Biochemical and Biophysical Research Communications **283,** 57–63 (2001)
doi:10.1006/bbrc.2001.4730, available online at http://www.idealibrary.com on IDEAL®

Time-Resolved FT-IR Spectroscopic Investigation of the pH-Dependent Proton Transfer Reactions in the E194Q Mutant of Bacteriorhodopsin

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Received March 19, 2001

The photoreaction of the E194Q mutant of bacteriorhodopsin has been investigated at various pH values by time-resolved step-scan Fourier-transform infrared difference spectroscopy employing the attenuated total reflection technique. The difference spectrum at pH 8.4 is comparable to the N-BR difference spectra of the wild type with the remarkable exception that D85 is deprotonated. Since the retinal configuration is not perturbed by the E194Q mutation, it is concluded that there is no interaction of D85 with retinal during the lifetime of the N state. At pH 6, a consecutive state to the O intermediate is detected in which D212 is transiently protonated. The comparison with wild-type bacteriorhodopsin reveals that protonation of D212 represents an intermediate step during proton transfer from D85 to the proton release group in the final stage of the reaction cycle. The described effects are more pronounced in the E194Q mutant than in the E204Q mutant demonstrating different roles of these two glutamates/glutamic acids at least in the final stages of the catalytic cycle of bacteriorhodopsin.

Key Words: **proton transfer; Fourier transform infrared; photocycle; purple membrane; attenuated total reflection spectroscopy.**

Bacteriorhodopsin (bR) is a 26 kDa integral membrane protein which is capable of proton pumping (for recent reviews see (1–3)). Light absorption by the chromophore retinal starts the reaction cycle which involves several intermediate states (J, K, L, M, N, and O). The structure of unphotolyzed bR (4–7) and of several photocycle intermediates (8–12) have recently been solved by X-ray crystallography. Although the resolution is extraordinary, the location and the dynamics of the protons are still not resolved. Time-resolved infrared spectroscopy has been proven to be a valuable tool to directly detect protonation changes of internal amino acid side chains (13–16). Moreover, the dynamics of structural changes involving the retinal and the protein moiety are also observable with FT-IR spectroscopy.

The first change in the protonation states occurs in the L to M transition. The proton of the Schiff base which links retinal to the side chain of K216, is transferred to D85 (17). At the same time scale another proton appears at the extracellular surface (18, 19). At pH values below 5–6, proton release is delayed until the O to bR reaction (20, 21). Thus, the pK_a of the proton release group is between 5 and 6 in the M intermediate. In the unphotolyzed state its pK_a is about 9.5 (22, 23).

To identify the proton release group several mutants have been investigated. Late proton release at neutral pH is observed in the single point mutants E204Q (24), E194C (25), and E194Q (26). In contrast, the photoreactions of E9A (27) and the double mutant E9Q/E74Q (28) are unperturbed as compared to the wild-type reaction. Therefore, it can be concluded that E194 and E204 play a crucial role in proton release and E9 as well as E74 are not necessary for normal proton transfer to the extracellular membrane surface. Recently, several authors suggested that the released proton originates from E204 (or several groups including E204: the E204 site), whereas E194 acts as the acceptor of the proton from the E204 site and as the proton donor to the membrane surface (27, 29, 30). Because a band due to deprotonation of E204 (28, 31) or E194 (28) is not observed in time-resolved infrared difference spectra it was concluded that the proton is released by a hydrogen-bonded network connecting D85 and E204 and possibly including E194 and bound water. Although normal proton release is prevented at low pH,

Abbreviations used: ATR/FT-IR, attenuated total reflection Fourier-transform infrared; bR, bacteriorhodopsin; E_4Q, quadruple mutant E9Q/E74Q/E194Q/E204Q.

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0006-291X/01 $35.00

at the end of the photoreaction D85 donates its proton to the extracellular membrane surface, allowing vectorial proton transport across the membrane even at low pH. At high pH, D85 reprotonates the proton release group which is protonated throughout the photoreaction at low pH. Whether the proton pathway is the same at high and low pH is unknown. The recently published structural model of the bR ground state based on X-ray data with 1.55 Å resolution (5) includes a complex branched network of 11 residues, seven water molecules, and 23 hydrogen bonds in the extracellular region. Among others the Schiff base, D85, D212, R82, E194, and E204 are participating.

In a previous study, we have shown that a carboxylic side chain is transiently protonated during the final stage of the photoreaction of wild-type bR (32). Recently, evidence was provided that it is D212 which becomes transiently protonated during deprotonation of D85 as observed in mutants containing substitutions of either E194 or E204 (33). In this study, we present infrared difference spectra of the late part of the photoreaction of the single-site mutants E194Q and E204Q at several pH values as well as of the quadruple mutant E9Q/E74Q/E194Q/E204Q (E_4Q) at pH 5.1. Our results are in line with the assignment of D212 to the difference bands resulting from protonation of a carboxylic acid investigated by Dioumaev *et al.* (33). However, the appearance of a strong band of D212 is only observed in the E194 mutant. The amplitude of this band is strongly dependent on the pH of the surrounding medium with an optimum around pH 6. The results presented here contradict the widely assumed view that D212 remains deprotonated throughout the photocycle (see, e.g., 34). In addition, we demonstrate that the E194Q mutant forms an N intermediate at elevated pH where D85 is deprotonated but the retinal configuration is not influenced as compared to wild-type bR. This result provides evidence that the interaction of the counterion D85 with retinal in the ground state of bR is lost in the N state.

MATERIALS AND METHODS

Wild-type bR, the point-mutants E194Q, E204Q and the quadruple mutant E9Q/E74Q/E194Q/E204Q were obtained as reported previously (28). Infrared spectra were recorded on a Bruker IFS 66v equipped with a MCT (mercury cadmium telluride) detector and a horizontal "out-of-compartment" attenuated total reflection (ATR) accessory. A 45° ZnS trapezoidal internal reflection element was used. The sample was flashed by the second harmonic of a Nd:YAG-laser (532 nm, 8 ns, 3 mJ/cm^2). Difference spectra have been measured with the rapid-scan and the step-scan technique (5 μs time resolution, for details see (32, 35)). If not otherwise stated, experiments have been performed at 20°C. The bulk buffer was an aqueous solution of 1 M KCl and either 25 mM citrate, phosphate, or carbonate for the respective pH. For H/D-exchange, the dry salts have been dissolved in D_2O. The pD was adjusted by taking into account the altered autoprotolysis of heavy water.

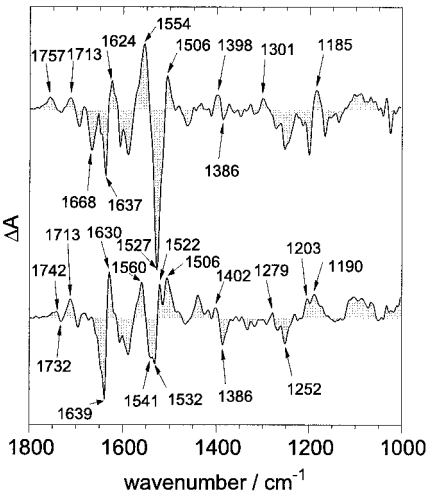

FIG. 1. Infrared difference spectra of the E194Q mutant at pH 6, 20°C. Upper trace, 20–35 ms; lower trace, 100–200 ms after photoexcitation. The lower trace is magnified by a factor of 2 to facilitate the comparison.

RESULTS

The infrared difference spectra between the intermediate states (positive bands) and the initial state (negative bands) of bR are sensitive for changes of the structure and the environment of the chromophore, for changes of the structure of the amide backbone of the protein, and even for changes in the protonation state of single amino acid side chains (14, 36, 37). Therefore, simultaneous observation of all processes important for proton transfer across bR is possible with light-induced FT-IR difference spectroscopy. Here, we focus on the late part of the photoreaction of the E194Q mutant.

Time-resolved FT-IR experiments have been performed at pH 6 and 20°C and the resulting difference spectra are presented in Fig. 1. In the top spectrum (20–35 ms after laser excitation), bands appear that are typical for the O intermediate (marker band at 1506 cm^{-1}) with an admixture of the N state (see for instance the negative band at 1668 cm^{-1}). Remarkably, a positive band at 1713 cm^{-1} shows up. This band has already been detected in the O-BR difference spectrum of the wild type (32) but the intensity is much stronger in the E194Q mutant. The band at 1757 cm^{-1} demonstrates, that D85 is still protonated. Likewise to the M intermediate (28), this band is up-shifted by 2 cm^{-1} in the E194Q mutant as compared to the N and to the O states of the wild type (32).

In the 100–200 ms difference spectrum (bottom spectrum in Fig. 1) absorbance changes above 1750 cm^{-1} have disappeared indicating that D85 is deprotonated at that time. Instead, another carboxylic acid is formed from a carboxylate. This proton transfer reaction is deduced from the concomitant appearance of bands at

1713 cm^{-1} (C═O stretch vibration, positive) and 1386 cm^{-1} (COO^- symmetric stretch vibration, negative). Besides the symmetric vibration, the asymmetric stretching vibration of the carboxylate should also appear in the spectral region between 1500–1600 cm^{-1}. Indeed, a difference band is discernible at 1541 cm^{-1}.

Protonated carboxylic acids can be assigned by isotopic replacement. H_2O/D_2O exchange shifts the band at 1713 cm^{-1} down to 1708 cm^{-1} (spectrum not shown but see Fig. 3B). The downshift of 5 cm^{-1} is not as large as for protonated D96 (in the ground-state of bR) and D85 (in the M, N, and O state) where it is about 10–12 cm^{-1} (28, 32). The smaller isotope effect is characteristic for a strongly hydrogen-bonded carboxylic acid, or in other terms, for a medium pK_a of D212 in the O′ state. As expected for carboxylate vibrations, the bands at 1386 cm^{-1} and 1541 cm^{-1} are not affected by H/D exchange (data not shown).

The differential band feature at 1742 cm^{-1} (positive) and 1732 cm^{-1} (negative) in the 100–200 ms difference spectrum (bottom spectrum in Fig. 1) originates from a band shift of the C═O stretch of (protonated) D115 (38). The absence of a band around 1670 cm^{-1} (amide I, negative) demonstrates that there are no contributions from the N intermediate. The negative band at 1639 cm^{-1} can be assigned to the C═N—H group of the Schiff base in the unphotolyzed state (39). This band is shifted to 1630 cm^{-1} during the lifetime of the late intermediate. The downshift of 15 cm^{-1} in D_2O for both of these bands (data not shown) supports the assignment to the C═N—H stretching vibration. The C═C stretching modes of the chromophore can be attributed to bands at 1532 (negative), 1522 (positive), and 1506 cm^{-1} (positive). The latter originates from contributions of the O intermediate. Other chromophore bands are located at 1440 cm^{-1} (CH_3 bending mode, positive), and 1252 (C—C stretch, negative), 1203 (C—C stretch, positive), and 1190 cm^{-1} (C—C stretch, positive). It is yet difficult to decide whether these bands are due to contributions from O or not. The absence of C—H and N—H in-plane bending vibrations (at 1301 and 1398 cm^{-1}, see top spectrum) indicates a relaxed retinal configuration. In summary, a late photocycle intermediate is found whose structure significantly deviates from the preceding O state. This final intermediate is named O′ in agreement with the definition provided by Dioumaev *et al.* (33).

The positive difference band at 1713 cm^{-1} of O′ is of particular relevance since it allows to represents a proton transfer step during the final stage of the reaction cycle of the E194Q mutant. As mentioned above, it is assigned to the protonation of a carboxylate (D or E). Since E194 is close to E204 in the ground-state structure of bR (5, 6), one might expect a perturbation of E204 in the E194Q mutant of bR. However, the difference spectrum of the mutant E204Q does not exhibit the strong band at 1713 cm^{-1}. Rather, the difference spectrum is very similar to the wild-type O-BR spectrum (Fig. 2) where the band is low in amplitude. This points towards a specific effect by the replacement of E194.

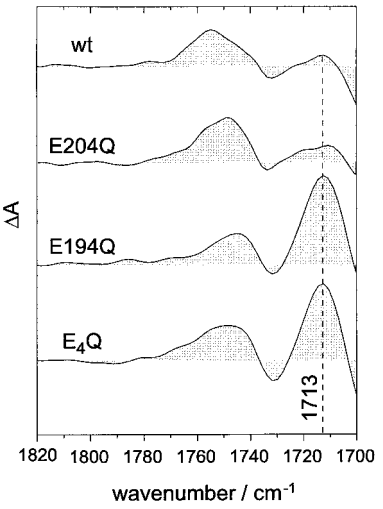

FIG. 2. Comparison of FT-IR difference spectra in the carbonyl region of wild-type BR (pH 4.0, 40°C, taken and averaged at 5–10 ms after photoexcitation), the E204Q mutant (pH 6.0, 20°C, 200–400 ms), the E194Q mutant (pH 6.0, 20°C, 100–200 ms), and the quadruple mutant E9Q/E74Q/E194Q/E204Q (E_4Q, pH 6.0, 20°C, 300 ms).

The assignment of the band at 1713 cm^{-1} to a particular amino acid residue was done by using the quadruple mutant where all glutamates (or glutamic acids—the protonation state of these residues is not yet certain) are replaced by glutamine. Since the band at 1713 cm^{-1} can still be observed in the quadruple mutant E9Q/E74Q/E194Q/E204Q (E_4Q in Fig. 2) it is straightforward to conclude that it is due to D212, the only acidic residue left at the extracellular side of bR.

The kinetics of the acid/base reaction of D212 is depicted in Fig. 3. The time course of the band at 1713 cm^{-1} tally with that at 1386 cm^{-1} albeit with opposite amplitudes (Fig. 3A). This substantiates the assignment of these bands to the C═O stretch and the respective symmetric COO^- stretching vibration of D212. It is evident from the time trace at 1713 cm^{-1} and 1754 cm^{-1} that the protonation of D212 occurs with about the same time constant as the deprotonation of D85 (Fig. 3A). Thus, we conclude that D85 donates the proton directly to D212 during the O to O′ transition. The kinetic isotope effect (KIE = $\tau_{(D2O)}/\tau_{(H2O)}$) for both the protonation and the deprotonation reaction of D212, is about 2 (Fig. 3B). This corroborates with other proton transfer reactions during the slow part of the photocycle (40, 41) where the proton transfer is rate-limited by structural changes of the protein.

Characteristic for a proton pump, proton transfer reactions are influenced by the pH of the surrounding medium. Therefore, difference spectra of the E194Q

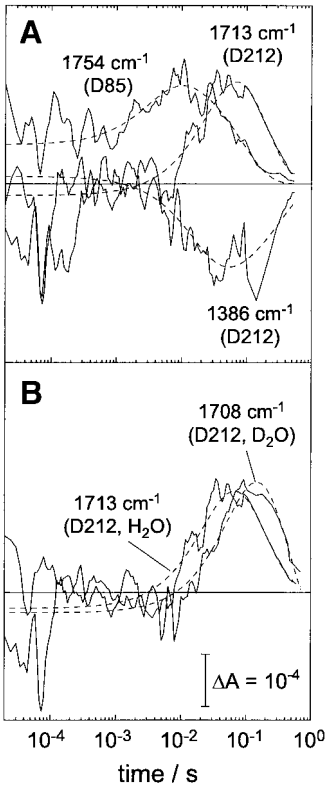

FIG. 3. Time-resolved absorbance changes at 1754, 1713, and 1386 cm^{-1}. Data are plotted on a logarithmic time scale. The kinetics of the E194Q mutant have been extracted from step-scan experiments. At 100 ms after light excitation, rapid-scan data have been appended. The dashed lines are biexponential fits to the respective time trace. Resulting time-constants are: $\tau_1 = 5.8$ ms, $\tau_2 = 68$ ms for 1754 cm^{-1}, $\tau_1 = 34$ ms, $\tau_2 = 167$ ms for 1713 cm^{-1}, and $\tau_1 = 17$ ms, $\tau_2 = 323$ ms for 1386 cm^{-1}. (B) Kinetic isotope effect of the protonation/deprotonation reaction of D212. Measurements have been performed in H_2O and D_2O, respectively.

mutant have been recorded at various pH values (Fig. 4). The strong band at 1713 cm^{-1} is clearly observed at pH 4 and 6, but only weak at pH 7.4 and completely missing at pH 7.8 and 8.4. This behavior indicates an optimum around pH 6 for the transient accumulation of protonated D212. The negative band at 1743 cm^{-1} in the difference spectra at pH 7.4 and higher is representative for the deprotonation of D96 (42). Like in the wild type, this band is missing at pH 6 and 4 because the transient pK_a of D96 is 7.1 (28). This demonstrates that the E194Q mutation does not influence the reprotonation of the retinal Schiff's base. Surprisingly because not observed in wild-type bR, the intensity of the band of the C═O stretch of D85 (located between 1763 and 1755 cm^{-1} depending on the respective photocycle intermediate, (32)) decreases at alkaline pH. This culminates in the complete deprotonation of D85 at pH 8.4 in the late stage of the photocycle of the E194Q mutant.

Figure 5 depicts the difference spectrum in the range 1850–1000 cm^{-1} of the E194Q mutant (top spectrum). It is very similar to the N-BR difference of the wild type

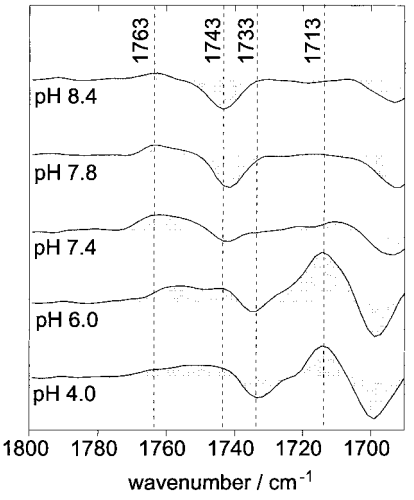

FIG. 4. Infrared difference spectra in the carbonyl region of the E194Q mutant at pH 8.4, 7.8, 7.4, 6.0, and 4.0 (from top to bottom) detected 70 ms after photoexcitation.

(middle spectrum) taken under the same conditions (32). Specifically, the bands in the fingerprint region (1250–1150 cm^{-1}) are identical in both spectra providing evidence for the same retinal conformation. The transient concentration of the N state is slightly higher in wild-type bR as can be deduced from the larger absorbance at 1185 cm^{-1}. The band at 1553 cm^{-1} comprising amide II and the C═C stretching vibration of the chromophore, is also congruent. The C═C stretch of retinal in the ground-state bR is slightly shifted in the mutant (1529 cm^{-1}) as compared to the wild type (1523 cm^{-1}, Fig. 5). The shift correlates with the reported blue-shift in the visible wavelength region (25). The difference spectrum of the E194Q mutant deviates significantly from that of wild-type bR in the amide I region. Whereas the positive band at 1648 cm^{-1} is not

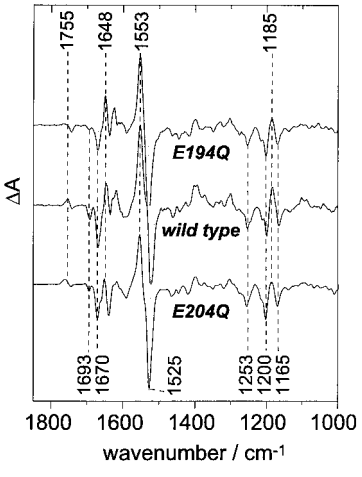

FIG. 5. Time-resolved infrared difference spectra of the E194Q mutant, wild-type bR, and the E204Q mutant (from top to bottom) at pH 8.4 and 70 ms after photoexcitation.

altered in the mutant, the negative band at 1670 cm^{-1} is smaller in size. These amide I changes have been attributed to transient change in the backbone conformation of the transmembrane α-helices. Even more obvious (Fig. 5) is the almost complete disappearance of the negative band at 1693 cm^{-1} in the E194Q mutant. We have previously attributed this band to a backbone change of a β-turn type I motif in the vicinity of E194 (32). The bottom spectrum in Fig. 5 corresponds to the E204Q mutant taken under the same conditions. Most of the bands are at the same frequency as in the E194Q mutant and in the wild-type spectrum, albeit with slightly different intensities. Remarkably, the positive amide I band at 1648 cm^{-1} has lost intensity whereas the negative band at 1670 cm^{-1} is present as compared to the wild type and the E194Q mutant. The very different effects of the mutations of E194 and E204 on the amide I difference bands point to a rather specific influence of these residues on the changes in secondary/tertiary structure during catalytic activity of bR. Yet, the most intriguing deviation from the E194Q spectrum is the presence of the C═O stretching mode of protonated D85 in the E204Q difference spectrum. The fact that the protonated D85 is observed both in wild-type bR and in the E204Q mutant but not in the N-BR difference spectrum of the E194Q mutant demonstrates that only the mutation of E194 decreases the high pK_a of D85 which eventually leads to deprotonation of D85 during the lifetime of the N state.

DISCUSSION

The present study deals with the role of E194 and E204 both being located close to the extracellular surface, in proton transfer during the final stages of the catalytic cycle of bR. It has been previously shown that the exchange of either of these residues by a non-protonatable residue severely impairs the proton release reaction. As we demonstrate here, this functional similarity is suspended in the final proton transfer steps where the E194Q mutation exerts distinct effects hardly observed in the E204Q mutant.

Proton release by bR is initiated by proton transfer from the retinal Schiff's base to D85. Thereafter, R82 delivers the information from D85 via a Domino effect to the still unknown group to release a proton to the extracellular surface. In the late stage of proton translocation across bR, D85 needs to be deproteinated to reset bR to the original state. The proton of D85 is supposed to be transferred to the proton release group or it is directly released into the extracellular medium. The latter proton translocation reaction corresponds to late proton release. It is observed if the proton release group remains protonated in M (pH <5–6 depending on the ionic strength (22, 43)) or if the proton release complex has been disturbed as in mutants involving residues R82 (44), E194 (25, 27), or E204 (24).

By time-resolved infrared spectroscopy on the E194Q mutant, we could visualize a proton transfer step during the late reaction steps (Fig. 1). The data suggest that D85 directly donates the proton to the nearby D212 (Fig. 3). The frequency of the C═O stretching vibration of D212 is at 1713 cm^{-1} in this state (coined O′ by Dioumaev *et al.* (33)). The corresponding carboxylate modes are at 1386 cm^{-1} (symmetric stretch) and 1541 cm^{-1} (asymmetric stretch) for the ground state. The assignment of the C═O stretch and the symmetric COO^- stretch agrees with previous work by Dioumaev *et al.* on the double mutant E9Q/E194Q who used the labeling approach with [4-^{13}C]aspartic acid (33). However, the low content of label and the overlap of the thereby shifted bands with other difference bands prevented a clear-cut interpretation of the data. Since the difference band at 1713 cm^{-1} appears also in the quadruple mutant E9Q/E74Q/E194Q/E204Q we are able to exclude that this band is due to protonation of one of the glutamates at the extracellular surface. The only residue with a carboxylic side chain that is left in the extracellular domain of bR, is D212. With this procedure of exclusion, we substantiate the assignment of a protonation change of D212 in the final stage of proton translocation of bR. At this point, it would be desirable to employ a D212 mutant for band assignment. However, the exchange of D212 leads to a functionally strongly impaired enzyme (45, 46) prohibiting such a strategy.

In a previous study on wild-type bR, we were able to detect the bands at 1713, 1386, and 1541 cm^{-1} in the difference spectrum of the O intermediate, albeit with weak intensity (32). This is probably due to less transient accumulation of this intermediate in the wild type. We are now able to conclude that after the well-known O intermediate a consecutive intermediate is formed in wild-type bR whose peculiar property is a protonated D212 with a deprotonated D85. This leads to a different interaction of these residues in O′ as compared to the preceding O state. The altered electrostatic environment in the vicinity of retinal Schiff base leads to a slight red-shift of the absorption of this intermediate as compared to ground-state bR (1522 cm^{-1} in O′ versus 1527 cm^{-1} in ground-state bR). The largest bands in the O′ difference spectrum (at 1639/1630 cm^{-1}) are due to a shift of the C═N—H group of the Schiff base indicating different environments (hydrogen-bonding and/or electrostatic interaction) of the Schiff base in O′ as compared to the ground state. Since the retinal configuration is supposed to be very similar as can be judged from the absence of large difference bands in the fingerprint region, the main difference between O′ and the ground state is the change in interaction between D212 and the retinal Schiff base. The structure of the surrounding protein

matrix is identical in both states since difference bands in the amide region are absent.

The time course of protonation and subsequent deprotonation of D212 is only slightly influenced by replacing the proton by a deuteron. We find a kinetic isotope effect (KIE) of about 2 in the E194Q mutant (Fig. 3). This value is typical for proton transfer reactions taking place in the slow part of the bR photocycle. For example, the proton uptake reaction of wild-type bR exhibits a KIE of 1.6 (40). For the rise and the decay of the O intermediate it is around 2 (32, 47). The low KIE's suggest that the kinetics are not rate-limited by the actual proton transfer reaction. However, large KIE's have been reported for the decay of the O intermediate in the mutants E204Q, Y57F and E194Q as detected by visible spectroscopy (48). In light of our results on the E194Q mutant where we have been able to directly determine the kinetics of the protonation of D212, the high KIE reported for the E194Q mutant (48) is surprising.

The appearance of the protonated D212 in the late stage of the photoreaction of the E194Q mutant exhibits a pH-optimum at around pH 6 (Fig. 4). In the alkaline pH region, reprotonation of the Schiff base by D96 is rate-limiting for the subsequent proton transfer steps. Reprotonation of the Schiff base corresponds to the formation of the N intermediate. The pK_a of D96 drops from higher than 11 down to 7.1 during this reaction (28). The pK_a of 7.1 represents the upper pH-limit for the transient accumulation of the protonated D212. The lower limit can be the deprotonation reaction of the proton release complex. In wild-type bR, it was determined to be between pH 5 and 6 (depending on the ionic strength). However, the proton release complex is impaired by the exchange of E194. Consequently, we are forced to conclude that it is the isomerization of retinal from *13-cis* to *all-trans* that triggers proton transfer from D85 to D212. Indeed, the observation that the O′ intermediate succeeds the O intermediate (see Figs. 1 and 3) supports this view. The physical origin of the pH-dependence of the retinal re-isomerization is not clear. It can be driven by a change in the local electric field around the retinal and/or by a slight change in the conformation of residues lining the retinal binding pocket that drives the relaxation of retinal back to the all-trans state.

In contrast to previous work (33), we find that O′ can be significantly accumulated only by the replacement of E194. In E204 mutants as well as in wild-type bR, the protonated state of D212 is hardly observed (Fig. 2). This fact points to a specific (long-distant) interaction of E194 with D85 and/or D212. This finding agrees well with the conclusion drawn by Lazarova *et al.* (49).

At elevated pH, an N-BR difference spectrum is observed that is nearly identical to that of wild-type bR with the surprising exception that the band due to protonation of D85 is missing (Figs. 4 and 5). This experimental fact provides evidence that the proton acceptor of the retinal Schiff's base (D85) does not interact with retinal during the lifetime of the N intermediate. Otherwise, vibrational bands of the chromophore would be altered which can be definitely ruled out from the vibrational spectra displayed in Fig. 5. This finding agrees with resonance-Raman spectroscopy on wild-type bR in which only the chromophore vibrations are selectively enhanced. The spectra of the L and the N intermediate are very similar (50) although the counterion D85 is deprotonated during the L state while it is protonated in N. Obviously, the influence of the ionization state of D85 on the vibrational spectrum of the chromophore is only of minor importance in these states. It might be concluded that when the retinal is in *13-cis* configuration with a protonated Schiff's base then the electronic state of the retinal is not susceptible to the interaction with the counterion.

The comparison with the data obtained from mutants where E204 was exchanged (Fig. 5), clearly shows that the effect is specific for the E194Q mutation, again. It is concluded that E194 controls the decrease in acidity of D85 to deprotonate in the final stage of the wild-type photoreaction. The long range interaction between E194 and D85 is most probably mediated by the hydrogen-bonded network in the extracellular proton pathway (9, 31).

Finally, it should be pointed out that the methodology used in this work is superior to previous studies on the photoreaction of the E194Q mutant. Lazarova *et al.* (49) trapped the M and N intermediate at low temperature. This procedure suffers from the fact that the kinetics of the E194Q mutant is different from wild-type bR and hence the composition of the trapped states at a given temperature is altered as compared to the wild type. Dioumaev *et al.* (33) employed rapid-scan spectroscopy with millisecond time-resolution. To fully record the relevant kinetics with the low time-resolution, the sample was cooled down to 8°C. Moreover, both of the studies used transmission FT-IR spectroscopy. The ATR technique applied in our work allows for the precise adjustment of the sample conditions (pH, water content, salt concentration) which is crucial for drawing conclusions on specific details of the reaction mechanism. In addition, we could investigate the sub-millisecond kinetics of the E194Q mutant with the high temporal resolution of the step-scan technique.

ACKNOWLEDGMENTS

We thank J. Tittor and D. Oesterhelt (Max-Planck-Institute for Biochemistry, Martinsried) for providing the mutants E194Q and E204Q. We acknowledge the generous support by Dr. G. Büldt and the skillful technical assistance by I. Ritter. This work was partly financed by a grant from the Deutsche Forschungsgemeinschaft (SFB 189/C6 to J.H.).

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