Resonance Raman spectroscopy on microbial rhodopsins

vorgelegt von
M.Sc.
Sara Bruun
aus Blomskog

von der Fakultät II – Mathematik und Naturwissenschaften
der Technischen Universität Berlin
zur Erlangung des Akademischen Grades
Doktor der Naturwissenschaften
Dr. rer. nat.

genehmigte Dissertation

Promotionsausschuss:

Vorsitzender: Prof. Dr. Reinhard Schomäcker
Berichter: Prof. Dr. Peter Hildebrandt
Berichter: Prof. Dr. Peter Hegemann

Tag der wissenschaftlichen Aussprache: 11.04.2013

Berlin 2013
D83
“He woke up, the room was bare
He didn’t see her anywhere
He told himself he didn’t care, pushed the window open wide
Felt an emptiness inside to which he just could not relate
Brought on by a simple twist of fate”

Bob Dylan “Simple twist of fate” 1974
Abstract

Resonance Raman spectroscopy has been employed to determine the chromophore structure in the dark and intermediate states of various microbial rhodopsins, i.e., channelrhodopsin-2 (ChR2), histidine kinase rhodopsin-1 (HKR1), and xanthorhodopsin (XR). Assignment of the structure was mainly accomplished by the comparison with bacteriorhodopsin (BR) spectra of states with known conformation of the retinal. ChR2 was studied both at ambient and cryogenic temperatures, using different excitation conditions to preferentially probe the dark state and the intermediates P500 and P390. Global analysis of the spectra in the C=C stretching region, using Lorentzian functions, revealed that the dark state (D470) consists of two different isomers, presumably all-trans, 15-anti and 13-cis, 15-syn, both including a protonated Schiff base, as in the case of dark-adapted BR and Rh-BI in HKR1. The photocycle is predominantly initiated by 13-trans/cis isomerisation to afford 13-cis, 15-anti retinal in the photocycle intermediates as found for the P390 state in the slow-cycling mutant ChR2-C128S-D156A. Photoisomerisation of the 13-cis isomer of the dark state appears to be less efficient if it takes place at all. Analysis of the C=NH+ stretching modes of both dark state isomers indicate a closer proximity of a water molecule to the Schiff base bond in the case of the dark state all-trans isomer as compared to the 13-cis isomer. The photocycle of ChR2-C128T includes a side-reaction involving the reversible hydrolysis of the retinal Schiff base bond (P353). Regardless of the similarities in the fine-structured absorption pattern, the retinal configuration in P353 is not identical to a reduced and UV-treated chromophore state in BR as shown by resonance Raman spectroscopy. The retinal configuration in the dark state (Rh-UV) of the histidine kinase rhodopsin-1 (HKR1) was found to be 13-cis, 15-anti with a deprotonated RSB, similar to the M-intermediate of BR but thermally stable. Photoconversion of Rh-UV to a stable meta state (Rh-BI) is associated with a 13-cis/trans or 15-anti/syn isomerisation resulting in two retinal isomers (all-trans, 15-anti and 13-cis, 15-syn) as identified in the resonance Raman spectra. XR contains a photoinactive carotenoid, salinixanthin, in addition to the retinal chromophore, with an absorption spectrum that largely obscures that of the retinal chromophore. Using an excitation line at the long-wavelength side of the absorption envelop identifies most of the resonance Raman bands of the retinal to an all-trans, 15-anti configuration with a protonated retinal Schiff base in accordance with most microbial rhodopsins.
Zusammenfassung

Publications

Parts of this work have been published in:

Luck, M., Mathes, T., Bruun, S., Fudim, R., Hagedorn, R., Nguyen, T. M. T., Kateriya, S., Kennis, J. T. M. Hildebrandt, P. and Hegemann, P. *A photochromic histidine kinase rhodopsin (HKRI) that is bimodally switched by UV and blue light*. JBC 287 (2012) 40083-90


# Index

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>1</td>
</tr>
<tr>
<td>Zusammenfassung</td>
<td>2</td>
</tr>
<tr>
<td>Publications</td>
<td>4</td>
</tr>
<tr>
<td>Introduction and motivation</td>
<td>9</td>
</tr>
<tr>
<td>1. Theoretical background</td>
<td>13</td>
</tr>
<tr>
<td>1.1 Microbial rhodopsins</td>
<td>14</td>
</tr>
<tr>
<td>1.1.1 Bacteriorhodopsin</td>
<td>18</td>
</tr>
<tr>
<td>1.1.2 Xanthorhodopsin</td>
<td>20</td>
</tr>
<tr>
<td>1.1.3 Rhodopsins in <em>Chlamydomonas reinhardtii</em></td>
<td>21</td>
</tr>
<tr>
<td>1.1.3.1 Channelrhodopin-2</td>
<td>22</td>
</tr>
<tr>
<td>1.1.3.2 Histidine kinase rhodopsin-1</td>
<td>26</td>
</tr>
<tr>
<td>1.2 Raman spectroscopy</td>
<td>28</td>
</tr>
<tr>
<td>1.2.1 Vibrational spectroscopy</td>
<td>28</td>
</tr>
<tr>
<td>1.2.2 Resonance Raman scattering</td>
<td>30</td>
</tr>
<tr>
<td>1.2.3 Resonance Raman spectroscopy on microbial rhodopsins</td>
<td>33</td>
</tr>
<tr>
<td>1.2.3 Vibrational modes in retinal proteins</td>
<td>35</td>
</tr>
<tr>
<td>2. Materials and Methods</td>
<td>37</td>
</tr>
<tr>
<td>2.1 Heterologous expression of channelrhodopsin-2</td>
<td>38</td>
</tr>
<tr>
<td>2.1.1 Transformation in <em>Pichia pastoris</em></td>
<td>38</td>
</tr>
<tr>
<td>2.1.1.1 Construction of plasmid</td>
<td>38</td>
</tr>
<tr>
<td>2.1.1.2 Transformation</td>
<td>39</td>
</tr>
<tr>
<td>2.1.1.3 Phenotype and multicopy test</td>
<td>39</td>
</tr>
<tr>
<td>2.1.2 Cell cultivation</td>
<td>39</td>
</tr>
<tr>
<td>2.1.3 Purification of channelrhodopsin-2</td>
<td>40</td>
</tr>
<tr>
<td>2.1.3.1 Exchange of the buffer for the spectroscopic measurements</td>
<td>41</td>
</tr>
<tr>
<td>2.1.3.2 Deglycosylation protocol</td>
<td>41</td>
</tr>
<tr>
<td>2.2 Reduction of the retinal in bacteriorhodopsin</td>
<td>43</td>
</tr>
<tr>
<td>2.3 Optical and vibrational spectroscopy</td>
<td>44</td>
</tr>
<tr>
<td>2.3.1 UV-vis absorption spectroscopy</td>
<td>44</td>
</tr>
<tr>
<td>2.3.2.1 Conventional setup</td>
<td>44</td>
</tr>
<tr>
<td>2.3.2.2 Flash photolysis</td>
<td>44</td>
</tr>
<tr>
<td>2.3.2 The Raman setup</td>
<td>45</td>
</tr>
</tbody>
</table>
3. Results and discussion ................................................................. 53

3.1 Channelrhodopsin-2 ................................................................. 54
  3.1.1 ChR2-H134R ........................................................................ 55
    3.1.1.1 Optimisation of the biochemical procedures .................. 55
    3.1.1.2 N-glycosylation of channelrhodopsin-2 ......................... 57
    3.1.1.3 Kinetics of the P500, P390 and P520 intermediates ......... 59
    3.1.1.4 pH-dependent kinetics of the P480$_{ab}$ intermediates ...... 61
    3.1.1.5 Trapping of early intermediates at cryogenic temperatures .. 63
    3.1.1.6 Resonance Raman spectroscopy at cryogenic temperatures .. 65
    3.1.1.7 Resonance Raman spectroscopy at room temperature ....... 70
    3.1.1.8 Summary ..................................................................... 80
  3.1.2 ChR2-C128T ....................................................................... 82
    3.1.2.1 The UV-absorbing intermediates .................................. 83
    3.1.2.2 The chromophores of P380 and P353 ............................ 85
  3.1.3 ChR2-C128S-D156A ............................................................. 90
    3.1.3.1 The dark and UV-absorbing states ................................ 90
    3.1.3.2 Cryo-trapping of P500 .................................................. 92
    3.1.3.3 Resonance Raman spectroscopy at room temperature ...... 93
    3.1.3.4 Resonance Raman spectroscopy at cryogenic temperature .. 97
  3.1.4 Discussion ........................................................................... 98
    3.3.4.1 Isomeric configuration of the retinal chromophore in D470 .. 98
    3.3.4.2 Water and RSB interactions in D470 all-trans and 13-cis .... 101
    3.3.4.3 Introducing mutations close to the retinal ..................... 103

3.2 Histidine kinase rhodopsin-1 ...................................................... 108
  3.2.1 Retinal chromophores in Rh-UV and Rh-BI .......................... 108
  3.2.2 Light-induced structural changes of the protein backbone ...... 111
  3.2.3 Discussion .......................................................................... 112

3.3 Xanthorhodopsin ............................................................... 114
  3.3.1 The salinixanthin and retinal chromophores ....................... 114
  3.3.2 Accumulation of the L-intermediate in the laser spot .......... 116
  3.3.3 Summary .......................................................................... 117
Resonance Raman spectroscopy on microbial rhodopsins
Introduction and motivation

Photoreceptors can be divided into three main classes of proteins, namely rhodopsins, phytochromes and blue-light receptors including cryptochromes, LOV and BLUF proteins. With the onset of light stimuli, they initiate a wide range of functions such as vision in animals, the growth in the direction of the light in plants (phototropism), the locomotory movement of an organism (phototaxis) or are involved in the circadian clock which is the oscillations of biological processes in dependence of day and night [1]. Rhodopsins are membrane proteins consisting of seven α-helices and a retinal co-factor and they appear in the entire phylogenic tree of life. The first rhodopsins were found in the retina of animals. All so far known animal rhodopsins function as G-protein coupled receptors where the absorption of light is the initial step in a signal transduction leading to vision. Over 40 years ago, Walther Stoeckenius and Dieter Oesterhelt discovered that the archeon Halobacterium halobium contains a rhodopsin arranged in a hexagonal pattern in the membranes [2,3]. It was named bacteriorhodopsin (BR) and it functions as a proton pump creating a proton gradient that drives the synthesis of ATP, thus it facilitates the conversion of light into chemical energy. Since the discovery of BR several other rhodopsins have been found in archa but also in bacteria, fungi and algae. Many of these microbial rhodopsins, like BR, function as proton pumps but some are classified as sensory proteins which can bind to transducer proteins. In the beginning of this century Georg Nagel, Ernst Bamberg and Peter Hegemann proved that two rhodopsins, named channelrhodopisin-1 and -2, from the microalga Chlamydomonas reinhardtii function as light-gated cation channels with the ability to depolarise the plasma membrane in the eyespot which triggers a photophobic response [4,5]. A few years later Karl Deisseroth and co-workers managed, after lentiviral transfection, to express channelrhodopsin-2 (ChR2) in hippocampal neurons of rats and observed neuronal spiking with the onset of light pulses [6]. Shortly after, ChR2 was expressed in embryonic chick spinal cords [7] and in the mechanosensory neurons of the nematode (worm) Caenorhabditis elegans which caused a withdrawal behaviour upon light stimuli [8]. This pioneering work has now grown into a new broad research field called optogenetics and it offers advantages compared to the traditionally used techniques in neuroscience which rely on electrical stimulation or drugs [9]. The lentivirus including the ChR2 gene can be designed to induce expression in specific neurons whereas electrodes cannot discriminate between cells. When compared to the slow effect of drugs, ChR2 can trigger neuronal firering with the temporal...
Resonance Raman spectroscopy on microbial rhodopsins

precision defined by the light pulse. ChR2 as well as other microbial rhodopsins (BR and halorhodopsin) are now used to activate or deactivate neurons in tissues or living animals such as mice or rats [10,11], Drosophila (fly) larvae [12], and zebrafish [13]. Expression and activation of ChR2 in neurons of mice have helped researchers to understand different functions and diseases of the brain, in medium spiny projection neurons in mice suffering from Parkinson’s it reduced the symptoms of the disease [14], in a part of the brain normally assigned to the reproduction it triggered an attack behaviour [15], in the medial prefrontal cortex of the brain it had an anti-depressive like effect [16] and in inner retinal neurons it restored perception of light in blind mice [17]. ChR2 has also been used to stimulate heart muscle [18]. The list of examples is long and the discovery of ChR2 and the implementation in neuroscience is likely to contribute to the development of therapy for neuronal diseases.

ChR2 undergoes a photocycle after the absorption of blue light of the retinal chromophore. The light-induced all-trans to 13-cis isomerisation of the retinal leads to structural rearrangements of the entire protein where at one point in the photocycle a cation conducting pore is formed. The cations are passively transported to the intracellular side of the plasma membrane under physiological conditions. The channel spontaneously closes and the retinal re-isomerises correlated with the re-formation of the dark state. [19–21] In the native alga, it was found that the configuration of the functional chromophore is all-trans retinal [22]. Extraction of the retinal in heterologously expressed and purified ChR2 and following HPLC analysis showed additional contributions of an 13-cis isomer in dark state which cannot be assigned to an unbound retinal due to the fine-structure in the absorption spectrum [23,24]. However, it might be related to the BR dark-state, which consists of two different isomers, beside all-trans, 15-anti also additional 13-cis, 15-syn that differs from the 13-cis, 15-anti present in the photocycle intermediates [25–27]. Light-adaptation of BR removes 13-cis, 15-syn and only the all-trans form is observed in this so-called light-adapted dark state, as demonstrated by extraction experiments and UV-vis absorption spectra as a shift of the absorption maximum [25,26]. Dark-adaptation of BR re-establishes the mixed isomeric state due to a thermal equilibrium between the two isomers. ChR2, on the other hand, shows almost no difference in the extraction or resonance Raman experiments after light-adaptation [23,24] as well as in the UV-vis absorption and FTIR spectra after long periods of dark-adaptation [19–21]. It implies that the 13-cis form is either inactive or both isomers are immediately formed after completing the photocycle. Beside the extraction experiments, the configuration of the retinal can be determined by using NMR spectroscopy or X-ray diffraction on crystals.
However, this depends on the availability of crystals which are often hard to obtain. Recently, the first crystal structure of C1C2, a chimera of channelrhodopsin-1 and 2, was published which showed an all-trans, 15-anti retinal [28]. Thus, the involvement of the second isomer in the dark state is still not clear.

Raman spectroscopy selectively probes chromophores and might be applied as an additional technique for assigning the structure of the retinal. It is based on the excitation of molecular vibrations by using monochromatic (laser) light. This leads to a shift in the wavelength of the incident photons correlated with a vibrational transition which in turn depends on the configuration of the retinal. The intensity of the vibrational bands can be enhanced by choosing a wavelength matching the transition into the first electronically excited state, i.e. close to the absorption maximum of the retinal. This is called resonance Raman spectroscopy, for a review on the application of vibrational spectroscopy on biological samples see [29]. Several disadvantages concerning Raman spectroscopy have to be taken into consideration. Unless isotopically labelled retinals are available the assignment of the structure often requires that the spectra show similarities to other well-characterised rhodopsins. Furthermore, large amounts of protein is needed since the laser will trigger the photocycle, thus the sample has to be exchanged in a flow system. However, the main advantage of Raman spectroscopy is that only the chromophore is being detected and the intermediate states can be followed by using a time-resolved pump-probe setup. In this work, resonance Raman spectroscopy was employed to study the chromophores in the dark and intermediate states of ChR2 mutants widely used in the optogenetic research, with the main objective to identify the retinal configuration in these states. The work was also extended to other microbial rhodopsins, the carotenoid-containing xanthorhodopsin from the eubacterium Salinibacter ruber, a reduced chromophore in BR and the histidine kinase rhodopsin-1 from Chlamydomonas reinhardtii.
1. Theoretical background
1.1 Microbial rhodopsins

Retinylidene proteins, also called rhodopsins, belong to a family of photoreceptors consisting of seven α-helices stretching throughout the cell membrane. The helices are connected by intra- and extracellular loops. Rhodopsins absorb visible light corresponding to an electronic excitation of a retinal (vitamin A aldehyde) chromophore. The retinal is covalently linked to a lysine (Lys, K) in helix VII via a Retinal Schiff Base (RSB) and embedded within the helices in a cavity referred to as the chromophore pocket. Free retinal absorbs in the UV whereas for retinal bound to rhodopsin the absorption is shifted towards the red, denoted as the opsin-shift. The isomerisation of the retinal induces changes in the arrangement of the helices which in turn allows the protein to transform light into chemical energy or sensory and regulatory signalling of the organism. The wavelength for the maximum absorption of the rhodopsin depends mainly on the electrostatic interactions between the positively charged protonated RSB and a counter ion which consists of a negatively charged and deprotonated aspartic or glutamic amino acid residue. However, also the polarity of the amino acids surrounding the entire chromophore is important since it defines the planarity and the level of electron delocalization in the retinal chain. Based on the amino acid sequence and the arrangement of the helices, the rhodopsins are divided into two different sub-groups. [30]

![Figure 1. Schematic representation of the microbial rhodopsin channelrhodopsin-2 (ChR2) from the alga Chlamydomonas reinhardtii. Adapted with permission from Peter Hegemann.](image)
Animal or type II rhodopsins are found in the eyes, brain or other tissues and they are involved in vision of vertebrates and invertebrates. Type II rhodopsins belong to the family of so-called G-protein coupled receptors (GPCR). The best studied example is rhodopsin from the bovine outer rod segment membranes in the eye retina. Light (absorption maximum is around 500 nm) induces an 11-cis to all-trans isomerisation of the retinal (linked to K296) (Figure 2) and the photoinduced process includes several intermediate states before the RSB is hydrolysed and all-trans retinal is released from the protein. Enzymes are required for the re-assembly of opsin and 11-cis retinal to recover the functional photoreceptor. One of the intermediates in the photocycle, Meta II, has an all-trans deprotonated RSB. In this state rhodopsin binds to a G-protein and cGMP is hydrolysed. The reduction of the cGMP level in the cell leads to the closing of a cGMP-gated Na⁺/Ca²⁺ channel and the photoreceptor cell is hyperpolarized. This signal is then transferred to the brain via the ganglion cells. Other rhodopsins with different absorption maxima enable the discrimination of different colours. [31–33]

Microbial or type I rhodopsins are found in archaea and bacteria but also in eukaryote such as fungi and algae. The first discovered microbial rhodopsin was the light-driven proton-pump bacteriorhodopsin (BR) from the archeon Halobacterium salinarum. BR pumps one proton out of the cell per photocycle and the resultant electrochemical gradient drives the synthesis of ATP by ATP synthase. Other rhodopsins were later found in Halobacterium salinarum, i.e. halorhodopsin (HR) and sensory rhodopsin (SR). The structures and functions of these rhodopsins have been thoroughly characterised. Halorhodopsin is an inward-directed chloride pump with the ability to hyperpolarize the cell membrane. The sensory rhodopsins I or II are involved in the phototaxis, the ability of the organism to sense changes in the intensity of the light and respond to different wavelengths and thus move towards or away from the light-source. The signal transferred to the flagella motor is induced during the photocycle by the formation of a complex between the sensory rhodopsins and transducer proteins. Unlike visual rhodopins, the retinal in microbial rhodopsins isomerises from all-trans to 13-cis (Figure 2). No hydrolysation of the RSB is involved and the chromophore remains inside the protein throughout the entire photocycle. [1,30,34,35]

Analogues to BR with the ability to translocate protons using light have been found in various organisms. Examples are proteorhodopsin (PR) from the γ-Proteobacteria (SAR86) [36], Archaerhodopsin-1 and -2 (aR-1 and -2) from the archeon Halorubrum sp. aus -1 and -2 [37],
Gleobacter rhodopsin (GR) from the cyanobacterium *Gleobacter violaceus* [38], Leptosphaeria rhodopsin (LR) from the fungus *Leptosphaeria maculans* [39,40] and Acetabularia rhodopsin (AR) from the alga *Acetabularia acetabulum* [41]. The following chapter includes an introduction to four microbial rhodopsins with quite different functions. The already mention BR as well as another proton-pump xanthorhodopsin from the archaeon *Salinibacter ruber*. The latter shows an extended absorption range due to a second carotenoid chromophore. Furthermore, two rhodopsins from the alga *Chlamydomonas reinhardtii* will be described; the newly discovered histidine kinase rhodopsin-1 and the cation conducting channelrhodopsin-2.
Figure 2. Isomeric configurations of the retinal chromophore that are naturally found in type I and type II rhodopsins. The retinal is covalently linked to a lysine (Lys) via a Schiff base bond and all structures are shown with a protonated Schiff base. The dark-adapted state of the microbial (type I) bacteriorhodopsin consists of both all-trans, 15-anti and 13-cis, 15-syn whereas the retinal in most photocycle intermediates are found in the 13-cis, 15-anti form. The dark-adapted state of bovine rhodopsin (type II) show instead a 11-cis, 15-anti retinal (note the different orientation of the ionone ring) and the photocycle is initialised by 11-cis to all-trans isomerisation.
1.1.1 Bacteriorhodopsin

The proton-pumping (26.8 kDa) membrane protein bacteriorhodopsin (BR), which is the most widely studied microbial rhodopsin, was discovered and first studied by Allen E. Blaurock, Dieter Oesterhelt and Walther Stoeckenius in the 70’s [2,42]. The light-driven proton translocation and resultant proton gradient is used to drive the synthesis of ATP (phosphorylation of ADP) which acts as an energy source in the cell metabolism [3]. BR is organised in a hexagonal arrangement mixed with lipids in membrane patches of the archeon *Halobacterium salinarum* and these so-called purple membranes can be readily isolated. Due to the extensive research conducted on this protein it is often used as a reference when the function, structure and photocycle are being investigated of newly discovered microbial rhodopsins.

The BR photocycle is initialised by absorption of (green) light leading to an electronic excitation of the retinal. The next steps include isomerisation and thermal relaxation of the chromophore ($J_{625}$, $K_{590}$ and $L_{550}$) as well as deprotonation of the Schiff base ($M_{410}$) that covalently links the retinal to Lys216 (Figure 3). The proton from the RSB is transferred to the aspartic acid 85 (Asp85, located at the extracellular side compared to the retinal) via a water molecule. This is the start of a translocation of protons facilitated by a complex network of charged residues and water. Eventually the Schiff base is again re-protonated ($N_{560}$) by Asp96 which requires a so-called switch of the RSB towards the intracellular side in the later M-state [43,44]. The net transfer per photocycle (and absorbed photon) is one proton being released on the extracellular and one taken up on the intracellular side of the membrane. Deprotonation at one residue leads to a coupled protonation at another and this transfer of charge also induce structural alterations of the protein backbone and changes in the pKa values. [45–48]

Extraction of the retinal and NMR spectroscopy showed that dark-adapted BR$_{560}$ have two different isomeric configurations of the retinal, namely all-trans, 15-anti and 13-cis, 15-syn, both with a protonated RSB [25–27]. However, light-adaptation leads to the presence of only one isomer in the dark state. In this so-called light-adapted dark-state (BR$_{57}$) the configuration of the retinal is exclusively in the all-trans form. The light-induced isomerisation of all-trans, 15-anti into 13-cis, 15-anti (different from 13-cis, 15-syn in the dark-adapted state) initialises the photocycle. At the end of the cycle this process is reversed.
(in O$_{640}$), by thermal $13$-cis to trans isomerisation. Long periods of dark-adaptation are necessary to thermally recover the mixed isomeric dark state of BR seen as a shift in the absorption maximum [25]. The structure of the chromophores in the dark-state of BR can be easily characterised since these isomers are thermally stable. The description of the retinal configuration throughout the photocycle is more complicated. The full $13$-trans/cis isomerisation is strictly speaking only achieved in the later M-state when the Schiff base is facing towards the intracellular side whereas the preceding intermediates are in a distorted $13$-cis configuration. The crystal structures of the intermediates do not always provide a clear answer to the exact structure of the retinal, for instance in the case of the L intermediate where different experiments generated different configurations [49–52]. However, many crystal structures are now available for the dark-adapted [53,54] and intermediate states [55] of BR which, together with other techniques, has contributed to the elucidation of the proton translocation during the photocycle.

![Figure 3. Photocycle of BR showing the sequential order of the intermediates after the light-induced isomerisation of the retinal and the proton release and uptake from the extracellular and intracellular side, respectively. The isomeric state of the retinal in the dark-states is $13$-cis, $15$-syn in bR$_{555}$ and all-trans, $15$-anti in bR$_{568}$, both with protonated RSB. Adapted with permission from [56]. Copyright (2008) National Academy of Sciences, USA.](image)
1.1.2 Xanthorhodopsin

Xanthorhodopsin (XR) shares homology with and has a similar photocycle as BR. It functions as a proton pump in the eubacterium *Salinibacter ruber* and it can be purified directly from the native membranes [57]. However quite distinctive for XR is the presence of a second chromophore, a C₄₀ carotenoid called salinixanthin containing a β-D-glycoside and an acyl tail, that is not covalently linked to the protein (Figure 4A) [58]. The Schiff base bound retinal and the salinixanthin are found in the protein with a 1:1 stoichiometry. The crystal structure revealed that the keto ring of the salinixanthin is located within *van der Waals* distance to the ionone ring of the retinal [59]. This facilitates the so-called light-harvesting function – the energy absorbed by the salinixanthin is transferred with around 40% quantum efficiency from the S₂ state in salinixanthin to the S₁ state in retinal which eventually leads to isomerisation of the latter and the photocycle is initialised [57,60]. Thus, salinixanthin broadens the spectral absorption of the protein beyond that of the retinal alone. The retinal absorbs in the green (~560 nm) and is seen as a shoulder of the salinixanthin fine-structured absorption with peaks at 458, 486 and 521 nm (Figure 4B). For review see [61].

![Figure 4](image_url)

*Figure 4.* (A) The molecular structure of salinixanthin includes a carotenoid with a 4-keto group, a β-D-glycoside and an acyl tail. Reprinted with permission from [58]. Copyright (2002) American Chemical Society. (B) Absorption spectrum of xanthorhodopsin in the native membranes of *Salinibacter ruber*. The solid and dotted lines represent the absorption spectra before and after hydroxylamine treatment, respectively. The retinal chromophore is removed by hydroxylamine due to the transformation into a retinal oxime correlated with a shift of the absorption from ~567 to 367 nm. The fine-structured pattern with peaks at 458, 486 and 521 nm corresponds to the salinixanthin and it is partly lost upon removal of the retinal. Adapted with permission from [60]. Copyright (2008) Elsevier.
1.1.3 Rhodopsins in *Chlamydomonas reinhardtii*

Eight rhodopsin genes (chlamyopsins or COP) are found in the genome of the single cellular alga *Chlamydomonas reinhardtii*. Whereas COP1 and 2 (both transcribed from the COP1 gene) show homology to vertebran rhodopsins, COP3 and 4 are more microbial-like and functions as light-gated cation channels (channelrhodopsin-1 and 2). COP5-8 are enzymatic rhodopsins and they include a histidine kinase, response regulator and sometimes a cyclase at the c-terminal end. At least COP1-5 are located in the plasma membrane of the *C. reinhardtii* eyespot (Figure 5). [62,63] Channelrhodopsin-1 and 2 (ChR1 and ChR2) and the histidine kinase rhodopsin-1 (HKR1, COP5) have been heterologously expressed in for instance *Xenopus laevis* oocytes, human embryonic kidney (HEK) cells, COS (monkey kidney) cells or the yeast *Pichia pastoris* which enabled researchers to characterise these proteins using electrophysiology, spectroscopy, microscopy and recently X-ray diffraction.

![Figure 5](image_url)

*Figure 5.* The microalgae *Chlamydomonas reinhardtii*. The chlamyopsins are localized in the orange eyespot (white arrow). Under physiological conditions Ca\(^{2+}\) ions are transported into the cell facilitated by light-gated cation channels and it activates voltage sensitive channels in the flagella (black arrow) membrane. The subsequent influx of Ca\(^{2+}\) into the flagella leads to a switch of the movement (phototaxis). [63] Scale bar 10 µm. Reprinted with permission from [64]. Copyright (2009) Springer.
1.1.3.1 Channelrhodopin-2

Channelrhodopsin-1 and 2, located in the eyespot of the *C. reinhardtii*, have the ability to depolarize the cell membrane upon light stimuli and are responsible for the absorption of light connected to the phototaxis of the alga – forcing it towards or away from the light source depending on the light intensity and colour [65–72]. Studies in blind *C. reinhardtii* cells report that the main configuration of the chromophore for the rhodopsins involved in the phototaxis is all-trans retinal [22]. Light isomerises all-trans into 13-cis [73,74] although some literature suggest an alternative with a polarisation of the amino acids in the chromophore pocket caused by the excited retinal initiating the photocycle rather than the isomerisation [75,76]. In either case, the light-induced depolarisation of the cell membrane triggered by light-absorption of the retinal is the initial step in a signal transduction that in the end induces a switch in the flagella motion of the alga. The depolarisation is facilitated by a protein channel selective for cations, in the case of channelrhodopsin-1 (ChR1) primarily H⁺ and for channelrhodopsin-2 (ChR2) others as well such as K⁺, Na⁺ and Ca²⁺ [4,5]. Under physiological conditions, the cations are transported to the intracellular side of the plasma membrane. ChR2 consists of, beside the seven trans-membrane (TM) helices, a cytosolic tail at the c-terminus which may be important for the localization of the protein inside the eyespot [77]. However, the photocurrent of the full-length ChR2 1-737, expressed in *Xenopus laevis* oocytes and recorded by voltage-clamp experiments, is indistinguishable from the truncated version ChR2 1-315 [5]. Thus the shorter version is sufficient for studies where the interest lies in the structure and dynamic of the seven membrane helices, the cation channel and the retinal chromophore. Over the years, several mutants [78–82] and channelrhodopsin chimeras [83–85] have been developed for the new and rapidly growing field of optogenetics. Wild-type ChR2 as well as these specially designed channelrhodopsin variants are used to efficiently depolarize the cell membranes in neuronal tissue or living animals in order to trigger signalling between cells with the temporal precision of the light source [86–88], for instance in hippocampal neurons of rats [6] or in the nematode *Caenorhabditis elegans* [8]. The virus including the ChR2 sequence used for the transfection can be constructed such that specific neurons are targeted. ChR2 has also been shown to be a promising candidate in the first attempts for the restoration of vision [17].

An electron microscope projection study using 2D crystals provided a structure with 6 Å resolution of recombinant ChR2-C128T purified from the yeast *Pichia Pastoris* [89]. Recently, the crystal structure was solved at 2.3 Å resolution using X-ray diffraction for the
chimera C1C2 expressed in insect cells [28]. C1C2 consists of the first five TM helices from ChR1 and the last two from ChR2. However, the high homology between ChR1 and ChR2 implies that the positions of the amino acids are likely to be the same. Both studies found channelrhodopsin as a dimer with connections at the TM3 and TM4. Calculations of the electrostatic surface potential indicate that the cation channel is constituted of an electronegative pore located between TM1, TM2, TM3 and TM7 [28]. E90 and E87 (in ChR2) are important residues for the ion flux [90–93] and ChR2 lacks several serines responsible for intrahelical interactions in BR which may be the reason for the large pore-diameter necessary for the channeling function [94]. Furthermore, the position of TM1 and TM2 are quite different with respect to BR and many of the negatively charged residues from the pore are found in TM2 [28,95].

![Figure 6](image_url)

*Figure 6. Left; crystal structure of C1C2 focussed on the environment of the protonated Schiff base (blue) in the *all-trans, 15-anti* retinal chromophore (purple). Right; the same region in the crystal structure of BR. Reprinted with permission from [28]. Copyright (2012) Nature Publishing Group.*

The X-ray crystal structure gives detailed information about the retinal chromophore covalently bound to Lys296 (Lys257 in ChR2) and its hydrophobic pocket consisting of five aromatic residues. The best fit was obtained using a chromophore in the *all-trans, 15-anti* configuration consistent with extraction and resonance Raman experiments [22–24]. The carboxylic amino acids E162 (E123 in ChR2) and D292 (D253 in ChR2) are located at a distance of 3.4 and 3.0 Å from the retinal Schiff base (RSB), respectively, which is closer than the adjacent water molecule (4.4 Å) (Figure 6; left). This is different from BR where the water
Resonance Raman spectroscopy on microbial rhodopsins is closer to the RSB and it indicates that in ChR2 there is a direct proton transfer from the RSB to one of the carboxylic acids without involvement of the water. Calculations showed that E162 but not D292 might be protonated. Thus only D292 is negatively charged and can act as the counter-ion for the protonated RSB [28]. In contradiction, ultra-fast spectroscopy showed that E123 (E162 in ChR1) is negatively charged [96]. Regardless of the protonation state of E162, electrophysiological studies of C1C2 with mutations at these positions point out D292 to be the proton acceptor rather than E162 [28]. D292 in C1C2 is located 1.3 Å closer to the RSB than its counterpart in BR (D212) which may be one of the reasons for the blue-shifted absorption (hypsochromic shift) of ChR1 and ChR2 compared to BR (Figure 6) [28,97]. The cysteine-167 (C128 in ChR2) in TM3 and aspartic acid-195 (D156 in ChR2) in TM4 are located in the chromophore pocket. Mutations at these positions drastically reduce the kinetics of the photocycle [21,24,78,98] which was suggested to be related to an interhelical hydrogen bond between the sulfhydryl in C128 and carbonyl in (the protonated) D156 which is disrupted in the mutants [98,99]. However, this hydrogen bond could not be confirmed either by QM-MM calculations [95] or the crystal structure [28] and it is inconsistent with electrophysiological data [83].

The photocycle of ChR2 has been extensively studied using absorption and vibrational spectroscopy [19–21,100]. The photocycle is initiated by light absorption correlated with electronic excitation and polarisation of the retinal chromophore (Figure 7; top). The chromophore relaxes by isomerising, most likely from all-trans to 13-cis resulting in the first intermediate P500 which is slightly red-shifted compared to the dark state D470 (the numbers denotes the absorption maximum) [23]. On the time-scale of microseconds the RSB is deprotonated upon formation of the UV-absorbing P390 intermediate (counterpart of M_{410} in the BR photocycle) which is also connected to structural changes of the protein backbone. P390 is in an equilibrium with the conducting P520 state where the RSB is again re-protonated. Beside the passive transport of cations, which is under physiological conditions directed towards the intracellular side, ChR2 has been proposed to act as a weak proton translocator where the proton on the RSB leaves on the extracellular side and upon formation of P520 taken up on the intracellular side [101,102]. The closing of the channel (in milliseconds) involves the two intermediates P480_a and P480_b with absorptions similar to D470 and the structural rearrangements of the protein helixes (enabling the conduction of cations) are reversed. The photocycle is complete after roughly a minute which is considerably slower than the proton-pumping rhodopsins. A two-cycle model has been
proposed on the basis of the channelrhodopsin photocurrents and it involves two open states of the channel with different ion selectivities [103–107] (Figure 7; bottom). So far spectroscopy provided no evidence for multiple cycles, thus the sequential order and the absorption and vibrational properties of the intermediates may be almost identical in the two cycles [20].

Figure 7. Top; photocycle of ChR2 showing the sequential order of the photocycle intermediates (P500, P390, P520 and P480\textsubscript{ab}) after the retinal absorption of light in the dark-state (D470). Adapted with permission from [20]. Copyright (2008) American Society for Biochemistry and Molecular Biology. Bottom; the two-cycle model involving two dark-states (D470 and desensitised Des480) as well as two open states (O\textsubscript{1} and O\textsubscript{2}). Reprinted with permission from [106]. Copyright (2010) John Wiley and Sons.
1.1.3.2 Histidine kinase rhodopsin-1

Histidine kinase rhodopsin-1 (HKR1) consists of the rhodopsin, histidine kinase, response regulator and cyclase domains [108]. The histidine kinase is phosphorylated by ATP and the phosphate group transferred via the response regulator to produce cyclic AMP or GMP. Whether HKR1 function as a guanylyl or adenylyl cyclase is not known yet since so far it has not been possible to reconstitute the functional full-length protein in either Pichia Pastoris or Xenopus laevis oocytes. However the rhodopsin part of HKR1 has successfully been expressed in P. Pastoris, purified, solubilised in detergent and characterised using absorption spectroscopy, stationary as well as time-resolved in the range from femtoseconds up to minutes [108]. The rhodopsin part of HKR1 shows an interesting bimodal behaviour switching from the dark state absorbing in the UV (Rh-UV; \( \lambda_{\text{max}} = 380\text{nm} \)) to a stable meta state absorbing in the blue (Rh-Bl; \( \lambda_{\text{max}} = 490\text{nm} \)) (Figure 8). Rh-UV is transferred to Rh-Bl within milliseconds after UV exposure via an intermediate with similar absorption (Rh380`). The back-reaction triggered by illumination in the blue follows, compared to other microbial rhodopsins, a more classical photocycle pathway, including two red-shifted intermediates (P560 and P570) (Figure 9). The large photochromic shift between these two stable states and the fact that the dark state is absorbing in the UV has previously not been reported for microbial rhodopsins. The light-induced conversion of Rh-UV and Rh-Bl may represent an on/off switching of the enzymatic activity. Photochromism has previously been observed in retinochromes [109]. UV-absorbing retinol acts as an additional pigment in fly rhodopsin where the absorbed energy is transferred to the bound retinal [110]. Anabena sensory rhodopsin shows a small photochromic shift between the dark- and light-adapted states consisting predominantly of an all-trans (\( \lambda_{\text{max}} = 549 \text{ nm} \)) and 13-cis (\( \lambda_{\text{max}} = 537 \text{ nm} \)) retinal, respectively. The 13-trans/cis isomerisation is initiated by orange light and the 13-cis/trans by blue light. However after light-adaptation, this microbial rhodopsin thermally decays into the dark state in the time-scale of hours [111]. Bimodal photoconversion of the dark (\( \lambda_{\text{max}} = 590 \text{ nm} \)) and UV-absorbing intermediate (\( \lambda_{\text{max}} = 370 \text{ nm} \)) is reported for the slow-cycling sensory rhodopsin-1 in archaea. However, the UV-absorbing state is also not thermally stable and decays into the dark-state within a second [112].
Resonance Raman spectroscopy on microbial rhodopsins

Figure 8 UV-vis absorption spectra of Rh-UV and Rh-Bl. Reprinted with permission from [108]. Copyright (2012) American Society for Biochemistry and Molecular Biology.

Figure 9. Photocycle of HKR1 derived from flash photolysis, UV-vis and ultrafast absorption spectroscopy. UV absorption of the dark state Rh380 (Rh-UV) gives the intermediate Rh380' prior to formation of Rh485 (Rh-Bl). Subsequent blue-light exposure leads to accumulation of the first intermediate I1 correlated with two time-constants for the decay of the retinal excited state followed by two red-shifted intermediates (P560 and P570). The time-constants for the transitions are indicated. Reprinted with permission from [108]. Copyright (2012) American Society for Biochemistry and Molecular Biology.
1.2 Raman spectroscopy

1.2.1 Vibrational spectroscopy

Molecular vibrations are the periodic oscillations of the atoms within a molecule. Vibrational spectroscopy, i.e. infrared and Raman spectroscopy, can be implemented to characterise the frequencies of different molecular vibrational transitions which in turn depend on and can provide useful information concerning the structure of proteins. In infrared (IR) spectroscopy the molecular vibrations are excited by polychromatic light and the photons with the energy matching a vibrational transition are absorbed. Raman spectroscopy, on the other hand, requires monochromatic light and the photons are scattered by the electron cloud of the molecule. Most of the photons are left at the same energy (elastic scattering) referred to as the Rayleigh scattering (Figure 10). The remaining photons either gain or lose energy (inelastic scattering) correlated with a transfer to a lower (anti-Stokes) or higher (Stokes) vibrational state of the molecule, respectively. The law of conservation of energy implies that the difference in the energy of the incident \( (h\nu_0) \) and scattered photon \( (h\nu_R) \) represent that of the vibrational transition \( (h\nu_k) \).

\[
(1.1) \quad h\nu_0 - h\nu_R = h\nu_k
\]

In the measured IR or Raman spectrum, the absorption of photons or the intensity of the scattered photons depend on the probability of the vibrational transition. Absorption requires a change of the dipole moment whereas the inelastic scattering depends on the polarisability. Thus in IR and Raman spectroscopy, different molecular vibrations may be observed. Furthermore, the probability of inelastic scattering can be increased enormously by tuning the energy of the photons such that it matches the energy of a transition to an electronically excited state, referred to as the resonance Raman (RR) effect. In cofactor-containing proteins it can be achieved by choosing a wavelength close to the absorption maximum of the cofactor which in the case of retinal proteins is the \( \pi \rightarrow \pi^* \) transition of the polyene chain. The vibrational modes of rhodopsins can be studied by applying both techniques which generates complementary data. The strongest vibrational bands seen in the IR spectra represent the amide-I and amide-II vibrations of the protein backbone, whereas only the vibrational bands of the chromophore are observed in the resonance Raman spectra. In resonance Raman spectroscopy, the spectra are depicted by defining the x-scale as \( \Delta \nu \) (in cm\(^{-1}\)) where the
frequency of the incident light is zero. These can then be directly compared to the IR spectra. Normally the Stokes scattered light is detected since most of the molecules are already in the vibrational ground state.

Figure 10. The absorption and inelastic scattering of light leading to the vibrational transitions detected in infrared and Raman spectroscopy. Figure is taken from [113].
1.2.2 Resonance Raman scattering

The Raman effect was discovered in an experimental study performed by the Indian scientist Sir Chandrasekhara Venkata Raman in 1928 [114]. The Raman effect can be theoretically described using second order perturbation theory. The following derivations are adopted from [29,115,116].

A dipole moment ($\vec{\mu}$) is induced in a molecule by the magnetic field ($\vec{E}$) of an electromagnetic wave with the frequency $v_0$.

$$\vec{\mu} = \alpha(v) \cdot \vec{E}(v_0)$$  \hspace{1cm} (1.2)

It depends on the polarisability ($\alpha$) tensor of the molecule which varies with the movements of the electron cloud brought about by the oscillation of the nuclei. The polarisability tensor can be described by a Taylor series of normal coordinates $Q_k$, in this case for a non-linear molecule with 3N-6 normal modes

$$\alpha = \alpha_0 + \sum_{k=1}^{3N-6} \left( \frac{\delta \alpha}{\delta Q_k} \right)_0 Q_k + \cdots$$  \hspace{1cm} (1.3)

where the main Rayleigh scattering is described by $\alpha_0$. The intensity of the (frequency-shifted) Raman scattered light ($I_{\text{Raman}}$) is strong when the molecular polarisability changes along its normal coordinate.

$$I_{\text{Raman}} \propto \left( \left( \frac{\delta \alpha}{\delta Q_k} \right)_0 Q_k \right)^2$$  \hspace{1cm} (1.4)

The Raman cross section for the vibrational transition $i \rightarrow j$ is defined by the intensity of the incident ($I_0$) and scattered light ($I$).

$$\sigma_{ij} = \frac{I}{I_0}$$  \hspace{1cm} (1.5)

For the description of the Raman intensities and specifically for the resonance Raman effect, a quantum mechanical treatment of the polarisability or scattering tensor in Eq. (1.2) is
required. According to the Kramers-Heisenberg-Dirac’s dispersion theory, the matrix elements
of the scattering tensor are defined as

\[
(1.6) \quad [\alpha_{\rho \sigma}]_{if} = \frac{1}{\hbar} \sum_{r,R} \left( \frac{\langle Gi | M_\rho | Rr \rangle \langle Rr | M_\sigma | Gf \rangle}{v_{Rr} - v_0 + i\Gamma_R} + \frac{\langle Gi | M_\sigma | Rr \rangle \langle Rr | M_\rho | Gf \rangle}{v_{Rr} - v_0 + i\Gamma_R} \right)
\]

where \( \hbar \) is the Planck’s constant, \( R \) denote the electronic and \( r \) the vibrational states subject to
the summation, and \( Gi \) the initial and \( Gf \) the final vibrational state of the electronic ground state. \( M_\rho \) is the dipole moment of the electronic transition in the \( \rho \)-direction \( (\rho = x, y, z) \) and
\( \Gamma_R \) is a damping constant which is connected to the lifetime of the vibronic state \( Rr \). The sum
describing the scattering tensor includes the transitions \( Gi \rightarrow Rr \) and \( Rr \rightarrow Gf \). Consequently,
the Raman intensity depends on the transition probability of all vibronic states \( Rr \).

In resonance Raman spectroscopy, the excitation frequency \( v_0 \) is close to the frequency of an
electronic transition and the summation is reduced to the vibrational states \( r \) of the resonant
electronically excited state and Eq. 1.6 is simplified to

\[
(1.7) \quad [\alpha_{\rho \sigma}]_{if} \cong \frac{1}{\hbar} \sum_{r} \left( \frac{\langle Gi | M_\rho | Rr \rangle \langle Rr | M_\sigma | Gf \rangle}{v_{Rr} - v_0 + i\Gamma_R} \right)
\]

where the second non-resonant term is neglected. The Born-Oppenheimer approximation
separates the electronic and nuclear coordinates as given by

\[
(1.8) \quad \langle Gi | M_\rho | Rr \rangle = \langle ir \rangle \langle G | M_\rho | R \rangle = \langle ir \rangle M_{GR,\rho}
\]

where the transition dipole moments can be expanded in a Taylor series with respect to the
normal coordinates \( Q_k \).

\[
(1.9) \quad M_{GR,\rho}(Q_k) = M_{GR,\rho}^0 + \sum_k \left( \frac{\partial M_{GR,\rho}}{\partial Q_k} \right)_0 Q_k + \cdots
\]

Higher order terms are neglected within the harmonic approximation. Combination of Eq. 1.7-9 gives the scattering tensor as the sum of the two so-called Albrecht A- and B-terms.
(1.10) \[ [\alpha_{\rho\sigma}]_{if} \approx A + B \]

where

(1.11) \[ A \approx \frac{1}{\hbar} M_{GR,\rho}^0 M_{GR,\sigma}^0 \sum_r \frac{\langle i|r|f \rangle \langle r|f \rangle}{\nu_{RR} - \nu_{GR} + i\Gamma_R} \]

(1.12) \[ B \approx \frac{1}{\hbar} \sum_r \left[ \frac{\langle r|Q_k|i\rangle \langle i|r|f \rangle (\frac{\partial M_{GR,\rho}}{\partial q_k}) M_{GR,\sigma}^0}{\nu_{RR} - \nu_{GR} + i\Gamma_R} + \frac{(\frac{\partial M_{GR,\rho}}{\partial q_k}) M_{GR,\sigma}^0}{\nu_{RR} - \nu_{GR} + i\Gamma_R} \right] \]

Different scattering processes are represented by the A- and B-terms, however, both terms increase, and thus the intensity of the resonance Raman scattered light increase, when \( \nu_0 \) approaches the frequency of an electronic transition. The A-term becomes the leading term when the transition dipole moment \( M_{GR}^0 \) is large. The enhancement of a normal mode then depends on the Franck-Condon enhancement, i.e. the Franck-Condon factor products \( \langle i|r|f \rangle \langle r|f \rangle \). This enhancement is most pronounced for normal modes including internal coordinates that experience a shift of their equilibrium values upon the resonant electronic transition (excited state displacement). In the case of the retinal chromophore, the excited state displacement is particularly large of the C=C stretching coordinates upon excitation of the \( \pi \rightarrow \pi^* \) transition. The B-term increases when \( \nu_0 \) is close to an electronic transition into a state which is vibronically coupled to another state. The B-term may also become important under pre-resonance conditions.
1.2.3 Resonance Raman spectroscopy on microbial rhodopsins

Resonance Raman spectroscopy has been employed to study both animal and microbial rhodopsins as well as free retinals (for reviews see [117–119]). In animal rhodopsins (visual pigments), the photoinduced reaction sequence is terminated by the hydrolysis of the retinal Schiff base and the subsequent release of the chromophore from the protein. The irreversible character of the photoconversion imposes severe restrictions of the resonance Raman spectroscopic measurements of these rhodopsins. In this respect, microbial rhodopsins are more convenient research targets since the chromophore remains attached to the protein and the photoinduced reaction sequence eventually leads to the recovery of dark state (photocycle).

There are various strategies that can be adopted for the identification of the vibrational bands in the experimental resonance Raman spectrum of microbial rhodopsins and thus the structure of a retinal in an unknown configuration. In the simplest case, there is a match of the overall band-pattern compared to the resonance Raman spectrum of a retinal protein for which the isomeric state of the chromophore is known. In fact, in the literature this is the most common approach [38,120–125]. The spectrum can also be compared to those of model systems, such as free retinal in organic solvent. However, the chromophore pocket surrounding the retinal and especially the counter ion of the protonated Schiff base has a considerable effect on the vibrational modes [126]. Calculations of the normal mode frequencies can be used to assign the experimental bands to certain vibrational modes and have been extensively employed for BR and model compounds although it relies on the availability of isotopically labelled retinals [119,126–129]. If the spectra are retrieved from QM-MM simulations, the accuracy of the crystal structure or structural model is important [130]. In the end, it is also constructive to consider complementary data concerning the retinal configuration, obtained for example from retinal extraction combined with HPLC analysis or NMR and X-ray crystallography. This was actually the case for BR where it was already known that the dark-adapted state consisted of two isomers, all-trans, 15-anti and 13-cis, 15-syn, when Mathies and co-workers successfully assigned the vibrational bands in the resonance Raman spectra to different normal modes using a combination of isotopic labelling and calculations [127,128]. Thus, before analysing a Raman spectrum of a new rhodopsin one should at least perform a retinal extraction to determine the isomeric states involved, even though the extraction procedure is associated with considerable uncertainties. During the extraction the retinal Schiff base is hydrolysed.
which may promote thermal isomerisation reactions. Furthermore, it is not possible to retrieve any information concerning the 15-syn/anti conformation.

Time-resolved resonance Raman spectra are available for the bacteriorhodopsin intermediates, $L_{550}$ [131,132], $M_{410}$ [129], $N_{560}$ [133] and $O_{640}$ [134], but also of retinal analogues incorporated into BR such as 13-desmethyl [135,136]. The dark and intermediate states of other microbial rhodopsins have been characterised using Raman spectroscopy and they can be divided into two groups. Dark states obtained after light-adaptation, in accordance with bacteriorhodopsin, that contain an all-trans, 15-anti (protonated RSB) which partially and thermally relax into 13-cis, 15-syn (protonated RSB) after dark-adaptation. Examples are halorhodopsin [120], Neurospora rhodopsin [124], Leptosphaeria rhodopsin [123] and sensory rhodopsin II from *Halobacterium salinarum* [122]. The other group shows in the dark state only the all-trans, 15-anti (protonated RSB) and no additional isomer is thermally formed after long periods in the dark. Examples of these are Gleobacter rhodopsin [38], green and blue absorbing proteorhodopsin [125] and sensory rhodopsin I from *Halobacterium halobium* [121]. Both groups have in common that the light-induced isomerisation to 13-cis, 15-anti triggers the photocycle. The above mentioned rhodopsins come from different organisms such as archaea, cyanobacteria and fungi and in both groups pumps as well as sensors are represented. Thus, it is tempting to assume that all newly discovered microbial rhodopsins exhibit the same isomeric states, either with or without thermal isomerisation in the dark.
1.2.3 Vibrational modes in retinal proteins

The most important vibrational bands of retinals, in crystalline form or dissolved in organic solvent as well as bound to proteins, are found in the region between 800 and 1700 cm$^{-1}$. In resonance and pre-resonance Raman spectroscopy this region includes the molecular vibrations located within the conjugated system since only the vibrational transitions related to the $\pi\rightarrow\pi^*$ electronic transition of the polyene chain are enhanced. Thus, the vibrations of the ionone ring are normally not detectable. The molecular vibrations of the retinal can be described by the internal coordinates which are the C=N (in Schiff base bound retinal), C=O (in free retinal), C=C and C-C stretching’s as well as CH$_3$ deformation, C-H and CH$_3$ rocking and C-H out of plane (HOOP) wagging (Figure 11). [119]

*The ethylenic stretch (C=C) stretch* vibration is the most intense band in the rhodopsin spectra and is found around 1520-1570 cm$^{-1}$ (1570-1580 in free retinal). This band normally constitutes of more than one normal mode where each is dominated by the C=C stretching coordinates of the polyene chain. The most intense mode is attributed to the in-phase combination of individual stretching coordinates. There is a linear dependence of the C=C stretching frequency and the absorption maximum of rhodopsins, i.e a blue-shift in the absorption leads to a shift of the C=C stretching frequency towards higher frequencies [137].

*The C=N stretching* is found above 1600 cm$^{-1}$ and the frequency of this vibration strongly depends on the protonation state of the Schiff base, showing a higher frequency for a protonated compared to a deprotonated Schiff base. The mode of the protonated Schiff base has been shown to be involved in intramolecular energy transfer to adjacent water molecules (if available), leading to a broadening of the bandwidth [138]. Furthermore, H/D exchange at the retinal Schiff base causes a shift of the C=N stretching frequency towards lower frequencies. This shift is used to confirm that the RSB is protonated. The frequency of the C=N vibration also depends on the strength of the N-H bond, strong hydrogen bond interactions afford a higher C=N frequency [121].

The fingerprint region, 1100-1400 cm$^{-1}$, is reflected by a distinct pattern depending on the isomeric state of the retinal and can be used to identify the configuration. The underlying modes include the C-C stretching and the C-H rocking but also the N-H rocking coordinate as reflected by shifts upon H/D exchange. Between 1400-1500 cm$^{-1}$ one finds the methyl (CH$_3$)
Resonance Raman spectroscopy on microbial rhodopsins

deformation. The C-CH₃ rocking gives rise to a medium intense band at circa 1010 cm⁻¹. Below 1000 cm⁻¹, the C-H out of plane wagging (HOOP) modes give rise to resonance Raman bands that are normally relatively weak. However, a twisted chromophore leads to increased intensity of these modes compared to the C-C and C=C stretching vibrations [119].

Figure 11. Resonance Raman spectrum of BR₅₇₀ measured with 514 nm laser excitation. The coordinates dominating in the different frequency regions are indicated. Figure is taken from [116].
2. Materials and Methods
2.1 Heterologous expression of channelrhodopsin-2

In contrast to BR and XR, not all microbial rhodopsins can be extracted from the membranes of the native organisms in the amounts required for spectroscopic studies. When the heterologous expression of microbial rhodopsins in the bacterium *Escherichia coli* fails, the methylotrophic yeast *Pichia pastoris* has been shown to be a satisfactory alternative, for example in the case of NOP-1 from *Neurospora crassa* [139] and Leptosphaeria rhodopsin [39]. For a review on heterologous expression of proteins in *P. pastoris* see [140]. Channelrhodopsins have previously been expressed in COS-1 cells (fibroblast cells from the kidney of monkey) [20,141] as well as in *P. pastoris* [19,142] in amounts sufficient for purification. COS cells are cultured at the surface of flasks, which may be a limitation when large-scale production of protein is requested. Yeast cells, on the other hand, grow in solution and can be cultivated in large batch cultures on a medium simpler than the one used for the COS cells. Thus in this work *P. pastoris* was chosen for the preparation of ChR2 samples.

2.1.1 Transformation in *Pichia pastoris*

2.1.1.1 Construction of plasmid
cDNA encoding ChR2 1-309 (H134R mutant, accession number AF461397), including c-myc and 12 His tags at the c-terminal end, with the codon optimised for yeast, was ordered from Mr. Gene GmbH and inserted into the plasmid pPICZ-C (Invitrogen) by using the restriction sites EcoRI and AgeI. A yeast consensus sequence (encoding an extra methionine and serine) was introduced at the N-terminus in order to achieve proper initiation of translation. The plasmid consist of, *inter alia*, the AOX1 promotor and the Zeocin™ resistance gene [143]. Mutations were introduced by using the QuickChange® Site-Directed Mutagenesis kit (Stratagene). Following PCR and digestion with DPN1, transformation of supercompetent XL1-Blue was performed and the cells were grown in LB medium (see Appendix, BD Biosciences/Invitrogen) containing 25 µg/mL Zeocin. The plasmid was purified using the NucleoSpin® Plasmid kit (Macherey-Nagel GmbH) and digested with the restriction enzymes EcoRI and NotI. Gel chromatography facilitated the separation of the cDNA strands and the insert containing the mutated ChR2 sequence was purified using the NucleoSpin® ExtractII kit (Macherey-Nagel GmbH). The insert was introduced into a fresh pPIC-Z vector and the plasmid sent for sequencing in order to confirm that the mutation was successfully introduced.
2.1.1.2 Transformation

Competent cells were prepared by cultivating *Pichia pastoris* strain SMD1163 in 500 mL YPD medium (see Appendix, BD Biosciences/Invitrogen) until OD$_{600}$ = 1.3-1.5. The cells were repeatedly washed with ice-cold sterile water and finally re-suspended in ice-cold 1M sorbitol solution. The plasmid was linearized using the restriction enzyme PmeI (MssI) and transferred into the competent cells by electroporation (1500 V, 200Ω and 25µF). Ice-cold sorbitol (1 M) was immediately added and the cells stored for 1 hour on ice followed by 1 hour at 30 °C. The transformed cells were then plated on YPD medium (1 M sorbitol) containing 500, 1000 or 2000 µg/mL Zeocin and left at 30°C for around 1 week. Clones were picked, re-plated and finally transferred to 25 mL YPD medium in 250 mL flasks and left to grow over-night at 250 rpm and 28-30°C. The cells were finally transferred to fresh YPD medium with 30% glycerol, shock-frozen in liquid N$_2$ and stored at -80°C. [143]

2.1.1.3 Phenotype and multicopy test

SMD1163 belongs to the strains with wild-type methanol metabolism which is called the Mut$^+$ phenotype. If the promoter AOX1 is disrupted the cells can still metabolise methanol, however, at a much slower rate and by using the AOX2 promoter. This phenotype is called Mut$^S$ (methanol utilization slow). [143] Transformed *P. pastoris* cells were checked for phenotype by plating them on the MMH medium (Minimal Methanol with Histidine, see Appendix, BD Biosciences/Invitrogen) and comparing the growth to that of GS115 Mut$^S$ albumin and GS115/pPICZ/lacZ Mut$^+$. However, none of the clones showed Mut$^S$ growth and were all assigned to the Mut$^+$ phenotype.

Upon electroporation several plasmids can enter the cell and integrate into the genome. Generally, more copies of the plasmid lead to a higher protein expression. Transformed cells were plated on YPD medium (1 M sorbitol) containing 2000 µg/mL Zeocin whereby clones showing the best growth, and thereby potentially containing most inserted plasmids and Zeocin resistance, were chosen for further cultivation and expression. [143]

2.1.2 Cell cultivation

Transformed cells were transferred to 25 mL BMGY medium (see Appendix, BD Biosciences/Invitrogen) in 250 mL flasks and the pre-culture was left to grow over-night at
250 rpm and 28-30°C. Parts of the pre-culture was inoculated into 500 mL BMGY medium in 5 L baffled flasks and grown until the optical density at 600 nm (OD$_{600}$) reached 4-6. The initial optical density (OD$_{600}$(ini)) and thus the amount of the pre-culture required for the inoculation was calculated according to

$$\text{(2.1)} \quad OD_{600}(t) = OD_{600}(\text{ini}) \cdot 2^{t/d}$$

where $t$ is the time of the cell growth and $d$ is the cell doubling time experimentally found to be roughly 2.5 hours in the logarithmic growth phase. After the cell growth the old media was removed by centrifugation. The cell pellet was resuspended in BMMY medium (see Appendix, BD Biosciences/Invitrogen) containing 2.5% methanol and 10 µM all-trans retinal such that OD$_{600} = 1$. The medium was removed after 12 hours of induction and the cells washed in breaking buffer pH 7.4 (see Appendix, containing 1 mM PMSF) by repetitive centrifugation steps. The induced cells were stored in breaking buffer and at - 80°C after rapid-freeze with liquid N$_2$.

### 2.1.3 Purification of channelrhodopsin-2

The disruption of the induced cells was achieved using a French Press (G. Heinemann Ultraschall und Labortechnik) and the cell suspension (0.2-0.3 g cells / mL breaking buffer) was pressed twice under 20000 Psi. This was followed by two centrifugation steps, first to remove intact cells (10 min, 4000×g, 4°C) and the acquired supernatant further separated into the membrane and cytosol fractions by the means of ultracentrifugation (1 hour, 180000×g, 4°C). The final pellet comprising of the membranes was pottered in solubilisation buffer consisting of 20 mM HEPES, 100 mM NaCl, 10 vol% glycerol, 10 mM imidazole and 0.1 mM PMSF at pH 7.4 (see Appendix). The total amount of proteins was determined using the DC Protein Assay (BioRad). The volume was adjusted and the detergent dodecyl maltoside (DDM) added such the final concentration corresponded to 1 w/w% DDM, 250 mM arginine, 3 µM all-trans retinal and 10 mg proteins / mL solubilisation buffer. The proteins in the membranes were solubilised overnight at 4°C while the solution was being stirred and unsolubilised protein was then removed by ultracentrifugation (1 hour, 180000×g, 4°C). The supernatant was added onto a 1 mL Ni-NTA column (HisTrap™ FF crude, GE Healthcare) connected to an ÄKTA™ purifier 10 system (Amersham Pharmacia Biotech). If larger amounts of protein were purified (i.e. more than 1 L cell culture), two columns were
connected linearly. The column was washed using 15 column volumes of solubilisation buffer containing 25 mM imidazole and 0.03 w/w% DDM. The flow-rate of the system was typically set to 1 mL/min. A linear gradient of imidazole ranging from 25 to 500 mM, with a slope of around 30 mM/min, eluted the proteins attached to the column. The release was monitored by absorption changes at 280 and 470 nm. The purified protein was dialysed in solubilisation buffer (0.03 w/w% DDM, pH 7.4) without the imidazole and concentrated in an Amicon ultra (100000 MWCO, Millipore). Finally the protein purity and functionality was checked by UV-vis absorption spectroscopy before and after illumination in the blue. The yield was calculated on the basis of the absorption at 450 nm using Lambert-Beer law

\[
A = \varepsilon \cdot c \cdot d
\]

where \(A\) is the absorption, \(c\) the concentration, \(d\) the optical path length and \(\varepsilon\) the extinction coefficient which was adopted from the \textit{Volvox carteri} channelrhodopsin (VChR) with an experimentally determined value of \(\varepsilon_{450} = 45500 \text{ M}^{-1}\text{cm}^{-1}\) [141]. The molar mass of ChR2 is roughly 39000 g/mol not taking into consideration any attached carbohydrates.

\subsection{2.1.3.1 Exchange of the buffer for the spectroscopic measurements}
Protein in D\textsubscript{2}O buffer was a requirement in the resonance Raman experiments since the proton-to-deuterium (H/D) exchange of the retinal Schiff base leads to isotopic shifts most clearly seen for the C=N stretching mode. The sample was first measured in the normal solubilisation buffer at pH 7.4. The same sample was diluted 1:10 in the equivalent buffer, except containing D\textsubscript{2}O at pD 7.4, and then concentrated. The dilution and concentration step was repeated once in order to achieve a 1:100 dilution of the H\textsubscript{2}O. Note that when measuring pD using a standard pH-meter one must take into account that pD equals the measured pH + 0.4. The change of the pH was performed by using the solubilisation buffer where the HEPES was replaced with 20 mM phosphate or 20 mM tris with pH adjusted to 6.0 and 9.0, respectively.

\subsection{2.1.3.2 Deglycosylation protocol}
Roughly 10 \(\mu\)g ChR2-H134R in a solution containing 1 w/w% SDS was denatured at 95\degree C for 10 min. The solution (14 \(\mu\)L) was diluted in 20 mM sodium phosphate buffer (pH 7.2, 1 w/w% DDM) such that the concentration of SDS was reduced by a factor of 7.
Deglycosylation was performed by addition of 2 µL N-Glycosidase F and the sample was stored for 20 h at 37ºC. The enzyme was then denatured at 90ºC for 10 min. The deglycosylated sample was analysed using SDS-PAGE and Western blot and compared to glycosylated (untreated) ChR2-H134R.
2.2 Reduction of the retinal in bacteriorhodopsin

Reduced and UV-irradiated BR displays a fine-structured pattern in the absorption spectra and is proposed to contain a retro-retinyl chromophore corresponding to a one-bond shift of the conjugated system towards or away from the ionone ring [144]. Reduced and UV-irradiated BR was prepared in a protocol similar to [144,145]. Purple membrane expressed in *Halobacterium halobium* strain S9 was purified according to [3].

Purple membrane (OD$_{570} = 1.5$, 0.1 M NH$_4$CO$_3$, pH ~10) was reduced by adding 20 mg NaBH$_4$ per 1 mL solution followed by exposure to yellow light (Xe-lamp 220W, Schott filter OG 435) for 10-45 minutes until the sample fully and irreversibly bleached. The reaction was performed while the sample was being stirred and cooled in an ice-bath. The initial step is proposed to reduce the retinal Schiff base upon formation of a C-N single bond [144]. Following exposure for 3-10 minutes to the UV-fraction of white light (simply by removing the Schott filter) leads to the fine-structure in the absorption spectrum presumably due to a shift of the entire conjugated system [144]. The reaction was performed using both NaBH$_4$ and NaBD$_4$ (Sigma-Aldrich®), as well as in H$_2$O and D$_2$O buffers. Prior to the resonance Raman experiments, the buffer was exchanged by diluting the samples in 5 mL sodium phosphate buffer (150 mM, pH 7.0), followed by centrifugation at 8000 g for 2 hours and re-suspending the pellet in sodium phosphate buffer.
2.3 Optical and vibrational spectroscopy

2.3.1 UV-vis absorption spectroscopy

2.3.2.1 Conventional setup

A Cary 50 Bio spectrophotometer (Varian Inc.) or Cary 4000 UV-vis spectrophotometer (Agilent Technologies) was used for measuring stationary or time-resolved (in seconds to minutes) absorption spectra. The absorption spectroscopy at cryogenic temperatures were performed according to [146]. Illumination of the samples was facilitated by a blue ($\lambda_{\text{max}} \sim 455$ nm, LEDC2, Thorlabs) or a green ($\lambda_{\text{max}} \sim 525$ nm, LIU002, Thorlabs) LED.

2.3.1.2 Flash photolysis

The experiments and data evaluations were done in collaboration with Rolf Hagedorn. The setup for the transient spectroscopy was based on the LKS.60 flash photolysis system (Applied Photophysics Ltd., Leatherhead, UK) as described in [20,108,147]. The photocycle was initiated using a Rainbow OPO/Nd:YAG laser (10 ns pulse, 470 nm, 16 mJ/shot) and the absorption differences, comparing before and after the laser flash, was detected by a 150 W Xe-lamp and an Andor iStar ICCD Camera with a Shamrock-spectrograph of 2 nm (Andor technology, Belfast, Northern Ireland). The measurements were performed at 19°C and a set of difference spectra was recorded covering a time-range from 1 µs to 1 s after the laser flash. Singular value decomposition (using Matlab, The MathWorks, Natick, MA) identified the significant components of the time-resolved difference spectra. The kinetic constants, component spectra and reconstructed data set were obtained by target analysis with a sequential kinetic model. As a simple example, a model involving three states (A, B and C) with the kinetic constants $k_1$ and $k_2$ can be written according to:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

In this case the mean life time ($\tau$) of one state represent the reciprocal value of the kinetic constant.
2.3.2 The Raman setup

2.3.2.1 Lasers and spectrometers
Excitation of the various rhodopsins investigated in this work was facilitated by monochromatic light at 351, 458 and 514 nm of an Argon laser (Coherent, Santa Clara C.A., U.S.A) as well as at 413 and 647 nm of a Krypton laser (Coherent, Santa Clara C.A., U.S.A). The Raman signal was detected in a backscattering configuration (180°) by a confocal LabRamHR spectrometer (Horiba, Villeneuve, France). Depending on the excitation line an edge filter or a holographic notch filter was used to suppress the Rayleigh scattered light. The LabRam spectrometers are not ideally suited for pump-probe experiments or for detection in the UV and therefore, a U1000 double grating spectrometer (Horiba Jobin Yvon, Longjumeau, France) in 90° sample geometry was used in the case of excitation at 351 nm and for certain time-resolved measurements. A plasma filter corresponding to the wavelength of the laser was mounted in the beam to remove the plasma lines. The spectrometers were equipped with either a Peltier-cooled CCD (Andor) camera or a liquid N2 cooled back-illuminated CCD (Symphony) camera with higher efficiency in the blue/violet. The alignment of the laser beam in the LabRam spectrometers was verified by spectral accumulation of a silicon wafer and comparison to reference measurements with optimal alignment at the wavelength of interest.

2.3.2.2 Sample containers
The Linkam cryostat (Linkam Scientific Instruments, Surrey, UK) allowed for Raman measurements at liquid nitrogen temperature. The cell was slightly heated and purged with N2 gas prior to cooling in order to remove condensed water. After the required temperature was reached the sample was quickly inserted into the cell under dimmed red-light in order to avoid photoactivation of the protein before freezing. The laser was focussed onto the surface of the frozen sample via a BK7 optical glass window of the cryostat and using a 20× objective (Nikon, NA 0.35). The sample was moved through the laser beam by a computer controlled XY stage (OWIS GmbH, Germany) to reduce the heating of the sample in the laser spot.
A rotating cuvette, originally developed by Kiefer in 1973 [148], was used for the room-temperature measurements performed at the LabRam spectrometers. The rotation centrifuges the sample towards the walls and thus creates a film of the solution defined by the inner parameters of the cuvette (Table 1). Ideally the photocycle of the protein should be faster than the reciprocal value of the rotational frequency \( \nu_0^{-1} \). In order to create a uniform film one needs at least a \( \nu_0 \) of 10 s\(^{-1}\), hence the duration of the photocycle should be less than 0.1 s in order for photolyzed sample to relax to the dark state before being excited again. In the case of ChR2, the photocycle is considerably slower, i.e. around a minute. Therefore, concurrent mixing of the sample during the rotation is necessary in order to dilute the already photolyzed fraction. This is done by using a steel ball positioned by a magnet located outside of the cuvette (Figure 12). The laser was focused on the sample as close as possible to the cuvette wall (20× objective) by accumulating short spectra (1 s) and moving the cuvette until maximum signal of the chromophore was achieved.

<table>
<thead>
<tr>
<th>Dimension</th>
<th>Length / mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner diameter</td>
<td>21.3</td>
</tr>
<tr>
<td>Outer diameter</td>
<td>26.3</td>
</tr>
<tr>
<td>Inner height</td>
<td>3.0</td>
</tr>
<tr>
<td>Outer height</td>
<td>5.5</td>
</tr>
</tbody>
</table>

*Table 1. Dimension of the rotating cuvette used for the measurements at the LabRam spectrometers*

*Figure 12. Schematic representation of the rotating cuvette and displacement of the pump and probe laser beams in \( \Delta x \) and \( \Delta z \), also given by the angle \( \alpha \). During the rotation mixing is achieved by a steel ball and a magnet. Figure is taken from [116]. Only the probe beam was used when measuring at the LabRam spectrometer.*
2.3.2.3 Data evaluation
The spectra were evaluated using the free numeric software Octave (GNU General Public Licence, version 2.1.73) and by running modified scripts originally developed by Hendrik Naumann for the analysis of the Raman data on sensory rhodopsin-II from *Natronomonas pharaonis* (NpSRII) [116]. In some cases it was possible to remove some of the background and bands not originating from the chromophore by a simple subtraction procedure. An example is shown in Figure 13, where LED UV-illumination is needed to accumulate Rh-BI of HKR1 (blue). The absence of the UV-light leads to laser-induced photo-excitation of Rh-BI and subsequent formation of Rh-UV (red) with very small Raman cross-section at 514 nm. Thus the subtraction, UV-background minus without, generates a spectrum free of buffer bands (black). Next, the spectra were calibrated according to the toluene standard and, depending on the spectral window, either to the 1003.6, 1211.4 or 1605.1 cm\(^{-1}\) toluene bands (see example of a fit in Figure 14). A rough calibration of the spectrometer was done before the measurements but toluene spectra were also recorded throughout the entire experiment to verify the stability of the spectrometer. Normally the spectra were shifted, if at all, in the range of one wavenumber or less. Finally a baseline was subtracted using a polynomial function (Figure 15).

![Figure 13. Resonance Raman spectra of HKR1 measured using the 514 nm laser line (1 mW) with (blue) and without (red) UV-LED irradiation. Subtracted spectrum ‘with minus without LED’ (black) represents Rh-BI where the contributions of the buffer are removed.](image-url)
Figure 14. Raman spectra of toluene (black), in the region of the 1003.6 cm$^{-1}$ band, measured at 413 nm and the fitted Gaussian function (grey). The difference of the true value and the band position of the fit (1003.2 cm$^{-1}$) is 0.4 cm$^{-1}$.

Figure 15. Polynomial function (green) used for the baseline subtraction of the resonance Raman data on ChR2-H134R measured at 458 nm (red).
2.3.2.4 Time-resolved probe-only resonance Raman spectroscopy

The LabRam spectrometer with a 20× objective gives a beam radius $r_{\text{las}}$ of the Gaussian laser profile on the sample of roughly 20 µm. 500 µL sample in a rotating cuvette with a cell radius $r_{\text{cell}}$ of 13.15 mm was continuously mixed via the magnetic ball technique. The rotational frequency of the cuvette $\nu_0$ in these experiments was $\sim 15$ s$^{-1}$.

The photochemical rate constant can be calculated using

$$ l_0 = 4.81 \cdot 10^{-10} \cdot \frac{\gamma \varepsilon_{\lambda_{\text{las}}} P_{\text{las}} \lambda_{\text{las}}}{r_{\text{las}}^2} [\text{s}^{-1}] $$

where $\gamma$ is the quantum yield, $P_{\text{las}}$ the power of the laser and $\varepsilon_{\lambda_{\text{las}}}$ the extinction coefficient for the chromophore at the laser wavelength $\lambda_{\text{las}}$. For ChR2, $\varepsilon_{458} \approx 45500$ M$^{-1}$ cm$^{-1}$ at the 458 nm laser line. The residence time of the sample in the laser beam is given by

$$ \Delta t_{\text{las}} = \frac{r_{\text{las}}}{r_{\text{cell}} \pi \nu_0} \approx 30 \mu\text{s} $$

Combining equation 2.3 and 2.4 – after inserting the experimental values and if we assume that $\gamma = 0.1$ – the photoconversion parameter $(\Delta t_{\text{las}} \cdot l_0)$ now only depends on the power of the laser.

$$ \Delta t_{\text{las}} \cdot l_0 = 4.81 \cdot 10^{-10} \cdot \frac{\gamma \varepsilon_{\lambda_{\text{las}}} P_{\text{las}} \lambda_{\text{las}}}{r_{\text{las}}^2 r_{\text{cell}} \pi \nu_0} = 809 \cdot P_{\text{las}} $$

0.1 mW laser power gives a photoversion of less than 10 %. If we assume that due to the mixing ball we have an efficient exchange of the sample, the theoretical limit for the total time $t_{\text{meas}}$ the sample can be measured until a photolysed sample is photolysed a second time is given by the ratio of the cell volume $V_{\text{cell}}$ and the irradiated volume $V_{\text{las}}$ according to

$$ V_{\text{las}} = r_{\text{las}}^2 \pi \cdot d_{\text{pen}} $$

where $d_{\text{pen}}$ is the penetration depth of the laser in the sample which may be approximated by $r_{\text{las}}$. For $V_{\text{cell}} = 0.5 \cdot 10^{-3}$ L one obtains under the assumption of a complete mixing of the
sample

\[
(2.7) \quad t_{\text{meas}} = \frac{V_{\text{cell}}}{V_{\text{las}}} \cdot \frac{1}{\nu_0} = \frac{V_{\text{cell}}}{\tau_{\text{las}}^3 \pi \nu_0} \approx 370 \text{ hours}
\]

as a theoretical limit. Thus preferential enhancement of the dark state should be possible by using 0.1 mW laser power, a rotational frequency of 15 s\(^{-1}\), 500 µL sample mixed in the rotating cuvette by a magnetic ball and upon an hour of spectral accumulation at 458 nm excitation. Dark-adaptation corresponding to the recovering of the protein dark-state (~1 min) is then necessary before further laser exposure. Increasing the laser power, i.e. to 10 mW increases the photocoversion by a factor of 10 which should allow detectable amounts of intermediates in the laser spot. The residence time of the laser spot on a fraction of the sample and thus the window for the intermediate life time is 30 µs as defined by equation 2.4.

2.3.2.5 Time-resolved pump-probe resonance Raman spectroscopy

Resonance Raman spectra of photoreceptor intermediates can also be obtained by using a pump and probe two laser system. The photocycle is initiated in the pump laser spot, the photolysed sample is then transported according to the rotational frequency of the cuvette and probed with a spatially displaced laser beam (Figure 12). The difference in time between the pump and probe, defined by the rotational frequency of the cuvette and the displacement of the laser beams, determines what intermediate is being probed. Furthermore, the pump laser should be tuned to the absorption maximum of the dark state in order to achieve high photocconversion whereas the probe wavelength should correspond to the absorption maximum of the intermediate.

Since the BR-M\(_{410}\) spectrum is used to be compared with deprotonated states of other microbial rhodopsin, a (514 nm) pump (413 nm) probe experiment was performed. The experimental conditions, with modifications, were adapted from [129]. The measurement was performed on the U1000 spectrometer using a cuvette with a rotational frequency \(\nu_0\) of 20 s\(^{-1}\) and a cell radius \(r_0\) of 21 mm. The delay \(\Delta t\) between the pump and probe laser beams should be 1 ms which is in order of the BR-M\(_{410}\) life time, and the angle \(\alpha\) correlated to this delay is calculated according to...
(2.8) \[ \alpha = 2\pi \cdot \Delta t \cdot v_0 \]

Thus the displacement of the lasers in \( \Delta x \) and \( \Delta z \) (Figure 12) is

(2.9) \[ \Delta x = r_0 \cdot \left( 1 - \cos(2\pi \cdot \Delta t \cdot v_0) \right) = 0.17 \text{ mm} \]

(2.10) \[ \Delta z = r_0 \cdot \sin(2\pi \cdot \Delta t \cdot v_0) = 2.63 \text{ mm} \]

The lasers were initially aligned such that they both passed through a 5 \( \mu \)m pinhole. The pinhole was then moved by \( \Delta x \) and \( \Delta z \) and the probe beam adjusted to the new position.
Resonance Raman spectroscopy on microbial rhodopsins
3. Results and discussion
3.1 Channelrhodopsin-2

This work included the biochemical characterisation and optical spectroscopy of different channelrhodopsin-2 mutants, all of them used in the field of optogenetics, with the main focus on the (wild-type like) ChR2-H134R. The histidine at position 134 in ChR2 (H173 in ChR1) is located close to the intracellular side and is most likely part of the ion-conduction pore in the so-called inner gate [28]. ChR2-H134R shows increased steady-state currents compared to wild-type ChR2 [8,149] – due to a prolonged opening time of the channel [150] – and has been used to activate neurons in various organisms such as C. elegans [8,151], Drosophila larvae [12], mice and rats [149,152–156]. Step function rhodopsins are a group of well-expressing ChR2 mutants with prolonged conducting state compared to the wild-type protein [24,78,98,99,150]. They are commonly used in optogenetic applications since stable and effective depolarisation of the cell membrane is a desired property when activating mammalian neurons in tissues or living animals [81]. Step function rhodopsins include mutations at position C128 and/or D156; both amino acids are located close to retinal chromophore (C167 and D195 in Figure 16) [28], and ChR2-C128T as well as ChR2-C128S-D156A were studied in this work. The cysteine at position 128 was exchanged by a serine or threonine to alter the strength of the hydrogen bonding and/or steric interactions. The aspartic acid at position 156 was replaced by the neutral alanine which alters the hydrogen bonding, protonation reactions and steric interactions.

![Figure 16. Chromophore pocket in the C1C2 crystal structure showing the residues C128 (C167 in ChR1) and D156 (D195 in ChR1) that are mutated in the step-funtion rhodopsins. Reprinted with permission from [28]. Copyright (2012) Nature Publishing Group.](image-url)
3.1.1 ChR2-H134R

3.1.1.1 Optimisation of the biochemical procedures
After stable integration of the PICZ-C plasmid containing ChR2-H134R (amino acids 1-310, c-myc and 12 histidine tags) in the *P. pastoris* genome, expression tests were performed in order to determine the optimum time of induction. 20 mL cell suspension (in BMMY media), with absorbance (optical density) at 600 nm (OD$_{600}$) = 1 at the start of the induction, was sampled after 6, 12, 24, 29 and 32 hours of growth and the expressed protein in the membrane fraction detected by Western blot using a c-myc antibody (Figure 17). A negative control consisting of cells grown without methanol (BMGY media) was sampled as well. At time zero a band around ~100 kDa was detected which is also seen in the negative control and not related to any expressed channelrhodopsin. The same band is found for purified protein in stained gels and is one of the main impurities. After 6 hours two monomer channelrhodopsin bands appear at around ~40 kDa which probably represent glycosylated and deglycosylated protein. With time, i.e. at 24 hours, the lower molecular weight band disappears which indicates complete glycosylation. The band at ~80 kDa is most likely a channelrhodopsin dimer band but in order to properly identify all bands, digested gels were analysed using MALDI-ToF, see section 3.1.1.2.

![Western blot](image)

*Figure 17.* Western blot of the membrane fraction of induced *P. pastoris* (in BMMY), 0 h represent the start of induction. Negative control (NC) is cells grown under the same conditions but without methanol (in BMGY).
Next the cell growth was studied by measuring the optical density during the induction. The growth curves of 500 mL cell culture in BMMY (5 L bottles, 28°) with initial OD$_{600}$ = 1 showed that best growth is achieved at 250 rpm and using a sterile cloth to cover the bottle (Figure 18). The supply of oxygen is important since both lowering the speed of shaking (to 150 rpm) as well as using a plug reduced the cell growth. Although the OD is constantly increasing, the amount of dead cells is as well seen as a sand-like precipitation. This is qualitatively observed in the Western blot as the intensity of the channelrhodopsin monomer band is largely unchanged at 12, 24 and 29 hours but slightly decreased at 32 hours (Figure 17). The time of induction was therefore fixed to 24 hours when the glycosylation is complete and the cell culture still in a healthy state.

The amount of purified protein was typically 1-2 mg per litre cell culture. The purity of the samples can be evaluated by comparing the absorbance of the aromatic amino acids at 280 nm and the maximal absorbance of the retinal chromophore around 470 nm. Depending on the preparation, the ratio of absorbance at 280 and 470 nm varied around 2-3. Although the samples contain impurities – seen in the SDS-PAGE after Coomassie staining – no other proteins with chromophores were detected in the UV-vis absorption spectra (Figure 19). Thus for optical spectroscopy the amount and purity of the samples were sufficient.
3.1.1.2 N-glycosylation of channelrhodopsin-2

The trypsin digestion, extraction of peptides and MALDI-ToF measurements and analysis were performed by Christoph Weise. The objective was to identify the protein bands in the SDS-PAGE and N-glycosylation sites of ChR2-H134R.

Gels containing purified glycosylated and deglycosylated ChR2-H134R were stained with Coomassie blue and the bands of interest cut out and digested using trypsin. The same samples were separately analysed by Western blot to better illustrate the difference in molecular mass (Figure 20). Trypsin digests the protein after a lysine or arginine and the theoretical m/z for each peptide was calculated to identify the corresponding band in the MALDI-ToF spectra. Not all peptides could be extracted from the gel. In the case of ChR2-H134R, roughly half of all peptides were detected which was enough for identification and both the monomer and dimer bands in the glycosylated and deglycosylated samples could be ascribed to channelrhodopsin. The analysis further showed that the N-terminal methionine is removed and the N-terminal peptide (m/z = 1325.57) starts with an acetylated serine. The impurity and the band at ~100 kDa (see section 3.1.1.1) was analysed and identified as the P. pastoris membrane protein gi 254571875 (94607 Da) confirmed by MS/MS sequencing of two peptides (m/z 738.4 and 1536.8) [157]. This protein contains an above-average amount of histidine residues, 6% as compared to 2.3% average of all known and sequenced proteins [158] which may explain the co-purification on the Ni-NTA resin.

![Figure 20. Western blot of solubilised and purified ChR2-H134R before (A) and after (B) enzymatic deglycosylation.](image)

To promote the N-linked glycosylation, asparagine (N) has to be included in the sequence N-X-T/S where X can be any amino acid except proline (P) [159]. O-glycosylation is not considered at this point. There are two potential sites for N-glycosylation in ChR2 (Table 2). The first is found at position N24 (NGS) and located in the peptide 13-43 which was indeed
only detected in the deglycosylated protein in MALDI-ToF. Since this peptide is not found for the glycosylated protein, its molecular mass has been altered; hence the protein is N-glycosylated at N24. N104 found in the peptide 104-115 is followed by a proline (NPS) excluding N-glycosylation at this position. The final site N137 (NLT) is included in the peptide 135-147 which was detected in both the glycosylated and deglycosylated samples and, therefore, was considered not to be N-glycosylated. The analysis was repeated twice with different samples and in both cases the results were the same. Thus ChR2 is N-glycosylated at position N24 which is located close to the N-terminus and on the extracellular side of the plasma membrane. This is reasonable since glycosylation takes place after protein translation in the endoplasmic reticulum on the extracellular side only.

Finally the N-glycosylation site was removed by a mutation at position S26 to the hydrophilic aspartic acid (S26D). Although functional protein was expressed and purified, the yield was extremely low. This indicates that the sugar attached to the protein is important, possibly for the structural stability of the protein [160].

<table>
<thead>
<tr>
<th>Peptide</th>
<th>m/z</th>
<th>Glycosylated</th>
<th>Deglycosylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>13 - 43</td>
<td>3550.73</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>135 - 147</td>
<td>1439.71</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Table 2.* Peptides detected (+) and not detected (-) in MALDI-ToF MS of in-gel trypsin-digested glycosylated and deglycosylated ChR2-H134R.
3.1.1.3 Kinetics of the P500, P390 and P520 intermediates

After biochemical characterisation and optimisation, optical spectroscopy was used to elucidate the photocycle and chromophore structure of ChR2-H124R. First the absorption of intermediates formed in the μs to ms time-range was studied using flash photolysis and samples in H₂O and D₂O (at pH and pD 7.4) were compared. The protein was excited with a 470 nm laser flash. The measurements were performed in cycles, each cycle including a 120 s dark-time followed by a reference measurement (without the laser flash) and a 120 s dark-time prior to the laser flash and measurement. The reference measurement was needed in order to subtract light-induced effects caused by the Xe-lamp. Spectra were accumulated in the interval from 1 μs to 1 s after the laser flash and each 10³ logarithmic interval contained five data points. The time constants (τ) of the different intermediates were determined by target analysis generated from a model including four states (Figure 21). The model starts with the first intermediate P500 since detection of the decay of the excited state would require a resolution in the pico-second time scale [100].

The component spectra were compared to reference data on ChR2 [20,100]. The first intermediate is red-shifted

\[ A\ (P500) \rightleftharpoons B \rightleftharpoons C \rightleftharpoons D \rightleftharpoons A \]

**Figure 21.** Photocycle model used for the fit to the experimental flash photolysis data.

**Figure 22.** The reconstructed spectra generated by target analysis of the flash photolysis data on ChR2-H134R in H₂O. Top; the difference in the absorption after the laser flash in a logarithmic time-scale, the red trace represents positive and the blue trace negative absorption. Bottom; difference absorption spectra of the components A (green), B (red), C (cyan) and D (blue) compared to D470. The data evaluation was performed by Rolf Hagedorn.
compared to the dark state D470 and is assigned to P500 (Figure 22; green). The decay but not the formation of this state is observed and it is followed by accumulation of the UV-absorbing P390 intermediate associated with the deprotonation of the RSB (red). Re-protonation then acquires another red-shifted species, P520 which is the conducting state (cyan). P520 decays into an intermediate with absorption similar to D470 which represents the P480\textsubscript{a/b} states (blue). The last step of the reaction and decay of P480\textsubscript{a/b} is, according to the model, the recovery of P500 which in the reality is not the case. P480\textsubscript{a/b} would decay into D470 and further excitation is needed to form P500. However, a five-state model did not change the fit and time constants since P480\textsubscript{a/b} decays on a time-scale which is much slower than kinetics probed by flash photolysis.

The decay of P500 is almost the same in H\textsubscript{2}O and D\textsubscript{2}O buffers (τ\textsubscript{1} ~10 \, µs) within the error margin (Table 3). The subsequent intermediates, on the other hand, show significant differences, P390 with time constants τ\textsubscript{2} of 1.7 (H\textsubscript{2}O) and 3.7 ms (D\textsubscript{2}O) and P520 with τ\textsubscript{3} of 9.6 (H\textsubscript{2}O) and 22.9 ms (D\textsubscript{2}O), corresponding to a slowdown of roughly a factor 2 in D\textsubscript{2}O. Similar kinetic isotopic effects of the photocycle were found for BR and SRII [161–163]. The time constant of P480\textsubscript{a/b} (τ\textsubscript{4}) is beyond the time-resolution and was therefore set to be infinitely large.

<table>
<thead>
<tr>
<th>Time constant</th>
<th>\text{H}_2\text{O}</th>
<th>\text{D}_2\text{O}</th>
</tr>
</thead>
<tbody>
<tr>
<td>τ\textsubscript{1} (P500)</td>
<td>6.5 ± 0.8 , µs</td>
<td>8.9 ± 0.6 , µs</td>
</tr>
<tr>
<td>τ\textsubscript{2} (P390)</td>
<td>1.7 ± 0.5 ms</td>
<td>3.7 ± 0.3 ms</td>
</tr>
<tr>
<td>τ\textsubscript{3} (P520)</td>
<td>9.6 ± 1.2 ms</td>
<td>22.9 ± 1.2 ms</td>
</tr>
<tr>
<td>τ\textsubscript{4} (P480\textsubscript{a/b})</td>
<td>Infinity</td>
<td>Infinity</td>
</tr>
</tbody>
</table>

*Table 3.* Time constants derived from the flash photolysis data.
3.1.1.4 pH-dependent kinetics of the P480\textsubscript{a/b} intermediates

The late intermediates in the photocycle of ChR2-H134R were not resolved in the flash photolysis experiments and the decay of P480\textsubscript{a/b} therefore investigated using a conventional UV-vis absorption spectrometer. The differences between the two P480 states is only seen in the FTIR spectra and interpreted in terms of small structural changes of the protein backbone [20]. Separation of these intermediates using UV-vis absorption spectroscopy is not possible.

![UV-vis absorption difference spectrum of ChR2-H134R at pH 7.4](image)

Figure 23. UV-vis absorption difference spectrum of ChR2-H134R at pH 7.4 (grey squares). The sample was illuminated in the blue for ~20 seconds (blue line) and a mono-exponential function, $\Delta \text{Abs} = \Delta \text{Abs}_0 + A_1 \cdot e^{-t/\tau_4}$ (black curve), was fitted to the decay at 520 nm.

UV-vis absorption spectra (250 - 650 nm) of ChR2-H134R at pH 4.5, 5.0, 6.0, 7.4, 8.0 and 9.0 were measured after at least 2 minutes of dark-adaptation. In contrast to ChR1 from *Volvox carteri* [141] and *C. reinhardtii* [164] but in agreement with wild-type ChR2 [20], the absorption of the dark state does not depend on the pH (data not shown). The time constants for the decay at 520 nm after 30 seconds of blue-light exposure were determined by fitting a mono-exponential function to the time-resolved spectra. Figure 23 shows the difference spectrum and the fit for the sample at pH 7.4. Since P520 decays in the millisecond time scale (flash photolysis data, section 3.3.1.3), the decay at 520 nm only represents the P480\textsubscript{a/b}
intermediates which are slightly red-shifted compared to D470. The time constant for the decay of P480\textsubscript{a/b} at pH 4.5 is \(~\)50 s but decreases when pH is increased and at neutral pH \(\tau_4\) is \(~\)30 s (Table 4).

<table>
<thead>
<tr>
<th>pH</th>
<th>Time constant ((\tau_4)) / s</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>47.9 (\pm) 0.2</td>
</tr>
<tr>
<td>5.0</td>
<td>26.0 (\pm) 0.1</td>
</tr>
<tr>
<td>6.0</td>
<td>33.2 (\pm) 0.2</td>
</tr>
<tr>
<td>7.4</td>
<td>33.0 (\pm) 0.1</td>
</tr>
<tr>
<td>8.0</td>
<td>22.4 (\pm) 0.3</td>
</tr>
<tr>
<td>9.0</td>
<td>7.1 (\pm) 0.2</td>
</tr>
</tbody>
</table>

*Table 4.* Time constants for the decay at 520 nm in seconds.
3.1.1.5 Trapping of early intermediates at cryogenic temperatures

To further investigate the early intermediates in the photocycle, ChR2-H134R was measured using UV-vis absorption spectroscopy at liquid nitrogen (77 K) and liquid helium (5 K) temperatures. Intermediates could be trapped either by illumination at these temperatures or by increasing the temperature to 220 K.

Comparing the absorption spectra at room temperature and 5 K we note that lowering of the temperature causes an enhancement of the fine-structured absorption pattern (Figure 24). Six Gaussian functions and a linear baseline were fitted to the spectrum measured at 5 K (Figure 25). The difference in energy between the individual band components, i.e. Gauss 2-3, 3-4 and 4-5 is 1554, 1595 and 1436 cm\(^{-1}\), respectively, which represent the main vibronic transitions that are in the range of the C=C stretching modes (see section 3.1.1.6).

Blue-illumination (455 nm) of dark-adapted ChR2-H134R at 5 K induces a red-shift of the UV-vis absorption spectrum (Figure 26; top). The difference spectrum (blue-illuminated and dark-adapted sample) shows negative bands at 390, 414, 443 and 474 nm and a positive at 505 nm (Figure 26; bottom). The negative bands can be correlated to depletion of the D470 and the positive one to the accumulation of the primary photoproduct. These results show that photoisomerisation of the retinal takes place at temperatures as low as 5 K. It is possible to recover D470 by green light [20]. The difference spectrum (green- and blue-illuminated sample) with positive bands at 387, 411, 439 and 470 nm and a negative band 502 nm, is almost a mirror image of the difference spectrum between blue-illuminated and dark-adapted sample. Except for some residual absorption above 500 nm – probably caused by accumulation of the late intermediates P480\(_{a/b}\) – no other effects were observed when the sample was light-adapted before freezing (data not shown).

The difference spectrum of illuminated and dark-adapted sample at 77 K shows the same difference spectrum as at 5 K (data not shown). The difference spectrum of the sample illuminated at 220 K and the dark-adapted sample (both measured at 77 K) shows, besides the depletion of D470 and the accumulation of P500, also accumulation of an intermediate with difference bands at 366 and 387 nm (Figure 27). This UV-absorbing intermediate found after illumination at elevated temperatures corresponds to the P390 state. Thus deprotonation of the Schiff base requires elevated temperatures in order to overcome the energy barrier of this transition.
Figure 24. UV-vis absorption spectra of ChR2-H124R measured at 5 K (black) and room temperature (grey).

Figure 25. Gaussian functions (dotted) fitted to the UV-vis absorption spectrum of ChR2-H134R at 5 K (solid). Insert shows the calculated energy differences based on the absorption maximum of the Gaussian band components.

Figure 26. UV-vis absorption spectra of ChR2-H134R at 5 K. Top; absorption spectra of dark-adapted (dotted) and blue-illuminated (455 nm) (straight) sample. Bottom; difference absorption spectra of blue-illuminated and dark-adapted sample (black), and green- (525 nm) and blue-illuminated sample (grey).

Figure 27. UV-vis absorption spectra of ChR2-H134R at 77 K. Top; absorption spectra of dark-adapted (dotted) and blue-illuminated (455 nm) at 220 K (straight) sample. Bottom; difference absorption spectrum of blue-illuminated (at 220 K) and dark-adapted sample (black).
Resonance Raman spectroscopy on microbial rhodopsins

Figure 28. Resonance Raman spectra of dark-adapted ChR2-H134R D470 measured at 78 K. The black and grey traces represent the spectra with 458 and 514 nm excitation, respectively.

3.1.1.6 Resonance Raman spectroscopy at cryogenic temperatures

The data obtained using low temperature UV-vis absorption spectroscopy was the basis for understanding the resonance Raman spectra acquired at the same temperatures. ChR2-H134R was measured in a cryostat (78 K, 1 hour spectral accumulation) at 458 (1 mW) and 514 nm (4 mW) excitation to probe D470 and P500, respectively. As UV-vis absorption experiments have shown retinal photo-isomerisation already at 5 K (section 3.3.1.5; Figure 26), the laser will unavoidably induce the transition from D470 to P500, particularly due to the high photon flux through laser irradiation such that a photostationary mixture of the two states is obtained. However, the retinal vibrational bands for instance at 1113, 1158, 1188, 1205 and 1275 cm\(^{-1}\) are observed at both 458 and 514 nm excitation (Figure 28). Especially the main C=C stretching band at 1560 cm\(^{-1}\) is same in position and shape at both excitation wavelengths. The UV-vis absorption spectra revealed that D470 will not completely deplete upon light exposure and is therefore preferentially detected at 458 nm excitation. At 514 nm excitation, however, P500 is efficiently photoconverted back to D470 (section 3.1.1.5; Figure 26). The
signal-to-noise ratio at 514 nm is poor compared to that at 458 nm due to low absorption of D470 at this wavelength. The spectral features that are not seen at 458 nm but at 514 nm can be ascribed to fluorescence and the buffer. Probing at 413 nm (4 mW) gave a comparable spectrum to those at 458 and 514 nm (data not shown). Thus, the resonance Raman spectra accumulated at 413, 458 and 514 nm at 78 K represent largely a pure D470 state.

In a previous study, the pre-resonance Raman spectra of ChR2, obtained with 647 nm excitation, were measured at 77 K and analysed by a band fitting procedure to disentangle the spectral contributions of an all-trans as well as a 13-cis retinal, according to the results of extraction experiments of the dark state D470 [23]. Here a similar procedure was applied to the resonance Raman measured with the three excitation wavelengths at 77 K. Four Lorentzian functions were fitted to the C=C stretching envelope, centred at ca. 1560 cm$^{-1}$, taking into account that all-trans and 13-cis isomers each exhibit two C=C stretching modes contributing to the most prominent peak in the spectrum [127,128]. The 13-cis retinal with a protonated Schiff base in the dark-adapted and intermediate states of BR has clearly separated C=C stretching modes [128,132,133]. Thus, as a first assumption, the Lorentzian functions with the highest and lowest frequency was assigned to the 13-cis and the two remaining to the all-trans retinal. The fit was initially preformed for the D470 spectrum obtained at 458 nm excitation. The positions and the half-widths of the four Lorentzians were then fixed (Table 5). Due to the fact that the vibrational modes are differently enhanced when the excitation wavelength is changed, the intensities of the Lorentzian functions were allowed to vary for the analysis of the spectra measured with 413 and 514 nm (Figure 29; left panel). The fits at all excitation wavelengths showed that the major isomeric contribution in the resonance Raman spectra of D470 is all-trans (red curves) with a smaller fraction of 13-cis (blue curves), consistent with the isomeric composition (70% all-trans and 30% 13-cis) derived from extraction experiments of the dark-adapted state [23]. However, one has to take into account the inherent uncertainties of the fitting procedure despite the restrictions imposed on the variations of frequencies and half-widths.

The sample was then heated to 223 K, illuminated in the blue, cooled to 78 K, and measured at 413, 458 and 514 nm. Elevated temperature and illumination induces changes in the resonance Raman spectra at all excitation wavelengths. The peaks exhibit asymmetric band-shapes and especially at 413 nm a distinct shoulder, indicating the involvement of more than one state. The spectra after heating and illumination represent the additional contributions
besides those from D470 which according to cryogenic UV-vis absorption spectroscopy are the P500 and P390 intermediates. The Lorentzian functions representing the D470 all-trans and 13-cis were fitted to the spectra and the intensity ratio of the two conjugate bands of each isomer, as well as the respective frequencies and half-widths, were kept constant as for the fits to the D470 spectrum at the same excitation wavelength. However, the ratio of all-trans and 13-cis was allowed to vary and additional Lorentzian functions were added representing the accumulated intermediate states (Figure 29; right panel). Taking into account the partial photoconversion due to the exciting laser beam, the preferential enhancement of the state with absorption maximum close to the excitation wavelength, and the inverse relationship between the C=C stretching frequency and the absorption maximum, one may thus conclude that the spectra obtained with 458 and 514 nm excitation represent a mixture of D470 and P500 whereas the 413 nm excited spectra may reflect a mixture of D470 and P390. In the case of the P500 intermediate, the band positions and widths of the Lorentzian functions were determined during the fitting procedure of the spectrum at 514 nm and then fixed for the fit of the spectrum at 458 nm. The P390 intermediate is only represented by one mode consistent with the highly symmetric C=C stretching band of BR-M410 with a 13-cis deprotonated RSB [129]. Besides accumulation of intermediates, a significant reduction of the D470 all-trans compared to 13-cis was observed at all excitation wavelengths. The subtraction procedures did not generate pure intermediate spectra (data not shown) due to the fact that the ratio of all-trans and 13-cis is different in D470 before and after the heating and illumination.
Resonance Raman spectroscopy on microbial rhodopsins

<table>
<thead>
<tr>
<th>Component</th>
<th>Position / cm$^{-1}$</th>
<th>Bandwidth / cm$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D470 all-trans</td>
<td>1556.0</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>1560.1</td>
<td>7.4</td>
</tr>
<tr>
<td>D470 13-cis</td>
<td>1545.8</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>1564.3</td>
<td>6.3</td>
</tr>
<tr>
<td>P390</td>
<td>1570.9</td>
<td>15.2</td>
</tr>
<tr>
<td>P500</td>
<td>1540.1</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>1551.3</td>
<td>12.3</td>
</tr>
</tbody>
</table>

*Table 5.* Band positions and widths of the fitted Lorentzian functions on the 458 nm excited spectrum of D470 as well as of the functions related to the P500 and P390 intermediates accumulated after heating (to 223 K) and illumination at 514 and 413 nm excitation, respectively.
Figure 29. Left panel: the D470 C=\( \text{C} \) stretching region of the spectra measured at 78 K and 413, 458 and 514 nm excitation wavelengths. Fitted Lorentzian functions representing the all-trans isomer are shown in red and 13-cis in blue. For the sake of simplicity, only the sum of the two bands representing one isomer is plotted. The black curves are the sum of all functions. The band positions and widths were determined for the fit of the 458 nm spectrum and kept fixed for the fitting on the spectra at 413 and 514 nm. Right panel: spectra accumulated after heating (to 223 K) and illumination including the D470 all-trans and 13-cis as well as additional fitted Lorentzian functions representing P500 and P390 (dashed). The band positions and widths for the P500 and P390 intermediates were determined for the fit at 514 and 413 nm, respectively.
3.1.1.7 Resonance Raman spectroscopy at room temperature

In contrast to the resonance Raman measurements at cryogenic temperatures, room-temperature measurements allow photoexcited channelrhodospin to complete the photocycle, such that static experiments would sample a photostationary mixture involving all possible (intermediate) states, depending on the respective lifetime. However, if the experiments are carried out with a flow-system such as a rotating cuvette, the relative concentration of a given state in the Raman probe beam can be controlled by the excitation conditions (laser power and wavelength), the irradiated sample volume (laser beam diameter), and the flow rate of the sample [148]. The respective methodology has been established specifically for studying retinal proteins such as bacteriorhodospin or sensory rhodopsin [165]. Adaptation of this approach to ChR2, however, faces the challenges that the long recovery time, i.e. the slowest step of the photocycle, is in the range of tens of seconds such that the “fresh sample” condition is difficult to fulfil. This condition implies that the proteins of an irradiated volume element are completely back-converted to the dark state once the same molecules pass through the laser beam again. In the case of ChR2, the traditional technique would require a flow system with large sample reservoir which was beyond the preparation capacity, or, using the gated-cw technique, long dark times which drastically lowers the signal-to-noise ratio. As a consequence, the present work focusses on probe-only experiments that are sufficient for measuring the spectrum of the dark state but do only provide limited access to the spectra of the intermediates.

Dark state of ChR2-H134R. To measure the D470 state, the sample (typically 500 µL OD$_{470}$ ≈ 3) was deposited in a rotating cuvette, such that upon rotation the sample was continuously moved through the exciting laser beam. In addition, the sample included a metallic ball held at a fixed position by a magnet (Figure 12). In this way, the entire solution of the cuvette was thoroughly mixed and as a consequence the time between two irradiation events for the same molecule was substantially increased. This device together with a low laser power has been estimated to afford a high contribution of non-photolysed proteins in the exciting laser beam. Furthermore, an excitation wavelength of 458 nm was chosen that is close to the absorption maximum of D470, thereby ensuring preferential enhancement only of this state.

Besides for different signal-to-noise ratio, accumulation (1 hour) at excitation powers of 0.01 and 0.1 mW resulted in identical spectra. These low-power spectra are therefore assumed to represent mostly D470 with no significant contributions of any photocycle intermediates. This
Figure 30. Resonance Raman spectra accumulated at 458 nm excitation and 0.1 mW of ChR2-H134R in H$_2$O (black) and D$_2$O (grey).

Figure 31. Comparison of the resonance Raman spectra of ChR2-H134R accumulated at 458 nm and at 78 K (grey; 1 mW) and room temperature (black; 0.1 mW).
Resonance Raman spectroscopy on microbial rhodopsins is consistent with the estimates made in section 2.3.2.4. The main ethylenic stretch (C=C) mode is located at 1556 cm\(^{-1}\), as seen in the 0.1 mW spectrum (Figure 30). The C=N stretching band at 1662 cm\(^{-1}\) is shifted to 1633 cm\(^{-1}\) in D\(_2\)O indicating the presence of a protonated Schiff base. The Lorentzian fit to the C=N band showed a reduction of the half-width going from 17.0 (H\(_2\)O) to 15.1 cm\(^{-1}\) (D\(_2\)O) (Figure 32). The fingerprint region (1150-1350 cm\(^{-1}\)) consists of C-C stretchings at 1158, 1186 and 1203 cm\(^{-1}\) with shoulders at 1175 and 1211 cm\(^{-1}\) as well as a C-H rocking band at 1274 cm\(^{-1}\) (Figure 30). This region is similar to the spectrum obtained at 78 K indicating that the same state (D470) is being probed at 78 K as well as at room temperature (Figure 31). Changing the pH to 6 or 9 had no influence on the D470 spectra and, besides for different band intensities, also excitation with 413 and 514 nm afforded similar spectra (data not shown). Lorentzian functions were fitted to the C=C stretching region of the D470 spectra at the different wavelength in the same procedure as for

*Figure 32. C=C and C=N stretching bands of ChR2-H134R accumulated at 458 nm and 0.1 mW in H\(_2\)O and D\(_2\)O. Several Lorentzian functions (dashed; black is the sum) were fitted to the spectra and the band positions are labelled.*
the data obtained at 77 K (section 3.1.1.6) and including the all-trans and 13-cis components. The fitting was first carried out for the 458 nm accumulated spectrum, the band positions and widths were then fixed for the fits to the spectra accumulated at 413 and 514 nm excitation (Figure 33; left panel). Compared to the fits for the 77 K data, the D470 all-trans and 13-cis functions were shifted to lower frequencies and the half-widths increased (Table 6) The shape of the sum of the two functions of one component is similar to its equivalent at 77 K, i.e. D470 all-trans consists of two closely positioned functions (1549.9 and 1555.8 cm$^{-1}$) with comparable intensities whereas 13-cis has two separated Lorenzians (1540.2 and 1561.2 cm$^{-1}$) and, except for the fit for the 514 nm spectrum, with a higher intensity for the function at 1561.2 cm$^{-1}$. The ratio of the different isomers shows, in good agreement with the fits for the 77 K spectra, that the major contribution comes from the all-trans configuration. Also, the ratio of all-trans/13-cis is comparable at 413 and 458 nm excitations whereas the all-trans form is dominating at 514 nm indicating a red-shift of the absorption of the all-trans species compared the 13-cis form consistent with the isomers present in the dark-adapted state of BR.

<table>
<thead>
<tr>
<th>Component</th>
<th>Position / cm$^{-1}$</th>
<th>Bandwidth / cm$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D470 all-trans</td>
<td>1549.9 (-6.1)</td>
<td>13.9 (+4.6)</td>
</tr>
<tr>
<td></td>
<td>1555.8 (-4.3)</td>
<td>11.5 (+4.1)</td>
</tr>
<tr>
<td>D470 13-cis</td>
<td>1540.2 (-5.6)</td>
<td>17.1 (+1.3)</td>
</tr>
<tr>
<td></td>
<td>1561.2 (-3.1)</td>
<td>10.1 (+3.8)</td>
</tr>
<tr>
<td>P390</td>
<td>1566.0 (-4.9)</td>
<td>19.0 (+3.8)</td>
</tr>
</tbody>
</table>

*Table 6. Band positions and widths of the fitted Lorenzian functions for the D470 spectrum accumulated at 458 nm, 0.1 mW and room temperature, the values in brackets represent the difference to the fits for the 77 K data. The fit of the P390 intermediate was performed for the spectrum accumulated at 10 mW and 413 nm and the band position was fixed to 1569.0 which is the position of the C=C stretching frequency in BR-M$_{410}$.**
**Figure 33.** Left panel: the D470 C=C stretching region of the spectra measured at room-temperature, 0.1 mW and 413, 458 and 514 nm excitation wavelengths (black). Fitted Lorentzian functions representing the *all-trans* isomer are shown in red and *13-cis* in blue. For the sake of simplicity, only the sum of the two bands representing one isomer is plotted. The black curves are the sum of all functions. The band positions and widths were determined for the fit on the 458 nm spectrum and kept fixed for the fits to the spectra at 413 and 514 nm. Right panel: spectra accumulated at 10 mW including the D470 *all-trans* and *13-cis* as well as an additional fitted Lorentzian function to the spectrum at 413 nm representing P390 (dashed).
Resonance Raman spectroscopy on microbial rhodopsins

Figure 34. Subtracted resonance Raman spectra (10 – 0.1 mW) of ChR2-H134R accumulated at 458 nm in H$_2$O (black) and D$_2$O (grey).

Intermediate accumulated in the laser spot. Compared to the low-power spectra, accumulation at 1 or 10 mW (458 nm) excitation resulted in a broadening of bands. Subtracting the 0.1 mW spectrum representing D470 from the 10 mW spectrum (maximum subtraction but without generating negative bands) gives a spectrally different species compared to D470 (Figure 34). The C=C stretching band is found at 1560 with a shoulder at ~1540 cm$^{-1}$ which match the positions of the D470 13-cis Lorenzian functions fitted to the 0.1 mW spectrum. The C=N stretching at 1633 cm$^{-1}$ is shifted to 1623 cm$^{-1}$ in D$_2$O. The decreased intensity around 1331 cm$^{-1}$ in D$_2$O indicates a shift of the N-H bending, correlated to an increase of intensity at around 954 cm$^{-1}$ of the N-D bending similar as in the cases of HKR1 (section 3.2.1; Figure 59) and XR (section 3.3.1; Figure 62A). Thus the state observed in the difference spectrum refers to a chromophore with a protonated Schiff base. The C=N stretching region in H$_2$O displays a broad band suggesting that there is probably more than one species present in the subtracted spectrum. These states either represent photocycle intermediates existing on the µs time scale which is the residence time of the sample in the
laser beam (~30 µs; section 2.3.2.4) or they are extremely long-lived. It might also be possible that the subtracted spectrum reflects an increase of the D470 13-cis component compared to the corresponding all-trans form. The two states that decay and are formed on the µs timescale are P500 and P390. The concentration of each intermediate after the laser flash was evaluated by assuming an equilibrium between P500 and P390 when fitting the kinetic scheme in Figure 35 to the flash photolysis data (section 3.1.1.3). The other steps in the photocycle were assumed to consist of the forward reaction only. The rate constants for the forward and backward reaction P500 ⇌ P390 are roughly the same in H₂O (Table 7) (~78000 s⁻¹) giving equal ratio of P500 and P390 at around 10 µs to 1 ms after the laser flash (Figure 36). In D₂O, however, the reaction is slightly shifted towards P500, with ~47000 s⁻¹ for the forward and ~67000 s⁻¹ for the backwards reaction. If P500 and P390 were equally enhanced at 458 nm and the resonance Raman and flash photolysis experimental conditions comparable, a 0.5:0.5 mixture of P500 and P390 would be observed in the resonance Raman difference spectrum in H₂O but a slightly higher contribution of P500 in D₂O.

\[
\begin{align*}
&k_1 \quad k_2 \\
P500 \rightleftharpoons P390 \\ &k_3 \quad k_4 \\
P390 \rightarrow P520 \rightarrow P480
\end{align*}
\]

*Figure 35. Sequential model of the intermediates.*

<table>
<thead>
<tr>
<th>Rate constant / s⁻¹</th>
<th>H₂O</th>
<th>D₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>k₁ (P500 → P390)</td>
<td>7.8 ± 0.2 × 10⁴</td>
<td>4.7 ± 1.3 × 10⁴</td>
</tr>
<tr>
<td>k₂ (P500 ← P390)</td>
<td>7.8 ± 0.1 × 10⁴</td>
<td>6.7 ± 1.3 × 10⁴</td>
</tr>
<tr>
<td>k₃ (P390 → P520)</td>
<td>4.7 ± 4.2 × 10²</td>
<td>4.1 ± 3.3 × 10²</td>
</tr>
<tr>
<td>k₄ (P520 → P480)</td>
<td>4.5 ± 2.5 × 10²</td>
<td>1.6 ± 1.2 × 10²</td>
</tr>
</tbody>
</table>

*Table 7. Rate constants obtained from fitting the flash photolysis data using the model in Figure 35.*
Figure 36. Relative concentration of P500 (green), P390 (red), P520 (cyan) and P480a/b (blue) in H₂O (top) and D₂O (bottom) based on the rate constants from Table 1. Time after laser flash is given in a logarithmic scale.

Figure 37. Comparison of the subtracted resonance Raman spectrum of ChR2-H134R (10 – 0.1 mW) accumulated at 458 nm (black) and the SRII -L₄₉₅ from [116] (grey).
The BR-M\textsubscript{410}, deprotonated state and counterpart of P390, shows a symmetric C=C stretching band at 1566 cm\textsuperscript{-1} (section 3.4.2) [129]. The main C=C stretching in the subtracted resonance Raman spectrum of ChR2-H134R is located 6 cm\textsuperscript{-1} below and includes a second band seen as a shoulder at around 1640 cm\textsuperscript{-1}. The splitting of the C=C stretching modes agrees well with the resonance Raman spectrum of the L\textsubscript{550} and L\textsubscript{495} intermediates of BR and SRII, respectively (counterparts of P500) (Figure 37) [116,131,132,166–168]. Also the shift of the C=N band in D\textsubscript{2}O shows that the RSB is protonated as in the case of the chromophore in BR-L\textsubscript{550}.

The resonance Raman measurements were repeated at 413 and 514 nm to selectively enhance P390 and P500, respectively. However, except for small shifts in the intensity of different modes, the subtracted spectra looked similar at all excitation wavelengths (data not shown). For a further analysis, Lorentzian functions were fitted to the 10 mW spectra, (Figure 33; right panel). As in the analysis of the low-temperature spectra, two component spectra were generated including the conjugate bands of the all-trans and 13-cis isomer as determined from the spectra measured at low power. Then, the two component spectra were fitted to the experimental spectra measured with 10 mW, allowing only their relative contributions to vary. For the spectra measured with 458 and 514 nm, this procedure afforded a satisfactory fit whereas only for the fit to the 413-nm spectrum an additional Lorentzian function had to be added. The position of this extra band was fixed to 1566 cm\textsuperscript{-1} which is the C=C stretching frequency of BR-M\textsubscript{410} (i.e., the P390 counterpart) (Table 6). At all wavelengths, the D470 13-cis fraction increased relative to the all-trans when the power increased, but – except for the contribution of P390 in the 413-nm spectrum, there was no indication for the P500 intermediate, in contrast to the cryogenic measurements.

Since even the strongest band in the resonance Raman spectrum at 458 nm excitation does not provide an indication for the involvement of appreciable amounts of photocycle intermediate, the unique broadening of the much weaker C=N stretching mode at 1632.5 cm\textsuperscript{-1} in H\textsubscript{2}O must be an intrinsic property of D470 13-cis (Figure 38). On the basis of the 10 mW spectra, a line-shape analysis has to be treated with caution due to the over-lap of the D470 all-trans and 13-cis which may lead to artefacts in the resultant band profile. Thus, there are two possible explanations that may account for this effect. First, assuming a heterogeneous broadening, the Schiff base may exist in two states differing with respect to the hydrogen bonding interactions and thus causing subtle frequency shifts that lead to a broad overall band profile. This
interpretation, however, implies that the hydrogen bonding interactions are distinctly different in D$_2$O where the C=ND stretching displays a narrow band profile. Thus, as a more plausible explanation, one may assume a homogeneous broadening mechanism for the D470 13-cis C=N band. For both D470 all-trans and 13-cis, the broader C=N band in H$_2$O compared to that in D$_2$O is due to vibrational energy transfer between the protonated RSB and a water molecule.

Figure 38. C=C and C=N stretching region of the ChR2-H134R spectra accumulated at 10 mW and 458 nm excitation in H$_2$O and D$_2$O. The fitting was performed using the same band positions and widths ($\Delta \nu$) of the Lorentzian functions as for the fitting of the 0.1 mW spectra. Also the intensity-ratio of the two functions of one component (all-trans in red or 13-cis in blue) was fixed and only the ratio of the two isomers varied. The numbers in brackets (in red and blue) represent the multiplied factor to the fit at 0.1 mW. The C=N stretching modes, represented by a new Lorentzian function ($\nu_{C=N\ (13-cis)}$) as well as the original function ($\nu_{C=N\ (all-trans)}$) where the latter is the result of the fit performed for the spectra where the all-trans configuration dominated. The black curve is the sum of all Lorentzian functions.
3.1.1.8 Summary

ChR2-H134R could successfully be expressed in *P. pastoris* to a yield of 1-2 mg purified protein per litre cell culture based on a protocol similar to [19,142]. Induction tests showed that the glycosylation is complete after 24 hours and that sufficient supply of oxygen is important for the cell growth. MALDI-ToF experiments identified a N-glycosylation site at N24 which is crucial for the folding and/or transport of the protein to the membrane since the removal (by the mutation NGS to NGD) drastically decreased the yield of purified ChR2. One of the main impurities in the purified samples is a *P. pastoris* membrane protein with a high amount of histidine residues which is likely to be the reason for the co-accumulation during Ni-NTA chromatography. However most importantly for optical spectroscopy, no other co-purified chromophore-containing proteins were detected in the UV-vis absorption spectra.

The kinetics of the ChR2-H134R photocycle at pH 7.4, derived from UV-vis absorption and flash photolysis experiments, resulted in time constants for the decay of P500, P390, P520 and P480a/b of 7 µs, 2 ms, 10 ms and 30 s, respectively which is comparable to the data of the wild-type ChR2 [19,20,98,100,102]. Since the obtained kinetic data did not show a significant increase of any of the time constants, the increased conductivity of ChR2-H134R seen in the electrophysiological measurements [150] might instead be related to a higher expression level of this mutant. Note that the kinetics may depend on the protein lipid or detergent environment. Samples in D2O showed a significant decrease of the P390 and P520 time constants related to the slower diffusion and transport of the heavier deuterium. A shift towards alkaline pH showed decreased time constants of P480a/b possibly related to a generally accelerated photocycle.

The primary photochemical process of D480, i.e. the isomerisation of the retinal chromophore, is induced by illumination in the blue already at 5 K. The fine-structure in the absorption spectrum at low temperatures, caused by restrictions in the movement of the chromophore, is related to the vibronic transitions. This is demonstrated by the calculated energy differences of the Gaussian band positions which correlates with the energy of the main vibrational C=C stretching modes. Heating of the sample and exposure to blue light leads to accumulation of the deprotonated and UV-absorbing P390 state.

Resonance Raman experiments of dark-adapted ChR2-H134R at cryogenic temperatures (78 K) mainly probed D470 since the spectral appearances were independent on the excitation
wavelength. Spectra accumulated after heating and illumination gave a mixture of the D470, P500 and P390 states, perceived as shifts of the main C=C stretching vibration when comparing spectra at 413, 458 and 514 nm. Room-temperature resonance Raman experiments generated a largely pure D470 spectrum at 458 nm excitation and 0.1 mW. The fitting procedure using Lorenzian functions indicated that D470 consists of two isomers, most likely the all-trans and 13-cis configurations. Increasing the laser power afforded accumulation of the D470 13-cis isomer in the laser spot.
3.1.2 ChR2-C128T

The photocycle of ChR2-C128T has been thoroughly studied using UV-vis, flash photolysis, infrared and resonance Raman spectroscopy and shows the same intermediates as wild-type ChR2 plus an additional side-chain consisting of two UV-absorbing states P380 and P353 (Figure 39) [21,24,98,99]. The initial intermediates P500 and P390 have similar kinetics as wild-type ChR2 [20,100]. The following conducting state P520 decays more than 500 times slower and due to bi-exponential behavior it is believed to consist of two species with identical absorption. The closing of the channel and return to the dark state probably include the two P480α/β intermediates. Two UV-absorbing intermediates P380 and P353 were found on the same time-scale as the P480’s, their origin is presumably P480α and the branching ratio of P380/P353 depends on the pH [24].

*Figure 39.* Photocycle of ChR2-C128T based on [24]. Reprinted with permission from [169]. Copyright (2011) Elsevier.
3.1.2.1 The UV-absorbing intermediates
At pH 6.0, in accordance with previous experiments [21,24,98], a fine-structured pattern with maxima at 338, 356 and 377 nm is found in the UV-vis absorption spectra after illumination in the blue of dark-adapted ChR2-C128T (Figure 40; black). This absorption spectrum is attributed to the P353 intermediate. At pH 7.4 the spectrum is similar (blue) but increasing pH to 9.0 (red) leads to an absorption band without fine-structure in this region corresponding to a relative increase of P380 but also to protein degradation as seen by an increased background absorption. The fine-structure corresponds to resolved vibronic transitions of the chromophore. Fitting Gaussian functions to the UV-bands in the spectrum of ChR2-C128T at pH 6 afforded band positions at 356.4 and 377.1 nm (Figure 41) corresponding to an energy difference of ~1540 cm⁻¹ similar to the frequency of the C=C stretching mode in the resonance Raman spectrum (section 3.1.2.2).

Fine-structured absorption spectra have also been found for other retinal proteins such as the reduced and UV-treated BR. It was proposed to contain a retro-retinyl chromophore (retro-BR, section 3.4.1). In this retinal derivative the conjugated double bonds are shifted towards or away from the ionone ring by one unit (Figure 42) [144,145]. The question arose whether or not the same chromophore structure could be assigned to the long-lived intermediate P353 [24]. The difference absorption spectrum of retro- and dark-adapted BR shows the depletion of BR₅₆₀ and an increase in the UV with maxima at 344, 362 and 383 nm representing retro-BR (Figure 43; blue). The difference spectrum of illuminated and dark-adapted ChR2-C128T (black) shows a similar increase in the UV with maxima at 340, 356 and 376 nm representing P353, as well as accumulation of P520 and P480ₐ/ₚ and the depletion of D480. Despite the comparable UV-absorption features, it is important to note that retro-BR is irreversibly created after reduction and UV-irradiation whereas P353 is part of the ChR2-C128T photocycle and eventually relaxes into D480. To further study the chromophores of retro-BR and P353 and to possibly confirm the retro-retinyl structure of retro-BR, resonance Raman experiments were carried out.
Figure 40. UV-vis absorption spectra of dark-adapted (pH 6.0, grey) and illuminated ChR2-C128T at pH 6.0 (black), 7.4 (blue) and 9.0 (light blue).

Figure 41. Absorption spectrum in the UV of illuminated ChR2-C128T at pH 6.0 (black). Fitted Gaussian functions are represented by the dashed and blue lines.

Figure 42. Top; structure of all-trans protonated RSB. Bottom; all-trans retro-retinyl lysine.

Figure 43. Difference UV-vis absorption spectra of illuminated and dark-adapted ChR2-C128T at pH 6.0 (black), and retro- and dark-adapted BR (blue). BR spectra were normalised before subtraction.
3.1.2.2 The chromophores of P380 and P353

The UV-absorbing intermediates were investigated using resonance Raman spectroscopy with laser excitation at 351 nm (10 mW, 2 hours accumulation, 500 µL, OD$_{480}$ ~1-2, rotating cuvette) to preferentially enhance the chromophore bands of P390, P380 and P353. However, the decay of P390 (in ms) is too fast compared to P380 and P353 (in min) to expect contributions in the spectra [24]. The ratio of P380 and P353 was shifted by variation of the pH (6.0, 7.4 and 9.0). Continuous irradiation with blue LED light (455 nm) was applied during acquisition of spectra in order to ensure sufficient amounts of these intermediates in the laser beam.

The resonance Raman spectrum of the buffer at pH 6.0 was the same as at pH 7.4 and 9.0, and used to identify and separate buffer and retinal bands (Figure 44; grey). In fact, the bands assigned to the retinal chromophore in ChR2-C128T are the same at all pH, indicating that the chromophores of P380 and P353 are structurally very similar. At pH 9.0 the contributions of the retinal compared to the buffer bands was lower than at pH 6.0 and 7.4, which is related to the instability of the protein at alkaline pH as seen in the UV-vis absorption spectra (section 3.1.2.1; Figure 40). Thus the loss of fine-structure at high pH in the UV-vis absorption spectra is not correlated to any changes in the resonance Raman spectra.
Figure 44. Resonance Raman spectra of ChR2-C128T at pH 6.0, 7.4 and 9.0 measured with 351 nm excitation (10 mW), under continuous irradiation with a blue LED (455 nm). Retinal bands were identified based on the spectrum of the buffer at pH 6.0 (grey). Bands of the buffer are marked by “B”.
The resonance Raman spectrum of ChR2-C128T at pH 6.0 representing P353 (Figure 45B) displays no similarities to the spectrum of retro-BR (Figure 45A). The position of the C=C stretching is different, 1570 and 1579 cm\(^{-1}\) for retro-BR and P353, respectively, and there is no match in the pattern or position of the bands in the fingerprint region (1150-1350 cm\(^{-1}\)). Although the fine-structure in the UV-vis absorption spectra is comparable, the resonance Raman experiments rule out that retro-BR and P353 include chromophores of the same geometry. On the other hand, the resonance Raman spectrum of P353 match that of free all-trans retinal in CCl\(_4\) (Figure 45C) with a C-C stretching at 1163, C-H rocking at 1268 and 1333 and C=C stretching at 1579 cm\(^{-1}\). Only minor shifts for the methyl rocking at 1011/1008 cm\(^{-1}\) and C-C stretching at 1202/1198 cm\(^{-1}\) are observed and can be related to the different protein or solvent environment of the chromophores. The band at 1665 cm\(^{-1}\) representing mostly the C=O stretching mode in all-trans retinal [119], is not seen in P353 which can be attributed to the different experimental conditions. While the spectrum of all-trans retinal is measured under pre-resonance conditions (647 nm excitation) in order to avoid photo-destruction of the free chromophore, the P353 spectrum is measured at full resonance (351 nm). The C=C and C-C stretching modes are known to be preferentially enhanced when the excitation wavelength is in resonance with the \(\pi \rightarrow \pi^*\) transition of the polyene chain [170]; hence the band containing the C=O stretching mode is low in intensity in the P353 spectrum. Except for a one-wavenumber difference of the band at 1663 cm\(^{-1}\), H\(_2\)O to D\(_2\)O exchange did not induce any other shifts of the retinal bands in the buffer-subtracted spectra in agreement with a free and uncharged retinal (Figure 46).

The polarity and electrostatics of the chromophore surroundings strongly influence the C=C stretching modes of retinal derivatives. The C=C stretching is shifted towards lower frequencies if the polarity of the solvent is increased [171]; in the case of all-trans retinal it is found at 1579 cm\(^{-1}\) in CCl\(_4\) (Figure 45C) but shifted to 1574 cm\(^{-1}\) in ethanol. The match of the C=C stretchings in P353 and all-trans retinal in CCl\(_4\) indicates that the chromophore in P353 is embedded within a mainly non-polar protein environment.
Figure 45. Resonance Raman spectra of retro-BR (A) and ChR2-C128T P353 at pH 6.0 (B) measured at 351 nm and 10 mW, as well as the resonance Raman spectrum of all-trans retinal in CCl₄ (C) measured at 647 nm and 20 mW. Reprinted with permission from [169]. Copyright (2011) Elsevier.
Figure 46. Resonance Raman spectra of ChR2-C128T P353 (pH 6.0) in H₂O and D₂O measured at 351 nm excitation (10 mW). The buffer spectra were subtracted from the protein spectra prior to baseline subtraction.
3.1.3 ChR2-C128S-D156A

The ChR2-C128S-D156A photocycle was characterised by UV-vis absorption prior to resonance Raman spectroscopy to study slow intermediate/s as well as mutation-induced effects on the structure of the chromophore in the dark state. The transition from the conducting state P520 to P480\textsubscript{a,b} has been shown to be blocked in this mutant [81].

3.1.3.1 The dark and UV-absorbing states

The absorption spectrum of dark-adapted ChR2-C128S-D156A with maximum at 480 nm is slightly red-shifted compared to wild-type ChR2 [20] and therefore named D480 (Figure 48; top; grey). Illumination in the blue (480 nm) (top; blue) depletes D480, seen as a negative absorption at 479 nm in the difference spectrum (bottom; blue). The absorbance is increased in the UV at 387 nm indicating the accumulation of a deprotonated state corresponding to P390. This state is stable for hours as seen in the kinetic measurements (Figure 47; left). The mono-exponential fitting resulted in a time constant of around 80 min for the decrease of absorption at 390 nm after illumination as well as the increase at 480 nm (Figure 47; right). The decay of P390 can be influenced by illumination in the UV (375 nm) (Figure 48; top; black), and the difference spectrum, UV- minus blue-illuminated sample, mirrors the difference spectrum blue-illuminated minus dark-adapted (bottom; black). Thus blue light induces the transition D480 → P390 and UV accelerates the back-reaction P390 → D480.

![Figure 47. Absorbance changes of ChR2-C128S-D156A (grey) after illumination in the blue (blue arrow) measured at 390 nm (left) and 480 nm (right). A mono-exponential function (black curves) was fitted to the time-resolved data. The time constants derived from both fits was 88.3 ± 0.5 for the decay at 390 nm and 75.8 ± 0.3 for the rise at 480 nm.](image-url)
UV-exposure also reduces the absorption in the UV to a level which is below the dark-adapted spectrum and it leads to a red-shift of the maximum intensity in the difference spectrum (486 nm for P390 → D480), implying that the dark-adapted state already contains small amounts of P390 which apparently cannot thermally relax to D480. Furthermore, the absorption of the conducting state (P520) might overlap with the absorption of the dark state in this mutant. The blue-illuminated sample then includes a mixture of P390 and P520 in equilibrium with each other as in the wild-type protein but with considerably slower decay kinetics [81].

![Figure 48](image-url)

*Figure 48.* Top; UV-vis absorption spectra of dark-adapted ChR2-C128S-D156A (dotted) and after blue- (blue) and following UV-illumination (black). Bottom; difference spectra displaying UV-illuminated minus dark (black; D480 → P390) and blue- minus UV-illuminated (grey; P390 → D480) sample.
3.1.3.2 Cryo-trapping of P500

So far UV-vis absorption spectroscopy at room temperature showed the existence of D480 and P390 only. Similar to ChR2-H134R (section 3.1.1.5), dark-adapted ChR2-C128S-D156A was measured at 77 K followed by illumination in the blue (also at 77K) to trap the early P500 state (Figure 49; top). Indeed, the difference spectrum ‘blue-illuminated’ minus ‘dark-adapted’ (bottom) shows the depletion of D480 with negative bands at 416, 445 and 478 nm and an increase of absorption at around 522 nm, most likely representing the P500 state. This finding leads to the conclusion that the early photocycle after light-excitation of ChR2-C128S-D156A resembles that of wild-type ChR2, including retinal isomerisation (P500) followed by deprotonation of the Schiff base (P390).

![Figure 49](image.png)

*Figure 49.* Top; UV-vis absorption spectroscopy at 77 K of dark-adapted ChR2-C128S-D156A (dotted) and subsequent exposure to blue light (straight). Bottom; difference absorption spectrum of ‘blue-illuminated’ minus ‘dark-adapted’.
3.1.3.3 Resonance Raman spectroscopy at room temperature

The dark state of ChR2-C128S-D156A (D480) was measured using 458 nm excitation in a rotating cuvette under mixing (500 µL, OD480 = 2-3, pH 7.4). Under these conditions, predominantly the resonance Raman spectrum of the dark state is enhanced whereas significant contributions from the P390 and P520 states are not expected due to the lower resonance enhancement (section 3.1.3.1). Varying the laser power (0.1, 1 or 10 mW) did not change the spectra (data not shown), such as broadening of bands as seen for ChR2-H134R (section 3.1.1.7). The resonance Raman spectrum of D480 displays high intensity of bands in the HOOP region (800 - 1000 cm\(^{-1}\)), i.e. at 951 cm\(^{-1}\), which is unique for this mutant and not found in the spectrum of ChR2-H134R D470 (Figure 50). There are also differences in the fingerprint region; the 1186 band in ChR2-H134R D470 is split, giving an additional band at 1175 cm\(^{-1}\) for D480. The band at 1224 cm\(^{-1}\) in D480 is not seen in D470. The C=C and C=N stretchings at 1556 and 1662 cm\(^{-1}\), respectively, are almost the same in shape and position in both mutants. The C=N stretching shifts to 1630 cm\(^{-1}\) in D\(_2\)O, thus the RSB of ChR2-C128S-D156A D480 is protonated (Figure 51).

![Resonance Raman spectra](image-url)

Figure 50. Resonance Raman spectra measured at 458 nm excitation of ChR2-C128S-D156A (black; 1 mW) and ChR2-H134R (blue; 0.1 mW). The insert shows an expanded view of the fingerprint region dominated by the C=C stretching modes.
Figure 51. Resonance Raman spectra measured at 458 nm excitation (1 mW) of ChR2-C128S-D156A in H$_2$O (black) and D$_2$O (grey).
The UV-absorbing P390 intermediate in the ChR2-C128S-D156A photocycle was measured at 413 nm excitation. Here the intensity of different bands depended on the power of the laser as seen in the spectra measured at 2 and 10 mW (Figure 52). According to the resonance Raman spectrum at 458 nm, the band at 1556 cm\(^{-1}\) in the broad C=C stretching at 413 nm can be assigned to D480. The higher frequency band at around 1570 cm\(^{-1}\) might be related to P390 in analogy to the spectrum of M\(_{410}\) in BR with a C=C stretching at 1566 cm\(^{-1}\) [129]. At 2 mW the ratio of D480 and P390 is shifted towards P390, seen by an increase in intensity at 1570 compared to at 1556 cm\(^{-1}\). A subtraction of the spectra (2 – 10 mW) gave a spectrum with C=C stretching at 1570 cm\(^{-1}\) representing P390 (Figure 53). Other bands, i.e. 1232, 1308 and 1376 cm\(^{-1}\), also match the time-resolved resonance Raman spectrum of BR-M\(_{410}\) (section 3.4.2). However, the subtracted spectrum still includes residual contributions of D480; the HOOP, C-C and C=N stretching’s at 956, 1200 and 1662 cm\(^{-1}\) (with a shift to 1630 cm\(^{-1}\) in D\(_2\)O), respectively. The likely origin of this unexpected behaviour will be discussed below. Nevertheless, even though the subtracted spectra do not represent the pure spectra of the P390 state, the similarities with the spectrum of M\(_{410}\) of BR point to the same chromophore structure, a 13-cis, 15-anti deprotonated RSB as in the case of the Rh-UV state of HKR1 (section 3.2.1; Figure 57B/C).

![Figure 52. C=C stretching region of the resonance Raman spectra measured at 413 nm of ChR2-C128S-D156A at 2 (black) and 10 mW (grey) excitation energies.](image-url)
Figure 53. Subtracted resonance Raman spectra (2 - 10 mW) of ChR2-C128S-D156A in H₂O (top; black) and D₂O (top; grey) measured at 413 nm compared to the resonance Raman spectrum of BR-M₄₁₀ (bottom; section 3.4.2). The bands at 956, 1200 and 1662 cm⁻¹ represent residual contributions of D₄₈₀.
3.1.3.4 Resonance Raman spectroscopy at cryogenic temperature

D480 in ChR2-C128S-D156A (OD$_{480} = 1.4$) was measured in the cryostat (78 K) at 458 nm and 1 mW excitation. Compared to the room temperature measurements, the modes in the HOOP region are not as pronounced although a band at 956 cm$^{-1}$ is seen in the spectrum of D480 but not in ChR2-H134R D470 (Figure 54). The C=C stretching with a maximum at 1558 cm$^{-1}$ for ChR2-C128S-D156A D480 is broader than the band of ChR2-H134R D470 at 1560 cm$^{-1}$. Beside a band at 1668 cm$^{-1}$ seen in both dark states, ChR2-C128S-D156A D480 displays a second band at 1646 cm$^{-1}$ in the region of the C=N stretching (Figure 54; insert). The two C=N stretching’s indicate two chromophore states (i.e., all-trans and 13-cis) that are – unlike to D470 of ChR2-H134R – populated to a similar extent. This interpretation is consistent with the broadening of the C=C stretching envelope in D480 compared to D470. Note that these findings for D480 are similar to those for the Rh-Bi form of HKR1 (section 3.2.1; Figure 57A).

![Resonance Raman spectra](image)

*Figure 54.* Resonance Raman spectra measured at 78 K, 458 nm and 1 mW of D480 in ChR2-C128S-D156A (black) and D470 in ChR2-H134R (blue).
3.1.4 Discussion

3.3.4.1 Isomeric configuration of the retinal chromophore in D470

Unlike dark-adapted BR$_{560}$ and Rh-BI in HKR1 (section 4.2) with mixed all-trans, 15-anti and 13-cis, 15-syn isomeric compositions, the D470 room temperature spectrum of ChR2-H134R reveal no obvious asymmetries of the peak profiles of the C= C and C= N stretching bands (section 3.1.1.7; Figure 30). However, a global fit of all D470 spectra measured with 413, 458 and 514 nm excitation at 77 K and at room temperature with very low power, showed that two different isomeric states are involved.

On the other hand, UV-vis absorption spectroscopy has demonstrated that a photochemical reaction of the dark state is possible even at 5 K, implying a transition essentially without any thermal activation barrier. Furthermore, this reaction is fully reversible such that it is assigned to the primary photochemical process. The product displays the same absorption characteristics as the P500 intermediate which has been shown to be formed within 3 ps [100] and it decays within 6.5 µs as determined in this work (section 3.1.1.3; Table 3). Thus the lifetime is much longer than those of the photoproducts of BR or HR [172], suggesting that P500 represents not only the primary photoproduct, denoted as P500a, but also subsequent thermal relaxation products (P500i) which all exhibit very similar UV-vis absorption spectra. According to this hypothesis, we can rationalize both the UV-vis absorption and the resonance Raman spectroscopic results obtained at cryogenic temperatures. At 4 K (UV-vis) and 77 K (UV-vis and resonance Raman) only the primary photoreaction can take place whereas subsequent thermal reactions are blocked. Whereas in the UV-vis absorption experiment, this photoreaction must be initiated by extra blue-light irradiation, in the resonance Raman experiment the photoreaction is inevitably induced already by the Raman probe beam. Since this photoreaction is reversible (section 3.1.1.5; Figure 26), the resonance Raman spectrum of D470 measured at 77 K represents in fact the photostationary equilibrium between the true dark state and P500a.

Upon blue-light irradiation of ChR2-H134R at 220 K, thermal relaxation processes can take place such that P500a can thermally decay to P500i and even further to P390 as demonstrated by the UV-vis absorption (section 3.1.1.5; Figure 27) but also the resonance Raman spectra measured at different excitation wavelengths (section 3.1.1.6; Figure 29). P500i exhibits a protonated Schiff base with the retinal chromophore most likely in the 13-cis, 15-anti
configuration as this is the chromophore configuration of the subsequent decay product P390. This conclusion is derived from the comparison of the resonance Raman spectra of M410 state of BR with that of the long-lived P390 state of ChR2-C128S-D156A (section 3.1.3.3). The starting point of this photoinduced reaction sequence is hence attributed to a chromophore in an all-trans, 15-anti configuration.

The proposed interpretation is also consistent with the analysis of the resonance Raman spectra obtained at room temperature with high laser power. Under these conditions, the photocycle is efficiently initiated and, in view of the residence time of the sample in the laser beam (30 µs), the fraction of the photolysed protein is expected to be in the P390 state. This state was in fact detected in the resonance Raman spectrum obtained with 413 nm excitation but, due to the lack of sufficient resonance enhancement, not with 458 and 514 nm excitation. In all spectra, however, we noted a distinct increase of the relative contribution of the 13-cis species at the expense of the all-trans isomer compared to the spectra obtained at low laser power (section 3.1.1.7; Figure 33). A similar redistribution between the all-trans and 13-cis species are observed by comparing the low-temperature spectra obtained with and without blue-light irradiation at 220 K (section 3.1.1.6; Figure 29).

The Raman spectra of the dark state of other microbial rhodopsins such as green and blue absorbing proteorhodopsin [125], gleobactar rhodopsin [38] and sensory rhodopsin I and II from Halobacterium [121,122] are comparable to BR570 where the chromophore configuration has been identified as all-trans, 15-anti [127]. The fingerprint region of ChR2-H134R D470 with peaks at 1158, 1186, 1203 cm\(^{-1}\) and shoulders at 1175 and 1210 cm\(^{-1}\) is very similar to the blue-absorbing proteorhodopsin with peaks at 1162, 1185 and 1200 and a shoulder at 1172 cm\(^{-1}\) [125].

These findings indicate that the D470 state includes a mixture of a photochemically active all-trans, 15-anti isomer and a 13-cis species that is not or only to a much lower extent capable to undergo a photochemical conversion. If this 13-cis species has some photochemical activity at all, it is likely to lead to the same photoproduct as the photoconversion of the all-trans, 15-anti species, i.e. to a 13-cis, 15-anti configuration. This would imply that the photoisomerisation refers to the Schiff base double bond. In fact, such a scenario is not unlikely in view of a similar reaction pathway identified for HKR1 (section 3.2.3).
Resonance Raman spectroscopy on microbial rhodopsins

ChR2 shows no indication for a dark-adaptation like BR or HR [25,27,120,127,128], because this should be reflected by time-dependent changes in the UV-vis absorption and FTIR spectra [19–21]. However, 13-cis is detected in the extraction and pre-resonance Raman experiments for the so-called “dark-adapted” ChR2 [23] and ChR2-C128T [24], to a ratio of around 7:3 all-trans to 13-cis. The ratio remains fairly similar after “light-adaptation”. Contrary to this, extraction experiments from [142] showed 100% all-trans for “dark-adapted” ChR2 and 5:1 all-trans to 13-cis after “light-adaptation”. It appears to be that this scattering of the extraction data reflects the intrinsic inaccuracy of the method. Nevertheless, in BR only all-trans is found in the extraction experiments after light-adaptation [25] showing that the 13-cis isomer in the dark state of ChR2 behaves differently from that in BR.

Dark-adapted BR includes two chromophore isomers, i.e. an all-trans, 15-anti and a 13-cis, 15-syn configuration. In both cases, the protonated RSB is assumed to face the counterion Asp85 and a water molecule [54]. A similar arrangement was suggested for the Rh-BI state of HKR1 after homology modelling [108]. Such a scenario may also hold for the two isomers in D470 of ChR2. In fact, the reduction of bandwidth for both (D470 all-trans and 13-cis) upon deuteration showed that a water molecule is located in the near vicinity in agreement with previous Raman measurements on wild-type ChR2 [23] and the C1C2 crystal structure [28] (see section 3.3.4.2.).
3.3.4.2 Water and RSB interactions in D470 all-trans and 13-cis.

The natural line width $\Delta\nu$ (in cm$^{-1}$) is related to the natural life time $\Delta t$ of an excited state according to

$$
\Delta t \Delta E = \frac{\hbar}{4\pi} = \Delta t \cdot \hbar \cdot \Delta\nu c
$$

and thus

$$
\Delta t = \frac{1}{4\pi \Delta\nu c}
$$

where $c$ is the velocity of light and $\hbar$ the Planck constant. If we take the half width of the C=ND stretching in a first approximation as a transition lacking intermolecular energy transfer, the half width is mainly controlled by intramolecular energy transfer, corresponding to the decay to the ground state given by the decay constant $k_0$ (the reciprocal value of $\Delta t_{D_2O}$). As the specific structure of the chromophore in the binding pocket is largely the same in H$_2$O and D$_2$O, we can further assume that $k_0$ is the same for both the C=ND and C=NH stretching. Then the line broadening for the C=NH stretching and thus the respective decreased life time ($\Delta t_{H_2O}$) can be used to determine the rate constant for the energy transfer to the adjacent water molecule ($k_{ET}$) according to

$$
\Delta t_{H_2O} = \frac{1}{k_{H_2O}} = \frac{1}{k_0 + k_{ET}}
$$

The calculated natural life times and rate constants for the all-trans and 13-cis isomers in D470 in H$_2$O and D$_2$O are shown in Table 8.

<table>
<thead>
<tr>
<th></th>
<th>$\Delta\nu$ / cm$^{-1}$</th>
<th>$\Delta t / 10^{13}$ s</th>
<th>$k / 10^{13}$ s$^{-1}$</th>
<th>$k_{ET} / 10^{12}$ s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D470 all-trans H$_2$O</td>
<td>17.0</td>
<td>1.560</td>
<td>0.641</td>
<td></td>
</tr>
<tr>
<td>D470 all-trans D$_2$O</td>
<td>15.1</td>
<td>1.757</td>
<td>0.569</td>
<td>0.716</td>
</tr>
<tr>
<td>D470 13-cis H$_2$O</td>
<td>34.8</td>
<td>0.762</td>
<td>1.312</td>
<td>4.901</td>
</tr>
<tr>
<td>D470 13-cis D$_2$O</td>
<td>21.8</td>
<td>1.217</td>
<td>0.822</td>
<td></td>
</tr>
</tbody>
</table>

Table 8. Half widths (section 3.1.1.7; Figure 38) and calculated life times and rate constants for D470 all-trans and 13-cis in the H$_2$O and D$_2$O.
Resonance Raman spectroscopy on microbial rhodopsins

On the basis of the Förster mechanism for the intermolecular (collisionless) energy transfer [173], the different values for $k_{ET}$ in D470 all-trans and 13-cis should be primarily due to different distances of the water molecule with respect to the Schiff base. Accordingly, one may derive the change in the distance R in the two states D470 all-trans and 13-cis by

\[
\frac{k_{ET}(\text{D470 all-trans})}{k_{ET}(\text{D470 13-cis})} = \frac{R_{D470 13-cis}^6}{R_{D470 all-trans}^6}
\]

which leads to $R_{\text{all-trans}} = 1.38 \cdot R_{13-cis}$. In the C1C2 dark state, the distance of the RSB nitrogen to the closest H$_2$O is 4.4 Å [28]. Assuming comparable structures of C1C2 and ChR2 D470 all-trans, the equivalent distance in D470 13-cis is 3.2 Å corresponding to a 1.2 Å decrease.
3.3.4.3 Introducing mutations close to the retinal

The slow photocycle of ChR2-C128S-D156A. The dark state of ChR2-C128S-D156A (D480) has a slightly red-shifted absorption spectra compared to wild-type ChR2 (D470). UV-vis absorption spectroscopy at 77 K and room temperature showed that the first part of the photocycle of ChR2-C128S-D156A includes the same intermediates as ChR2. D480 is photoconverted to P500 upon retinal isomerisation followed by the deprotonation of the RSB giving P390. UV-vis absorption spectroscopy at room temperature showed no indications for the presence of the late intermediates P520 and P480a/b unless their absorption is obscured by the absorption of the dark state. Electrophysiology data showed that the channel in ChR2-C128S-D156A is open long after illumination, thus the conducting state P520 is likely to be in equilibrium with P390, and the involvement of P520 in the photocycle is further substantiated by the fact the channel off-kinetics can be accelerated by exposure to red light [81,150]. The decay of P390 matches the recovery of D480 with time constants of around 80 min implying that the thermal decay of P390 is much slower than the decay of the subsequent intermediates P480a/b (section 3.1.3.1; Figure 47). The recovery of D480 can be accelerated by illumination in the UV evidently due to a photoconversion of P390 similar to, for example, the M to the dark state ppR phototransformation in pharaonis phoborhodopsin [174].

In terms of the kinetics, the early events up to the formation of P390 are quite similar in ChR2-C128S-D156A as in the wild-typ like protein ChR2-H134R. This is most likely also true for the isomeric composition of the dark state D480 in terms of an all-trans, 15-anti and a 13-cis, 15-syn species. However, details of the chromophore structures are likely to be different and the relative contribution of the 13-cis species is presumably higher than in the D470 state of ChR2-H134R. This conclusion is derived, inter alia, from the clearly detectable second C=N stretching mode, the broadening of the C=C stretching band profile, and the changes in the fingerprint region as well as in the HOOP region. Specifically, the relatively high resonance Raman activity in the HOOP region which has no counterpart in the spectrum of the dark state of ChR2-H134R suggests that the mutation in the chromophore pocket of ChR2-C128S-D156A causes a distortion of the retinal polyene chain with substantial deviations from planarity [119].

If we assume the same photochemical behaviour of the two isomers of the dark state as in ChR2-H134R, photolysis within the exciting laser beam of the high-power resonance Raman spectrum should lead to the accumulation of the P390 state whereas the all-trans species of
the dark state is preferentially depleted. In fact, formation of P390 is observed upon 413-nm excitation and its spectrum exhibits basically the same vibrational pattern of the M\textsubscript{410} state of BR, confirming the assignment of the chromophore to a 13-cis, 15-anti retinal with a non-protonated Schiff base (section 3.1.3.3; Figure 53). Also in analogy to BR, the P390 can be photochemically converted back to the dark state as indicated by the decrease of its relative contribution in the spectrum obtained with high laser power. This effect corresponds to the observation that recovery of the dark state is promoted under UV irradiation (vide supra).

Since the difference spectrum “high power-minus-low power” does not correspond to the pure P390 spectrum, one must conclude that either an additional intermediate is involved or the isomeric composition of the “residual dark state” is different in the high power spectrum compared to the low-power spectrum. As the only intermediate on the pathway from D480 to P390 is the short-lived P500 with a life time shorter than the residence time of the sample in the laser beam (section 3.3.4.1), the former interpretation can be discarded. Instead, the present observations nicely support the view that photoconversion preferentially occurs with the all-trans form of D480 as discussed in detail for the ChR2-H134R variant. However, it cannot be excluded that also a P520-like state contributes to the spectra if this state is blue-shifted compared to the wild-type P520 and thus enhanced at 413 nm.

In this context it is interesting to compare the difference UV-vis absorption spectrum of ChR2-H134R “illuminated at 220 K minus dark state” (section 3.1.1.5; Figure 27) with the difference spectrum “ChR2-C128S-D156A D480 minus ChR2-H134R D470” (Figure 55) which are very similar. Whereas the former spectrum reflects the formation of the intermediate states P500 and P390 at the expense of the dark state D470, the latter spectrum demonstrates the different isomeric composition in the dark state. Residual P390 intermediate was observed in the dark state of ChR2-C128S-D156A since the absorption in the UV after UV-irradiation was depleted to below the level of the dark-adapted spectrum (section 3.1.3.1; Figure 48). The red-shifted absorption of this mutant can be rationalised if we assume that the altered isomeric composition in the dark state of ChR2-C128S-D156A corresponds to an increased 13-cis contribution since the UV-vis absorption spectrum of this isomer (rather than the all-trans species) should be more closely related to the absorption spectrum of P500, carrying a 13-cis chromophore as well, albeit with a presumably different Schiff base configuration (i.e. syn vs. anti). The similarity of these spectra reflecting the dark state with a 13-cis retinal and the P500 intermediate, respectively, indicates that UV-vis absorption
spectroscopy is not necessarily very sensitive towards structural details of the 13-cis chromophores in ChR2. The situation is different in the corresponding resonance Raman difference spectra (Figure 56) which display differences due to the conformational perturbations induced by the mutation in the retinal binding pocket, i.e. in the C-C (1150-1250 cm\(^{-1}\)) and C=C (1550-1570 cm\(^{-1}\)) stretching vibration difference bands.

**Figure 55.** Difference UV-vis absorption spectra measured at 77 K. Black; ChR2-H134R illuminated at 220 K minus the dark state. Blue; dark state of ChR2-C128S-D156A minus dark state of ChR2-H134R.

**Figure 56.** Difference resonance Raman spectra measured at 458 nm (1 mW) and 78 K. Black; ChR2-H134R illuminated at 223 K minus the dark state. Blue; dark state of ChR2-C128S-D156A minus dark state of ChR2-H134R.
The side-reaction of the photocycle of ChR2-C128T involves hydrolysis of the retinal Schiff base. ChR2-C128T shows a full photocycle and an additional side-reaction including the two UV-absorbing intermediates P380 and P353 [24]. Different to P380, P353 displays a fine-structured UV-vis absorption spectrum [21,24,98]. Resonance Raman spectroscopy at different pH revealed that there are no structural differences between the chromophores in P380 and P353. The differences in the absorption pattern of these states is therefore rather related to the environment of the chromophores. The retinal vibrational bands of P380 and P353 matched those of free all-trans retinal in the hydrophobic solvent CCl₄. Thus the formation of P380 and P353 involves the hydrolysis of the Schiff base and the created free all-trans retinal is embedded within a hydrophobic protein pocket.

The resonance Raman spectra of retro-BR and P353 excluded any similarities of the chromophores, not unexpectedly since P353 is part of the ChR2-C128T photocycle whereas retro-BR is irreversibly created after reduction and UV-treatment (section 3.4.1). Beside retro-BR, vibronic fine-structure in the UV has been found in the absorption spectra of BR and blowfly rhodopsin containing an unbound retinol located in the chromophore pocket [110,145,175]. The fine-structure is proposed to origin from environmentally-induced geometric constraints such as hydrogen bonding leading to a coplanar ring-chain organisation of the chromophore. For the retinol bound in BR, the fine-structure is lost at acidic pH presumably due to protonation of an amino acid in the chromophore pocket leading to loss of a hydrogen bond to the retinol hydroxyl group [176]. The opposite pH-dependence is observed in the case of P380 and P353 and may analogously be explained by deprotonation of an amino acid at alkaline conditions and loss of hydrogen bonding to the aldehyde in retinal.

According to the C1C2 crystal structure [28], a water is located 4.4 Å from the RSB which is also seen in the resonance Raman spectra of ChR2 [23] and ChR2-H134R (section 3.1.1.7). It is conceivable that this water is responsible for the hydrolysis if the structural changes during the photocycle bring the water closer to the RSB. This argument may hold for both ChR2-H134R and ChR2-C128T but the latter variant exhibits much longer life times of the intermediates formed after re-protonation of the Schiff base corresponding to a much longer reaction time. For instance, the decay time of P520 is ca. 500 times slower than in ChR2-H134R [24]. On the first sight, this interpretation may be questioned by the fact that the recovery time of the photocycle of ChR2-C128S-D156A is even much longer than that of ChR2-C128T but does not provide any indication for the transient hydrolysis of the Schiff
base. However, this unusually long recovery time in ChR2-C128S-D156A is caused by the slow decay of P390 and P520, whereas the lifetime of the subsequent intermediate P480 \textsubscript{a} at which branching to the P380/P353 intermediates in C128T occurs must be significantly shorter although it cannot be determined.

P380 and P353 decays into D470 upon reformation of the RSB; hence the free all-trans retinal in these intermediates must remain inside the protein in contrary to human rhodopsins where the all-trans retinal – formed after RSB hydrolysis in metarhodopsin II – escapes from the protein via a “retinal channel” [177]. Enzymes are needed for the reformation of functional phororeceptor by isomerising the released chromophore and reconstituting opsin with 11-cis retinal (see [31] for a review). The free retinal in ChR2-C128T is apparently stronger bound in the chromophore pocket than in the case of visual rhodopsins consistent with the vibronic fine-structure and thus steric confinement as well as the hydrophobic environment of the free all-trans retinal in P353.
3.2 Histidine kinase rhodopsin-1

The structure of the retinal chromophore and the protein backbone of the two stationary states Rh-UV and Rh-Bl in the histidine kinase rhodopsin-1 (HKR1) photocycle were investigated using resonance Raman and ATR-FTIR difference spectroscopy. The samples contained a truncated version of the protein, i.e. only the rhodopsin part without histidine kinase, response regulator and cyclase domains was purified [108].

3.2.1 Retinal chromophores in Rh-UV and Rh-Bl

In an approach similar to the resonance Raman measurements of ChR2-C128S-D156A (section 3.1.3.3), the laser line was chosen to selectively enhance one of the two states; 413 nm in the case of Rh-UV and 514 nm for Rh-Bl. Laser excitation inevitably promotes photochemical conversion of the respective states such that a continuous background of LED irradiation in the blue (480 nm) and UV (375 nm) region was necessary to revert the phototransformation of Rh-UV and Rh-Bl, respectively. The spectra were accumulated for 1 hour at 1 mW using the rotating cuvette containing a mixing ball (500 μL sample, OD380 ~3). Spectra measured without LED-background at 514 nm excitation showed only buffer bands whereas at 413 nm excitation, both buffer and Rh-UV contributions were detectable although the intensities of the latter bands were lower compared to the spectra taken with a LED-background. Spectra with and without LED irradiation were subtracted from each other to obtain spectra free of buffer bands. Unfortunately, the rhodopsin part of HKR1 was not stable in D_{2}O resulting in rather poor spectra compared to sample in H_{2}O.

The resonance Raman spectrum of Rh-UV displays bands at 972 and 1010 cm\(^{-1}\) (C-CH\(_{3}\) rock), 1173, 1187, 1196 and 1223 (C-C stretch), 1303 and 1378 cm\(^{-1}\) (C-H rock), 1566 cm\(^{-1}\) (C=C stretch) and 1613 cm\(^{-1}\) (C=N stretch) (Figure 57B). No shifts of bands are observed after H\(_{2}\)O to D\(_{2}\)O exchange indicating that the RSB is deprotonated (Figure 58). The overall band-pattern almost accurately resembles the spectrum of the M\(_{410}\) intermediate of BR, with exceptions for the C-C at 1173/1179 and C=N stretchings at 1613/1620 cm\(^{-1}\) in Rh-UV/BR-M (Figure 57B/C). Thus it is concluded that Rh-UV contains a retinal in the same configuration as in the BR M-state, i.e. a 13-cis, 15-anti deprotonated RSB [129].

There are no spectral similarities when comparing the spectra of Rh-Bl and Rh-UV (Figure 57A/B). The Rh-Bl C=C stretching region consists of two bands at 1540 and 1550 cm\(^{-1}\)
resemblant of the split C=C stretchings of the K, L or N intermediate in the BR-photocycle (Figure 57A) [132,133,166]. However Rh-Bl displays two C=N stretching bands at 1633 and 1652 cm\(^{-1}\) – shifted to 1620 cm\(^{-1}\) in D\(_2\)O (Figure 59) – which can only be explained by the coexistence of two different isomers with a protonated RSB. The intensities of the two C=C stretchings in Rh-Bl are similar indicating an almost fifty-fifty ratio of the isomer distribution, involving an \textit{all-trans} and a \textit{13-cis} configuration in analogy to the dark-adapted state of BR.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{resonance_raman_spectra.png}
\caption{Resonance Raman spectra obtained with 514 nm excitation under continuous LED irradiation in the UV (375 nm) (A) and with 413 nm excitation and blue (480 nm) LED irradiation (B), representing Rh-Bl and Rh-UV, respectively. The time-resolved resonance Raman spectrum of M\textsubscript{410} of BR obtained by the pump (514 nm) – probe (413 nm) technique is shown in (C) for comparison. The contribution of the buffer has been subtracted from all states. Adapted with permission from [108]. Copyright (2012) American Society for Biochemistry and Molecular Biology.}
\end{figure}
Figure 58. Resonance Raman spectra using 413 nm excitation (and blue LED irradiation) to probe Rh-UV in H$_2$O (black) and D$_2$O (grey).

Figure 59. Resonance Raman spectra using 514 nm excitation (and UV LED irradiation) to probe Rh-Bl in H$_2$O (black) and D$_2$O (grey).
3.2.2 Light-induced structural changes of the protein backbone

A concentrated HKR1 sample (30 μL, OD_{380} ~ 20-30) was illuminated in the blue to initially accumulate the pure Rh-UV state and ATR-FTIR difference spectra of the illuminated state minus the dark state were recorded. Spectra were accumulated while continuously exposing the sample to UV (375 nm) light to induce the photoreaction to Rh-Bl and blue (480 nm) light to induce the back-reaction to Rh-UV. The difference spectra going from Rh-UV → Rh-Bl and Rh-Bl → Rh-UV are mirror images indicating that the light-induced structural changes are fully reversible (Figure 60). The amide-I and amide-II difference bands — corresponding to the structural rearrangements of the protein backbone — are found at 1656/1673 and 1552/1535 cm⁻¹, respectively. The Rh-UV → Rh-Bl spectrum (Figure 60; grey) has a higher level of noise and there is also a drift in the background which could be caused by temperature shifts induced by the LED irradiation. The difference band at 1726 cm⁻¹ in the region of the C=O stretch vibration is negative going from Rh-UV to Rh-Bl, related to the deprotonation of a carboxylic acid [178]. The subsequent protonation of the carboxylate in the transition Rh-Bl to Rh-UV is seen as positive absorbance (black). The mirrored C=O band can be assigned to one or more aspartic and/or glutamic acids potentially acting as a proton donor and acceptor for the RSB.

![Figure 60](image_url)
3.2.3 Discussion

The excellent spectral agreement between Rh-UV of BR-M_{410} indicates that the most stable dark state of the rhodopsin part in HKR1 includes a chromophore in the 13-cis, 15-anti configuration with a deprotonated RSB. Although the function and photocycle of BR and HKR1 are different, the amino acid sequence in the retinal pocket is highly homologous (supplementary info in [108]) supporting the conclusion of similar structures of the chromophores. Homology modelling using structures of other microbial rhodopsins such as BR, SRII [179], HR [180] and C1C2 [28] implied that the free electron pair of the RSB nitrogen is facing away from the bona fide counterion Asp-239 [108]. Thus, it may explain why the protein is stable in this state since protonation facilitated by the Asp-239 is sterically unfavourable (Figure 61A). In order to stabilise Rh-BI, the RSB proton should instead face towards the negatively charged Asp-239 which could be achieved by a retinal in the all-trans, 15-anti or 13-cis, 15-syn configuration (Figure 61B/C). Furthermore, introducing the mutation D239E caused a hypsochromic (blue) shift in absorption of Rh-BI implying that this residue interacts with the protonated Schiff base [108]. The proton transfer of D239 might be assigned to the C=O difference bands at 1726 cm\(^{-1}\) in the FTIR spectra which reflects the deprotonation of the carboxylic acid (Rh-UV to Rh-BI) or protonation of the carboxylate (Rh-BI to Rh-UV) although mutational removal of this residue is necessary to unambiguously confirm this interpretation. Unfortunately, no functional protein could be purified without a carboxylic acid at position 239 (Roman Fudim, personal communication). The mirrored amide-I and amide-II in the difference FTIR spectra further indicates that the structural rearrangements of the protein helixes are reversible between the two states.

Rh-BI includes two different isomeric states seen as a pair of C=C and C=N stretching bands in the resonance Raman spectra. H/D exchange confirmed that the RSB is protonated in both species. Retinal extraction experiments and HPLC analysis (supplementary info in [108]) detected a mixture of all-trans and 13-cis in both Rh-BI and Rh-UV. The all-trans found in Rh-UV (which was less than in Rh-BI) may be attributed to thermal relaxation of the chromophore during or after the extraction procedure since the resonance Raman spectra showed no indications for the presence of a second isomer. Assuming all-trans and 13-cis are the two isomers in Rh-BI, the transition to this state can then only be achieved by 13-cis/trans or 15-anti/syn isomerisation prior to the protonation of the RSB. This would generate a mixture of all-trans, 15-anti and 13-cis, 15-syn, which are the two isomeric configurations
found in the dark-adapted BR<sub>570</sub>. The electronically excited state of Rh-UV is unusually long (~60 ps) which might reflect the dual option of isomerisation [108]. Anti/syn isomerisation is also involved in the photochemical transition from the Meta-II to the Meta-III state of bovine rhodopsin [181]. Ultrafast spectroscopy revealed that the excited state after light-activation of Rh-BI decays with two different time constants which might be assigned to the two Rh-BI isomers [108].

![Diagram of retinal configuration and isomers](image)

**Figure 61.** The retinal configuration in Rh-UV (A) as well as the proposed isomers involved in Rh-BI (B & C). Light-induced 13-cis/trans isomerisation is indicated in blue and 15-anti/syn in green. A thermal equilibrium (T) presumably exists between the Rh-BI isomers. Reprinted with permission from [108]. Copyright (2012) American Society for Biochemistry and Molecular Biology.
3.3 Xanthorhodopsin

3.3.1 The salinixanthin and retinal chromophores

Xanthorhodopsin (XR) displays a broad UV-vis spectrum covering the absorption of salinixanthin and retinal [57]. The structure of the chromophores was investigated using resonance Raman spectroscopy with excitation lines at 458, 514 and 568 nm (1 mW). Probing at 458 and 514 nm should preferentially enhance the spectrum of the salinixanthin and at 568 nm that of the retinal chromophore. 500 µL (OD486 ~3) XR in the native membrane of the bacterium Salinibacter ruber (1 mM tris, 15 mM KCl, pH 7.0) was measured in a rotating cuvette using the mixing ball. The exchange of sample in the laser spot combined with the short photocycle (~1 s; [57]) assure detection of only the dark state. The accumulation time was 10 min at 458/514 nm and 30 min at 568 nm. In order to increase the frequency-window at 568 nm, spectra were accumulated in two different segments – covering an interval of 800 – 1800 cm\(^{-1}\) with an overlap of around 200 cm\(^{-1}\) – and finally combined.

The bands assigned to the retinal chromophore in the 568 nm spectrum (Figure 62A; black) are located at 825 and 879 (HOOP), 957 and 1006 (C-CH\(_3\) rock), 1171, 1186, 1200 and 1206 (C-C stretch), 1254 and 1275 (C-H rock), 1530 and 1578 (C=C stretch) and 1644 cm\(^{-1}\) (C=N stretch) and show the almost identical counterparts of resonance Raman spectrum of BR\(_{570}\) [127]. In D\(_2\)O (Figure 62A; grey) the C=N stretching at 1644 shifts to 1628 cm\(^{-1}\) and the N-H bending at around 1350 to 976 cm\(^{-1}\), showing that the RSB is protonated (Figure 4A/B in [127]). The bands at 1156 and 1516 cm\(^{-1}\) are assigned to the C-C and C=C stretchings of salinixanthin as seen in the 458 and 514 nm spectra (Figure 62B/C) as well as in the FT-Raman spectrum of salinixanthin in deuterated methanol (Figure 62D), consistent with the characteristic vibrational pattern of carotenoids [170]. The salinixanthin spectrum taken at 514 nm does not change in D\(_2\)O (data not shown) and the excitation wavelength 458 or 514 nm only affected the intensities of the various modes.
Figure 62. Resonance Raman spectra of XR measured at 568 (A) (black H\textsubscript{2}O, grey D\textsubscript{2}O), 514 (B) and 458 nm (C). All spectra were accumulated at 1 mW excitation energy and normalised to the 1156 cm\(^{-1}\) band. FT-Raman on salinixanthin (D) measured at 1064 nm, the spectrum of the solvent (CD\textsubscript{3}OD) has been subtracted.
3.3.2 Accumulation of the L-intermediate in the laser spot

Increasing the power of the laser to 10 mW did not induce any changes in the salinixanthin spectra at 458 and 514 nm but caused a shift of the relative intensity of the C=C stretching bands at 568 nm (Figure 63; top). The intensity decrease of the C=C stretching of the retinal (1530 cm\(^{-1}\)) compared to that of salinixanthin (1516 cm\(^{-1}\)) is seen in the subtracted spectrum (“10 mW spectrum minus 1 mW spectrum”) (Figure 63; bottom). The carotenoid C-C and C=C stretchings at 1156 and 1516 cm\(^{-1}\) are still detectable but two new bands in the C=C stretching region at 1539 and 1553 cm\(^{-1}\) indicate the presence of an L-intermediate [131,132,167]. Thus, the photocycle is initiated in the laser spot although the contribution of the L-intermediate in the spectrum at 10 mW is relatively low.

*Figure 63.* Top; Resonance Raman spectra of XR measured at 568 nm excitation at 1 (black) and 10 mW (grey). Bottom; subtracted spectrum 10 − 1 mW applying maximal subtraction while avoiding generation of negative bands.
3.3.3 Summary

The selective enhancement by tuning the laser wavelength to the absorption maximum of a certain state or chromophore is nicely demonstrated in the case of XR. The salinixanthin chromophore can be detected at 458, 514 and 568 nm whereas sufficient enhancement for the retinal is only obtained at 568 nm excitation. This allows for separation and identification of salinixanthin and retinal bands. The salinixanthin spectra at 458 and 514 nm did not display a dependence on the laser power showing the photochemical stability of the chromophore. The resonance Raman spectrum with 514 nm excitation displayed no isotopic shifts in D₂O since there are no exchangeable protons on salinixanthin.

The retinal bands at 568 nm as well as the shifts of the C=N stretching and N-H bending in D₂O, revealed almost identical features compared to the light-adapted BR₅₇₀. Thus the retinal chromophore in the dark state of XR is in the all-trans, 15-anti geometry with a protonated RSB [127]. The marker bands at 800 (HOOP) and 1183 cm⁻¹ (C-C stretching) of the second isomer (13-cis, 15-syn protonated RSB) in dark-adapted BR₅₆₀ [128] was not found in the XR resonance Raman spectrum. However if present original, it might not be detected due to laser-induced isomerisation of the chromophore with the onset of the resonance Raman measurement. Unlike to the salinixanthin spectra at 458 and 514 nm, higher laser powers induced spectral changes at 568 nm seen by a shift of the intensity ratio of the salinixanthin and retinal C=C stretching bands. At high power, a species similar to the BR L-intermediate is accumulated in the laser spot, albeit with low concentrations.
3.4 Bacteriorhodopsin

3.4.1 Reduced and UV-treated bacteriorhodopsin

The retinal chromophore in BR, after reduction and UV-treatment according to [144, 145] (see material and methods, section 2.2), was investigated using UV-vis and resonance Raman spectroscopy. The spectra were compared to those of the UV-absorbing intermediates in the ChR2-C128T photocycle (section 3.1.2.2) as well as to calculated spectra.

The reduction of the retinal chromophore in BR using NaBH₄ had to be carried out under yellow illumination to induce the transition into the M-state. It is therefore assumed that the chromophore being reduced is in the configuration of 13-cis, 15-anti deprotonated RSB. The proposed mechanism for the reduction reaction includes hydride transfer to carbon 15 and a shift of one electron pair to the nitrogen (Figure 64). The now negatively charged nitrogen receives a proton from the environment, probably from Asp96, the amino acid responsible for the re-protonation of the RSB in the BR photocycle [46, 47, 178]. The reduced chromophore has an absorption maximum at around 370 nm and, after irradiation in the UV, it is transferred into a state displaying a fine-structured UV-vis absorption spectrum (Figure 65). The reduction and UV reaction is irreversible and the chromophore un-extractable [144, 145]. The fine-structure obtained after UV-irradiation or by acidic pH is proposed to be related to a shift of the conjugated bonds either towards or away from the ionone ring to afford a so-called retro configuration (Figure 64) [144, 145]. In retro-retinyl the angle of the ionone ring is fixed with respect to the polyene chain via the double bond between carbons 6 and 7 which leads to steric constraints in the conformational flexibility and thus to a fine-structured absorption pattern. The reduced and UV-treated BR will, for the sake of simplicity, be called retro-BR.

Under the assumption of a reduction mechanism as proposed in Figure 64, performing the reaction in NaBD₄ should give a deuterium at carbon 15 and in D₂O at the nitrogen. The reduction and UV-treatment was carried out in a) H₂O/NaBH₄, b) H₂O/NaBD₄, c) D₂O/NaBH₄ and d) D₂O/NaBD₄ followed by exchange to 150 mM sodium phosphate buffer, pH 7.0 (in H₂O). The four reaction mixtures gave the same UV-vis absorption spectra with maxima at 344, 363 and 383 nm (Figure 65B). Unfortunately, also the resonance Raman spectra measured at 351 nm were identical in all of these cases. The spectrum of retro-BR showed bands at 1014 (C-CH₃ rock), 1156, 1190 and 1216 (C-C stretch), 1278 and 1323 (C-H
rock) and 1570 cm\(^{-1}\) (C=C stretching) (Figure 66). The band at 1638 cm\(^{-1}\) cannot be assigned to a C=N stretching since the C=N bond was reduced. However, the frequency is in line with that of a stretching mode of a poorly conjugated C=C bond. It is conceivable that carbon 15 and the nitrogen are no longer part of the conjugated system, such that these vibrations are therefore not enhanced and deuterated-induced shifts not detected which argues in favour of a shift of the conjugated system towards the ionone ring. To evaluate this, the Raman spectrum of retro-retinyl ethyl amine was calculated using density functional theory [116] (Figure 66). The bands at 1156, 1190, 1216, 1278 and 1323 cm\(^{-1}\) in the fingerprint region of retro-BR show a comparable pattern in the calculated spectrum. However the main C=C stretching frequency is too high in the calculated spectrum (1594 cm\(^{-1}\)) compared to in the experimental spectrum (1570 cm\(^{-1}\)) to safely confirm the structure. The calculation includes a hexane solvent model and the work has to be continued with advanced hybrid techniques in order to consider the protein environment of the chromophore.
Figure 64. Proposed reaction mechanism for the reduction (using NaBH₄) of BR-M₄₁₀, containing a 13-cis, 15-anti deprotonated RSB. Following irradiation in the UV or by lowering the pH presumably shifts the conjugated double bonds, although the final configuration is not known [144,145].
Figure 65. (A) Preparation of reduced and UV-treated (in 0.1 M NH₄CO₃H, pH ~10) (grey) using NaBH₄ and exposure to yellow light to accumulate the M₄₁₀ (black) followed by UV irradiation (blue). NaBH₄ reacts with water under the formation of hydrogen gas and the bubbles created during the reaction are causing the noise in the spectra. (B) Retro-BR (in 0.1 M NH₄CO₃H, pH ~10) after complete reaction with NaBH₄.

Figure 66. Black; resonance Raman spectra of retro-BR excited with 351 nm. The reaction was carried out in H₂O/NaBH₄ followed by exchange to 150 mM phosphate buffer (pH 7.0). Red; calculated spectrum of 13-cis, 15-anti retro-retinyl ethyl amine (insert) using the program package Gaussian03, B3-P86, 6-31(d) basis set and a uniform frequency scaling by a factor of 0.9644 [116]. The calculation was performed by Hendrik Naumann.
3.4.2 The M_{410} state

BR-M_{410} is used in comparison with deprotonated states of other microbial rhodopsin and a pump probe experiment was performed in order to acquire a fresh data-set. 2 mL purple membrane from *Halobacterium halobium* strain S9 (OD_{570} ~2, 10 mM Tris-buffer, pH 8.0) in a rotating cuvette was measured using a 25 mW 413 nm probe beam. The photocycle was initiated using a 250 mW 514 nm pump beam which was spatially displaced with respect to the probe beam so that the delay of probe after pump was circa 1 ms. The accumulation time was 4 hours and the buffer contribution was removed prior to polynomial baseline subtraction. The measured spectrum agrees very well with those previously published (Figure 67) [129]. Bands are found at 976 and 1012 (C-CH$_3$ rock), 1179, 1187, 1197 and 1226 (C-C stretch), 1274, 1307, 1334 and 1376 (C-H rock), 1566 (C=C stretch) and 1620 cm$^{-1}$ (C=N stretch). The chromophore was assigned to a 13-*cis*, 15-*anti* configuration with a deprotonated RSB [129].

![Spectrum of BR-M_{410}](image)

*Figure 67.* Pump (514 nm) and probe (413 nm) resonance Raman experiment of purple membrane representing BR-M$_{410}$. 
3.4.3 Summary
The preparation of reduced and UV-exposed BR (retro-BR) according to [144,145] was successfully reproduced. Retro-BR absorbs in the UV and shows a distinct fine-structured pattern believed to originate from a shift of the conjugated double bonds. This involves fixation of the ionone ring with respect to the polyene chain leading to restrictions in movement of the chromophore out of the co-planar structure. Resonance Raman experiments of retro-BR afforded a unique spectrum which remained unchanged when the reaction was performed in NaBD₄ and/or D₂O. The shift of the conjugated system during UV-irradiation is therefore most likely moving in the direction of the ionone ring since the deuterated parts of the chromophore are then separated from the conjugated region. The DFT calculated spectrum of this structure was comparable to the experimental spectrum in the C-C stretching and C-H bending region. However a 24 cm⁻¹ difference was observed for the main C=C stretching which might be explained by polarity and/or electrostatic effects related to the surroundings that are not adequately considered by the present approach.
Summary and outlook

In this work, resonance Raman spectroscopy has been used to specifically target and elucidate the structure of the chromophore of several microbial rhodopsins, among them XR, various ChR2 mutants and HRK1, which have not yet been characterised by resonance Raman spectroscopy in depth so far. Special emphasis has been laid on the analysis of the chromophore structure in the dark states which in most cases display heterogeneous isomer composition. This is certainly one of the reasons for the quite complex phototransformation pathways which are by far not fully understood. Although the present work already provides some information about the photoisomerisation steps via determining the retinal configuration in photoconversion products, future studies have to be focussed on the vibrational spectroscopic characterisation of the intermediate states.

a) HKR1 Rh-UV and Rh-Bl could be studied by the means of two different laser lines. The chromophore of Rh-UV was assigned to a 13-cis, 15-anti deprotonated RSB whereas Rh-Bl consists of two different isomers (with shifted frequencies of the C=C and C=N stretching’s), most likely corresponding to an all-trans, 15-anti and 13-cis, 15-syn retinal, both with protonated Schiff bases. The phototransformation pathways, however, do not resemble that of BR and related bacterial rhodopsins, specifically due to the lack of detectable intermediate states with characteristic UV-vis absorption maxima and life times on the micro- and millisecond time scale. On the other hand, it is reasonable to assume that protein structural changes occur on this time scale which hence should be explored by IR difference spectroscopy.

b) The analysis of the resonance Raman spectra at 78 K and at room temperature showed that the ChR2 dark state (D470) consists of two different isomeric states with predominant contributions of the all-trans form. The contributions of the D470 13-cis isomer was found to increase upon increased laser intensity at room temperature and thus increasing the extent of photoconversion, or by heating and illuminating the sample in the cryogenic experiments and thus allowing the thermal transition into later intermediates. The shift of the isomeric distribution might be related to inactivity or lower photo-reactivity of the D470 13-cis isomer based on the observation that the photocycle intermediates were not enhanced and therefore not detected at 458 nm excitation in contrary to at 413 nm (P390; 78 K and room temperatures) and 514 nm (P500; 78 K). Future experiments would include pump-probe resonance Raman spectroscopy to obtain high quality spectra of the
intermediates P500, P390 and the conducting state P520 to explicitly determine the isomeric configuration of the retinal in these states but also to study, for instance, the interaction of the retinal Schiff base with the protein/solvent environment.

c) Furthermore, the recent C1C2 crystal structure now enables QM-MM calculations which can provide calculated spectra for the ChR2 dark state. This theoretical technique has to be implemented to the reduced chromophore in BR as well.

d) The P353/P380 intermediates in the side-chain of the ChR2-C128T photocycle could be assigned to an unbound free all-trans retinal. Thus, the side-reaction corresponds to the hydrolysis of the RSB under the formation of free retinal and subsequent reformation of the covalent linkage to the protein.

e) ChR2-C128S-D156A was found to include small amounts of the P390 intermediate in the dark-adapted sample. P390 can be further accumulated by exposing this mutant to blue light and P390 decays, correlated with the formation of the dark state, on the time-scale of hours. However, the transition into the dark state can be accelerated by irradiation with UV light. The retinal structure of P390 was studied by subtracting the low and high power resonance Raman spectra. Although the subtracted spectrum included residual contributions of the dark state, probably due to a shift of the all-trans and 13-cis isomer distribution in the dark state, the remaining bands could be assigned to a 13-cis, 15-anti deprotonated retinal Schiff base, in view of the similarities with the resonance Raman spectrum of BR-M410. However, time-resolved resonance Raman spectroscopy, where the transition into the P390 state can be effectively induced by a pump beam, would be required to improve the P390 spectrum.
Acknowledgements

I have had the privilege to work in two groups, being co-supervised by Prof. Dr. Peter Hildebrandt at the TU Berlin (Raman spectroscopy) and Prof. Dr. Peter Hegemann at the HU Berlin (protein expression and characterisation) which has been a rewarding experience. I would like to express my deepest gratitude to both supervisors for offering the opportunity to work on this topic as well as for the expertise and inspiring feedback. To Peter Hildebrandt for bringing clarity into a sometimes diffuse topic and to Peter Hegemann for always supporting and critically evaluating the project and thereby accelerating the progress.

Additional supervision was provided by Dr. Katja Stehfest at the HU Berlin and Dr. Hendrik Naumann at the TU Berlin, who excellently taught and guided me in the practical aspects of protein expression/purification and resonance Raman spectroscopy on photoreceptors.

ChR2 expression and purification at the TU Berlin was performed by Claudia Schulz, who provided high quality samples for the resonance Raman experiments. The general handling of the Raman setup was done under supervision of Dr. Uwe Kuhlmann. Without both of them, progress would have been much too slow.

I would also like to thank Meike Luck at HU Berlin for the nice collaboration concerning HKR1 and Miriam Colindres and Prof. Dr. Rolf Diller at the TU Kaiserslautern for providing the XR samples.

Valuable data was generated in collaborations with Dr. Christoph Weise at the FU Berlin (MALDI-ToF), Dr. Rolf Hagedorn and Christina Mrosek at the HU Berlin (flash photolysis) and P.D. Dr. Eberhard Schlodder and Marianne Çetin at the TU Berlin (absorption spectroscopy at cryogenic temperature).

The undergraduate students Alexander Wolkow, Stefan Wahlefeld and Stefanie Kathe all contributed greatly to the studies on the slow mutant ChR2-C128S-D156A.

Special thanks to Dr. Khoa Hoang Ly at the TU Berlin for reading and correcting the thesis as well as helping with the data fitting procedure, to Margrit Michalsky, Heide Evers and Gabi
Reinke at the HU Berlin for always helping out with a friendly smile and to the group of P.D. Dr. Ullrich Keller for offering the possibility to use the French Press.

IR spectra and interpretations, although all were not shown here, were provided by Francisco Velazquez Escobar, Prof. Dr. Friedrich Siebert and Dr. Ingo Zebger. Financial support and exciting seminars were offered within the framework of the Cluster of Excellence UniCat and BIG-NSE by Claudia Nasrallah, Dr. Jean-Philippe Lonjaret and Prof. Dr. Reinhard Schomäcker.

Many thanks to

my colleagues at the TU Berlin: Anke Keidel, Dr. Diego Millo, Elisabeth Siebert, Dr. Friedhelm Lendzian, Dr. Gal Schkolnik, Dr. Grazia Daminelli-Widany, Dr. Jacek Kozuch, Ina Seuffert, Dr. Inez Weidinger, Johannes Salewski, Jürgen Krauss, Lars Paasche, Prof. Dr. Maria Andrea Mroginski, Marina Böttcher, Marius Horch, Dr. Murat Sezer, Norbert Michael, Tillmann Utesch, Wiebke Meister and Yvonne Rippers

my colleagues at the HU Berlin: Altina Klein, Dr. André Berndt, Andre Greiner, Angela Piater, Franziska Schneider, Kyoko Tsunoda, Maila Reh, Manuela Stierl, Dr. Matthias Prigge, Roman Fudim, Dr. Satoshi Tsunoda, Dr. Tilo Mathes and Tra My Tran Nguyen

my former teachers John Granberg at Solbergagymnasiet in Arvika, Prof. Dr. Rolf Berger and Prof. Dr. Mats Boman at Uppsala Universitet for the inspiration and encouragement to continue in the field of chemistry

Johanna Von Münchhofen and Dr. Thomas Hauß for helping me out in a new country and a new city

Jesper Gunnarsson, Dan Engelbrektson, Jonas Hallberg, Jonas Gerborn, Ole Martin Larsen, Anita Hamre, Sven Ödman, Trude Warner, Johan Lindberg, Petter Nyberg, Anna Henriksson, Emma Zaars and all the others at “Arvika nation” at Dragarbrunngatan in Uppsala

my ”Mitbewohnerinnen” Tina Eichendorf and Gema Montaner Rios
Resonance Raman spectroscopy on microbial rhodopsins

my “Ersatzeltern“ Elke Ganschow and Manfred Töpfer

hele familien S. B. Hansen and my aunts and uncles with families

Lasse & Malou Johanson, Anne-Marie Lindberg, Stina Andersson, Karin & Per Tidlund

Annzi Danielsson & Anne-Sofie Bäck på ”affärn”, saknar dig Conny

Agata Pomorska, Felix Daume and Dr. Susanne Perlt from the HMI summer school and my friends at the BIG-NSE, especially Dr. Subhamoy Bhattacharya and Dr. Kirstin Hobiger for pushing me in the right direction when times were tough

my girls for all the fun we had: Dr. Anabel Molero, Anna Dahlgren, Amandine Guiet, Catrin Andersson, Helena Saarela, Julia Hellmich, Kathinka Leine, Klara Flink, Nina Heidary and Xiao Xie

my grandparents and Arnevi, I wish that you were still around

my parents, my brothers with families and Søren B. Hansen for the all the love and support throughout the years.
Bibliography


Resonance Raman spectroscopy on microbial rhodopsins


M. Sumii, Y. Furutani, S.A. Waschuk, L.S. Brown, H. Kandori, Strongly hydrogen-bonded water molecule present near the retinal chromophore of Leptosphaeria rhodopsin, the bacteriorhodopsin-like proton pump from a eukaryote, Biochemistry, 44 (2005) 15159–15166.


134 | Resonance Raman spectroscopy on microbial rhodopsins


[92] K. Ruffert, B. Himmel, D. Lall, C. Bamann, E. Bamberg, H. Betz, V. Eulenburg, Glutamate residue 90 in the predicted transmembrane domain 2 is crucial for cation flux through channelrhodopsin 2, Biochemical and Biophysical Research Communications, 410 (2011) 737–743.


Resonance Raman spectroscopy on microbial rhodopsins


Resonance Raman spectroscopy on microbial rhodopsins


Appendix

Media recipes

The Media were prepared according to reference [143]. 20 g agar/L was added in the Media used for plates. All solutions were autoclaved or filter sterilized. Percentages represent weight per weight.

Low salt LB (Luria-Bertani)
1 % tryptone
0.5 % yeast extract
0.5 % NaCl
pH 7.5 (adjusted with 1 M NaOH)

YPD – Yeast Extract Peptone Dextrose
1 % yeast extract
2 % peptone (tryptone)
2 % dextrose (D-glucose)

MMH – Minimal Methanol with Histidine
1.34 % YNB (Yeast Nitrogen Base with ammonium sulfate without amino acids)
$4\times10^{-5}$ % biotin
0.5 % methanol
$4\times10^{-3}$ % histidine

BMGY/BMMY – Buffered Glycerol/Methanol-complex
1 % yeast extract
2 % peptone
100 mM potassium phosphate buffer, pH 6.0
1.34% YNB
$4\times10^{-5}$ % biotin
1 % glycerol or 2.5 % methanol
Buffers recipes

Breaking buffer
11.3 mM NaH$_2$PO$_4$
100 mM NaCl
1 mM EDTA
5 vol% glycerol
pH 7.4 (adjusted with NaOH)

Solubilisation buffer
100 mM NaCl
20 mM HEPES
10 vol% glycerol
1 w/w% or 0.03 w/w% DDM
pH 7.4