Chapter 3

Reaction pathways of photoexcited retinal in Proteorhodopsin

Abstract

Proteorhodopsin (pR) is a membrane-embedded proton pump from the microbial rhodopsin family. Light absorption by its retinal chromophore initiates a photocycle, driven by trans/cis isomerization on the femtosecond to picosecond time scales. Here we report a study on the photoisomerization dynamics of the retinal chromophore of pR, using dispersed ultrafast pump-dump-probe spectroscopy. The application of a pump pulse initiates the photocycle, and with an appropriately tuned dump pulse applied at a time delay after the dump, the molecules in the initial stages of the photochemical process can be de-excited and sent back to the ground state. In this way we resolved an intermediate on the electronic ground state that represents chromophores that are unsuccessful in isomerization. In particular, the fractions of molecules that undergo slow isomerization (20 ps) have a high probability to enter this state rather than the isomerized K-state. On the ground-state reaction surface, return to the stable ground state conformation, via a structural or vibrational relaxation, occurs in 2-3 ps. Inclusion of this intermediate in the kinetic scheme led to more consistent spectra of the retinal excited state and to a more accurate estimation of the quantum yield of isomerization ($\Phi = 0.4$ at pH 6).

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3.1 Introduction

Proteorhodopsins (pR) are members of the large family of microbial rhodopsins. They are widely distributed in marine proteobacteria and spectrally tuned to the light climate in their oceanic environment, which has led to the evolution of blue and green variants. Proteorhodopsin can be co-assembled with charged lipids spontaneously in vitro, creating a long-range-ordered 2D array that makes it a promising material for technological developments.

Green-light absorbing proteorhodopsin, the subject of this investigation, functions as a light-driven proton pump creating a proton gradient from the extracellular to the cytoplasmic side of the membrane. Similar to bacteriorhodopsin, the absorption of light by the chromophore of pR, a retinal molecule covalently linked to the apo-protein through a protonated Schiff base, initiates E/Z (or all-trans to 13-cis) isomerization of the chromophore. This leads to a series of thermal transitions during which the proton from the Schiff base is transferred to the counterion formed by Asp97, Asp227 and a bound water molecule. The Schiff base is re-protonated by a proton from Glu108. Proteorhodopsin lacks the ionisable residues corresponding to Glu194 and Glu204 of bacteriorhodopsin, thought to be involved in the proton release pathway at the extracellular surface, which leaves the exit route of pumped protons in pR uncertain.

The light-induced isomerization in pR has been studied by femtosecond fluorescence and pump-probe spectroscopy in the visible and in the mid-IR. The quantum yield of the reaction is \( \sim 0.65 \) at pH 9.5 and \( \sim 0.5 \) at pH 6.59, meaning that the initially excited population can evolve either to the isomerized product, or relax back to the original trans ground state. Most of the product is formed on the faster time scales (\( \sim 0.2 - 2 \) ps), and a small fraction is formed on a \( \sim 20 \) ps time scale. The isomerization is sensed by the protein backbone, which possibly undergoes a conformational change on an early time scale (\( \sim 1 \) ps), as revealed by a change in amide band absorption.

Interestingly, the mutation of a single residue close to the chromophore, Gln105, can shift the absorption of retinal in blue pR to mimic the absorption in green pR. The role of the protein environment is also important in the chromophore photoreactions. Small structural differences between different rhodopsin proteins have a
significant effect on the retinal isomerization rates and mechanism. The isomerization in solution is slower and non-selective, around several double bonds, with the photoproduct being formed in 1-2 ps\textsuperscript{74} and the quantum yield 2-3 times less than in bacteriorhodopsin.\textsuperscript{75,76} In the chromophore pocket of rhodopsin, photoisomerization is completed in $\sim$0.2 ps,\textsuperscript{77} and in bacteriorhodopsin in 0.55 ps.\textsuperscript{65} In pR the formation of the 13-\textit{cis} state (K-state) occurs multiexponentially, on time scales spanning from hundreds of femtoseconds to tens of picoseconds. Proteorhodopsin at alkaline pH, close to pR's natural environment, has a slightly higher quantum yield and faster rate constants. At pH 6, where the Schiff base counterion Asp97 is protonated, the reaction is less efficient, probably due to the different charge distribution in the retinal pocket.\textsuperscript{6,9,72} However, the reaction mechanism is not altered by the pH of the environment, and the transient absorption data of pR in alkaline and acidic environment can be interpreted using the same model for the dynamics of the isomerization.\textsuperscript{72}

Although the excited state evolution of retinal in proteorhodopsin has been characterized, more information on what determines the quantum yield of photoisomerization can be obtained from monitoring relative progress on the excited state potential and looking at the dynamics on the ground state potential energy surface, using pump-dump-probe spectroscopy. The pump-dump-probe experimental technique consists of the administration of an additional (dump) pulse to the sample, in the spectral region of the stimulated emission, delayed with respect to the excitation pulse.\textsuperscript{78–82} This pulse causes depletion of the excited state population, sending part of it back to the ground state. During the delay time between the two pulses the excited state population may evolve on the reaction surface. In this way the dynamics of the ground state and of the excited state can be accessed. Pump-dump-probe spectroscopy probing at a single wavelength in the region of the stimulated emission has been applied to bacteriorhodopsin, resolving induced absorption in the near-IR, attributed to the excited state previously hidden by overlap with the stimulated emission.\textsuperscript{83} In another study on bacteriorhodopsin, it was found that the delay times between the pump and the dump pulses do not change the product formation yield and that the dumped population is biologically inactive.\textsuperscript{78}

Application of dispersed pump-dump-probe (or stimulated emission depletion) spectroscopy to photoactive yellow protein (PYP), a bacterial photoreceptor from
Halorhodospira halophila,\textsuperscript{84} demonstrated a ground state intermediate (GSI) in PYP, the detection of which until then was hindered by overlap with stimulated emission.\textsuperscript{31} Molecules that are not able to enter the photocycle can return from the excited state to the ground state either directly, or indirectly via this intermediate on the relaxation pathway. Vibrational ultrafast spectroscopy revealed that the GSI in photoactive yellow protein results from a transition of a molecule in the excited state towards a transient ground-state in a twisted (partial torsion around the bonds of the chromophore), \textit{cis} conformation\textsuperscript{28} but where the H-bond of the chromophore C=O with Cys69 is intact. In the protein environment the chromophore is sterically restricted and stabilized by H-bonding with the neighboring residues.\textsuperscript{28,85} This showed therefore that an important event required for successful entry into the photocycle is the breaking of the H-bond between the chromophore C=O and the backbone N-H of Cys69.\textsuperscript{28} Here we report a pump-dump-probe study of proteorhodopsin in an acidic (pH 6), detergent-containing buffer. By applying dispersed pump-dump-probe spectroscopy we are able to monitor the dump-induced changes at wavelengths from 450-750 nm. Simultaneous global analysis of pump-probe data and pump-dump-probe data, collected at different dump delays, enables us to develop a more complete reaction model that includes the ground state dynamics, and reveals the reaction pathway of those retinal chromophores that are not able to accomplish full isomerization.

3.2 Materials and methods

Proteorhodopsin was isolated from \textit{E. coli} UT5600 / pBeta-caro / pKJ829-proteo, a strain kindly provided by Dr. K.H. Jung (Department of Life Science, Sogang University, Seoul, Korea). Membranes containing holo-pR were isolated by resuspending harvested \textit{E. coli} cells in about 1/100 culture volume of 20 mM Tris buffer pH 7.5, containing 500 mM NaCl, 0.1% (w/v) dodecyl-maltoside (DM) and 20 mM imidazole. After thawing and addition of 1mg/mL lysozyme, 25 \(\mu\)g/ml ml DNAse I, 25 \(\mu\)g/ml RNAse and a protease inhibitor cocktail, cells were disrupted by sonication. Cell debris was removed by low speed centrifugation (10 min at 3000 x g) and the cytoplasmic membranes in the supernatant were solubilized in detergent (1.5% (w/v) DM) overnight via slow stirring 4°C. After 10 min centrifugation at 10,000 x
g, pR was purified using a HisTrap FF crude column (GE Healthcare, Eindhoven, The Netherlands), in combination with an imidazole gradient. Finally, the purified pR was dialyzed against 0.5 M NaCl, 0.1% (w/v) DM, plus 25 mM MES buffer at pH 6.72.

Samples consisted of a protein solution contained in a 2 mm quartz cuvet. The OD of the sample was 0.3 at the maximum of the absorption spectrum (530 nm). This absorption maximum is consistent with values reported in literature for pR at pH 6.27.

The experimental setup consisted of an integrated Ti:sapphire laser system (oscillator and regenerative amplifier, Coherent Legend-UHP) operating at 1 kHz and at a central wavelength of 800 nm, producing 85-fs pulses of 2.5 mJ. Part of this 800-nm light was introduced into a commercial OPA (Coherent OPERA) system to generate the 510 nm excitation pulse. Second portion of the 800 nm light was focused into a laterally rotating CaF$_2$ plate to generate white light for the probe. The probe pulses were spatially overlapped with the excitation beam in the sample using reflective optics. After overlap in the sample, the probe was dispersed by a 15 cm focal length spectrograph (Oriel) onto a home-built photo-diode array detector. The polarization of the excitation pulse was set to the magic angle (54.7°) with respect to the probe pulses. A phase-locked chopper, operating at 500 Hz, ensured that at every other shot the sample was excited and an absorbance difference spectrum could be calculated. To ensure a fresh spot for each laser shot, the quartz cuvet was repositioned by shaking. The power of the excitation pulses was 200 nJ. The instrument response function was $\sim$130 fs. For the three-pulse PDP measurements, part of the pump line was branched off before the OPERA, and the 800 nm pulses were introduced to a second commercial OPERA system (Coherent) to produce a 700 nm dump pulse of 200 nJ. A second delay line in the path of the dump pulse allowed for the timing of the dump pulse with respect to the pump pulse. A second chopper, placed in the path of the dump pulse, was used to ensure the acquisition of dumped and non-dumped spectra. This chopper, together with the pump chopper, was set to rotate asynchronously, resulting in a quasi-simultaneous measurement of a set of four discrete signals: A. Pump on and dump on (referred to as pump-dump-probe data set, PDP); B. Pump on and dump off (referred to as pump-probe data set, PP); C. Pump off and dump on; D. Pump
off and dump off. This way of collecting data ensured that all measurements were collected under the same conditions. We performed measurements at dump delays of 0.5, 2, 5 and 10 ps, respectively.

The data were subjected to global analysis. Cross-phase modulation (xpm) was modeled by a component with the time profile of the instrument response function (IRF).

### 3.3 Results

An overlay of the steady state spectrum of pR with the pump pulse (510 nm), the dump pulse (700 nm), and the transient absorption of a sample, taken at 0.55 ps after excitation, is shown in figure 3.1. The dump pulse was chosen to overlap with the stimulated emission band, but not with the steady state absorption of the protein. Upon dumping two possible outcomes are anticipated: if there is no higher excited-state absorption that coincides with the dump wavelength, a fraction of the molecules in their excited state will be de-excited and sent back to their ground state. Alternatively, molecules in their excited state can absorb another photon, to be sent to a higher excited state. Therefore, the wavelength of the dump pulse is very important for the outcome of the dumping. None of the ultrafast studies of pR report excited-state absorption in the spectral region around 700 nm,\(^6,9,72\) see also figure 3.1.

We applied dump pulses at delay times of 0.5 ps, 2, 5 and 10 ps between the pump- and the dump pulse. Representative time traces are shown in figure 3.2, together with reference time traces recorded without the application of the dump pulse.

Following the application of the dump pulse, a clear instantaneous depletion of the excited state absorption signals is observed, and the appearance of induced absorption signals, which are red shifted compared to the ground state absorption (see the time trace at 623 nm). These signals disappear in ~5 ps, and the dump pulse leads to lower final-product absorption, as can be seen from the lower signals at long delay times in figure 3.2B and 3.2C. This difference, most pronounced in the spectral region of the product state, implies that the dumped population does not feed into the "productive" reaction pathway.
3.3 Results

Figure 3.1 The steady state absorption spectra of pR, overlaid with the transients at t=0 and the spectra of the pump and the dump pulses. All spectra are normalized.

Figure 3.2 Pump-dump-probe time traces at dump delays of 2, 5 and 10 ps, together with pump-probe time traces at selected wavelengths for pR at pH 6. The fits and the residuals are derived from the application of the target model, shown in figure 3.4.

Sequential analysis of pump-probe data

First, we analyzed the pump-probe time traces of all data sets using a simple sequential scheme, with increasing exponential lifetimes, which yielded time constants of 0.4 ps, 6 ps, 30 ps, and a non-decaying component. In our previous study, similar time constants were sufficient to describe the transient absorption changes with a sequential model. The corresponding evolution associated difference spectra (EADS)
are shown in figure 3.3. The initial EADS (black), with a lifetime of 0.4 ps, is characterized by induced absorption, blue-shifted from the ground state bleach, peaking at \( \sim 470 \) nm, a ground state bleach, which is maximal at 560 nm, and a broad stimulated emission band at wavelengths above 580 nm. The signal in the stimulated emission region is reduced after the first 0.4 ps and ranges from 650 to 760 nm.

Figure 3.3  Evolution-associated difference spectra, derived from a sequential analysis of the data. The decay times of the EADS are shown in the inset to the figure.

The excited state absorption and the stimulated emission decay multiexponentially with time constants of 0.4, 6 and 30 ps, accompanied by the rise of the positive signal at \( \sim 625 \) nm (red and blue EADS). In the final (dash-dot-dashed line) spectrum the positive signal has developed into a band peaking at \( \sim 625 \) nm. This final spectrum can be attributed to the sum of the bleached ground state absorption and absorption due to product formation. This spectrum corresponds to the absorption-difference spectrum of the K intermediate in the photocycle of pR,\(^{38}\) which has an isomerized, 13-\(cis\) retinal chromophore. These decay times of the excited state of pR at pH 6 are slightly slower than the decay times of 0.2 ps, 2 ps, 18 ps observed in our pump-probe study,\(^{72}\) due to the lower pH of the sample, but are nevertheless in fairly good agreement with previously reported time constants associated with the pR multiexponential excited state decay, derived from pump-probe experiments in the visible (1 ps and 16 ps at pH 6\(^6,9\)) and in the mid-IR (0.5 ps, 2 ps and 11 ps at pH 9.5\(^{41}\)).
Target analysis of pump probe and pump-dump-probe data

The spectra obtained by analysis with a sequential scheme are mixtures of the intermediates that exist simultaneously for periods of time, defined by the decay rate of each intermediate, rather than spectra associated with physically real species. We therefore applied a physically relevant model to the data to obtain the pure species-associated difference spectra (SADS). In this analysis all the pump-dump-probe and pump probe data sets were analyzed simultaneously, which led to significant improvement of the signal-to-noise ratio of the results. The model, shown in figure 3.4, is partially based on the model that we employed previously.\textsuperscript{72}

![Diagram](image)

**Figure 3.4** A) Schematic representation of the target model used for the analysis of the data. The arrows represent transitions with associated rate constants in ps\(^{-1}\), the squares represent states, color coded to the SADS shown in figure 3.5; B) Sketch of the potential energy surfaces, corresponding to the target model that was applied for analysis of the data. The green arrow indicates the excitation of molecules in the ground state due to the pump pulse; the red arrow indicates the de-excitation of molecules in the excited state due to the dump pulse.

It comprises three excited state species (ES1, ES2, ES3) to account for the multiphasic decay of the excited state, and a species corresponding to the product state. Each of the three excited states, denoted ES1, ES2 and ES3 can form a product, K, or decay to the ground state via the transient ground state intermediate, GSI.
To simplify the model, and because in a first try the spectra of ES2 and ES3 were virtually identical, the spectra of the ES2 and ES3 are equated (violet SADS in figure 3.5), and they are characterized by a red shift of the stimulated emission, as compared to the SADS of ES1. This heterogeneous model provides a direct way to assess the amount of product formed with each time constant and from each ES component. Physically, it may represent heterogeneity in the retinal environment that is evident after the initial fast ES1 relaxation. The species associated difference spectra of each component are shown in figure 3.5.

The instantaneous depletion of the ES absorption at time delays of 0.5, 2, 5 and 10 ps after time zero, induced by the dump pulse, is modeled as an instantaneous loss of population from the excited states ES2 and ES3 and a gain of population of a newly formed intermediate, referred to as a ground state intermediate (GSI). The spectrum of this species is characterized by a ground state bleach and positive absorption band at wavelengths longer than \( \sim 600 \) nm (short-dashed line SADS in figure 3.5). The GSI has a lifetime of 2.5 ps and its absorption is red-shifted with respect to the ground state, overlapping with the stimulated emission. If the GSI is not taken into account in the analysis of pump-probe data, it manifests itself as a positive feature centered at 600 nm in the SADS of the excited states.\(^{72}\) Taking it into account improves significantly the spectral shapes of these SADS in the region of the stimulated emission.

ES1, ES2 and ES3 exhibit pure excited state spectra (ground state bleach, blue shifted positive absorption and stimulated emission), without a contribution from the absorption of the product state, see figure 3.5. The branching parameters of all transitions are shown in table 3.1. Note that the product state is formed mainly on a sub-picosecond to picosecond time scale, from ES1 and ES2. Note that only 1% of the product yield originates from the long-lived ES3. We estimated previously the quantum yield of the reaction to be \( \sim 0.5 \), based on target analysis and on deconvolution of the product state difference spectrum.\(^{72}\) However, now that the pump-dump-probe measurements revealed the involvement of the GSI in the de-activation pathway, a more reliable estimate of the quantum yield by target analysis can be given: 0.4.

We also tested a model allowing for direct transitions to the ground state on the relaxation pathway, and we found that it does neither improve the SADS nor the
Figure 3.5 Species associated difference spectra (SADS), estimated from the simultaneous application of the target model shown in figure 3.4A to pump-probe- and pump-dump-probe data, at multiple delay times of the dump pulse. The inset in the figure shows the lifetimes of the species.

![Figure 3.5](image)

<table>
<thead>
<tr>
<th>population</th>
<th>ES2</th>
<th>ES3</th>
<th>K</th>
<th>GSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES1 1</td>
<td>0.39</td>
<td>0.52</td>
<td>0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>ES2 0.39</td>
<td></td>
<td></td>
<td>0.79</td>
<td>0.21</td>
</tr>
<tr>
<td>ES3 0.52</td>
<td></td>
<td></td>
<td>0.03</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Table 3.1 Contributions of each excited state to the product yield (K yield) and the ground state intermediate yield (GSI yield), estimated from target analysis of the data with the model shown in figure 3.4.

quality of the fits. We further attempted to analyze our mid-IR data with this model. However, due to the simultaneous coexistence of the ground state intermediate and the excited and product states, the spectral shape of the GSI could not be unambiguously assigned. Therefore, future pump-dump-probe experiments, probing in the mid-IR, are necessary to obtain the mid-IR spectrum of the GSI.
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3.4 Discussion

The simultaneous acquisition of pump-probe and pump-dump-probe data have allowed for a more detailed model for the reaction dynamics of photosomerization of retinal in pR. A previously undetected intermediate state on the ground state potential has now been resolved and more reliable ES spectra have been obtained, which has led to the improved estimate of the isomerization quantum yield of 0.4.

In pR, the excited state decay and the formation of the cis-product state are multiphasic processes. Since we do not observe spectral evolution in the excited state absorption after the initial subpicosecond phase, we assign the multi-exponentiality of the excited state decay to structural heterogeneity of the retinal and the surrounding protein. Heterogeneity in bacteriorhodopsin has been suggested previously to result from conformational plasticity of the protein that would modify the structure around the chromophore and result in a distribution of rate constants for the decay of the excited state. Recently, the simultaneous existence of several 13-cis, 15-syn states (representing slightly different conformations in the neighborhood of the Schiff-base linkage) in dark-adapted bacteriorhodopsin has been directly observed by solid state NMR experiments, as well as heterogeneity within several of the cryotrapped intermediates.

Our measurements can be described by a model for the excited state dynamics that is similar to that for other retinal molecules, except for the fact that the heterogeneity in pR is larger: Upon absorption of a photon, the wave packet that is created initially oscillates on the high vibrational levels of $S_1$, and, within 0.3 ps, relaxes via intramolecular vibrational energy redistribution, due to structural evolution of the excited-state population along multiple low-frequency modes that carry the molecule out of the harmonic photochemically inactive Franck-Condon region, into the photochemically active geometry. A similar sequence of reactions has been observed in bacteriorhodopsin using stimulated Raman spectroscopy, and is responsible for the observed shift of the stimulated emission. Red-shifted and broad bands, corresponding to vibrationally hot states, are also observed in the mid-IR spectra of the excited states of pR. During the lifetime of the initial vibrationally hot excited state, the wave packet may cross the ground state potential (figure 3.4) and subsequently form either a cis-isomerized product, or it can return via the ground state intermediate,
to the ground state conformation. The same scenario holds for the two excited states formed after relaxation of the initial excited state: they can either undergo transition to the cis state, or return to the initial ground state conformation via the ground state intermediate. The long lifetime of ES3 (24 ps) cannot be explained by vibrational cooling, and points to the crossing of a significant potential-energy barrier on the excited state potential surface that slows down the reaction, indicating that ES3 is structurally different from ES1, as indicated by the vibrational spectra of the excited states of pR.\textsuperscript{72} As much as 50% of the excited states have this exceptionally long lifetime, and these states have a relative K-yield of only 3%, and therefore contribute only 1.5% to the total product yield. The rate to the K-state of ES3 we find is only 0.001 ps\textsuperscript{−1}, as compared to 0.16 ps\textsuperscript{−1} for ES2 (and 0.27 ps\textsuperscript{−1} for ES1). The barrier for K-formation from ES3 is therefore significantly higher than for ES2, and can be estimated, using these rates and the Boltzman expression, to \(\sim\)125 meV. This may point to structural rearrangements that hinder the isomerization, as our previous mid-IR studies of pR isomerization showed that the proteins in the long-living excited state were characterized by a spectral change in the Amide II region.\textsuperscript{41,72}

The SADS of the GSI that we resolve upon the simultaneous analysis of pump-probe and pump-dump probe data, has the shape of the sum of the ground state bleach plus a positive absorption band, red-shifted with respect to the ground state absorption, but blue-shifted with respect to the stimulated emission (short-dashed line SADS in figure 3.5). The population of this state is significantly enhanced in the pump-dump-probe data but hidden by overlap with the stimulated emission and the product state absorption in the pump-probe data. We note that the GSI nevertheless is also present on the normal relaxation pathway of the retinal molecules (without forced de-excitation due to the dump pulse), but that it can only lead to the re-formation of the GS, and not to K-state formation. This is similar to the observations made in bacteriorhodopsin and PYP, i.e. that the dumped state is biologically inactive.\textsuperscript{24,78}

The blue shift of the GSI absorption with respect to the stimulated emission may indicate that upon dumping a very fast relaxation occurs on the ground state, which we have not time-resolved in the current experiment. The red-shifted absorption of the GSI with respect to the ground state absorption, notably even further red-shifted than
the K-state absorption, would correspond to an intermediate with a lower frequency of the C=C stretch vibration in the mid-IR, according to a reported correlation of the vibrational frequencies of retinal with visible absorption maxima. This might indicate that the GSI is also in a cis-conformation, similar to the product state K. Considering the GSI lifetime of only 2.5 ps, and its absorption properties, it can be related to molecules that undergo unsuccessful attempts to fully isomerize and decay to the ground state from a distorted or twisted cis-conformation that needs to relax in order to assume the ground state conformation. Moreover, a distorted cryo-trapped K-like state of bacteriorhodopsin has recently also been observed by solid-state NMR spectroscopy. On the other hand, via the dumping, a non-equilibrium ground state population, i.e. a vibrationally "hot" all-trans ground state, may be formed. In both cases the 2.5 ps dynamic process represents evolution on the ground state potential energy surface, represented in figure 3.4 as a potential barrier on the S₀ surface. It will be revealing to measure the characteristics of the structure of the GSI state with mid-infrared transient spectroscopy in future experiments.

The explanation why the ground state intermediate is involved in the return to the ground state might also be related to the protein surroundings of the chromophore. A conformational change of the protein on a picosecond time scale present in all excited states was observed in ultrafast pump-probe studies. This conformational change is due to a response of the protein backbone, following the excitation of the retinal molecule, due to the isomerization, which in turn might be related to storage of photon energy for proton transport. As this amide spectral change is persistent on a time scale longer than the time of relaxation of the excited state molecules, it creates an environment for the chromophore, different from the initial ground state environment, possibly imposing steric or electrostatic constraints on the chromophore, which could translate into a barrier on the relaxation pathway.

We can now summarize the dynamics of the light-induced isomerization of retinal in pR. The initially excited molecules can follow either a productive pathway, and form a cis-isomer, or a relaxation pathway, returning towards the initial all-trans ground state via a ground state intermediate. Most of the cis-isomers are formed on a sub-picosecond to picosecond time scale, from short-lived excited states. Conformational heterogeneity accounts for the presence of a long-lived excited state population that
is characterized by dramatically reduced product formation and evolves mainly on the relaxation pathway.