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Distortion of the L → M transition in the photocycle of the bacteriorhodopsin mutant D96N: a time-resolved step-scan FTIR investigation

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Abstract The D96N mutant of bacteriorhodopsin has often been taken as a model system to study the M intermediate of the wild type photocycle due to the long life time of the corresponding intermediate of the mutant. Using time-resolved step-scan FTIR spectroscopy in combination with a sample changing wheel we investigated the photocycle of the mutant with microsecond time resolution. Already after several microseconds an intermediate similar to the M_N state is observed, which contrasts with the M state of the wild type protein. At reduced hydration M and N intermediates similar to those of wild type BR can be detected. These results have a bearing on the interpretation of the photocycle of this mutant. A mechanism is suggested for the fast rise of M_N which provides some insight into the molecular events involved in triggering the opening of the cytosolic channel also of the wild type protein.

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Key words: Bacteriorhodopsin; D96N mutant; Step-scan Fourier transform spectroscopy; Time-resolved infrared spectroscopy

1. Introduction

Proton translocation in the light driven proton pump bacteriorhodopsin (BR) from the cytoplasmic to the extracellular medium is initiated by the isomerization of the retinal chromophore upon absorption of a photon from the all-*trans* to the 13-*cis* configuration. The chromophore is covalently bound to lysine 216 via a protonated Schiff base. Different intermediate states such as K, L, M, N, O have been first characterized by their spectra in the visible which reflect the environment of the chromophore. The most important steps for proton translocation are the L → M and the M → N transitions involving the Schiff base and the amino acid residues Asp-85 and Asp-96. The proton from the Schiff base is transferred to Asp-85 during the rise of the M intermediate. Due to Schiff base deprotonation, the visible absorption maximum of the M intermediate is shifted near 412 nm. The M decay is related to reprotonation of the Schiff base by proton transfer from Asp-96 (for reviews on BR see [1–4]). Therefore, studying the M state is of particular interest for the understanding of the BR photocycle. In order to ensure the vectoriality of the proton transport, during the lifetime of the M intermediate a change of the accessibility of the Schiff base has to occur between the extracellular half channel where the proton acceptor Asp-85 is located, and the proton donor Asp-96 in the cytoplasmic half channel.

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In the D96N mutant residue 96 has lost its function as a proton donor for Schiff base reprotonation resulting in a photocycle, whose turnover time is slowed down from about 20 ms to more than 2 s at pH 7 [5] and to extremely long times at pH 10 [6]. This is due to the fact that reprotonation of the Schiff base has to occur directly from the aqueous phase and therefore a long-living state with a deprotonated Schiff base can be observed [5,7–9]. For this reason the D96N mutant has been employed as a model system to study the photocycle of wild type BR with time-resolved UV-vis spectroscopy [10,11], and especially for structural investigations of this long-living M state [12,13].

The BR photointermediates derived originally from UV-vis spectroscopy can be also recognized by FTIR spectroscopy providing information on changes in the retinal as well as in the protein. In the case of the D96N mutant an intermediate has been detected which cannot be observed in the wild type protein: the so called M_N state represents a combination of an M-like chromophore configuration with a deprotonated Schiff base and an N-like protein conformation [6]. Since this intermediate has so far only been detected under static conditions at high pH and low temperatures, it is important to investigate also the circumstances of its formation with appropriate time resolution to clarify its role in the photocycle of the D96N mutant. In the present study, we have carried out time-resolved step-scan FTIR measurements and time-resolved rapid-scan experiments to study molecular changes in the mutant photocycle at pH 7 as well as its relation to that of the wild type protein in a time domain ranging from 500 ns to several seconds.

2. Materials and methods

Preparation of the D96N bacteriorhodopsin by site directed mutagenesis has been described earlier [10]. For all measurements hydrated films in phosphate buffer (pH 7) on BaF_2 windows at ambient temperature (298 K) were used. Partially dehydrated samples with 66% relative humidity (r.h.) (293 K) were obtained by vapor exchange over saturated sodium nitrite solution.

The spectrometer used for step-scan measurements has been described previously [14]. Since with the conventional step-scan technique measurements of slow cycling systems cause impractically long measuring times, the experimental setup was extended by a sample changing wheel [15]. This device allows successive positioning of 10 different samples into the infrared beam and averaging their signals repetitively at each sampling position of the interferometer mirror.

For sample excitation an excimer laser pumped dye laser (Lambda Physik, Germany) operated at 530 nm with a pulse energy of 1.5 mJ was used. Kinetic signals were recorded with a transient recorder PAD82 (Spectrum, Germany). Data were recorded in the time interval from 100 ns to 3.2 ms after laser flash with 100 ns time spacing, and transformed to a quasi-logarithmic timescale with about 25 time points per decade. Final spectra were obtained by coadding four sep-

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arate measurements with 10 flashes per sampling position each (spectral resolution=8 cm^{-1}). The slower part of the reaction cycle starting at 2 ms was measured with the same spectrophotometer in the rapid-scan mode using OPUS software by Bruker.

3. Results

We report here the first infrared difference spectra of the earlier part of the photocycle of the D96N mutant with a time resolution of about 500 ns. A series of difference spectra at various times (1.4 μs, 28 μs, 204 μs, 2.25 ms) after the laser flash is shown in Fig. 1. Negative bands are caused by unphotolysed BR, positive ones by the photoproducts. The transition BR → KL/L (mainly L) is reflected by the first spectrum, while the spectra at 28 μs, 204 μs and 2.25 ms show the BR → L/M transition with decreasing amplitude of the L intermediate.

Indicative bands for the rise of the M intermediate are located at 1761 cm^{-1} and at 1184 cm^{-1}. The first one is due to the C=O stretching vibration of protonated Asp-85, the primary proton acceptor of the Schiff base [16,17]. Since Asp-85 remains protonated in N, this band does not disappear in this intermediate, but shifts to lower frequencies as a consequence of changes in the protein environment [18,19]. The other spectral position where the rise of the M intermediate can be followed is around 1184 cm^{-1}: the positive band of the C-C stretch vibration of the protonated 13-*cis* chromophore in L disappears and the absorbance reaches its most negative value with maximum M amplitude only at 2.25 ms. This clearly indicates a slowed down L decay in the D96N mutant. As the absorbance of the band at 1184 cm^{-1} in Fig. 1 drops continuously up to 2.25 ms, there is no significant rise of the N intermediate detected in this time domain, i.e. the Schiff base remains unprotonated. In contrast, the shift of the band at 1762 cm^{-1} already takes place before reprotonation of the Schiff base as revealed in the insert of Fig. 1. For comparison, the corresponding difference spectra of the wild type photocycle are shown in Fig. 2. Again, the shift of the protonation signal of Asp-85 can be seen, which now correlates with the reprotonation of the Schiff base. At 280 μs, when the M intensity reaches its largest amplitude, the Asp-85 band is still found at 1761 cm^{-1}. It shifts towards lower frequencies when the Schiff base is reprotonated and the positive band at 1185 cm^{-1} reappears.

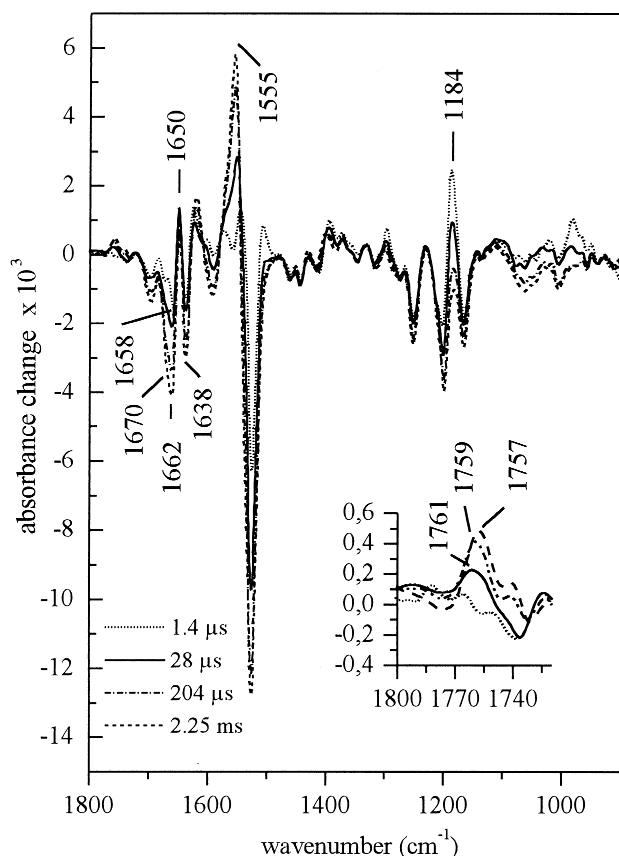

Fig. 1. Time-resolved FTIR difference spectra of the photocycle of the D96N mutant of bacteriorhodopsin at pH 7 and 298 K at indicated times after photolysis (100% r.h.). The inset shows the expanded region of protonated carboxyl groups.

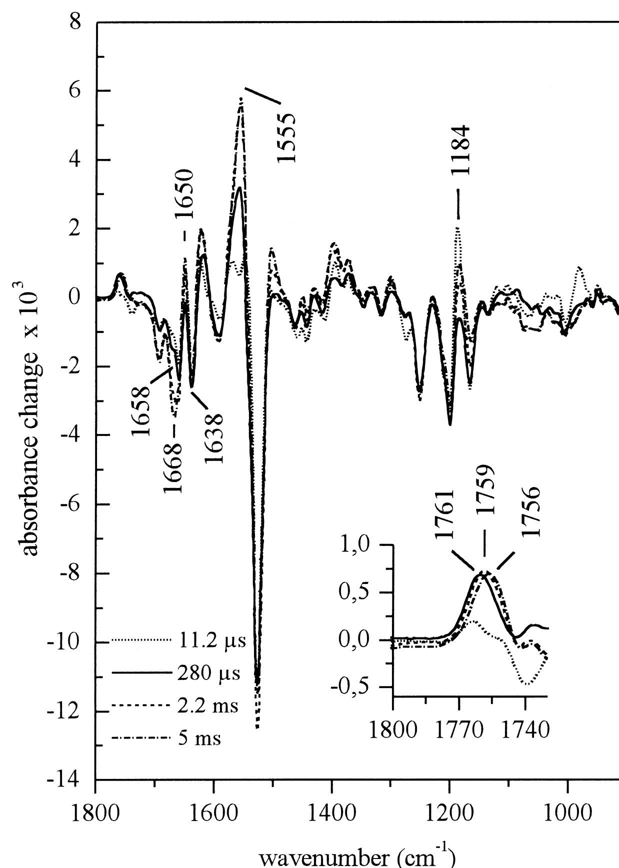

Fig. 2. Time-resolved FTIR difference spectra of the photocycle of wild type bacteriorhodopsin at pH 7 and 298 K at indicated times after photolysis (100% r.h.). The inset shows the expanded region of protonated carboxyl groups. In order to compensate for the decrease of the signal amplitude at later times, the wild type spectrum at 2.2 ms is normalized at 1250 cm^{-1} with a factor of 1.2.

Major differences between mutant and wild type protein are also obvious in the amide-I region, where changes in the secondary structure can be observed. In the wild type spectrum at 1.4 μs there are two negative bands located at 1658 cm^{-1} and 1638 cm^{-1}. Whereas the position of the latter which is attributed to the C=N stretching mode of the protonated state of the Schiff base in unphotolysed BR [20] remains almost unchanged during the observed time domain, the first one

shows a shift towards higher frequencies as well as a strong increase in intensity when the N intermediate is formed. The temporal evolution of the amide I bands can be followed in more detail in Fig. 3. In the wild type M intermediate (280 μs), there is no positive band at 1650 cm^{-1} typical of N [18,19], the band at 1658 cm^{-1} shows somewhat smaller intensity than the band at 1638 cm^{-1} and there is very little negative intensity observed at 1668 cm^{-1}. A dramatic change is observed with the rise of the N amplitude. Positive and negative bands appear at 1650 cm^{-1} and 1668 cm^{-1} respectively, while the intensity around 1660 cm^{-1} decreases. Thus, the ratio of the peak amplitudes of these two negative bands at 1658 and 1670 cm^{-1} is reversed in the N intermediate. These observations are in agreement with published data [21], where the peak amplitude ratio of these bands was taken as an indicator for the M and N intermediates. Inspecting the amide-I region of the mutant on the right panel in Fig. 3 reveals a completely different behavior. Not only when N is formed, but also during the formation of the M intermediate, the amide-I bands of the D96N mutant show characteristics of the N state of wild type BR. The positive band at 1650 cm^{-1} is already present in the early microsecond time range as well as considerable intensity at 1670 cm^{-1}. As the bands at 1658 cm^{-1} and 1670 cm^{-1} are not well separated (similar to the 2.2 ms spectrum of wild type BR), and comparable intensities are found at both frequencies, the maximum of the negative band is located near 1662 cm^{-1} from the beginning of formation of M. We corrected the earlier spectra for the not negligible contribution of L to exclude any effects of the L decay (data not shown). The results confirmed the observations described above. Thus, in time-resolved measurements a state similar to the so called M_N intermediate of the D96N mutant described by Sasaki et al. [6] is clearly observed, which is characterized by a deprotonated Schiff base and an N-like protein conformation. It seems to replace in the mutant photocycle to some extent the wild-type M state. As shown in Fig. 4 (top) the reprotonation of the Schiff base of the mutant (transition to the N state) involves only minor changes in the amide I region. Since a modified behavior of the mutant in partially dehydrated films has been reported [13], we investigated its photocycle under similar conditions (66% r.h.). The result is shown in Fig. 4 (bottom). It becomes clear by comparison with Figs. 2 and 3 (left panel) that most of the properties of the wild type M intermediate are restored under reduced water content of the mutant sample. Especially the narrow band at 1660 cm^{-1} with a much smaller intensity than the band at 1670 cm^{-1} can be observed together with the negative absorbance at 1184 cm^{-1} (spectrum 2–12 ms, right panel)). The large changes in the amide-I range of the spectrum only occur with the reprotonation of the Schiff base (spectrum 600–720 ms). As in the spectra of wild type BR during the transition to the N intermediate, the partially dehydrated sample shows a shift of the Asp-85 band to lower frequencies, an increase of the band intensity at 1670 cm^{-1}

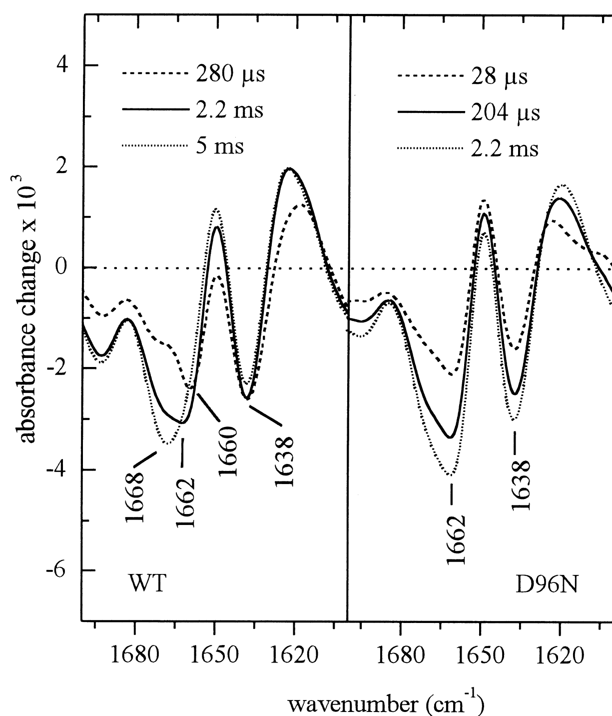

Fig. 3. Expanded amide-I region of time-resolved FTIR difference spectra of the photocycle of wild type bacteriorhodopsin (left panel) and the D96N mutant (right panel) at pH 7 and 298 K at indicated times after photolysis. In order to compensate for the decrease of the signal amplitude at later times, the wild type spectra are normalized at 1250 cm^{-1} with factors of 1.2 (2.2 ms) and 1.6 (5 ms), respectively.

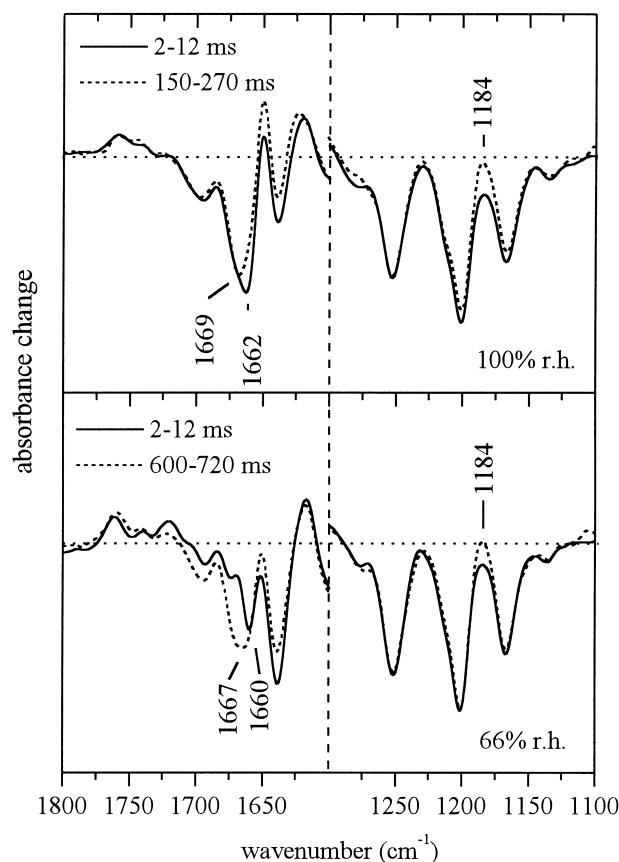

Fig. 4. Time-resolved FTIR difference spectra of the photocycle of the D96N mutant of bacteriorhodopsin at pH 7 and 298 K at indicated times after photolysis and different levels of hydration (upper part: 100% r.h., lower part 66% r.h.). For clarity, only the amide-I range (left panels) and the fingerprint range (right panels) are shown. The spectra at 150–270 ms (100% r.h.) and 600–720 ms (66% r.h.) are normalized with a factor of 3 at 1250 cm^{-1}.

and a reversal of the peak intensity ratio of the negative bands at 1658 cm^{-1} and 1670 cm^{-1}. This behavior is in contrast to that of the fully hydrated mutant sample shown in Fig. 4 (top). Note that the photocycle at 66% r.h. is slowed down by a factor of about 10, so the spectra at 100% r.h. and 66% r.h. compared in Fig. 4 were taken at different times.

One additional peculiarity of the D96N mutant at 66% r.h. can be observed. While at the lower hydration level the N-like broad bandshape around 1760 cm^{-1} of the fully hydrated mutant sample [6] is replaced by a more narrow band typical of wild type BR, there appears a new positive band with relatively large intensity at 1720 cm^{-1}. This band is neither observed in wild type M or N intermediates nor in the fully hydrated mutant spectra. Interestingly, such a band has been described for the M intermediate of the E194D mutant [22].

4. Discussion

Our time-resolved FTIR data provide clear evidence for the presence of an intermediate similar to the M_N first described by Sasaki et al. [6] in the photocycle of the bacteriorhodopsin mutant D96N at pH 7 and 298 K (Figs. 1 and 2). Throughout the whole lifetime of the state with deprotonated Schiff base essentially an N-like protein conformation is observed. Only under partially dehydrated conditions protein bands typical of the wild type M intermediate (Figs. 2 and 3) are detected in the mutant photocycle (Fig. 4).

It is important to note that this behavior of the D96N mutant is observed at neutral pH in our measurements, whereas M_N has previously been stabilized in static measurements at pH values above 9.5 [6,13]. Thus, the presence of the M_N intermediate seems to be characteristic of the mutant photocycle regardless of the pH value. Therefore, when the mutant with its long-living M intermediate is used to derive information about the BR photocycle, one has to take into account that also during its rise the M state observed in D96N BR is different from that of the wild type protein.

Furthermore, our results suggest, that the presence of the M_N intermediate, i.e. the formation of the N-like protein conformation together with a deprotonated Schiff base, is a result of the timing between the conformational changes and reprotonation of the Schiff base, both occurring with variable time delay after the deprotonation of the Schiff base, which appears to be a precondition for the conformational change to take place. Both N-like structural changes and reprotonation of the Schiff base can be affected by varying the hydration level: the equilibrium between M, M_N and N in the wild type photocycle [9,23–25] as well as in that of the mutant [13,25,26] is strongly influenced by the hydration level. The reason for the observation of an M_N state in the D96N mutant has been assumed to be the slow reprotonation of the Schiff base due to the replacement of proton donating residue Asp-96 [5,8,27]. This would be in agreement with the photocycle of the D85E mutant, where the reprotonation of the Schiff base is also slowed down and a large N-like conformational change is observed before the reprotonation step [17].

However, since our time-resolved measurements show that this structure is detected earlier in the mutant than in wild type protein, the fast formation of an N-like protein structure seems to be facilitated by the mutation. We observe in the fully hydrated D96N sample an N-like conformational change from the beginning of M formation (Fig. 1) which is essentially completed before Schiff base reprotonation (Figs. 3 and 4 (top)). Only small changes occur at later times. At pH 7 especially the completion of the shift of the amide-I mode from 1662 to 1669 cm^{-1} takes place together with the reprotonation of the Schiff base. Nevertheless, measurements at pH 9.5 (data not shown) indicate that also this shift is independent of reprotonation of the Schiff base. The facilitated formation of the N-like conformation in the mutant contributes to the observation of the M_N state.

In all published structures of BR, the only specific interaction of the carboxyl group of D96 is hydrogen bonding to the OH group of T46 [28–32], although the structures differ considerably with respect to the exact positioning of the side chains. Thus it appears that the alteration of the hydrogen bond between the two residues facilitates the structural changes occurring in the N intermediate. This is supported by the observation of a considerably faster M decay in the T46V mutant [33,34]. Electron, neutron and X-ray diffraction experiments show that the N state (as well as the M_N state) is characterized by tilts of the helices F, and G, and to a lesser degree also of helix B, whereas in the M state, the structural changes appear to be smaller and mainly involve helix B, although there are some discrepancies among these data [13,26,35–38]. Since T46 is located at the upper part of helix B, a change in the interaction with the residue at position 96 (helix C) could influence the movement of helix B, and in turn those of helices F and G. It is interesting to note that in the D96N mutant the activation energy for reprotonation of the Schiff base is lowered, whereas the activation entropy is reduced, the latter explaining the slowing down of the photocycle [5]. This is in agreement with our observation of facilitated N-like structural changes: since these occur very early, they are no longer the rate-limiting step regulating Schiff base reprotonation. It appears that in both D96N and T46V mutants the facilitated structural changes are not effective since the proton transfer from the cytosol is slowed down [33].

At reduced hydration of the sample (66% r.h.) the restoring of a spectral behavior similar to the that of wild type BR is observed. This means that although the photocycle at this hydration level is slowed down by a factor of about 10, the kinetics for Schiff base deprotonation, protein conformational changes and Schiff base reprotonation are separated, so that intermediates similar to the wild type M and N states are formed (Fig. 4). Thus, the formation of the N-like protein structure in the D96N mutant is not prevented by partial removal of water, but its kinetic is affected in a way that it occurs later in the photocycle together with the reprotonation of the Schiff base.

With respect to the effect of dehydration of the D96N samples our data qualitatively agree with recently published data from Kamikubo et al. [13] and Sass et al. [26]. In both studies, parallel static FTIR spectroscopic and x-ray diffraction experiments have been performed. However, both investigations differ from each other in experimental results as well as in the molecular interpretation. This is possibly due to differing experimental conditions such as hydration level.

As for the D96N mutant, it has been recently reported that several agents are able to inhibit the blue shift from 412 nm to 404 nm of the difference spectra maximum of the M intermediate [11,39]. Such a blue shift cannot be observed in wild type BR. Thus, the wild type properties could be restored by treatment with these agents (glycerol, lutetium ions and glu-

taraldehyde). This result was interpreted in terms of shifting the equilibrium between two states corresponding to protein conformations with absorption maxima at 412 nm and 404 nm, respectively, which differ in the accessibility of the Schiff base for azide and water molecules. It was concluded that the latter intermediate must be the same as M_N. This observation coincides well with our finding of an N-like protein conformation present already in the early M intermediate. Thus, an increased absorption at 404 nm in the visible spectrum would correspond to the early phase of the rise of the negative band around 1670 cm^{-1} in the infrared difference spectrum. Also the removal of this early conformational change by glycerol agrees with our data from partially dehydrated samples, when the osmotic properties of glycerol are taken into account [9].

Also for the wild type photocycle an M_N-like state has been described. FTIR and solid-state NMR measurements on BR treated with guanidinium HCl indicate the prevalence of such an intermediate at high pH [26,40]. In addition static FTIR investigations of the photoreaction of partially dehydrated BR molecules embedded in glucose exhibit formation of an M_N intermediate at 260 K while at 240 K only the typical M was observed [23,24]. This indicates that M_N may indeed be part of the wild type photocycle, but it does not accumulate kinetically.

In conclusion, comparing the mutant D96N and wild type BR, our time-resolved studies show significant differences in the decay of the L state and in the development of the M intermediate with accompanying structural changes. These results together with the data on the T46V mutant indicate the importance of the specific interaction between the residues T46 and D96 for effective proton transfer, and are therefore also of relevance for the wild type photocycle. It is further demonstrated that the hydration level of the sample plays a crucial role in the timing between the occurrence of N-like structural changes and the reprotonation of the Schiff base. Our results demonstrate clearly the restrictions of the use of the long living state with deprotonated Schiff base of the mutant to derive information about the wild type M intermediate.

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