INSIGHTS INTO THE ACTIVATION AND THE DEACTIVATION MECHANISM OF G-PROTEIN COUPLED RECEPTOR - RHODOPSIN - A STUDY BY FTIR SPECTROSCOPY

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Photochem Photobiol 85:437-441

Standfuss J, Zaitseva E, **Mahalingam M**, Vogel R.
Structural impact of the E113Q counterion mutation on the activation and deactivation pathways of the G protein-coupled receptor rhodopsin.
J Mol Biol 380: 145-157

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**CONFERENCE PRESENTATION**

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Presented in 13th International Conference on Retinal Proteins (ICRP) held in Barcelona, Spain June 15th - 19th 2008

**Interaction of a G protein coupled receptor with different domains of its G protein**
**Mohana Mahalingam** and Reiner Vogel
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Mohana Mahalingam, Friedrich Siebert, Gerald Mathias, Paul Tavan, Guibao Fan, Xin-Yu Zhang, Mudi Sheves, and Reiner Vogel
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## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DDM</td>
<td>n-Dodecyl-(\beta)-D-Maltoside</td>
</tr>
<tr>
<td>OG</td>
<td>n-Octyl-(\beta)-D-Glucoside</td>
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<td>FTIR</td>
<td>Fourier Transform Infra Red</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>Con-A</td>
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<tr>
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<tr>
<td>UV-Vis</td>
<td>Ultra Violet Visible</td>
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<tr>
<td>ROS</td>
<td>Rod Outer Segments</td>
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<tr>
<td>Batho</td>
<td>Bathorhodopsin</td>
</tr>
<tr>
<td>Lumi</td>
<td>Lumirhodopsin</td>
</tr>
<tr>
<td>Meta I</td>
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<tr>
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</tr>
<tr>
<td>Meta III</td>
<td>Metarhodopsin III</td>
</tr>
<tr>
<td>BTP</td>
<td>Bis Tris Propane</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino) ethanesulfonic acid.</td>
</tr>
<tr>
<td>CIT</td>
<td>Citrate buffer</td>
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<tr>
<td>PC</td>
<td>Phosphatidyl Choline(L-(\alpha)-lecithin) from egg yolk</td>
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<td>cGMP</td>
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</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<tr>
<td>PDE</td>
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</tr>
<tr>
<td>HOOP</td>
<td>Hydrogen-out-of-plane</td>
</tr>
<tr>
<td>Gt</td>
<td>G protein Transducin</td>
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### SYMBOLS FOR AMINO ACIDS

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<tr>
<td>Valine</td>
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1. General Introduction

1.1 Signal transduction – a necessity for life

The existence of any living organism depends on its ability to detect the surrounding environment and adapt itself according to the changes. Unicellular organisms respond to signals generated in the environment as well as from other organisms. For the eukaryotes, sensing the extracellular environment is as important as coordinating intracellular metabolic and developmental signals. The pathways involved in the translation of environmental ‘cues’ to a cellular response are known as the ‘signal transduction pathways’. The signaling molecules are of two types: a) molecules that bind to cell surface receptors and b) membrane permeable components which are usually lipophilic and bind to cytosolic receptors. Signal transduction takes place either by activating enzymes that produce high amounts of second messengers, which amplify signals as well as activate other intracellular components or by protein-protein interactions, which might involve covalent modifications or intramolecular conformational changes leading to activation or both.

1.2 Signaling of vision

Analysis of the visual world depends on the information coming from the retina, the initial stage of processing that sets our limits for the perception. The neural retina consists of several layers organized in a way that seems perverse. The neurons that transmit signals from the eye to the brain (called retinal ganglion cells) lie closest to the external world, so that the light focused by the lens, must pass through them to reach the photoreceptor cells (Fig 1.1). The photoreceptors which are classified as cones and rods lie with their photoreceptive ends or outer segments partly buried in the pigment epithelium. Rods and cones contain different photosensitive complexes of protein with visual pigment: rods are especially sensitive at low light levels while cones which are of three types (each with different spectral responses) detect color and fine details. The remainder of the outer segment is almost entirely packed with intense stack of membranes in which the photosensitive complexes are embedded; light absorbed here produces an electrical response, as described below. At their opposite ends the photoreceptors form synapses on interneurons, which relay the signal to the retinal ganglion cells.
Fig 1.1 Anatomy of eye and retina (Figure derived from Neuroscience tutorial- Washington University School of Medicine.

The pathway relevant to the study presented in this work is the rod photoreceptors (rods) in the vertebrate retina. Rods are responsible for monochromatic vision in dim light whereas cone cells confer color vision in bright light. Rods are highly specialized cells with an outer segment, inner segment, a cell body and a synaptic region where the rod cell passes the chemical signal to a nerve cell, which relays the signal along the visual pathway. Outer segment of the rod cell has the photo transduction apparatus which contains the stack of discs, each formed by a closed sac of membrane in which photo sensitive
rhodopsin molecules (photon receptor molecules) are embedded. Outer segment is surrounded by plasma membrane which has cyclic GMP gated $\text{Na}^+$ channels. In the dark these $\text{Na}^+$ channels are kept open by the channel bound cGMP molecules causing the depolarization of the plasma membrane. Light causes hyperpolarisation of the plasma membrane because the activation of rhodopsin by light leads to the closure of $\text{Na}^+$ channels and decrease in the level of cyclic nucleotide.

Fig 1.2 Schematic picture of rod and cone photoreceptor cells. Rhodopsin is present in the disc membrane of the rod cells. (Figure derived from Forschungszentrum Jülich - Institute of Structural Biology and Biophysics website)
Rhodopsin, a seven transmembrane molecule is a prototypical G-protein coupled receptor. Like the other G-PCRs rhodopsin also acts through a trimeric G protein. However the activating extracellular signal is not a molecule but a light photon. Each rhodopsin molecule consists of protein moiety called opsin. A chromophore 11-cis retinal is covalently linked to opsin. Chromophore 11-cis retinal isomerizes to all-trans retinal upon the absorption of photon. The isomerization alters the shape of the retinal forcing conformation changes in the protein- opsin. The activated protein then binds to the G- protein transducin (Gt) causing the α-subunit of the transducin to dissociate and to activate cyclic GMP phosphodiesterase(by relieving the constraint imposed by the inhibitory subunit PDEγ), which hydrolyses the cyclic GMP so that cyclic GMP levels in the cytosol drop. As a result of this cyclic GMP dissociates from the Na+ channels, causing them to close. In this way the signal passes from the disc membrane to the plasma membrane and a light signal is converted to an electrical signal. The Na+ channels are also permeable to Ca2+, so that when they close, the normal influx of Ca2+ is inhibited, causing the Ca2+ concentration in the cytosol to decrease. The fall in the Ca2+ stimulates the guanylyl cyclases to replenish the cyclic GMP, thereby returning the cell towards the state in which it was before the incident of light. The activation of guanylyl cyclases by the fall of Ca2+ is mediated by a Ca2+ sensitive protein called recoverin, which in contrast to calmodulin, is inactive when Ca2+ is bound to it. It stimulates the guanylyl cyclase when Ca2+ is low after the light response. This Ca2+ dependent mechanism is of crucial importance in two ways. First it allows the photoreceptor to quickly revert to its resting, dark state in the after math of a flash of light, making it possible to perceive the shortness of the flash. Second it helps to enable the photoreceptor to adapt, stepping down the response when it is exposed to light continuously.
Figure 1.3: Details of phototransduction in rod photoreceptors. The second messenger cascade of phototransduction. 1. Light stimulation of rhodopsin in the receptor discs leads to the activation of a G-protein (transducin). 2. The GTP-bound alpha subunit of transducin activates a phosphodiesterase (PDE) by relieving the constraint imposed by the inhibitory subunit PDE γ. 3. The activated phosphodiesterase hydrolyzes cGMP into GMP, reducing its concentration in the outer segment and leading to the closure of sodium channels in the outer segment membrane. (Neuroscience, Purves et al., 2001)

In the visual signal transduction pathway, a single activated rhodopsin molecule activates hundreds of molecules of transducin at a rate of about 1000 transducin molecules per second. Each transducin molecule activates a molecule of cyclic GMP phosphodiesterase, each of which hydrolyses about 4000 molecules of cyclic GMP per second. This catalytic cascade lasts for about one second and results in the hydrolysis of about more than $10^5$ cyclic GMP molecules for a single quantum of light absorbed, which transiently closes hundreds of Na$^{2+}$ channels in the plasma membrane.
1.3 Rhodopsin

Rhodopsin is an integral membrane protein and is a main component of the Disc membranes in the Rod cells. It is a seven transmembrane segment receptor and belongs to the largest family of proteins called G protein coupled receptors. G-PCRs are classified into three major subfamilies out of which Rhodopsin belongs to the Family A Rhodopsin/β adrenergic receptor like molecules.

In general visual pigments have a significant specialization which is not found in other receptor families. These pigments are made up of opsin apoprotein plus chromophore. The chromophore is linked via a protonated Schiff base bond to a specific lysine residue in the membrane embedded domain of the protein. The chromophore of most vertebrate visual pigment is the aldehyde of vitamin A, \(11\text{-cis} \) retinal. The chromophore of few invertebrates, fish and amphibian pigments is the aldehyde of vitamin A\(_2\), \(11\text{-cis} -3\text{-dehydroretinal}\). An important structural feature of the retinal chromophore in rhodopsin, in addition to its Schiff base is its extended polyene structure which accounts for its visible absorption properties. Rhodopsin is an extremely sensitive protein, enabling vision in low light. Rhodopsin has a broad visible absorption maximum (\(\lambda_{\text{max}}\)) at about 500 nm. Upon the photoisomerization of the chromophore from \(11\text{-cis}\) to \(\text{all-trans}\), protein is converted to metarhodopsin II (Meta II) with a \(\lambda_{\text{max}}\) value of 380 nm. Upon the formation of Meta II the Schiff base gets deprotonated. Meta II is the active form of the receptor which then catalyses the guanine nucleotide exchange by transducin. This photoisomerization of the 11-cis to all trans is the only light dependent step and the first event in the visual signal transduction. The activated form of rhodopsin, meta II then interacts with G protein, transducin which then leads to the activation of cyclic GMP phosphodiesterase and then the closing of cyclic GMP gated ion channels in the plasma membrane of the rod cell. Bovine rhodopsin is the most studied G-PCR. A large amount of this protein is purified by the sucrose gradient centrifugation technique from the rod outer segment disc membranes (refer chapter 2.2).
Fig 1.4 Dark state crystal structure of Rhodopsin. This figure is based on Protein Data Bank ID code 1GZM.
1.4 Structure of Rhodopsin

Like all the G-PCRs the core structure consists of seven transmembrane segments (H1 to H7) which are predominantly \( \alpha \) helical. The N terminal region is located intradiscally (extracellularly—for rhodopsin in the plasma membrane) and the C terminal region is cytoplasmic. A pair of highly conserved Cys residues (cys110 and cys187) is found on the extracellular surface of the receptor and forms a disulphide bond. An ERY tripeptide sequence (highly conserved in Family A G-PCR) is found at the cytoplasmic border of H3. Sites of light dependent phosphorylation at Serine and Threonine are found at the carboxy-terminal tail of most visual pigments. These sites are analogous to phosphorylation sites found on the carboxy terminal tails of other G-PCRs.

The extracellular surface domain of Rhodopsin has the amino terminal tail and the three interhelical loops (E1, E2 and E3). An extracellular domain has several secondary structures and several intra and inter domain interactions. Amino terminal tail extends from the amino terminus to Pro34 and has fine distorted strands (B1, B2, S3, S4 and S5). Amino terminus is glycosylated at Asn2 and Asn15. The oligosaccharides extend away from the extracellular domain and do not seem to interact with any part of the molecule. The B4 strand on E2 is positioned to form extracellular roof for the retinal binding pocket. The B4 band runs nearly parallel to the length of the polyene chain from about C9 to the Schiff base imine Nitrogen.

The crystal structure of the membrane embedded domain is remarkable for the number of kinks and distortions of the individual transmembrane(TM) segments, which are otherwise \( \alpha \)- helical in secondary structure. The binding site of the retinal chromophore lies within the membrane embedded domain of the receptor close to the extracellular side of the TM domain. The chromophore polyene from C6 to C11 runs almost parallel to H3. The polyene chain facing towards the extracellular side of the receptor is covered or capped, by the amino acid residues from the B4 sheet of the E2 loop. The carboxylic acid side chain of Glu181 in the B3 sheet of the E2 loop points towards the center of the polyene chain. Glu113 on H3 serves as Schiff base counterion. The 3-D crystal structure has confirmed hydrogen bonding networks between helices in the region of the cytoplasmic interface. The network between helices 3 and 6 is of great interest for the work discussed in this thesis. It connects
the side chains of Glu134 and Arg135 in the ERY motif on helix 3 with those of Glu247 on helix 6. Because of the involvement of its charged groups this network is called ionic lock.

The cytoplasmic domain of the Rhodopsin comprises three cytoplasmic loops and the carboxy terminal tail. Carboxy terminal tail has a \( \alpha \) helical stretch, H8 which lies perpendicular to H7 and together with NPXXY motif in H7, it is one of the highly conserved long stretches of primary structure of rhodopsin in family A G-PCRs. The environment around H8 is mainly hydrophobic which increases the stability and H8 is a cationic amphipathic \( \alpha \) helix. H8 points away from the center of the rhodopsin and it appears that the palmitoyl group linked to the Cys322 and Cys323 by thiester bonds may be anchored in the membrane bilayer.

1.5 The Rhodopsin photocycle

The photoisomerization of the chromophore takes place in ultra fast time scale and at low temperatures using variety of spectroscopic techniques many photo intermediates have been trapped. In rhodopsin dark state, the 11-cis isomer of the retinal chromophore serves as inverse agonist which locks rhodopsin in completely inactive receptor conformation. Activation of the rhodopsin is achieved by light dependent isomerization of the chromophore 11-cis into all-trans retinal (Fig 1.5). The several short lived species of the rhodopsin photocycle are bathorhodopsin (543 nm) \( \rightarrow \) blue shifted intermediate (BSI) (477 nm) \( \rightarrow \) lumirhodopsin (497 nm) \( \rightarrow \) Meta I (480 nm) \( \leftrightarrow \) Meta II (380 nm). In Meta II the Schiff base linkage between the all-trans-retinal and Lys296 is still intact but deprotonated. The signaling state of the rhodopsin is rapidly quenched by the action of the rhodopsin kinase and arrestin. The metabolic cycle, in which the all-trans –retinal is taken away and replaced by 11-cis-retinal, thus regenerating the pigment is equally important for the vision. This release of the activating ligand from its binding pocket allows opsin to adopt again an inactive protein conformation. Besides this direct pathway from Meta II to opsin, there is another decay pathway which involves formation of species absorbing at around 470 nm (in a time scale of minutes) which also decays ultimately into opsin. This 470 nm species is called as Metarhodopsin III (Meta III) which also finally hydrolysis and releases all-trans retinal. Dissociation and re-association of Retinal, dephosphorylation of rhodopsin and release of
arrest in all return rhodopsin to its ready state, prepared once again to respond to light. Illumination can now initiate another reaction cycle.

The released all trans retinal undergoes the Retinoid Cycle and gets converted to 11-cis retinal. All-trans-retinal is reduced to all-trans-retinol by NADPH-dependent all-trans-retinol dehydrogenase, a membrane-associated enzyme that belongs to the large gene family of short-chain alcohol dehydrogenases (SCAD). All-trans-Retinol translocates to the Retinal Pigment Epithelial cells (RPE). Esterification in the RPE involves the transfer of an acyl group from lecithin to retinol and is catalyzed by lecithin: retinol acyltransferase (LRAT). The isomerase, RPE-specific 65 kDa protein (Rpe65) uses an all-trans-retinyl ester as substrate to form 11-cis-retinol plus a free fatty acid. The Rpe65 isomerase activity may be regulated by the non-photoreceptor opsin, RGR (RPE-retinal G protein coupled receptor). 11-cis-Retinol may be esterified by LRAT to yield 11-cis-retinyl esters, a stable storage-form of pre-isomerized retinoid (Travis GH et al 2007). 11-cis-retinyl esters are hydrolyzed to 11-cis-Retinol for synthesis of chromophore by 11-cis-retinyl ester hydrolase. 11-cis-Retinol would then be oxidized to 11-cis-retinal in a reaction catalyzed by NAD- and NADP-dependent 11-cis-retinol dehydrogenases, which are other short chain dehydrogenase family members. Finally, 11-cis-retinal moves back to the rod photoreceptors, where it joins with opsin to regenerate visual pigment.

![Retinal and Retinol Structures](image-url)

*Fig 1.5 Photo isomerization of the retinal chromophore from 11-cis to all-trans.*
Fig 1.6 Scheme of light induced and thermal reactions of rhodopsin. Light induced (wavy arrows) and thermal reactions (straight arrows) between the dark state and its photointermediates are summarized in this simplified scheme. Receptor conformation (active and inactive) and protonation state of the retinal Schiff base are indicted for the intermediate
states. Activation is triggered by light induced isomerization of retinal chromophore from 11-cis 15-anti in the dark state to all-trans 15-anti leading to the photoproduction equilibrium between the inactive Meta I and active Meta II. The transition to Meta II from its precursor Meta I involves most of the conformational changes during the receptor activation, which depend on proton uptake from the solvent and disruption of salt bridge between the protonated Schiff base and the counterion. Meta II decays on two pathways, to opsin by hydrolysis of the Schiff base or Meta III (via Meta I) by thermal isomerization of the Schiff base C15=N double bond. Meta III also decays to opsin. Complete deactivation of the receptor is achieved only after binding of new 11-cis retinal supplied by the retinoid cycle which is an inverse agonist stabilizing the inactive receptor conformation.

1.6 Molecular mechanisms involved in activation of rhodopsin

The ligand of rhodopsin 11-cis retinal is covalently bound via a protonated Schiff base to the side chain of Lys296 on helix 7 in the transmembrane core of the receptor. Activation is achieved by the light dependent isomerization of the chromophore from 11-cis to all trans and subsequent thermal relaxation of the protein on millisecond time scale to reach active conformation. As described above the activation of the receptor takes place via several intermediate and the key event is the transition of the still inactive Meta I to the active Meta II state, absorbing at 480 and 380 nm respectively. These two receptor states form a conformational equilibrium that can be shifted both ways by altering pH and/or temperature. The global helix arrangement in Meta I is largely the same as in the dark state, while small but distinct changes are observed at switch region at helix 6 (Rubrecht, Mielke at al 2004). Most of the conformational changes during the receptor activation take place during the transition from Meta I to Meta II.

Evidence for changes in interhelical interactions upon the receptor activation was provided by various techniques like Fourier transformed infrared spectroscopy (FTIR), site-directed spin labeling together with EPR spectroscopy. FTIR spectroscopy has been a valuable technique for studying the light induced changes. For example among the membrane embedded carboxylic acid groups, light induced changes of the protonation states or H-bond strengths were deduced from characteristic frequency shifts of C=O stretching vibrations of
protonated carboxylic acid groups on FTIR spectra. Activation of rhodopsin has two main steps: first isomerization of the retinal chromophore followed by the protein conformational changes that enable the receptor to activate its G protein. The salt bridge is broken in the transition from Meta I to Meta II by a net proton transfer from the protonated Schiff base to Glu113. These protein changes involve helix rigid body movements relative to each other and a breaking of ionic and hydrogen bonding networks that otherwise keeps the protein in inactive conformation. This is also reflected in the pH dependence of the transition from the Meta I to Meta II, both containing the all-trans chromophore. This indicates the necessity of the protonation of an amino acid residue for activation and is consistent with the disruption of an intra or interhelical salt bridge. The rigid body movement of the receptor involves especially helices 3 and 6. One of the interhelical microdomains which undergo changes in hydrogen bonding properties are Asp83 in helix 2 and Glu122 in Helix 3. Double electron electron spectroscopy result showed some conclusive outward displacement of helix 6 at the cytoplasmic surface which is a hallmark of the activation and some smaller changes involving relative motions of helix 1, 7 and the C terminal domain accompany activation.

These light induced changes which are initially constrained to the chromophore binding pocket in the transmembrane core are then transmitted to the cytoplasmic side of the receptor. In milliseconds the cytoplasmic side of the receptor is rearranged to the active receptor state R* which is termed Meta II to allow the recognition and binding of the G protein transducin.

1.7 Aim and scope of the current study

1.7.1 Study of the deactivation pathway

The active Meta II receptor decays by two fundamentally different pathways. One involves hydrolysis of the retinal Schiff base in Meta II, leading to the release of all-trans-retinal from its binding pocket. The other pathways involves thermal isomerization of the C15=N double bond of the retinal Schiff base from an extended 15-anti (trans) geometry to 15-syn (cis), leading to the formation of Meta III and consequently deactivation of the
receptor. Some spectroscopic studies that have contributed substantially to the understanding of Meta III is addressed here. In this current study by FTIR spectroscopy it is shown that the Meta III decay rate is pH dependent. The apparent pK_a of the active/inactive state equilibrium is downshifted to 5.1 from above 8 for the Meta I/Meta II equilibrium. But this still above the pKa of the opsin which indicates that the all-trans-15-syn-retinal ligand of Meta III is not an inverse agonist, but a classical partial agonist.

1.7.2 Study of the activation pathway- Functional role of the “ionic lock”

In family A G-PCRs, the cytoplasmic network between the E(D)RY motif on H3 and H6 is thought to form an ionic lock that is disrupted during the receptor activation, releasing constraints on relative movement of the two helices. Mutations on both sides of the network were shown to increase basal activity in many receptors. Glu134 of the ERY motif in rhodopsin forms an intrahelical salt bridge with Arg135 in dark state crystal structure of rhodopsin. Also there exist interhelical interactions of Glu247 and Glu249 on H6 with H3 and H7 respectively. In order to study the significance of these interactions and to answer which poses important constraint on the receptor for the activation, several site directed mutants of these residues of rhodopsin were investigated using FTIR spectroscopy. All the mutants studied here (E134Q, R135L, E247Q, E249Q) were incorporated into lipid bilayers before investigated using FTIR spectroscopy. Since the detergents which are commonly used for the purification alter the energetics of the receptor activation pathway considerably and particularly abolish the pH dependence of the Meta I/ Meta II pathway reconstitution into a native-like environment was mandatory.

1.7.3 Rhodopsin activation controlled by two protonation switches in membranes.

By using rhodopsin purified in flexible detergents such as dodecyl maltoside, Hofmann and coworkers (Arnis S et al 1993) showed by kinetic UV–visible spectrophotometry and proton-uptake experiments that the transition from Meta I to Meta II proceeds in a sequential manner, with Schiff base deprotonation preceding the cytoplasmic
proton uptake reaction, resulting in multiple Meta II states. Hubbell and collaborators (Knierim B et al 2007) also extended this scheme by using time-resolved EPR spectroscopy of spin labeled detergent-solubilized rhodopsin, revealing that Meta II$_a$ converts to Meta II$_b$ by movement of H6, and to Meta II$_b$H$^+$ by the cytoplasmic proton uptake. Notably, the activating helix movements in the transition to Meta II$_b$ take place independently of pH and, hence, the proton uptake step under these conditions. This behavior differs considerably from the classical anomalous pH dependence of the Meta I/Meta II equilibrium in native membranes (Matthews RG et al 1963), reflecting a substantial perturbation of the energetics of the metarhodopsin equilibrium induced by the detergent. Alternative models include a branched scheme where early intermediates with a protonated or deprotonated Schiff base evolve in a parallel manner on the time scale of Lumi decay (Thorgeirsson TE et al 1993). In this work, a comprehensive thermodynamic model that allows the description of rhodopsin activation in both detergent and membrane environments within a single conceptual framework were developed.
2. Materials and Methods

2.1 Preparation of Disc membranes from Retinae

Materials required

Buffer A  
- 60 mM KCl
- 30 mM NaCl
- 20 mM Tris-HCl (pH 7.3)
- 2 mM MgCl2
- 1 mM DTT
- 0.1 mM PMSF

Buffer E  
- 5 mM Tris-HCl (pH 7.3)
- 0.5 mM MgCl2
- 1 mM DTT
- 0.1 mM PMSF

Rhodopsin in native isotonically washed disc membranes was prepared from cattle retinae (Lawson) according to the standard procedure (Papermaster 1982).

Around 100 Retinae were thawed at 4 °C overnight, mixed strongly in the magnetic stirrer for 3 minutes and homogenized by passing it through a doubled nylon mesh (0.1 mm) with rubber police man. During this the outer segment breaks off and re-seal. Buffer A (without DTT and PMSF) was added to the homogenate and the volume was made upto 80 ml. 14 ml of the homogenate is aliquoted onto the 6 sucrose cushions (37.7 % sucrose in buffer A) in the centrifuge tubes. This was then spun in the ultra centrifuge (Beckman OPTIMATM L series) for 30 minutes at 15000 rpm, 4 °C in SW28 rotor. Rod Outer Segment (ROS) floats above the sucrose cushions while the heavier particles sediment through the 37.7 % sucrose solution. Supernatant was then discarded and the ROS band was then collected with a syringe and the volume was then made upto 90 ml with buffer A. 22.5 ml of this was then added into 4 centrifuge tubes and was spun for 20 minutes at 30,000 rpm, 4 °C in Ti50-2 rotor. Pellets were then suspended in 1-2 ml of sucrose solution of density 1.100 in buffer A and loaded onto the 4 step gradients of density 1.110 g/cm³ (6 ml),
1.130 g/cm³ (10 ml), 1.150 g/cm³ (6 ml) and was spun for 60 minutes at 28,000 rpm, 4 °C in SW 28 rotor. Then the middle band at the interface of densities 1.11 and 1.13 was recovered using a syringe with care and then the volume of the recovered sample was made to 36 ml using buffer A. At this step the concentration of the sucrose is brought down to below 20 %. 9 ml of the sample was then added to the 4 linear gradients (20-35 % w/w, these gradients were made using the gradient mixer) and spun for 60 minutes at 28,000 rpm, 4 °C in SW28 rotor. ROS band (at 29 % sucrose) was then collected, volume was made to 100 ml with buffer A and then spun in 4 centrifuge tubes for 20 minutes at 25,000 rpm, 4°C in Ti50-2 rotor. Pellet was then suspended and then spun for 20 minutes at 25,000 rpm, 4°C in Ti50-2 rotors (during which the ROS is disrupted osmotically and the disc membranes were released. Pellets were then washed 2 times in buffer E and then washed thrice with 1mM phosphate buffer pH 7. Concentration was then measured by UV-Vis spectroscopy by difference in the absorption between the illuminated and dark spectra at \( \lambda_{\text{Max}} = 500 \text{ nm} \), \( \epsilon = 40000 \text{ L/ (mol-cm)} \). Sample was then stored at -80 °C.

2.2 Purification of Rhodopsin from Disc membranes

Materials required

<table>
<thead>
<tr>
<th>Buffer D</th>
<th>250 mM Na₂CO₃ (pH 8.8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer H</td>
<td>3 M NaCl</td>
</tr>
<tr>
<td>Buffer F</td>
<td>300 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>40 mM PIPES /NaOH (pH 6.5)</td>
</tr>
<tr>
<td></td>
<td>2 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>2 mM CaCl₂</td>
</tr>
<tr>
<td></td>
<td>2 mM MnCl₂</td>
</tr>
<tr>
<td></td>
<td>0.5 mM EDTA</td>
</tr>
<tr>
<td>Buffer G</td>
<td>300 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>40 mM PIPES /NaOH (pH 6.5)</td>
</tr>
<tr>
<td></td>
<td>2 mM MgCl₂</td>
</tr>
</tbody>
</table>
2 mM CaCl₂
0.2 mM EDTA

Detergent I 1.2 mM DDM (n-Dodecyl-β-D Maltoside; Biomol) (0.06 %W/V) equivalent to 6x Critical Micellular Concentration (CMC) OR 82.1 mM β-OG (n-Octyl-β-D-Glucoside; Biomol) (2.4 %W/V) equivalent to 4x CMC

Wash buffer G:I (1:1)

Solubilisation buffer 20 mM DDM (1 %W/V) in buffer G OR 68 mM OG (2 %W/V) in buffer G

Elution buffer 0.2 mM Methyl-α-D-Manno pyranoside in wash buffer

2.2.1 Column Equilibration

The required amount of Concanavalin A-sepharose was packed onto the column (≈1 ml settled gel for 100 nmole Rhodopsin) and then washed with the following buffers at the speed of 10 ml/hour
1. D:H (2:1) 3 volumes
2. F:H (2:1) 7 volumes
3. F:H₂O (1:1) 3 volumes
4. G:I (1:1) 3 volumes

Concanavalin A is a lectin with preference for α-D-derivatives of mannose and to a lesser extent, glucose. For activity Con A requires metal ions Ca²⁺ and Mn²⁺ which are present in the equilibration buffers.

2.2.2 Protein Preparation, binding and Elution

Disc membranes (volume made to 6ml with the buffer G: H₂O) were spun for 20 minutes at 40,000 rpm, 4 °C. Pellet was then solubilised in the required amount of Solubilisation buffer (1 ml per 50 nmole rhodopsin which is 300 molecules of detergent per
molecule of Rhodopsin) at 4 °C overnight. Solubilised disc membranes were then spun for 20 minutes at 4 °C, 40000 rpm. Supernatant was then loaded onto the ConA column at the speed of 8 ml/hour. Column was then washed with 10 volumes of the buffer G:I at the rate of 10 ml/hour to get rid of the unbound materials. Rhodopsin was then eluted with the elution buffer at the rate of 4.5 ml/hour as 1 ml fractions. Concentration of Rhodopsin containing fractions were then measured using UV-Vis spectroscopy. Purity can also be determined by A280/A500 ratio.

2.2. Dialysis

Salt and sugar from the eluates can be removed from the sample by dialysis. DDM purified Rhodopsin samples were dialyzed using 8-10 KDa dialysis membranes (Zellutrans Roth) against 1 mM phosphate buffer (1000 ml) with 0.01 %DDM for 18 hours with three buffer exchanges. Concentration was again measured by UV-Vis spectroscopy. OG purified samples need not be dialyzed since they were taken for the reconstitution into the lipid vesicles where salt and sugar were anyways dialyzed out.

2.3 Preparation of Rhodopsin Mutants

Rhodopsin mutants studied here were cloned and expressed in Professor Thomas Sakmar’s laboratory, Rockefeller University, New York or in Professor Gebhard F. X Schertler, MRC Laboratory of Molecular Biology, Cambridge, England. Mutants studied in this work were E134Q, E134D, R135L, R135A, E249Q and Y136F. All the mutants described here was self expressed and the Site directed point mutagenesis was done using quick change (stratagene) method. E249Q was mutated and cloned onto the expression vector pMT4 while the rest of the mutant clones were already made by the seniors in the laboratory.

2.3.1 Expression vector pMT4

Wild type rhodopsin (1058 base pairs) was cloned onto the expression vector pMT4. pMT4 is a plasmid gene of 6187 base pairs. pMT4 is a derivative of the pMT2 mammalian expression vector and additionally carries the sequence of the bovine 1D4 epitope.
2.3.2 Site directed mutagenesis

Appropriate primer was designed and ordered in Gene link

Wildtype Rhodopsin-pMT4 Vector

5’ AG AAG GCC GAG AAG GAG GTC ACG CGT ATG 3’
3’ TC TTC CGG CTC TTC CTC CAG TGC GCA TAC 5’

Primer for E249Q

5’ AG AAG GCC GAG AAG CAG GTC ACG CGT ATG 3’
3’ TC TTC CGG CTC TTC GTC CAG TGC GCA TAC 5’

These oligonucleotides were dissolved in sterile TE (Tris-EDTA) at 1 ug/ul. Sense and antisense oligonucleotides were mixed and diluted to 1:10 in sterile double distilled H₂O (100 ng/ul each). Template DNA was diluted to 10 ng/ul in sterile TE.

Reaction mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligonucleotide-Mix (100 ng/ul)</td>
<td>1.25 ul</td>
<td>125 ng</td>
</tr>
<tr>
<td>DNA-Template (10 ng/ul)</td>
<td>2.5 ul</td>
<td>25 ng</td>
</tr>
<tr>
<td>dNTP-Mix (4 mM total/ 1 mM each)</td>
<td>5 ul</td>
<td>100 uM</td>
</tr>
<tr>
<td>Reaction buffer 10x</td>
<td>5 ul</td>
<td>1X</td>
</tr>
<tr>
<td>H₂O dd</td>
<td>6.25 ul</td>
<td>50 ul final</td>
</tr>
<tr>
<td>Pfu DNA-Pol. (2,5 U/ul)</td>
<td>1 ul</td>
<td>2.5 U</td>
</tr>
</tbody>
</table>

Mix is prepared without the oligonucleotides and then the oligonucleotide mix was added and finally the Pfu was added.

PCR reaction

<table>
<thead>
<tr>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 s</td>
<td>95 °C</td>
</tr>
<tr>
<td>14 cycles were performed</td>
<td></td>
</tr>
<tr>
<td>30 s</td>
<td>95 °C (Denaturation)</td>
</tr>
<tr>
<td>1 min</td>
<td>55 °C (Annealing)</td>
</tr>
<tr>
<td>2 min/kbp</td>
<td>68 °C (Elongation)</td>
</tr>
<tr>
<td>∞</td>
<td>4 °C</td>
</tr>
</tbody>
</table>
DNA was digested with Dpn I (20 U/ul). 0.5 – 1 ul of the enzyme was added to each reaction and incubated for 2 h at 37 °C. 5 µl of the sample was analysed using Agarose gel electrophoresis.

2.3.3 Transformation of the mutant DNA into Competent E. Coli cells.

Materials required

Top 10 competent E.coli cells (Invitrogen, Carlsbad, CA)
SOC medium (having Trypton, Yeast extract, NaCl, KCl, Glucose)
LB- Medium (having Trypton, Yeast extract, NaCl)
Ampicillin 50 mg/ml
Ampicillin containing agar plates

Ampicillin containing LB agar plates were poured and stored at 4 °C. Before transformation they were brought to 37 °C. 1 µl of the Dpn digested PCR reaction was mixed with 25 µl of the Top 10 E.coli competent cells. This was then incubated for 30 minutes in ice which was then followed by a heat shock for 30 seconds at 42 °C. Then the sterile SOC medium was added and incubated for 1 hour at 37 °C. Cells were then spun down, resuspended in 100 µl and plated onto the LB Amp plates. Plates were incubated over night at 37 °C incubators.

2.3.4 Plasmid DNA preparation and DNA sequencing

Single colonies were then streak purified, and then colonies were picked in a 5 ml LB medium for Plasmid DNA mini preparation using commercial kit(QIAGEN, Hilden). The procedure has three main steps 1. Bacterial cells were lysed in alkaline conditions, subsequently neutralized and adjusted to high salt binding conditions. 2. Adsorption of DNA onto the silica membrane. 3. Washing and elution of the DNA from the membrane. The concentration of the eluates were then determined using Nanodrop.
The purified plasmid DNA was then sent into sequencing to GENEWIZ (South Plainfield, NJ) with the primers. The mutant clone was then identified and taken for a maxi DNA preparation also using QIAGEN kit.

**2.3.5 HEK293T cell culture**

**Materials required**
- HEK293T cells
- Cell culture bottles (BD-Falcon)
- DMEM (Dulbecco’s Modified Eagle’s Medium)
- PBS (Phosphate buffer Saline)
- Fetal Bovine Serum
- Full medium DMEM with 10 % Fetal Bovine Serum

HEK 293T cells are Human Embryonic Kidney 293 cells transformed by the expression of the large T antigen from SV 40 virus (that inactivates pRb -Retinoblastoma) that allows for episomal replication of transfected plasmids containing the SV40 origin of replication. This allows for amplification of transfected plasmids and extended temporal expression of the desired gene products.

**2.3.6 Transient transfection and the expression of the Rhodopsin mutants in HEK293T cells**

**Materials required**
- 80 % confluent HEK293T cell culture in bottles
- Plus™ reagent (Invitrogen, Carlsbad, CA)
- Plasmid DNA
- Lipofectamine reagent (Invitrogen)
- DMEM, serum free
- DMEM with 10 % Fetal Bovine Serum
- DMEM with 20 % Fetal Bovine Serum
- PBS
HEK293 T cells were split on the day before the transfection so that the cells were 80 % confluent on the day of the transfection. In a polystyrene falcon tube, 3.5 µg/plate of DNA was diluted with 750 µl/plate of serum free DMEM. To the diluted DNA 10 µl/plate of PLUS reagent was added and incubated for 15 minutes. In another Polystyrene falcon tube, 17 µl/plate of Lipofectamine reagent is diluted with 500 µl/plate of DMEM. Diluted Lipofectamine reagent was then mixed to the DNA-Plus reagent and incubated at room temperature for 15 minutes. Culture medium was then aspirated from the cells. DNA-Lipofectamine-Plus cocktail was then diluted to a total volume of 4 ml/plate. Diluted cocktail was then added to the cells and incubated at 37 °C for 3-5 hours. 3-5 hours post transfection, one volume of medium containing 20 % FBS was added and incubated overnight. 24 hours post transfection cells were fed with fresh full medium (10 %FBS). 48 hours later, cells were harvested.

Culture medium was aspirated from the plates and washed with 1 ml PBS containing 10 µg/ml protease inhibitors Aprotinin and Leupeptin. Cells were then wiped with rubber police men. Cells were then regenerated in dark with 5.5 µl/plate with1.4 mM 11-cis Retinal (stored in ethanol) for 3 hours at 4 °C. Then the cells were pelleted down and stored at -20 °C.

### 2.4 Purification of Rhodopsin mutants using Immunoaffinity Chromatography

Monoclonal antibody 1D4 coupled Sepharose column

**Solubilisation buffer:** 50 mM Tris pH6.8

- 100 mM NaCl
- 1 mM CaCl2
- 0.1 mM PMSF
- 2 % OG / 1 %DDM

**Wash buffer I:** 20 mM Tris pH6.8

- 100 mM NaCl
- 1.5 %OG / 0.1 %DDM

**Wash buffer II:** 50 mM Phosphate buffer

- 140 mM NaCl
- 1.5 %OG/ 0.1 %DDM
Elution buffer 0.18 mg/ml 1D4 peptide (carboxy terminal segment of Rhodopsin) in Wash buffer II

The protein produced in the cells binds exogenously supplied 11-cis-retinal which is advantageous in maintaining the protein in the correct folded state. Then the cells were taken for the affinity purification procedure for the purification of Rhodopsin in fully functional form. The efficient purification is resulted from a combination of the specific, high-affinity binding of rhodopsin to the 1D4 antibody coupled-Sepharose and its selective elution from the antibody with the synthetic peptide.

Frozen cell pellet was taken out and detergent containing solubilisation buffer was added on to it. Cells were broken down by pipeting up and down. Then it was left for Solubilisation for 4 hours at 4 °C. After solubilisation, cells were spun at 30,000 rpm for 30 minutes in Beckman type 45 Ti rotor. Then the supernatant was left overnight for binding with 1D4 coupled Sepharose gel. Resin was then spun down and the supernatant was removed. Resin was washed with 15 ml of wash buffer I once and thrice with 15 ml of wash buffer II. Then 1.5 ml of elution buffer was added and the resin was allowed to sit for 1 hour and spun down and supernatant was collected which is the I eluate. Similarly II eluate was also collected after 1 hour. Concentration of Rhodopsin was then determined by UV-Vis spectroscopy. If needed the sample was concentrated by Centricon YM 30 concentrator (Millipore, Billerica, MA) before taking into reconstitution onto lipid bilayers.

2.5 Reconstitution of Wild type Rhodopsin and Rhodopsin Mutants in Lipid Vesicles.

Reconstitution from OG

Materials required

Dialysis buffer: 1mM Phosphate buffer pH 6.5 Rhodopsin: from purification

Detergent stocks: β-OG 6 % W/V (Molecular weight is 292. Critical Micellular Concentration (CMC) 0.6 % ≈ 20 mM

Lipids: PC (Phosphotidyl Choline) from egg yolk (Sigma, Avanti polar-Lipids., Inc)
Molecular weight ≈ 788.5

- Lipid was aliquoted in chloroform in stocks of 5 mg in 1.5 ml glass vials, dried under vacuum overnight to remove chloroform, overlaid with Argon, closed, parafilmed and frozen
- Before use, dried lipid films stored in glass vials were taken out, 800 µl of the buffer was added to the lipid film, incubated for 1 hour at 40 °C, vortexed to produce Large Multilamellar Vesicles (LMV). 200 µl of OG stock was added to dissolve the lipids which are finally then 5 mg/ml in 1.2 %OG buffer.

Dialysis membrane unit: This was decided based on the volume of the sample
For 100-500 µl of sample, slide- A-Lyzer dialysis cassette(Pierce) MWCO 10 KDa was used. Larger volumes dialysis bags were used with large surface area (MWCO 14 KDa)

Rhodopsin and the lipid were mixed in an appropriate ratio (1:100 molar ratio) and the mix was incubated for 1 hour at room temperature. Then sample was transferred onto a dialysis bag with large surface area. And the dialysis was carried out against phosphate buffer pH 6.5 for 72 hours with buffer exchange every 8-10 hours. Sample was then collected and spun for one hour in Beckman type 50 Ti 50 rotors at 30,000 rpm, 4 °C. Supernatant was checked if it was clear of protein and pellet was resuspended well and the concentration of Rhodopsin was determined by UV-Vis spectroscopy.

2.6 Fourier Transform Infrared Spectroscopy

Fourier Transform infrared (FTIR) Spectroscopy is one physical method that is readily applicable to the study of structural organization of integral membrane proteins in their natural membrane environment. This technique involves only a very little protein. In most cases data acquisition is relatively rapid and simple such that detailed insights into membrane structure protein can be obtained.

2.6.1 Infrared spectroscopy

IR spectroscopy is the study of the interaction of infrared light with matter. Light is composed of electric and magnetic field. These two fields oscillates perpendicular to each other and light wave moves through space in a direction perpendicular to planes.
containing the electric and magnetic vectors. It is the electric part of light, called the electric vector, that interacts with molecules. The amplitude of the electric vector changes over time and has the form of a sine wave. The wavelength ($\lambda$) of a light wave is the distance between adjacent crests or troughs. The wave number of a light wave is defined as the reciprocal of the wavelength or

\[ W = \frac{1}{\lambda} \]

where $W$ is wavenumber. If $\lambda$ is measured in cm, then $W$ is reported as cm$^{-1}$, sometimes called reciprocal centimeters. $W$ is really a measure of the number of waves (counted as the number of crests or troughs) there are in one centimeter. Wavenumbers are the units used to denote different kinds of light. The wavenumber of light is directly proportional to energy as follows

\[ E = h c W \]

Where $E$ is light energy

- $c$ is the speed of light (3x10$^8$ meters/second)
- $h$ is Planck’s constant (6.63x10$^{-34}$ Joule-second)
- $W$ is the wavenumber

Thus, high wavenumber light has more energy than low wavenumber light. Mid-infrared radiation is the light between 4000 and 200 cm$^{-1}$. When infrared radiation interacts with matter it can be absorbed, by molecular vibrations. Characteristics vibrations of covalently bonded atoms can be classified as stretching which involves changes in the bond length and bending corresponding to changes in the bond angles. Chemical structural fragments within molecules known as functional groups, tend to absorb infrared radiation in the same wavenumber range regardless of the structure of the rest of the molecule that the functional group is in. For instance, the C=O stretch of the carbonyl group occurs around 1700 cm$^{-1}$ in ketones, aldehydes and carboxylic acids. This means there is a correlation between the wave numbers at which a molecule absorbs infrared radiation and its structure. This correlation allows the structure of unknown molecules to be identified from the molecule’s infrared spectrum. Thus infrared spectroscopy is a useful chemical analysis tool. The introduction of Fourier Transformed IR spectroscopy revolutionized the field which is explained below.
2.6.2 Introduction to FTIR Spectroscopy

A plot of measured infrared radiation intensity versus wavenumber is known as an infrared spectrum. Y axis is normally the absorbance and X axis is wavenumber. Traditionally, infrared spectra plotted with high wavenumber on the left and the low wavenumber on right. This means that as spectrum is read from left to right one is looking from high energy to low energy. The upward pointing peaks in such a spectra will represent the wavenumbers at which the sample absorbed infrared radiation. The Y axis can also be plotted in transmittance in which case the peaks would point down and represent wavenumbers where the sample transmitted less infrared radiation. Regardless of the Y axis unit, it is the wavenumber positions of these peaks that correlated with molecular structure.

In addition to chemical structures, infrared spectra can provide quantitative information as well, such as the concentration of the molecule in a sample. The basis of all quantitative analysis in FTIR is Beer’s law, which relates concentration to absorbance

\[ A = \varepsilon l c \]

Where \( A \) is the absorbance
\( \varepsilon \) is Absorptivity
\( l \) is pathlength
\( c \) is the concentration

The absorbance is measured as a peak height, peak height ratio, peak area or peak area ratio from the FTIR spectrum. The absorptivity is the proportionality constant between concentration and absorbance. It changes from molecule to molecule and from wavenumber to wavenumber for a given molecule. For example the absorptivity of acetone at 1700 cm\(^{-1}\) is different than the absorptivity for acetone at 1690 cm\(^{-1}\). However for a given molecule and wavenumber, the absorptivity is a fundamental physical property of the molecule. For example the absorptivity of acetone at 1700 cm\(^{-1}\) is as invariant as its boiling point or molecular weight. The units of \( \varepsilon \) are usually given in \( \text{(concentration} \times \text{pathlength})^{-1} \), so the absorptivity cancels the units of the other two variables in Beer’s law, making absorbance a
unitless quantity. The width of an infrared band gives information about the strength and nature of molecular interactions. Thus an infrared spectrum provides a great deal of information.

Infrared spectrometer is the instrument used to obtain an infrared spectrum. There are several kinds of instrument used to obtain infrared spectra. Here for the current work Fourier Transform Infrared Spectrometers were used. Conventional dispersive IR instruments consists of an infrared source that is focused on the sample, the output IR beam is dispersed by a grating or a prism onto a slit that blocks all but a narrow range of frequencies from reaching the detector. Therefore the resolution depends on the width of the slit. The result is a low IR intensity spectrum making the method highly insensitive.

Fourier transform infrared technique has distinct advantages over this method in terms of spectral bandwidth, resolution and sensitivity for both static and time resolved measurements. The incorporation of an interferometer in the time resolved allows a broad band of vibrational frequencies to be monitored simultaneously. With this tool, processes like ligand binding and dissociation, protein folding, photocycles, reaction kinetics can all be measured and characterized.

The heart of an FTIR spectrometer is the Michelson interferometer (Fig 2.1)

Michelson interferometer is a device that can divide a beam of radiation into two paths and then recombine the two beams after a path difference has been introduced. It consists of two perpendicular place mirrors, one of which can move along an axis that is perpendicular to its plane. Between the fixed mirror (M1) and the movable mirror (M2) is a beam splitter, where a beam of radiation from an external source can be partially reflected to the fixed mirror and partially transmitted to the movable mirror. After the beam return to the beam splitter, they recombine and are again partially reflected and partially transmitted. At the detector, the two beams from the two arms of the interferometer interfere.
Fig 2.1. Michelson interferometer which consists of a fixed mirror, a moving mirror and a beamsplitter. Beam splitter divided the IR spectrum into two parts and subsequently recombines them and directs the combined beam to the output. The two combined beams interfere constructively or destructively depending on the wavelength of the light (or frequency in wavenumbers) and the optical path difference introduced by the moving mirror. Before the output beam is focused onto the detector, it passes through the sample.

The movement of M2 at constant velocity will then give a cosine wave for a source of monochromatic radiation of frequency $\nu$ (See derivation). Because of the effect of interference, the intensity of the combined beam passing to the detector $I'(\delta)$ depends upon the optical path difference $\delta$ (retardation) between the light beams in the two arms of the
interferometer and it is given by

\[ \Gamma'(\delta) = 0.5I(W) \left( 1 + \cos 2\pi W \delta \right) \]

where \( W = 1/\lambda \) is the wavenumber of the radiation and \( I(W) \) is the intensity of the source. One sees that \( \Gamma'(\delta) \) has two components, a constant term equal to \( 0.5I(W) \) and a modulated term equal to \( 0.5I(W) \cos (2\pi W \delta) \). Only this modulated component is important in spectroscopic measurements, and is generally referred to as the interferogram \( I(\delta) \). In practice, the amplitude of the interferogram observed after detection and amplification is proportional not only to the intensity of the source, but also to the beam splitter efficiency, detector response and amplifier characteristics. These contributions define a single wavenumber-dependent correction factor \( H(W) \) to give

\[ I(\delta) = 0.5H(W)I(W)\cos (2\pi W \delta) = S(W) \cos(2\pi W \delta) \]

Where \( S(W) = 0.5H(W)I(W) \) is the single beam spectral intensity.

When the source is a continuum the interferogram can be represented by:

\[ I(\delta) = \int_{-\infty}^{\infty} S(W) \cos(2\pi W \delta) dW \]

\( I(\delta) \) is the cosine Fourier transform of \( S(W) \), which accounts for the name given to this spectrometric technique.

The other half of the cosine Fourier pair is:

\[ S(W) = \int_{-\infty}^{\infty} I(\delta) \cos(2\pi W \delta) d\delta \]

The Fourier transform converts the information in the interferogram to an \( S(W) \) plot of intensity versus wavenumber which is more easily interpreted by the scientist. This measure represents in fact the single beam spectrum.

In an absorption experiment there are two steps that are carried out sequentially: first, an interferogram is recorded with no sample present, and in the second step the sample is placed in the beam, and one records its interferogram with a smaller amplitude due to light absorption by the probe. After Fourier transform, the former gives the single beam reference
S_0(W) and the latter gives the single beam sample S(W). The desired absorbance spectrum can be calculated with the formula:

$$A(W) = -\log \left[ \frac{S(W)}{S(W)_0} \right]$$

In addition to the infrared light, a He-Ne laser beam traverses the same optical path as the IR light. Being a monochromatic light it generates a cosine-squared interferogram pattern when modulated by the interferometer. Its interferogram is used by the spectrometer to monitor mirror position during the course of the scan: every time a $\lambda/2$ of He-Ne laser is completed, the electronics are triggered to read the detector output and to store the value. As a result of this process one obtains the digital interferogram, which is built from N discrete, equidistant mirror points. In this case the interferogram is depicted in its discrete form:

$$I(n\Delta\delta) = \frac{1}{N} \sum_{n=0}^{N-1} S(K\Delta W) \exp(-2\pi ink/N)$$

And its corresponding single beam spectrum is

$$S(k\Delta W) = \sum_{n=0}^{N-1} I(n\Delta\delta) \exp(2\pi ink/N)$$

where the continuous variables $\delta$ and $W$ were replaced by the discrete digitization points $n\Delta\delta$ and $n\Delta W$.

The complex discrete fourier transform algorithm decreases considerably the computing time for obtaining the spectrum, but results in a loss of the photometric accuracy of FTIR spectrometry since rarely the computed datum corresponds to the wavenumber of maximum absorption. Generally, if $(2r-1)N$ zeros are added to an N-point interferogram, where r is an integer, the resulted spectrum looks far smoother, and the procedure is known as zero filling.

In practice, the interferogram cannot be measured to a retardation of $\infty$ like stated in above equation. The measurement of the signal over a limited retardation will give a finite
resolution. Resolution in FTIR instruments depends on the maximum retardation of the interferometer $\Delta_{\text{max}}$, that is on the length of the mirror scan away from the zero path difference (ZPD).

$\Delta W = (\Delta_{\text{max}})^{-1}$

A larger length of the mirror scan would improve the resolution but in the same time would increase the noise and data acquisition time.

This restriction of the maximum retardation of the interferogram to $\Delta$ centimeters is equal to multiplying the complete integral by a truncation function (boxcar) $D(\delta)$, that is:

$D(\delta) = 1$, if $-\Delta \leq \delta \leq +\Delta$

$D(\delta) = 0$, if $\delta > |\Delta|$

Further, this represents the convolution of the real spectrum $S(W)$ with the Fourier transform of $D(\delta)$, i.e. $2\Delta \text{sinc}2\pi\delta\Delta$. When a single spectral line of wavenumber $W$ is convolved with the sinc function the sharp line will appear in spectrum broader than the half-width of the line. So, the sinc function is not exactly a useful line shape in FTIR technique because has fairly large amplitude at wavenumbers well away from the peak maximum. Suppression of the magnitude of these oscillations is known as apodization and functions like

$$T(\delta) = 1 - \frac{\delta}{\Delta}, \quad \text{if} \ -\Delta \leq \delta \leq \Delta$$

$$T(\delta) = 0, \quad \text{if} \ \delta > |\Delta|$$

are known as apodization function (in this particular case is called triangular apodization function)

The sampling of the interferogram by means of the He-Ne laser, and further optical and electronic effects lead to a wavenumber dependent phase shift between the real and detected interferogram. The latter one is not anymore symmetrical.

The above mentioned equations can be rewritten in the exponential notations

$$I(\delta) = \int_{-\infty}^{\infty} S(W) \exp(2\pi i W \delta) dW$$
The other half of the cosine Fourier pair is:

\[ S(W) = \int_{-\infty}^{\infty} I(\delta) \exp(2\pi i W \delta) d\delta \]

In light of Euler’s formulas, the component of the spectrum computed using the cosine Fourier transform is called the real part of the spectrum \( \text{Re}(W) \), whereas the component computed using the sine transform is called the imaginary part of the spectrum \( \text{Im}(W) \). The process of removing these sine effects from the spectrum is called phase correction.

In the most commonly used method of phase correction, known as Mertz method, the phase spectrum \( \Phi(W) \) is computed at a lower resolution from a region of the interferogram measured symmetrically on either side of the ZPD. Data collection is started at a short distance to the left of the centerburst, and continued until the desired resolution is attained. The short double-sided interferogram is used to compute the sine and cosine transforms and further to calculate the phase spectrum

\[ \Phi(W) = \arctan \frac{\text{Im}(W)}{\text{Re}(W)} \]

The number of digital points in an interferogram is a function of spectral bandwidth and resolution, and of the acquisition mode. Because, sometimes FTIR experiments are long (step-scan), it is often practical to minimize the number of points in the interferogram.

The Nyquist theorem says that in order to correctly sample any frequency in interferogram space, there must be more than two sampling points per every sinusoidal period in order to record the signal to the correct spectral range. The problem is that if one covers a larger spectral range without increasing the sampling rate in the interferogram, any spectral features in a domain frequency \( \nu \geq \nu_{\text{Nyquist}} \) would appear at incorrect frequencies. They would be as matter-of-fact back reflected in the allowed spectral domain \( \nu \leq \nu_{\text{Nyquist}} \). These artifacts are referred to as aliasing.

The highest sampling rate that can be obtained in an interferometer that uses a He-Ne laser is to sample every \( \frac{\lambda_{\text{He-Ne}}}{2} \), that is a sampling distance of 316 nm, i.e. 15800 cm\(^{-1}\). Actually for the studied proteins a smaller spectral domain, down to 2000 cm\(^{-1}\) is only
necessary so that one could record the signal at every $8 (\lambda_{\text{He-Ne}}/2)$. The higher frequencies are blocked by corresponding optical low-pass filters (germanium filter).

**Advantages of FTIR**

Some of the major advantages of FTIR over the dispersive technique include:

- **Speed**: Because all of the frequencies are measured simultaneously, most measurements by FTIR are made in a matter of seconds rather than several hours. This is sometimes referred to as the Felgett Advantage.

- **Sensitivity**: Sensitivity is dramatically improved with FTIR for many reasons. The detectors employed are much more sensitive, the optical throughput is much higher (referred to as the Jacquinot Advantage) which results in much lower noise levels, and the fast scans enable the coaddition of several scans in order to reduce the random measurement noise to any desired level (referred to as signal averaging).

- **Mechanical Simplicity**: The moving mirror in the interferometer is the only continuously moving part in the instrument. Thus, there is very little possibility of mechanical breakdown.

- **Internally Calibrated**: These instruments employ a HeNe laser as an internal wavelength calibration standard (referred to as the Connes Advantage). These instruments are self-calibrating and never need to be calibrated by the user.

These advantages, along with several others, make measurements made by FTIR extremely accurate and reproducible. Thus, it is a very reliable technique for positive identification of many sample. The sensitivity benefits enable identification of even the smallest of contaminants. This makes FTIR an invaluable tool for quality control or quality assurance applications whether it be batch-to-batch comparisons to quality standards or analysis of an unknown contaminant. In addition, the sensitivity and accuracy of FTIR detectors, along with a wide variety of software algorithms, have dramatically increased the practical use of infrared spectroscopy for quantitative analysis. Quantitative methods can be easily developed and calibrated and can be incorporated into simple procedures for routine analysis. Thus, the Fourier Transform Infrared (FTIR) technique has brought significant practical advantages to
infrared spectroscopy. It has made possible the development of many new sampling
techniques which were designed to tackle challenging problems which were impossible by
older technology. It has made the use of infrared analysis virtually limitless.

![Single beam spectrum of rhodopsin in disc membranes which has many protein bands overlapped. Apart from the contributions of the protein there are contributions of lipid, water and buffer to the spectra. Only the amide I and amide II bands are directly recognizable.](image)

**Fig 2.2** Single beam spectrum of rhodopsin in disc membranes which has many protein bands overlapped. Apart from the contributions of the protein there are contributions of lipid, water and buffer to the spectra. Only the amide I and amide II bands are directly recognizable.

### 2.6.3 Light induced FTIR difference spectroscopy

The study of molecular mechanism of proteins from IR absorption spectra which in almost all cases involves only a small fraction of the system, is impeded by the large background signal. If only a single side chain is involved in a certain reaction step, its
identification is lost in the large palette of overlapped absorption bands. In order to still be able to resolve it, the spectrometer should have a high sensitivity. Practically, if the background absorbance is around 0.4, it is desirable to resolve absorbance changes smaller than $10^{-4}$ (Siebert, 1993). This drawback can be overcome by the IR difference spectroscopy: the spectrum of the sample where the protein is in state A is subtracted from the spectrum of sample where the protein is in state B. An important prerequisite of this procedure is that the probe- and measurement- parameters are in irreproachable agreement.

The reaction of the protein can be initiated directly in the cuvette, and one computes the reaction induced difference spectra between the state before the start (ground state) of reaction and different states after triggering the reaction. These difference spectra originate only from those molecular groups that are affected by reaction. The reaction can be induced by different methods like stopped and continuous flow, temperature and pressure jump, photolysis activation of caged compounds, and in the case of photobiological systems like rhodopsin, by light (Kotting and Gerwert, 2005a-b; Siebert, 1997). Conventionally, the difference spectra are so calculated that the negative bands are characteristic for the initial state, while the positive bands reflect changes in the protein during the reaction.

### 2.6.4 Making of the probe

The functionality of the proteins is strongly correlated to the medium where they reside, water especially influencing the proper behaviour of proteins as well as their interactions. We adopted a type of IR sample called sandwich samples. Samples of rhodopsin and rhodopsin mutants (0.05-0.2 nmol pigment) was suspended on bottom BaF$_2$ window within a circular groove and was dried under low-flow Nitrogen. Then 40 µl of appropriate buffer was overlaid. After a reasonable 1 minute equilibration time counter window is pressed over the sample. A counter window squeezes this construct forming a reservoir with the thickness between 3 and 7 µm. Normally 200 mM citrate buffer was used for pH <5.5, MES (Morpholinoethansulfonsäure) for pH 6.0-7.0 and BTP (Bis-Tris propane) for pH> 7.5 is used. Then the sample containing windows was packed in a specially designed sample holder with screws.

For the making samples in D$_2$O-buffer the sample film was first equilibrated twice with D$_2$O
and to remove H2O, followed by overlaying of buffer made in D2O.

Samples in DM (Dodecylmaltoside) film samples were also measured. There the samples were suspended on a window and breathe on to hydrate the sample.

![Diagram of buffer, sample window, and IR beam]

*Fig 2.3 Specially designed sandwich cuvette for biological samples*

### 2.6.5 FTIR Spectrometer and measurements

FTIR difference spectroscopy was performed with a Bruker Vertex 70 Spectrometer or with IFS 28 (Bruker, Karlsruhe). The spectrometer unit consists of three components: Globar the middle infrared source, interferometer and the detection systems which are mostly semiconductor detectors, (mercury-cadmium-telluride detector used for this present work). The probe chamber is flushed with dry air in order to avoid the influence of the water vapour in the spectra. MCT detector is operated at low temperature (77 K). They are cooled by using liquid nitrogen. Water spectra in the range of 5000 cm\(^{-1}\) and 500 cm\(^{-1}\) was always taken for all the samples to determine the homogeneity of the film and the water content in the sample. A germanium filter is fixed in front of the MCT detector which obstructs the wavelengths above 1975 cm\(^{-1}\). Therefore the DTGS (Deuterated Triglycine Sulfate) detector is used to evaluate the water content in the sample. The temperature of the samples was controlled by cooling bath (-7 to 30 °C) by a Peltier element (-30 to -10 °C) or a nitrogen-cooled cryostat (-170 to -20 °C). Before any measurement, it was made sure that the background absorbance of the sample was stable. This was done by recording multiple
spectra in time interval of 1 minute. This is called baseline test. For a static difference spectrum roughly about four dark spectra were recorded in blocks of 512 scans with an acquisition time of 1 minute. Sample was then photolyzed using slide projector or by LEDs depending on the need. Illumination time also varies depending on the need. Photochemical generation of Meta III was achieved by illumination of rhodopsin for 20 s with orange light >550 nm (through fiber optics fitted to a 150 W tungsten lamp equipped with a Schott OG 530 filter) to produce Meta II, followed by a 5s near UV illumination using an LED array consisting of six 10 mW LEDs with an emission peak at 395 nm, to produce mostly Meta III. For Meta II, 10 s photolysis using 150 W tungsten lamp and a 530 nm long pass filter was performed. After light excitation spectra were recorded again in blocks of 512 scans with an acquisition time of 1 minute. Difference spectra were then calculated

$$\Delta \text{Absorption} = -\log \frac{I_{\text{after ill}}}{I_{\text{before ill}}}$$

For baseline correction all the spectra recorded before the illumination were averaged and used.

2.6.6 Time resolved rapid scan FTIR spectroscopy

Rapid scan FTIR spectroscopy employs the fact that the sampling time for an interferogram could be pretty short (depending on the pathlength of the mirror). It takes only 5-20 ms for the movable mirror of the interferometer to travel over the required distance (typically 0.5 cm or less). Thus a complete interferogram is measured within that time. Most of the FTIR spectrometers provide the means to synchronize the data acquisition of the first interferogram to an external trigger linked to an event in the sample, that is, the initiation of a reaction. Thus spectral changes induced by the event can be monitored in a time resolved manner on the basis of successively measured interferograms. The spectral changes are calculated with respect to the spectrum before the event or with respect to the spectrum a long time after the event when the final state of the sample has been reached. Since the signal to noise ratio of such a single measurement is not sufficient to detect the spectral changes, this procedure has to be repeated several times and averaging is always done. For longer reaction times that allow reduction of the time resolution, several successive spectra can be co-added, thereby increasing the signal to noise ratio. (Siebert et al Vibrational
spectroscopy in life sciences). A practical way to achieve such a progressive slowing down of the effective time resolution is the implementation of a quasi logarithmic time base. For example, the first 8 spectra are linearly spaced for the next 16 scans, 2 consecutive interferograms are averaged, resulting again in 8 linearly spaced spectra, however with a doubling of the time spacing; and for the next 32 scans 4 consecutive interferograms are averaged. Such a quasi-logarithmic time base is often used in time resolved studies. In addition to the improvement of the signal to noise ratio for longer times it allows a much larger time range to be covered with a still manageable number of data points.

Following figure explains the principle of rapid scan FTIR spectroscopy

![Diagram of FTIR technique](image)

**Fig 2.4 Principle of rapid scan FTIR technique.**

*The time pattern of rapid scan FTIR technique. The first two reference (R) interferograms represent the ground state (A), while the following three interferograms are taken during the reaction pathway (B to C). The first interferogram will mainly represent the spectrum of B.*
and in the following the ratio of C will increase according to the kinetics of the observe reaction.

In this current study especially where the protonation switches of rhodopsin (chapter 5) was studied IR spectra were recorded by using time resolved rapid scan FTIR spectroscopy with a spectral resolution of 4 cm$^{-1}$. An acquisition time of 6s was used for the preillumination spectrum and a time of 240 ms for the single post illumination spectra.

2.6.7 Normalization of the FTIR difference spectra

The FTIR difference spectra are presented generally in the spectral range from 1900 to 900 cm$^{-1}$. Because the absorption changes with varying sample concentration, and the illumination conditions, the spectra get difficult to compare. For the comparison of several difference spectra, spectral normalization was achieved by using the intense 1237 cm$^{-1}$ fingerprint mode of the dark state which was normalized to 0.002 OD. Every tickmark represents 0.001 OD in all the FTIR spectra shown here.

2.7 UV-Vis spectroscopy

Both qualitative and quantitative studies on rhodopsin and rhodopsin mutants were carried out by UV-Vis spectroscopy. The measurements were done on a Perkin Elmer Lambda 17 spectrometer. Illumination conditions were performed similar as in FTIR experiments. Spectrometer was attached to a temperature controlled sample holder. Sandwich samples are made just like it was mentioned for FTIR spectroscopy. UV-Vis spectra of sandwich rhodopsin samples with dark state absorption peak at 500 nm and Meta II peak at 380 nm was obtained as in figure 2.5. Concentration determination of the rhodopsin samples were often determined using UV-Vis spectroscopy. We used a quartz microcuvette for this purpose. In order to minimize scattering effects, an aliquot of a membrane sample was first solubilised in Dodecylmaltoside and then in 100 mM MES buffer pH 6.0, with 10 mM hydroxylamine contained. The hydroxyl amine dissociates retina from opsin. Spectra were recorded before and after the illumination. The difference in absorption at 500 nm was then calculated. Concentration was then determined
by using Beer-Lambert law by using 40,000 L / (mol • cm) as molecular coefficient or absorptivity of rhodopsin.

![UV-Vis spectrum of rhodopsin in Disc membranes. Measurement was done at 10 °C, pH 5. Absorption maximum of dark state is 500 nm and Meta II is 380 nm.](image)

**Fig 2.5 UV-Vis spectrum of rhodopsin in Disc membranes. Measurement was done at 10 °C, pH 5. Absorption maximum of dark state is 500 nm and Meta II is 380 nm.**

### 2.8 FTIR difference spectroscopy to photointermediates of rhodopsin

FTIR difference spectroscopy can be employed for elucidating the functional mechanism of the protein. By forming the difference spectra between functionally well defined states of the protein, only those groups that are altered in the transition show up in the difference spectrum. Since rhodopsin is light triggered, it is relatively easy to be studied by FTIR spectroscopy. In the absorption spectra of rhodopsin, since many vibrational bands
overlap it is difficult to derive any meaningful information. The amide I/II bands of rhodopsin can easily be identified in absorption spectra. The band at 1735 cm\(^{-1}\) is caused by the C=O stretching mode. In a difference spectrum negative bands belong to the initial or dark state, whereas the positive bands are caused by the respective photoproduct. In rhodopsin photoproducts can be trapped at different temperatures. Interpretation of Meta II difference spectra (fig 2.6) is described here because of the relevance to the current work.

The greatest challenge in the interpretation of the FTIR difference spectra is the assignment of the bands to specific groups of the protein. Chromophore, amino acid side chains and the peptide group of the protein backbone will all contribute to the bands making it complicated to interpret the data. Site directed mutagenesis and isotopic labeling have been useful in band assignment of FTIR difference spectra.

In the Meta II spectrum (fig 2.6), the negative bands of the chromophore of rhodopsin can be recognized between 1300 and 1100 cm\(^{-1}\). The lack of strong positive bands in this region is explained by Schiff base deprotonation, which reduces charge alteration and therefore the IR intensity of the fingerprint modes. The ethylenic modes of rhodopsin and Meta II cannot be identified due to the superposition by amide II bands. Bands at 1600cm\(^{-1}\) are the signature bands for the transition to the active state and they are interpreted as amide I changes. These intense changes in the amide I range (1600 – 1680 cm\(^{-1}\)) reflects the large backbone changes of the protein as compared to the other earlier intermediates like Batho, Lumi and Meta I. This might correspond to the relative movement of helices 3 and 6 detected by spin labeling technique, which increases the distance between the cytoplasmic ends. The protonated carboxylic acids appear in the region of 1800 cm\(^{-1}\) and 1700 cm\(^{-1}\). The bands represents the C=O symmetric stretching vibration coupled with O-H in plane bending mode. The bands here were attributed to the intra membrane carboxyl groups (Asp83, Glu113, Glu122) since these bands were present in DDM solubilised rhodopsin at pH 8. (peripheral carboxyl groups get deprotonated in this pH). By mutagenesis studies Glu113 was identified as the counter ion of the protonated schiff base in rhodopsin. The absorption changes centered at 1550 cm\(^{-1}\) (amide II) represents the NH in plane bend in combination with CN stretch vibration.
Meta II minus dark state spectrum

Fig 2.6 Meta II minus dark state spectrum
3. Study on the deactivation pathway of rhodopsin

3.1 Introduction

The key steps involved in the visual perception are the activation and deactivation of the visual pigment rhodopsin. Activation is achieved by light dependent isomerization of the chromophore and the subsequent thermal relaxation of the receptor on the millisecond time scale to the active receptor conformation. As discussed before this relaxation of this receptor proceeds via several spectroscopically resolvable intermediates and the key event is the transition from Meta I to Meta II. Meta I absorbs at 480 nm and Meta II absorbs at 380 nm. These two intermediates form a conformational equilibrium that can be shifted from Meta I to Meta II by decreasing pH or increasing temperature. The active Meta II state binds and activates the G protein transducin, thereby triggering the visual signaling cascade. This coupling of Meta II to G protein is rapidly shut off on the time scale of 100 ms by interaction with rhodopsin kinase and arrestin. However the active protein conformation persists and decays only on the time scale of minutes, mainly by the hydrolysis of the retinal Schiff base. The decay of the Meta I/Meta II photoproduct equilibrium is heterogeneous and two pathways occur in parallel. 1. Direct dissociation of the active receptor into opsin and free all-trans retinal, which is the predominant pathway at acidic and neutral pH. 2. Decay of the Meta I/Meta II to Meta III involving re-protonation of the retinal Schiff base and shift of the absorption maximum to 470 nm. This happens mainly at alkaline pH.

Fig 3.1 (Bartl et al)
During the first mentioned pathway, all-trans retinal which is released may form peripheral protonated Schiff bases with amino groups of the protein or lipid head groups which also absorb in a similar spectral range as that of Meta III. Due to this spectral overlap, Meta III can be hardly distinguished using the conventional UV-Vis spectroscopy. But an alternative way to examine Meta II selectively is by taking advantage of the light sensitivity of Meta III. The photoproduct Meta I/Meta II equilibrium decays via two pathways to form opsin and all-trans retinal and to form Meta III leading to an increase in the absorption at 470 nm. Applying another illumination with light >475 nm to the decay products converts only Meta III efficiently back to Meta I/Meta II while the other decay products remain largely insensitive to the second illumination. Light sensitivity of the Meta III has been shown already in Matthews et al. This offers a good approach to investigate the properties of Meta III despite the background of other products. Using this approach as discussed below we have shown that the decay of Meta III is strongly pH dependent. This approach is also extended to study the properties of Meta III by other spectroscopic methods like FTIR.

The first hint of the retinal geometry came from the retinal extraction experiments and the subsequent HPLC experiments. It was monitored that the thermal decay of the Meta I/Meta II equilibrium to Meta III did not significantly change the contribution of the all-trans isomer to the extracted retinal (F.J.Bartl et al 2001, R.Vogel et al 2004). This was surprising as the chromophore bands in Meta III were very different from those of rhodopsin photoproducts containing the all-trans isomer of retinal. In the all trans photoproducts of rhodopsin, the trans configuration of the retinal polyene extend to the Schiff base C15=N bond, which is also in an extended anti configuration (corresponding to trans in double bonds formed between same atoms). An all-trans chromophore, in which the C15=N Schiff base double bond is syn (corresponding to cis) instead of anti could therefore be possibly account for the peculiar FTIR signature of the Meta III chromophore. The isomeric state of the Schiff base was determined by taking advantage of the coupling pattern of the C14-C15 stretching mode of the chromophore with the C15=N stretch and the NH bending mode of the Schiff base. In a 15-syn geometry (but not in 15 anti) the strong coupling between the C14-C15 stretch (at around 1200 cm⁻¹) and the higher frequency NH bending mode (at around 1400 cm⁻¹) shifts the NH bending up and shifts the C14-C15 stretch down considerably (S.O.Smith et al 1984). This coupling is reversed upon deuteration of the Schiff base leading to a very
strong upshift of the C14-C15 stretch in D2O compared with H2O. Using isotopic labeling it was also identified (Franz J Bartl et al 2005) the C14-C15 stretch in Meta III in H2O at 1181 cm\(^{-1}\). In D2O this band shows a tremendous upshift by approximately 60 cm\(^{-1}\) (Fig 3.2A), which is accompanied by the disappearance of the NH bending mode of the Schiff base at 1349 cm\(^{-1}\). Accompanying quantum chemical DFT calculations of the retinal fingerprint modes performed by Mathias and Tavan showed good agreement with the experimental data for the all-trans 15-syn isomer, but not for other isomers. (R.Vogel, Siebert, P Tavan et al 2003) These results confirmed that the chromophore in Meta III is indeed all-trans 15-syn and that the thermal decay of the Meta I/Meta II photoproduct equilibrium is triggered by a thermal isomerization of the Schiff base C15=N double bond. This further implies that the 15-anti/syn isomerization of the Schiff base renders the shape of the chromophore much more compatible with an inactive protein structure and therefore allows the transition from the active Meta II conformation to the inactive conformation of Meta III.
As discussed above, the decay pathway to Meta III is favored at alkaline pH, which also favors Meta I over Meta II in the Meta I/Meta II photoproduction equilibrium. On the other hand, presence of a G protein-derived peptide analogue, which is known to bind and stabilize Meta II, prevented formation of Meta III as presented in M.Heck at al (2003). In the detergent digitonin, which tends to stabilize Meta I at the expense of Meta II, the Meta III pathway contributed roughly similarly to the Meta I/Meta II decay as in lipid membranes. In the detergent dodecyl maltoside, on the other hand, in which the Meta I/Meta II equilibrium is
completely forward-shifted to Meta II, no formation of Meta III was observed at all. (R. Vogel et al 2004) These experiments indicate that Meta III will not be formed under conditions that favor exclusively the active Meta II receptor state and instead the presence of the inactive receptor state Meta I is required for allowing the thermal isomerization of the Schiff base. These experiments were further extended to conditions where formation of the active Meta II receptor state is completely blocked. This can be achieved by preparing rhodopsin samples under very low hydration conditions, or by reconstituting rhodopsin into rigid lipid bilayers, as for instance saturated dipalmitoyl PC. In either case, the sequence of photo intermediates of rhodopsin is blocked prior to the transition to the active state, between Lumi and Meta I, or between Meta I and Meta II, respectively. In both cases, the resulting inactive photoproduct state decays by thermal isomerization of the Schiff base C15=\text{N} bond. This indicates that the trigger, which is responsible for formation of Meta III in the decay of Meta I/Meta II, is functional in these inactive photoproduct states. In the decay of the Meta I/Meta II pool, the thermal C15=\text{N} isomerization appears therefore to be driven by a steric mismatch between the inactive protein conformation of Meta I and the activating all-\text{trans} 15-\text{anti} chromophore. This incompatibility, which is also the driving force for the transition to the active receptor conformation Meta II, is relieved at least partly by the isomerization of the chromophore to the all-\text{trans} 15-\text{syn} isomer and the transition to Meta III. These results further support that the transition from the Meta I/Meta II photoproduct equilibrium to Meta III proceeds via Meta I and not via Meta II. Under conditions where the Meta I/Meta II equilibrium is fully on the Meta II side, Meta I is formed only as a transient species with a life time in the range of milliseconds, which is not sufficient to catalyze the Meta III pathway appreciably. However, Meta III itself is not a stable species either, but decays likewise by hydrolysis of the Schiff base and release of all-\text{trans} retinal. This reaction proceeds under neutral to alkaline pH and is very slow.

Earlier in our laboratory it has been revealed (Vogel et al 2004) that the photoreactions of Meta III are characterized by a quite complicated process, involving a branched reaction scheme: Meta III may be photo converted directly to Meta II by a light-dependent isomerization of the protonated Schiff base of Meta III from 15-\text{syn} to 15-\text{anti}. Alternatively, Meta III may be photoconverted by a light-induced isomerization of either C9=C10 or C11=C12 of the polyene of the chromophore, followed by a thermal isomerization of the protonated Schiff base C=\text{N} bond, yielding isorhodopsin and dark state rhodopsin as stable products. Under continuous illumination with visible light, dark state rhodopsin and
isorhodopsin may not accumulate and Meta III is therefore quantitatively converted to Meta II.

In an attempt to understand Meta III more, in this study we have examined the conformation of Meta III over a wider pH range and found that Meta III exists in a pH dependent conformational equilibrium between this inactive conformation at neutral to alkaline pH and an active conformation similar to that of Meta II, which is however assumed only at very acidic pH only.

3.2 RESULTS

3.2.1 Synopsis

In this study, we show by FTIR spectroscopy that this pH dependence of the Meta III decay rates reflects a conformational transition of Meta III to an active state conformation at low pH, which is similar to that of Meta II. Meta III forms therefore a pH-dependent equilibrium between active and inactive conformations corresponding to the Meta I/Meta II photoprodut equilibrium, which is, however, shifted considerably to the side of the inactive conformation. This shift to the inactive side is reflected in a downshift of the apparent $pK_a$ of the equilibrium from above 8 for the Meta I/Meta II equilibrium to around 5.1, implying that the active conformation of Meta III is attained only at quite acidic pH. Still, the apparent $pK_a$ of the Meta III conformational equilibrium between active and inactive conformations is above that of the corresponding equilibrium of opsin in the absence of ligand, which was found to be 4.1 (Vogel et al 2001). The all-trans-15-syn-retinal ligand of Meta III is therefore not an inverse agonist, which stabilizes the inactive conformation of the receptor as compared with ligand-free opsin, but a classical partial agonist, which is capable of activating the receptor, yet with a considerably decreased efficiency as compared with the full agonist all-trans-15-anti-retinal. The isomeric state of the Schiff base C15=N double bond acts thus as a functional molecular switch between full and partial agonism. The general principle behind this functional switch could be a requirement for a certain minimal effective length of the ligand between its anchoring points at the ring and the $\varepsilon$-carbon of Lys 296 next to the Schiff base in order to achieve maximal activation of the receptor. This effective length of the ligand would be increased in the extended 15-anti and decreased in the 15-syn state of the Schiff base. This general principle is also discussed in regard to other partial agonists that have deletions at the $\beta$-ionone ring at the opposite end of the ligand.
3.2.2 The Decay Rate of Meta III is pH dependent.

The Meta III pathway contributes significantly to the thermal decay of the Meta I/Meta II equilibrium only at neutral to alkaline pH (G. Cohen at al, M. Heck et al 2003). In order to study Meta III over a broader pH range, we have used a photochemical protocol for generation of Meta III (Figure 3.3 A): First, we illuminate rhodopsin in disc membranes (λ\text{max} 500 nm, black spectrum) for 20 s by orange light (>550 nm) to produce the Meta I/Meta II photoproduct equilibrium. At 30 °C, the apparent pK of this equilibrium is above 8, such that up to pH 7.0 essentially only Meta II (λ\text{max} 380 nm, red spectrum) is produced. Meta II is then photolyzed for 5 s by near-UV light (395 nm), which produces besides small amounts of rhodopsin and isorhodopsin mainly Meta III (λ\text{max} ≈470 nm, blue spectrum) (Matthews R et al 1963 and Ritter E at al 2004). We followed the thermal decay of Meta III to opsin and all-trans-retinal at 30 °C in the pH range from 4.0 to 7.0. This decay leads in the first line to an absorption decrease at 470 nm (Figure 3.3B). As the disc membranes are aligned in our samples with their membrane normal largely parallel to the measuring beam, the concurrent absorption increase at 380 nm due to released all-trans-retinal is marginal, as released retinal is oriented preferentially parallel to the membrane normal and thus parallel to the beam (Kolesnikov A et al 2003). The decay kinetics show a marked pH dependence with time constants in the range of more than 1 h at pH 7.0 down to less than 2 min at pH 4.5 (red crosses in Figure 3.3C), which roughly equals the decay rate of Meta II under otherwise similar conditions (R. Vogel et al 2001). At pH 4.0, the decay rate increased again, presumably due to some long-term structural instability of the photoproduct (not shown). The decay rates can be fitted to a Henderson-Hasselbalch equation with an apparent pK\textsubscript{a} roughly around 5.1. Due to the uncertainty regarding the end point of the titration curve at low pH, pK\textsubscript{a} values in the range between 4.9 and 5.3 were obtained, depending on the precise assumptions regarding this end point. The blue points in Figure 3.3C were derived from a conformational analysis of FTIR difference spectra, which will be discussed further below, and show similar pH dependence.

In the following, it has been shown that this pH dependence of Meta III decay reflects a pH-dependent equilibrium of Meta III between two conformations: an active conformation at low pH, which decays by rapid hydrolysis of the retinal Schiff base similar to Meta II, and a stable inactive conformation at high pH, to some extent similar to that of Meta
I. To avoid complication, we will retain the term Meta III for both all-trans 15-syn species and distinguish between both species by specifying for the active low-pH form and the inactive high-pH form.

Fig 3.3: The decay kinetics of Meta III are pH-dependent. (A) For studying Meta III over a broad pH range, Meta III was produced by photochemical conversion of the dark state to Meta II by orange light (>550 nm), followed by photochemical conversion of Meta II to Meta III by near-UV light (395 nm). UV-visible spectra were recorded at pH 5.0 and 10 °C, where the respective photoproduct states were stable during the time required for spectrum acquisition. (B) The decay of Meta III was studied at 30 °C. UV-visible spectra of Meta III
The decay kinetics of Meta III (red crosses, left axis) show a marked pH dependence, which follows a Henderson-Hasselbalch titration curve. The transition from a stable Meta III species at alkaline pH to a rapidly decaying Meta III species at low pH has an apparent pKa roughly around 5.1 at 30 °C. A conformational analysis of FTIR difference spectra Meta III minus dark state (blue dots, right axis), as described in the text, shows a similar pH dependence. (B) Meta III was produced in a membrane suspension by the same protocol as in (A).
photochemical protocol as in (A) at 30 °C in 20 µl of buffer at pH 5.0 (20 mM citrate, 180 mM NaCl), and the pH was then shifted by adding 180 µl of 200 mM citrate buffer (pH 4.0) or of 200 mM MES buffer (pH 7.0). The subsequent decay of Meta III was monitored at t ) 1, 2, 4, and 8 min. By switching the pH, the initially identical Meta III product is converted to the Meta III species with slow decay at more alkaline pH and to the Meta III species with rapid decay at more acidic pH, indicating that both species form a conformational equilibrium.

3.2.3 Low- and High-pH conformations of Meta III form a pH-dependent equilibrium.

Amongst the first thing that was examined was whether these two species can be interconverted by changing the pH. 1 nmol of rhodopsin was illuminated by the same protocol as above to produce Meta III in 20 µL of 20 mM citrate buffer at pH 5.0, added then 180 µL of either 200 mM citrate buffer at pH 4.0 or MES buffer at pH 7.0, and followed the decay kinetics of Meta III by UV-visible spectroscopy. As evident from Figure 3.4 B, the Meta III state produced under identical conditions was converted to the rapidly decaying and to the slowly decaying Meta III form by switching the pH of the sample to more acidic or more alkaline values, respectively.

3.2.4 Low- and high-pH forms of Meta III Have active and inactive receptor conformations.

In order to study the conformational changes involved in the two light reactions from the dark state to Meta II and from Meta II to Meta III, we followed these reactions by FTIR difference spectroscopy (Figure 3.6). In order to avoid the implications of rapid photoproduct decay at higher temperatures, these experiments were performed at 10 °C. Control experiments performed with UV-visible spectroscopy on identical samples as used for FTIR spectroscopy confirm both at pH 6.0 and at pH 4.0 the conversion of the dark state to mostly Meta II by orange light and the subsequent conversion to Meta III by near-UV light (Figure 3.5). The small peaks around 480 nm in the Meta II spectra are due to small contributions of Meta I at pH 6.0 and to pH-induced formation of some Meta II with protonated Schiff base at pH 4.0. The Meta III spectra appear very similar at both pH values
and indicate a protonated Schiff base for both the low- and high-pH form of Meta III.

Figure 3.5. High- and low-pH forms of Meta III were produced at 10 °C at pH 6.0 (left panel) and pH 4.0 (right panel), respectively, by the same photochemical protocol as in Figure 3.3A, involving successive orange (>550 nm) and near-UV (395 nm) illumination steps. The UV-visible spectra of the product (blue spectra), consisting mainly of Meta III, are at both pH values very similar and indicate in particular a protonated Schiff base in both cases.
Fig 3.6 The low-pH form of Meta III adopts an active state conformation. FTIR difference spectra photoproduction minus initial state were recorded for the transitions from the dark state to Meta II (red spectra) and from Meta II to Meta III (blue spectra), induced by the orange and near-UV illuminations, respectively. At both pH 6.0 and pH 4.0, the red spectra of the transition from the dark state to Meta II reveal the conformational changes due to the activation of the receptor, leading to the distinct carboxylic acid band pattern in the range above 1700 cm$^{-1}$ and the pronounced amide I marker band of Meta II at 1644 cm$^{-1}$. The transition from Meta II to Meta III (blue spectra) involves a deactivation of the receptor at pH 6.0, as evident from the reversal of the conformationally sensitive Meta II bands. At pH 4.0, on the other hand, the active state conformation of Meta II is maintained in Meta III, as evident from the absence of this band reversal. The high-pH form of Meta III corresponds therefore to an inactive receptor conformation, while the low-pH form of Meta III has an active state conformation similar to that of Meta II.
The light-induced FTIR difference spectra are shown as photoproduct minus initial state spectra, such that photoproduct bands are positive, while those of the respective initial state are negative. In Figure 3.6, the difference spectra of the first illumination with orange light (red spectra) show both at pH 6.0 and at pH 4.0 the distinct band pattern of the transition from the dark state to Meta II, reflecting the conformational changes going along with receptor activation. These band patterns comprise that of the C=O stretches of protonated carboxylic acids above 1700 cm\(^{-1}\). They include the positive band at 1712 cm\(^{-1}\) due to protonation of Glu113 on transmembrane helix H3, the counterion to the protonated Schiff base in the dark state, as well as a distinct band pattern with positive and negative bands at 1727, 1747, and 1768 cm\(^{-1}\), which reflect hydrogen-bonding changes of Glu122 on transmembrane helix H3 and Asp83 on H2 (Siebert F 1995) due to conformational changes in interhelical hydrogen-bonded networks between H3 and H5 and between H1, H2, and H7, respectively (Menon S at al 2001, Lüdeke S et al 2005) (compare Figure 3.10), and of a lipid molecule (M. Beck at al 1998) There is further a prominent positive amide I marker band of Meta II at 1644 cm\(^{-1}\), reflecting changes of the protein backbone during receptor activation.

At pH 6.0, conversion of Meta II to Meta III by near-UV light (blue spectrum) reverts most of these conformational changes, as evident from the mirror symmetry of the spectra in the range between 1600 and 1800 cm\(^{-1}\). This is in line with previous experiments (Bartl F et al 2001), indicating that the transition from Meta II to Meta III involves a deactivation of the receptor at this pH. The band patterns in the range between 900 and 1400 cm\(^{-1}\), which include in particular vibrational modes of the chromophore, are not mirror symmetric, indicating that not the dark state with its 11-cis 15-anti chromophore is restored with its characteristic fingerprint band at 1238 cm\(^{-1}\) but mainly Meta III with the all-trans 15-syn isomer of the chromophore with characteristic positive bands at 1348 and 1180 cm\(^{-1}\).

At pH 4.0, this reversal of conformationally sensitive bands in the range between 1600 and 1800 cm\(^{-1}\) is not observed in the transition from Meta II to Meta III (blue spectrum). Instead, hardly any changes are observed in the range above 1700 cm\(^{-1}\) of protonated carboxylic acids and at 1644 cm\(^{-1}\) at the position of the amide I marker band of Meta II. This clearly indicates that the active state conformation of Meta II persists in Meta III. In particular, the receptor micro domains between H3 and H5 around Glu122 and between H1, H2, and H7 around Asp 83 are not substantially changed by the transition. Further, Glu113
on H3 remains protonated as in Meta II, and the protein backbone changes responsible for the amide band at 1644 cm\(^{-1}\) in Meta II, which had been tentatively assigned to those associated with relative movement of H3 and H6 (Vogel R et al 2006), also persist in Meta III at this low pH.

At both pH 4.0 and pH 6.0, the near-UV illumination of Meta I/Meta II produces besides Meta III also 11-\textit{cis}-rhodopsin and 9-\textit{cis}-isorhodopsin, which can be quantified by photolysis of these stable products after complete decay of Meta III. At pH 4.0, this contribution is small, amounting to less than 20 % of the total photoproduct and consisting predominantly of rhodopsin. At pH 6.0, this contribution increases to a total of about 45 % of the photoproduct of the near-UV illumination, consisting of about equal amounts of both isomers. This will be important when turning to the high-pH form of Meta III, as will be detailed below. Interestingly, rhodopsin and isorhodopsin appear to be formed not only by a single light isomerization of the C11 or C9 polyene double bond but to some extent also by light isomerization of both the Schiff base C15=N and one of either the C11 or C9 polyene double bonds, followed by a thermal back-isomerization of the C15=N to 15-\textit{anti} on a time scale of few minutes. Similar combinations of consecutive light-induced and thermal isomerizations had been reported previously in the photoreactions of Meta III (Vogel R et al 2001). The low- and the high-pH forms of Meta III, which had been identified above by their distinctly different decay properties, correspond therefore to different protein conformations of Meta III. The stable high-pH form, which is also formed during the thermal decay of Meta I/Meta II at neutral to alkaline pH, corresponds to an inactive receptor conformation, while the low-pH form corresponds to an active receptor conformation similar to that of Meta II, with which it also shares the rapid decay pathway by Schiff base hydrolysis.

\subsection*{3.2.5 E134Q Mutation does not favor the active Meta III conformation.}

In order to study the influence of the E134Q mutation on the conformation of Meta III, the experiments described in the previous section were repeated with the E134Q mutant of rhodopsin reconstituted into PC lipid membranes. As evident from Figure 3.7, the conformational changes of the transition from the dark state to Meta II (red spectrum) are
reverted in the transition to Meta III by the near-UV illumination at pH 6.0 to a similar extent as in the native pigment at the same pH (Figure 3.6). Similar results were obtained for the wild-type pigment in PC lipid membranes, revealing little influence of the specific lipid environment. The E134Q mutation does therefore not significantly extend the pH range in which Meta III is present in its active low-pH form to higher pH values, which bears similarity to other partial agonists (Vogel R et al, 2005 and 2006),

\[ \text{Fig 3.7. The experiments of Fig 3.6 were repeated with the E134Q mutant of rhodopsin reconstituted into lipid membranes. Similar to the native pigment, a deactivation of the receptor is observed in the transition from Meta II to Meta III (blue spectrum) at pH 6.0. The E134Q mutation does therefore not significantly increase the apparent pK}_a \text{ of the Meta III conformational equilibrium.} \]

\[ \text{3.2.6 Analysis of the chromophore in the active Meta III conformation at low pH.} \]

The chromophore marker bands of Meta III are at slightly different positions (1343 and 1177 cm\(^{-1}\)) in the low-pH form of Meta III as compared with Meta III obtained at pH 6.0 or with classical Meta III obtained by thermal decay of Meta I/Meta II at alkaline pH. For classical Meta III, the isomerization of the Schiff base to 15-syn in the transition to Meta III had been revealed using isotopic labeling in combination with a quantum chemical
vibrational analysis (Vogel R et al 2007), which had been reproduced as well for Meta III formed photochemically by near-UV illumination of Meta II at neutral pH (Ritter E et al 2004). In a 15-syn geometry, the NH in-plane bending mode of the Schiff base and the C14–C15 stretching mode of the polyene are kinetically coupled, leading to a downshift of the C14–C15 stretch in H2O and an upshift in D2O (Smith S.O et al 1984). In Meta III with its 15-syn geometry of the Schiff base, the C14–C15 stretch experiences therefore an enormous upshift upon H/D exchange by more than 50 cm\(^{-1}\) (Vogel R et al 2007).

In Figure 3.8, the positions of the chromophore bands are compared for Meta III produced photochemically at pH 4.0 (Figure 3.8B, bands belonging to Meta III being positive) with those of Meta III produced in the thermal decay of Meta I/Meta II (Figure 3.8A, bands belonging to Meta III being negative), both in H2O (red) and in D2O (blue). The band at 1343 cm\(^{-1}\) in the active state conformation of Meta III at pH 4.0 in H2O is the NH bending mode of the Schiff base, which is slightly downshifted from 1349 cm\(^{-1}\) in inactive thermal Meta III obtained at pH 8.0 and which disappears in D2O. The band at 1177 cm\(^{-1}\) is the C14–C15 stretching mode, which had been assigned using \(^{13}\)C-labeling of C14 and C15 of the retinal (not shown) and which is as well slightly downshifted as compared with the inactive Meta III form at alkaline pH. Upon H/D exchange, the C14–C15 stretch in the low-pH form of Meta III shows the same \(\approx 50 \text{ cm}^{-1}\) upshift as in the high-pH form. In the high-pH form, the C14–C15 stretch appears to be split in D2O into two bands at 1247 and 1236 cm\(^{-1}\), which had been proposed to be due to an interfering Meta II protein band of opposite sign (Vogel R et al 2007). In the low-pH form of Meta III, which shares the active state conformation of Meta II, this interfering band is lacking, such that the C14–C15 stretch appears in D2O as a single band at 1238 cm\(^{-1}\). This confirms the implicit assumption that low- and high-pH forms of Meta III share the same 15-syn chromophore geometry.
Figure 3.8 The active state conformation of Meta III shares the all-trans 15-syn chromophore geometry of classical Meta III. (A) Classical (thermal) Meta III, which had been produced by thermal decay of Meta I/Meta II at 30 °C and pH 8.0, was photo converted back to Meta I/Meta II, producing a Meta I/Meta II minus Meta III difference spectrum (bands of thermal Meta III being negative). This difference spectrum reveals in H$_2$O (red spectrum) the characteristic chromophore bands of the 15-syn chromophore of Meta III, the NH bending mode of the Schiff base at 1349 cm$^{-1}$, and the C14–C15 stretching mode of the polyene at 1181 cm$^{-1}$, of which the NH bending mode disappears in D$_2$O (blue spectrum), while the C14–C15 stretch shows a pronounced upshift to a doublet at 1247/1236 cm$^{-1}$ (Vogel et al 2003). (B) Meta III minus Meta II difference spectra of the photochemical production of Meta III by near-UV illumination of Meta II at pH 4.0 (Meta III bands being positive) reveal a very similar band pattern with NH bending and C14–C15 stretching modes slightly downshifted to 1343 and 1177 cm$^{-1}$, respectively, in H$_2$O, while the C14–C15 doublet in D$_2$O is replaced by
a single band at 1238 cm\(^{-1}\). This confirms that the low-pH active Meta III form has indeed the same all-trans 15-syn chromophore isomer as classical inactive Meta III.

### 3.2.7 Analysis of active and inactive conformations of Meta III.

As shown above in Figure 3.6, the active Meta III conformation corresponds to that of Meta II and features in particular a protonated Glu113, a similar hydrogen bonding of Asp83 and Glu122 in the H1/H2/H3 and the H3/H5 microdomains, respectively, and a similar structure of the protein backbone.

For a characterization of the inactive high-pH form of Meta III, we will not use the photochemical protocol for generation of Meta III, as this protocol produces in particular at alkaline pH also substantial amounts of rhodopsin and isorhodopsin, which might interfere with a structural analysis. We therefore generated Meta I/Meta II by orange illumination of rhodopsin in disc membranes at 30 °C and pH 8.0 (conditions under which this photoproduct equilibrium is largely on the Meta II side) and let this photoproduct equilibrium decay completely over 20 min by the thermal decay pathways to Meta III (in its inactive high-pH conformation) and opsin. Meta III was then selectively photolyzed for 20 s by a >475 nm long pass filter, which converted it back to Meta I/Meta II, as described before (Vogel et al 2006 and Ritter E et al 2004). All of these reactions were followed by FTIR and UV-visible spectroscopy, yielding two light-dependent difference spectra: the Meta I/Meta II minus dark state spectrum of the first illumination and the Meta I/Meta II minus Meta III difference spectrum of the second illumination. Importantly, the position of the Meta I/Meta II photoproduct equilibrium does not depend on the initial state and is therefore identical in both difference spectra. We could now subtract the latter difference spectrum of the second illumination from that of the first illumination, such that contributions of Meta I/Meta II cancel out, ending up with a Meta III minus dark state difference spectrum. The spectrum of the second illumination is, however, substantially smaller than the spectrum of the first illumination, as only part of the initial Meta I/Meta II photoproduct of the first illumination decays to Meta III. We therefore derived a normalization parameter for the subtraction by analyzing the amplitude of the 380 nm photoproduct peak of Meta II in the two light reactions by UV-visible spectroscopy. Under our experimental conditions, this normalization parameter was found to be 35–40 %, corresponding to the contribution of the Meta III pathway in the
thermal decay of Meta I/Meta II.

Using this scaling factor, a Meta III minus dark state difference spectrum is obtained that can be compared to a Meta I minus dark state difference spectrum for comparison of the protein conformation in these two inactive product species (Figure 3.9). The Meta III minus dark state spectrum is shown with two limiting scaling factors in light blue and dark blue, corresponding to a 35 % and 40 % yield of Meta III in the thermal decay, respectively. Glu122 experiences an increase of its hydrogen bonding in Meta I as compared to the dark state, leading to a difference band with absorption at 1735 and 1728 cm⁻¹ in the dark state and a shoulder at the high-frequency side of the peak at 1704 cm⁻¹ in Meta I. In the inactive Meta III state, the absorption of Glu122, which is positioned close to the ring of retinal (Figure 3.10), is upshifted toward 1741 cm⁻¹, indicating a weakening of its hydrogen bonding in inactive Meta III and a perturbation of the H3/H5 network different from that in Meta I. Also, the absorption change of Asp83 is in Meta III hardly changed as compared to the dark state, while a small but distinct negative absorption peak is produced in the transition to Meta I at 1769 cm⁻¹. Further, the amide I band of Meta I at 1664 cm⁻¹ is absent in inactive Meta III.

Fig 3.9 An FTIR difference spectrum Meta I minus dark state (gray spectrum), obtained at
10 °C and pH 9.0, is compared to difference spectra of inactive high-pH Meta III minus dark state (blue spectra). The latter spectra were obtained at 30 °C and pH 8.0 from Meta III produced in the thermal decay of Meta I/Meta II and show two limiting cases, corresponding to 40 % (dark blue) and 35 % (light blue) yield of Meta III in the thermal decay process (see text for details). The high-pH form of Meta III shows the general features of an inactive receptor conformation with some distinct differences to Meta I in the range above 1700 cm\(^{-1}\) due to changed interactions of Asp 83 in the H1/H2/H7 microdomain and Glu122 in the H3/H5 microdomain.

Fig 3.10 A structural model of the retinal binding pocket with the protonated retinal Schiff base (PSB) and Glu113 on H3, the H3/H5 microdomain with Glu122, and the H1/H2/H7 microdomain with Asp83 is shown, which is based on the coordinates 2HPY (Nakamichi H et al 2006) of the inactive Lumi conformation with its all-trans 15-anti chromophore (the cytoplasmic side points upward).
The pH dependence of the Meta III conformation, which was characterized in Figure 3.4A already by monitoring the decay kinetics, was also studied by FTIR spectroscopy by producing Meta III by the photochemical protocol with orange and near-UV illumination at a series of pH values. This approach is somewhat hampered by the side reactions in the near-UV illumination step, which produce besides Meta III also rhodopsin and isorhodopsin in a pH-dependent manner, as reported above. Nevertheless, the obtained spectra can be evaluated to obtain a rough estimate of the apparent pK_a of the conformational equilibrium of Meta III, using the conformationally most sensitive spectral range between 1800 and 1600 cm⁻¹ of the FTIR spectra as described previously (Vogel R et al 2005). This analysis indicates a pK_a of the transition roughly around 4.7 at 10 °C and 5.1 at 30 °C. The data obtained at 30 °C are shown in Figure 3.4A in blue and are in agreement with the pH dependence of the decay kinetics presented in the same graph in red.

3.3 Discussion

Meta III is formed during the thermal decay of the Meta I/Meta II photoproduct equilibrium at neutral to alkaline pH in parallel to decay of Meta II by hydrolysis of the retinal Schiff base. Meta III can likewise be produced photochemically by near-UV illumination of Meta II. In both cases the transition to Meta III is happens by an isomerization (either thermal or light-induced) of the Schiff base C15=N double bond leading to an all-trans 15-syn isomer of the chromophore. In this study, we have shown that Meta III exists in a pH-dependent conformational equilibrium between active and inactive conformations similar to the Meta I/Meta II photoproduct equilibrium, however, with an apparent pK_a that is considerably downshifted into the range around 5.1. The inactive Meta III conformation formed at neutral to alkaline pH is stable and corresponds despite some distinct differences to a Meta I-like conformation. The active conformation of Meta III formed at pH values below the pK_a, on the other hand, corresponds to that of Meta II, with conformational rearrangements of receptor microdomains, protonation of Glu113, and decay properties similar to that of Meta II. The Schiff base in this active form of Meta III remains, however, protonated, at least in the low-pH range in which this receptor conformation is stabilized.
Also, the apoprotein opsin forms in the absence of a ligand a conformational equilibrium between active and inactive conformations. This equilibrium is as well pH-dependent (Vogel R et al 2006), indicating that the pH dependence of rhodopsin's photoprotein conformational equilibria is an intrinsic property of the receptor protein (Cohen G et al 1992). The presence of a ligand in the retinal binding pocket merely modulates the position of this intrinsic equilibrium by specific ligand-protein interactions, which alter the energetics of the associated conformational states and thereby the apparent proton affinity (Vogel R et al 2006). A shift of the position of the equilibrium is therefore synonymous to changing the apparent pKₐ of the equilibrium. In the case of the Meta III conformational equilibrium, the apparent pKₐ is at 30 °C around 5.1 and thus higher than that of opsin in the absence of ligand, which was found to be 4.1, but lower than that of Meta I/Meta II in the presence of the full agonist all-trans-15-anti-retinal, where the apparent pKₐ is above 8. As compared with the full agonist all-trans-15-anti-retinal, the all-trans 15-syn ligand of Meta III is therefore a classical partial agonist. In the thermal decay of the Meta I/Meta II equilibrium to Meta III at neutral pH, the thermal isomerization of the Schiff base C15=N double bond is therefore sufficient to switch the receptor from an active to an inactive conformation. Such an isomerization is, however, not sufficient to completely lock the receptor in an inactive conformation, as inverse agonists like 11-cis or 9-cis ligands would do.

How does the C15=N isomerization to 15-syn achieve this switch from a full to a partial agonist? Recent solid state NMR studies revealed a longitudinal movement of retinal toward H5 during receptor activation (Patel A et al 2004). The β-ionone ring of retinal had been shown to interact closely with the interhelical network between Glu122 and His211 on TM3 and TM5, respectively (Figure 4B). This key interaction couples allosterically the disruption of the salt bridge between protonated Schiff base and Glu 113 on TM3 in the transmembrane core of the receptor with proton uptake by a cytoplasmic microdomain between H3 and H6 around Glu134 (Vogel R et al 2006). Partial or complete removal of retinal's β-ionone ring weakens this key interaction, uncouples the Schiff base and the cytoplasmic microdomains, and renders receptor activation inefficient. This uncoupling becomes obvious in a deficiency of the E134Q mutation to counteract inefficient receptor activation by acyclic or 9-demethyl partial agonist ligands (Vogel R et al 2006), which is observed similarly here for the all-trans 15-syn partial agonist.
In the all-trans 15-anti full agonist, the chromophore maintains its extended geometry of the polyene beyond the Schiff base C15=N double bond to the ε-carbon of Lys 296 (Figure 4B), before the lysine side chain turns sharply in the direction to the cytoplasmic side, as suggested by the published structure of lumirhodopsin (Nakamichi et al 2006). In this structure, the ε-carbon of Lys 296 is docked between Met 44 on H1, Phe 91 on H2, and Phe 293 on H7. The all-trans 15-anti chromophore represents therefore a quite rigid linker connecting the H1/H2/H7 domain at one end close to the Schiff base with the ring binding pocket and the H3/H5 domain.

In the all-trans 15-syn isomer of Meta III, this fully extended geometry is lost, and the effective length of the chromophore becomes shorter. This shortening of the rigid part of the chromophore could impair receptor activation by weakening tight interactions between the ring and H3/H5 microdomain at the opposite end. Such a change in interaction is seen in the altering of the hydrogen bonding of Glu 122 in this microdomain (Figure 3.10). In line with such an effective shortening of the ligand, the network between H1, H2, and H7 at the Schiff base end of the ligand appears to be unperturbed by the all-trans 15-syn chromophore as compared with the dark state, while the extended all-trans 15-anti geometry of the full agonist leads to a small, but distinct signature in the absorption of Asp83 (H2) in the Meta I minus dark state difference spectrum (Figure 3.9).

Comparison of inactive Meta III and Meta I. Obviously, the partial agonist behavior of the all-trans 15-syn ligand in Meta III could further be expected to be influenced considerably by the reorientation of the protonated Schiff base in the 15-syn isomer. However in Lumi, the farthest evolved photoproduct state of which an atomic resolution structure is available up to now, Glu 113 is oriented to the side of the polyene plane (Nakamichi et al 2006) (Figure 3.10), such that the influence of the isomeric state of the C15=N double bond on a Protonated schiff base–Glu 113 salt bridge could in fact be rather marginal. This situation might be somewhat changed again in Meta I or in the inactive Meta III conformation, as retinal's motion seen in the transition to Meta II (Patel A et al 2004) might be anticipated to some extent already in these inactive conformations and as the influence of Glu 181 on extracellular loop 2 to the counterion function would become stronger (Lüdeke S et al 2005), (Yan E:C et al 2003).
The general principle of a minimal effective length of the ligand required for maximal activation of the receptor is likely also relevant for other partial agonists that have, for instance, deletions at the β-ionone ring at the opposite end of the ligand. As mentioned above, deletions of ring methyl groups or of the closed ring structure of retinal severely impaired receptor activation and rendered the ligands classical partial agonists (Vogel et al 2005). The partial agonism of these acyclic or ring-demethylated retinal analogues could be at least partially caused by an effective shortening of the length of the ligand similar to that of the 15-syn ligand of Meta III. Of course, the precise molecular interactions at the ring binding pocket interface are different for the ring-modified ligands as compared with the Meta III ligand. This is evident from the entirely different hydrogen-bonding pattern of Glu122 (Fig 3.10), showing an extremely strong hydrogen bonding in the inactive conformation of the acyclic analogues, which is not seen in the inactive Meta III state. Nevertheless, we observe with both ligand systems a similar shift of the conformational equilibrium to the inactive side going along with the characteristic breakdown of the allosteric coupling between the Schiff base region and the cytoplasmic H3/H6 microdomain around Glu134. This suggests that a shortening of the effective length of the ligand could be a common mechanism for partial agonism that is shared by both the acyclic ligands and the all-trans 15-syn ligand of Meta III.

In summary, results show that the 15-anti to 15-syn isomerization of the all-trans chromophore Schiff base in the thermal decay from Meta I/Meta II to Meta III accomplishes a partial deactivation of the receptor by switching the ligand from a full agonist to a partial agonist. An effective shortening of the ligand could be the molecular mechanism behind this functional switch. This switch leads to a silencing of the receptor at neutral to alkaline pH that does, however, not restore the complete, pH-independent inactivity of the dark state. Complete deactivation of the receptor is achieved only after release of all-trans-retinal from its binding pocket and binding of 11-cis-retinal supplied by the visual cycle, which act as an inverse agonist locking the receptor in its inactive conformation.
4. Functional role of the ionic lock during the activation of Rhodopsin

4.1 Introduction

Activation of rhodopsin which is triggered by the isomerization of the 11-cis retinal is known to involve significant helix motion. In particular, a movement of the cytoplasmic end of transmembrane helix 6 relative to helix 3 outward of the helix bundle is one of the hallmark event leading to the active Meta II state. This movement is thought to open up a cleft in the cytoplasmic domain of rhodopsin which is a part of G protein binding site.

In the dark state crystal structure of rhodopsin there is an interhelical hydrogen bond network between the ERY motif on transmembrane helix 3 and the residues on helix 6 (also conserved in family A G protein coupled receptors) which is commonly termed as ionic lock. Activation of Rhodopsin is known to involve rearrangement of this ionic lock. The transition from Meta I to Meta II is pH dependent, indicating proton uptake as a determining step of the conformational change. Mutagenesis studies have suggested that the residue Glu134 in H3 is involved in the proton-uptake reaction (Fahmy K et al 1993) (Fig 4.1). Glu134 is part of the E(D)RY motif at the interface between H3 and cytoplasmic loop 2 (Fig. 1), which is conserved within family A G-protein coupled receptors (G-PCRs) and controls receptor activation and interaction with G protein. Replacement of Glu134 in helix 3 by a neutral glutamine was shown to abolish proton uptake during formation of the active state and to extend the pH range of efficient interaction with G protein into the alkaline range. In the crystal structures of the dark state, Glu134 forms an intrahelical salt bridge with neighboring Arg135, (Li J et al 2004) which maintains further interhelical interactions with Glu247 on H6.
Fig 4.1 Molecular model of the cytoplasmic domain of the dark state of rhodopsin. In rhodopsin, Arg135 of the conserved E(D)RY motif on H3 forms an intrahelical salt bridge to neighboring Glu134 and interacts closely with Glu247 on H6, while Glu249 on H6 interacts with the backbone NH of Lys311 at the kink from H7 to the small cytoplasmic helix 8. The models and the distances (in Angstroms) are based on the coordinates 1GZM. The cytoplasmic side is toward the top. H1, H2, and H4 have been omitted for clarity.

The transition to the active state of rhodopsin also appears to change the H6/H7 interface, as shown by the exposure of the cytoplasmic terminus of H7 to a specific antibody in Meta II. (Abdulaev et al 1998). In the crystal structures of the dark state, we identified Glu249 on H6 to interact with the backbone NH of Lys311 at the kink from H7 to cytoplasmic helix 8 (Fig. 4.1). In the β2AR, the homologous residues Lys270 and Asp331 are switched but do not appear to interact in the β2AR structure (Fig. 4.10). Here the functional roles of these putatively ionic interactions during receptor activation is investigated using Fourier transform infrared (FTIR) difference spectroscopy on site-directed mutants of rhodopsin reconstituted into lipid bilayers. The incorporation of the purified receptors into a
native-like environment appears critical, as the detergents commonly used for purification of rhodopsin, such as the neutral detergent DDM (n-dodecyl-β-D-maltoside), alter the energetics of the receptor activation pathway considerably and abolish, in particular, the native pH dependence of the transition from inactive Meta I to active Meta II. Some of the conclusions are discussed in context to structural data of the β2 adrenergic receptor.

4.2 Results

4.2.1 Expression of the rhodopsin mutants

Mutants E134Q, R135L, E247Q, and E249Q were expressed, regenerated with 11-cis retinal, purified in n-octyl β-D-glucoside (OG), and reconstituted into phosphatidyl choline (PC) lipid membranes. All mutants were stable and could be expressed at a yield similar to that of wild-type pigment, except for R135L, where the yield was reduced to 25 %. For all mutants, the position of the dark-state visible absorption peak was unchanged from that of wildtype pigment.

4.2.2 Glu134 is the proton-uptake group responsible for pH-dependent receptor activation

Fig 4.2 shows light-induced FTIR difference spectra “photoproduct minus dark state” obtained from wild-type rhodopsin at 0 °C for the transition to the inactive Meta I conformation at pH 8.6 and to active Meta II at pH 5.1 with their characteristic marker bands.(vogel et al 2005). Characteristic Meta I bands include an amide I band at 1662 cm⁻¹, a chromophore fingerprint band at 1200 cm⁻¹, and the pronounced C11=C12 hydrogen out-of-plane band of the chromophore at 952 cm⁻¹. The Meta II difference bands in the range of the C=O stretch mode of protonated carboxylic acids between 1700 and 1780 cm⁻¹ have been assigned previously by mutational analysis.(Fahmy et al 1993, Jäger F et al 1994, Rath P et al 1993,). The band pattern can be decomposed into a 1768 (−)/1749 (+) cm⁻¹ difference band due to the downshift of the C=O stretch of Asp83 during the transition from the dark state to
Meta II, a 1728 (−)/1745 (+) cm\(^{-1}\) difference band reflecting an upshift of Glu122, and positive band at 1712 cm\(^{-1}\) due to protonation of Glu113. Superimposed on the dominant band pattern is a 1727 (−)/1744 (+) cm\(^{-1}\) difference band due to an absorption change of a lipid ester carbonyl.

By decomposing difference spectra obtained as a function of pH into a linear combination of Meta I and Meta II reference spectra, (Vogel R et al 2005) we can plot the pH dependence of formation of the active Meta II state, which follows a regular Henderson–Hasselbalch function (Fig. 4.3). A recent study using UV–visible spectrometry suggested an additional titration step of the rhodopsin photoproducts in the alkaline pH range involving a deprotonation of the retinal Schiff base in Meta I, which is not observed in this study.
Fig 4.2 FTIR difference spectra, photoproduct minus dark state (dark-state bands are negative, while photoproduct bands are positive), obtained from wild-type rhodopsin in PC membranes at 0 °C reveal the pH dependence of the Meta I/Meta II equilibrium due to uptake of a proton from the cytoplasm, with Meta II being formed at acidic pH (red) and Meta I at alkaline pH (blue), each with its characteristic band pattern. The E134Q mutant in PC membranes, on the other hand, forms a Meta II photoproduct state irrespective of pH.

Fig 4.3 Titration curves showing the fraction of the active Meta II conformation in the photoproduct equilibrium follow, in the case of wild-type rhodopsin, a regular Henderson–Hasselbalch function with an apparent pKA of 6.3, but not for the E134Q mutant. The pH dependence of the Meta I/Meta II equilibrium due to cytoplasmic proton uptake is abolished in the E134Q mutant of rhodopsin.
In contrast to wild type, difference spectra of the E134Q mutant reveal formation of the Meta II photoproduct state at both acidic and alkaline pH (Fig. 4.2 and 4.3). This pH-independent formation of Meta II persists down to -10 °C. Only at -20 °C and below, where the transition to Meta II is thermally blocked, is a Meta I state stabilized—irrespective of pH—that is virtually identical to that of wild type (Fig. 4.4). The Meta II difference spectrum of the E134Q mutant is similar, although not identical to that of wild type, showing several small but distinct deviations (Fig. 4.4b). These can be extracted in the double difference spectrum “wild type minus E134Q” shown in Fig. 4.4c. Besides several bands in the range between 1600 and 1700 cm⁻¹—of which the bands at 1664 and 1693 cm⁻¹ possibly include the amine C=O stretch of Gln134 in the dark and in Meta II of the mutant—there is a distinct triad of bands marked in blue, which is the signature of a protonation change of a carboxylate in wild type that is absent in the mutant. This protonation signature together with the absence of a pH-dependent Meta I/Meta II equilibrium indicates that Glu134 in wild type is deprotonated in the dark state, showing the symmetric and the antisymmetric COO− stretch at 1394 and 1562 cm⁻¹, respectively. Glu134 becomes protonated in Meta II with the C=O stretch absorbing at 1713 cm⁻¹ (Barth et al 2000 for amino acid side-chain absorption properties). The position of the Glu134 C=O stretch in Meta II at 1713 cm⁻¹ coincides with that of Glu113, which becomes likewise protonated in Meta II by internal proton transfer from the protonated Schiff base. (Jäger F et al 1994) and indicates a carboxylic acid with one strong or two weaker hydrogen bonds. (Nie B et al 2005)
Fig. 4.4  Glu134 in the ERY motif is the cytoplasmic proton acceptor in the transition from Meta I to Meta II. FTIR difference spectra of wild type (gray) and the E134Q mutant (black) are essentially identical for the transition from the dark to the inactive Meta I state (cryotrapped at −20 °C, upper spectra), and similar, although not identical, for the transition
to Meta II (0 °C, middle spectra). Instead, the double-difference spectrum wild type minus E134Q (lower spectrum) reveals the signature of protonation of Glu134 in wild type with characteristic bands at 1714 (+), 1562 (−), and 1394 (−) cm⁻¹ marked in blue. Other differences are due to Gln134 in the mutant (green) or reflect other alterations introduced by the mutation.

To detect this protonation signature of Glu134 between 1750 and 1350 cm⁻¹, MES (2-(Nmorpholino) ethanesulfonic acid), which has no interfering protonation-sensitive bands in this range, was used as buffer. MES has, however, protonation sensitive bands in the range between 1300 and 1000 cm⁻¹, which overlap with the dark-state chromophore band at the 1238 cm⁻¹ band normally used for spectral normalization (Fig 4.5). We accounted for this interference of the MES buffer with this receptor marker band at 1238 cm⁻¹ by measuring Meta II minus dark state spectra of wild-type rhodopsin in two different buffers (MES and citrate) to extract the deprotonation band pattern of the buffers. These buffer deprotonation bands were largely reduced or absent in the corresponding spectra of the E134Q mutant, indicating the implication of this mutation in the proton-uptake reaction in the transition to Meta II. These buffer deprotonation band patterns were further confirmed by adding NaOH to pure buffer solutions in supplementary experiments using an attenuated total reflection FTIR setup and allowed for a buffer independent normalization using the dark-state chromophore band at 1238 cm⁻¹ (Fig 4.5). Alternatively, we used the dark-state band of Asp83 at 1768 cm⁻¹ for normalization, yielding consistent results.
Figure 4.5: Buffer deprotonation bands. (A) Band patterns due to protonation changes of the buffer molecules were determined using a diamond plate micro ATR unit (Sens-IR, Danbury, CT, USA) by forming the difference between spectra recorded before and after adding 5 μl aliquots of 1 M NaOH to 200 μl of 200 mM MES (red) or citrate buffer (blue) at
pH 5.0 (bands of the protonated buffer are negative and of the deprotonated buffer positive). These spectra were corrected for the ATR-specific wavelength-dependence of the penetration depth to make them compatible with spectra obtained in transmission mode.

(B) Meta II minus dark state difference spectra of wildtype rhodopsin obtained in transmission mode in MES (red) and citrate buffer (blue) were recorded under corresponding conditions, revealing slight buffer-dependent alterations. (C) The double difference spectrum of the buffer deprotonation spectra citrate minus MES from panel A is shown in green and is compared to the corresponding double difference spectrum obtained from the Meta II minus dark state difference spectra of wildtype rhodopsin from panel B (in black). In particular the difference bands due to deprotonation of the MES buffer between 1000 and 1300 cm\(^{-1}\) are well reproduced in the black spectrum and allow to account for the contribution of the buffer to the absorption change at 1238 cm\(^{-1}\) for spectral normalization. Alterations in the range around 1650 and 1550 cm\(^{-1}\) possibly reflect altered amide I and II difference bands of the protein in the presence of the two buffer molecules. Note that the dark state band of Asp83 at 1768 cm\(^{-1}\) is free from buffer contributions. In comparison to the double difference spectrum obtained from wildtype rhodopsin, the buffer deprotonation band pattern is largely absent in the corresponding double difference spectrum obtained from mutant E134Q (gray spectrum), in agreement with a reduced or absent net proton uptake by the mutant.

4.2.3 Characterization of the R135L mutant

In the R135L mutant, the positively charged side chain of the arginine was replaced by a neutral leucine of approximately the same volume. Similar to the E134Q mutant, the pH-dependent Meta I/Meta II equilibrium was abolished in R135L in favor of Meta II (Fig. 4.6). The Meta II minus dark state difference spectrum of the mutant reveals several significant alterations as compared with wild type and E134Q (Fig. 4.7). The amide I marker band at 1644 cm\(^{-1}\), which had been previously shown to reflect structural changes specifically associated with the Glu134 proton uptake in a pigment analogue, (Vogel R et al 2006) is considerably reduced in R135L. The reduced intensity of this amide I band is observed similarly in D2O, excluding compensating effects of superimposed
bands of the arginine guanidyl group, which otherwise would lead to strong deuteration-induced shifts. (Barth A 2000) In the absence of the positive charge of the arginine side chain as a countercharge, Glu134 is possibly already protonated in the dark state, which is in agreement with the similarity of the Meta II difference spectrum of R135L to that of E134Q in the range of the protonation-sensitive bands at 1394 (−) and 1713 (+) cm⁻¹. In this scenario, one would still expect to observe a difference band of Glu134 in the range above 1700 cm⁻¹ if it undergoes a change of hydrogen bonding in the transition from the dark state to Meta II.

![Graph showing pH vs. active conformation for wildtype and R135L](image)

*Fig 4.6 Similar to the E134Q mutant, R135L forms Meta II irrespective of pH.*

Compared with wild type (and also E134Q), the positive absorption at 1747 cm⁻¹ is substantially reduced and the negative absorption at 1728 cm⁻¹ is slightly smaller. While the latter might be caused by Glu134, the alteration at 1747 cm⁻¹ is at a position that appears too high for a carboxylic acid close to the cytoplasmic interface. The apparent reduction of the 1747 cm⁻¹ band in the mutant could instead be associated with a smaller spectral shift of Glu122 in the membrane-embedded part of H3 during activation of the R135L mutant than in wild type, (Fahmy et al 1993) or alternatively indicate the reduction or

Fig 4.7 Neutralization of Arg135 in the ERY motif distorts the Meta II conformation and abolishes proton uptake. The Meta II minus dark state difference spectrum of the R135L mutant (blue) obtained at pH 5.1 is compared to corresponding spectra of wild type (gray) and the E134Q mutant (red) and reveals substantial differences regarding the amide I marker band of Meta II at 1644 cm\(^{-1}\) and a C=O stretch absorption at 1747 cm\(^{-1}\).
4.2.4 Characterization of the E247Q and E249Q – These mutations favor Meta II but maintain a pH-dependent Meta I/Meta II equilibrium

The Meta II minus dark state difference spectra of E247Q and E249Q mutants show no substantial alterations from that of the wild-type spectrum (Fig. 4.8). The similarity of the spectra in particular in the range above 1700 cm\(^{-1}\) indicates that both groups are present as carboxylates in both the dark and the Meta II state, which is in agreement with their position at the protein solvent interface. In contrast to E134Q and R135L, both H6 mutants maintain a pH-dependent Meta I/Meta II equilibrium at 0 °C, which is, however, shifted to the side of the active receptor conformation as compared with wild type. This shift of approximately 1.5 pKA units corresponds to a shift of the equilibrium constant by a factor of 30. (Fig 4.9) Pure Meta I states of the two mutants could not be stabilized at 0 °C due to the pronounced upshift of the apparent pKA values of the titration curves. Meta I states stabilized at -20 °C were also similar to those of wild type.
Fig 4.8 Meta II minus dark state difference spectra of E247Q (red) and E249Q (blue) mutants are very similar to the corresponding spectrum of wild type (gray).

Fig. 4.9. Neutralization of Glu247 and Glu249 involved in interaction of H6 with H3 and H7, respectively, favors active Meta II, but does not abolish its pH dependence. Both E247Q and E249Q reveal a pH-dependent Meta I/Meta II equilibrium with a pKa value that is upshifted approximately 1.5 units compared with wild type (gray). With the mutant data, the pH points plotted here were less because of the limitations on the availability of the sample.

4.3 Discussions

Relevant site directed mutants of rhodopsin were engineered and studied pH dependent activation using FTIR difference spectroscopy to test and refine the ionic lock hypothesis of G-PCR activation. The mutant pigments were expressed, purified, and reconstituted into PC lipid membranes to maintain conditions similar to those in the native membrane environment. The structural sensitivity of FTIR spectroscopy is exploited to monitor directly the conformational equilibrium between agonist bound inactive Meta I and active Meta II states, which allows to separate the effects of the mutations on the conformation of the receptor from their potential impact on binding and activation of G protein or other downstream effectors.
Glu247 and Glu249 on H6, which maintain interhelical interactions with H3 and H7, respectively (Fig. 4.1), were examined in the mutants E247Q and E249Q. As shown in the results both mutants maintained Meta I and Meta II states that were essentially identical to those of wild type. The equilibrium between both states was, however, shifted to the active receptor conformation by a factor of approximately 30, corresponding to an upshift of the pKA value of the pH dependent titration curve by roughly 1.5. Weakening of these interhelical interactions therefore facilitates the transition to the active receptor conformation Meta II, showing that these constraints need to be broken during activation of the wild-type receptor as suggested earlier in the following published articles. (Sheikh S.P et al 1999, Farrens D et al 1996 and Janz J et al 2004).

Glu134 and Arg135, on the other hand, are part of the conserved E(D)RY motif on H3 and form an intrahelical salt bridge in the crystal structures of the dark state. Neutralization of either residue in the E134Q and R135L mutants abolished the pH dependence of the transition from Meta I to Meta II by shifting the equilibrium completely to the side of Meta II. In agreement with previous proton-uptake studies, we show that Glu134 becomes protonated in this transition by the lack of the respective protonation-sensitive bands of a carboxylate in the E134Q mutant. The position of the C=O stretch of protonated Glu134 in Meta II at around 1713 cm⁻¹ indicates that this residue is well hydrogen-bonded in the active receptor state. Aside from these protonation-dependent alterations, the Meta II state shows no substantial conformational alterations due to the mutation. The conformations of the Meta I state and other inactive precursor states, such as Batho and Lumi, were also unchanged. This result seems to contradict a previous spin-labeling study that reported a partially activated conformation of the dark state in the E134Q mutant. (Kim J et al 1997). Possibly, conformational alterations sensed by the spin labels in the cytoplasmic domain in the E134Q mutant have no detectable signature in vibrational spectra. On the other hand, the spin-labeling study was performed with rhodopsin solubilized in DDM detergent, while the present study employed a more physiological membrane environment, which might better stabilize a native-like protein conformation of the dark state in the absence of a salt bridge between Glu134 and Arg135.

Also these results show that the cytoplasmic ionic lock, which stabilizes the inactive receptor conformation, consists primarily of the charge pair Glu134/Arg135 of the E(D)RY motif on H3. Neutralization of either residue abolishes the protonation
dependence of receptor activation. The E134Q mutation appears to have considerable energetic effect, but only small structural impact, on the Meta I/Meta II equilibrium, altering substantially the position of the equilibrium but not the structure of the endpoint species. The R135L mutation, however, affects both energetics and structure. Compared with the impact of Glu134 or Arg135 mutations, neutralization of either Glu247 or Glu249 has much less pronounced effects, albeit still with forward shifts of the equilibrium toward Meta II by a factor of 30. Although the interactions between both Glu249 and the backbone of H7, and between Arg135 and Glu247, appear to be broken during activation of rhodopsin, the decisive step is the disruption of the intrahelical salt bridge at the cytoplasmic terminus of H3.

The complete forward shift of the Meta I/Meta II equilibrium of rhodopsin to Meta II by the E134Q and R135L mutation is in agreement with previous studies using rhodopsin mutants purified in digitonin detergent,(Weitz C et al 1993) while the influence of the H6 mutations on the equilibrium have not been studied before in rhodopsin. Similar mechanisms as observed here for rhodopsin appear to govern activation of other rhodopsin-like G-PCRs. In the β2AR, replacement of Asp130 on helix 3 by Asn and Glu268 on helix 6 by Gln increased the partial agonist-dependent receptor activation, with the effect of Asp130 on helix 3 mutation being considerably more pronounced than that of Glu268 mutation.(Ballesteros J.A et al 2001) The effect of mutation of Glu on helix 3 by Ala had been tested in dose–response curves of agonist-dependent receptor stimulation of the 5HT2A serotonin receptor and the muscarinic acetylcholine Hm1 receptor, revealing a 4.5- and 6-fold higher agonist potency, respectively. (Hogger P et al 1995, Shapiro D et al 2002). A similar shift was observed in the α1b adrenergic receptor by mutation of Glu289 on helix 6 to Ala, but not to Gln, (Greasley P et al 2002) indicating that a Gln can at least partially compensate for Glu.

These results are consistent with the structure of the Fab-stabilized human β2AR bound to the inverse agonist carazolol solved recently from microcrystals grown in lipid/detergent bicelles. (Rasmussen et al 2007). In the Fab-β2AR structure, Asp130 and Arg131 form a similar intrahelical salt bridge as in the inactive dark state of rhodopsin, with a distance of 3.1 Å that is only slightly larger than that of rhodopsin, despite the shorter side chain of the Asp residue as compared with Glu in rhodopsin (Fig. 4.10). The distance between Arg131 and Glu268, on the other hand, is increased to 6.4 Å as compared with 2.9 Å in
rhodopsin. In addition, Lys270 on helix 6 and Asp331 on helix 7 appear not to interact in contrast to the homologous, switched pair Glu249 on helix6 and Lys311 on helix 7 in the dark state of rhodopsin. The altered packing between H3 and H6 might relate to the conformation of the intracellular loop 2 (IC2), which extends to the cytoplasm in rhodopsin, but is folded back into the helix bundle in the β2AR structure (Fig. 4.10). As IC2 in the β2AR makes several crystal contacts with the constant region of the Fab antibody fragment used in the crystallization, this altered conformation of IC2 possibly reflects crystal packing effects. The altered conformation of IC2 positions the side chain of Tyr141 between Arg131 and Glu268 and brings Gln142 on IC2 within 3 Å of Arg131, allowing for efficient hydrogen bonding and directing Arg131 away from H6. An asparagine at a similar position in rhodopsin, Asn145, does not make this interaction, but is exposed to the solvent due to the different conformation of IC2.

Fig 4.10 In the molecular model of the β2AR, the intrahelical salt bridge between D130 and R131 is preserved, while the interhelical interactions between homologous residues on H3, H6, and H7 appear to be broken. The models and the distances (in angstroms) are based on the coordinates 2R4R. The cytoplasmic side is toward the top. H1, H2, and H4 have been omitted for clarity.
In the second solved structure of the β2AR in which the IC3 loop had been replaced by T4 lysozyme (T4L- β2AR), (Cherzov et al 2007) Glu268 makes a salt bridge to an Arg on the T4L moiety and is directed away from Arg131 on helix 3. This construct has a backbone conformation of IC2 and a more open H3/H6 interface quite similar to the Fab-β2AR structure, although the positions of the side chains (as, for instance, of Tyr141 and Gln142) vary considerably. According to its ligand-binding properties, the T4L-β2AR has the phenotype of partial constitutive activity. (Rosenbaum D et al 2007). The loosened H3/H6 interaction and the resulting more open conformation in this interface in the structures of both Fab-β2AR and T4L-β2AR had been interpreted to signify possibly a partially active conformation. (Rosenbaum D et al 2007) On the other hand, fluorescence quenching data showed that partial agonists are as effective as full agonists in opening the H3/H6 interface in the β2AR, indicating that disruption of the conformational constraint is necessary but not sufficient for activation. (Yao X et al 2006).

In summary, these structural studies strongly suggest that in the β2AR the H3/H6 interaction is much more easily broken than the internal salt bridge between Asp130 and Arg131 in the DRY motif. This correlates very well with the findings presented here for rhodopsin, where the homologous Glu134/Arg135 salt bridge imposes a much stronger constraint than the interaction between the ERY motif and Glu247 on H6.

In this study, the activation profiles of rhodopsin mutants reconstituted into lipid membranes was analysed to characterize the contribution of charged amino acid side chains to the cytoplasmic ionic lock, which stabilizes the inactive receptor states. While disruption of interhelical interactions of H6 with H3 and H7 contributes to the energetics of opening this ionic lock during activation, the most important contribution stems from the neutralization of the Glu134/Arg135 intrahelical salt bridge in the E(D)RY motif. This indicates that the role of an H3/H6 interhelical salt bridge is much less pivotal than put forward in previous models of receptor activation. Using the E134Q mutant, FTIR signature of proton uptake by Glu134 in the transition to Meta II has been defined in this study. Remarkably, despite its pronounced effect on the energetics of the Meta I/Meta II equilibrium, the E134Q mutation does not substantially alter the overall receptor conformation of the involved states.
5. Rhodopsin activation controlled by two protonation switches in membranes

5.1 Introduction

The ligand for rhodopsin a 7-helical transmembrane visual pigment is the retinal chromophore, which is covalently linked to a lysine on transmembrane helix H7 by a protonated Schiff base (PSB) (Menon ST et al 2001). The 11-cis retinal chromophore of the dark state is an inactivating inverse agonist that is converted by photoisomerization to the all-trans agonist, driving the conformational transitions leading to receptor activation. Within milliseconds several inactive intermediates are formed, such as Batho, BSI, Lumi, and Meta I, that can be examined by using time-resolved or cryotrapping techniques (Schichida Y et al 1998). The early transitions involve mainly a relaxation of the isomerized retinal chromophore (Struts AV et al 2007, Salgado GF et al 2006), with only minor changes to the helix bundle of the receptor protein as revealed by electron crystallography of the Meta I state (Schertler GFK et al). Only in the transition from Meta I to the active receptor conformation Meta II is a rearrangement of the helix bundle observed, involving tilt movements of H6 (Sheikh SP et al 1996, Altenbach C et al 2008, Farrens DL et al 1996) and presumably also of H5 (Park JH et al 2008).

Activation of the receptor is proposed to involve 2 distinct protonation switches (Fig. 5.2). The first switch entails disruption of a salt bridge between the retinal PSB on H7 and its complex counterion, consisting of Glu113 on H3 and Glu181 on extracellular loop 2 in the transmembrane part of the receptor (Lüdeke S et al 2005), by deprotonation of the PSB and internal proton transfer to Glu-113 (Jäger F et al 1994). The second switch is proton uptake by Glu134 of the conserved E(D)RY motif at the cytoplasmic terminus of H3 (Arnis S et al), forming a salt bridge with Arg135 that is part of an interhelical network between H3 and H6 in the cytoplasmic domain. Protonation of Glu134 in Meta II from the solvent has been shown recently by FTIR spectroscopy (Vogel R et al 2008). This second protonation switch leads to the anomalous pH-dependence of the Meta I/Meta II conformational equilibrium, favoring deprotonation of the PSB and the transition to active Meta II at acidic pH (Matthews RG et al 1963, Arnis S et al 1984).
Fig 5.1 Previous studies (Arnis et al, Knierim et al) have proposed a sequential reaction scheme of coupled equilibria of photoproduct states for rhodopsin activation in a detergent environment, involving PSB deprotonation in the transition to Meta IIa, activating helix movements in the transition to Meta IIb, and cytoplasmic proton uptake by Glu134 in the transition to Meta IIbH+. (The terms inactive and active are used to describe the receptor conformations before and after helix movements, respectively, and do not necessarily specify activity toward G protein.)

By using rhodopsin purified in flexible detergents such as dodecyl maltoside, Hofmann and coworkers (Arnis S et al 1994) showed by kinetic UV–visible spectrophotometry and proton-uptake experiments that the transition from Meta I to Meta II proceeds in a sequential manner, with Schiff base deprotonation preceding the cytoplasmic proton uptake reaction, resulting in multiple Meta II states. Hubbell and collaborators (Knierim B et al) also extended this scheme by using time-resolved EPR spectroscopy of spin labeled detergent-solubilized rhodopsin, revealing that Meta IIa converts to Meta IIb by movement of H6, and to Meta IIbH+ by the cytoplasmic proton uptake (Fig 5.1). Notably, the activating helix movements in the transition to Meta IIb take place independently of pH and, hence, the proton uptake step under these conditions. This behavior differs considerably from the classical anomalous pH dependence of the Meta I/Meta II equilibrium in native membranes (Matthews RG et al 1963), reflecting a substantial perturbation of the energetics of the metarhodopsin equilibrium induced by the detergent. Alternative models include a branched scheme where early intermediates with a protonated or deprotonated Schiff base evolve in a parallel manner on the time scale of Lumi decay (Thorgeirsson TE et al 1993). A comprehensive thermodynamic model that allows the description of rhodopsin activation in
both detergent and membrane environments within a single conceptual framework were developed with the experimental results as described below.

Fig 5.2 The structure of the dark state of rhodopsin [based on Protein Data Bank ID code 1GZM] includes 2 protonation switches. (A) The cytoplasmic network around Glu134 in the ERY motif of H3 is involved in proton uptake from the solvent, and (B) the interhelical salt bridge between the protonated retinal Schiff base (PSB) linked to H7 and Glu113 on H3 in the transmembrane domain is disrupted by internal proton transfer during receptor activation.

5.2 Synopsis

Here the transition from Meta I to Meta IIbH\(^+\) are treated as a set of coupled equilibria with corresponding thermodynamic parameters. For native membranes, this reaction scheme reproduces the classical Henderson–Hasselbalch-like equilibrium between Meta I and Meta IIbH\(^+\) at lower temperatures. At higher temperatures, more complex titration curves are predicted with non-zero alkaline endpoints, reflecting in particular a significant population of the entropy-stabilized Meta IIb state. The validity of this framework is tested and verified experimentally by using a combination of UV-visible and FTIR spectroscopy of
rhodopsin in native and synthetic membranes, by which deprotonation of the PSB and activating conformational changes of the helix bundle can be accessed separately. These data are used to define the function of the second switch, protonation of Glu134, which is shown to be a thermodynamic prerequisite to achieve full receptor activation in a membrane environment.

5.3 RESULTS

5.3.1 Thermodynamic Model of Receptor Activation.

Starting from the extended model as shown in Fig. 5.1 (Arnis S et al. 1993, Knierim B et al 2007), titration scheme involving the different photoproduct species were derived. At very alkaline pH, where the proton uptake by Glu134 and, hence, formation of Meta IIbH⁺ is absent, the extended reaction scheme is reduced to Meta I, Meta IIa, and Meta IIb, which form a set of two coupled, pH-independent equilibria. At lower pH, these intrinsically pH-independent equilibria are coupled to the pH-dependent equilibrium with Meta IIbH⁺, resulting in the complex titration behavior.

Previous studies of the activation of rhodopsin analogues in membranes have suggested that disruption of the PSB salt bridge as the first protonation switch involves a relatively large unfavorable enthalpy change $\Delta H^\circ$, which is only partially offset by a favorable positive entropy change $T\Delta S^\circ$ (Vogel R et al. 2006, Thorgeirsson TE et al. 1993). The resulting free energy change $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$ for the first switch is compensated by the favorable $\Delta H^\circ$ of the second switch, protonation of Glu134, such that the transition to Meta II is strictly coupled to cytoplasmic proton uptake. At lower temperature, this coupling therefore reduces the extended scheme to the classical 2-state equilibrium between Meta I and Meta IIbH⁺. At higher temperature, because of the explicit temperature dependence of the entropic contribution to the free-energy change, triggering of the first switch should become favorable and, thus, independent of the second switch. With increasing temperature, we would therefore expect the classical 2-state equilibrium to evolve into the full 4-state equilibrium.
5.3.2 Schiff Base Deprotonation in the Meta I/Meta II Equilibria in Native Membranes.

By using UV–visible spectrophotometry, the protonation state of the Schiff base in the photoproducts can be determined as can the concentration of the Meta II states. The Meta I photoproduct with PSB absorbs at 485 nm, whereas the Meta II states with deprotonated Schiff base absorb at 380 nm (Fig. 5.3 A and B). The contribution of Meta II states with deprotonated Schiff base, θ_{UV-vis}, to the photoproduct equilibrium can be monitored in the photoproduct minus dark state difference spectra in Fig. 5.3C by decomposing them into a linear combination of difference spectra corresponding to the transitions to pure Meta I and Meta II states. At 10 °C, the resulting titration curve follows closely a regular Henderson–Hasselbalch function $\theta_{UV-vis} = 10^{pK_a-pH/(1 + 10^{pK_a-pH})}$ with acidic and alkaline endpoints at 1 and 0, respectively, reflecting the classical 2-state equilibrium between Meta I and Meta IIbH⁺ (Fig. 5.4, green curve)
Fig. 5.3. UV–visible spectroscopic characterization of the PSB salt bridge in the Meta I/Meta II equilibrium in native dic membranes. (A and B) At 20 °C and pH 5.0, photolysis converts the dark state of rhodopsin (black) to the Meta IIbH⁺ photoproduct state with deprotonated Schiff base (red), whereas, at 10 °C and pH 9.5, the Meta I photoproduct with protonated Schiff base (blue) is formed. (C) Difference spectra for the photoproduct minus dark state obtained at 10 °C reveal the pH dependence of the Meta I/Meta IIbH⁺ equilibrium, which is completely on the side of Meta IIbH⁺ at pH 5.0 and of Meta I at pH 9.0 and 10.0, as evident from the complete lack of absorption at 380 nm. (D) At 30 °C, the equilibrium is not fully shifted to Meta I at alkaline pH; instead a 380 nm Meta II photoproduct contribution is observed at very alkaline pH, which becomes pH-independent at pH 9.0 and above.

Fig 5.4 Schiff base deprotonation (θ_{UV–vis}) and, hence, the fraction of Meta II were
determined from UV–visible difference spectra. At 10 °C the titration curve follows a regular
Henderson–Hasselbalch function, whereas, at higher temperature, the alkaline endpoint does
not reach zero because of increasing population of the Meta IIa and Meta IIb states.

However, in the temperature range from 20 to 37 °C, the 380 nm absorption of
Meta II persists even at very alkaline pH (Fig. 5.3D), and the titration curves follow the
modified phenomenological function $\theta_{\text{UV-vis}} = 10pK_a-pH + \theta_{\text{alkUV-vis}} / (1 +10pK_a-pH)$,
with non-zero values for $\theta_{\text{alkUV-vis}}$, the alkaline endpoint values of the titration curves (Fig.
5.4). In the scheme depicted in Fig. 5.10, $\theta_{\text{UV-vis}}$ corresponds to the blue line and the alkaline
endpoint value $\theta_{\text{alkUV-vis}}$ is determined by the combined contributions of Meta IIa and Meta
IIb to the photoproduct equilibrium. As these experiments involved a combination of elevated
temperature and extremely alkaline pH values, photoproduct stability needed to be controlled
carefully. The sampling time after photolysis in both UV-visible spectroscopy and FTIR
experiments were minimized by using a 100 ms photolysis pulse followed by time-resolved
spectral acquisition over 240 ms intervals as described below. Consecutively recorded spectra
verified the photoproduct stability in the initial spectra obtained immediately after photolysis
and allowed spectral averaging (Fig. 5.5).
Fig. 5.5. Confirmation of photoproduct stability in time-resolved FTIR experiments. While the dark state is stable in the range of conditions covered in this study, photoproducts are less stable, in particular at the highest temperature and pH values. To confirm the integrity of the photoproduct states in the difference spectra used for data analysis, the photoproduct spectra were acquired in successive time intervals of approximately 240 ms duration, using
quasi-logarithmic averaging for longer time scales. Potential photoproduct decay was assessed using the temporal evolution of spectral marker bands above 1700 cm\(^{-1}\) of membrane-embedded carboxylic acids Glu122 and Asp83 involved in interhelical-hydrogen bonded networks, and of amide I and II marker bands at 1633 and 1548 cm\(^{-1}\), respectively, that were characterized in previous studies (Vogel R et al 2006). In order to increase the signal to noise ratio of the difference spectra used for spectral fitting, subsequent post-illumination spectra were averaged over the time range for which no photoproduct destabilization could be observed. (A and B) Time-resolved photoproduct minus dark state difference spectra that were obtained with rapid-scan FTIR spectroscopy at 37 °C at pH 7.6 and 9.6, respectively. Photoproduct spectra were acquired during time intervals between 0 and 240 ms (green), 250 and 500 ms (turquoise), 760 and 1250 ms (purple), and 2780 and 3780 ms (gray), starting after the 100 ms photolysis flash. At pH 7.6, the photoproduct remains stable over the entire time range covered in the experiment. At pH 9.6, on the other hand, some destabilization is observed on the longest time scales. Characteristic marker bands of this long-term destabilization are tagged by arrows. (C) For data analysis, photoproduct spectra were averaged over time intervals where complete photoproduct stability was warranted by the absence of these spectral changes, which covered, in this particular case, the time interval between 0 and 240 ms. This difference spectrum is decomposed into a linear combination of Meta I and Meta II\(_{2}\)H\(^{+}\) minus dark state reference spectra.

**Spectral recording** IR spectra were recorded by using time-resolved rapid-scan FTIR spectroscopy with a spectral resolution of 4 cm\(^{-1}\). An acquisition time of 6 s was used for the pre illumination spectrum and a time of 240 ms for the single post illumination spectra. Alternatively, at 10 and 20 °C, conventional FTIR difference spectroscopy was used with acquisition times of 12 s for pre illumination spectra and between 1.5 and 12 s for post illumination spectra, depending on temperature and stability of the photoproduct states. In all experiments, thermal stability of the photoproduct states was confirmed in series of postillumination spectra (Fig.5.5). Samples were photolyzed with an array of 6 ultrabright green (max, 520 nm) 5-mm LEDs (Nichia) with nominal 16 candela at 20 mA current and 15° emission half angle. Illumination time was 100 ms at 100 mA in rapid scan experiments, by which \(\approx\)70 % of rhodopsin was photolyzed. By using its fingerprint bands as spectral
markers, the contribution of isorhodopsin to the photoproduct equilibria was found to be small under these conditions (≈10 % of that of Meta I). The resulting small overestimation of the Meta I contribution was accounted for in the calculation of $\Delta G^\circ$ values. In conventional FTIR experiments, illumination was 2 s at 20 mA, which led to complete photolysis. Spectral normalization was achieved by using the intense 1237 cm$^{-1}$ fingerprint mode of the dark state.

5.3.3 Changes in Interhelical Hydrogen-Bonded Networks in the Meta I/Meta II Equilibria in Native Membranes.

Whereas UV–visible spectrophotometry determines the protonation state of the retinal Schiff base, FTIR difference spectroscopy can be used to follow the conformation of the receptor in the photoproduct equilibria. FTIR difference spectra photoproduct minus dark state of rhodopsin in native disc membranes are decomposed into a linear combination of Meta I and Meta IIbH$^+$ reference (basis) spectra, as shown in Fig. 5.8, corresponding to inactive and active receptor states, respectively. This decomposition yields $\theta_{\text{FTIR}}$ as the fraction of the active conformation in the photoproduct equilibrium. Spectral decomposition was performed in the conformationally most sensitive spectral region between 1,800 and 1,600 cm$^{-1}$ (marked in green in fig 5.6). This range comprises the amide I vibrations of the protein backbone and the C=O stretch of protonated carboxylic acids Glu122 and Asp83, which are involved in hydrogen-bonded networks between H3 and H5 and between H1, H2, and H7, respectively (Park J et al 2008, Okada T et al 2004, Li J et al 2004, Palczewski et al 2000), and are sensitive markers for the conformational changes during receptor activation (Lüdeke S et al 2005, Fahmy K et al 1993, Vogel R et al 2006).

The titration curve follows a regular Henderson–Hasselbalch function at 10 °C but not at higher temperature, where the alkaline endpoint value, $\theta_{\text{FTIR}}^{\text{alk}}$, is different from zero (Fig. 5.7). Notably, at 30 and 37 °C, $\theta_{\text{FTIR}}^{\text{alk}}$ is slightly lower than $\theta_{\text{UV-Vis}}^{\text{alk}}$. According to the reaction scheme in Fig. 5.11, $\theta_{\text{FTIR}}^{\text{alk}}$ corresponds to the contribution of Meta IIb at alkaline pH. The small difference between $\theta_{\text{UV-Vis}}^{\text{alk}}$ and $\theta_{\text{FTIR}}^{\text{alk}}$ therefore determines the contribution of Meta IIa to the photoproduct equilibrium.
at the alkaline endpoint, amounting to only \( \approx 11\% \) at 37 °C and even less at lower temperatures. Alkaline endpoint and apparent pKa values are tabulated below.

<table>
<thead>
<tr>
<th>Membranes</th>
<th>Temperature, °C</th>
<th>UV-visible</th>
<th>FTIR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( pK_a )</td>
<td>( \Theta_{UV\text{-}vis} )</td>
</tr>
<tr>
<td>Native disk</td>
<td>10</td>
<td>7.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>7.7</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>7.8</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>7.8</td>
<td>0.58</td>
</tr>
<tr>
<td>POPC</td>
<td>10</td>
<td>5.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>6.0</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>6.1</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>5.9</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Estimated errors are ±0.05 for \( \Theta \) values and ±0.2 for pKa values.
Fig 5.6 Meta I and Meta IIbH⁺ minus dark state FTIR difference spectra were obtained from rhodopsin in native disc membranes at 10 °C at pH 9.5 and 20 °C at pH 5.0, respectively, under identical conditions as in Fig. 5.3 A and B. By using the conformationally sensitive range marked in green, they served as basis spectra for inactive and active conformations, respectively, to determine the pH dependence of activating conformational changes.

Fig 5.7 The FTIR-based titration curves of receptor conformation, θ_{FTIR} (filled circles and
solid lines), reveal a similar deviation from classical Henderson–Hasselbalch behavior as the UV–visible data, with a temperature-dependent increase of the alkaline endpoint $\theta_{\text{FTIR alk}}$. The alkaline endpoints $\theta_{\text{UV-Vis alk}}$ of the UV–visible-based titration curves (open symbols, broken lines, reproduced from Fig. 5.4) at 30 and 37 °C are slightly higher than $\theta_{\text{FTIR alk}}$, revealing the additional contribution of inactive Meta IIa.

Calculation of titration curves: FTIR difference spectra were decomposed into a linear combination of Meta II and Meta I reference spectra obtained at 10 °C at pH 5.0 and pH 9.0 by a spectral fitting procedure using the conformationally sensitive range between 1800 and 1600 cm$^{-1}$. To account for an apparent temperature sensitivity of an amide at around 1660 cm$^{-1}$, the range between 1655 cm$^{-1}$ and 1666 cm$^{-1}$ was excluded from the analysis.

5.3.4 Rhodopsin Activation in Synthetic 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC) Membranes.

Experiments were also extended to rhodopsin reconstituted into POPC membranes (Fig. 5.8), which differ from native disc membranes in the composition of both head groups and chains. These recombinant membranes reveal a generally similar titration scheme as native membranes, yet with drastic shifts in the associated equilibria, in agreement with previous studies (Wang Y et al 2002, Gibson NJ et al 1993). The presence of an alkaline plateau region in POPC membranes as observed here deviates from results of a previous study that used a considerably longer acquisition time of $\approx 60$ s after illumination (Botelho AV et al 2002), such that photoprodct instability might have distorted the data obtained at very alkaline pH. Interestingly, although the alkaline endpoint values in POPC recombinant membranes are only slightly lower, corresponding to shifts of the associated equilibrium constants by a factor of $<2$, the apparent pKa values of the titration curves are reduced by $\approx 1.5$ units, corresponding to a downshift of the apparent protonation-dependent equilibrium constants by a factor of $\approx 30$. 
Fig 5.8 Rhodopsin reconstituted into synthetic POPC membranes shows the same general activation scheme but with shifted equilibria because of altered properties of the lipid bilayer. Both in native disc membranes and synthetic POPC membranes, proton uptake by Glu134 and, hence, the transition to protonated Meta IIbH$^+$ is necessary for full receptor activation.

5.3.5 Protonated and Unprotonated Meta IIb Share a Similar Active State Receptor Conformation.

Neglecting the small contribution of Meta IIa, the alkaline endpoint FTIR difference spectra represent primarily the transition from the dark state to the Meta I/MetaIIb photoproduct equilibrium. In Fig. 5.9, we compare these spectra with synthetic Meta I/Meta IIbH$^+$ minus dark state spectra calculated as a linear combination of the corresponding reference spectra obtained at 10 °C. The remarkable agreement between synthetic and experimental spectra indicates similar FTIR signatures of Meta IIb and Meta IIbH$^+$. This agreement includes in particular the intense bands of conformationally sensitive carboxylic acids above 1700 cm$^{-1}$ (and hence the H3/H5 and H1/H2/H7 interhelical
networks) and the amide I marker band of Meta II at 1,644 cm\(^{-1}\). Note that the FTIR signature of protonation of Glu134 should contribute to Meta II\(_b\)H\(^+\) but not to Meta II\(_b\). This pattern consists of a positive band at 1713 cm\(^{-1}\) and negative bands at 1562 and 1394 cm\(^{-1}\) (Vogel R et al 2008), which are small compared with the pronounced spectral changes of Asp83, Glu122, and Glu113 (Jäger F et al 1994, Fahmy F et al 1993), and are therefore not clearly resolved in this approximation.

**Fig. 5.9.** Meta II\(_b\) and Meta II\(_b\)H\(^+\) share a similar active state FTIR signature. FTIR difference spectra for the photoproduct minus dark state obtained for disc membranes at the alkaline endpoint reveal the increasing contribution of the active conformation of unprotonated Meta II\(_b\) to the Meta I/Meta II\(_a\)/Meta II\(_b\) photoproduct equilibrium at higher temperature. Synthetic Meta I/Meta II\(_b\)H\(^+\) minus dark state spectra (red) were calculated as a linear combination of the Meta I and Meta II\(_b\)H\(^+\) basis spectra shown in Fig. 5.6 by using the endpoint values \(\theta\) \(_{UV-Vis\ alk}\) (neglecting the small contribution of Meta II\(_a\)). These
synthetic spectra show a remarkable agreement with the experimental endpoint spectra, indicating similar FTIR signatures of Meta IIb and Meta IIbH+.

5.3.6 Thermodynamic Parameters of the Meta I/Meta II Equilibria.

The equilibrium constants $K_1$, $K_2$, and $K_3$ of the single equilibria (Fig.5.1) can be expressed in terms of the experimental data $\theta_{UV-Vis}$ and $\theta_{FTIR}$ as derived here.

**Derivation of the thermodynamic parameters from UV-Visible and FTIR data**

Adopting the scheme from fig 5.1 the transition from Meta I to Meta IIb H$^+$ is described as a set of three coupled equilibria.

Meta I + H$^+$ $\leftrightarrow$ Meta IIa H$^+$ $\leftrightarrow$ Meta IIb + H$^+$ $\leftrightarrow$ Meta IIbH$^+$

With equilibrium constants

$$K_1 = \frac{[\text{Meta II}_a]}{[\text{Meta I}]}$$

$$K_2 = \frac{[\text{Meta II}_b]}{[\text{Meta II}_a]} \quad \text{and}$$

$$K_3 = \frac{[\text{Meta IIbH}^+]}{[\text{Meta II}_b][H^+]$$

And the normalization $[\text{Meta I}] + [\text{Meta II}_a] + [\text{Meta II}_b] + [\text{Meta IIbH}^+] = 1$

The concentrations of the individual photoproduct species can be expressed by the equilibrium constants

$[\text{Meta I}] = 1 / Z$, $[\text{Meta II}_a] = K_1 / Z$, $[\text{Meta II}_b] = K_1K_2 / Z$ and
$[\text{Meta II}_b H^+] = K_1 K_2 K_3 [H^+]$

Where $Z = 1 + K_1 + K_1 K_2 + K_1 K_2 K_3 [H^+]$

Using UV-Vis spectroscopy, $\theta_{\text{UV-Vis}}$ was determined which is the fraction of the photoproducts with deprotonated schiff base. The according to the reaction scheme $\theta_{\text{UV-Vis}}$ can be expressed as follows

$$\theta_{\text{UV-Vis}} = \frac{K_1 + K_1 K_2 + K_1 K_2 K_3 [H^+]}{1 + K_1 + K_1 K_2 + K_1 K_2 K_3 [H^+]}$$

$$= \frac{K_1 + K_1 K_2 + K_1 K_2 10^{pK_{a3} - pH}}{1 + K_1 + K_1 K_2 + K_1 K_2 10^{pK_{a3} - pH}}$$

With $pK_{a3} = - \log_{10} (1/K_3)$. The alkaline end point of the UV-Vis titration curves is given by

$$\theta_{\text{UV-Vis alk}} = \frac{K_1 + K_1 K_2}{1 + K_1 + K_1 K_2}$$

Similarly by FTIR spectroscopy, $\theta_{\text{FTIR}}$ was determined which was the fraction of the photoproducts with FTIR signature of an active state confirmation. As per the reaction scheme $\theta_{\text{FTIR}}$ can be expressed as

$$\theta_{\text{FTIR}} = \frac{K_1 + K_1 K_2 + K_1 K_2 K_3 [H^+]}{1 + K_1 + K_1 K_2 + K_1 K_2 K_3 [H^+]}$$

$$= \frac{K_1 + K_1 K_2 + K_1 K_2 10^{pK_{a3} - pH}}{1 + K_1 + K_1 K_2 + K_1 K_2 10^{pK_{a3} - pH}}$$
And also similar to UV-Vis alkaline end point of the FTIR titration curves is given by

$$\theta_{\text{FTIR alk}} = \frac{K_1 K_2}{1 + K_1 + K_1 K_2}$$

The $K_i$ values were obtained by least square fits to the spectroscopic observables. With the alkaline end point values given above for the UV-Vis and FTIR titration curves, this description is equivalent to the description by the phenomenological Henderson-Hasselbalch functions $\theta = (\theta \text{ alk} + 10^{pK_a-pH})/(1+10^{pK_a-pH})$. The apparent $pK_a$ values of these phenomenological Henderson-Hasselbalch titration curves are related to $pK_{a3}$ by

$$pK_a = \log_{10} \left[ \frac{(K_1 K_2)}{(1+K_1+K_1 K_2)} \right] + pK_{a3}$$

$\Delta H^\circ$ and $\Delta S^\circ$ values of the equilibria could not be obtained from the thermodynamic description of the full scheme because of the marginal population of Meta IIa. Therefore a reduced reaction model (where the Meta I to Meta IIb was treated as a single step) was used to derive values for $\Delta H^\circ$ and $\Delta S^\circ$

Meta I + H$^+$ $\leftrightarrow$ Meta IIb + H$^+$ $\leftrightarrow$ Meta IIbH$^+$

With equilibrium constants

$$K_{12} = \frac{[\text{Meta IIb}]}{[\text{Meta I}]}$$

and

$$K_3 = \frac{[\text{Meta IIbH}^+]}{[\text{Meta IIb}][H^+]}$$

And the normalization $[\text{Meta I}] + [\text{Meta IIb}] + [\text{Meta IIbH}^+] = 1$
According to this reduced model, the observables $\theta_{\text{FTIR}}$ and $\theta_{\text{UV-Vis}}$ are defined identically as the sum of the concentrations of the Meta IIb and Meta IIbH$^+$ states and are designated $\theta$ which is expressed as

$$\theta = [\text{Meta IIb}] + [\text{Meta IIbH}^+] = \frac{K_1 + K_{12}K_3 [H^+]}{1 + K_{12} + K_{12}K_3 [H^+]} = \frac{K_{12} + K_{12}10^{pK_{a3} - pH}}{K_{12} + K_{12}10^{pK_{a3} - pH}}$$

Using this reduced model, the alkaline end point is $\theta_{\text{alk}} = \frac{K_{12}}{1 + K_{12}}$

In this reduced model, the free energy change was calculated as $\Delta G_{12}^\circ = \Delta H_{12}^\circ - T \Delta S_{12}^\circ$

$$= -RT \ln K_{12}$$

and $\Delta H_{12}^\circ$ and $\Delta S_{12}^\circ$ were obtained from a Van’t hoff analysis over the temperature range from 20 and 37°C.

Free-energy changes were calculated at 37 °C from $\Delta G_{i}^\circ = -RT \ln K_i$, for the individual equilibria, with $R$ being the gas constant and $T$ the absolute temperature. This calculation indicates an increase of $G^\circ$ in disc membranes by $\approx 3.1 \pm 1.9$ kJ.mol$^{-1}$ in the transition from Meta I to Meta IIa and a subsequent decrease by $-3.7 \pm 1.6$ kJ.mol$^{-1}$ in the transition to Meta IIb (4.6 $\pm$ 2.3 kJ.mol$^{-1}$ and -4.0 $\pm$ 2.5 kJ.mol$^{-1}$, respectively, in POPC membranes). The large errors reflect the uncertainty in determining a precise value of the low amount of Meta IIa present. Values of $pK_{a3}$ of the Meta IIb$^+ H^+ \leftrightarrow$ Meta IIbH$^+$ proton uptake reaction were determined to be 8.0 in native and 6.2 in recombinant POPC membranes (at 37 °C). Note that $pK_{a3}$ is different from the apparent $pK_a$ in the phenomenological Henderson–Hasselbalch description used above. This result yields a $\Delta G_{3}^\circ$ of $-48 \pm 2$ kJ.mol$^{-1}$
for disc membranes and -37 ± 2 kJ.mol⁻¹ for POPC membranes by using the solute standard state including the hydronium concentration. Furthermore, \( \Delta H^\circ \) and \( \Delta S^\circ \) values of the equilibria could not be obtained from the thermodynamic description of the full scheme because of the marginal population of Meta IIₐ. We therefore used a reduced reaction scheme in which the transition from Meta I to Meta IIₐ was treated as a single step with an equilibrium constant \( K_{12} \), neglecting the small contribution of Meta IIₐ. The \( \theta_{\text{UV-vis}} \) and \( \theta_{\text{FTIR}} \) data were then merged and \( K_{12} \) was obtained by a fit to the observables. The free-energy change was calculated as \( \Delta G^\circ_{12} = \Delta H_{12}^\circ - T \Delta S_{12}^\circ = -RT \ln K_{12} \), yielding \( \Delta H_{12}^\circ = 88 \pm 16 \) kJ.mol⁻¹ and \( \Delta S_{12}^\circ = 285 \pm 53 \) J.mol⁻¹K⁻¹ (84±22 kJ.mol⁻¹ and 269±74 J.mol⁻¹.K⁻¹, respectively, in the case of POPC membranes) from a van ’t Hoff analysis. The relatively large errors are because of the small temperature range of the data between 20 and 37 °C. Additionally, \( \Delta G^\circ_{3} \) consists primarily of a negative enthalpic term corresponding to a decrease of pKa3 with increasing temperature. A more precise analysis is, however, precluded by large error margins imposed by the limited temperature range of the van’t Hoff analysis.

### 5.3.7 Titration curves of the two state and four state equilibrium

The following two titration curves of the experiments explains the classical 2-state equilibrium and the full 4-state equilibrium with increase in temperature.
Fig 5.10 The reaction scheme depicted here (see also fig 5.1) predicts a complex titration behavior of photoproducts in a membrane environment, with Meta I, Meta IIa, and Meta IIb forming an equilibrium at alkaline pH. This intrinsically pH-independent equilibrium is coupled to a pH-dependent equilibrium with Meta IIbH⁺ at lower pH, reflecting proton uptake by Glu134 from the solvent.

Fig 5.11 The reaction scheme depicted here predicts a titration behavior of photoproducts in a membrane environment, with Meta I and Meta IIbH⁺ forming an equilibrium at acidic to alkaline pH. Because Meta IIa and Meta IIb are populated only at higher temperature, this 4 state scheme reduces to the classical 2 state Meta I/Meta IIbH⁺ scheme at lower temperatures.
5.4 Discussion

5.4.1 Activation mechanism of Rhodopsin in native membranes.

In this study, the role of the 2 protonation switches for the activation of rhodopsin has been investigated in its native membrane environment in the physiologically relevant temperature range. With UV-visible spectroscopy, the internal proton transfer leading to disruption of the PSB salt bridge has been monitored, whereas FTIR spectroscopy allowed to follow the conformational rearrangement of transmembrane helical networks during receptor activation. Moreover, the pH dependence of the spectral changes allowed to map these changes to the cytoplasmic proton uptake step by Glu134. It has been shown that at lower temperatures, exclusive triggering of the PSB-Glu113 switch is disfavored and depends on the enthalpically downhill cytoplasmic proton uptake by Glu134. This assistance by the Glu134 protonation switch previously was shown to be facilitated by an allosteric coupling mechanism depending on the C9-methyl group and the ß-ionone ring of all-trans retinal (Vogel R et al 2006, Meyer CK et al 2000). Coupling of the 2 protonation switches establishes the classical 2-state equilibrium between Meta I and protonated Meta IIbH⁺ (Matthews RG et al, Parkes JH et al 1984) at 10°C and below. The key result of these experiments is that at higher temperatures the 2 switches become partially uncoupled, leading to population of the unprotonated Meta IIb state (and a marginal population of Meta IIa), in which the PSB – Glu113 salt bridge is broken, but cytoplasmic proton uptake by Glu134 is absent.

5.4.2 Role of Glu 134 protonation for conformational changes in Rhodopsin activation.

Glu134 is part of the conserved E(D)RY and, in the dark state, forms an intrahelical salt bridge with neighboring Arg135 at the cytoplasmic terminus of H3 (Fig. 5.2) (Palczewski et al 2000, Okada T et al 2001). A similar intrahelical salt bridge is assumed to control activation of other G-PCRs (Kobilka BK et al 2007) and also is present in the recently solved adrenergic receptor structures (Rasmussen et al 2007, Warne T et al 2008 and Cherezov et al 2007). Neutralization of position 134 in the E134Q rhodopsin mutant abolishes proton uptake during Meta II formation (Arnis et al 1994) and leads to pH-independent Meta II formation even in a membrane environment (Vogel R et al 2008). In our
studies on rhodopsin in membranes, both unprotonated Meta IIb and protonated Meta IIbH⁺ are shown to share a very similar active receptor conformation, despite their differing protonation states regarding the Glu134 switch. This finding agrees with previous studies on detergent-solubilized rhodopsin, showing that activating movement of H6 require disruption of the PSB salt bridge (Kim JM et al 1997) but are not per se dependent on proton uptake by Glu134 (Knierim B et al 2007).

Protonation of Glu134 is enthalpically downhill, as shown here and in previous studies (Arnis S et al 1994) and renders more favorable an electrostatically unfavorable environment in the cytoplasmic H3/H6 microdomain. This view is supported by the pKa3 of 8.0 for the proton uptake reaction, which is substantially higher than the pKa < 5 of a carboxylate in solution. Such a hydrophobic environment could be created by a movement of Arg135 away from Glu134 in Meta II, in keeping with the recently solved structure of the active conformation of opsin (Park JH et al 2008). Notably, in the native membrane environment, the proton uptake function of Glu134 also is relevant at 37 °C, where the pH-independent conformational equilibrium between Meta I and Meta IIb is only halfway on the active side. From an energetic point of view, protonation of Glu134 is therefore a prerequisite for complete receptor activation under physiological conditions.

5.4.3 Glu134 Protonation and Signal Transduction.

Protonation of Glu134 probably serves a dual role by additionally improving the binding of the activated receptor to the G protein. The E134Q mutation extends G protein activation of detergent-purified pigment into the alkaline range, although the detergent environment favors pH-independent Meta II formation already in the wild-type receptor (Fahmy K et al 1993). This finding suggests that a protonated Glu134 side chain facilitates productive interaction with the G protein transducin. Fluorescence spectroscopy has further revealed the H3/H6 microdomain to contribute to the binding site of the C-terminal part of the G protein α-subunit (Janz JM et al 2004). Because this C-terminal part of transducin contains three potentially charged carboxylates, protonation of Glu134 on the receptor side would reduce electrostatic repulsion or allow a repositioning of neighboring Arg135 to provide for efficient binding.
6. Summary

6.1 Study on the deactivation pathway

Rhodopsin is considered as a prototype of the G protein coupled receptor (G-PCR) superfamily and a complete understanding of its activation mechanisms is therefore of high relevance. Vertebrate rhodopsin consists of the apoprotein opsin and the chromophore 11-cis retinal covalently linked via a protonated schiff base. Photoisomerization of the chromophore to all trans retinal triggers the large scale helix movements leading to the formation of the active state protein Meta II. The Meta II thus formed decays which represents an essential step in maintaining a functional photoreceptor cell. This decay happens by fundamentally two different pathways, either forming the apoprotein opsin by release of the activating all-trans retinal ligand from its binding pocket, or by a thermal isomerization of this ligand to a less activating species in the transition to meta III. Later pathway is favored at neutral to alkaline pH invitro. Meta III is formed by thermal isomerization of the retinal Schiff base C15=N bond, converting the ligand from all-trans 15-anti to all-trans 15-syn. Thereby the induced change of ligand geometry switches the receptor to inactive conformation. In this work the conformation of Meta III was examined over a wider range of pH range and found that Meta III exists in a pH-dependent conformational equilibrium between this inactive conformation at neutral to alkaline pH and an active conformation similar to that of Meta II, which is however achieved at more acidic pH. The apparent pKa of this transition is around 5.1 and thus several units lower than that of the Meta I/Meta II equilibrium with its all-trans-15-anti ligand, but still about 1 unit higher than that of the opsin conformational equilibrium in the absence of the ligand. The all-trans-15-syn chromophore is therefore not an inverse agonist like 11-cis or 9-cis retinal, which locks the receptor in inactive conformation, but a classical partial agonist which is capable of activating the receptor, yet with an efficiency considerably lower than the full agonist all-trans 15-anti. As the Meta III chromophore differs structurally from this full agonist only in the isomeric state of C15=N bond, this ligand represents an excellent model system to study principal mechanisms of partial agonism which are helpful to understand the partial agonist behavior of other ligands.
6.2 Study on the activation pathway

Activation of rhodopsin triggered by isomerization of the 11-cis retinal leads to the conformational changes of the protein ultimately to form active Meta II state. The activation of rhodopsin involves rearrangement of a conserved interhelical cytoplasmic hydrogen bond between the ERY motif on transmembrane helix 3 (H3) and some residues on helix 6, which is commonly termed as cytoplasmic ionic lock. Glu134 of the ERY motif forms an intrahelical salt bridge with neighboring Arg135 in the dark state structure of rhodopsin. Here we have examined the roles of Glu134 and Arg135 on H3 and Glu247 and Glu249 on helix 6 by mutating these residues. These wild type and mutant pigments were studied using FTIR spectroscopy. These pigments were reconstituted onto lipid membranes. Activation of rhodopsin is a pH dependent which signifies proton uptake reaction during activation. Using FTIR protonation signature it has been concluded that Glu134 is the proton accepting group during activation. When Glu134 was neutralized by mutating E to Q the pH dependence of receptor activation was completely abolished. Neutralization of R135 to Leucin also leads to a complete pH independent receptor activation. R135L Meta II spectra showed considerable structural alterations to that of wildtype unlike E134Q. Neutralization of E247 and E249 on H6 (which are involved in interhelical interactions with H3 and H7 respectively) led to a shift towards Meta II while retaining the pH sensitivity of the equilibrium. The pKa value gets upshifted by approximately 1.5 units. These results suggest that neutralization of the Glu134 and Arg135 is considerably more critical on shifting the photoproduct equilibrium than the disruption of the interhelical interaction of Glu247 and Glu249 on H6 with H3 and H7. This indicates that role of the H3/H6 interhelical salt bridge is much less pivotal than the intrahelical interaction in ERY motif.

Also in the conformational transition to the active Meta II state light induced isomerization of the retinal ligand triggers two protonation switches. First switch involves disruption of an interhelical salt bridge by internal proton transfer from the retinal Protonated Schiff base to its counterion Glu113 in the transmembrane domain. Second switch consists of a uptake of a proton from the solvent by Glu134 of the conserved ERY motif at the cytoplasmic terminus of helix 3, leading to the pH dependent receptor activation as discussed above. By using UV-Vis and FTIR spectroscopy, the activation mechanisms of rhodopsin has been studied in this work and shown that these two protonation switches become partially
uncoupled at physiological pH. This partial uncoupling allows formation of an entropy-stabilized Meta II state, in which the conformational changes associated with receptor activation have taken place despite the lack of protonation of Glu134. Disruption of the salt bridge between the PSB and its complex counterion is therefore a structural prerequisite for the activating helix movements, whereas protonation of Glu134 is a thermodynamic requirement for shifting the conformational equilibrium completely to the active Meta II conformation. Although the structural role of PSB salt-bridge disruption is similarly observed for rhodopsin in detergent (Kim J et al 2004), the thermodynamic function of the second switch is not required in a detergent environment (Knierim B et al 2007) because of a lowered enthalpy change in the transition to Meta II. Therefore, in native and synthetic membranes, a sequential destabilization of both salt bridges is required for full receptor activation. The similar structure of the intrahelical salt bridge in the DRY motif in the β2 adrenergic receptor structures (Rasmussen SG et al 2007, Warne T et al 2008 and Cherezov V et al 2007) suggests a similar thermodynamic role of this second switch in the activation of other members of the rhodopsin-like receptor family.
7. Zusammenfassung

7.1 Untersuchungen zum Deaktivierungsmechanismus

Der Lichtreceptor Rhodopsin wird als Prototyp für die Familie der G-Protein gekoppelten
Rezeptoren betrachtet, weshalb das vollständige Verständnis seiner
Aktivierungsmechanismen von höchster Bedeutung ist. Rhodopsin setzt sich in Wirbeltieren
aus dem Apoprotein Opsin und dem Chromophor 11-cis-Retinal zusammen, welches über
eine protonierte Schiff-Base (PSB) kovalent an das Protein gebunden ist. Starke Helix-
Verschiebungen, die durch die Photoisomerisierung des Chromophors zu all-trans-Retinal
ausgelöst werden, führen letztendlich zur Ausbildung des aktiven Rezeptor-Zustands
Meta II. Der darauf folgende Zerfall des Meta II-Zustands stellt seinerseits einen wichtigen
Schritt dar, um die Funktionstüchtigkeit der Photorezeptor-Zelle aufrecht zu erhalten. Dieser
Zerfall kann auf zwei grundverschiedene Weisen erfolgen: Entweder wird der aktivierende
Ligand all-trans-Retinal aus der Bindungstasche freigesetzt, wodurch das Apoprotein Opsin
entsteht, oder es kommt durch thermische Isomerisierung einer Doppelbindung des
gebundenen Liganden zur Ausbildung einer weniger aktivierenden Spezies und somit zum
Übergang nach Meta III. Letzteres wird in vitro bei neutralem bis alkalischen pH bevorzugt
gebildet. Meta III entsteht durch thermische Isomerisierung der Schiff-Basenbindung des
Retinals (C15=N). Hierbei wird der gebundene Ligand von all-trans 15-anti in das all-trans
15-syn-Isomer umgewandelt. Dadurch ergibt sich eine Änderung in der Liganden-Geometrie,
die wiederum eine inaktive Konformation des Rezeptors bewirkt. In dieser Arbeit wurde die
Meta III-Konformation über einen größeren pH-Bereich untersucht und aufgeklärt, dass
Meta III in einem pH-abhängigen Gleichgewicht existiert. Dieses Gleichgewicht stellt sich
zwischen der inaktiven Konformation bei neutralem bis alkalischem pH und einer aktiven,
dem Meta II-Zustand ähnlichen Konformation bei eher saurem pH ein. Der scheinbare pKₐ-
Wert dieses Übergangs liegt bei etwa 5,1. Dies stellt einen um einige Einheiten niedrigeren
pKₐ-Wert dar als der Wert, der für das Meta I/Meta II-Gleichgewicht, in welchem ein 15-anti-
Ligand vorliegt, bestimmt wurde. Dennoch ist er um etwa eine Einheit höher als der pKₐ-Wert
für das Konformationsgleichgewicht des Opsins, d. h. wenn überhaupt kein Ligand anwesend
ist. Beim all-trans-15-syn-Chromophor handelt es sich also um keinen inversen Agonisten,
der wie 11-cis oder 9-cis-Retinal eine inaktive Konformation des Rezeptors erzwingt, sondern
um einen klassischen partiellen Agonisten. Dieser ist zwar in der Lage den Rezeptor zu
aktivieren, hat aber eine deutlich geringere Effizienz als der volle Agonist all-trans-15-anti-
Retinal. Da sich der Meta III-Chromophor von dem vollen Agonisten strukturell nur über die Isomerie der C15=N-Bindung unterscheidet, stellt er ein ausgezeichnetes Modellsystem dar, um prinzipielle Mechanismen des partiellen Agonismus zu untersuchen. Das Verständnis dieser Mechanismen kann letztendlich sehr hilfreich sein, um den partiellen Agonismus anderer Liganden zu verstehen.

7.2 Untersuchungen zum Aktivierungsmechanismus


8. References


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