difference in photoresponse between the two cell types has been related to a more efficient inactivation mechanism in cones (45).

1.3 Structure and function in rhodopsin

1.3.1 Towards a structural model

The fact that visual pigments are expressed in very large quantities in an easily isolated cell-organelle facilitated numerous biochemical and biophysical studies leading to the structural model discussed above for the topography of rhodopsin. The macroscopic anisotropy of the rod outer segment was recognized already in the mid nineteen thirties. Using a polarizing microscope, it was observed that rod outer segments appear pink in transversely polarized white light, while they appear colorless in axially polarized light [reviewed in (46)]. Later, linear dichroism studies indeed showed that the electronic transition moment of rhodopsin’s chromophore was oriented preferentially perpendicular to the long axis of the rod cell (47). X-ray diffraction studies on membrane bound rhodopsin indicated that it is a monomeric transmembrane protein (48). From UV-circular dichroism (49) and infrared studies (50) it was concluded that over 55% of rhodopsin’s secondary structure is α-helical. Infrared linear dichroism studies on magnetically oriented rod outer segments in D2O indicated that these helical structures were axially oriented, and that they form the hydrophobic core of the protein since they were found to be insensitive to deuterium exchange (51). Clearly, their exact orientation in the membrane make the pigments a highly efficient target for the light passing through the disc stack.

The primary sequence of bovine rod ops in was published in the early eighties (52, 53). Sequence analysis indicated that the 348 amino acid sequence contains alternate hydrophobic and hydrophilic stretches suggesting the presence of seven transmembrane domains (54). Accessibility studies using immunochemical, proteolytic, and labeling techniques (55, 56), together with the biophysical data discussed above, finally culminated in the proposal of the familiar seven-transmembrane topography (Figure 1.3). Post-translational modifications include the glycosylation of Asn2 and Asn15 (57), thio-palmitoylation of Cys322 and Cys323 (58), and a disulfide-bridge between Cys110 and Cys187 (59). The transducin interaction sites have been inferred from a variety of experimental data [reviewed in (24)]. Only when the first projection structure became available in the early nineties, based on electron cryo-microscopy data on 2-D crystals of rhodopsin (60), the proposed seven-transmembrane topography could be directly confirmed.

Only recently, the arrangement of the transmembrane segments in (frog-) rhodopsin
could be clearly distinguished in a new low-resolution density map with an effective resolution of 7.5 Å in the membrane plane and 16.5 Å normal to it (61). It was found that the arrangement of the helices at the intracellular side is significantly more compact than it is on the extracellular side, where a possible retinal binding site was observed between helix 3, 4, 5, 6 and 7. Based on sequence analysis of over 500 receptors in the rhodopsin family of G-protein coupled receptors, and guided by the new rhodopsin structure, an alpha-carbon template was proposed for the arrangement of the seven helices of these receptors [(1), see Figure 1.4]. The retinal chromophore in rhodopsin lies at an angle of about 16° to the plane of the membrane (47); the estimated $z$ coordinate of the Schiff base consistent with this model would be about -8.5 Å, that of the center of the ionone ring portion about -5 Å (1). The residues predicted to outline the retinal binding pocket in rhodopsin (section 1.3.2) were indeed observed to modulate ligand-protein interactions in a variety
Figure 1.4: Stereo-views of the GPCR model’s alpha-carbon positions [adapted from (1)].
(a), View from the intracellular side of the membrane; (b), from within the membrane looking from helix 5; (c) from within the membrane looking from helices 2 and 4. In (b) and (c), the intracellular side of the molecule is at the top of the Figure. The levels $-12\ \text{Å}$ and $+12\ \text{Å}$ from the center of the lipid bilayer are indicated.

To date, bacteriorhodopsin, the light-driven proton-translocating pump in the plasma-membrane of *Halobacterium salinarium*, is the only seven helix transmembrane protein for...
which the structure is known from X-ray crystallography to significant (2.5 Å) resolution (62). However, to our knowledge, 3-D crystals suitable for X-ray diffraction analyses have not yet been produced for any GPCR. Solution-state NMR has been successfully applied to polypeptide models of the cytoplasmic loops and the C-terminal domain, and based on these data a three dimensional structure of the cytoplasmic face of rhodopsin was proposed (63). For transducin, a 2 Å crystal structure has already been reported (64).

In order to understand visual transduction at a mechanistic level, more detailed structural as well as dynamical information on visual pigments is a prerequisite. It will be clear that a high resolution structure per se is not sufficient to resolve questions relating structure and function in the photoactivation process. To date, EPR, NMR, UV/visible and vibrational spectroscopic studies slowly start to unravel some of the intriguing features of rhodopsin activation.

### 1.3.2 A spectroscopic model of rhodopsin

**Photoactivation of rhodopsin**

Photoactivation of rhodopsin leads to the exposure of signaling sites for transducin at the metarhodopsin II stage. However, this process is not a single conformational rearrangement, but rather proceeds through a series of spectrally and structurally distinct intermediates (photocascade: rhodopsin ➔ bathorhodopsin ➔ lumirhodopsin ➔ metarhodopsin I ➔ metarhodopsin II) of which only the first transition is light-dependent (see Figure 1.2a). The subsequent transitions are dark-reactions in which the photon energy, stored in the all-trans retinylidene chromophore, is transferred to the protein. These intermediate conformations have been identified by low temperature steady state, and time-resolved spectroscopic techniques. Obviously, the presence of such intermediate states offers unique access to study the molecular mechanism of this activation process.

UV/visible time resolved spectroscopic studies indicated the existence of additional photointermediates, not observed by low-temperature trapping, suggesting the following scheme: rhodopsin ➔ bathorhodopsin ↔ blue-shifted-intermediate ➔ lumirhodopsin (20). Moreover, additional isochromic (380 nm) intermediate states were detected in the decay of lumirhodopsin to metarhodopsin II (65, 66). It has further been suggested that there may be two different forms of metarhodopsin II, which must be successively formed to allow binding of, as well as catalysis of guanine nucleotide exchange in transducin (67).

The metarhodopsin II intermediate is in equilibrium with its precursor metarhodopsin I. The transition between metarhodopsin I and II is characterized by the deprotonation of the Schiff base linkage, net proton uptake from the aqueous phase, and has been related to a transient change in membrane potential. A prerequisite for the formation of the active intermediate is the presence of water. To date, the equilibrium between metarhodopsin I and II is known to be sensitive to pH, temperature, pressure, ionic strength, and the composition of the hydrophobic environment of the protein [see for recent reviews (68, 69)]. It is the first step in the photocascade showing considerable dependence on the microenvironment, consistent with the idea that the largest structural change in the protein moi-
ety takes place during this transition. On a much longer time-scale, metarhodopsin II decays to metarhodopsin III, or opsin and free retinal (Figure 1.2a). The proposed function of the metarhodopsin III intermediate is still a matter of debate. The equilibrium between metarhodopsin I and II will be discussed in more detail in chapter 5.

To date, the most detailed structural information on rhodopsin has been obtained by studying the photocascade using vibrational spectroscopy (section 1.4). FTIR difference spectroscopy of the various transitions proved to be especially powerful since it simultaneously monitors light-induced structural changes in the chromophore and the protein, which are accompanied by changes in vibrational frequency, band width or band intensity [see Figure 1.5 and refs. (70–72)]. Isotope labeling of the chromophore and site directed mutagenesis facilitated the identification of some of the difference bands in these spectra. In this way, it was shown that the majority of peaks in the rhodopsin to bathorhodopsin difference spectra derives from the chromophore, while the subsequent transitions present a gradual increase in protein activity (73–75). This supports the concept that discrete conformational transitions in the protein propagate and culminate at the metarhodopsin II stage. Time resolved studies FTIR studies indicated that the structural changes in the protein moiety are partially reversed during the decay of metarhodopsin II (76, 77). Importantly, the rhodopsin to bathorhodopsin difference spectrum already presented unequivocal evidence that, at this stage, the 11-cis / all-trans isomerization has been completed and that the all-trans chromophore is in a highly strained conformation, providing a highly efficient means of photon energy storage (see below).

**The transmembrane region**

**Wavelength sensitivity.** An important factor in the regulation of the wavelength sensitivity is the protonation state of the retinylidene-opsin Schiff base linkage. Its protonation has been suggested to stabilize the bond against hydrolysis and against thermal isomerization, which, as noted previously, may strongly increase dark-noise. More importantly, however, the resulting electronic charge distribution can be relatively easily modulated by the protein environment, allowing the wavelength of maximal sensitivity of the pigment to shift away from that of the protonated retinylidene Schiff base in model compounds (ca. 440 nm) all over the 360–600 nm range. This provides the basic requirement of color vision.

UV/visible studies indicated that the binding pocket is electrically neutral (78). Consequently, the positively charged Schiff base region has to be balanced by a negative counterion. In bovine rhodopsin, the most likely candidates were D83, E113, E122 and E134. Site directed mutagenesis studies identified E113, highly conserved among the visual pigments, to be the counterion (79–81). This conclusion was based primarily on observations that mutant E113Q displayed a dramatically lowered Schiff base pKₐ value of around 6, compared to that in rhodopsin of over 16 (34). This suggests that the Schiff base protonation state is in fact stabilized by the interaction with its counterion. The location and orientation of the ionized side-chain of E113 with respect to the Schiff base has recently been addressed using solid state NMR (82).

Interestingly, with the introduction of a negative charge at position 90 in helix 2 [mu-
Figure 1.5: FTIR difference spectra of the various transitions in the rhodopsin photocascade (e.g. Figure 1.2). The spectra were taken at the indicated temperature to arrest the cascade at that specific intermediate and calculated by subtracting the Rho spectrum from the photoproduct spectrum. Hence, negative bands represent vibrational bands originally present in Rho; positive peaks represent bands newly arising in the photointermediate.

In the biogenic amine receptors, a negative charge (Asp) is conserved at (equivalent) position 117, which is supposedly functioning as counterion to the positively charged nitrogen in the ligands of these receptors. In the case of rhodopsin, the above mentioned counterion mutations were shown to affect both kinetic and structural aspects of the mutant G90D (83), see below}, or 117, just one turn above 113 [double mutant E113Q/A117D (84)], it could be shown that a functional counterion to the Schiff base could also be located at these positions. The latter finding is of special interest since it parallels the situation in the biogenic amine receptors. In these receptors, a negative charge (Asp) is conserved at (equivalent) position 117, which is supposedly functioning as counterion to the positively charged nitrogen in the ligands of these receptors. In the case of rhodopsin, the above mentioned counterion mutations were shown to affect both kinetic and structural aspects of the
1.3 Structure and function in rhodopsin

On the basis of resonance Raman data on (recombinant) pigments (15, 88), a wavelength regulation mechanism has been proposed to involve i) dipolar interactions between the retinal, bound water molecules, and hydroxyl bearing residues lining the binding site, and ii) the hydrogen bonding strength of the Schiff base proton to, most likely, a bound water molecule (see also chapter 6). Mutagenesis studies revealed that the introduction of hydroxyl group bearing residues at equivalent positions (A)292 in helix 7, (F)261 and (A)269 in helix 6, and (A)164 in helix 4 had the predominant effect on spectral tuning, and suggested that these residues are responsible for tuning the wavelength of maximal sensitivity from the blue to the red [reviewed in (14)].

The primary photochemical event and photon energy storage. Femtosecond time-resolved transient visible absorption spectroscopy demonstrated that the formation of the bathorhodopsin intermediate is complete in only 200 fs (89). Photocalorimetric studies revealed that about 145 kJ/mol is stored in the batho conformation, which is approximately 60% of the incoming photon energy (90). Quantitative bleaching experiments indicated that the quantum yield of the process is 0.67 (91), i.e. two out of three photons absorbed induce an isomerization. In contrast to what may be, naively, expected upon binding the retinal to the protein moiety, its photochemistry becomes more efficient. Indeed, in methanol, 11-cis-retinal protonated Schiff base models show a photoproduct formation time of somewhat less than 8 ps (40 times slower!), with a quantum yield of 0.15 (92). These data raise important questions regarding the mechanism behind the ultrafast photoisomerization process in rhodopsin.

An important clue comes from visible circular dichroism studies. In methanol, retinal does not display visible circular dichroism, whereas a significant CD signal is observed after binding to opsin (49), indicating that the protein accommodates only one chromophore conformer. Indeed, the conformational arrangement of the retinal polyene chain has recently been uniquely specified from CD spectroscopy (93). Thus, the picture emerges that by 'selective solvation', opsin gets the most out of its ligand. Of course, such a mechanism requires well defined chromophore-protein interactions.

Several models have been proposed to explain the mechanism behind the ultrafast photoisomerization process, and photon energy storage in the batho photoproduct. Several lines of evidence indicate that a strained conformation of the chromophore in the dark state of the receptor is one rate-determining factor; however, the exact mechanism is still a matter of debate (92, 94). For the mechanism of energy storage in the batho intermediate basically two mechanisms were proposed, first, charge separation between the Schiff base and its counterion (95), and second, a storage mechanism involving torsional strain in the all-trans chromophore (96). Hybrid models were proposed as well (97). The latest evidence indicates that if charge separation contributes at all, it will be only of minor importance [(82), see also chapter 4].

Intramolecular signaling. Numerous retinal analog studies have addressed the role of chro-
mophore-protein interactions in the activation mechanism of rhodopsin. However, direct evidence for the interaction between the retinal and a specific residue has only recently been reported for the 9-methyl group and G121 in helix 3 (98–100). Interestingly, some of the effects of introducing a residue with a bulky side chain at position 121 in the mutant G121L were found to be counteracted by reducing the size of the side chain at only one specific position in helix 6 [double mutant G121L/F261A, (101–103)]. Since these residues seem to be too far apart to allow direct contact, the interaction between these two residues was supposed to be relayed by the retinal. In an earlier study using FTIR, it has been shown that the 9-methyl group probably is one of the crucial ‘contact-points’ between the retinal and opsin since removal of this group resulted in a pigment showing a completely disturbed photocascade (104).

Cross-linking studies indicated that W265 (helix 6) had to be close to C3 in the ionone ring portion [(105), see Figure 4.1 for numbering]. UV difference spectroscopy (106) and linear dichroism (107) studies indicated that W126 (helix 3) and W265 are structurally active and transiently adopt an altered orientation in the activation step of rhodopsin. These movements were correlated to that of the retinal during the formation and decay of the metarhodopsin II intermediate (108). In addition, it was found that a rhodopsin analog in which the ionone ring was replaced by two ethyl groups did not generate metarhodopsin II (109). Together, these data suggest that the reorientation of these Trp residues, triggered by the retinal ring portion, participates in or directs the formation of the active intermediate.

These, and related observations fit the “steric trigger model” of rhodopsin activation [see refs. (69, 110) and chapter 4]. A model of the retinal binding site, in general agreement with the above data, has recently been proposed by Smith and coworkers (110). Unfortunately, models of different origin still differ considerably [compare e.g. refs. (110) and (111)] and for the time being reliable high-resolution structural information cannot be extracted from such structures.

A wealth of site directed mutagenesis data has become available over the past years. Several mutations were shown to affect the photocascade, and in some cases this approach led to the identification of specific FTIR difference bands (chapter 2). Several of the reported point mutations also lead to altered biosynthesis, membrane translocation and glycosylation of opsin [reviewed in (112)]. Not unexpectedly, amino acid replacements involving highly conserved residues often show mosaic effects on rhodopsin biosynthesis, structure and function (113). Clearly, these effects have to be taken into account when highly sensitive techniques like FTIR are employed.

Of special interest are the point mutations causing constitutive activity of the receptor, i.e. significant activity of the apo-protein in the absence of both ligand and light. So far, three naturally occurring mutations were identified involving K296, causing Retinitis Pigmentosa (a disease leading to severe retina degeneration), and G90 and A292, related to congenital night blindness. Mutations of E113 also induce constitutive activity. These data led to the proposal of the “salt bridge model” in which a salt bridge between K296 and E113, in helix seven and three, respectively, was considered to be essential to
keep rhodopsin in its inactive conformation. This subject has been recently reviewed in (114). Interestingly, in mutant G90D and A292E, D90 (helix 2) and E292 (helix 7) were found to compete with E113 for electrostatic interactions with the protonated Schiff base. FTIR studies on mutant G90D suggest that E113 is neutral, and indicate significantly altered chromophore protein interactions in this pigment (85, 86). Regarding the salt bridge concept, it should be noted that a negative charge at position 134 (E134) also seems to be essential for keeping opsin in its inactive conformation (115). Furthermore, constitutive activation has recently also been reported for M257 mutants (116). Hence, the salt bridge concept as such probably is too simplistic to account for the low basal activity of opsin.

**The intracellular surface**

As noted before, rhodopsin’s signaling sites, the interface of rhodopsin with transducin, presumably comprise the cytoplasmic i2, i3 and i4 loop (Figure 1.3). Direct evidence for light induced conformational rearrangements in the cytoplasmic domain was obtained from recent spin labeling EPR studies. For this, site specific spin labeling techniques were employed to introduce spin labels into the cytoplasmic boundaries of helix 3 through 7 and the i2 and i3 loop. Changes in EPR line-width upon photoactivation of labeled rhodopsin were interpreted to indicate structural changes in the inter-helical loops (117, 118), and rigid body movement of helix 3 and 6, relative to the other transmembrane helices (119). Interestingly, in the dark state of mutant E134Q, showing considerable constitutive activity, these movements seem to be decoupled (120). In the dark, this mutant shows only the characteristics of the activated surface in the boundaries of helix 3 and 7, while the EPR signals indicate a normal dark state structure of helix 6. This observation suggests that subdomains in the intracellular surface of rhodopsin can be activated independently, either by photoinduced steric interactions or by neutralization of E134.

Most G-protein coupled receptors show the E/DR pair at the cytoplasmic border of helix 3 (E134 and R135 in bovine rhodopsin), implying a role for this site in a more general scheme of signal transduction. In rhodopsin, reversal of this charged pair hampers binding and activating of transducin. In fact, all R135 mutants have impaired ability to bind and activate transducin, while the effects of E134 substitutions depend on whether a neutral or negatively charged group is introduced. Mutant E134D does not show constitutive activity, but shows a 50 % reduction in transducin activating ability. Mutant E134Q is constitutively active, which is suppressed upon binding of 11-cis-retinal, and shows a 50 % increase in ability to activate transducin (24). Furthermore, in this mutant, proton uptake at the metarhodopsin II stage is abolished, suggesting that E134 is one of the proton acceptor sites (121). Thus, together with the EPR data discussed above, neutralization of the E134 site seems a determining factor in completing the light induced cytoplasmic rearrangements necessary to bind and activate transducin. In fact, from the above arguments, modulation of the cytoplasmic surface charge, mediated by protonating E134, may be relevant in a general mechanism of G-protein activation. While FTIR difference spectroscopy, in combination with site-directed mutagenesis, has been successfully employed to study the protonation states of the membrane embedded carboxyl group bearing residues
in bovine rhodopsin (D83, E113, E122, D90 in mutant G90D), the protonation of E134 could not be confirmed (112, 122).

1.4 Vibrational spectroscopy

1.4.1 Background

Transitions between vibrational levels of the electronic ground state of a molecule are much lower in energy than those between the electronic levels. While the latter are responsible for the UV/visible characteristics of a molecule, the former give rise to infrared absorbance. The absorption bands for these transitions generally lie in the range from 2.000 nm (5.000 cm\(^{-1}\)) to 50.000 nm (200 cm\(^{-1}\)). The principles that underlie infrared absorbance are essentially similar to those underlying UV/visible spectroscopy. Infrared spectroscopy is considered to be complementary to Raman spectroscopy, which also senses molecular vibrations albeit in a fundamentally different way (see below). Vibrational band frequencies, in general, depend strongly on the molecular conformation and hence provide a molecular fingerprint. Since the vibrational transitions are very fast, i.e. in the order of \(10^{-14}\)s, most biologically relevant processes can, in principle, be time-resolved using these techniques (123).

Raman and infrared vibrational spectra are based on fundamentally different phenomena, and are governed by different selection rules. In short, the vibrational Raman effect is an inelastic scattering process: light at a given frequency falling onto the sample is scattered, and the scattered light is of a different frequency. The frequency difference between the incoming and scattered radiation matches the energy corresponding to a ‘Raman active’ vibrational transition in the molecule. A vibration is said to be Raman active when it is accompanied by a change in molecular polarizability. Infrared spectroscopy, on the other hand, detects the absorbance of infrared radiation with a frequency that matches that of an oscillating permanent electric dipole moment, which accompanies an ‘infrared active’ molecular vibration. The rule of mutual exclusion states that in any molecule with a true center of symmetry, bands which are infrared active are Raman inactive and vice versa.

A classical example is set by the linear CO\(_2\) molecule: O=C=O. Simultaneous, symmetric stretching of both C=O bonds will cause the electro-negative oxygen atoms to move apart, therewith changing the polarizability of the molecule. This molecular vibration will thus cause Raman intensity. In contrast, as viewed from the carbon atom, this vibration is not accompanied by a net change in permanent dipole moment. Thus, the symmetric C=O stretch vibration is Raman active and infrared inactive. The reverse holds for the asymmetric stretch and the bending mode of the molecule, both of which are Raman inactive and infrared active.

It is common practice in dealing with molecular vibrations to regard a molecule in a simplified way as a set of masses held together by springs; this forms the basis of molecular mechanics. Applying a simple harmonic oscillator approximation to a chemical bond
between atoms A and B of mass $m_A$ and $m_B$, respectively, the vibration frequency is given by

$$\nu = \frac{1}{2\pi} \sqrt{\frac{k}{\mu}}$$

(1.1)

where $k$ is the Hooke’s law force constant, and $\mu$ is the reduced mass of the system: $\mu = m_A \cdot m_B / (m_A + m_B)$. The frequencies of these vibrations can be seen to be directly related to the atomic masses and molecular geometry (124). For example, characteristics like bond strength, length, and torsion angles will affect $k$. The sensitivity of a molecular vibration to the atomic mass forms the basis of the most widely exploited technique in assigning bands to a certain vibration, i.e. stable isotope labeling (with e.g. $^2$H, $^{13}$C, $^{15}$N, or $^{18}$O). Furthermore, since a spring bends more easily than it expands, this qualitative description immediately indicates, that bending modes are lower in energy and thus absorb at lower frequency than stretching modes.

In principle, and in fact feasible for small molecules, there are direct spectra-structure relations which can be obtained from combined vibrational analyses and molecular mechanics calculations [see for instance (125)]. For large biological molecules, attempts to this end have not yet been very successful. Nevertheless, vibrational analyses can provide highly detailed information on localized structures within these molecules. This will be discussed below.

Due to the inherent weakness of the Raman effect, this technique has mainly focussed on the chromophore upon application to visual pigments (chapter 4). Using laser resonance Raman spectroscopy, the signal of the retinal moiety can be immensely enhanced by using light at or near the main absorbance band of rhodopsin. Recently, UV resonance Raman spectroscopy was exploited, which enhances the signal of aromatic residues in the protein moiety (126). However, when it comes to study the light induced structural changes in the chromophore, protein and lipid phase simultaneously, FTIR spectroscopy is the method of choice.

### 1.4.2 FTIR as a tool in molecular biophysics

The introduction of the Fourier transform infrared spectrometer in the mid seventies revolutionized infrared spectroscopy. This 'technology push' increased the sensitivity of the IR instrumentation with three orders of magnitude compared to the previous generation of dispersive instruments (127). Since that time, 'IR’ has become one of the most versatile spectroscopic techniques for molecular level analyses.

A major advantage of infrared spectroscopy is that samples of practically any substance may be analyzed, without a need for labeling, in practically any morphological form. This is largely facilitated by the wide range of sampling methods available. For instance, the infrared absorbance of biological samples can be studied in solution using transmittance spectroscopy, and in a (single) crystalline or an amorphous solid state using
specular or diffuse reflectance (micro)spectroscopy (128). Data can be readily compared to that obtained in the other modes.

The very strong infrared absorbance bands of water, nature’s most popular solvent, has long been a problem in IR applications, since they obscure important bands in biological substances. To circumvent this problem, $^2\text{H}_2\text{O} (\text{D}_2\text{O})$, instead of $\text{H}_2\text{O}$, is often used. From the above qualitative description of molecular vibrations, the O–D modes are expected to be considerably down-shifted with respect to the O–H modes. It should be noted, however, that due to the fact that H/D exchange itself is very sensitive to pH and temperature, the analysis of kinetic parameters, like e.g. protein denaturation and enzymatic activity, is seriously complicated in D$_2$O solutions. On the other hand, the sensitivity of a protein to hydrogen-deuterium exchange itself and especially the exchange kinetics, can offer valuable insight in solvent accessibility and protein dynamics.

Biomembranes are preferentially studied in thin films, formed by drying an aqueous suspension onto a suitable infrared substrate, in either transmission or attenuated total reflectance (ATR) mode [see (129–132) and Figure 1.6)]. In ATR, the sample is dried onto an infrared transparent crystal, and the infrared light is directed into the crystal under conditions where total internal reflectance is obtained. At the surface of the crystal, the re-

![Figure 1.6: Experimental configuration of the attenuated total reflectance (ATR) sampling technique. Incident radiation reflects within the internal reflection element and partially penetrates the sample (gray area). The reflected beam therefore is attenuated by the absorption of the sample. Since the penetration depth is typically only a few microns this configuration allows for perfusion of the sample with buffer, an important advantage over transmission spectroscopy. When using plane polarized light, this technique can also be fruitfully applied to monitor sample orientations; this is indicated with (∥) and (⊥), see also chapter 3.](image-url)
1.4 Vibrational spectroscopy

A reflected beam partially penetrates into the surrounding medium, and is attenuated by the absorption of the sample. The penetration depth of the infrared beam into the surrounding medium is typically only a few micrometers. Thus, ATR is especially suited for the analysis of biomembranes since it allows perfusion of the sample, without introducing too large a disturbance caused by the strong water bands. Furthermore, using linearly polarized infrared light, ATR spectroscopy can be fruitfully applied to obtain information on the relative orientation of specific components in the membrane film under study (chapter 3).

Accessible information in the mid-infrared region

Big macromolecular structures are expected to exhibit a large number of infrared active vibrations. However, particular modes, for example C–H stretching modes in lipids, detergents, protein and sugars, show considerable overlap and absorb at so called characteristic group frequencies. Thus, most absorption bands can be found at more or less discrete positions (133), resulting in a limited set of bands (Table 1.1). On the other hand, the band posi-

<table>
<thead>
<tr>
<th>Frequency (cm$^{-1}$)</th>
<th>Assignment</th>
<th>Main source</th>
</tr>
</thead>
<tbody>
<tr>
<td>± 3600–3000</td>
<td>$\nu$ (OH)</td>
<td>water</td>
</tr>
<tr>
<td>± 3000–2800</td>
<td>$\nu_{as,s}$ (CH$_2$, CH$_3$)</td>
<td>lipids</td>
</tr>
<tr>
<td>± 1740</td>
<td>$\nu$ (C=O)</td>
<td>lipids (esters), overlap with Asp and Glu $\nu$ (COOH)</td>
</tr>
<tr>
<td>± 1650</td>
<td>HOH scissoring</td>
<td>water</td>
</tr>
<tr>
<td>± 1650</td>
<td>$\nu$ (C=O)</td>
<td>protein (amide I)</td>
</tr>
<tr>
<td>± 1540</td>
<td>$\delta$ (N–H)</td>
<td>protein (amide II)</td>
</tr>
<tr>
<td>± 1450</td>
<td>$\delta$ (C–H)</td>
<td>lipids</td>
</tr>
<tr>
<td>± 1240</td>
<td>$\nu_{as}$ (PO$_2^-$)</td>
<td>phospholipids, nucleic acids</td>
</tr>
<tr>
<td>± 1100</td>
<td>$\nu$ (C–OH)</td>
<td>carbohydrates</td>
</tr>
</tbody>
</table>

Table 1.1: Band positions of molecular groups commonly found in biological samples (e.g. see Figure 3.1) Stretching vibrations are denoted by $\nu$, bending modes by $\delta$. Subscripts (as, s) denote anti-symmetric and symmetric vibrations, respectively.

Tensions are highly sensitive to molecular structure, as well as to the micro-environment (solvent, pH, temperature, pressure). Of particular importance for the analysis of proteins are the amide I (1710–1590 cm$^{-1}$) and amide II (1590–1480 cm$^{-1}$) vibrations. These bands arise from the amide backbone vibrations of the polypeptide chain, and are sensitive to hydrogen bonding and hence the conformation (secondary structure) of the protein. Many of the modes in these regions have been correlated with specific secondary structure elements (134, 135), although the spectra-structure relationships are not fully water-tight. The C–H stretching modes of lipids have been found to be sensitive markers of the lipid acyl chain conformation (e.g. lipid phase transitions, membrane fluidity).
Of special interest, since it forms the basis of the most powerful applications of infrared spectroscopy, is the sensitivity of vibrational bands to hydrogen bonding. Hydrogen bonding causes a down-shift in frequency and a broadening of the stretching bands of both donor and acceptor.

Temperature induced spectral changes can often be directly related to specific sample components. This offers unique access to study protein (or nucleic acid) folding and denaturation, lipid phase transitions and lipid-protein interactions. The information obtained is complementary to that from differential scanning calorimetry; however, the obvious advantage of infrared spectrometry is that, besides transition temperatures, detailed structural information can be obtained simultaneously.

A complicating factor in the analysis of infrared spectra is that many of the bands show considerable overlap. Assignment of spectral components to specific secondary structure elements, for example, requires careful spectral decomposition of the amide I band [see e.g. (136, 137)]. For this, component bands can be identified using the second derivative of the band shape, or by applying deconvolution techniques. These results may then be used in curve fitting analyses of the band shape. Thus, the complex infrared band shape is described as the sum of a set of component bands, each representing a certain secondary structure element. The final fractional areas of the bands provide an estimate of the secondary structure of the protein. Alternatively, global analysis may be employed [see e.g. (138)]. However, these methods are not without flaws, and complementary spectral data, e.g. from UV and vibrational circular dichroism, vibrational linear dichroism, and H/D exchange studies may be needed to verify the assignments to specific secondary structure elements. For critical evaluations, see (139–141).

In the case of rhodopsin, FTIR spectroscopy has provided a predominant contribution to the present knowledge on its global structure. The position and shape of the amide I band, its sensitivity to deuterium exchange, and the infrared dichroism of both the amide I and amide II bands indicated the presence of deeply buried helical structures, oriented preferentially perpendicular to the membrane plane (section 1.3). Recently, this picture has been refined using limited proteolytic treatment (142). It was shown that the C-terminal domain, and the i3 loop, connecting helix 5 and 6, contain significant amounts of double-stranded β-sheet. Significantly, this assignment was recently supported by solution state NMR analyses of model peptides for this region (63), illustrative of the power of the infrared technique.

### Difference Spectroscopy

Since biochemically relevant processes often involve only small rearrangements occurring in the active site(s) within the protein, fine-tuning is needed to study structure-function relationships on this scale by FTIR spectroscopy. Structural rearrangements involving changes in local environment, orientation or protonation state will be accompanied by changes in vibrational band position, width or intensity. Since FTIR is sensitive enough to detect changes on a single bond level (ΔA/A of 10^{-4}–10^{-5} can be obtained after extensive signal averaging), visualization of these processes can, in principle, be achieved by calculating
infrared difference spectra. Although this type of analysis has been initialized and matured in the application to the light sensitive retinal proteins, numerous examples have been described for other systems as well.

Photo induced ligand release from biological inactive derivatives, caged ligands, has been used to study structural changes and ATP hydrolysis in Ca\(^{2+}\)-ATPase (143). Redox reactions triggered either photochemically, or directly by applying a potential to the sample, have facilitated studies on photosynthetic reaction centers (144, 145) and peroxidases (146). Furthermore, direct interactions can be studied using ATR, which allows perfusion of the sample with buffer alternatively with or without reactant. This kind of experiment has been described on the nicotinic acetylcholine receptor (147, 148). Clearly, time-resolved measurements make these technique even more powerful in their applications to elucidate structure-function relationships in complex protein systems (149, 150).

Of course, visualization of structural changes by infrared difference spectroscopy is one thing, assigning the differences to specific molecular groups is another. For this, multi-disciplinary research combining biochemical and biotechnological techniques is essential. For example, in the case of rhodopsin limited proteolysis (151, 152), site-directed mutagenesis (reviewed in chapter 2), isotope labeling of the ligand (74, 153) and the introduction of isotope labeled amino acids in the protein (154) proved to be powerful tools for the assignment of FTIR difference bands. Furthermore, comparison with Raman and infrared data of model compounds has led to several band-assignments (chapter 4). The ultimate tool in this respect, is site directed isotope labeling, i.e. the introduction of a stable-isotope labeled amino acid at a specific position in the protein. This method has already been successfully applied in the case of bacteriorhodopsin, which can be expressed in a cell free expression system and can subsequently be properly folded (155, 156). However, the latter has not yet been accomplished for eukaryotic membrane proteins like rhodopsin that are folded in the endoplasmic reticulum and need specific post-translational modifications for proper functioning.

### 1.5 Aim and outline of this thesis

Visual pigments are complex integral membrane proteins and are the prototypical members of a large family of G-protein coupled receptors. These receptors mediate the actions of extracellular signals as diverse as light, odorants, peptide hormones and neurotransmitters. Detailed knowledge on their structure and intramolecular signaling pathways is still fairly limited. Activation of these receptors is generally believed to require conformational changes in the protein moiety; the exposure of signaling sites in the cytoplasmic domains then facilitates protein-protein interactions between the receptors and their respective G-proteins.

Vibrational spectroscopy, and especially FTIR difference spectroscopy in combination with well-defined protein modification is, at present, the only way to obtain significantly detailed information on structure and function in these systems.

The aim of the work presented in this thesis was to further identify protein activity and
Introduction

protein-ligand interactions during the photoactivation process of rhodopsin using FTIR difference spectroscopy. We especially focussed on the formation of the active state of the receptor: metarhodopsin II. Novel band-assignments in the rhodopsin to metarhodopsin II difference spectrum to specific intramolecular groups are presented in chapter 2. Chapter 3 describes the use of polarized infrared (ATR) spectroscopy to obtain information on the relative orientation of structurally active groups within the protein. In addition, this approach facilitated the identification of previously unobserved bands in the rhodopsin to metarhodopsin II infrared difference spectrum. Ligand-protein interactions are the subject of chapter 4. Here, the photoactivation of rhodopsin regenerated with the 10-methyl homolog of 11-cis-retinal is described. Several lines of evidence indicate that there is a major electrostatic component to the photoactivation mechanism of rhodopsin. In chapter 5, this subject is addressed by studying the effects of ionic strength on the formation of metarhodopsin II.

Most of what we know on the photoactivation of visual pigments stems from work on rhodopsin, the rod pigment. Recently, in our group, the functional expression and purification of the human green cone pigment has been accomplished on a large enough scale to allow spectroscopic studies. Chapter 6 describes initial FTIR investigations on the photocascade of this pigment.

The work presented in this thesis is summarized and discussed in chapter 7.

References

References

95. Honig, B., Dinur, U., Nakanishi, K., Balogh-Nair, V., Gawinowicz, M.A., Arnaboldi, M., and
References

Chapter 2

Band-assignment strategies: site-directed mutagenesis and isotope editing


FTIR difference spectroscopy, protein engineering and isotope labeling techniques were combined in order to identify structural activity of (bound) water molecules and specific protein residues in the rhodopsin to metarhodopsin II transition. Bands associated with the OH stretch modes of water are identified in the 3700–3200 cm$^{-1}$ region by a characteristic down-shift upon substitution of H$_2^{18}$O for H$_2$O. It is found that at least two water molecules undergo an increase in hydrogen bonding upon formation of the metarhodopsin II intermediate. The role of cysteines has been addressed by studying the effects of single cysteine → serine replacements on the 2600–2500 cm$^{-1}$ spectral region, highly characteristic of the SH stretch mode. Here, four mutant pigments were prepared: C167S, C185S, C122S and C264S. In a previous study, a positive band near 2550 cm$^{-1}$ was identified and associated with the formation of metarhodopsin II. Here, we show that this band may be assigned to the SH stretch mode of C167. Interestingly, the C185S replacement was found to affect the spectral changes due to one of the newly observed structurally active water molecules. On this basis, we argue that this water molecule may be localized in direct vicinity of C185, at the extracellular surface of rhodopsin. Finally, we present the first FTIR analysis of a stable-isotope edited rhodopsin. [ring-$^{2}$H$_{4}$]Tyr labeled recombinant rhodopsin was studied in order to investigate the possible involvement of tyrosines in the photoactivation of rhodopsin. Analysis of the 1800-1000 cm$^{-1}$ region revealed clear changes in bands that correspond to tyrosine and tyrosinate vibrational modes. It is discussed that at least part of these changes may reflect activity of Tyr268 in helix 6.
2.1 Introduction

As detailed in chapter 1, rhodopsin is a 7-helix integral membrane protein found in the outer segments of the retinal rod cells and is the primary photoreceptor responsible for scotopic vision in vertebrates (1, 2). The rapid light induced 11-cis to all-trans isomerization of the retinylidene chromophore (3) is followed by a series of spectral changes which correspond to the different bleaching intermediates (Batho → Lumi → Meta I → Meta II) in the photoactivation cascade (4, 5). Signal transduction occurs upon formation of the Meta II intermediate, the only intermediate known to bind and activate the G-protein transducin (6). On a much longer time-scale, Meta II decays to Meta III or to opsin and all-trans retinal (7). An important goal in vision research is to understand the key molecular events that underlie rhodopsin activation.

Chemical characterizations revealed that the formation of the physiologically active Meta II intermediate leads to an increased accessibility of water-soluble substances to the chromophore binding site, and that it is associated with enhanced digestibility of the cytoplasmic loops. Meta II formation has further been shown to depend on pH, ionic strength [(8), see chapter 5] and hydration state [see for a recent review (9)]. Furthermore, presumably in Meta II, additional SH groups of rhodopsin are exposed (10, 11). Important progress has been made with the introduction of biotechnological methods. This led to the identification of amino acid residues that are involved in the photoactivation of rhodopsin [reviewed in (9, 12)]. Information about the possible changes in local environment and protonation state of these residues during photoactivation have so far mainly been obtained by methods based on FTIR difference spectroscopy.

The success of FTIR difference spectroscopy resides in the sensitivity of vibrational modes of a molecule to structural perturbations and local interactions. In addition, IR is a not a highly selective technique and thus allows the simultaneous detection of light induced structural changes in the retinal chromophore, in the protein moiety and in the lipid phase. Detailed assignment of the observed difference bands to individual groups may eventually disclose the nature of the corresponding conformational rearrangements in the receptor. An initial assignment may in some cases already be accomplished by noting that certain regions in the mid-infrared are characteristic of certain types of vibrations (13). In particular, OH and NH stretching vibrations (in water, amino acid side chains or peptide bonds) may be identified in the 3700–3200 cm\(^{-1}\) region, the (cysteine) SH stretching mode between 2600 and 2500 cm\(^{-1}\), and C=O stretching vibrations between 1800 and 1600 cm\(^{-1}\) (Asp and Glu carboxyl groups, and lipid ester carbonyl modes in the 1800–1700 cm\(^{-1}\) region, peptide carbonyls between 1700–1600 cm\(^{-1}\). The region below 1700 cm\(^{-1}\) is spectrally very crowded precluding direct assignments). More specific assignments, i.e. to specific groups or residues, may be accomplished by studying changes in the difference band pattern caused by well-defined modification of the photoreceptor complex, e.g. by limited proteolysis, by incorporation of isotope labeled chromophores, or by site-directed mutagenesis or isotope-labeling of rhodopsin.

In this chapter, novel band-assignments in the Rho → Meta II difference spectrum of bovine rhodopsin are presented based on site-directed mutagenesis and stable-isotope edit-
ing of rhodopsin. In addition, bands reflecting a conformational rearrangement involving internal water molecules are identified by a characteristic $\pm 10$ cm$^{-1}$ shift of the water OH stretching mode upon substitution of H$_2$O with H$_2^{18}$O. Evidence is presented for the involvement of at least two water molecules in the structural transition to metarhodopsin II. The involvement of cysteine residues has been shown in an earlier study describing the appearance of a positive 2550 cm$^{-1}$ difference band, assignable to a cysteine SH stretching mode, with kinetics that correlate with the formation and decay of Meta II (14). By elimination, four out of a total of ten cysteine residues in the primary sequence of rhodopsin were selected as possible candidates to be responsible for this band and were replaced by serines: C167, C185, C222 and C264$^1$. Only the C167S replacement was found to significantly affect the 2550 cm$^{-1}$ band, and we therefore assign this band to the SH stretch mode of C167. Interestingly, the C185S replacement caused complete disappearance of bands associated with a bound water molecule. This suggests that this water molecule may be located in direct vicinity of C185, at the extracellular surface of the receptor. Through the years, site-directed mutagenesis of rhodopsin has become a well-established strategy to identify the origin of FTIR difference bands and to study possible interactions between groups in the photoreceptor complex. However, when key residues are involved, even the replacement of a single amino acid residue may result in disfunctional protein and alternative strategies are required. Here, we report a first step in a new direction: stable-isotope editing of rhodopsin. The possible involvement of tyrosine residues in the photoactivation process has been investigated by analyzing [ring-$^2$H$_4$]Tyr labeled rhodopsin (17). This revealed clear isotope induced changes in difference bands assignable to tyrosine/tyrosinate vibrational modes. By analogy to the case of the bR $\rightarrow$ M transition in bacteriorhodopsin, it is discussed that at least part of these spectral changes may reflect activity of Tyr268, located in the center of helix 6.

2.2 Experimental Procedures

All manipulations with rhodopsin were performed under dim red light (RG645, Schott, Mainz, FRG).

Preparation of rhodopsin membranes. Rhodopsin membranes were prepared from bovine rod outer segments (ROS) according to methods previously described (18). The $A_{280}/A_{500}$ ratio of the resulting washed photoreceptor membranes was typically 2.0 ± 0.1. Membrane suspensions at a concentration of 55 nmoles/ml of rhodopsin were stored under argon at $-80^\circ$C until further use.

Preparation of recombinant rhodopsin. Wild-type hexahistidine tagged rhodopsin derived from bovine rhodopsin was produced and purified as described previously (19). Large scale production of cysteine mutants C167S, C185S, C222S and C264S was performed as described (M.J.M. VanDeLuitgaarden et al., in preparation). For the purpose of isotope labeling, Spodoptera frugiperda

$^1$C140 and C316 were ruled out as possible candidates on the basis of FTIR results on NEM labeled rhodopsin (14), C110 and C187 because they form a disulfide bond (15), and C322 and C323 since these are the palmitoylation sites for rhodopsin (16).
(Sf9) cells (ATCC: CRL-1711) were grown and subsequently infected in spinner flasks (Bellco, Vineland, USA) containing customized serum-free and protein-free medium partially depleted in L-Tyr (Biowhittaker, Walkersville, MD, USA) to which an additional 2 mM L-[ring-2H4]Tyr (CIL, Andover, MA, USA) was added. To determine label incorporation levels, total proteoliposome fractions were treated with Pronase to generate free amino acids. Subsequent GC-MS analyses of two different samples revealed that 70 ± 3% of membrane protein incorporated tyrosines were labeled (C.H.W. Klaassen et al., in preparation). The wavelength of maximal absorbance in the visible region (λ_{max}) of the pigments was determined in 20 mM dodecylmaltoside in buffer A (20 mM Pipes, 130 mM NaCl, 5 mM KCl, 2 mM CaCl2, 0.1 mM EDTA, 1 mM dithioerythreitol, pH 6.5) [(20), chapter 4]. The relative amount of 380 nm photoproduct for med at 10 °C, representing metarhodopsin II, was determined in proteoliposomes in buffer A as described [(8), chapter 5].

FTIR spectroscopy. Rhodopsin films for transmission IR spectroscopy were prepared by isopotential spin-drying (21, 22) a 1 ml aqueous suspension of membranes containing approximately 3 nanomoles of rhodopsin (based on A_{500}) onto an AgCl window. In case the films contained residual sucrose² from the isolation procedure (19), two additional wash steps were performed in 1 ml doubly distilled water. Subsequently, the films were redried as described above. Rehydration of the films (using ~1.5 µl of doubly concentrated buffer A) prior to insertion into a sealed transmittance cell was performed as described [(8), chapter 5]. The H₂O content of the sample was monitored by measuring the intensity ratio of the 3400 cm⁻¹ band (O–H stretch mode) to the methyl and methylene C–H stretch bands of the protein and lipids in the 2800-3000 cm⁻¹ region, or alternatively, the amide II band near 1545 cm⁻¹. These ratios were adjusted close to two (2.1 ± 0.2). This results in a maximal yield of Meta II upon illumination of the sample and minimizes interference due to the strong water bands.

Native rhodopsin was also measured in H_{18}²O and D₂O. Hereto, the rhodopsin film was first dried for more than 12 hours in a dry-air box in order to remove residual H₂O. The dried film was then exposed to bulk H_{18}²O or D₂O for more than 24 hours by putting a 10 µl drop directly onto the film and then sealing it with a second AgCl window. It was then redried in a dry-air box and assembled into a sealed IR cell using a second AgCl window containing small drops (± 3 µl) of H_{2}O / D₂O placed outside of the IR beam path. The D₂O or H_{18}²O content of the sample was monitored by measuring the intensity ratio of the 3400 cm⁻¹ band (O–H stretch mode) or 2600 cm⁻¹ band (O–D stretch mode) to the methyl and methylene C–H stretch bands, as described above.

Transmission FTIR difference spectra of the rhodopsin to metarhodopsin II transition were recorded at 10 °C using methods similar to those previously reported (23, 24). Briefly, the Rho → Meta II difference spectra were obtained as follows: the sample was photobleached for 3 minutes using light from a 150-Watt tungsten illuminator (Model 180, Dolan-Jenner industries, Lawrence, MA) filtered by a 500 nm long-pass filter (Corion Corp., Holliston, MA) and several heat filters, and transmitted to the sample with an annular optical fiber. Spectra were recorded at 8 cm⁻¹ resolution and 5 minute intervals for several hours before and after illumination (1350 scans for each spectrum) on a BioRad FTS-60A spectrometer (BioRad, Digilab Division, Cambridge, MA) equipped with a Mercury-Cadmium-Telluride (MCT) detector. Each difference spectrum shown represents a subtraction of the spectrum recorded immediately before the light is turned on from the spec-

²The sucrose content of the films was checked by FTIR, monitoring the carbohydrate C–O stretch band between 1000 and 1100 cm⁻¹.
2.3 Results

2.3.1 Water Molecules That Change Upon Formation of Meta II

The 3700-3200 cm\(^{-1}\) region of the Rho \(\rightarrow\) Meta II difference spectra for rhodopsin exposed to H\(_2\)O, H\(_{18}\)O and D\(_2\)O are shown in Figure 2.1. In the region above 3525 cm\(^{-1}\) all

![Figure 2.1: FTIR difference spectra of the Rho \(\rightarrow\) Meta II transition for rhodopsin films hydrated with H\(_2\)O, H\(_{18}\)O and D\(_2\)O. The Y-scale is for the H\(_2\)O data.](image)

of the bands can be assigned to the OH stretch mode of water on the basis of a \(\pm\)10 cm\(^{-1}\) down-shift in the H\(_{18}\)O spectrum. Furthermore, all of the bands in this region disappear due to H/D exchange. Compared to the Rho \(\rightarrow\) Meta I difference spectrum [not shown, see (25)], the 3659/3645 cm\(^{-1}\) pair of bands significantly increases in intensity and is therefore assigned to a water molecule which is rather altered during formation of the Meta II intermediate. A second set of bands which may be associated with Meta II formation appears at 3614/3587 cm\(^{-1}\). On the other hand, bands at 3562/3536 cm\(^{-1}\) found in the Rho
→ Meta II spectrum are also present in the Rho → Meta I spectrum but are less intense and may be due to residual amounts of Meta I. In an earlier report, the positive $H_{18}O$ sensitive component at 3645 cm$^{-1}$ was already identified but the other bands were not clearly seen (26). Thus, we conclude that the Meta I to Meta II transition involves at least one (3659/3645 cm$^{-1}$) and possibly a second water molecule (3614/3587 cm$^{-1}$). Whether one or two molecules are involved, the pattern of bands, i.e. negative/positive pairs with the negative the highest frequency component, is consistent with an increase in the hydrogen bonding of the water molecule(s).

Several bands are also observed below 3525 cm$^{-1}$ which appear to be insensitive to both $H_{18}O$ and $D_2O$. Most notable is the band near 3480 cm$^{-1}$ (−)$^3$ which is also observed in the Rho → Meta I spectrum and has been previously assigned to the NH stretch mode of a tryptophan indole group by analogy to bacteriorhodopsin (26). A positive band at 3500 cm$^{-1}$ may also be associated with this NH group in the Meta I intermediate. However, this band becomes less prominent in the Rho → Meta II difference spectrum, possibly due to overlap with a new negative band at 3518 cm$^{-1}$. The latter does not shift in $H_{18}O$ but is absent in $D_2O$ and thus is associated with an NH or OH group which can undergo H/D exchange in unphotolyzed rhodopsin. A second positive broad band near 3446 cm$^{-1}$ is also present in the Rho → Meta I and Meta II difference spectra and absent only in the case of Meta II in $D_2O$. Hence this band may represent an additional NH or OH group which becomes accessible for H/D exchange only upon formation of Meta II.

The largest change in this region is the appearance of a previously unreported intense negative band at 3287 cm$^{-1}$ which is present already in Meta I and intensifies upon Meta II formation. This band falls in the region of the amide A mode (NH stretch vibration) of peptide groups (13). For example, the major band in the absolute absorption spectrum of rhodopsin in this region appears at 3305 cm$^{-1}$ at 10 °C. On this basis, the bands below 3400 cm$^{-1}$ were discussed to reflect (yet unidentified) peptide groups which undergo changes in hydrogen bonding pattern upon photoactivation (25).

### 2.3.2 Cysteine Structural Changes in the Formation of Meta II

The UV/visible characteristics of the mutant pigments C185S, C222S and C264S are similar to that of wild-type ($\lambda_{max}$: 498 ± 2 nm, n = 3 in all cases). Only the C167S mutation induces a slight blue-shift of the $\lambda_{max}$ to 492 ± 2 nm (n = 3). The relative amount of Meta II formed upon illumination at pH 6.5 and 10 °C is similar for all pigments (75 ± 10 %; M.J.M. VanDeLuijtgaarden et al., in preparation).

Figure 2.2 compares the FTIR results obtained on the wild-type and mutant pigments in the range from 3700–3450 cm$^{-1}$ (Figure 2.2A), 2700–2400 cm$^{-1}$ (Figure 2.2B) and 1800–800 cm$^{-1}$ (Figure 2.2C). panels. The spectra in Figure 2.2C show very similar features, mainly reflecting changes of the retinylidene chromophore, which undergoes an 11-cis to all-trans conformational transition, as well as structural changes of the protein. For exam-

$^3$The sign shown next to the frequency in parenthesis (e.g. + or −) indicates whether the band is positive or negative; sh denotes shoulder
Figure 2.2: The effect of cysteine replacements C167S, C185S, C222S, and C264S on the Rho → Meta II difference spectrum in the 3700–3450 cm\(^{-1}\) region (A), the 2700–2400 cm\(^{-1}\) region (B) and the 1800–800 cm\(^{-1}\) region (C). Spectra are scaled with respect to the 969 cm\(^{-1}\) chromophore band. Y-scales are for the WT spectrum: note the differences in OD/div. in every panel.
ple, negative bands have been assigned to the Rho chromophore on the basis of isotope labeling and comparison with results from resonance Raman spectroscopy at 1548 cm$^{-1}$ (−) (C=C ethylenic stretch), 1238 cm$^{-1}$ (−) (C$_{12}$–C$_{13}$ stretch mode), and 969 cm$^{-1}$ (−) (HC$_{11}$ =C$_{12}$H hydrogen-out-of-plane mode) (27–31). In contrast, few bands have been assigned thus far to specific amino acids except in the 1700–1800 cm$^{-1}$ region [C=O stretch modes of Asp and Glu carboxyl groups (32, 33)]. The bands near 2550 cm$^{-1}$ (Figure 2.2B) have been tentatively assigned to the SH stretch mode of cysteine residues (14). The frequency of this mode varies only in a narrow range from 2525–2600 cm$^{-1}$, depending on hydrogen bonding strength and, to a lesser extent, side-chain conformation (34).

The cysteine → serine replacements can be seen to induce only minor effects on the 1800–800 cm$^{-1}$ spectral region (Figure 2.2C). In fact, only a slight shift of a positive band near 1042 cm$^{-1}$ has been reproducibly observed for the C185S pigment (n = 2). Changes in the amide I and II regions, e.g. the shift of the band near 1550 cm$^{-1}$ (−) and differences in the 1600–1690 cm$^{-1}$ range, probably reflect a slight variability in sample preparation and cannot be considered specific for any of the cysteine substitutions. Variability in the highly absorbing amide I and II regions may also be due to baseline effects or differences in sample orientation which can give rise to polarization effects (chapter 3). The overall similarity of the spectra in this region strongly suggests that the photoinduced structural changes in the mutant pigments are indeed very similar and that the cysteine replacements do not interfere with the structural and functional integrity of the receptor.

Upon comparing the relative contribution to the 2700–2400 cm$^{-1}$ region in Figure 2.2B, it can be seen that only the C167S replacement significantly affects the intensity of the 2550 cm$^{-1}$ difference band. We estimate that C167 is responsible for about 70 % of the difference intensity, and we therefore assign this band to the SH stretch mode of C167 in Meta II. The remaining 30 % may be due to residual Meta I (see above). This suggests that other cysteines are involved in the late phase of photoactivation as well. Unfortunately, due to slight variations in the relative amount of photoproduct formed upon illumination (± 10 % on the basis of UV/visible characterizations) and the related difficulties in scaling of the infrared difference spectra, the present results do not allow definitive assignment of the remainder of the 2550 cm$^{-1}$ band to a specific cysteine in Meta I. This requires further analysis of the Rho → Meta I transition at −15 °C of the cysteine mutants described in this paper, which is in progress.

Interestingly, the OH stretch region (Figure 2.2A) of the C185S spectrum does not show the 3659/3645 cm$^{-1}$ (−/+) pair assigned above to a weakly hydrogen-bonded water molecule. This supports the conclusion that this pair of bands actually originates from the same water molecule and allows a tentative localization of this structurally active water molecule near C185, at the extracellular surface of the receptor.

### 2.3.3 Tyrosine Structural Changes in the Formation of Meta II

Figure 2.3 shows a comparison of the Rho → Meta II difference spectra from unlabelled wild-type and [ring-$^2$H$_4$]Tyr-labeled rhodopsin in the region from 1800–1000 cm$^{-1}$. The spectra are scaled with respect to the 969 cm$^{-1}$ chromophore band. The introduc-
Figure 2.3: FTIR difference spectra of the Rho → Meta II transition for unlabeled and [ring-$^2$H$_4$]Tyr-labeled WT-rhodopsin, hydrated with H$_2$O. Inset, expansion of the 1290–1190 cm$^{-1}$ region (note the compressed Y-scale). The [ring-$^2$H$_4$]Tyr spectrum is shown as bold line. The Y-scale is for the [ring-$^2$H$_4$]Tyr spectrum.

ion of [ring-$^2$H$_4$]Tyr into Rho produces several clear changes in this region. In particular, reproducible changes in band intensity, reflecting isotope induced shifts in frequency, are observed for bands near 1595, 1570, 1517, 1423, 1373, 1275, 1253, 1221 (sh) and 1178 cm$^{-1}$ [n = 3, (17)]. We note that, for similar reasons as described above, there are bands near 1643 and 1530 cm$^{-1}$ whose intensities are not reproducible and are therefore not assignable to tyrosine vibrations.

In general, the changes we have assigned to tyrosine vibrations can be caused either by an isotope induced frequency shift of a tyrosine/tyrosinate vibrational mode out of a
particular region or by a shift of a labeled tyrosine/tyrosinate mode into that region of the spectrum. Detailed assignments can be made on the basis of comparison with Tyr and \([\text{ring-}^2\text{H}_4]\)Tyr model compounds \((17, 35, 36)\). For example, a decrease in intensity at 1595 cm\(^{-1}\) and an increase at 1570 cm\(^{-1}\) due to \([\text{ring-}^2\text{H}_4]\)Tyr incorporation agrees well with the isotope induced down-shift of the 1598 cm\(^{-1}\) ring stretching mode of tyrosinate to 1569 cm\(^{-1}\) in L-[\text{ring-}^2\text{H}_4]\)Tyr measured at pH 12 \((17)\). Similarly, a drop in intensity near 1275 cm\(^{-1}\) and increase near 1253 cm\(^{-1}\) (Figure 2.3, inset) (this increase appears as a maximum at 1245 cm\(^{-1}\) in a double difference spectrum, not shown) agrees qualitatively with the isotope induced shift of a band at 1271 cm\(^{-1}\) in L-tyrosinate to 1238 cm\(^{-1}\) in L-[\text{ring-}^2\text{H}_4]\)Tyr \((17)\). Previously this band has been assigned to the C–O\(^-\) stretch mode of tyrosinate \((35, 36)\). Overall, these data, and in particular the appearance of positive bands characteristic of (labeled) tyrosinate, indicate that a tyrosine residue is partially deprotonated in the Meta II state. This might reflect a partial deprotonation of a tyrosine residue in Rho upon formation of Meta II (see below) or, alternatively, a change in environment of an existing tyrosinate in Rho upon Meta II formation.

Additional bands which exhibit isotope induced changes are assignable to vibrational modes found in tyrosine or \([\text{ring-}^2\text{H}_4]\)Tyr. However, the pattern is complex and may reflect one or more tyrosines which undergo a change in environment or protonation state during Meta II formation. For example, the positive 1517 cm\(^{-1}\) band which drops in intensity is close to the frequency of an intense tyrosine ring vibration in L-Tyr \((35, 37)\) and therefore reflects a tyrosine in the Meta II state. Other bands which change and match known tyrosine frequencies are at 1373 cm\(^{-1}\) (not assigned), 1250 cm\(^{-1}\) (bending mode of tyrosine hydroxyl group), and 1179 cm\(^{-1}\) (ring CH bending mode). The 1373 cm\(^{-1}\) and 1179 cm\(^{-1}\) bands are positive and therefore also reflect tyrosine(s) in Meta II. The 1373 cm\(^{-1}\) band shifts to 1348 cm\(^{-1}\) in \([\text{ring-}^2\text{H}_4]\)Tyr-Rho in agreement with published spectra \((36)\). However, the increased intensity at 1250 cm\(^{-1}\) may reflect a change in a tyrosine residue in the unbleached state of rhodopsin (Rho). Part of the intensity changes in this region were already attributed to the down-shift of a positive tyrosinate band from 1277 cm\(^{-1}\) to near 1248 cm\(^{-1}\) in the Meta II state (see above). However, a prominent tyrosine mode due to the bending vibration of the tyrosine hydroxyl also occurs at this frequency and the down-shift in L-[\text{ring-}^2\text{H}_4]\)Tyr to 1226 cm\(^{-1}\) in good agreement with the change observed near 1221 cm\(^{-1}\) in \([\text{ring-}^2\text{H}_4]\)Tyr-Rho. This band is not likely to be due to residual Meta I, which exhibits a band near 1215 cm\(^{-1}\) \((8, 24)\), since no increase in the corresponding Meta I specific band at 950 cm\(^{-1}\) is observed (not shown). Whether a Tyr C–O\(^-\) stretching or a COH bending mode is involved may be resolved by incorporation of tyrosines \(^{13}\)C labeled at the ring carbon atom binding to the hydroxyl group and/or carrying an \(^{18}\)O labeled hydroxyl.

Finally, a prominent drop in intensity at 1423 cm\(^{-1}\) in \([\text{ring-}^2\text{H}_4]\)Tyr-Rho might be due to the isotope induced down-shift of a negative band due to a tyrosine ring mode from the 1515 cm\(^{-1}\) region. A band due to such a ring mode has been previously identified in the Tyr\(_D\) to Tyr\(_D^*\) FTIR difference spectrum of \([\text{ring-}^2\text{H}_4]\)Tyr labeled Photosystem II \((36)\). Alternatively, this change might be due to a mode observed at 1418 cm\(^{-1}\) in L-[\text{ring-}
$^2$H$_4$]Tyr at high pH which down-shifts from 1498 cm$^{-1}$ in L-Tyr at high pH (17). This would indicate that a (partially) ionized tyrosine exists in Rho. However, the loss of the corresponding unlabeled tyrosinate vibration at 1498 cm$^{-1}$ would lead to an increase in intensity near 1498 cm$^{-1}$ in [ring-$^2$H$_4$]Tyr-Rho, which is not clearly observed, but could be partially masked by the loss of the positive band at 1517 cm$^{-1}$.

Strikingly, there is a similar pattern of tyrosin(at)e related spectral changes observed in the bR $\rightarrow$ M difference spectrum of bacteriorhodopsin (bR) [not shown, (17, 38)] and the Rho $\rightarrow$ Meta II difference spectrum of bovine rhodopsin when [ring-$^2$H$_4$]Tyr is incorporated, although the sign of the bands is reversed. Similar to the results reported above, [ring-$^2$H$_4$]Tyr labeling causes a down-shift of a band from 1590 cm$^{-1}$ to 1570 cm$^{-1}$ in the bR difference spectrum, although the down-shifted bands are positive in the Rho spectrum and negative in the bR spectrum. Also, similar to rhodopsin a band at 1276 cm$^{-1}$ appears to shift to near 1248 cm$^{-1}$ in the bR $\rightarrow$ M difference spectrum, although the down-shifted bands are positive in the Rho spectrum and negative in the bR spectrum. Additional peaks [1517 (−, tyrosine) and 1419–1410 cm$^{-1}$ (+, [ring-$^2$H$_4$]-tyrosine)] were also previously assigned to tyrosine vibrations in the bR $\rightarrow$ M spectrum. Here again, the frequencies are similar to bands observed in the Rho $\rightarrow$ Meta II difference spectrum, but the signs of the bands are reversed. Interestingly, the similarity of the Tyr related difference bands suggests that, upon photoactivation, similar Tyr related conformation and protonation changes occur in both systems.

2.4 Discussion

Identification of bands in the 3700–3200 cm$^{-1}$ region. The structural importance of water to guide the photocascade transitions of rhodopsin is well established. For example, in dehydrated membranes, under conditions otherwise favoring the formation of metarhodopsin II, the photocascade is blocked at a 480 nm absorbing stage. FTIR difference spectroscopy indicated that this intermediate shows many of the Meta I and actually also Lumi structural characteristics [our own unpublished observations and (39)]. Any water which is structurally active, i.e. undergoes a change in hydrogen bonding or orientation, should give rise to bands in the FTIR difference spectra in the region above 3200 cm$^{-1}$ where the OH stretch mode of water is found. D$_2$O and H$_2^{18}$O substitutions allow the assignment of bands first to all groups accessible for H/D exchange and, second, specifically to water molecules. However, the low signal/noise obtained in a previous study prevented the assignment of negative bands to water molecules in the Rho $\rightarrow$ Lumi, MetaI and Meta II transitions, thereby making it difficult to determine how water is altered in rhodopsin in response to photoactivation (26).

The present results\(^4\) show that one, and possibly another, water molecule undergoes an increase in hydrogen bonding during the formation of Meta II. In the previous paper (25),

\(^4\)Parallel with this investigation, the Maeda group obtained high quality data in this spectral region and came to a similar conclusion regarding the involvement of water in the late photocascade transitions (40).
the extra water molecule could not be unambiguously assigned because of the possible detection of multiple bands from a single water molecule. This can occur for example if a water molecule has one strongly and one weakly bonded hydrogen. In this case, band splitting may amount to 100 cm\(^{-1}\), with the lower-frequency band arising from the stronger hydrogen bonded oscillator (41). However, since the C185S replacement described in the present work seems to affect the 3659/3645 cm\(^{-1}\) pair of bands only (see below), we now argue that most likely two water molecules are involved.

The localization of the structurally active water molecules in rhodopsin will be an important element in ascertaining their role in the photocascade reactions. Following earlier work on bacteriorhodopsin (42, 43) and the Rho \(\rightarrow\) Batho transition in rhodopsin (44), this is possible by examining whether substitutions of specific residues induce changes in the assigned water OH bands. On this basis we were already able to exclude the possibility that D83, which has previously been found to undergo a change in hydrogen bonding during Meta II formation (29, 30), interacts with any of the assigned water molecules at this stage in the photocascade. In particular, we were able to show that the D83N substitution did not affect any of the assigned water bands in the Rho \(\rightarrow\) Meta II spectrum (25). Significantly, the present results allow a tentative localization of the water molecule that gives rise to the 3659/3645 cm\(^{-1}\) OH stretch bands at the extracellular side of rhodopsin, in the vicinity of C185. Replacement of this residue results in complete disappearance of the 3659/3645 cm\(^{-1}\) pair. This may be due either to the removal of the water molecule from its binding site, to an increase in hydrogen bonding which might cause a significant down-shift and broadening of these bands, or to the absence of a structural change in the photoreaction of the mutant pigment. On the other hand, in addition to the loss of the water OH bands, a reproducible shift of a band near 1042 cm\(^{-1}\) is observed. Possibly, the structural change which is sensed by the water OH mode in native rhodopsin may now be detected as a change in the serine side chain C–O stretch mode, absorbing at or near 1042 cm\(^{-1}\) in the mutant pigment.

A possible candidate for the interaction with the second structurally active water molecule detected in the present work is Glu113, the putative proton acceptor and counterion of the Schiff base (45–47). In the case of bacteriorhodopsin, FTIR evidence indicates that a water molecule displaying an OH stretch mode at 3642 cm\(^{-1}\) interacts with the analogous residue Asp85 (43). In support of such an interaction in rhodopsin, under ultra-high vacuum a shift in \(\lambda_{\text{max}}\) is induced from 500 \(\rightarrow\) 390 nm (48), suggesting that water is involved in stabilizing the protonated Schiff base in the dark. Further indications for water located in the retinal binding pocket have been inferred from recent solid state NMR studies on \(^{15}\)N-lysine labeled rhodopsin (49), and the rapid H/D exchange kinetics of the Schiff base proton observed in resonance Raman studies (50). This led to the proposal of an internal water molecule bridging the Schiff base and its counterion [e.g. see the recent rhodopsin models in (51–53)]. Direct evidence for water in the retinal binding site has been obtained from the Rho \(\rightarrow\) Batho FTIR difference spectrum which shows several bands [six in total, (40)] assignable to water OH stretching modes (54). In a subsequent study, two of these bands [located at 3538/3525 cm\(^{-1}\) (−/+) ] were shown to disappear upon replace-
ment of E113 by Gln and were therefore assigned to the water molecule that is in direct contact with E113 and stabilizes the protonated Schiff base (44). Alternatively, the water molecule that gives rise to the 3614/3587 cm\(^{-1}\) bands may be located in the vicinity of E122. The light-induced structural rearrangements involving this residue have been proposed to be mediated by internal water based on an FTIR study on dehydrated rhodopsin samples. It was shown that the alterations typically observed in the carboxyl-region upon formation of Meta I, and tentatively assigned to E122, did not appear in the Meta I like state formed upon illumination of a dehydrated membrane sample (39). It has been suggested that the structural changes involving E122 are reversed during formation of Meta II (33, 55), which could be related to the water activity observed here.

In addition to structurally active water, our results demonstrate that a variety of other OH and NH groups (including peptide NH) undergo distinct changes in hydrogen bonding during photoactivation. Almost all of these groups were inaccessible for H/D exchange in both the unphotolyzed rhodopsin and the Meta I and Meta II intermediates. This indicates that a significantly large core of rhodopsin remains buried in the membrane interior during the photoactivation cascade. However, we also detect the existence of an NH or OH group(s), which becomes available for H/D exchange only upon formation of the Meta II intermediate. This is consistent with recent evidence from FTIR that upon photoactivation buried portions of the rhodopsin backbone become more accessible during Meta II formation (56).

The cysteine SH stretch mode; the 2600–2500 cm\(^{-1}\) region. The cysteine SH stretch mode absorbs in the 2600–2500 cm\(^{-1}\) spectral region (57). This region is essentially devoid of interference from other sample vibrations, and the appearance of bands in this region in the Rho \(\rightarrow\) Meta II difference spectrum thus is a distinctive marker for photoinduced changes involving cysteine residues. The observed positive bands near 2550 cm\(^{-1}\) in the Rho \(\rightarrow\) Meta II difference spectrum have been assigned to cysteine SH modes in metarhodopsin II on the basis of, firstly, H/D exchange studies which revealed that these bands show the expected down-shift to near 1850 cm\(^{-1}\), and secondly, kinetic analyses showing that the rise and decay of these bands correlates with the appearance and decay of other metarhodopsin II characteristics [e.g. the bands at 1767/1747 cm\(^{-1}\) (–/+)] (14).

The present work allows the assignment of the 2550 cm\(^{-1}\) band to SH stretch mode of C167 in metarhodopsin II. However, replacement of this residue revealed another band absorbing at a similar frequency, which we propose to be due to a residual cysteine change originating in Meta I. Hence, in addition to C167, other cysteines may be sensing conformational rearrangements during receptor activation as well. Indeed, bands assignable to cysteine SH modes have also been identified in the Rho \(\rightarrow\) Batho difference spectrum obtained at 80 K [2574 (+)/2556 cm\(^{-1}\) (sh)] and the Rho \(\rightarrow\) Meta I spectrum obtained at \(-15^\circ\)C [2576/2553 cm\(^{-1}\) (–/+), our own unpublished observations]. The identification of the cysteine(s) responsible for these bands is in progress.

The appearance of only a positive band in this region of the Rho \(\rightarrow\) Meta II difference spectrum, with no detectable negative component, precludes direct determination of how
C167 is altered in the photoactivation process. On the basis of the correlation between frequency and hydrogen bonding strength, one would expect to observe a negative/positive pair, like that in the Rho → Meta I spectrum, if an SH group undergoes a change in hydrogen bonding environment during this transition. It has previously been argued that the appearance of only a positive band may nevertheless reflect an increase in hydrogen bonding because the molar extinction coefficient of the SH stretch mode may increase markedly upon an increase in hydrogen bonding (14). However, the absence of a negative component may also be due to a reorientation of an SH group towards the plane of the membrane in the Rho → Meta II transition. Polarized infrared difference spectroscopy should allow to distinguish between the latter possibilities.

Interestingly, the structural activity which is sensed by the SH stretch mode of C167 may be related to changes in interaction between helix 3 and 5 during Meta II formation. Recent molecular models predict the interaction between these helices and indicate that this interaction may be mediated by E122 (helix 3) and H211 (helix 5) (52, 53). In the model of Mosberg and coworkers (53) the polar side chains of E122 and H211 were suggested to be shielded from the highly nonpolar environment by a shell of residues of intermediate polarity, among others including C167 (helix 4) and W126. Spectroscopic evidence for interaction between E122 and H211 during the formation of Meta I has recently been obtained in a study combining site-directed mutagenesis and FTIR difference spectroscopy (55). As noted above, the conformational rearrangement involving E122 during formation of Meta I may be reversed in the transition to Meta II. Significantly, both C167 and W126 have been shown to undergo some type of structural rearrangement upon formation of Meta II [this work and (58)].

**Tyr modes in the 1800–800 cm⁻¹ region.** Our results clearly demonstrate that a set of bands in the Rho → Meta II difference spectra can be assigned to vibrational modes of tyrosine and tyrosinate. On this basis, it can be concluded that one or more tyrosines participate in the rhodopsin photocascade up to formation of the Meta II intermediate, and that at least a partial deprotonation of a tyrosine is involved. A possible involvement of tyrosine residues was previously proposed on the basis of UV-difference spectroscopy (59), site-specific mutagenesis (60), and was also suggested in a recent paper describing UV-resonance Raman studies of rhodopsin (61).

The similarity between the pattern of isotope induced changes observed in bacteriorhodopsin and rhodopsin is particularly interesting. Earlier studies based on tyrosine isotope labels (38), site-directed mutagenesis (62) and site-directed isotope labeling (63) concluded that tyrosine bands identified in the FTIR difference spectra of bacteriorhodopsin arose from protonation changes in Y185 located in the F-helix. Subsequent FTIR measurements on bR containing a ¹³C isotope label in the C1 position of Y185 indicated that the Y185 backbone undergoes some type of structural rearrangement (64). An interesting possibility is that the Y185/P186 region serves as a hinge which gives rise to the apparent tilting of the F-helix late in the bR photocycle (65, 66).

The present findings indicate that a similar, although reversed, pattern of tyrosine/tyrosi-
nate changes occurs also upon photoactivation of rhodopsin. While on the basis of this study we cannot assign these changes to any particular tyrosine(s) in rhodopsin, an interesting candidate is Y268 in helix 6\(^5\). This residue is also positioned adjacent to a Pro residue (P267). It is well known that Pro residues break \(\alpha\)-helical structure and can function as kinks allowing independent movement of adjacent helical segments. If indeed these similarities in Tyr activity arise in changes in the sixth transmembrane helix, then it is possible that the Pro/Tyr region in helix 6 in rhodopsin acts similarly as a hinge for movements in response to chromophore isomerization. Indeed, several lines of evidence have been presented that a rigid-body movement involving part of helix 6 takes place during photoactivation of rhodopsin (67, 68). Also, recent structural models for rhodopsin (52, 69) place Y268 close to E113, suggesting a possible role in proton transfer from the Schiff base upon formation of Meta II, which could be related to the Tyr protonation changes indicated by our data. In fact, mutagenesis studies show that replacement of Y268 by Phe perturbs ligand-binding and activation of rhodopsin (60), and that W265 also is involved structurally in formation of Meta II (58, 70). Actually, this region probably is one of the hot-spots in rhodopsin activation, since in the vicinity of Y268 are also residues G121 and F261, which form a synergistic functional unit with the 9-methyl group of the chromophore (71). Mutation of these residues in most cases has a profound effect on dark and/or rest activity of rhodopsin (71, 72). However, in order to definitely identify Y268 as (one of) the residue(s) responsible for the changes we observe additional site-specific mutagenesis and/or site-directed isotope labeling will be required. Since mutagenesis tends to perturb functionally or structurally active sites, isotope labeling will be the preferred method.

**Conclusion.** To date, FTIR difference spectroscopy of rhodopsin proved to be the most versatile approach to extract atomic-level structural data from this receptor. The level of detail to be reached, however, strongly depends on the accuracy of the assignments of the IR difference bands. In order to identify protein activity, initially limited proteolysis techniques were employed. At the present time, site-directed mutagenesis strategies offer the highest level of detail. This already allowed the assignment of several bands in the Rho → Meta II difference spectrum to individual amino acids and the nature of the corresponding conformational changes to be disclosed. Furthermore, interactions between residues have been studied. However, even very conservative replacements of key-residues were reported to show mosaic effects on rhodopsins structure and function. Obviously, in such cases also a many-fold of (non-specific) changes may be expected in the IR difference band pattern, and an alternative band-assignment strategy is required. The recent achievement to incorporate stable-isotope labeled amino acids into rhodopsin allowed the clear identification of tyrosine activity in the photocascade transitions, as described in the present work. An obvious advantage of this labeling approach is that it leaves the protein-structure

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\(^5\)Recent evidence (49) indicates that a Tyr residue is involved already in the Rho → Batho transition since isotope induced band-shifts were also detected at this stage for the \([\text{ring}\-^{2}\text{H}_4]\)Tyr-labeled pigment. Since only binding site residues are expected to participate in this transition, Tyr268 is the only candidate.
unaffected. A disadvantage, compared to site-directed mutagenesis, however, is that the isotope induced band-shifts cannot directly be assigned to a single residue. An important next step therefore is the site-specific introduction of isotope labels. However, although this has already been accomplished for bacteriorhodopsin via a cell-free expression system, it will be a formidable task to achieve in the case of eukaryotic membrane proteins like rhodopsin.

References

44 Band-assignment strategies

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Chapter 3

Probing Intramolecular Orientations in Rhodopsin and Metarhodopsin II by Polarized Infrared Difference Spectroscopy


The light-induced conformational changes of rhodopsin, which lead to the formation of the G-protein activating Meta II intermediate, are studied by polarized attenuated total reflectance infrared difference spectroscopy. Orientations of protein groups as well as the retinylidene chromophore were calculated from the linear dichroism of infrared difference bands. These bands correspond to changes in the vibrational modes of individual molecular groups that are structurally active during receptor activation, i.e. during the Rho → Meta II transition. The orientation of the transition dipole moments of bands previously assigned to the carboxyl (C=O) groups of Asp83 and Glu113 has been determined. The orientation of specific groups in the retinylidene chromophore has been inferred from the dichroism of the bands associated with the polyene C=C, C=C, and hydrogen-out-of-plane vibrations. Interestingly, the use of polarized infrared light reveals several difference bands in the Rho → Meta II difference spectrum which were previously undetected, e.g. at 1736 cm$^{-1}$ and 939 cm$^{-1}$. The latter is tentatively assigned to the hydrogen-out-of-plane mode of the HC$_{11}$=C$_{12}$H segment of the chromophore. Our data suggest a significant change in orientation of this group in the late phase of rhodopsin activation. On the basis of available site directed mutagenesis data, bands at 1406, 1583 and 1736 cm$^{-1}$ are tentatively assigned to Glu134. The main features in the amide regions in the dichroic difference spectrum are discussed in terms of a slight reorientation of helical segments upon receptor activation.


3.1 Introduction

Rhodopsin, the vertebrate visual pigment responsible for scotopic vision, is located in the retinal rod cells. It is a 7-helix integral membrane protein, and is considered to be a paradigm for the vast superfamily of G-protein coupled receptors (GPCRs) (1). It is generally assumed that the 7-helix bundle is arranged such that a cleft is formed in the interior part of the protein which accommodates the light sensitive ligand (and chromophore) 11-cis-retinal, covalently bound through a Schiff base linkage to a lysine in helix 7. Photoexcitation of rhodopsin is triggered by ultra-fast (< 200 fs) (2) 11-cis → all-trans photoisomerization of the chromophore, which subsequently initiates a series of thermal conformational transitions in the protein (Batoh ↔ blue-shifted intermediate (BSI) → Lumi → Meta I ↔ Meta II) (3, 4). At physiological temperature, Meta II, the active state of the receptor (5, 6), is formed within milliseconds after isomerization of the chromophore. Since activation of a GPCR is assumed to require protein conformational changes, understanding the molecular events underlying rhodopsin activation is not only extremely important for understanding the first step in vision, but is likely to hold significance for other GPCRs as well.

Fourier transform infrared (FTIR) difference spectroscopy has been extensively used to study the structural changes occurring in the photoreceptor membrane upon light-activation. With this technique, conformational changes in the chromophore, peptide backbone, lipid matrix and bound H$_2$O, as well as changes in protonation state and/or hydrogen bonding environment of Asp, Glu, Cys and Tyr residues have been detected at the various stages of rhodopsin activation (7–24). Assignment of vibrational bands to individual protein groups has been accomplished in combination with site-directed mutagenesis (14, 16, 23) and recently also by stable-isotope labeling of the protein (24).

Here, we describe the use of polarized attenuated total reflectance (ATR) FTIR difference spectroscopy to investigate the structural changes of bovine rhodopsin as it undergoes the transition from the dark-state to the active (Meta II) conformation. This method combines the sensitivity of infrared spectroscopy with the ability of polarized spectroscopy to probe the orientation relative to the membrane plane of the transition dipole moment associated with specific bands. This approach is complementary to earlier polarized FTIR studies of the absolute absorption which revealed information about the orientation of structural components of the protein, e.g. α-helices, relative to the membrane plane (25, 26). In the case of difference spectroscopy, however, information is obtained about the relative orientation of specific structurally active molecular groups including previously assigned protein residues and retinylidene groups (or vibrational modes). Our data also complement the results obtained using other experimental approaches which probe orientation of structural components of rhodopsin including the early visible dichroism studies (27–29) and, more recently, solid state NMR studies (30). The macroscopic orientation of secondary structure elements we calculate, basically agrees with earlier transmission FTIR studies on bovine rhodopsin (26). Our polarized difference spectra clearly reveal several new, previously unobserved bands throughout the entire spectral range (4000–800 cm$^{-1}$). The dominant features in the amide regions suggest that there occurs a small reorientation of helical
segments in the activation step of rhodopsin. A novel band at 939 cm$^{-1}$, assignable to a HOOP mode in Meta II, is considered to reflect a reorientation of the retinal HC$_{11}$=C$_{12}$H group in the Meta I to Meta II transition. Bands at 1406, 1583, and a previously undetected band near 1736 cm$^{-1}$ are tentatively assigned to the symmetric and asymmetric carboxylate and carbonyl stretching vibrations of Glu134, which was proposed to protonate upon formation of Meta II (31).

3.2 Experimental Procedures

Preparation of rhodopsin. ROS was prepared as previously described (32). The A$_{280}$/A$_{500}$ typically was between 1.9–2.1. Membrane suspensions in doubly distilled water (typically 130 nmol/ml in rhodopsin) were stored in an Argon atmosphere at −80 °C until further use. All manipulations involving rhodopsin were performed under dim red light (RG645 cutoff filter, Schott). Throughout the experiments a Mes H$_2$O/D$_2$O buffer (40 mM Mes, 260 mM NaCl, 10 mM KCl, 4 mM CaCl$_2$, 0.2 mM EDTA, pH/pD 6) was used.

ATR-FTIR measurements. Samples were prepared by slowly drying 30 µl of ROS suspension (about 4 nmol of rhodopsin), under a gentle stream of nitrogen, onto a 50 × 20 × 2 (l × w × h) mm$^3$ Ge internal reflection element (EJ3121, Harrick Scientific Corporation, Ossining, NY). Hereafter, the Ge element was placed in a vacuum dessicator for 15 min to remove residual water. Subsequently, the sample was mounted into a modified, temperature controlled, variable angle ATR unit (MEC-1W in combination with TMP-V, Harrick) and polarized dark spectra were recorded at ambient temperature. For the analysis of the Rho → Meta II transition the membranes were humidified with either H$_2$O or D$_2$O buffer. Deuteration of the sample was accomplished by putting 50 µl of D$_2$O on top of the dried sample, now placed in a nitrogen purged glove bag. The sample was left to exchange for 2–3 hours. After this period, the sample was redried before mounting it into the ATR cell, to allow a well defined humidification with D$_2$O buffer. Six µl of D$_2$O buffer was then placed on top of the sample and, in total, 50 µl of D$_2$O buffer on the inside of the sample cell’s covering lid. Data collection typically started 6 hours after sealing the cell. Thus, the samples had been in contact with bulk D$_2$O for at least 8 hours prior to data collection. Spectra were recorded at 10 °C, since under these conditions Meta II is sufficiently stable. Sample humidification with H$_2$O buffer, and all subsequent procedures were performed under identical conditions as humidification with D$_2$O.

Polarized ATR-FTIR spectra were recorded using a wire-grid BaF$_2$ polarizer (Graseby Specac, Kent, UK) placed in the IR beam of a Mattson Cygnus 100 FTIR spectrometer (Mattson, Madison, WI) equipped with a narrow band Hg-Cd-Te detector. Spectra were derived from 256 single beam scans (1 min acquisition time), taken at 8 cm$^{-1}$ resolution. Interferograms were doubly zero-filled, and apodized using a triangular function, prior to further processing. The polarized single beam spectra were ratioed against a correspondingly polarized background. The orientation of the polarizer could be controlled with the spectrometer computer and spectra were recorded with the polarization of the IR probe beam alternately parallel (∥) and perpendicular (⊥) to the plane of incidence. In this way, we were able to signal average the parallel and perpendicular data sets in the bleaching experiments over the same time span.

Photoactivation of the samples was accomplished by illumination with yellow light (40 s) using
a 20 W halogen lamp equipped with a KG1 heat filter and an OG530 cutoff filter (both filters from Schott). Difference spectra (Meta II — rhodopsin) were computed by subtracting the average of at least 8 spectra, recorded with the same polarization of the IR probe beam (at least 2048 co-added interferograms), taken before and after illumination, respectively.

Dichroic difference (LD) spectra were calculated by subtracting (difference) absorption spectra of the sample taken with perpendicularly polarized light (⊥) from those taken with parallel polarized light (∥) using the scaling factor c: LD = (∥) − c × (⊥). The factor c was determined interactively from the absolute absorption data in the dried or humidified state, by optimizing the sign and relative magnitude of the dichroic difference bands corresponding to the lipid methyl and methylene CH stretching modes, i.e. the νs(CH3) band at 2875 cm−1 and the νas and νs(CH2) bands at 2924 and 2853 cm−1, respectively. Since the CH2 vibrations are known to have their average transition moments perpendicular to the acyl chain long axis, i.e. perpendicular to the plane of incidence, in oriented fluid bilayers their respective LD signals are expected to be overall negative. The reverse holds for the CH3 symmetric stretching mode since its transition moment lies approximately parallel to the lipid acyl chains, and in oriented samples this band should give rise to positive LD (33). For presentation purposes, the same scaling was used to calculate the LD of the absolute absorbance spectra (in the dried and humidified state) as well as that of the light induced difference spectra of the sample. We emphasize, however, that the LD spectra were used only for visualization purposes; the actual calculations of the linear dichroism of specific bands were based on the original, unscaled, data.

3.2.1 Calculation of orientations from ATR-FTIR dichroism

The method applied here to determine the angle between the dipole moment of a specific vibrational mode corresponding to a dichroic band and the normal of the ATR internal reflection element, is essentially similar to that previously described (34–37). Briefly, a dichroic ratio, R_{ATR}, is defined as:

\[ R_{ATR} = \frac{\int A_{∥} (ν) dν}{\int A_{⊥} (ν) dν}, \]  

(3.1)

where \( \int A_{∥,⊥} (ν) dν \) are the integrated intensities of a certain vibrational band in the frequency range dν. From the dichroic ratio, and an estimation of the electric field amplitudes of the evanescent wave at the surface of the internal reflectance element (E_X, E_Y and E_Z; XY-plane parallel to the surface of the Ge element), an order parameter S is calculated for the orientation of the structural element (38):

\[ S = \frac{2(E_X^2 - R_{ATR}E_Y^2 + E_Z^2)}{[(3 \cos^2 \alpha - 1)(E_X^2 - R_{ATR}E_Y^2 - 2E_Z^2)]}. \]  

(3.2)

The average angle,  \( \theta \), between the main axis of symmetry of the structural element and the membrane normal, relates to S through the expression:

\[ S = \frac{1}{2} (3 \cos^2 \theta > -1). \]  

(3.3)

The order parameter is a time- and space-averaged function of  \( \theta \), which is symbolized with the brackets in equation 3.3. In equation 3.2, a scaling factor \( 1/S_\alpha = 2/(3 \cos^2 \alpha - 1) \) has been introduced to account for the angle \( \alpha \) between the transition dipole moment of the vibration and the main
3.2 Experimental Procedures

axis of symmetry of the structural element. Similar correction factors can be introduced to account for membrane disorder ($S_m$) and imperfect surface flatness ($S_f$) of the ATR crystal. However, here we take $S_m = S_f = 1$, i.e. basically a perfectly flat ATR surface and a perfectly oriented membrane multilayer is assumed. Note that due to this assumption we probably slightly underestimate the degree of orientation of the photoreceptor membrane segments (see results).

Electric field amplitudes were estimated based on the semi-infinite-bulk (or two-phase) approximation. In the case of unit incoming amplitude for both polarizations, the electric field amplitudes in the $X$, $Y$, and $Z$ direction (equation 3.2) are given by (39):

$$E_X = \frac{2\cos \phi \sqrt{\sin^2 \phi - n_{31}^2}}{\sqrt{(1 - n_{31}^2)[(1 + n_{31}^2)\sin^2 \phi - n_{31}^2]}}$$

$$E_Y = \frac{2\cos \phi}{\sqrt{1 - n_{31}^2}}$$

$$E_Z = \frac{2\sin \phi \cos \phi}{\sqrt{(1 - n_{31}^2)[(1 + n_{31}^2)\sin^2 \phi - n_{31}^2]}}$$

Here, $\phi$ is the angle of incidence between the IR beam and the Ge element (set to 45°, see e.g. Figure 1.6), and $n_{31} (= n_3/n_1)$ is the ratio between the refractive indices of the sample ($n_3 = 1.7$) (26) and the Ge crystal ($n_1 = 4$). Thus, throughout this work, we used $E_X = 1.38$, $E_Y = 1.56$ and $E_Z = 1.73$. The use of these estimates, specifically applying to the semi-infinite bulk case, is based on the assumption that the thickness of the sample [typically in the order of 10–20 µm (34)] is much larger than the penetration depth of the evanescent wave [about 1.4–2 µm in the spectral range from 2000–1000 cm$^{-1}$ (35)].

Data analysis. All spectral analyses were performed using the GRAMS/32 Spectral Notebase program suite (Galactic Industries, Nashua, NH). Dichroic ratios were determined from peak heights as well as from the integrated intensities of the bands in the absolute absorbance spectra. Baselines were calculated either between data-points limiting the integrating intervals, or a global (straight line) baseline correction was performed by zeroing the 3800, 2600, 1900 and 945 cm$^{-1}$ data-points. In addition, in the case of the amide I region, curve-fitting analyses were performed in order to resolve the relative contribution of the $\alpha$-helices to this band. For this, spectra were baseline corrected in the region from 1900–945 cm$^{-1}$, and a maximum of 13 bands were used to curve-fit the 1790–1415 cm$^{-1}$ region (40). The dichroic ratios corresponding to the $\alpha$-helical structures were determined from the integrated intensity of the band centered at 1655 cm$^{-1}$. For the analysis of the rhodopsin → Meta II difference spectra, dichroic ratios were determined from the integrated intensities of the difference bands. Integrating intervals were chosen such that only one dichroic difference band fell in that region, thus minimizing interference from overlapping bands. Throughout this work, the average or apparent tilt angle $\theta$ is calculated from equation 3.3 by equating $<\cos^2 \theta> = \cos^2 <\theta>$, i.e. by assuming an infinitely narrow distribution of TDM orientations contributing to the absorption band under study.
3.3 Results

Figure 3.1 compares the linear dichroism (LD) of an oriented ROS membrane film in darkness in the dried (a) and humidified state (b). The sample presented in Figure 3.1b was also used for the photoactivation experiments, presented in figures 2 and 3. Panels b1 and b2 derive from the same sample as shown in Figure 3.1a, and the same scaling factor ($c=1.70$) was used to calculate the LD spectrum in the humidified state ($LD = (//) − 1.70 \times (\perp)$). Panels b3 and b4 show the absorbance data from a sample in $D_2O$ ($c=1.54$). In all cases, the LD spectra are dominated by the positive (+) amide A (3287 cm$^{-1}$) and amide I (1654 cm$^{-1}$) contributions and negative (−) amide II (1545 cm$^{-1}$) contribution, typical for a net transmembrane orientation of $\alpha$-helical structures (26, 37, 41−43). Moreover, LD signals from the lipid moiety are observed at 1740 (−), 1219 (−) and 1060 cm$^{-1}$ (+). The latter bands are assigned to the phospholipid ester carbonyl stretch and the antisymmetric and symmetric PO$_2^-$ double bond stretch, respectively, and their relative dichroism is in agreement with previously published data on oriented phospholipid containing membranes (33, 34, 44). Clearly, the qualitative orientation of the dried membrane film is well preserved upon humidification with $H_2O$/ $D_2O$ buffer.

Hydrogen-deuterium exchange. As previously shown, the FTIR-ATR spectra of ROS in $D_2O$ exhibit a characteristic down-shift of the amide II band from 1545 to near 1450 cm$^{-1}$ (amide II') (45). The amide II mode is assigned to (primarily) N–H bending vibrations of peptide amide groups (46), therefore, the isotope induced shift of this band provides a measure of the fraction of peptide groups undergoing hydrogen-deuterium (H/D) exchange. The relative amount of peptides undergoing H/D exchange was estimated by comparing the ratio of the (residual) amide II band intensity to the amide I band intensity in $D_2O$, $w_D$, to that before H/D exchange in a dehydrated sample, $w_H$. Integrating intervals ranged from 1710–1587 cm$^{-1}$ and 1580–1520 cm$^{-1}$ for the amide I and amide II bands, respectively. The fraction of unexchanged peptides, $f_u$, was estimated from $f_u = w_D / w_H$ (47). Calculations using different approaches (choice of baseline, peak intensity, peak area etc.) indicate that 60 ± 9 % of the peptide groups did not exchange under the experimental conditions, in good agreement with previously reported values (45, 47, 48).

In contrast to the marked changes observed in the absolute absorption spectra in the $H_2O$ and $D_2O$ condition as a result of H/D exchange, the corresponding LD spectra (Figure 3.1 b) are found to be very similar. Significantly, the relatively intense amide II’ band is found to be essentially non-dichroic. Thus, similar to an earlier polarized FTIR study of bacteriorhodopsin (42), the combination of polarized light spectroscopy with H/D exchange is able to reveal more information on the structural origin of the exchanging groups of rhodopsin and shows that the latter are located in secondary structures showing little net orientation. Such a differential H/D exchange in rhodopsin was recently also proposed on the basis of unpolarized infrared measurements (45), and has also been observed in the early polarized infrared experiments of Michel-Villaz and coauthors (25) on oriented intact (frog) rod outer segments.
Figure 3.1: FTIR spectra of ROS membranes using either parallel (∥) or perpendicular (⊥) polarized IR light. (a) Dried ROS membranes, (b1–b4) humidified ROS membranes: (b1 and b2) ROS in H$_2$O buffer and (b3 and b4) ROS in D$_2$O buffer. Amide A, I and II bands are indicated in (a). LD denotes the difference spectrum between ∥ and ⊥, identifying dichroic bands (LD = (∥) − c × (⊥)). Factor c (see text) was 1.70 in panels a, b1 and b2, and 1.54 in panels b3 and b4. Traces shown are the average of 1280 scans. Note the differences in the frequency scale between (a) and (b) and the different absorbance scales for all panels. In all panels, the absorbance scales are those corresponding to the ∥-spectra. In (a) and panel b1, the LD signal has been scaled by a factor of 2 for better comparison.
3.3.1 Orientation of structural elements

In order to calculate the average orientation of the rhodopsin helix bundle with respect to the membrane normal, we used previously determined \( \alpha \) values, i.e. values for the angles of the transition dipole moments (TDMs) relative to the helix long-axis. For example, we used 35° for the amide I band, 75° for the amide II and II’ bands and 28° for the amide A band (34, 36). The lipid acyl chain orientation was calculated from the dichroism of the methylene \( \nu_s \) and \( \nu_{as} \) modes, setting \( \alpha = 90° \) (33, 36, 49). The lipid ester carbonyl band was analyzed using \( \alpha = 0° \) (49). The results of these calculations are shown in Table 3.1.

<table>
<thead>
<tr>
<th>Band</th>
<th>frequency (cm(^{-1}))</th>
<th>dried H(_2)O</th>
<th>D(_2)O</th>
<th>dried H(_2)O</th>
<th>D(_2)O</th>
</tr>
</thead>
<tbody>
<tr>
<td>amide A</td>
<td>3289</td>
<td>2.7</td>
<td>n.d.</td>
<td>3.50</td>
<td></td>
</tr>
<tr>
<td>( \nu_{as} ) (CH(_2))</td>
<td>2924</td>
<td>1.68</td>
<td>1.52</td>
<td>1.43</td>
<td>47</td>
</tr>
<tr>
<td>( \nu_s ) (CH(_2))</td>
<td>2853</td>
<td>1.68</td>
<td>1.41</td>
<td>1.34</td>
<td>47</td>
</tr>
<tr>
<td>( \nu_{C=O} ) lipids</td>
<td>1739</td>
<td>1.57</td>
<td>1.26</td>
<td>1.46</td>
<td>60</td>
</tr>
<tr>
<td>amide I</td>
<td>1654</td>
<td>2.24</td>
<td>2.24</td>
<td>2.20</td>
<td>50</td>
</tr>
<tr>
<td>amide II (^b)</td>
<td>1545</td>
<td>1.48</td>
<td>1.46</td>
<td>1.32</td>
<td>39</td>
</tr>
<tr>
<td>amide II’</td>
<td>1450</td>
<td>n.a.</td>
<td>n.a.</td>
<td>1.74</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Table 3.1: Values for the average dichroic ratio, \( R \), and the average angles \( \theta \) for selected vibrational modes of oriented photoreceptor membrane films in the dried (\( n = 4 \)) and humidified state (\( n = 2 \)). Standard deviations were smaller than 7% in \( R \), resulting in a smaller than 3° deviation in \( \theta \). \(^b\): There was a slight variability (see text) in the amide II results of 4, 5 and 7° in \( \theta \) for the dried and H\(_2\)O/D\(_2\)O humidified membranes, respectively. (n.d.): Not determined, (n.a.): not applicable.

The listed dichroic ratios are based on integrated intensities, using baselines calculated between data-points. Integrating intervals ranged from 3400–3225 cm\(^{-1}\) for the amide A band; from 2950–2903 cm\(^{-1}\) and 2867–2839 cm\(^{-1}\) for the methylene \( \nu_{as} \) and \( \nu_s \) bands; from 1769–1709 cm\(^{-1}\) for the lipid ester \( \nu_{C=O} \) band and from 1710–1587 cm\(^{-1}\), 1580–1520 cm\(^{-1}\), and 1500–1390 cm\(^{-1}\) for the amide I, amide II and amide II’ bands, respectively.

First, we note that the lipid acyl chain ordering seems to be slightly higher in the humidified membranes. This might reflect a partial bilayer to H\(_{II}\) phase transition in the phospholipid membranes upon dehydration, resulting in a decrease in dichroism of the methylene CH\(_2\) stretch bands. The coexistence of these two phases in dried ROS samples has been inferred from X-ray and electron microscopy (50, 51), as well as from \(^{31}\)P NMR studies on ROS membranes (52). Second, the average orientation of the rhodopsin helix-bundle in the photoreceptor membrane, obtained from the dichroism of the amide bands in the dried/and H\(_2\)O/D\(_2\)O humidified state (45 ± 5 / 44 ± 7 / 40 ± 8°) is in good agreement with
values of $40 \pm 6^\circ$ and $38^\circ$ obtained from polarized infrared transmission spectroscopy on dried samples (26) and from measurements on intact rod outer segments (25), respectively. Third, the orientation calculated from the dichroism of the amide II and amide II band in D$_2$O, indeed suggests that the peptide groups which were subject to H/D exchange are located in protein segments exhibiting considerable less net orientation (see above). Having separated the distinct contributions by deuteration, we believe that the best estimate for the overall orientation of rhodopsin should be rather based on the results obtained for the amide A and II bands in D$_2$O buffer. Hence, we conclude that the average tilt angle of the seven helical segments in rhodopsins membrane domain is at $30 \pm 40^\circ$ with respect to the membrane normal, while the accessible surface regions have a more random distribution.

The above mentioned qualitative results were found to be only weakly dependent on the choice of the input parameters ($S_{m,f}$, $n_3$ and $\omega$) and the way the spectral parameters were extracted (integrated vs. peak intensities, curve-fitting; not shown). In some cases (amide A, amide II) a slight dependence on the choice of the baseline was observed, however, this variability ($< 7^\circ$ in $\theta$) does not affect the general trend of our data. We further note that the internal consistency of the orientations calculated from the amide band dichroisms and the good correspondence to the literature values indicates that the most important assumption in our analysis, i.e. the estimation of the electric-field amplitudes using the two-phase approximation, is indeed valid for both hydrated and dehydrated membranes. Importantly, this gives us a realistic basis to extend our analysis to the case of rhodopsin $\rightarrow$ Meta II difference spectra.

### 3.3.2 Polarized infrared difference spectroscopy

Figure 3.2 shows the difference spectra of the Rho $\rightarrow$ Meta II transition recorded with the IR beam polarized either parallel or perpendicular to the plane of incidence. Since these spectra are calculated by subtracting a rhodopsin spectrum from a Meta II spectrum, positive bands represent Meta II and negative bands rhodopsin. These spectra reflect changes of the retinylidene chromophore, which undergoes an 11-cis to all-trans conformational change, as well as structural changes of the protein including those required to expose signaling sites for the G-protein transducin (7, 10, 12). For example, negative bands at 1558, 1237, 1214, 1190, and 969 cm$^{-1}$ have been assigned to the retinylidene chromophore in rhodopsin upon comparison with resonance Raman (53, 54) and FTIR analyses of rhodopsin analogs containing isotope labeled chromophores (10, 55). In contrast, few bands have been assigned thus far to specific protein residues except in the 1700–1800 cm$^{-1}$ region (C=O stretch modes of Asp and Glu carboxylic acid groups) (14, 16, 18, 23). The LD spectrum has been calculated using a scaling factor of 1.71, and the spectrum recorded with perpendicular polarization has been scaled with the same factor to allow easier visual comparison. The traces shown represent the average of 2–4 independent experiments, in order to compensate for slight variations in the baseline. However, we note that the band profiles in the LD spectrum, reflecting the dichroism of the difference bands, was reproducibly observed in every experiment. Obvious differences between the polarized difference spectra are best observed in the LD spectrum.
Figure 3.2: Polarized FTIR difference spectroscopy of the Rho $\rightarrow$ Meta II transition. Traces shown are the averaged results of 4 samples. $LD = (\parallel) - c \times (\perp)$. The $\perp$-spectrum has been scaled with a factor 1.71 for better comparison. The absorbance scale is for the $\parallel$-spectrum. The inset shows the averaged result of two samples in the 3500–3100 cm$^{-1}$ region; the scaling is similar to that in the main panel.

The relatively strong LD bands at 1657 (+) and 1537 cm$^{-1}$ (−) can be seen to derive from positive contributions in the parallel and perpendicular polarized spectra, respectively. On this basis, these bands may be assigned to the Meta II intermediate. The
dichroism of this pair qualitatively agrees with the amide I and amide II LD characteristics of \(\alpha\)-helical structure, cf. Figure 3.1, and may thus reflect a small reorientation of helical segment(s) upon activation of rhodopsin. The positive band observed at 3275 cm\(^{-1}\), which lies in the amide A region (assigned to peptide NH stretch vibrations) (56) supports this assignment. This band shows significant overlap with a negative band at 3308 cm\(^{-1}\), which is prominently present in the \(/\)-polarized spectrum only, reducing the intensity of the 3275 cm\(^{-1}\) band. On the whole, these data suggest a slight reorientation of helical segments in the photoactivation step of rhodopsin, resulting in a smaller angle between the \(\alpha\)-helix axis and membrane normal in the Meta II intermediate.

Bands appearing in the 1700–1800 cm\(^{-1}\) region are highly characteristic of C=O stretching modes in esters and protonated carboxylic acid groups in Asp or Glu side chains. By utilizing rhodopsin reconstituted into an ether-phospholipid which lacks the ester carbonyl group it was shown that contributions from phospholipids in this region of the spectrum is unlikely (12). Indeed, the bands observed in the 1780–1710 cm\(^{-1}\) range in the Rho \(\rightarrow\) Meta II difference spectrum have thus far all been tentatively assigned to specific Asp or Glu residues on the basis of mutagenesis data. In this way, the 1767 (−) / 1745 (+) and the 1730 (−) / 1745 (+) pairs have been assigned to the Asp83 (14, 16) and putatively to the Glu122 (16, 18) COOH group, respectively. Part of the positive band at 1713 cm\(^{-1}\) has been assigned to Glu113 (57), the counterion to the protonated Schiff base. According to this assignment Glu113 bears a carboxylate group in rhodopsin and the corresponding vibrations have been tentatively assigned to bands at 1590 and 1395 cm\(^{-1}\) (57).

Interestingly, the major band observed in this region of the LD spectrum is at 1736 cm\(^{-1}\) (−), at a position where a specific band has not yet been detected in the unpolarized difference spectrum of the Rho \(\rightarrow\) Meta II transition. Furthermore, this LD band is sensitive to H/D exchange as discussed below, and is therefore assigned to a protein carboxyl group. Only much smaller LD bands are observed at the position of the Asp83 bands (1767/1745 cm\(^{-1}\)) indicating that this group is not strongly immobilized or is located near the magic angle (54.6°) where the dichroism is 0°. Two strongly dichroic bands are also observed at 1407 and 1583 cm\(^{-1}\), in the frequency ranges expected for the symmetric and asymmetric stretching vibrations of a carboxylate group. Combination of the latter two bands with the band at 1736 cm\(^{-1}\) could represent either the deprotonation or the protonation of a previously undetected Asp or Glu residue.

A moderately strong LD band is also observed at 1247 cm\(^{-1}\) (−). In a previous paper, the role of tyrosines in the Rho \(\rightarrow\) Meta II transition has been specifically addressed using stable-isotope labeling [ring-deuterated tyrosine, see chapter 2 (24)]. Among other things, it was found that there is a negative band at 1248 cm\(^{-1}\) assignable to the bending vibration of a tyrosine hydroxyl function in rhodopsin. The 1247 cm\(^{-1}\) LD band observed in the present study could therefore represent a reorientation of this tyrosine side-chain upon photoexcitation. We are presently in the process of incorporating single-\(^{13}\)C-labeled tyrosine into rhodopsin; this should facilitate definite assignment of tyrosine vibrations to specific tyrosine modes.

The difference spectrum taken with parallel polarization further shows an additional
band at 939 cm\(^{-1}\), which lies within a region characteristic of hydrogen-out-of-plane modes. In previous FTIR studies (55), using rhodopsin analogs containing a stable-isotope labeled chromophore, bands at 947 and 950 cm\(^{-1}\) have been identified to derive from the retinal HC\(_{11}\)=C\(_{12}\)H \(\text{A}_{\alpha}\) HOOP combination in lumirhodopsin and Meta I, respectively. In Meta II, a band assignable to this mode has never been reported. This vibration is the best candidate for the 939 cm\(^{-1}\) (\(+\)) band of Meta II. The presence of this band only in the spectrum taken with parallel polarization would then indicate a significant change in orientation of the HC\(_{11}\)=C\(_{12}\)H segment during the final activation step of rhodopsin. A definitive assignment, however, requires the use of selectively labeled chromophores, and this work is in progress.

In Figure 3.3, the polarized difference spectra recorded in \(\text{H}_2\text{O}\) are compared to those obtained in \(\text{D}_2\text{O}\) buffer. As in the case of the absolute absorbance spectra, the difference spectra also exhibit characteristic isotope induced band shifts (16, 17). Overall, the net intensity of the LD spectrum in \(\text{D}_2\text{O}\) is significantly smaller than that in \(\text{H}_2\text{O}\). For example, the bands in the amide A, I and II regions (3275 (+), 1657 (+) and 1537 cm\(^{-1}\) (−), respectively) are reduced in intensity. Assuming the above assignment of these bands to amide modes is correct, the H/D sensitivity of the amide A and II bands indicate that the involved helical structures are positioned in a region accessible to H/D exchange, implying a localization near the membrane surface. The loss of intensity in the amide I region, however, is somewhat puzzling. Clearly, the reduced sensitivity to the polarization of the IR probe beam cannot be ascribed to a reduced net orientation of the samples (cf. Table 3.1). Indeed, the orientation obtained from the bands assigned to D83 in \(\text{D}_2\text{O}\) was found to be the same as in \(\text{H}_2\text{O}\) (see below). On the other hand, additional H/D exchange triggered by bleaching, extensively discussed in a previous paper (45), produces additional difference bands near 1660/1632 cm\(^{-1}\). Therefore, we currently believe that the reduced 1657 cm\(^{-1}\) LD contribution in \(\text{D}_2\text{O}\) is rather caused by mixing with down-shifted, non-helical, amide I contributions.

The dichroic difference spectra in Figure 3.3 further indicate that the 1736 (−) band shifts down to ± 1720 cm\(^{-1}\) in \(\text{D}_2\text{O}\), effectively cancelling the positive, H/D insensitive part of the peak at 1713 cm\(^{-1}\). The H/D sensitive part of the band at 1713 cm\(^{-1}\), assigned to E113, shifts down to 1705 cm\(^{-1}\), in agreement with earlier studies (57). Notably, the 1407 and 1583 cm\(^{-1}\) bands are still present in the \(\text{D}_2\text{O}\) data, whereas the 1736 cm\(^{-1}\) band clearly shows an isotope induced shift, in general agreement with the assignment discussed above.

### Intramolecular orientations from polarized difference spectroscopy

Table 3.2 shows the apparent orientations calculated from the dichroism of selected bands in the \(\text{H}_2\text{O}\) data-set, taking \(\alpha = 0^\circ\). Integrating intervals were chosen such that only one dichroic difference band (LD) was located in that region. Since the bands in the 1630–1680 and 1550–1490 cm\(^{-1}\) regions are prone to lead to erroneous results due to extreme overlap, they were not taken into account. For similar reasons, we were not able to determine the dichroic ratio of the 1736 cm\(^{-1}\) and 1583 cm\(^{-1}\) bands. The dichroic ratio of the 1407 cm\(^{-1}\)
Figure 3.3: Comparison of the FTIR difference spectra of the Rho \( \rightarrow \) Meta II transition in \( H_2O \) or \( D_2O \) (thicker lines). LD spectra were calculated using subtraction factors of 1.70 (\( H_2O \)) and 1.58 (\( D_2O \)), and the corresponding \( \perp \)-spectra were scaled using the same factors. All traces are shown on the same scale; the 1780–1680 cm\(^{-1}\) region of the LD spectra have been expanded on the y-scale for clarity. The inset shows the LD spectra in the 3500–3100 cm\(^{-1}\) region; the scaling is similar to that in the main panel.

band was found to be 4.8 \( \pm \) 0.1, from which we calculated that its transition moment lies at 38\(^\circ\) with respect to the membrane normal.
Table 3.2: Dichroic ratio, R, and orientation angle θ, for selected absorption bands in the rhodopsin Rho → Meta II transition in H₂O buffer (n = 4, except for the 3308 cm⁻¹ band: n = 2). All analyses were performed using α = 0°. Standard deviations were smaller than 3°, except for the 1748 cm⁻¹ band (7°).

As an additional check on sample orientation in D₂O, orientations were calculated from the dichroism of the 1757 (−) / 1740 (+) pair, assigned to Asp83. Here, it was assumed that the loss of coupling of the O–H bending mode to the C=O mode has a negligible effect on the orientation of the C=O TDM (58). Orientations of 56° (1757 cm⁻¹ band) and 57° (1740 cm⁻¹) were obtained for the D83 side chain C=O band in rhodopsin and Meta II, respectively, i.e. essentially identical to those obtained from the data in H₂O.

3.4 Discussion

In this paper, we report the first application of polarized ATR-FTIR difference spectroscopy to investigate the orientation of individual groups in bovine rhodopsin. Previously, only the macroscopic orientation of rhodopsin in its native membrane has been investigated using polarized transmission FTIR spectroscopy (26). In the case of bacteriorhodopsin infrared linear dichroism studies, in combination with difference spectroscopy, already provided detailed information on intramolecular orientations including the orientation of the retinylidene polyene plane relative to the membrane plane and specific protein groups (58–62).

In infrared LD measurements based on transmission spectroscopy, a tilt angle series is normally conducted where the sample plane is tilted with respect to the direction of incident radiation (26, 60, 63). Extensive data acquisition and averaging is often necessary.
in these experiments since the dichroism tends to be small at low angles. However, for analysis of \textit{Rho} $\rightarrow$ \textit{Meta II} difference spectra, which are essentially obtained from a single photolytic reaction, a tilt series analysis is not readily accomplished on the same sample. This problem does not exist in the ATR-mode, where both polarization angles can be alternately monitored. The ATR technique offers the additional advantage over transmission spectroscopy that the sample is probed with the maximal difference in polarization of $90^\circ$ with respect to the sample-plane, whereas in transmission mode the maximal tilt angle usually is about $60^\circ$. This is an important advantage since it significantly increases the sensitivity of the measurements.

**Macroscopic orientation and H/D exchange.** The macroscopic orientation of rhodopsin in its native disk membrane was determined and compared to literature data in order to check on the validity of the method of analysis. The membranes were studied in both the dried and humidified state (H$_2$O and D$_2$O). The present estimate of $45 \pm 5^\circ$ (Table 3.1) for the average orientation of rhodopsins helix bundle is in good agreement with the previously reported value from polarized transmission experiments on (dried) oriented samples (26).

When combined with hydrogen-deuterium exchange polarized absorbance spectra can also provide information on the regions of the protein most accessible to the aqueous phase. It is found that the inaccessible helical core of the protein has an average orientation of 30–40$^\circ$ with respect to the membrane normal, while the more accessible parts have a more random distribution. These results substantiate the predictions based on previously reported unpolarized infrared studies (45).

**Polarized IR difference spectroscopy.** The \textit{Rho} $\rightarrow$ \textit{Meta II} difference spectra obtained with the polarization of the IR light perpendicular to the plane of incidence most closely resemble the unpolarized transmission mode difference spectra previously reported from our laboratories (12, 14, 17, 18, 24, 64). This is not unexpected, since, in transmission experiments, the polarization of the incident light will be in the plane of the membrane for the well oriented samples we routinely use. The photoreceptor membrane samples we use for transmission FTIR are routinely prepared by spin-drying a photoreceptor membrane suspension onto an IR transparent substrate, which yields well-oriented membrane films (65–67). On the other hand, the parallel polarized difference spectrum is obtained using a polarization direction that should not be present in transmission measurements on oriented samples. Indeed, these difference spectra exhibit several new bands such as the one at 939 cm$^{-1}$, which we tentatively assign to the HC$_{11}$=C$_{12}$H hydrogen-out-of-plane (HOOP) mode of the all-trans retinylidene chromophore in Meta II.

**Orientation of the chromophore.** The present work yields information on the relative orientation of specific groups in the retinylidene chromophore. The data in Table 3.2 show, that the C=C and C–C TDMs for the chromophore polyene chain in rhodopsin have tilt angles in the range 56–75$^\circ$, in qualitative agreement with visible LD studies indicating that the electronic dipole moment of the 498 nm transition, oriented approximately along
a vector connecting the Schiff base nitrogen and the ionone-ring portion, lies at 16° with respect to the membrane plane (27). It should be noted, however, that obtaining global chromophore orientations from individual chromophore bands relies on accurate knowledge on the orientation of the infrared dipole moment (which may be the sum of various contributing normal modes) relative to the retinal plane. Mixing of normal modes may cause the net vibrational TDM of the band to deviate from the C–C or C=C bond-directors (58, 60–62). Furthermore, the retinal moiety in rhodopsin adopts a skewed 11-cis conformation and exhibits considerable twists around the carbon-carbon bonds in the isomerization region (C_{10}· · ·C_{13}) (68, 69). Due to these twists, the HOOP modes may mix with in-plane modes (e.g. C=C and C–C stretch and C–CH bending modes) by which they loose their pure out-of-plane character (58, 61, 62). More quantitative analyses of the retinal conformation therefore must await further theoretical (normal-mode) and experimental efforts.

It has been suggested from visible dichroism studies (28), that the chromophore in Meta II transiently adopts a more in-plane orientation of 5°±4°. However, we could not address such overall effects, since, apart from the newly assigned band at 939 cm^{-1}, few bands have been identified to derive from the all-trans chromophore in Meta II. Using 13C labeled retinals, we are currently trying to identify these bands which should facilitate orientation studies of the chromophore in this late phase of the photocascade.

The discovery of a new band in the Rho → Meta II difference spectrum deserves special mention. The intensity of the 939 cm^{-1} (+) band is significantly lower than that of the HOOP bands in lumirhodopsin and Meta I, where it is approximately as intense as the 969 cm^{-1} (−) rhodopsin band. This may be due to the more relaxed local structure, or to secondary effects caused by the deprotonation of the Schiff base linkage in the Meta I to Meta II transition (55). In any case, if the assignment of this band to an A_u-HOOP mode proves to be correct, the fact that in Meta I this mode strongly responds to perpendicular polarized light (i.e. obvious in ″unpolarized″ FTIR difference spectra of oriented membrane films), but in Meta II only to parallel polarized light, indicates that the HC_{11}=C_{12}H group significantly reorients during this transition. Possibly, this represents a second essential conformational change in the chromophore, permitting proton-transfer from the Schiff base and finally leads to the rearrangements in the protein necessary to expose its signaling sites. This would agree with the observation that an additional methyl group at the 10-position of the retinal strongly retards the Meta I to Meta II transition (64).

**Intramolecular (re-)orientation of protein groups during photoactivation.** The highest sensitivity to the direction of polarization in the Rho → Meta II transition was observed in the amide regions. In the linear dichroic difference spectrum, the amide I contribution is strongly positive whereas that in the amide II region is negative. The polarized difference spectra indicate that these bands stem from the Meta II intermediate. A corresponding positive amide A band may be located at 3275 cm^{-1}, although this band seems to be largely obscured by a strong band at 3308 cm^{-1}, possibly due to a specific peptide-bond NH stretch mode. The overall appearance of these bands in the dichroic difference spectra is strikingly similar to those characteristic of oriented helical structures (cf. the absolute spectra...
in Figure 3.1). Hence, the most direct interpretation is that these bands reflect a small reorientation of helical structure, and the pattern observed is consistent with the net tilting of α-helical structures towards the membrane normal during the Rho → Meta II transition. In a previous paper (24), we discussed that the fully conserved Pro/Tyr pair in helix 6 in all visual pigments may be acting as a hinge for movements in this helix in response to chromophore isomerization. Possibly in relation to this, the present work provides evidence for a tyrosine mode (1247 cm⁻¹) which changes its orientation in the Rho → Meta II transition. Overall, this agrees with and complements previous results, obtained from electron paramagnetic resonance spectroscopy on site directed spin labeled rhodopsin, which were interpreted to reflect rigid body movement involving parts of helix 3 and/or 6, resulting in a more expanded structure of the cytoplasmic side of rhodopsin in the activation step (70).

FTIR difference spectroscopy has been especially successful in identifying changes in the hydrogen bonding environment and protonation state of residues carrying carboxylic acid groups (e.g. Asp83, Glu113 and Glu122, see Results). The highly conserved Glu134 has been associated with light dependent proton uptake and signaling in rhodopsin (31) and, therefore, is also expected to become protonated in the activation step. However, to date, bands assignable to this protonation reaction could not be identified from FTIR studies on E134 mutants (16, 18). We now suggest that the strong bands observed in the dichroic difference spectrum at 1736 (carboxyl), 1583 and 1407 cm⁻¹ (carboxylate asymmetric and symmetric stretch, respectively) may represent the protonation of E134 in the Rho → Meta II transition. It would also follow that this group has restricted mobility and is oriented towards the plane of the membrane. In unpolarized difference spectra this band is probably obscured by the other strong carboxyl bands. Although definite assignment will require further studies involving site-directed mutagenesis, additional support comes from a recent study describing ATR-FTIR studies on complex formation between bovine rhodopsin and transducin, and transducin derived peptides (71). A band at 1735 cm⁻¹ was clearly observed in the double difference spectrum representing the Rho → Meta II transition in the absence or presence of transducin, and was tentatively assigned to protonation of Glu134 induced by transducin binding. The protonation of Glu134 does not depend, however, on the presence of transducin (31), and we rather propose that the band observable upon complex formation with transducin may reflect a reorientation of the carboxyl group of Glu134 protonated at an earlier stage, rather than the protonation of this residue.

Conclusion. In this work, we demonstrate the potential of polarized ATR-FTIR difference spectroscopy to obtain highly detailed information on intramolecular orientations in rhodopsin. Evidence is presented for a small reorientation of helical segments in the activation of rhodopsin as well as for the protonation of a previously undetected carboxyl-group-bearing residue. Furthermore, the dichroic properties of the bands in the HOOP region suggest a localized reorientation in the retinal polyene tail in the late phase of photoactivation. Significantly, the use of polarized probe light yields additional resolving power

¹These bands may have escaped detection in the difference spectra of mutants E134D and E134R because of the severe overlap in these regions (18).
and facilitates the analysis of component bands in spectral regions showing considerable overlap. Future perspectives include studies on oriented membranes of rhodopsin analogs and recombinant rhodopsin, which are in progress. In combination with site-directed mutagenesis and (site-directed) isotope labeling, this will allow both identification as well as orientation studies of additional protein groups involved in the activation process of rhodopsin.

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References

64. DeLange, F., Bovee-Geurs, P. H. M., VanOostrum, J., Portier, M. D., Verdegem, P. J. E., Lugtenburg,
Chapter 4

An additional methyl group at the 10-position of retinal dramatically slows down the kinetics of the rhodopsin photocascade


This chapter focuses on ligand-protein interactions in the rhodopsin analog generated from bovine opsin and the 10-methyl homolog of 11-cis-retinal. The resulting pigment displays a reduced α-band at 506 nm and a stronger β-band at 325 nm. Remarkably, the rotational strength of these bands was found to be similar for both native and 10-methyl rhodopsin. The quantum yield of the analog pigment was determined to be 0.55. The batho intermediate of the 10-methyl pigment is stable up to 180 K, and only slowly decays to the next intermediate between 180 and 210 K. As in native rhodopsin, the 10-methyl metarhodopsin I intermediate is generated at about 220 K, but its transition to the metarhodopsin II state is again shifted to a much higher temperature (> 293 K). Infrared analysis, nevertheless, shows that the conformational changes in the photointermediates of the 10-methyl pigment are basically identical to those observed in the native pigment. This is supported by a signal function assay, showing that the analog pigment is able to activate transducin. The dual effect of the 10-methyl group on the photocascade is attributed to steric interactions which, initially, hamper the relaxation of strain in the polyene chain of the chromophore and, eventually, interfere with the conformational rearrangements of the protein moiety required to adopt the active conformation of the receptor. These data provide direct support for the concept that the relaxation of strain in the retinal polyene chain acts as the major driving force of the photocascade dark reaction.
4.1 Introduction

Rhodopsin is the photosensor in the vertebrate retinal rod cell and consists of the protein opsins and a photosensitive group (11-cis-retinal), linked via a protonated Schiff base to Lys296 of opsin. Photoexcitation of rhodopsin initiates a series of at least four discrete, structurally and spectrally distinct, intermediates. The light dependent reaction, isomerization of the retinal group to the all-trans conformation, generates the first well-characterized photoproduct: bathorhodopsin. The subsequent dark reaction (batho- to lumirhodopsin and subsequently to metarhodopsin I and II) involves conformational rearrangements in both chromophore and protein. This entire process, called photocascade, culminates within milliseconds after illumination in the active conformation, metarhodopsin II (Meta II), which binds and activates the G-protein transducin (1, 2).

To date, the most detailed structural information on the photocascade of rhodopsin has been obtained by Resonance Raman (RR) and Fourier transform infrared (FTIR) spectroscopy. FTIR difference spectroscopy of the various transitions proved to be extremely powerful since it monitors structural changes in both chromophore and protein which are accompanied by shifts in vibrational frequency, band width or band intensity. Site directed mutagenesis and isotope labeling allowed identification of some of the difference bands in these spectra (3, 4, 5). In this way, it was shown that the majority of peaks in the Rho → Batho difference spectra derive from the chromophore, while the subsequent transitions present a gradual increase in protein activity (6, 7).

The intense hydrogen-out-of-plane (HOOP) vibrations in the Rho → Batho FTIR difference spectra are correlated with those observed in RR spectra of (batho-)rhodopsin and are regarded to reflect twisting around the single bonds in the chromophore induced by steric ligand-protein interactions (8, 9, 10). Torsional strain in the chromophore and charge separation between the Schiff base and its counterion have been proposed as potential mechanisms to store the photon energy in the Rho → Batho transition (11, 12, 13). Recent work has shown that out-of-plane distortions in the retinal polyene chain, apart from being a highly efficient means of energy storage at the Batho stage, also may contribute to the ultrafast isomerization kinetics in rhodopsin (14, 15). Moreover, since both RR and NMR data show that the environment of the Schiff base does not change significantly in the transition from Rho → Batho (16, 17) the concept that the photon energy is stored as torsional strain in the retinal polyene chain is gradually gaining support. The picture emerges that, at the batho stage, close contacts occur between the isomerized ligand and opsin which hold the former in a strained conformation and function as “contact points” by which the torsional strain in the polyene chain is mediated to drive the conformational changes in the protein moiety.

Potential candidates to mediate steric interactions between opsin and retinal are the protruding retinal side-chain methyl groups. Single demethyl analogs of rhodopsin (9-demethyl and 13-demethyl) have been examined before and show distinct effects on rhodopsin functionality [see for recent reviews (18, 19)]. We have taken the alternative approach and are in the process of evaluating the effects of single methyl group additions. We have started to analyze one of the most critical positions: C_{10} (Figure 4.1). Although the 10-
methyl homolog of 11-cis retinal has earlier been shown to produce a pigment (20), effects of the additional methyl group on the photocascade have never been reported. The data presented here show that the extra methyl group has a two-fold effect on rhodopsin’s photocascade: firstly, it stabilizes the strained conformation of the all-trans chromophore up to 210 K and, secondly, it interferes with the conformational rearrangements in the receptor necessary to adopt the active conformation (Meta II), which binds and activates transducin. These results emphasize the importance of finely tuned ligand-protein interactions to guide the photocascade of rhodopsin.

4.2 Experimental Procedures

The synthesis of 10-methylretinal was accomplished as described (21).

*Generation of 10-methyl rhodopsin.* Bovine rod outer segments in the opsin form (opsin membranes) were prepared from fresh, light-adapted, eyes as described (22). The regeneration capacity of these preparations was estimated from the $A_{280}/A_{500}$ ratio obtained upon subsequent incubation with a threefold excess of 11-cis-retinal, whereby a ratio of 2.0 was taken to represent membranes with maximal rhodopsin content. Rhodopsin and 10-Me rhodopsin were prepared from opsin membranes showing a regeneration capacity in the range 90–100%. All manipulations were done under dim red light (> 620 nm, Schott RG620 cutoff filter).

Analog pigments were generated by incubating opsin membranes with a 3-fold molar excess of 11-cis-10-methylretinal at room temperature. Opsi membranes were suspended in buffer A (20
photocascade of 10-methyl rhodopsin

Mm Pipes, 130 Mm NaCl, 5 Mm KCl, 2 Mm CaCl2, 0.1 Mm EDTA, 1 Mm dithioerythreitol, pH 6.5), to a final concentration in opsin of 100 µM. The retinals were added in a small volume of dimethylformamide (≤ 2% final volume) and the incubation was performed in an inert atmosphere (nitrogen or argon). Excess retinal was then converted into the oxime by addition of hydroxylamine to a final concentration of 10 Mm. To remove the large excess of oxime a novel procedure was developed. Extraction with 10 Mm solutions of heptakis (2,6-di-O-methyl)-β-cyclodextrin (Aldrich), which produces a soluble inclusion complex with retinals and retinaloximes, removes 70–80% of the excess retinoid (23). This procedure is almost as efficient as extraction by high concentrations of bovine serum albumin, but does not leave a difficult to remove protein residue nor does it affect the functionality of rhodopsin (VanOostrum and DeGrip, unpublished). To further reduce residual oxime, higher cyclodextrin concentrations were employed, but this also leads to partial lipid extraction. Three extractions with 50 Mm β-cyclodextrin in buffer A (4 °C) followed by washing twice with doubly distilled water removed over 95% of the excess retinal according to HPLC-analysis (24). To restore the native lipid/protein ratio, the resulting pigments were then dissolved in 20 Mm nonylglucose in buffer A and mixed with a 50 fold molar excess of retina lipids isolated as described (25). Reconstitution in proteoliposomes was accomplished by stepwise dilution (26). The resulting proteoliposomes were finally washed with doubly distilled water and stored at -80 °C until further use.

Spectral properties of the 10-methyl pigment. UV/visible spectra of proteoliposomes suspended in buffer A (either Mes, Pipes or Bis-Tris Propane, pH as indicated) to a final concentration of 1 µM in pigment were recorded on a Perkin Elmer λ15 double beam spectrometer equipped with an end-on photomultiplier detector. A circulating bath was used to control sample temperature. The sample was bleached with yellow light (Schott OG530 cutoff filter). The wavelength of maximal absorbance (λmax) of rhodopsin and the 10-Me analog was determined from the peak position in the difference spectrum obtained by subtracting the spectrum after illumination from the dark state spectrum, both taken in the presence of 10 Mm hydroxylamine in mixed micellar solution (20 Mm DoM in buffer A). The metarhodopsin I – metarhodopsin II equilibrium was studied in proteoliposomes as described for rhodopsin [chapter 5 (27)].

Circular Dichroism. UV/visible circular dichroism spectra were recorded for solubilized pigment samples (40 Mm DoM in buffer A, 10 °C) in order to compare the overall conformation of the 10-methyl chromophore with that of the native chromophore in the binding pocket of opsin. Solutions, containing about 22 µM pigment, were analyzed on a Jasco J-715 spectropolarimeter. Calibration of the instrument was checked before and after the measurements using a 0.06% (w/v) aqueous solution of d-10-camphor sulfonate.

Photosensitivity of the 10-methyl pigment. The quantum yield of 10-Me rhodopsin was determined relative to that of rhodopsin (28–30). The quantum yield (ΦRh = 0.67) and extinction coefficient at λmax (εRh = 40.600 ± 500 M−1 cm−1) of rhodopsin are both well characterized experimentally (28). Rhodopsin and 10-Me rhodopsin were solubilized in buffer A (Pipes, pH 6.5) with 10 Mm DoM and 10 Mm hydroxylamine to give an absorbance at 497 nm of 0.16 ± 0.007 OD/cm. Solutions were kept at 10 °C and illuminated under low bleaching conditions through a 497 ± 5 nm interference filter. The illumination conditions were such that the half-time of pigment bleaching was between 30–60 min. Spectra were taken as function of illumination time until the absorbance
at 497 nm was below 3% of its initial value. The half-time of pigment (absorbance) decay in the dark, providing a measure of pigment stability under the described experimental conditions, was determined to be > 20 h.

A plot of the function \( \log \left[ \frac{I_f}{(I_f - I_t)} \right] \) versus illumination time was shown to yield a straight line of which the slope \((S)\) is a measure of the photosensitivity \((\varepsilon \Phi)\) of a visual pigment \((28)\). Here, \(I_t\) is the excitation light transmission as a function of time and \(I_f\) is the transmission after complete bleaching. Since in our experiments absorbance data were collected, we converted the above function to \(A_f - \log (10^{A_t} - 10^{A_f})\). Here, \(A\) denotes the absorbance of the sample at the excitation wavelength. The quantum yield of the 10-methyl pigment \((\Phi_{An})\) was calculated from equation 4.1:

\[
\Phi_{An} = \frac{S_{An} \varepsilon_{Rh}}{S_{Rh} \varepsilon_{An}} \Phi_{Rh}
\]

As a control, a similar analysis was performed on isorhodopsin (opsin regenerated with 9-cis retinal). For the extinction coefficient of isorhodopsin at \(\lambda_{max}\) we used \(\varepsilon_{Iso} = 43.000 \text{ M}^{-1} \text{ cm}^{-1} \) \((31)\).

FTIR spectroscopy. FTIR analyses were performed on a Mattson Cygnus 100 spectrometer equipped with a liquid nitrogen cooled, narrow band, HgCdTe detector. Spectra were taken at 4 cm\(^{-1}\) resolution unless stated otherwise. Sample temperature was computer controlled using a variable temperature cell (Graseby Specac), which was evacuated and had a set of NaCl windows in the infrared light path. Samples, prepared by isopotential spin-drying 2–3 nmol of pigment on an AgCl window (Fisher Scientific Co.), were rehydrated and sealed using a second AgCl window and a rubber O-ring spacer as described \((3, 27, 32)\).

Samples were illuminated in the spectrometer using either a 20 W halogen lamp, equipped with a KG1 infrared filter (Schott Mainz, F.R.G.) in combination with a 497 ± 5 nm interference filter, or a modified (150 W halogen) fiberoptics ring illuminator (Schott) equipped with a filterwheel carrying a set of cutoff filters. Typically eight spectra (256 scans each, ∼65 s acquisition time per spectrum) were taken, and averaged, of the dark state receptor and, subsequently following illumination, of a particular photointermediate state. Difference spectra were calculated by subtracting these blocks of spectra.

To analyze the successive phototransitions of rhodopsin and the 10-Me analog, we selected the following illumination scheme. At 90 K, native and analog samples were illuminated for 40 seconds with light of 497 nm. The batho intermediates could be completely photoreversed by illuminating the sample for 130 seconds with light of \(\geq 610\) nm (Schott RG610 cutoff filter), as evidenced from the successive difference spectra. Subsequently, in the case of rhodopsin, the temperature was adjusted to 180 K and by illuminating the sample again with light of 497 nm (40 s) a stable Lumi photointermediate was produced. ‘Dark state’ rhodopsin was recovered from this Lumi sample by cooling down to 90 K and subsequent irradiation with red light for at least three minutes (RG610). From here, either the Lumi measurement was repeated or metarhodopsin I was produced by heating to 253 K and irradiation with yellow light (OG530) for two minutes. Metarhodopsin II (OG530, two minutes) was measured at 283 K on a separate sample at 8 cm\(^{-1}\) resolution (interferograms were doubly zero-filled prior to further processing yielding an apparent resolution of 4 cm\(^{-1}\)). A similar approach was used to collect the analog pigment data.
Signal transduction. Transducin activation was monitored using a fluorescence assay as described (33, 34). The enhanced intrinsic fluorescence of activated, i.e. GTP bound, bovine transducin was recorded on a Shimadzu RF-5301PC spectrofluorophotometer (excitation: 295 nm, bandwidth 1.5 nm; emission 337 nm, bandwidth 15 nm). Measurements were carried out at pH 7.4 and 20 °C in a buffer containing 20 mM HEPPS, 100 mM NaCl, 2 mM MgCl₂, 1 mM DTE and 0.01 % (w/v) DoM; the final volume was 2 ml and contained 100 nM transducin and 5 nM of (10-methyl) rhodopsin. A hypotonic extract of isotonically washed rod outer segments served as the source for transducin (35). Immediately before data acquisition the reaction mixture was bleached for 5 minutes in bright white light. After reaching a steady fluorescence level, GTP-γ-S (Boehringer) was added to a final concentration of 2.5 µM and the subsequent increase in tryptophan fluorescence of the α-subunit of transducin (Gtα) was monitored.

4.3 Results

Pigment formation. Regeneration of bovine opsin with 11-cis-10-methylretinal showed about six-fold slower kinetics at room temperature than with 11-cis-retinal at 15 °C. Nevertheless, the full equivalent of functional opsin produced analog pigment since the same final amount of analog pigment was produced with increasing molar excess (3 → 10) of 11-cis-10-methylretinal and subsequent incubation with 11-cis-retinal did not generate additional rhodopsin. The 10-methyl pigment showed a λmax at 506 ± 2 nm (n=5) as deduced from difference spectroscopy. Figure 4.2 shows the UV/visible absorbance spectrum of regenerated rhodopsin and the 10-methyl pigment based on identical amounts of opsin. It is obvious that the analog spectrum has a relatively high β-band, indicative of a distorted chromophore, and that the molar absorbance of the main band is much smaller than that of native rhodopsin. Moreover, by simulating these spectra using three gaussian band profiles (not shown), the β-band of the 10-methyl pigment was found to be shifted down relative to that of the native pigment by 15 nm to 325 nm. From our data we estimate the molar extinction coefficient of the analog at λmax to be 60 ± 5 % (n=5) of that of native rhodopsin, i.e. 24.000 ± 2.000 M⁻¹ cm⁻¹.

4.3.1 Circular Dichroism

Figure 4.3 shows the UV/visible circular dichroism (CD) spectra of the native and analog pigments corrected for the buffer contribution. Less than 1% of the pigments was bleached during the CD experiments. The CD spectrum of rhodopsin is in good agreement with data published previously (36–40). The rotational strengths of both the α- and β-band of the 10-methyl pigment were found to be essentially similar to those of native rhodopsin. The parameter [Θ]max/Αmax relates the maximal ellipticity to the absolute absorbance of a certain band and provides a measure for the conformation of the 10-Me and native chromophore in the binding pocket of opsin (Table 4.1). Whereas [Θ]max,α/Αmax,α has been shown to relate to the strain in the polyene chain of the retinal, the value for [Θ]max,β/Αmax,α relates to the distortion in the β-ionone ring portion of the chromophore (36–38, 40). In addition, the maximal ellipticity of the β-band has also been directly related to the absorbance of this
Figure 4.2: UV/visible absorbance spectra of rhodopsin (dashed line) and 10-methyl rhodopsin (solid line). These spectra were taken on the basis of identical amounts of opsin (see text). The difference in absorbance at 280 nm arises from different relative contributions of the β-band at this wavelength.

<table>
<thead>
<tr>
<th>Pigment</th>
<th>( \lambda_{\max([\Theta],\alpha)} )</th>
<th>( \lambda_{\max([\Theta],\beta)} )</th>
<th>([\Theta]<em>{\max,\alpha} / A</em>{\max,\alpha})</th>
<th>([\Theta]<em>{\max,\beta} / A</em>{\max,\beta})</th>
<th>([\Theta]<em>{\max,\beta} / A</em>{\max,\alpha})</th>
</tr>
</thead>
<tbody>
<tr>
<td>native</td>
<td>490</td>
<td>335</td>
<td>11.0</td>
<td>48.3</td>
<td>15.9</td>
</tr>
<tr>
<td>10-Me</td>
<td>500</td>
<td>330</td>
<td>15.4</td>
<td>25.2</td>
<td>21.7</td>
</tr>
</tbody>
</table>

Table 4.1: Relative circular dichroism properties of native and 10-methyl rhodopsin. \([\Theta]_{\max(\alpha,\beta)} = \text{measured ellipticity at } \lambda_{\max(\alpha,\beta)} \text{ in mdeg. } A_{\max(\alpha,\beta)} = \text{measured absorbance at } \lambda_{\max(\alpha,\beta)} \text{ (OD). For discussion of the significance of the } [\Theta]/A \text{ parameter, see text.}

4.3.2 Photosensitivity of the analog pigment

A comparison of the photosensitivity of 10-methyl rhodopsin, isorhodopsin and rhodopsin is shown in Figure 4.4. This figure shows a plot of \(-\log [10^{A_t} - 10^{A_f}]\) as a function of time. Both \(A_t\) and \(A_f\) were determined relative to a baseline set by the 650 nm absorbance in the same spectrum. The ratio of the slopes of these lines yields the photosensitivity of 10-methyl and isorhodopsin relative to rhodopsin. The photosensitivity of the 10-methyl
pigment was found to be 49 ± 6 % that of rhodopsin. The reported error is based on two independent experiments on different batches of pigment. Thus, taking the ratio of the molar extinction coefficients at 497 nm (0.60 ± 0.05) into consideration, the quantum yield of the 10-methyl analog was calculated to be 0.55 ± 0.07. In a similar way the quantum yield of isorhodopsin was determined to be 0.27 ± 0.04, in good agreement with previously published results (29, 30).

4.3.3 The photocascade in 10-methyl rhodopsin

Meta I → Meta II transition. In contrast to rhodopsin, illumination of the 10-methyl analog pigment at 10 °C only produced a Meta I like intermediate. This 10-methyl Meta I has its absorbance maximum at 490 nm and thus is blue-shifted to a similar extent relative to dark pigment as Meta I (480 nm) from rhodopsin (498 nm). Only at temperatures higher than about 20 °C the 10-methyl Meta I decayed to a photointermediate with absorbance characteristics of Meta II (unprotonated Schiff base, $\lambda_{\text{max}}$ at 380 nm). Figure 4.5 shows the UV/visible spectrum of 10-Me Meta I obtained at 25 °C. Interestingly, the absorbance of the 10-Me Meta I intermediate at 490 nm is significantly larger than that of 10-Me rho-
Figure 4.4: The photosensitivity of 10-Me rhodopsin (△) and isorhodopsin (◊) compared to that of rhodopsin (○). $F(A,t) = -\log[10^{A_t} - 10^{A_f}]$ where $A_t$ denotes the absorbance of the samples at the excitation wavelength (497 nm) as function of illumination time and $A_f$ is the 497 nm absorbance of the fully bleached sample. The initial absorbance at 497 nm was $0.16 \pm 0.007$ OD/cm, for all samples. The ratio of the slope of the lines describing the 10-Me rhodopsin, isorhodopsin and rhodopsin data ($r^2 > 0.998$ in all cases) is used to determine the quantum yield of the 10-Me and isorhodopsin pigments, relative to that of rhodopsin (equation 4.1).

dopsin at 506 nm, indicating that the extinction of the all-trans 10-Me chromophore is quite similar to that of native Meta I. The decay of the 10-Me Meta I to Meta II intermediate was followed kinetically at various temperatures. An Arrhenius plot of these data (Figure 4.6) yielded an activation energy ($E_A$) of $155 \pm 10$ kJ/mol for the Meta I to Meta II transition in the 10-methyl pigment, which is similar to that of rhodopsin [156 kJ/mol, (41)]. It is very striking that at physiological temperature, where native Meta I decays to Meta II in milliseconds, 10-Me Meta I still has a half-life of several minutes.

**FTIR difference spectroscopy.** Figure 4.7 shows the infrared difference spectra of the
rhodopsin to Batho, Meta I and Meta II transitions for both rhodopsin and 10-Me rhodopsin. In these spectra rhodopsin bands are negative. It is clear that, going from Batho to Meta II an increasing number of bands appear in the 1800–1550 cm$^{-1}$ region, indicative of a gradual increase in protein activity (6, 7). The major peaks in the spectral region from 1560 cm$^{-1}$ downwards mainly contain information about changes in conformation of the chromophore. Since especially the Rho $\rightarrow$ Batho difference spectra are dominated by changes in the vibrational state of the retinal chromophore due to isomerization and conformational strain, we expect that this transition will yield the most pronounced differences in the FTIR spectra of the two pigments. This indeed is evident from the spectra presented in Figure 4.7A. Most of the difference bands in the Rho $\rightarrow$ Batho difference spectrum have been assigned to specific vibrations of the retinal backbone (C=C and C–C stretch vibrations at $\sim$1550 and $\sim$1200 cm$^{-1}$, respectively) and hydrogen-out-of-plane (HOOP) vibrations, absorbing below 1000 cm$^{-1}$ (10, 16, 42). In the 10-Me Rho $\rightarrow$ 10-Me Batho difference spectrum only the small, yet very characteristic, changes in the amide I region (1700–1650 cm$^{-1}$) are reproduced. These difference bands probably represent subtle conformational changes in the protein moiety. Bands at 1558 cm$^{-1}$ (–) and 1535 cm$^{-1}$ (+) in the Rho $\rightarrow$ Batho spectrum have been assigned to the C$_7$=C$_8$ and the C$_{11}$=C$_{12}$ stretching modes (10) and are down-shifted in the 10-Me pigment spectrum to 1554 (–) and 1520 (+). The down-shift of these bands most likely reflects a more delocalized electronic structure across the retinal polyene chain. More pronounced differences are found
in the fingerprint (1350–1150 cm\(^{-1}\)) and HOOP (1000–700 cm\(^{-1}\)) region. Changes in the fingerprint region (a.o. the absence of the 1238 (−), 1192 (−) and 1166 cm\(^{-1}\) (+) bands) are probably caused by various contributions, as will be discussed below. The intensity of the bands in the HOOP region indicate that, like the native chromophore, the 10-methyl chromophore also contains out of plane distortions. The 969 cm\(^{-1}\) band of rhodopsin has been assigned to the strongly coupled C\(_{11}\)H and C\(_{12}\)H wags (HC\(_{11}\)=C\(_{12}\)H A\(_2\) HOOP), indicative of torsion in the C\(_{10}\)· · ·C\(_{13}\) region (9, 42). The positive bands at 921, 875 and 850 cm\(^{-1}\) in bathorhodopsin have been shown to contain contributions of the C\(_{14}\)H (848 cm\(^{-1}\)) and HC\(_7\)=C\(_8\)H B\(_8\) (838) and the (uncoupled) C\(_{11}\)H (921) and C\(_{12}\)H (858) and C\(_{10}\)H (874) wags (10). Tentative assignments of the bands in the 10-methyl Rho → Batho difference spectrum will be presented below.

The Rho → Meta I difference spectra (Figure 4.7B) are highly similar for rhodopsin and 10-methyl rhodopsin, indicating that this transition involves very similar conformational changes in both pigments. In particular, the region between 1800 and 1470 cm\(^{-1}\), which mainly represents structural rearrangements in the protein moiety, is almost identical. Similar to the rhodopsin to Batho transition, the most pronounced differences between
Figure 4.7: FTIR difference spectra of the (A) Rho → Batho (90 K), (B) Rho → Meta I (253 K) and (C) Rho → Meta II transition (taken at 283 K and 310 K for rhodopsin (Rho) and 10-Me rhodopsin (10-Me), respectively).
the two pigments are found in the fingerprint and HOOP region. Importantly, it is evident from the similarity of these spectra in the HOOP region that, as for native rhodopsin, the 10-Me chromophore has reached a relaxed all-\textit{trans} conformation at this stage.

The 10-methyl Rho $\rightarrow$ Meta II spectrum (Figure 4.7C) was taken at 37 $^\circ$C and shows band patterns which are nearly identical to those observed with native Meta II (10 $^\circ$C), indicating that the “active” 10-Me analog adopts a conformation very close to native Meta II. Native Meta II decays too rapidly at 37 $^\circ$C to be reliably measured by FTIR difference spectroscopy. Characteristic Meta II difference bands (1767 (−), 1750 (+), 1710 (+) and 1686 (+, cm$^{-1}$) are clearly evident in the 10-Me spectra as well.

In view of the very striking effect of the 10-methyl group on the Meta I $\rightarrow$ Meta II transition, we investigated whether the kinetics of earlier steps in the photocascade also were affected. Indeed we observed a significant change in the temperature dependence of the Batho $\rightarrow$ Lumi transition. Figure 4.8 shows the FTIR difference data obtained upon illuminating of 10-methyl rhodopsin at 90, 178, 203, 228 and 253 K. These spectra are scaled with respect to the 954 cm$^{-1}$ (−) band. At 178 K, difference spectra are obtained which are basically identical to those taken at 90 K. At 203 K, we observed a slow decay of the 894 (+) HOOP mode in favor of the 947 cm$^{-1}$ (+) band and a concurrent decrease of the 1520 (+) band in favor of the 1534 cm$^{-1}$ (+) band ($t_{1/2} \sim 15$ min, not shown), indicating that 10-Me Batho slowly decays to the next intermediate. It was also observed that with the loss of HOOP signal, photoregeneration after recooling to 90 K became less efficient. Only at 228 K the strong HOOP mode observed at 894 cm$^{-1}$ is completely absent, but, even at this temperature, the characteristic 1655 (−), 1635 (+) cm$^{-1}$ pair of native Lumi is not observed (7, 43). Whereas in rhodopsin the Batho $\rightarrow$ Lumi transition already occurs at 135 K and Lumi is characterized by the absence of the strong HOOP modes indicative of a relaxed all-\textit{trans} chromophore, the Batho intermediate of the 10-methyl pigment, including the strained conformation of retinal, seems to prevail up to unusually high temperature.

### 4.3.4 Transducin activation

The fluorescence increase of the $\alpha$-subunit of transducin upon binding of GTP to the receptor–G-protein complex was used to assess whether the 10-methyl pigment could act as a functional photoreceptor (33, 34). In Figure 4.9, the 10-methyl pigment is compared to native rhodopsin and indeed appears to be capable of activating transducin, albeit at an initial rate of about 20% of that of rhodopsin. The ability of the 10-Me pigment to activate transducin agrees with the FTIR difference data, which show that the structural features of 10-Me Meta II are nearly identical to those of native Meta II.

### 4.4 Discussion

\textit{Analog pigment formation.} The 10-methyl homolog of 11-\textit{cis}-retinal was shown before to form a pigment absorbing at 508 nm (20), for which a quantum yield of 0.32 $\pm$ 0.06 was reported (29). In our hands, 10-methyl-11-\textit{cis} retinal yields a pigment with a $\lambda_{\text{max}}$ of
Figure 4.8: FTIR difference spectra obtained upon illuminating 10-Me rhodopsin at 90, 178, 203, 228 and 253 K, displayed from top to bottom. The "Batho"-HOOP signals at 894 and 864 cm\(^{-1}\) are present even in the spectrum obtained at 203 K, however, at this temperature a slow decay of these bands was observed (see text). The spectra are scaled with respect to the 954 cm\(^{-1}\) (−) band of 10-Me rhodopsin. From 228 K on, the amide and fingerprint regions gradually show more of the Meta I characteristics.

506 ± 2 nm, and a quantum yield of 0.55 ± 0.07. While the difference in \(\lambda_{\text{max}}\) is within experimental error, the reported values of the quantum yield deviate to a significant extent. The discrepancy between the reported quantum yields appears to be largely caused by the different values used for the extinction coefficient of the 10-methyl pigment. Liu et al. (29) based their calculations on the extinction coefficient determined for the 10-fluoro analog of rhodopsin, which was reported to be 0.75 of that of rhodopsin (20). A separate value for the 10-Me analog was not reported. Our data show that the extinction coefficient of the 10-methyl pigment (0.60 of that of rhodopsin) is significantly lower. Taking our value would increase the quantum yield reported by Liu et al. to 0.40 ± 0.08, which
agrees within experimental error with our data. Interestingly, the reduction of the quantum yield of photoisomerization of the native pigment due to the addition of a methyl group at the C_{10} position of the chromophore (0.67 → 0.55) is also exhibited by the 13-demethyl pigment (0.47 → 0.35) (30). However, it should be noted that deviating values for the quantum yield of the 13-demethyl pigment have been reported by Gärtner and coworkers (44, 45).

**Spectral properties and chromophore conformation of 10-Me rhodopsin.** Both the observed red-shift of the \( \lambda_{\text{max}} \) in the UV/visible spectrum of the analog pigment, as well as the downshifted ethylenic stretching modes in the 10-Me Rho → Batho FTIR difference spectrum, are indicative of a more delocalized charge distribution in the conjugated retinal moiety (46). These effects may relate to the electro-donating properties of the methyl group. However, the \( \lambda_{\text{max}} \) of the protonated Schiff base of all-\( \text{trans} \)-10-methyl retinal (imine formed by reaction of all-\( \text{trans} \)-10-methyl retinal and a 30-fold molar excess of decylamine in hexane, protonated form studied in 1% Ammonyx-LO at pH 2) was observed at 438 ± 1 nm, only
slightly red-shifted from that of all-trans retinal (436 ± 1 nm, Bovee and DeLange, unpublished). Thus, the 8 nm red-shift of the absorbance maximum of 10-Me rhodopsin is more likely caused by an altered conformation and/or positioning of the 10-Me chromophore in the binding pocket of opsin, relative to that of 11-cis-retinal.

All available evidence indicates that the 10-Me chromophore conformation is more perturbed than that in rhodopsin. First, the slow pigment formation suggests sub-optimal accessibility of the retinal homolog into the binding pocket of opsin. Second, the 10-methyl group seems to impose an enhanced torsional strain in the chromophore, which is apparent from the stronger β-band in the UV/visible spectrum of the 10-Me pigment, from the CD spectra, as well as from the increased intensity of the (rhodopsin-) HOOP band in the infrared difference spectrum of the 10-Me Rho → Batho transition.

The β-band in CD spectra of visual pigments has been shown to be sensitive to distortions in the ionone ring portion of the retinal. Recently, Wada et al. reported that the planarization of the C₅—C₆—C₇—C₈ torsion angle in 11-cis-8,18-Methanoretinal doubles the value of $[\Theta]_{\max,\beta}/A_{\max,\alpha}$ (40). However, the reduced molar extinction of the α-band in the UV/visible spectrum of the 10-Me pigment makes a comparison of the $[\Theta]_{\max,\beta}/A_{\max,\alpha}$ parameter between native and 10-Me rhodopsin less useful. By relating the maximal ellipticity of the β-band directly to the absorbance of the same band we observed an almost 50% decrease of the $[\Theta]_{\max,\beta}/A_{\max,\beta}$ parameter for 10-Me rhodopsin, which may well reflect increased torsion in the C₆—C₇ bond. From the increased $[\Theta]_{\max,\alpha}/A_{\max,\alpha}$ value for 10-methyl rhodopsin, and the increase in intensity of the rhodopsin HOOP vibration at 954 cm⁻¹ relative to the 969 cm⁻¹ band in the native Rho → Batho spectrum (assignments discussed below), we infer that the polyene chain of the chromophore in the analog pigment is in a more strained conformation than 11-cis-retinal in native rhodopsin (30, 36, 37, 42). Indeed, recent solid-state $^{13}$C-NMR analyses of 10-methyl rhodopsin also indicate increased torsional strain in the C₁₀···C₁₃ region (Verdegem et al., in preparation).

**Photoactivation of the receptor.** The amide and carboxyl region (1800–1400 cm⁻¹) in the FTIR difference spectrum of the 10-Me Rho → Meta II transition, measured at 37 °C, differs only very slightly from that of rhodopsin at 10 °C. In fact, the subtle differences in these spectral regions may well be attributed to temperature effects. All available evidence indicates, that the accompanying conformational changes in the 10-methyl pigment are identical to those in rhodopsin. Our observation that the analog pigment is able to activate transducin corroborates this conclusion. The lower absolute rate of activation by the 10-methyl pigment is attributed to the relatively slow formation of 10-Me Meta II under the experimental conditions, which now has become the rate-limiting step. Notably, although the reaction rates of the Meta I to Meta II transition are quite different in the 10-methyl pigment, the Arrhenius activation energy for this transition is surprisingly similar to that determined for native rhodopsin. This further supports our conclusion, that similar protein conformational changes are involved. Moreover, the effect of the additional methyl group on the thermal activation of the Meta I to Meta II transition appears to be predominantly entropy related. A similar observation was reported for the effect of detergent solubiliza-
4.4 Discussion

4.4 Discussion on the reaction rate of the Meta I to Meta II transition in native rhodopsin (41).

Ligand-receptor interactions; FTIR analysis of the 10-Me rhodopsin photocascade. Our data show that the 10-methyl group, apart from the structural effects it imposes on the dark state conformation of the chromophore, also has a striking effect upon the photocascade up to at least Meta II. Although the 10-Me Rho → Batho difference spectrum differs strongly from that of rhodopsin, some of the differences in the ethylenic and fingerprint regions can be explained by the mere presence of the additional methyl group, and do not necessarily point at altered ligand-protein interactions. In contrast to the ethylenic stretch modes, the vibrational frequency of the fingerprint modes are shifted upwards at increasing charge delocalization of the retinal. Moreover, the 10-Me group is likely to affect the coupling between the fingerprint modes. The coupling of the $C_{10} - C_{11}$ polyene single bond stretch and the $C_{10} - C_{11}$ stretch, for example, is expected to result in an up-shift of the band by $\sim 100 \text{ cm}^{-1}$ (30), and may explain the absence of the native positive 1166 cm$^{-1}$ band in the analog spectrum. Further, the loss of coupling with the 10-H (by deuteration) can up-shift the $C_{8} - C_{9}$ band as much as 80 cm$^{-1}$ (16). Moreover, also in the 10-D pigment the $C_{14} - C_{15}$ band of rhodopsin (1192 cm$^{-1}$) was not observed, while that of Batho was shifted to 1220 cm$^{-1}$. Thus, we tentatively assign the 1216 cm$^{-1}$ (+) band to the $C_{14} - C_{15}$ stretch in 10-Me Batho. The absence of the 1238 cm$^{-1}$ (−) band, assigned to the $C_{12} - C_{13}$ vibration in rhodopsin (16), is also consistent with RR data on the 10-D pigment: the $C_{12} - C_{13}$ stretch vibrations of 10-D rhodopsin and Batho were shown to practically coincide at 1235 cm$^{-1}$. Therefore, we assign the 1230 cm$^{-1}$ (+) band to the partially cancelled $C_{12} - C_{13}$ stretching vibration of 10-Me Batho.

The strong HOOP vibrations observed in the 10-Me Rho → Batho FTIR difference spectrum indicate that there are out of plane distortions of the chromophore as there are in native rhodopsin. By analogy, we tentatively assign the 954 cm$^{-1}$ negative band to the $HC_{11} = C_{12}H A_{2}$ HOOP modes of 10-Me rhodopsin, again, in agreement with RR data on 10-D rhodopsin (9). In fact, the 954 cm$^{-1}$ band of 10-Me rhodopsin band is more intense than the 969 cm$^{-1}$ observed in native rhodopsin, consistent with a more strained conformation of the polyene chain of the 10-Me chromophore as discussed above. We further assign the 10-Me Batho band at 894 cm$^{-1}$ to the $C_{11}H$ wag. In native rhodopsin this mode absorbs at 921 cm$^{-1}$, a frequency characteristic for an isolated wag. The observed down-shift of this band, not observed in the 10-D Batho RR data, may be caused by a net displacement of the 10-methyl chromophore, such, that the $C_{11}$ gets in closer vicinity of a negatively charged protein residue. In native rhodopsin, the $C_{12}$ was tentatively placed near a negatively charged residue to explain the uncoupling of the $C_{11}H$ and $C_{12}H$ wags upon photoisomerization and the 60 cm$^{-1}$ down-shift of the latter (8, 9). On the basis of the 10-D Batho RR data, it is tempting to assign the 864 and 854 cm$^{-1}$ bands to the $HC_{7} = C_{8}H B_{x}$ and $C_{14}H$ HOOPs respectively (10). We note that these assignments are preliminary and await direct proof from labeling studies which currently are in progress. If the assignment of the 864 cm$^{-1}$ band proves to be correct, the strength of this band relative to the 862 cm$^{-1}$ band in 10-D Batho would indicate enhanced torsional strain in the $C_{7} = C_{8}$ bond.
in 10-Me Batho.

According to our present evidence, apart from the lowered quantum yield of isomerization, the 10-methyl group does not seem to interfere structurally with the photocascade up to formation of a Batho intermediate with a strained all-trans chromophore. Importantly, the fingerprint region in our FTIR difference spectra of the 10-Me Rho → Batho transition shows good correspondence with the RR data on 10-D labeled rhodopsin and bathorhodopsin (16). We conclude that the primary photoreaction in the analog pigment indeed is an 11-cis to all-trans photoisomerization. The observation that 10-Me Batho can be photoreversed without significant loss of signal or spectral shifts supports this conclusion.

In contrast to what is found for native rhodopsin, the 10-Me HOOP signals were detectable up to 210 K, indicating that steric interactions between the 10-Me retinal and opsin hamper the relaxation of strain in the retinal moiety. Likewise, the slow formation of 10-Me Meta II is attributed to steric interactions with another protein residue. Several bands in the infrared difference spectra hint at the involvement of protein residues, however, the exact interaction sites in the protein cannot yet be identified. For example, the 10-Me rhodopsin to Meta I and Meta II difference spectra show a shoulder at 1558 cm$^{-1}$ and a significant loss of intensity of the 1644 cm$^{-1}$ (+) band. Moreover, in the 10-Me Rho → Meta II spectra, the (native) 1655 cm$^{-1}$ (−) band is reproducibly observed at 1659 cm$^{-1}$, possibly due to the absence of a shoulder at 1663 cm$^{-1}$ in the native spectrum. We note, however, that an incomplete Meta I to Meta II transition, and the different experimental temperatures may induce small differences in the amide regions. Isotopic labeling should elucidate the nature of the ligand-protein interactions throughout the photocascade of the 10-methyl pigment.

**Relation to other (de)methyl rhodopsin analogs.** Together with previous studies on rhodopsins containing a demethyl-11-cis retinal analog, this work provides a consistent picture on the conformational freedom of the retinal in the binding pocket of opsin as well as on the mechanism of energy storage in the Batho intermediate of rhodopsin. In short, the 9-demethyl compound was reported to have a fully disturbed photocascade, in which a clear Batho intermediate could not be detected at 80 K (see e.g. (47)). Furthermore, the 9-demethyl Meta II like intermediate was found to have a protonated Schiff base, unusual structural features in the corresponding FTIR difference spectrum and was incapable of activating transducin. The 13-demethyl rhodopsin analog exhibits a basically normal photocascade [see e.g. (48)] and G-protein activity, and, interestingly, was also found to activate transducin in the dark (49). While the 13-methyl group seems to be of prime importance in suppressing dark activity of the receptor, the 9-methyl group seems to be an important factor for the correct positioning of the retinal in the binding pocket of opsin. Moreover, the 9-methyl group seems to provide the necessary fixation of parts of the chromophore, which, upon photoisomerization, gives rise to the torsional strain in the chromophore. Our results clearly show, that addition of a methyl group at the 10 position results in a completely different phenotype. Not unexpectedly the 10-methyl pigment behaves as a func-
tional photoreceptor, including activation of the G-protein transducin. However, the additional methyl group severely perturbs the kinetics of the photocascade at two stages (Batho → Lumi and Meta I → Meta II), without significant effects on the conformational changes in the various transitions. The most logical explanation is that sterical interaction of the 10-methyl group with the protein environment changes the free energy barriers of these two transitions and forces the system through a slightly different trajectory. In the Batho→Lumi transition it probably is the relaxation of strain in the chromophore which now becomes rate-limiting, and is obstructed by (a) protein residue(s). In the Meta I → Meta II transition the chromophore is fully relaxed and the 10-methyl group probably obstructs conformational changes in the protein, involving adjacent residues. It follows that most likely different protein residues are involved in the interaction with the 10-methyl group during these transitions. The available 3-D structure has not enough resolution to allow selection of potential candidates for these residues, but we are currently trying to identify these by scanning mutagenesis.

The current picture is that the photon energy is stored as torsional strain in the chromophore at the Batho stage (10, 50), and that the energy released upon relaxation of the strain is used to drive the protein conformational changes in the photocascade dark reaction. This is consistent with our observation that as long as the HOOP signals prevail, only very small changes in the amide I and amide II region are observed in the FTIR difference spectra of the 10-methyl pigment.

**Conclusion.** The salient result of this study is that the addition of a methyl group at position 10 of the chromophore strongly perturbs two stages of the photocascade. Interestingly, although the additional methyl group somewhat reduces the efficiency of the process, photoisomerization of this chromophore results in a "normal" Batho intermediate with a strained all-trans chromophore conformation. However, then, it first markedly retards relaxation of the photoisomerized chromophore and the occurrence of structural changes in the protein moiety. The important conclusion is, that relaxation of the chromophore drives the subsequent structural changes in the receptor. Subsequently, it severely interferes with the conformational transition of the Meta I intermediate into the "active" intermediate, Meta II. Clearly, the chromophore has not much conformational freedom and the interaction between ligand and protein has become very finely tuned in order to allow highly efficient photon capture as well as very selective triggering of conformational changes in the protein moiety towards receptor activation.
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References

References


Chapter 5

Modulation of the Meta I – Meta II equilibrium by ionic strength: evidence for a surface charge effect


The effects of ionic strength on formation and decay of metarhodopsin II (Meta II), the active photointermediate of bovine rhodopsin, were studied in the native membrane environment using UV/visible and Fourier transform infrared (FTIR) spectroscopy. It was found that, by increasing the concentration of KCl in the range from hypotonic to 4 M, the apparent pKₐ of the metarhodopsin I-metarhodopsin II equilibrium is shifted by approximately 3 pH units, in favor of the Meta II intermediate. In addition, the apparent rate of Meta II formation is enhanced by an increase in ionic strength (about two-fold in the presence of 2 M KCl). Meta II decay was found to be independent of the salt concentration. Attenuated total reflectance (ATR)-FTIR data show that the high salt conditions have no effect on the rigidity of the membrane matrix and do not induce structural changes in the intermediates themselves. Different salts were tested for their ability to shift the Meta I – Meta II equilibrium; however, no clear ion dependence was observed. We interpret these results as an indication for a direct involvement of the cytosolic surface charge in the regulation of the photochemical activity of bovine rhodopsin.
5.1 Introduction

Rhodopsin is the photoreceptor protein located in the disk membranes of retinal rod photoreceptor cells and is considered to be a model for the superfamily of G-protein coupled receptors (1, 2). It consists of the apoprotein opsin and the chromophore 11-cis-retinal, which is covalently linked through a Schiff base to Lys296 in bovine opsin. Photoexcitation of rhodopsin involves the rapid photoisomerization of the chromophore to the all-trans configuration. This primary photochemical event triggers a cascade of photointermediates involving a series of slower thermal transitions in the protein moiety. Under physiological conditions the active metarhodopsin II intermediate (Meta II) is formed on a millisecond time scale (3, 4). Meta II can bind and activate the G-protein transducin, which ultimately leads to hyperpolarization of the rod photoreceptor cell and further neuronal transduction of the signal.

The active intermediate Meta II is in equilibrium with its precursor Meta I (5). Therefore, to clarify the mechanism of visual excitation on a molecular level, understanding the factors that control the Meta I – Meta II transition is essential. To date, the main characteristics of the Meta I – Meta II transition are considered to be: (i) deprotonation of the retinal Schiff base and a net proton uptake of at least one proton (5, 6), (ii) relatively large structural changes in the protein moiety among other things involving carboxyl groups (7–10) and sulfhydryl groups (11), (iii) a transient change in charge distribution (12, 13). The Meta I – Meta II equilibrium has been shown to be sensitive to temperature, pH (5) and pressure (14, 15). Micro-environmental properties like lipid composition and membrane fluidity (16–21), the degree of hydration (22, 23) and the presence of detergents (14, 24) also affect the equilibrium. Furthermore, several mutations involving polar amino acid residues in rhodopsin have been reported, which shift the Meta I – Meta II equilibrium (25–27). These and other studies on analog or mutant rhodopsins (28–31) could suggest that the Meta I – Meta II transition is at least partially controlled by local electrostatic interactions and/or charge distributions. In general, it is evident that the Meta I – Meta II transition involves the largest structural change in the protein and that it strongly responds to the micro-environment. It is therefore essential to study local effects also in the native environment: the photoreceptor membrane.

If the Meta I – Meta II transition indeed would be basically controlled by electrostatic factors, it should respond to the ionic strength of the solvent. Salt effects on kinetics of rhodopsin bleaching have been reported for rhodopsin in detergent solution (32). However, since almost all detergents dramatically alter the pH dependence of the Meta I – Meta II equilibrium (5, 14, 24), any effects of ionic strength on the apparent pKₐ of this equilibrium have not been investigated. We have begun to analyze these aspects using both UV/visible and FTIR spectroscopy on the native ROS membrane. While UV/visible spectra primarily contain information about the chromophore and its electrostatic environment, FTIR spectra contain vibrational information of the entire complex and can be used to monitor conformational changes in chromophore, protein and lipid. By combining both techniques, we can establish whether the photo-induced electronical and vibrational transitions are coupled under various experimental conditions and, moreover, we have a
sensitive tool to monitor any salt-induced structural changes in the rhodopsin molecule. Here, we will demonstrate that several properties of the rhodopsin photocascade respond to an increase in ionic strength, without a marked ion-dependence: (i) the formation but not the decay of Meta II is accelerated, (ii) the pK_d of the Meta I – Meta II equilibrium is shifted upwards, increasingly favoring Meta II at increasing salt concentration. We will discuss the implications of these findings for a role of the protein surface charge in the regulation of rhodopsin function.

5.2 Experimental Procedures

Rod outer segments (ROS) were prepared as previously described (33). The resulting photoreceptor membranes had an A_{280}/A_{500} absorbance ratio of 2.0–2.2. All manipulations were performed under dim red light (RG645 cutoff filter, Schott, Mainz, FRG.). Standard buffer solutions contained 20 mM buffer, 130 mM NaCl, 10 mM KCl, 3 mM MgCl_2, 2 mM CaCl_2 and 0.1 mM EDTA. Buffers Mes, Mops, Hepps and Bistris-propane were used to cover the pH range 5.5–9.0.

UV/visible spectroscopy

All UV/visible analyses were performed on a Perkin Elmer λ15 double beam spectrophotometer equipped with an end-on photomultiplier detector. A circulating bath connected to the cuvette holder was used to control sample temperature.

*Calculation of the percentage Meta I formed after illumination.* The measurements were routinely performed at 10 °C since at this temperature the decay of Meta II is negligibly slow and does not interfere with our analyses. Samples contained a ROS membrane suspension (≈ 2 μM in rhodopsin) in buffer and salts as indicated. A spectrum was recorded between 750 and 250 nm (taking 170 s) of the initial, unbleached sample (spectrum 1). The sample was then illuminated for 10 seconds (OG530 filter, Schott, Mainz, FRG., 75W light bulb) and subsequently two or three spectra were recorded to check the stability of the photointermediate spectrum under the experimental conditions (spectrum 2). The relative amount of Meta I formed after illumination ([Meta I]/[Bleached Rhodopsin]) was derived from the linear relationship between the λ_max of the difference spectrum (spectrum 1 – spectrum 2) and the percentage photoproduct formed. In these difference spectra λ_max moves linearly from 498 nm (0% Meta I, 100% Meta II) to 530 nm (100% Meta I, 0% Meta II). Assuming [Bleached Rhodopsin] = [Meta I] + [Meta II] (34) the fraction Meta I (% Meta I) formed upon illumination was calculated from [(λ_{max} – 498) / (530 – 498)] (“difference spectrum approach”). The validity of this approach was checked using the “classical approach” which requires a third spectrum after addition of hydroxylamine to convert all photointermediates in the sample into opsin and retinaloxime [e.g. (17)]. The difference spectrum approach, which was also exploited for analyses in digitonin solution by (26), yields the same average percentage Meta I but generally gives better reproducibility than the classical approach.

*Kinetic analysis of the formation of Meta II.* The formation of Meta II was studied at −6 °C, with a time resolution of 0.1 s, using the rise in absorbance at 380 nm upon bleaching the samples with a short photoflash in the spectrophotometer. Photoreceptor membrane suspensions (≈ 2 μM in
rhodopsin) were studied in standard buffers (with or without 2 M KCl) containing 20% glycerol (mass/vol) to prevent freezing. Flash illumination, using a conventional photoflasher equipped with a Kodak 32 filter, resulted in approximately 40% bleaching per flash. Each sample was illuminated at least twice to evaluate reproducibility. Although the photomultiplier was protected from the photoflash by a Kodak 58 filter, reliable absorbance data could only be collected after 2 seconds. The apparent rate constants ($k_{obs}$) were obtained by fitting a mono-exponential equation to the absorbance data.

**Kinetic analysis of the decay of Meta II.** The decay of Meta II to Meta III was analyzed at pH 6, and at 15 °C, by measuring 50 spectra (170 s per spectrum) after bleaching the sample (10 s illumination). We selected 15 °C and a pH of 6 because under these conditions photoconverted rhodopsin decays almost fully and within several ms to Meta II, while Meta II decay is dominated by the transition of Meta II to Meta III. Other processes like Meta II → opsin + retinal and Meta III → opsin + retinal proceed only very slowly under these conditions compared to Meta II → Meta III ($35\pm37$). Standard buffer or standard buffer with 4 M KCl was used. We took the decrease in the absorbance difference between 380 nm and 418 nm [isosbestic point for the transition, (38)] as a measure for the decay of Meta II. Similarly, the rise in absorbance difference between 455 nm (Meta III) and 418 nm was used to measure the formation of Meta III. Fits of a mono-exponential function to these absorbance data were used to calculate the rates of Meta II decay and Meta III formation under both experimental conditions.

**FTIR spectroscopy**

FTIR analyses were performed on a Mattson Cygnus 100 spectrometer (Madison, WI., U.S.A.) equipped with a liquid nitrogen cooled narrow band HgCdTe detector. Operating control of the spectrometer, as well as all spectral manipulations were done using the Expert-IR software package (Mattson). All spectra were taken at 8 cm$^{-1}$ resolution. Samples were illuminated in the spectrometer for 20–30 seconds using a 20 W halogen lamp equipped with a KG1 infrared filter and an OG530 cutoff filter (Schott, Mainz, FRG.) in the transmission experiments and a fiberoptics ring illuminator (Schott, Mainz, FRG.) in combination with an OG530 filter in the ATR-FTIR experiments. Sample temperature was controlled using a circulating bath in the ATR-FTIR experiments and an immersion cooler in combination with a computer controlled variable temperature cell (Graseby Specac, Orpington, Kent, UK.) in the FTIR transmission experiments.

*Static FTIR difference spectroscopy.* For analysis of the rhodopsin to Meta I → Meta II transition, FTIR difference spectra were obtained in a similar way to that previously described (1, 39). Samples were prepared by isopotential spin drying of an aqueous suspension of photoreceptor membranes (containing 2–3 nmol of rhodopsin) on an AgCl window (Fisher Scientific Co., Pittsburgh P.A., U.S.A.). The photoreceptor membrane films were rehydrated with ≈ 2 µl ($2 \times$) buffer, with or without 4 M KCl, and subsequently sealed using a rubber O-ring spacer and a second AgCl window. The concentration of the buffer solution was doubled [denoted as ($2 \times$) buffer above] to enhance its buffering capacity. Difference spectra were obtained at 10 °C by subtracting the spectrum (256 scans, one min/spectrum) just before illumination from the spectrum immediately after sample illumination. Under the various experimental conditions the shape of the amide I band in the absolute infrared dark spectra did not significantly change, indicating that no significant changes
5.3 Results

In order to have better control of pH and ionic strength, ATR-FTIR experiments were performed as well. A suspension of photoreceptor membranes (containing \( \approx 40 \text{ nmol of rhodopsin} \)) was dried under a gentle stream of nitrogen to form a film on a horizontal, trough plate, germanium ATR accessory (Spectra-Tech, Birchwood, Warrington, UK.). The ATR accessory was mounted with a home built, perspex flow setup allowing in situ illumination. Standard buffer, Mes pH 6 or Bistris-propane pH 8.8 (with or without 2 M KCl), cooled to 10 °C, perfused the film at a rate of 12 ml/min. Difference spectra were calculated by subtracting blocks of spectra (1800 scans, \( \approx 7 \text{ min} \)) in order to improve the signal to noise ratio.

**Kinetic Analysis of the decay of Meta II.** The decay of Meta II was studied in the FTIR transmission mode at 15 °C in spin dried films that were rehydrated with (2 ×) Mes buffer pH 6.0 with or without 4 M KCl. The decay was analyzed by taking 60 subsequent spectra (256 scans, one min/spectrum) following illumination of the sample. Decay rates of the peak absorbance of various typical Meta II-bands in these difference spectra were obtained by fitting a mono-exponential function to the data. We monitored the absorbance decay of four bands: the negative peaks at 1390 cm\(^{-1}\) and at 1435 cm\(^{-1}\), the positive peak at 1687 cm\(^{-1}\) and the sum of the positive peak at 1750 cm\(^{-1}\) and the negative peak at 1768 cm\(^{-1}\) [these can be added because they were shown to originate from a frequency shift of the C=O vibration of the carboxyl group in the Asp83 residue and appear in the Rho \( \rightarrow \) Meta II difference spectrum only (9, 10)].

5.3 Results

5.3.1 The pK\(_a\) of the Meta I – Meta II equilibrium shifts to higher values at higher salt concentrations

We determined the relative amount of Meta I formed after bleaching ROS membrane suspensions at 10 °C as a function of pH at five different salt concentrations: hypotonic buffer solution only containing 20 mM buffer and 5 mM KCl; isotonic standard buffer (no extra KCl) and with an extra 1 M KCl, 2 M KCl and 4 M KCl respectively (Figure 5.1). Under isotonic conditions we find an apparent pK\(_a\) of 7.3, which agrees well with the pK\(_a\) reported by Parkes and Liebman (34) for rod disk membrane suspensions under similar conditions. Under hypotonic conditions the apparent pK\(_a\) is shifted downwards to 6.8. Increasing the concentration of KCl up to 4 M shifts the apparent pK\(_a\) of the equilibrium upwards to about 9.5. Due to the instability of Meta I at high pH under high salt conditions no reliable measurements at pH 9 could be performed. Except for the effect on the Meta I – Meta II equilibrium, the late photocascade in the presence of 4 M KCl is very similar to that under isotonic conditions: the absorbance maximum of rhodopsin is the same (498 nm) and, judging from to the \( \lambda_{\text{max}} \) in the UV/visible difference spectra, the same late intermediates (Meta I, Meta II and Meta III) are formed upon illumination (not shown). We did not observe significant effects of increasing salt concentration on the scattering, measured at 650 nm, of the membrane suspension, indicating that under our experimental
Figure 5.1: Effect of the KCl concentration on the apparent pK\textsubscript{a} of the Meta I – Meta II equilibrium. The relative amount of Meta I formed after bleaching a ROS membrane suspension at 10 °C presented as a function of pH at different KCl concentrations. (+) Hypotonic buffer (20 mM buffer and 5mM KCl; (○), isotonic buffer A; (▲), buffer A + 1 M KCl; (■), + 2 M KCl; (◇), + 4 M KCl. For clarity, standard deviations (n ≥ 3) are shown only for one curve (+ 1 M KCl). The SD. in the other curves is comparable. Solid lines show fits to the Henderson-Hasselbalch equation using two ‘fixation’ points at pH 4 (0% Meta I) and at pH 12 (100% Meta I).

FTIR difference spectroscopy at different KCl concentrations. To establish whether the light-induced structural changes in the presence of 4 M KCl are similar to those under isotonic conditions, we applied FTIR difference spectroscopy. Figure 5.2 shows the ATR-FTIR difference spectra of photoreceptor membrane films perfused with Bistris-propane buffer (pH 8.8) under isotonic (top spectrum) and 2 M KCl conditions (middle spectrum) and with standard Mes buffer (pH 6; lower spectrum). These spectra were smoothed using the Savitsky-Golay algorithm (13 point smooth). The ATR difference spectrum obtained at pH 6 looks very similar to that obtained under the same conditions in transmission mode (7, 40). From UV/visible spectroscopy (Figure 5.1), it is clear that at pH 8.8 in suspension
Figure 5.2: Analysis of the salt effect on the Meta I – Meta II equilibrium by infrared difference spectroscopy. ATR-FTIR difference spectra (dark spectrum subtracted from spectrum after illumination; 8 cm\(^{-1}\) resolution; 1792 scans/spectrum; 13 point smoothed, see text) of ROS membrane films perfused with Bistris-propane buffer (10° C; pH 8.8) at isotonic (top spectrum) and 2 M KCl (middle spectrum) conditions and with standard Mes buffer (pH 6; lower spectrum). The spectra were scaled with respect to the 1238 cm\(^{-1}\)-band, since the amplitude of this chromophore band does not change significantly during the Meta I – Meta II transition. Typical Meta II bands are indicated by vertical dotted lines. Y-scale is for the spectrum obtained at 2M KCl (pH 8.8).

essentially no Meta II is formed under isotonic conditions. Since the ATR trough contains at least 2 ml buffer, we believe that the conditions in the ATR-FTIR and UV/visible experiments can be considered to be identical. To our knowledge, these results are the first to confirm, by means of FTIR difference spectroscopy, that essentially no Meta II is formed
under these conditions. From Figure 5.2, it is obvious that at pH 8.8, in the presence of 2 M KCl, bands typically observed in Rho → Meta I difference spectra decrease, while those typical for the Rho → Meta II transition (indicated by vertical dotted lines in Figure 5.2) are enhanced. No marked shifts or alteration of band patterns are observed. From the increase in the typical Meta II bands it can be roughly estimated that $40 \pm 10\%$ is formed in the presence of 2 M KCl. This is in good agreement with the UV/visible data (Figure 5.1). FTIR transmission experiments yielded similar results as the ATR-FTIR experiments, albeit less pronounced due to the poorer control of pH and ionic strength (results not shown). From these data, the band shape of the amide I band in the absolute infrared spectra and the UV/visible spectral properties of rhodopsin and the photointermediates formed upon illumination, we infer that higher salt concentrations by itself do not induce structural changes and that the altered pH dependence of the Meta I – Meta II equilibrium in the presence of salt can indeed be ascribed to a change in the Meta I/Meta II ratio.

**Ion dependence of the observed $pK_a$ shift.** In principle, the KCl effect on the Meta I – Meta II equilibrium may either be the result of binding of potassium or chloride ions at specific binding sites or it may be an effect of the high ionic strength of the bulk solution on the local pH at the membrane surface and/or on the intramolecular charge distributions. In the case of specific binding, saturation of the binding sites is likely to occur at relatively low salt concentrations (isotonic) and one would not expect to observe the large difference in apparent $pK_a$ between isotonic and 4 M KCl shown in Figure 5.1. Hence, we have investigated the ability of other salts as well as of the zwitterions betaine and NDSB195, a sulphobetaine derivative (4I), to shift the equilibrium towards Meta II. This was assayed by measuring the percentage Meta I formed at pH 8 and 10 °C in the presence of 0.5 M of divalent salts, 1 M of monovalent salt and 2 M of zwitterions (Figure 5.3). Most salts induce a comparable shift in equilibrium towards Meta II. However, the two zwitterions and tetraethylammoniumchloride (TEACl) had no significant effect. 1 M of KCl, NaCl, RbCl, CsCl or 0.5 M of CaCl$_2$ or MgSO$_4$ all effectuate about the same shift ($45 \pm 5\%$) towards Meta II, so the effect is not highly cation-specific. Changing the anion in the series KCl, KNO$_3$, KBr, KI and KSCN shows that there is some anion-selectivity. The shift induced by KI and KSCN is significantly larger than for the other salts (1 M KSCN being about as effective as 2 M KCl, cf. Figure 5.1). On the whole, these results nevertheless indicate that the shift in the Meta I – Meta II equilibrium is primarily dictated by the ionic strength of the membrane suspension.

**5.3.2 High salt concentrations enhance the rate of Meta II formation**

The formation of Meta II, at $-6\, ^\circ C$, in photoreceptor membrane suspensions was monitored from the rise in absorbance at 380 nm. Values for $k_{obs}$ were obtained under isotonic conditions and in the presence of 2 M KCl at various pH values between 5 and 8.5 (Figure 5.4). Under isotonic conditions $k_{obs}$ reaches a minimal value near neutral pH in agreement with earlier reports (34, 42). At pH $\leq 7$, the presence of 2 M KCl enhances $k_{obs}$ about two-fold.
5.3 Results

Figure 5.3: Effects of various salts on the Meta I – Meta II equilibrium. The relative amount of Meta I formed after bleaching a ROS membrane suspension at pH 8.0 and 10 °C under isotonic conditions compared to that formed in the presence of various salts. (1 M for monovalent electrolytes, 0.5 M for CaCl$_2$ and MgSO$_4$ and 2 M for the zwitterions to correct for net ion charge or solute concentration). Error bars indicate the standard deviation in these experiments (n = 3).

The rate of Meta II decay is not affected by high salt concentrations. The decay of Meta II was analyzed at 15 °C and pH 6.0 with both UV/visible and FTIR spectroscopy (transmittance mode). The FTIR difference spectra we obtained (not shown) are very similar to those presented in other FTIR studies on Meta II decay (37, 39). All FTIR difference bands analyzed decayed at essentially the same rate. No significant differences were observed between the samples rehydrated with isotonic or with 4 M KCl buffer. The decay of all bands could be adequately described by a mono-exponential function to yield a half time of 18 ± 2 min under both conditions. Typical results obtained by UV/visible spectroscopy are presented in Figure 5.5, showing the decay of Meta II and the formation of Meta III as represented by (A$_{380nm}$ – A$_{418nm}$) and (A$_{455nm}$ – A$_{418nm}$) as a function of time.
Figure 5.4: Effect of 2 M KCl on the rate of Meta II formation. Calculated rate constants ($k_{obs}$) for the Meta II-formation at $-6.0 \pm 0.5^\circ C$ presented as a function of pH. (♦) buffer A; (○) buffer A plus 2 M KCl. Buffer solutions contained 20% glycerol (mass/vol) to prevent freezing. Standard deviation in these experiments is about 10% ($n = 4$). (Inset) Typical curves for the rise in 380 nm absorbance at pH 6.1 and $-6^\circ C$ for isotonic buffer (solid line) and for buffer with 2 M KCl (dashed line). Amplitudes were scaled for easier comparison. Mono-exponential functions were fitted to these data to obtain best estimates for $k_{obs}$.

Again, the kinetics under isotonic or 4 M KCl conditions are not significantly different. This type of experiment yielded half times of $19 \pm 3$ min. Our data provided no indica-
5.4 Discussion

In this study we investigated the effect of ionic strength on the late photocascade of bovine rhodopsin in the photoreceptor membrane. The most striking observation was that the apparent pK$_a$ of the Meta I – Meta II equilibrium strongly depends on the ionic strength of the membrane suspension. The shift in equilibrium position is primarily due to an ionic strength dependent increase in the rate of the Meta I $\rightarrow$ Meta II transition. For instance, from the data presented in Figure 5.4 and the shift in equilibrium position under these conditions we calculated that at pH 7, assuming a pseudo first order reaction scheme and defining $k_{obs} = k_{+1} + k_{-1}$, the rate of Meta II formation (Meta I $\rightarrow$ Meta II; $k_{+1}$) increases about ten-fold by this increase in ionic strength, while the reverse reaction (Meta II $\rightarrow$ Meta I; $k_{-1}$) is only accelerated about two-fold. Because the equilibrium shift is not highly ion-specific, we conclude that it is not primarily caused by specific binding of ions, but that it rather depends on the bulk ionic strength. A possible explanation for the different behavior observed for KI, KSCN and TEACl lies in the more lipophilic character of I$^-$, SCN$^-$, and tetraethylammonium ions relative to the other ions we tested. It seems unlikely that higher ionic strength causes structural changes in rhodopsin or its photointermediates, con-

Figure 5.5: Effect of 4 M KCl on the decay of Meta II and formation of Meta III as measured by UV/visible spectroscopy. $A_{380nm} - A_{418nm}$ and $(A_{455nm} - A_{418nm}$ (Change in Absorbance (%)), represent Meta II-decay and the formation of Meta III respectively. Traces were measured at 15°C and pH 6.0, under isotonic (solid line) and 4 M KCl (dashed line) conditions. Amplitudes are scaled for easier comparison.
considering that their visible and infrared spectral properties are independent of ionic strength. Small structural changes in the retinal binding pocket, for example, are likely to be of influence on both the UV/visible $\lambda_{\text{max}}$ and the characteristic band patterns in the FTIR difference spectra [see e.g. (27)]. The implications of the observed salt effects for models of the Meta I – Meta II transition will be discussed in the next sections.

**Membrane properties.** Biomembranes and lipid-protein interactions are well known to be sensitive to the ionic environment. For instance, several salts can influence membrane proteins indirectly by affecting the fluidity of the membrane matrix (17, 43). These effects, however, involve binding of ions to the lipid head-groups and therefore always show a clear preference for di- and trivalent cations (e.g. Ca$^{2+}$, Mg$^{2+}$ and La$^{3+}$) which we do not observe. Binding of divalent cations like Ca$^{2+}$ to phosphatidylserine bilayers, for example, has been shown to rigidify the membrane, which results in a shift of the Meta I – Meta II equilibrium towards Meta I (17, 20). The fluidity of the photoreceptor membrane, however, seems to be unaffected by high salt concentrations, as we noticed that the frequency of the methylene C–H symmetric stretch vibration remains at 2854.2 ± 0.2 cm$^{-1}$ in ATR-FTIR spectra of ROS-films perfused with standard Bistris-propane buffer with and without 2 M KCl (DeLange and DeGrip, unpublished observations). The frequency of this vibration is a good indicator of membrane packing (44, 45).

**Effects on surface pH.** Another explanation might be that the ionic strength of the bulk membrane suspension affects the surface pH. Because of the relatively high local charge density, the pH at the membrane surface usually differs from bulk pH. Surface charge effects were put forward to explain the ionic strength dependence of the purple-to-blue-transition in bacteriorhodopsin (46). Recently, Alexiev et al. (47) showed that the charge density calculated from the ionic strength dependence of the purple-to-blue transition in a bacteriorhodopsin mutant reconstituted in detergent/lipid mixed micelles equaled the surface charge density calculated from the ionic strength dependence of the apparent pK$_a$ of a pH indicator dye attached to the extracellular side of the protein. Thus, a direct relationship was established between the purple-to-blue transition and the surface potential on the extracellular side of bacteriorhodopsin. Another example of such a phenomenon is the ionic strength dependence of the equilibrium between acid and alkaline metarhodopsin in octopus photoreceptor membranes, which was interpreted to be due to screening of net negative charges at the extracellular membrane surface (48). In these two cases raising the ionic strength results in a decrease of the apparent pK$_a$. In order to explain the pK$_a$-increase for the Meta I – Meta II equilibrium in this way, the side from which we are titrating the Meta I – Meta II transition should have a net positive charge. As the cytosolic side of rhodopsin contains more basic than acidic residues, this side is probably positively charged (49, 50). Hence, part of the shift in pK$_a$ of the Meta I – Meta II equilibrium we observe might be explained by an ionic strength dependence of the surface charge and hence of the surface pH at the cytosolic side of the photoreceptor membrane. It should be noted that screening of the net negative lipid head-group charges is not expected to result in an altered equilib-
rium position since it is possible to recover full photochemical function of rhodopsin upon reconstitution in a neutral lipid environment alone (21). The results for the two zwitterions may now be interpreted to be due to less effective screening of these surface charges. Preliminary evidence from comparative titration studies as in Figure 5.1 on ROS membranes and partially digested (proteinase K) or modified (succinic anhydride) rhodopsin indeed suggests that changes in the cytosolic surface charge affect the Meta I – Meta II equilibrium (DeLange, Bovee-Geurts and DeGrip, unpublished data).

However, effects on the surface pH can only explain part of the observed shift in pK\textsubscript{a}. The cross-sectional area of rhodopsin is known to lie in the range of 8–12 nm\textsuperscript{2} (51). From the folding model of bovine rhodopsin [(49, 50), cf. Figure 1.3] we deduce that in our experimental pH region there is a net amount of maximally 5 positive charges at the cytosolic side of the protein. Applying the Gouy-Chapman and Boltzmann equations in modified form (48) we calculate that under such conditions this surface charge would already be effectively screened at a concentration of 2 M monovalent electrolyte. This calculated concentration actually very likely represents an upper estimate, since from the data obtained by Tsui et al. (51) it may be inferred that 0.2 M monovalent salt already is enough to screen the surface potential of disc vesicles. The apparent pK\textsubscript{a} of the Meta I – Meta II equilibrium, however, is still considerably shifted upwards upon raising the KCl concentration from 2 to 4 M. Therefore, high ionic strength might also affect buried residues which are involved in tuning the pH-sensitivity of the Meta I – Meta II equilibrium. This could also explain the stronger effect on the Meta I – Meta II equilibrium of the lipophilic anions I\textsuperscript{−} and SCN\textsuperscript{−} (Figure 5.3). The suggestion that a high ionic strength of the bulk membrane suspension can influence residues in the interior of rhodopsin is supported by the observation that at pH > 9 the absorbance maximum of rhodopsin is blue-shifted by 8 nm in rod disk membranes at 4 M KCl, while this shift does not occur under isotonic conditions (53).

To our knowledge this study is the first report of ionic strength effects on the apparent pK\textsubscript{a} of the Meta I – Meta II equilibrium of bovine rhodopsin in the native photoreceptor membrane. In this respect, it is important to note that almost all detergents dramatically alter the Meta I – Meta II transition. In fact, in most detergents the transition is no longer a pH dependent equilibrium in the experimental pH-range (pH 5–9), but fully proceeds to Meta II. Salt effects have been reported before only for rhodopsin in micellar solution. Matthews et al. (5) observed that in digitonin micelles Meta II is favored in the presence of neutral salts like lithium bromide or sodium and potassium phosphate, however, without affecting the apparent pK\textsubscript{a} of the Meta I – Meta II equilibrium. Another, more recent report describes ionic strength effects on proton movements during the formation of Meta II (32). In this study it is shown that in dodecylmaltoside or nonylglucoside micelles deprotonation of the retinal Schiff base (Meta I ⇔ Meta I\textsubscript{A}) precedes proton uptake by rhodopsin (Meta I\textsubscript{A} + n H\textsuperscript{+} ⇔ Meta II\textsubscript{B}). Schiff base deprotonation was found to be accelerated at high ionic strength. This agrees with our observation that Meta II formation is accelerated at 2 M KCl in ROS membranes.

Our finding that the pK\textsubscript{a} of the Meta I – Meta II equilibrium is sensitive to the ionic
strength of the membrane suspension up to very high KCl concentrations, supports the concept that the Meta I – Meta II equilibrium is at least partially controlled by electrostatic factors (26, 30, 31). It has been proposed that the pH dependence of the Meta I – Meta II equilibrium is mainly regulated by a histidine residue because the pKₐ of the imidazole group (in water) is within the range of the apparent pKₐ of the Meta I – Meta II equilibrium in digitonin solution [6.4 at 3.2 °C (5)]. Weitz and Nathans (25) suggested that this histidine is His211, as they found that its replacement by either cysteine or phenylalanine results in a complete blockade of the Meta I – Meta II transition in digitonin solution. Since the pKₐ of the Meta I – Meta II equilibrium can be shifted to at least 9 in the native photoreceptor membrane, we believe that other residues than histidines may also participate in coupling of the deprotonation of the retinal Schiff base to the structural changes resulting in Meta II formation. Actually, the fact that the apparent pKₐ can shift from as low as pH 6.8 to over pH 9 suggests that not a single residue may be involved, but rather that a consortium of residues like in a H-bonded network may be responsible (53).

In conclusion, we believe that the observed effects can be attributed to ionic strength effects on the protein itself, and do not as much involve the lipid matrix. While the ionic strength will vary only very little under physiological conditions, these studies seem to indicate that the charge asymmetry in bovine rhodopsin is finely tuned so as to produce significant amounts of the active Meta II intermediate under physiological conditions. It would be very interesting to investigate whether this concept of charge driven activation modulation could also hold for other members of the family of G-protein coupled receptors.

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Chapter 6

The photocascade in the human green cone visual pigment: variations on a common theme?


In this chapter we present a first FTIR analysis of the photocascade of a cone visual pigment, i.e. the human green cone pigment. FTIR difference spectroscopy was applied to address structural aspects in both the chloride (527 nm) and nitrate bound (508 nm) form of the pigment. At 80 K, a batho intermediate could be stabilized which shows strong hydrogen-out-of-plane (HOOP) vibrations, indicating that (similar to bathorhodopsin) this intermediate also contains a strained all-trans chromophore. However, as judged from the relative HOOP intensity, the torsional strain in the chromophore in the green cone pigment appears to be slightly reduced, compared to that in the binding pocket of rod opsin. In addition, at this early stage, the protein conformational changes observed in the amide I region appear to be smaller than in the case of rhodopsin. On this basis, we argue that green cone opsin accommodates the retinal moiety less tightly than its rod counterpart. The present results suggest that the anion dependence of the spectral sensitivity originates in subtle rearrangements in the hydrogen bonding network surrounding the Schiff base. As deduced from the amide I and amide II regions, relatively large changes in the protein moiety occur upon formation of the active intermediate, green cone Meta II (4 °C, 375 nm). The corresponding FTIR difference spectrum further shows bands at 1778, 1761, 1748 and 1738 cm⁻¹, which we tentatively assign to carbonyl stretch vibrations of the protonated side chains of Asp99 and Glu102 in helix 2.
6.1 Introduction

While human dim light (scotopic) vision is mediated by a single cell type, the rod cell, daylight (photopic) vision involves three cell types, the red, green and blue cone photoreceptor cells, respectively. These cells contain photosensitive membrane proteins, which, although they all have 11-cis-retinal as ligand and chromophore, show distinct absorption characteristics. The wavelength of maximal sensitivity ($\lambda_{max}$) of the cone cells ranges from 420 nm to 560 nm, thus providing the essential conditions for color discrimination. Since isolation and purification of the mammalian cone pigments has never been described, the present knowledge of the activation mechanism of this type of receptor primarily stems from work on the rod visual pigment rhodopsin.

On the basis of sequence homologies among a wide variety of vertebrate visual pigments, a phylogenetic tree of these pigments was constructed consisting of four groups of pigments, corresponding to their spectral sensitivity [group L, long wave sensitive, including the chicken red (iodopsin, 571 nm), human red (560 nm) and green cone (530 nm) pigments; middle wave sensitive groups M1 (including chicken blue, 455 nm) and M2 (e.g. chicken green 508 nm); and group S, short wave sensitive, including human blue (420 nm) and chicken violet (415 nm)]. A separate subclass, Rh, containing the rod pigments, was postulated to have evolved from the M2 class of cone pigments (1). Bovine and frog rhodopsin, by far the best studied members of the Rh class, serve as a model for all visual pigments (Figure 6.1).

The functional expression of human cone visual pigments in the early nineties (4, 5) paved the way for studies on the mechanism by which these pigments tune their spectral sensitivity. Since then, site directed mutagenesis studies revealed that, of the 15 amino acid differences between the human green and red pigments, only three replacements of non-polar by hydroxyl-bearing amino acids in the vicinity of the ionone ring portion of the retinal are largely responsible for the 30 nm red-shift: Ala180Ser (helix 5), Phe277Tyr and Ala285Thr (both in helix 6) [cf. Figure 6.1, see (6) for a recent review].

The physical aspects of the wavelength regulation mechanism have been addressed using resonance Raman spectroscopy studies on cone pigments by Mathies and coworkers (7–10). In a recent study on recombinant human visual pigments, these authors concluded that the 30 nm red-shift of the green pigment (530 nm) relative to rhodopsin (498 nm) arises from an effective decrease in hydrogen bonding of the Schiff base proton. The red-shift of the $\lambda_{max}$ of the red pigment relative to that of the green pigment could be modeled by dipolar interactions between protein-dipoles in the vicinity of the ionone ring portion of the retinal (the above mentioned hydroxyl bearing residues), and the change of dipole moment of the retinal upon excitation, resulting in an effective lowering of the excited-state energy level. Finally, the blue-shift going from rhodopsin to the blue pigment was proposed to arise from the stabilization of charge near the Schiff base terminus, again by dipolar interaction, related to substitutions G90S, A292S and A295S\(^1\) (10). However, in

\(^1\)Numbering based on the sequence of bovine rhodopsin, these replacements correspond to A106S, A308S and A311S in HGH.
6.1 Introduction

Figure 6.1: Schematic representation of HGH, the hexahistidine tagged human green visual pigment described in this paper. A180, F277 and A285 (see text) are indicated in bold font. Putative N-glycosylation site Asn34 is indicated (2). By analogy to rhodopsin, there is strong evidence for a disulfide bond between Cys126 and Cys203 (3), cf. Figure 1.3. Sequence data and positioning of the transmembrane domains are taken from the GPCR database Heidelberg (http://swift.embl-heidelberg.de/7tm/, entry OPSG_HUMAN).

contrast to the substantial information on the mechanism of wavelength regulation, receptor activation in the human cone pigments, i.e. the photocascade leading to the G-protein activating conformation, is still poorly resolved.

Recently, in our group, the functional expression, large scale production and purification of a hexahistidine tagged human green cone pigment (HGH, 527 ± 2 nm) has been accomplished (2, 11). Reconstitution of the pigment in a native-like lipid environment allowed the first detailed analysis of the late photocascade of a mammalian cone pigment, including the pH dependence of the Meta I - Meta II equilibrium and the kinetics of the late photocascade transitions, Meta I → Meta II and Meta II → Meta III (11). In this chapter, we describe a preliminary FTIR examination on this green pigment. Our results on
the HGH → HGH-Batho transition are in general agreement with resonance Raman data on the human green pigment (10). In particular, our data is consistent with a torsionally distorted conformation of the chromophore in both dark-state HGH and HGH-Batho; it further suggests that the overall interaction between the chromophore and its counterion is essentially similar in both pigments. Hence, mechanisms explaining the difference in $\lambda_{\text{max}}$ of the rod and green cone pigment involving a planarization of the chromophore or the presence of additional charged groups near the chromophore are also not supported by the present work. However, the reduced difference intensity observed in both the amide I and HOOP regions of the HGH spectrum does suggest a more loose fit of the chromophore in green cone opsin than in rod opsin. So far, several group L pigments were reported to show a dependence of the absorption maximum on the presence of anions such as Cl$^-$ and NO$_3^-$ (12–14). Here, this issue is addressed by studying the HGH → HGH-Batho transition of the chloride (527 nm) and nitrate (508 nm) bound form of HGH. The overall similarity of the spectra of both forms is considered to indicate that the difference in wavelength sensitivity primarily stems from an altered interaction between the Schiff base and its direct hydrogen-bonding environment. Finally, the structural rearrangements leading to the physiologically active HGH-Meta II intermediate are compared to those in bovine rhodopsin.

6.2 Experimental Procedures

Production, purification and reconstitution of recombinant pigment. Large scale expression, regeneration with 11-cis-retinal and purification of the hexahistidine tagged HGH pigment was performed as described (11). The pigments were reconstituted into a lipid environment by the addition of a 200 molar excess of bovine retina lipids, isolated as described (15). The proteoliposomes containing the green pigment were isolated from the 20 % – 45 % interface of a sucrose step gradient. Regeneration and all manipulations afterwards were performed in deep-red light (RG645 cutoff filter, all filters from Schott, Mainz, FRG.).

Sample preparation for FTIR spectroscopy. The HGH proteoliposomes were diluted in buffer A (20 mM PIPES, 130 mM NaCl, 5 mM KCl, 2 mM MgCl$_2$, 2 mM CaCl$_2$, 0.1 mM EDTA, 2 $\mu$g/ml leupeptin, pH 6.5) and were then pelleted as described (10). Subsequently, the pellet was resuspended in buffer B (2 mM PIPES, 2 mM KCl, pH 6.5), and films for transmission IR spectroscopy were prepared by isopotential spin-drying 5 nanomoles of HGH in buffer B, based on A$_{530}$, onto an AgCl window as described in chapter 2. In order to remove residual sucrose, the films were washed in 1 ml buffer C (20 mM PIPES, 130 mM KCl, pH 6.5). Hereafter, the washing buffer was carefully removed and the films were redried as described above. For the purpose of anion exchange KCl was replaced by KNO$_3$ in buffer C. Anion exchange, the stability of the pigments under these conditions, and their ability to form Meta II were checked by UV/visible spectroscopy in parallel experiments using HGH films prepared on cellulose-acetate cover slips (11).

FTIR measurements. FTIR analyses of the HGH → HGH-Batho and HGH → HGH-Meta II transi-
6.3 Results

The wavelength of maximal sensitivity of the HGH pigment films, prepared by drying a proteoliposome suspension onto cellulose-acetate coverslips, were similar to those of the solution spectra, described before [not shown, (11)]. The $\lambda_{\text{max}}$ of native HGH was observed at 527 ± 2 nm (n = 5), while the nitrate bound form absorbed maximally at 508 ± 2 nm (n = 3).

6.3.1 The photocascade in the green cone pigment

Figure 6.2 compares the FTIR results obtained with the dark-state to Batho and dark-state to Meta II transitions for HGH and (bovine) rhodopsin. As discussed in chapter 4, the region below $\sim 1550$ cm$^{-1}$ in the Rho $\rightarrow$ Batho difference spectrum mainly reflects changes in chromophore conformation, while bands appearing in the 1600–1800 cm$^{-1}$ region mainly reflect protein activity. Most of the bands in the Rho $\rightarrow$ Batho difference spectrum have been assigned to specific chromophore vibrations\(^2\) on the basis of FTIR and resonance Raman analyses of rhodopsin regenerated with stable-isotope labeled chromophores (18–20). The spectra presented in this chapter are scaled with respect to the dark-state chromophore band near 970 cm$^{-1}$. Rhodopsin spectra are reproduced from chapter 4.

\(^2\)C=C and C–C stretch vibrations at $\sim 1550$ and $\sim 1200$ cm$^{-1}$, respectively; hydrogen-out-of-plane (HOOP) vibrations below 1000 cm$^{-1}$. 
C=C stretch modes), 1269 cm$^{-1}$ (HC$_{11}$=C$_{12}$H $A_u$ rocking mode), 1234 cm$^{-1}$ (predominantly C$_{12}$–C$_{13}$ stretch), 1215 cm$^{-1}$ (C$_8$–C$_9$ stretch), and 974 cm$^{-1}$ (HC$_{11}$=C$_{12}$H $A_2$ HOOP mode). Assignments were based on comparison with resonance Raman data obtained on rhodopsin ($^{19, 20}$) and iodopsin ($^9$). The HGH $\rightarrow$ HGH-Batho difference spectrum in Figure 6.2 displays negative bands, representing dark-state HGH, at 1534, 1269, 1232, 1218 and 974 cm$^{-1}$, which we assign correspondingly. Positive bands, representing the Batho photointermediate, are observed at 1508 (C=C stretch), 1209 and 1168 cm$^{-1}$ (C±C stretch modes). Kochendoerfer et al. ($^{10}$) reported the green cone pigment’s Batho ethylenic double bond stretch mode at 1515 cm$^{-1}$. The appearance of this band at a lower frequency in our data may be due to the subtraction of bands showing considerable overlap in the dark-state and Batho form of the pigment$^3$. Although the good correspondence between the spectra of the rod and green cone pigment implies a similar coupling of the chromophore C–C modes, detailed assignments of these bands requires isotope labeling. In the case of rhodopsin, the positive band at 1166 cm$^{-1}$ has been assigned to the C$_{10}$–C$_{11}$ stretch in Batho and the 1207 cm$^{-1}$ band to a mixed mode with C$_8$–C$_9$ and C$_{14}$–C$_{15}$ character ($^{19}$).

The chromophore bands in the C=C and C–C stretch regions are well known to be sensitive to the charge delocalization across the retinal polyene backbone. The 30 nm red-shift of the $\lambda_{\text{max}}$ of the green pigment relative to rhodopsin, indicative of a more delocalized electronic distribution, thus has an infrared counterpart which appears as an approximately 30 cm$^{-1}$ down-shift of the ethylenic C=C bands. The C–C stretch bands are expected to shift to higher frequencies upon increasing charge delocalization. However, the effects on these modes are generally smaller ($^{9, 10}$). Indeed, the pattern in the 1300–1150 cm$^{-1}$ region is quite similar to that observed in the case of rhodopsin.

The position of the bands in the HOOP region at 912, 870, 849, 836 and 817 cm$^{-1}$ is also in good agreement with the occurrence of bands in the resonance Raman study: 914, 869, 850, 838 and 813 cm$^{-1}$, assigned to the C$_{11}$H, C$_{10}$H, C$_{12}$H, C$_{14}$H, and HC$_7$=C$_8$H $B_g$ combination, respectively ($^{10}$). On the basis of resonance Raman studies on the rhodopsin counterion mutant E113A ($^{25}$), the relatively small downshift of $\sim 10$ cm$^{-1}$ of the C$_{11}$H and C$_{14}$H modes in the green cone bathointermediate spectrum relative to Batho, was not considered to be indicative of a major change in interaction between the chromophore and its counterion in the green cone pigment ($^{10}$) but may be related to a slightly different position of the retinal moiety with respect to the counterion in HGH (see chapter 4). As discussed in previous chapters, HOOP intensity has been related to the non-planarity of the polyene chain in the all-trans chromophore in bathorhodopsin ($^{16, 20, 26}$). Similarly, the set of bands in the HGH spectrum is considered to derive from a twisted retinal backbone structure in HGH-Batho. The reduced HOOP intensity in the HGH-Batho spectrum is consistent with that observed in the resonance Raman experiments ($^{10}$). Especially the band assigned to the C$_{11}$H wag is significantly less intense, indicating that the torsional strain in the isomerization region (C$_{10}$···C$_{13}$) of the chromophore is less than that in rod

$^3$Conceivably, for similar reasons the rhodopsin C=C band is up-shifted by $\sim 10$ cm$^{-1}$ in FTIR difference spectra to 1558 cm$^{-1}$ ($^{16, 18, 21–23}$), compared to the value reported in resonance Raman studies of 1546 cm$^{-1}$ ($^{10, 19, 24}$).
Figure 6.2: FTIR difference spectra of the HGH → HGH-Batho and HGH → HGH-Meta II transitions. Corresponding rhodopsin (Rho) spectra are also shown. The traces are scaled with respect to the ∼970 cm$^{-1}$ (−) chromophore band. Y-scale is for the Rho → Batho spectrum.
bathorhodopsin.

The overall features in the HGH → HGH-Batho difference spectrum show a remarkable resemblance to their counterparts in rhodopsin. However, there are also some differences which hint at slightly altered chromophore-protein interaction in the green pigment. In addition to the reduced intensity in the HOOP region compared to Batho, the bands appearing in the 1700–1600 cm⁻¹ region are considerably less intense in the HGH → HGH-Batho spectrum. For rhodopsin, the bands in this region have been tentatively assigned to changes in peptide C=O modes (amide I), reflecting subtle rearrangements in the peptide backbone of several residues lining the retinal binding site (76). In principle, the Schiff base C=NH mode may also be observed in this region, however, since this mode is not altered in the Rho → Batho transition (19), it does not appear in the corresponding FTIR difference spectrum. In the green cone pigment the latter is expected to absorb near 1640 cm⁻¹ (10), and the lack of significant difference intensity observed in the HGH → HGH-Batho spectrum therefore suggests that also in the green cone pigment the Schiff base C=NH mode remains essentially unaffected during this transition. Together the reduced intensity in the amide I and HOOP region of the HGH spectrum suggests that possible protein backbone rearrangements are more subtle at this early stage, and that the steric constraints imposed on the chromophore are reduced in HGH. This is consistent with a less tight fit of the retinal moiety in the binding pocket of green cone opsin, relative to the situation in the rod pigment (see e.g. chapter 4).

The HGH → HGH-Meta II transition. The structural changes leading to the physiologically active Meta II conformation are compared in the bottom traces in Figure 6.2 and, on an expanded scale showing the 1800–1600 cm⁻¹ region, in Figure 6.3. The ability of HGH-Meta II to activate transducin has been reported elsewhere (11). Interestingly, the overall appearance of the bands in the amide I region (Figure 6.3) indeed shows many similarities to the Rho → Meta II spectrum. Also, as for rhodopsin (Figure 6.2), the HOOP bands which are dominantly present at the Batho stage are not observed in the HGH-Meta II spectrum, indicating that at this stage the torsional strain in the chromophore has essentially relaxed. In contrast, marked differences between rhodopsin and HGH may also be observed below 1550 cm⁻¹. However, since the late photocascade transitions primarily involve protein rearrangements, these differences most likely relate to dissimilarities in the primary structures of the pigments.

As discussed in previous chapters, some of the bands in the 1700–1800 cm⁻¹ region of the bovine Rho → Meta II difference spectrum (Figure 6.3) were assigned to C=O stretch modes of specific Asp and Glu carboxyl groups in the transmembrane domain using site-directed mutagenesis in combination with FTIR. Thus, the 1768/1748 cm⁻¹ (−/+ ) pair of bands has been assigned to D83 (22, 28); the band at 1728 cm⁻¹ (−) and part of the 1748 (+) band were tentatively assigned to E122 (28). Furthermore, part of the positive band at 1711 cm⁻¹ has been assigned to E113 (29), the counterion to and proton acceptor of the Schiff base proton in rhodopsin and metarhodopsin II, respectively (30–32). In HGH, the residue at the analogous position of Glu122 is Ile138 in HGH, and therefore cannot
6.3 Results

Figure 6.3: Expanded view of the 1800–1600 cm$^{-1}$ region of the HGH/Rho → Meta II spectra displayed in Figure 6.2. Y-scale is for the HGH spectrum.

contribute to this spectral region. The only Asp and Glu residues in the transmembrane domain of HGH are E129, at the equivalent position of E113 in bovine rhodopsin, D99 (D83 in Rho) and E102 (M86). It is tempting to assign the bands in this region of the HGH spectrum by correspondence: the band at 1711 cm$^{-1}$ is tentatively assigned to E129; D99 and E102 may then contribute to the 1728, 1738, 1761 and 1778 cm$^{-1}$ band-pattern.$^4$

Unfortunately, we were as yet unable to perform hydrogen-deuterium exchange studies on the HGH pigment, which could substantiate the above speculative assignments to Asp or Glu carbonyl vibrations.

We should note, however, that in a more recent experiment [Bovee-Geurts et al. (not shown)], although the bands below 1612 cm$^{-1}$ were clearly reproduced, the 1655 cm$^{-1}$ (−) band was absent and that rather a broad positive feature between 1687–1634 cm$^{-1}$ was observed. Moreover, the bands appearing above 1720 cm$^{-1}$ were only discernible as shoulders, while the 1778 (−)/1761 (+) cm$^{-1}$ pair was in fact not observed at all. At this point, we cannot yet fully explain these differences. Although variability in the strongly absorbing amide I region may be due to small differences in sample preparation, in the experiment shown here, the 1655 cm$^{-1}$ band gradually appeared in about 3 minutes after illumination. Hence, another possibility is that at 4 °C a significant change in protein conformation may still be in progress. This is supported by the recent observation that also at 0

$^4$Note that a band at 1736 cm$^{-1}$ has been observed in the Rho → Meta II difference spectrum using polarized IR light (chapter 3). This band was tentatively assigned to E134 (E150 in HGH).
°C a 380 nm intermediate is formed, and that the corresponding FTIR difference spectrum shows additional differences, especially in the (carboxyl?) C=O and the amide I and II regions. However, in view of the lifetime of the HGH-Meta II intermediate \([T_{1/2} = 5 \text{ min at } 4^\circ\text{C}]\) we cannot exclude the possibility that the slow formation of the 1655 cm\(^{-1}\) (−) band actually reflects Meta II decay. In rhodopsin, the decay of Meta II is believed to involve a partial refolding of the protein moiety, which may be monitored as a partial reversal of the infrared difference-band pattern during Meta II decay \((33, 34)\). This, however, was not observed in the present experiments on HGH. Future work will focus on these possible differences in the late photocascade in both systems.

### 6.3.2 Analysis of the effects of anion exchange

The effects of anion exchange on chromophore-protein interactions in HGH were studied in the HGH → HGH-Batho transition, since the corresponding difference spectrum presents the most detailed signature of the chromophore and its environment in the dark state of the receptor. Figure 6.4 compares the results obtained on chloride (taken from Figure 6.2) and nitrate bound HGH. Panels 4b and 4c present expanded views of the C−C stretch (b) and HOOP regions (c), and show the rhodopsin spectra for comparison. Although similar amounts of material were used, the maximal difference signal obtained in the nitrate bound form was about 1/3 of that of the chloride bound pigment (1/6 compared to the maximal signal in the Rho → Batho spectrum in Figure 6.2). In addition, photoregeneration of HGH-Batho → HGH was less efficient, as judged from the overall spectral intensity. This may relate to an increased instability of the nitrate bound HGH photoproduc-ult, or to the enhanced formation of 9-cis-HGH in the photoconversion of nitrate bound HGH-Batho (see below).

In Figure 6.4 it can be seen that the bands in the C−C stretch region appear at a slightly higher frequency in the spectrum of the nitrate bound form, as compared to those for the chloride bound form of HGH. Some of the C−C stretch bands, on the other hand, exhibit a slight down-shift. This is especially clear from the down-shift of the bands at 1232 (−), 1178 (+) and 1168 cm\(^{-1}\) (+), and the disappearance of the 1225/1217 cm\(^{-1}\) (+/−) pair in the spectrum of the nitrate bound pigment, which seems to be caused by the down-shift of the positive band at 1225 cm\(^{-1}\). As noted above, these shifts may represent the equivalent of the anion related 20 nm down-shift of the λ\(_{max}\) in the visible region. The magnitude of the shift of the C−C bands is indeed in general agreement with that observed going from rhodopsin to the green pigment, representing an almost identical shift in λ\(_{max}\). However, the up-shift of the C−C stretch bands, of only a few wavenumbers, is much smaller than what may be expected on the basis of comparison of the HGH → HGH-Batho spectrum (chloride form) with the corresponding Rho spectrum\(^5\). In rhodopsin, the C−C bands

\(^5\)Since the C=O mode of the 9-cis-retinal (or iso-) green cone (chloride bound) pigment has been observed at 1548 cm\(^{-1}\), the possibility that this is due to the presence of iso-HGH is not very likely. On the other hand, the presence of iso-HGH may be inferred from the additional negative band at 965 cm\(^{-1}\) (Figure 6.4c), which is a marker band for 9-cis-retinal conformers \((35)\). Note, however, that other bands characteristic for this isomer, e.g. at 1013 cm\(^{-1}\) \((23)\), are not clearly observed.]
Figure 6.4: The effect of anion exchange on the HGH → HGH-Batho transition. Overview of the 1800–800 cm$^{-1}$ region (a), and expanded views of the 1300–1100 (C–C) cm$^{-1}$ region (b), and HOOP region (c). Scaling similar to that in Figure 6.2. Y-scale is for the spectra obtained in nitrate buffer (see text).
have been primarily assigned to the $C_7=C_8$ and $C_{11}=C_{12}$ stretch modes. Conceivably, the very small effect on the HGH $C=\!C$ stretch modes implies that anion exchange alters the electronic properties of the chromophore mainly by affecting the $C_{13}$-$\cdots$-$C_{15}$ region of the retinal (i.e. the Schiff base terminus)$^6$. The overall similarity of the spectra of the chloride and nitrate bound forms of HGH suggest that the effects of anion exchange are indeed very subtle, and the possibility that major changes in ligand-receptor interactions are induced by anion exchange seems therefore unlikely.

### 6.4 Discussion

Vibrational spectroscopy has been extensively used in studies on structural aspects in the photocascade of rhodopsin. Resonance Raman spectroscopy, which probes the chromophore and its immediate environment, has given important clues regarding the mechanisms of photon energy storage at the Batho stage, and the mechanism behind the ultra-fast photoproduct formation time (reviewed in chapter 4). FTIR difference spectroscopy of the various transitions in the photocascade, on the other hand, proved to be very powerful since it monitors structural changes, not only in the chromophore, but also in bound water, and the protein and lipid phase of the sample. Until recently, the rod pigment rhodopsin was the only G-protein coupled receptor for which such analyses could be performed. Studies on related receptors had to await the achievement to obtain these proteins in large enough quantity and in a sufficiently pure form. The recently accomplished large scale functional expression, purification and reconstitution of the human green cone visual pigment ($2, 11$) allowed the initial FTIR studies presented in this chapter.

The FTIR results obtained for the HGH $\rightarrow$ HGH-Batho transition are in qualitative agreement with the results from resonance Raman studies published previously on human ($10$) and chicken cone pigments ($9$). This allowed an initial assignment of the observed FTIR difference bands by correspondence. Moreover, the overall band pattern of the HGH spectrum shows a striking similarity to that of the Rho $\rightarrow$ Batho transition. The main differences are the $\sim$30 cm$^{-1}$ downshift of the ethylenic $C=\!C$ stretch modes, and the slight upshift of some of the bands in the $C-C$ stretch region$^7$. However, these differences are likely caused by the altered charge distribution across the retinal backbone, and present the IR equivalent of the 30 nm red-shift of the UV/visible $\lambda_{\text{max}}$. Upon comparison of the pattern of bands observed in the HOOP region of HGH-Batho with that in resonance Raman

$^6$The observation that the effects of the altered charge delocalization differentially affect the chromophore $C=\!C$ and $C=\!C$ modes may be somewhat surprising. However, this is not without precedent, since for bovine rhodopsin mutant E122Q (481 nm) the $C=\!C$ modes, but not the $C=\!C$ and Schiff base $C=\!N$ (1657 cm$^{-1}$) modes were affected. ($25$). The latter is a rather localized coordinate ($9, 10$) and is due to coupling of the $C=\!N$ stretch mode and the $N=\!H$ rocking mode. It is therefore sensitive to the hydrogen bonding environment of the Schiff base, as well as the bond order of the Schiff base $C=\!N$ bond. Unfortunately, for reasons outlined above, this mode is not observed in FTIR difference spectra.

$^7$While the 1238 cm$^{-1}$ has its largest contribution from the $C_{12}-C_{13}$ stretching mode, this band is mixed with other chromophore stretching and rocking modes. The downshift of this band to 1232 cm$^{-1}$ in the green pigment has therefore been attributed to a slightly altered coupling pattern ($10$).
and difference FTIR spectra of rhodopsin counterion mutants E113A, E113Q, and G90D (25, 27, 36), it is argued that the interaction between the chromophore and its counterion in HGH is essentially similar to that in rhodopsin. Indeed, in agreement with the resonance Raman study by Kochendoerfer et al. (10), the present work does not support concepts involving chromophore planarization or the presence of additional charged groups to account for the difference in $\lambda_{\text{max}}$ between the rod and green cone pigment.

While the position and coupling pattern of the bands observed in the HOOP region suggests that the chromophore-counterion interaction is similar in both pigments, the observed decrease in intensity of especially the band assigned to the C$_{11}$H wag is indicative of a reduced steric interaction between all-trans retinal and HGH-opsin compared to the situation in bathorhodopsin. This decrease is even more pronounced in the resonance Raman spectrum of the human green cone pigment (10), where this band has almost completely disappeared in the background noise. Together these results are consistent with reduced torsional strain in the C$_{10}$· · · C$_{13}$ region of the all-trans chromophore in HGH-Batho (10, 20, 37, 38). Furthermore, the present IR difference spectra exhibit low intensity in the amide I region suggesting that possible changes in the peptide backbone occurring upon formation of HGH-Batho are smaller than in the case of rhodopsin. Hence, all available evidence indicates that the retinal moiety fits less tightly in the binding pocket of green cone opsin than of rod opsin.

A major point of discussion has been how photon energy is stored in Batho to drive the subsequent thermal transitions. Experimental evidence on rhodopsin seems to converge on a photon-energy storage mechanism involving a torsionally distorted conformation of the all-trans chromophore in bathorhodopsin (chapter 4). The present results and the previous resonance Raman study on the human green cone pigment (10) imply, however, that this mechanism is less efficient than in rhodopsin. Of course, other mechanisms, like charge separation between the protonated Schiff base and its counterion, may contribute to energy storage as well. A sensitive marker of the electronic environment of the Schiff base is provided by its C=NH(D) vibration (9, 10). In rhodopsin, neither the frequency nor the intensity of this mode changes significantly in the Rho $\rightarrow$ Batho transition, and already on this basis it has been concluded that charge separation only can have a minor contribution to energy storage in the primary photoproduct (19). For obvious reasons, the C=NH(D) mode is not readily observed in FTIR difference spectra of this transition. This mode is expected to absorb near 1640 cm$^{-1}$ in HGH (10). Hence, the absence of significant difference intensity in this region of the HGH $\rightarrow$ HGH-Batho spectrum suggests that the Schiff base environment in HGH also does not change significantly during this transition. If indeed less energy is stored in HGH-Batho, this would suggest that less energy is needed to reach the physiologically active conformation of the receptor. Conceivably, this forms one of the molecular determinants of the higher dark-noise of cone photoreceptor cells (linked to spontaneous thermal activation of the pigments, see e.g. chapter 1), and consequently the lower photosensitivity of cones compared to rods (39). Future resonance Raman studies or FTIR analysis of labeled pigments, in combination with photocalorimetric studies should provide further insight in the mechanism of energy storage in cone pigments.
Recently, H197 and K200, located in the extracellular loop connecting helix 4 and 5 (Figure 6.1), were identified as key constituents of the anion binding site in the human red and green cone pigments (14). It has been shown previously that anion exchange also affects the HGH-Meta I intermediate (490 nm → 463 nm) but not HGH-Meta II (11). Assuming that the binding site is not affected upon Meta II formation, this suggests that a protonated Schiff base is required for the anion sensitivity of the $\lambda_{max}$. Although the Schiff base C=NH mode is not observed in the HGH → HGH-Batho FTIR difference spectrum, the overall similarity of the chromophore C=C and C–C bands of the chloride and nitrate bound forms of the pigment indeed suggest that the effects of anion exchange on the chromophore involve predominantly the Schiff base terminus. It has been previously proposed that the removal of the electrostatic potential of the chloride ion provokes a reordering of water molecules in a hydrogen bonding network including the anion binding site and the Schiff base (10). This reordering might then alter the hydrogen bonding of the Schiff base proton and cause a blue-shift of the $\lambda_{max}$, by a similar mechanism as responsible for the shift observed going from rhodopsin to the green pigment. Studies combining Raman (C=NH mode) and (polarized) FTIR difference spectroscopy [(orientation of) water OH modes] could be used to test this hypothesis.

Since protein conformational changes dominate the late photocascade transitions, it is not surprising that the features in the HGH → HGH-Meta II spectrum differ substantially from those in the corresponding rhodopsin spectrum. Importantly, however, there are also several similarities to the case of rhodopsin, which especially become apparent in the 1800–1600 cm$^{-1}$ spectral region. Bands assignable to carboxyl vibrations of Asp and Glu residues are also observed in this region. We tentatively assigned these spectral features to D99 and E102. Both residues are located in helix 2, a segment that also participates in the last stages of rhodopsin activation. Conceivably, similar conformational rearrangements take place upon activation of the rod and the green cone pigment. However, we note that in the 380 nm photoproduc of HGH, under our experimental conditions, a substantial conformational rearrangement of the pigment may still be in progress. In this respect there may be a significant difference between the cone and rod pigment, where the largest conformational change appears to be correlated with Schiff base deprotonation. Further comparison between both systems should provide essential information on the basic mechanism of photoreceptor activation.

References

Chapter 7

General Discussion and Summary

The visual pigments form the best characterized subset in the super-family of G-protein coupled receptors (GPCRs). They share their main characteristic features: seven transmembrane segments (Tms) with strong α-helical character, that connect through loop structures of which the ones at the intracellular side of the membrane, upon activation of the receptor, generate high affinity binding domains for a heterotrimeric GTP-binding protein (G-protein). Thus, GPCRs mediate extracellular stimuli across the cell membrane in order to provoke a cellular response. Understanding of the structural aspects in the activation mechanism of these receptors, i.e. the conformational rearrangements following binding of the activating ligand (agonist) and leading to the active state (R*), is highly desirable since the GPCR clan includes receptors for neurotransmitters and hormones, and thus represent major targets for pharmacological intervention. However, in order to facilitate selective drug design, structural information about the receptor, ligand-binding and activation mechanism is required at atomic detail. The best prospects to achieve this level of detail for a GPCR in the near future are presently offered by the visual pigments, and especially the rod pigment rhodopsin.

As described in Chapter 1, in their evolution to adapt to a photosensory function, the visual pigments have developed some unique features among the GPCRs. They contain a photosensitive ligand (or chromophore: 11-cis-retinal), which is covalently bound to the protein (opsin) by means of a Schiff base linkage to a lysine residue, positioned in the trans-membrane domain. The Schiff base bond is stabilized by protonation, and this positively charged structure is stabilized by a local negative charge, originating in a nearby
glutamic acid carboxylate group. In rhodopsin this construction results in an extraordinary high pKₐ of the Schiff base of over 16 (from a normal range of 6–7), thus minimizing spontaneous deprotonation, and is believed to be responsible for the extremely low-noise characteristics which allows this receptor to function under very dim light conditions. In fact, the ligand acts as an inverse agonist: the basal activity of the receptor when bound to the retinal is orders of magnitude lower than that of opsin alone. Absorption of a photon triggers an ultra-fast isomerization of the chromophore to the all-trans conformation, and converts the ligand into a full agonist, resulting in rapid (msec), high power (about 10⁶ increase in activity over the dark state) activation of the receptor.

While to our knowledge 3-D crystals suitable for high-resolution X-ray diffraction analysis have not yet been produced for any GPCR, the visual pigments offer unique access to study the molecular mechanism of activation of a GPCR since discrete, spectrally and structurally distinct steps in the activation process [photocascade: Rho → Batho → Lumi → Meta I → Meta II (= R*)] can be isolated either thermally, or kinetically. EPR, (solid state) NMR, UV-visible and vibrational spectroscopic methods, in combination with well-defined modifications in the chromophore-protein complex, have been applied to address the intermediate conformations in rhodopsin activation, and several key observations are reviewed in chapter 1. To date, FTIR difference spectroscopy proved to be the most versatile approach to extract atomic-level structural data from this receptor. With this technique, changes in absolute structure, in interaction pattern, or in micro-environment can be monitored by the corresponding change in vibrational frequency and/or intensity.

The aim of this thesis was to apply FTIR spectroscopy to further identify protein activity and protein-ligand interactions in the various stages of receptor-activation, and especially during formation of the physiologically active intermediate: metarhodopsin II.

Identification of protein activity may be accomplished by assigning FTIR difference bands to specific protein chemical groups or bonds. A first identification of FTIR difference bands may sometimes be accomplished by noting that their frequency lies within a spectral region highly characteristic of a certain type of vibration. However, usually subtle modifications of the system are required which affect the difference band pattern and consequently allow the assignment of the original difference band to a specific residue or region in the protein. To date, this has been mostly accomplished using site-specific mutagenesis.

Chapter 2 (probably) illustrates the potential power of the combined IR–protein-engineering approach at its best: not only could changes in (bound) water OH stretch modes be identified in the Rho → Meta II difference spectrum by substitution of H₂¹⁸O for H₂O, indeed, a possible localization of this structurally active water was facilitated making use of a site-directed rhodopsin mutant (C185S). In addition, four cysteine mutants (C167S, C185S, C222S and C264S) were studied in an attempt to identify the 2550 cm⁻¹ FTIR difference band which was previously reported to be characteristic of the metarhodopsin II intermediate. This band lies within a spectral region characteristic of cysteine SH stretch modes. We found that only by replacement of C167 about 70% of the difference intensity disappeared, and we therefore assign this band to C167. The remaining difference inten-
sity is probably due to the spectral contribution of a residual cysteine change originating in Meta I, but, although C167, C185 and C222 can already be excluded, definitive assignment to (a) specific cysteine residue(s) requires further analysis. On the basis of the present results, since only a positive difference band is detected, we cannot yet fully specify the nature of the conformational change involving C167 in the Meta I → Meta II transition.

Several lines of evidence already indicated that tyrosine residues are involved in the photoactivation of rhodopsin. An interesting candidate is Y268, which in several rhodopsin models has been located in direct vicinity of the chromophore. However, the rather conservative mutation Y268F reportedly results in reduced capability to bind 11-cis-retinal as well as transducin activation ability. Clearly, given the mosaic effects this replacement has on functional properties of rhodopsin, this tyrosine is structurally important. However, the replacement of such key residues is also expected to show a manifold of effects on the Rho → Meta II FTIR difference spectrum, and if at all possible, it will be very hard to directly assign vibrational bands. The recent achievement to build in stable-isotope labeled amino acids allowed to circumvent this problem. FTIR studies on \([\text{ring-}^{2}\text{H}_4]\)Tyr-labeled rhodopsin, presented in chapter 2, provide clear evidence for the participation of several (at least two) tyrosin(at)e residues in receptor activation, possibly in proton transfer reactions. By analogy to the case of bacteriorhodopsin (bR → M transition), we argue that at least part of the changes we observe may be due to Y268. In bR, the analogous residue Y185 has been suggested to be part of a hinge region (Y185/P186) in helix 6, facilitating the apparent tilt of part of this helix late in the bR photocycle. In rhodopsin, the P267/Y268 pair may have a similar function, since also for rhodopsin movements of helix 6 have been proposed to occur upon photoactivation. However, to definitely identify Y268 as (one of) the residue(s) responsible for the changes we observe, further studies (and in preference site-directed isotope labeling) will be required.

Chapter 3 describes the use of polarized infrared light to obtain information on intramolecular orientations from infrared difference spectra. In this study we made use of the attenuated total reflectance (ATR) sampling technique because it offers important advantages over polarized transmission spectroscopy in the application to the Rho → Meta II transition. Apart from detailed information on relative orientations of both chromophore and protein chemical bonds in rhodopsin and metarhodopsin II, evidence is presented for a small reorientation of helical segments in the activation of rhodopsin as well as for the protonation of a previously undetected carboxyl-group-bearing residue. We suggest that the latter may be E134. Considerable evidence has accumulated that this residue becomes protonated during metarhodopsin II formation; however, from previous (unpolarized) difference FTIR studies on site-directed mutants this could not be directly confirmed. Finally, the dichroic properties of the bands in the HOOP region suggest a reorientation in the retinal polyene tail in the Meta I → Meta II transition. Conceivably, this movement places the retinal Schiff base moiety in a favorable position relative to its proton acceptor [either E113 directly or via intermediate group(s) (e.g. Y268)], and facilitates proton transfer from the Schiff base nitrogen in the Meta I → Meta II transition.

Symbiosis between opsin and chromophore in rhodopsin has pushed the efficiency of
its photochemistry to an incredible limit; a nearly complete stereo-specificity, sub-ps kinetics and a quantum yield of 0.67 are far beyond the values achieved in model systems. Since high resolution structural data of the binding pocket is still unavailable, chromophore-protein interactions are not readily addressed from the protein side. In this context, the study of rhodopsin analogs has offered the most significant progress. Chapter 4 describes the photochemistry of a rhodopsin analog regenerated with the 10-methyl homolog of 11-cis-retinal. This chromophore analog was chosen since it was expected to significantly increase the steric interference in the retinal by the interaction of the methyl groups at C_{10} and C_{13}, thereby increasing the strain in the isomerization region (C_{10} \cdot \cdot \cdot C_{13}), and consequently to affect the photoisomerization properties.

Indeed, the UV/visible absorbance and CD data, as well as the infrared difference spectrum of the Rho $\rightarrow$ Batho transition of 10-Me rhodopsin are all indicative of enhanced torsional strain in the 10-Me chromophore in rhodopsin. Furthermore, the additional methyl group strongly perturbs two stages of the photocascade. Interestingly, although the quantum efficiency of the process is somewhat reduced (0.67 $\rightarrow$ 0.55), photoisomerization of this chromophore results in a "normal" Batho intermediate with a strained all-trans chromophore conformation. However, then, it first markedly retards relaxation of the photoisomerized chromophore and the occurrence of structural changes in the protein moiety; a stable Batho intermediate was observed at temperatures as high as 180 K, where native rhodopsin adopts its Lumi conformation. The important conclusion is, that relaxation of the chromophore drives the subsequent structural changes in the receptor. Subsequently, the 10-methyl group interferes with the conformational transition of the Meta I intermediate into Meta II. An interesting possibility is that the additional methyl group interferes sterically with the reorientation of the retinal moiety in the Meta I $\rightarrow$ Meta II transition (chapter 3). Significantly, the rate of formation of Meta II is about 6 orders of magnitude slower in the 10-methyl pigment (21 °C), while the Arrhenius activation energy of this reaction is identical to that of rhodopsin. This suggests that there is a large entropic component to Meta II formation, that makes this reaction fast in spite of its high activation enthalpy. By compiling literature data, we calculate that this entropy factor is reduced by $\sim$30 kJ/mol in 10-Me rhodopsin at 21 °C. Future work will focus on the identification of the protein groups involved in the interaction with the 10-methyl group.

In native rhodopsin, the rate of formation of the metarhodopsin II intermediate depends strongly on the micro-environment (lipid environment, pH, temperature). This has been mainly related to the final step in receptor activation: the equilibrium between metarhodopsin II and its precursor metarhodopsin I. In addition, based on mutagenesis studies involving the replacement of uncharged for charged groups (and vice versa) in the putative transmembrane segments, it has been proposed that there is a major electrostatic component to the formation of Meta II from Meta I.

In Chapter 5 the effects of ionic strength on the Meta I – Meta II equilibrium are examined. It is found (by UV/visible spectroscopy) that the formation of Meta II in a native lipid environment is strongly enhanced at increasing concentrations of salt in the membrane suspension. Indeed, not only a tremendous shift in pK_{a} (3 pH units, 0 $\rightarrow$ 4 M KCl,
10 °C) of the equilibrium was observed, also the rate of formation of Meta II was found to increase about ten-fold (2 M KCl, pH 7, −6 °C). ATR-FTIR difference spectroscopy confirmed the shifted equilibrium position, and suggested that the salt-effect does not seem to be related to structural changes in protein conformation and/or membrane fluidity. A Gouy-Chapman model was tested for its ability to explain the altered equilibrium position by shielding of surface charges at the membrane interface. It was found that up to 1–2 M KCl the salt-effect may indeed be caused by shielding of a positive surface charge. We argue that these positively charged groups most likely reside on the cytosolic protein surface. On the whole, these results indeed support the concept that there is a significant electrostatic component to the Meta I – Meta II equilibrium. Finally, it is argued that the pH dependence of the transition most likely is regulated by several protein groups, in contrast to an earlier suggestion that the apparent pKₐ relates to the protonation of a specific histidine residue.

There is a clear correlation between progress in vision research and that in the fields of (large scale) functional expression and purification of recombinant pigments. While rhodopsin research by now is ready to step into the mg regime, i.e. the realm of NMR spectroscopy, the functional expression of mammalian cone pigments has already facilitated the first characterizations using vibrational spectroscopy. Of course, analysis of these pigments is significantly speeded up by comparing to their rod counterparts. Conversely, structural analyses of cone pigments are expected to enhance our understanding of the mechanisms of wavelength tuning, energy storage, and signal generation in visual pigments in general.

Chapter 6 presents the first application of FTIR spectroscopy to the photocascade of a cone pigment [recombinant hexahistidine tagged human green cone pigment (HGH, 527 nm)]. Previous resonance Raman studies on the human green cone pigment already indicated that the main difference in chromophore protein interaction in this pigment, relative to rhodopsin, resides in the hydrogen bonding environment of the protonated Schiff base terminus of the retinal. Indeed, the FTIR spectra of the dark state → Batho transition in the green pigment indicate that neither a planarization of the 11-cis-chromophore nor the presence of additional charged groups in its immediate vicinity need to be considered to explain the 30 nm red shift of the λmax. On the other hand, judged from the intensity of the bands in the HOOP region (1000–800 cm⁻¹), the all-trans chromophore in green cone bathorhodopsin seems less strained than in the case of rhodopsin. In addition, photoisomerization appears to induce smaller structural activity in the protein backbone, as judged from the low intensity in the amide I region in the HGH → HGH-Batho difference spectrum. This suggests that the retinal moiety has more conformational freedom in the binding pocket of the green cone pigment. In rhodopsin, torsional strain in the chromophore is believed to be the predominant storage mechanism for the incoming photon energy and this obviously requires a tight fit of the retinal moiety in the binding pocket of rod opsin. Since in HGH this mechanism seems less efficient, it appears that less energy is needed to reach the active conformation of the receptor. This would then render the cone photoreceptor cell more susceptible to spontaneous (thermal) activation, and may provide a framework for
understanding its lower photosensitivity relative to the rod cell. The substitution of nitrate ions for (native) chloride in the anion binding site, which reportedly results in an altered wavelength sensitivity of the green pigment (527 → 508 nm), appears to affect mainly the Schiff base terminus of the chromophore, possibly through the involvement of bound water molecules. Finally, the conformational rearrangements involving the formation of the 380 nm photoproduct were compared to those in rhodopsin. While several similarities in the FTIR difference band pattern were observed, we noted that in the 380 nm photoproduct of HGH, under our experimental conditions, a major conformational rearrangement of the pigment may still be in progress. Hence, in the green cone pigment, in contrast to the situation in rhodopsin, significant conformational changes may occur well after Schiff base deprotonation. This is a very interesting point and further comparison between both systems is expected to provide essential information on the basic mechanism of photoreceptor activation.

Having summarized the results obtained in the course of this project, we may now attempt to place them in the context of related studies on intramolecular signal transmission in visual pigments, reviewed in chapter 1. The signaling states of rhodopsin, in which it is able to interact with its partner proteins, are reached in a sequence of events initiated by the absorption of a photon. Somehow, the photon energy, stored in the retinal moiety upon photoactivation, is relayed to the surface of the receptor where all biochemically relevant interactions are thought to occur. Through the years, several key events were identified in the formation of the physiologically active (bovine) metarhodopsin II intermediate (deprotonation of the Schiff base, protonation of E113, uptake of protons from the surrounding medium), and functional roles were assigned to single residues. The concept of ‘salt-bridge breaking (salt-bridge between K296 and E113, protonation of E134) was introduced to explain the difference between the inactive and active states of rhodopsin. A steric trigger mechanism (steric trigger: G121 and the 9-Me group of the retinal) was postulated to account for the photon energy transfer into the protein moiety, leading to the conformational changes up to Meta I. The present work indeed confirms the importance of Coulombic interactions in the transition between the Meta states and that steric interactions are mainly responsible for signal transmission from the chromophore into the protein moiety. However, we believe that a clear separation between these two concepts, and especially the assignment of functional roles to individual residues, does not fully cover the complexity of the presently available experimental data.

Indeed, several lines of evidence argue for a mechanism involving more extended structures. First, site-directed mutagenesis studies have shown that position 113 is not unique to accommodate a functional counterion to the protonated Schiff base. Second, substantial evidence is accumulating in the literature that different (isochromic 380 nm) forms of the metarhodopsin II intermediate exist which show different activities towards partner proteins, and even transducin domains. Third, the effects of KCl on the apparent pKa of the Meta I – Meta II equilibrium argue for the involvement of several residues in tuning the pH dependence of this transition. Finally, we note that the results obtained on the 10-methyl pigment (chapter 4), as well as those from the IR linear dichroism study in chapter
3, clearly indicate that the interplay between chromophore and protein is important up to the formation of Meta II.

Following up on our earlier hypothesis, we believe that the most flexible way to accommodate the present results would be an extended, possibly modular organized, H-bonded network in which most likely also water molecules participate. Indeed, FTIR analyses have presented firm evidence for the participation of one or more water molecules in every photocascade transition. In addition, all protein residues identified so far to contribute to the photocascade (D83, E113, E134, C167, Y268, and additional Tyr and Cys residues) undergo protonation or H-bond interaction changes. As previously proposed, the role of E113 in this concept may be envisioned to sustain the structural integrity of the network, rather than to provide a unique counterion to the protonated Schiff base. This offers an attractive explanation for the observation that functional counterions to the Schiff base can also be located at positions 90 and 117, i.e. at nearby positions where negative charges can also stabilize the network. Receptor activation involves at least two protonation reactions which are generally accepted to be prerequisite for the formation of the fully active receptor conformation. Conceivably, the protonation of specific residues at key positions E113 and E134 rearranges the H-bonded network, and results in unlocking of the helices facilitating their relative movements. A modular organization of such a network would provide the flexibility to allow for partial steps, like those producing the isochromic Meta states. Importantly, a H-bonded network provides a more unified concept and explains how key events at a single amino acid level allow for efficient communication between the chromophore hot-spot and the cytoplasmic surface.

The concept of modular excitation in rhodopsin, was first postulated by Hofmann et al. by showing that in micellar solution Schiff base deprotonation [Meta I (480 nm) → Meta II α (380 nm)] precedes proton uptake from the aqueous phase [Meta II α (380 nm) → Meta II β (380 nm)]. Possibly in relation to this, more recent EPR spectroscopy studies by Hubbel and coworkers indicate that subdomains at the cytoplasmic surface can be activated separately. Our own unpublished observations seem to indicate that Schiff base deprotonation can be decoupled from the conformational changes leading to (some of) the IR characteristics of the Meta II intermediate. For example, partially delipidated rhodopsin (protein/lipid ratio of 1/30), which yields a 480 nm photoproduct at 10 °C, shows a typical Rho → Meta II difference spectrum. Furthermore, the structural changes involving D83, characteristic of Meta II formation, are already observed for the 10-methyl photopigment at 10 °C (480 nm), while the corresponding IR difference spectrum also shows many of the features characteristic of Meta I. Indeed, transducin binding kinetics of the 10-methyl pigment are much faster than expected on the basis of the Meta II formation rate we determined in chapter 4 (Hofmann and DeGrip, unpublished results), indicating that an active conformation, in this analog pigment, develops before Schiff base deprotonation.

Many years of rhodopsin research already disclosed some of the important molecular aspects of receptor activation. However, although it seems clear that all the observable events are somehow related, it is still largely unclear what is cause and what is consequence, or even what comes first. Future time resolved UV/visible, FTIR, and Raman
studies should provide important insight also into the temporal complexity of visual pigment activation.

**Conclusion.** The work presented in this thesis demonstrates that FTIR difference spectroscopy, in combination with protein engineering, stable-isotope labeling, and ligand modification, is a very sensitive tool to probe the conformational rearrangements leading to receptor activation in visual pigments. Not only does it facilitate the identification of structurally active groups within the complex, it also allows the nature of the structural changes and intramolecular interactions to be studied. It has further been demonstrated that polarized infrared difference spectroscopy in the ATR-mode yields additional resolving power and that it can be fruitfully applied to obtain mechanistic information on the relative orientation of functionally important groups within the ligand-protein complex. Clearly, the applicability of this approach is not limited to the case of visual pigments. With the achievement of large-scale functional expression of other (G-protein coupled) receptors and the availability of photo-activatable caged ligands, (ATR-)FTIR is expected to provide invaluable information on the mechanism of activation in a variety of other receptor systems as well.
Samenvatting

Het onderwerp van dit proefschrift is het moleculaire proces dat aan de basis staat van het visueel mechanisme: de interactie tussen licht en de visuele pigmenten. De visuele pigmenten zijn lichtgevoelige eiwitten en bevinden zich in de staafjes- en kegeltjescellen in het netvlies van het oog. Wanneer beelden via de ooglens op het netvlies worden geprojecteerd, leidt dit tot de foto-activatie van de visuele pigmenten. In de actieve vorm herkennen en activeren ze een zogenaamd G-eiwit, transducine, dat op zijn beurt een cascade van biochemische reacties initieert en wat uiteindelijk resulteert in het ontstaan van een electrochemische potentiaal over de celmembraan. Via de synaptische voet van de fotoreceptorcellen wordt dit signaal vervolgens doorgegeven aan de oogzenuw, die het signaal verder transporteert naar de hersenen waar de feitelijke visuele perceptie plaats vindt.

In de loop der jaren zijn de visuele pigmenten, en vooral het staafjespigment rhodopside (Rho), uitgegroeid tot een modelsysteem voor de zich nog steeds uitbreidende familie van G-eiwit gekoppelde receptoren (GPCRs). De eiwitten binnen deze familie hebben een aantal overeenkomstige uiterlijke kenmerken: ze bestaan uit zeven transmembran segmenten (TMs) die elk voor een belangrijke deel gevouwen zijn in een zogenaamde α-helix structuur. Deze segmenten zijn door lusstructuren verbonden aan de intra- en extracellulaire zijde van de membraan. Wanneer de receptor wordt geactiveerd, door binding van een activerende ligand (agonist), worden er aan de intracellulaire zijde signaaldomeinen blootgelegd die een hoge affiniteit hebben voor een GTP-bindend eiwit (G-eiwit). De GTP-binding, die slechts optreedt als het G-eiwit gebonden is aan de actieve receptor, activeert het G-eiwit dat vervolgens de receptor loslaat en de cellulaire respons aanschakelt. Naast lichtreceptoren bevat de GPCR-familie ook geurreceptoren en receptoren voor hormonen en neurotransmitters. Deze laatstgenoemden zijn belangrijke doelen voor farmacologische interventie. Om het ontwerpen van medicijnen mogelijk te maken is het echter noodzakelijk om over gedetailleerde structurele informatie te beschikken over het mechanisme van ligandbinding en receptoractivatie, liefst op atomair niveau. De beste vooruitzichten om dit niveau van detail voor een GPCR in de nabije toekomst te bereiken worden geboden door de visuele pigmenten, en vooral het staafjespigment rhodopsine.

De visuele pigmenten hebben een aantal, voor de GPCR-familie unieke, evolutionaire aanpassingen ondergaan die hun rol als fotoreceptor-eiwit mogelijk maakten. Zoals beschreven in hoofdstuk 1 zijn er vier soorten visuele pigmenten: het staafjespigment rhodopsine, en de pigmenten in de voor blauw, rood en groen licht gevoelige kegeltjescellen. De gevoeligheid voor (zichtbaar) licht van deze eiwitten is het gevolg van de covalente bin-
Samenvatting

ding van een lichtgevoelige ligand (of chromofoor; het Griekse chrōma betekent kleur): 11-cis-retinal. De chemische binding van deze groep aan het eiwit is een zogenaamde ge-
protoneerde Schiffse base. Deze constructie, die gestabiliseerd wordt door een negatief
geladen carboxylaat ion in het eiwit (E113)\(^1\), is hetzelfde in alle visuele pigmenten. Het
verschil in kleurgevoeligheid van de pigmenten onderling kan worden verklaard door sub-
tiele verschillen in de samenstelling van de aminozuurketens waarmee de eiwitten zijn opge-
bouwd. Als gevolg van belichting isomeriseert de retinal naar de trans conformatie. Door
deze fotochemische reactie wordt de kleurgroep omgevormd tot agonist. Het eiwit past
zich vervolgens aan aan de veranderde conformatie van de chromofoor [fotocascade: rho-
dopsine gaat achtereenvolgens over in bathorhodopsine (Batho), lumirhodopsine (Lumi),
metarhodopsine I (Meta I) en metarhodopsine II (Meta II)] en dit leidt, onder fysiologische
condities, binnen milliseconden tot de actieve vorm van de receptor (Meta II).

Niet alleen het relatieve gemak waarmee met name rhodopsine in grote hoeveelheden
can worden geïsoleerd, maar ook het feit dat het activeringsproces van de visuele pig-
menten kan worden gevolgd met diverse spectroscopische technieken, heeft ertoe geleid
dat de visuele pigmenten een modelpositie hebben verworven binnen de GPCR-familie.
EPR-, (vaste stof) NMR-, UV-licht-, en vibratiespectroscopie, in combinatie met
gecontroleerde veranderingen in het eiwit-ligand complex, heeft al geleid tot gedetailleerd
inzicht in het activeringsproces van rhodopsine. Enkele van de meest belangrijke observa-
ties staan beschreven in hoofdstuk 1. Tot op heden zijn de belangrijkste inzichten verkri-
gen met behulp van vibratiespectroscopie (Raman- en infraroodspectroscopie). In principe
kunnen met deze technieken de vibraties van individuele chemische bindingen worden
gemeten. Met name Fourier-transform-infrarood- (FTIR-) verschilspectroscopie bleek een
krachtig hulpmiddel. Met deze techniek kunnen veranderingen in absolute structuur, (in-
tra)moleculaire interacties en oriëntaties als gevolg van belichting worden bestudeerd door
de hiermee gepaard gaande subtiele veranderingen in vibratiefrequentie of -intensiteit te
registreren.

Het doel van het onderzoek beschreven in dit proefschrift was om met behulp van
FTIR-spectroscopie te komen tot verdere identificatie van eiwit-activiteit en ligand-eiwit
interacties in de verscheidene stadia van receptoractivatie, en met name tijdens de vorming
van de fysiologisch actieve Meta II-intermediair.

De identificatie van eiwit/ligand-activiteit kan worden bereikt door de effecten van
kleine modificaties van het receptorcomplex op het FTIR-verschilspectrum te bestuderen.
Omdat de structurele veranderingen die optreden als gevolg van belichting zo klein zijn,
dienen de aan te brengen modificaties zodanig subtiel te zijn dat de structurele integriteit
van het systeem niet wordt aangetast. Op eiwitniveau worden de mogelijkheden hiertoe
veelal aangereikt door biotechnologische technieken.

Waarschijnlijk wordt de potentiële kracht van deze gecombineerde aanpak het best
géïllustreerd in hoofdstuk 2. Niet alleen kon de betrokkenheid van individuele gebon-
den watermoleculen worden aangetoond door de identificatie van OH-strekvibraties in het

\(^{1}\)De gebruikte nummering voor de aminozuren in dit hoofdstuk is gebaseerd op de sequentie van
runderrhodopsine.
Rho → Meta II verschilspectrum, ook kon een mogelijke lokalisatie van één van deze watermoleculen worden verkregen door het bestuderen van de plaatsgerichte rhodopsine mutant C185S. Plaatsgerichte mutagenese is op het ogenblik de methode om te komen tot een identificatie van structurele activiteit van een bepaald aminozuur. Door zo alle cysteineresiduen in het transmembraandomein van rhodopsine één voor één te vervangen door een serine (de zijketen van dit aminozuur heeft een eindstandige OH groep, waar cysteine een SH functie heeft), kon verder worden aangetoond dat de SH groep van C167 verantwoordelijk is voor een voor metarhodopsine II karakteristieke verschilband bij 2550 cm⁻¹.

Omdat zelfs de substitutie van een enkel aminozuur al kan resulteren in een niet-functioneel eiwit, en omdat dit juist in het geval van de meest belangrijke residuen kan worden verwacht, is naast plaatsgerichte mutagenese voor een alternatieve weg gekozen: isotoop-labeling van rhodopsine. Door rhodopsine tot expressie te brengen in een medium dat in plaats van gewoon tyrosine (fenol [ring-1H₄]Tyr) isotoop-gelabeld tyrosine (fenol [ring-²H₄]Tyr) bevat is het mogelijk gebleken [ring-²H₄]Tyr-gelabeld rhodopsine te verkrijgen. Dit heeft geleid tot de identificatie van tyrosineactiviteit tijdens de vorming van de actieve Meta II-intermediair, waarbij op zijn minst twee tyrosineresiduen betrokken zijn, mogelijk in protonoverdrachtsreacties. Omdat chemisch gezien isotopen volkomen identiek zijn heeft deze methode als belangrijk voordeel dat de structuur van het eiwit op geen enkele wijze verschilt van de normale situatie. Een belangrijk nadeel is echter dat geen plaatsgerichte toekenning kan worden gedaan. Wel zijn er aanwijzingen dat een deel van de waargenomen activiteit wordt veroorzaakt door het tyrosineresidu op positie 268 (Y268). Dit is echter voornamelijk gebaseerd op de analogie met de situatie in het bacteriële pigment bacteriorhodopsine. Voor een definitieve toekenning zijn additionele studies noodzakelijk (bij voorkeur plaatsgerichte isotoop-labeling).

De experimenten beschreven in hoofdstuk 3 laten zien dat uit infraroodverschilspectra ook informatie over de relatieve oriëntatie van belangrijke groepen kan worden verkregen. Door met lineair gepolariseerd infraroodlicht te werken, en door gebruik te maken van de ”attenuated total reflectance” (ATR) sampling techniek, is het mogelijk gebleken de relatieve oriëntatie van individuele groepen in zowel het eiwit als in de chromofoor gedurende het fotoactiveringsproces te berekenen. Hierbij is gebruik gemaakt van het lineair dichroïsme van de pieken in het Rho → Meta II verschilspectrum. Op basis van deze gegevens kon verder worden geconcludeerd dat de vorming van de actieve Meta II-intermediair gepaard gaat met een heroriëntatie van helixsegmenten, en met de protonering van een tot dusverre niet gedetecteerde carboxylgroep. Deze laatste werd voorlopig toegedeeld aan glutaminezuur (E)134. Tenslotte vormen de dichroïsche eigenschappen van de banden in het zogenaamde HOOP-gebied van het spectrum een indicatie voor een heroriëntatie van de zijketen van de chromofoor in de Meta I → Meta II stap. Mogelijk is deze beweging noodzakelijk voor een correcte plaatsing van de Schiffse base ten opzichte van zijn proton acceptor (mogelijk E113, ofwel direct, danwel indirect via bijvoorbeeld Y268), hetgeen de deprotonering van de Schiffse base in de Meta I → Meta II stap mogelijk maakt.

²C185S: substitutie van cysteine (C) op plaats 185 in de eiwitketen voor een serine (S).
Het zijn de interacties tussen eiwit en ligand in rhodopsine die verantwoordelijk zijn voor de bijzonder hoge efficiëntie van deze biologische fotosensor; een bijna complete stereo-specificiteit, sub-picoseconde (\(< 10^{-12}\) s) kinetiek en een quantumopbrengst van 0.67 zijn veel efficiënter dan verwacht op basis van enig modelsysteem. Eiwit-ligand interacties worden bij voorkeur bestudeerd door gebruik te maken van rhopsineanalogen: rhodopsines geregeneereld met een homoloog van 11-cis-retinal. In hoofdstuk 4 wordt de fotochemie van 10-methyl rhodopsine (rhodopsine geregeneereld met 10-methyl-11-cis-retinal) beschreven. Voor deze gemodificeerde chromofoor is gekozen omdat door toegegno- nomen torsie in de isomerisatie regio (C_{10}· · ·C_{13}), veroorzaakt door sterische interactie tussen de methylgroepen op C_{10} en op C_{13}, verwacht werd dat de foto-isomerisatie-eigenschappen zouden worden beïnvloed, met mogelijke consequenties voor de verdere fotocascade.

Inderdaad kon met verschillende spectroscopische technieken [UV-zichtbaar licht (circulair dichroïsme-) en infraroodspectroscopie] worden aangetoond dat de 10-methyl chromofoor in een sterker getordeerde conformatie aanwezig is in de donkertoestand van het pigment. De meest opvallende verschillen tussen 10-methyl en natief rhodopsine werden echter waargenomen in de overgangstemperaturen van de fotointermediairen. Hoewel FTIR-analyses laten zien dat de structurele overgangen redelijk overeenkomen in beide systemen bleek dat in het geval van 10-methyl rhodopsine de Batho intermediair stabiel bleef tot ongeveer −90 °C, terwijl natief rhodopsine bij deze temperatuur de Lumi conformatie aaneemt. Bovendien bleek dat Meta II pas bij temperaturen boven 20 °C gevormd wordt, met een snelheid die echter ongeveer een miljoen keer langzamer is dan in natief rhodopsine bij deze temperatuur. Een mogelijke verklaring voor het tweeledige effect van de extra methyl groep is dat een sterische interactie met de eiwitomgeving de relaxatie belemmert van de energierijke chromofoor in de Batho \(\rightarrow\) Lumi overgang, en vervolgens de heroriëntatie van de retinalketen bemoeilijkt die nodig is voor de protontransfer van de Schiffs base in de Meta I \(\rightarrow\) Meta II stap (hoofdstuk 3). Met behulp van plaatsgerichte mutagenese kunnen de betrokken aminozuren mogelijk worden geïdentificeerd.

In natief rhodopsine hangt de snelheid waarmee Meta II gevormd wordt sterk af van de micro-omgeving [zuurtoregrad (pH), temperatuur, lipide-omgeving]. Het is algemeen aanvaard dat dit vooral samenhangt met de laatste stap in de fotocascade: het evenwicht tussen metarhodopsine I en metarhodopsine II. In hoofdstuk 5 worden de effecten van ionsterkte op dit evenwicht beschreven. De belangrijkste vinding was dat bij toenemende concentraties zout in de membraansuspensie het evenwicht drastisch verschuift ten gunste van de actieve Meta II-intermediair (de pK_a verschuift 3 pH eenheden, 0 \(\rightarrow\) 4 M KCl, 10 °C). Bovendien kon worden aangetoond dat de snelheid waarmee Meta II gevormd wordt sterk toeneemt in aanwezigheid van hoge concentraties KCl. Met behulp van ATR-FTIR-(verschil)spectra kon worden bevestigd dat het inderdaad gaat om een verschoven evenwicht en dat het zouteffect niet wordt veroorzaakt door een veranderde eiwitstructuur en/of membraanvloeibaarheid. Een belangrijk deel van het zouteffect kan worden verklaard door de afscherming van positief geladen aminozuren aan de intracellulaire zijde van het membraan. Dit bevestigt een eerdere conclusie dat de pH-afhankelijkheid van het
Samenvatting

evenwicht tussen Meta I en Meta II mede wordt bepaald door een elektrostatische compo-
nent. Tot slot beargumenteren we dat de pH-afhankelijkheid hoogst waarschijnlijk wordt
gereguleerd door meerdere groepen, in tegenstelling tot een eerdere suggestie dat de pKₐ
van het evenwicht gereguleerd wordt door één specifiek aminozuur.

Wat tot nu toe bekend is over de activatie van visuele pigmenten van zoogdieren is
voornamelijk gebaseerd op experimentele gegevens van de staafjespigmenten. In hoofd-
stuk 6 worden de eerste FTIR-resultaten beschreven van een kegeltjespigment [het (re-
combinante) humaan groene kegeltjespigment (HGH) met een maximale lichtgevoeligheid
(λ_max) bij 528 nm]. Op basis van het HGH → HGH-Batho infraroodverschilspectrum,
opgenomen bij −190 °C, lijkt het verschil in λ_max tussen rhodopsine (498 nm) en het groene
kegeltjespigment voornamelijk verband te houden met de waterstofbrugomgeving van de
Schiffse base. Dit is in overeenstemming met een recent door de groep van Mathies gepub-
liceerde Raman-studie. Bovendien lijkt ook het effect wat anionuitwisseling heeft op de
λ_max, typisch voor de klasse van pigmenten waartoe het groene pigment behoort, te re-
lateren aan subtiele veranderingen in de buurt van de Schiffse base. Verder vinden we
zowel in het amide I (eiwit) als in het HOOP (chromofoor) gebied van het spectrum aan-
wijzingen voor een grotere mate van bewegingsvrijheid van de chromofoor in het kegel-
tjespigment. Tenslotte is de structurele overgang die gepaard gaat met de vorming van
een bij 380 nm maximaal absorberend fotoproduct (4 °C, HGH-Meta II) vergeleken met
die in rhodopsine (10 °C, Meta II). Naast spectrale overeenkomsten zijn er ook duidelijke
verschillen waargenomen. Wat met name opviel is dat in het kegeltjespigment een signifi-
cante eiwitconformatieverandering nog steeds voortduurde, in tegenstelling tot de situatie
in rhodopsine. De verwachting is dat een nadere vergelijking belangrijke informatie zal
opleveren over het algemene werkingsmechanisme van visuele pigmenten.

Tenslotte worden in hoofdstuk 7 de huidige resultaten samengevat en bediscussieerd
in het kader van eerder gepubliceerde modellen voor de signaaltransmissie in visuele pig-
menten.
Publications


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Curriculum Vitae


Per 1 augustus 1999 werkt hij als postdoc op het Tumor Immunologie Laboratorium van de Katholieke Universiteit Nijmegen onder leiding van Prof. dr. C.G. Figdor aan een door de Stichting FOM gefinancierd onderzoek getiteld ’Dynamics of adhesisomes explored by near field optical microscopy’.