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Specific Effects of Chloride on the Photocycle of E194Q and E204Q Mutants of Bacteriorhodopsin As Measured by FTIR Spectroscopy†

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ABSTRACT: Low-temperature Fourier transform infrared spectroscopy has been used to study mutants of Glu194 and Glu204, two amino acids that are involved in proton release to the extracellular side of bacteriorhodopsin. Difference spectra of films of E194Q, E204Q, E194Q/E204Q, E9Q/E194Q/E204Q, and E9Q/E74Q/E194Q/E204Q at 243, 277, and 293 K and several pH values were obtained by continuous illumination. A specific effect of Cl^- ions was found for the mutants, promoting a N-like intermediate at alkaline pH and an O′ intermediate at neutral or acid pH. The apparent pK_a of Asp85 in the M intermediate was found to be decreased for E194Q in the presence of Cl^- (pK_a of 7.6), but it was unchanged for E204Q, as compared to wild-type. In the absence of Cl^- (i.e., in the presence of SO_4^{2-}), mutation of Glu194 or of Glu204 produces M- (or M_N, M_G)-like intermediates under all of the conditions examined. The absence of N, O, and O′ intermediates suggests a long-range effect of the mutation. Furthermore, it is suggested that Cl^- acts by reaching the interior of the protein, rather than producing surface effects. The effect of low water content was also examined, in the presence of Cl^-. Similar spectra corresponding to the M_1 intermediate were found for dry samples of both mutants, indicating that the effects of the mutations or of Cl^- ions are confined to the second part of the photocycle. The water O−H stretching data further confirms altered photocycles and the effect of Cl^- on the accumulation of the N intermediate.

Bacteriorhodopsin (BR),[1] a light-driven proton pump, contains a retinal-bound chromophore which upon absorption of a photon isomerizes and initiates a photocycle coupled to proton transfer from the cytoplasmatic to the extracellular side of the membrane (*1*−*3*). The simplified photocycle contains several intermediate states or photoproducts denoted as BR (unphotolyzed pigment), K, L, M, N, O, BR (*4*−*8*). After photon absorption, retinal isomerization from all-trans to 13-cis changes the active site of the chromophore, which in turn changes the pK_a values of the Schiff base (SB) and of Asp85, driving the transfer of the SB proton to Asp85 (*9*, *10*). At neutral pH, both processes, Asp85 protonation and proton release on the extracellular side, occur almost simultaneously, with a time constant of 80 μs. This temporal correspondence raises the necessity for other groups than Asp85, the proton release group (PRG), being the source of the released proton (*11*−*13*).

Experimental evidence indicates that the PRG is not a single residue but a complex set of groups, organized as a hydrogen-bonded network (*14*−*18*). Recent X-ray diffraction structural maps of BR have revealed the presence of a number of water molecules on the extracellular side and have indicated extensive rearrangements of the network of hydrogen-bonded residues and bound waters in M state (*19*, *20*). The inhibition of fast (normal) proton release upon mutation of side chains such as Glu194, Glu204, Arg82, or Tyr57 has provided experimental evidence that these amino acids are strongly involved in the network (*21*−*26*). However, despite the major advances of recent years, the exact functional or structural role of these amino acids in the proton-transfer mechanism is still not completely understood (*7*).

After proton release and reprotonation of the SB from the cytoplasmatic side, the initial state is recovered. At neutral pH, Asp85 remains protonated until the end of the photocycle, and it is only in the last step, the O−BR transition, that it loses its proton to reprotonate the PRG (*27*, *28*). Therefore, the O intermediate is the key to the final phase of proton pump mechanism. The slowed O−BR transition rate at low pH raised the hypothesis that the increased lifetime of the intermediate is due to the inability of the PRG

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[1] Abbreviations: BR, bacteriorhodopsin; PM, purple membrane; 3Glu, bacteriorhodopsin mutant E9Q+E194Q+E204Q; 4Glu, bacteriorhodopsin mutant E9Q+E74Q+E194Q+E204Q; PRG, proton release group; SB, Schiff base; FTIR, Fourier transform infrared; WT, wild-type.

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to deprotonate at low pH (*29*). In other words, the presence of deprotonated PRG in O would facilitate Asp85 deprotonation and the decay of O to BR. Replacement of Glu194 or Glu204 with uncharged residues results in an elevated accumulation of O. Moreover, in both mutants, the release of the proton correlates with O decay, while the uptake correlates with the O rise (*21*, *29*−*32*). Recently, a new species of O′ was described, in which Asp212 would protonate transiently from Asp85 (*30*, *33*), being a possible pathway for reprotonation of the PRG. On the other hand, from the occurrence of deprotonated Asp85 at alkaline pH of mutants containing E194Q, we were able to propose Glu194 as a group that controls the pK_a of Asp85 during the photocycle (*34*). This result has been confirmed using time-resolved FTIR spectroscopy (*33*). The important questions of proton transfers on the extracellular side are particularly related to the proton release group. First, regarding its identity (i.e., the groups directly involved in the proton release). Second, regarding the mechanism of coupling between Asp85 and the PRG. In this respect, it is important to know if PRG deprotonation, in turn, facilitates Asp85 deprotonation and the increase in O intermediate.

The aim of our study is to evaluate the role of the two key amino acids Glu194 and Glu204 in the photocycle. To do this, we have studied the effect of pH on the low-temperature FTIR difference spectra using E194Q, E204Q, E194Q/E204Q, E9Q/E194Q/E204Q (3Glu), and E9Q/E74Q/E194Q/E204Q (4Glu) mutants. The influence of water content on the state of protonation/deprotonation of certain groups in the M intermediate of the photocycle is also considered.

MATERIALS AND METHODS

The mutants E9Q, E74Q, E194Q, E204Q, E194Q/E204Q, 3Glu, and 4Glu were constructed and expressed as described previously (*35*). Purple membranes with mutated BR were prepared from *H. salinarum* using a standard method (*36*).

Samples for FTIR experiments were prepared as described elsewhere (*34*). pH was adjusted in 1 mL suspension of BR sample in 150 mM KCl, 1 M KCl or 75 mM Na_2SO_4, and either 3 mM sodium citrate (pH 3−4) and 3 mM sodium phosphate (pH 5−7) or 3 mM sodium carbonate buffer (pH 8−10). After centrifugation, an aliquot of about 50 μL of the sample was placed on a CaF_2 window and partially dried by applying a mild vacuum. To achieve the desired hydration level, the samples were kept overnight in a closed chamber with the appropriate solution of KNO_3, water, or P_2O_5. The hydration level of the film was controlled using the two previously described methods (*34*).

The steady-state spectra were recorded on Mattson Polaris or Bio-Rad FTS6000 FTIR spectrometers, at resolutions of 2 or 8 cm^{-1}. Sample temperature was controlled using a homemade water circulator and a cryostat. Illumination with light of >500 nm for 1 min at 293 K was used to obtain the light-adapted state of BR and then cooled to the desired temperature. Static low-temperature FTIR difference spectra were obtained at 230, 240, or 277 K. The difference spectra were obtained as described previously (*37*). The spectra shown are differences in the averages of at least five recordings. Experiments were repeated in at least three independent samples. For comparison purposes, difference spectra are scaled to the same intensities between the 1201 and 1210 cm^{-1} bands.

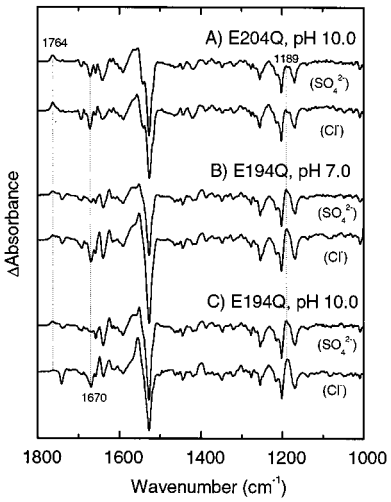

FIGURE 1: Steady-state FTIR difference spectra of hydrated films of E204Q (A) and E194Q (B, C) in 75 mM Na_2SO_4 or 1 M KCl at pH 7.0 (B) and pH 10.0 (A, C). The spectra were recorded at 243 K in the dark and under continuous yellow light illumination and were subtracted. All spectra are scaled to equal intensity between 1201 and 1210 cm^{-1} peaks.

RESULTS

Effect of Cl⁻ on the Photocycle of E194Q and E204Q Mutants: The M and N Intermediates Trapped at Low Temperatures. Figure 1 shows the 1800−1000 cm^{-1} FTIR region of difference spectra recorded at 243 K for E194Q and E204Q in the presence of Cl^- or SO_4^{2-}. At alkaline pH, the spectra of E204Q(Cl^-) and E204Q(SO_4^{2-}) are nearly identical, as was the case at neutral pH (not shown), and represent M state (*23*, *27*, *34*). The infrared difference spectra of E194Q reveal more complex shapes (Figure 1B and C). At neutral pH, the overall features of E194Q(Cl^-) and E194Q(SO_4^{2-}) spectra are similar, with small but distinct differences (Figure 1B). At alkaline pH, in the presence of Cl^- the 1762 cm^{-1} band corresponding to protonated Asp85 is almost absent, while a strong band at 1742 cm^{-1} indicates Asp96 deprotonation (Figure 1C; see also ref *34*). In the absence of Cl^-, however, the positive band at 1764 cm^{-1} is clearly seen (upshifted by about 2 cm^{-1} as compared to WT), accompanied by the bilobe at 1748 and 1740 cm^{-1} (Figure 1C), assigned to OH bonding changes of Asp96 (*9*, *10*, *38*). In the amide I region, as at neutral pH, E194Q(SO_4^{2-}) shows the 1670 cm^{-1} band of reduced intensity as compared to E194Q(Cl^-), indicating differences in the kinetic behavior. In the amide II, the main band at 1553 cm^{-1} shifts to 1556 cm^{-1} in the presence of Cl^-, suggesting that a N-like structure is accumulated (*34*).

When all of these features are combined, it can be deduced that, in the E194Q mutant at 243 K, (i) in the presence or in the absence of Cl^-, a mixture of M with another intermediate (L or N) is trapped; (ii) photocycle kinetics is salt dependent, and M accumulation is lower in the presence of Cl^-; (iii) the chromophore band at 1189 cm^{-1} remains positive and intense either in the presence or in the absence of Cl^-, suggesting a strongly disturbed chromophore as a result of mutation; and (iv) the second pK_a increase of Asp85 in the M intermediate in the presence of Cl^- is inhibited (*34*).

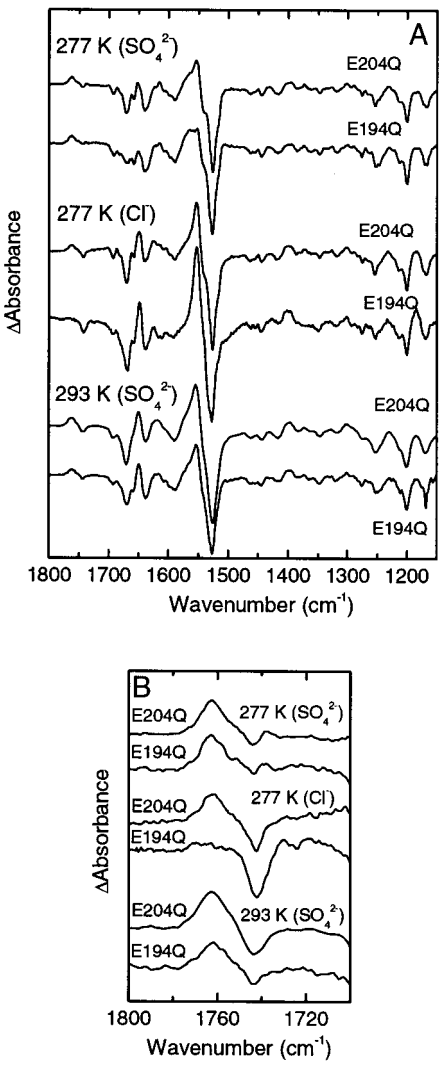

FIGURE 2: (A) FTIR difference spectra of hydrated films of E194Q and E204Q in 75 mM Na_2SO_4, recorded at 277 K and at 293 K (top and bottom). For comparison, the difference spectra of hydrated films of E204Q and E194Q in 1 M KCl at 277 K are also shown (*34*; middle). (B) Expanded view of the 1800−1700 cm^{-1} region of the spectra of panel A.

Figure 2A (top) presents FTIR difference spectra of E204Q and E194Q recorded at pH 10 and 277 K, in the presence of Na_2SO_4. These temperature and pH conditions, known to favor the N intermediate (*39*), are the same as in our previous work (*34*) apart from the salt used (KCl). For comparative purposes, the spectra of E194Q and E204Q in KCl are also shown (Figure 2A, middle). In the presence of Cl^-, both spectra represent a N-like intermediate, although E194Q has deprotonated Asp85. In contrast, in the absence of Cl^- (in 75 mM Na_2SO_4), they show very similar features, corresponding to an M-like intermediate (positive bands at 1764 and 1567 cm^{-1} and a band at 1186 cm^{-1} below the baseline). At a higher temperatures (293 K) and in the absence of Cl^-, the protein adopts the conformation typical of N in both mutants, seen by the bands at 1670 cm^{-1} and narrower amide II at 1556 cm^{-1} (Figure 2A, bottom). However, the positive band of Asp85 is at 1762 cm^{-1} and the chromophore shows the band at 1186 cm^{-1} below the baseline, indicating deprotonated SB in the M state. Therefore, the difference spectra at 293 K illustrate the previously described M_N or M_G intermediate (*40*−*42*). Remarkably, the small bands at

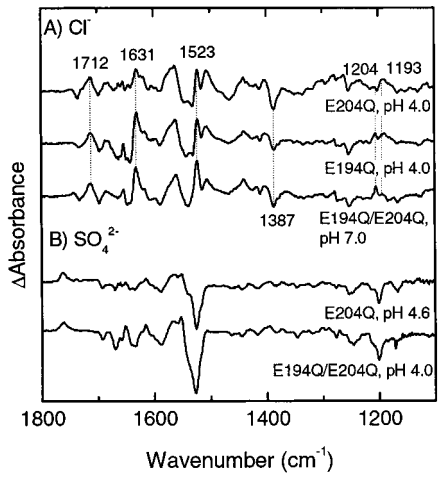

FIGURE 3: FTIR difference spectra recorded at 277 K of (A) hydrated films of E204Q, E194Q, and E194Q/E204Q in 1M KCl at pH 4.0 and 7.0 and (B) hydrated films of E204Q and E194Q/E204Q in 75 mM Na_2SO_4, at pH 4.6 and 4.0, respectively.

1738 and 1732 cm^{-1}, attributed in WT to perturbation of Asp115 (*43*, *44*), are not seen in the N-like difference spectra of both mutants either in the presence of Cl^- (*34*) or in its absence (Figure 2B).

Effect of Cl^- on the Photocycle of E194Q and E204Q Mutants: Accumulation of O and O' Intermediates. It has been described that the replacement of Glu204 or Glu194 produces an elevated accumulation of the O intermediate (*21*, *29*−*33*). Recently, an anomalous O state called O′ has been described for these mutants (*30*). Figure 3 shows the difference spectra of E194Q, E204Q, and the double mutant E194Q/E204Q recorded at 277 K, in the presence of Cl^- or SO_4^{2-}. In the presence of Cl^-, the difference spectra recorded at low pH represent a virtually pure O′ state (Figure 3A), as described by Dioumaev et al. (*30*). It is identified by the following characteristics: higher intensity of the positive band at 1523 cm^{-1} as compared to the 1508 cm^{-1} band (C═C stretching mode); a strong positive band at 1712 cm^{-1} (assigned to protonation of Asp212); a negative band at 1387 cm^{-1}, which appears simultaneously with the 1712 cm^{-1} band; and two positive bands at 1204 and 1193 cm^{-1} (C−C stretching mode of the chromophore). The spectra of E194Q/E204Q and 3Glu, representing O′, were recorded at pH 7.0 (Figure 3A; see also Figure 6), indicating that the pK_a of O′ appearance in the presence of Cl^- is raised in multiple mutants.

The aforementioned spectra corresponding to the O′ intermediate were obtained in the presence of Cl^-, as in previous works (*30*, *33*). Our attempts to obtain similar spectra in the same conditions but in the absence of Cl^- failed. As shown in Figure 3B, an M-like intermediate accumulates in E204Q or in E194Q/E204Q at low pH, and no bands for O or O′ are found. Similar spectra are obtained for E194Q (not shown). These results reveal that Cl^- plays an important role in the accumulation of O′ as well as of O in these mutants. On the other hand, it is important to emphasize that measurements performed under similar conditions as those for E204Q or E194Q, using the single E9Q or E74Q mutants, do not reveal any O′ intermediate (data not shown).

FTIR Spectra of E194Q and E204Q Mutants Recorded in the Presence of Cl^-. To further examine the effect of Cl^-

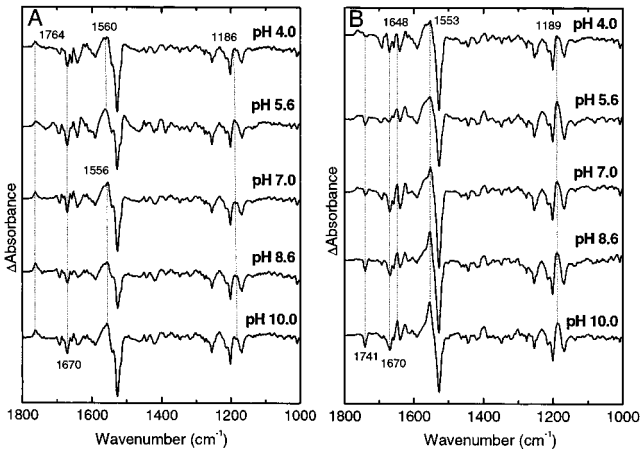

FIGURE 4: FTIR difference spectra of hydrated films of E204Q (A) and E194Q (B) in 1 M KCl recorded at 243 K and at different pH values, as indicated.

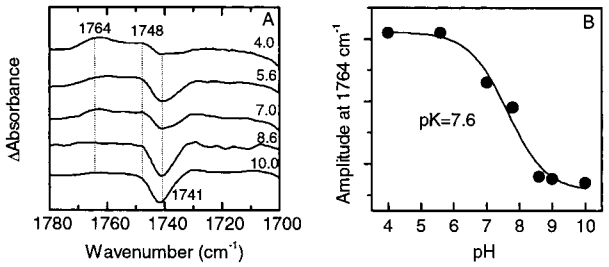

FIGURE 5: (A) Expanded view of the 1800−1700 cm^{-1} region of the spectra of Figure 4B. (B) Plot of the intensity at 1764 cm^{-1} as a function of pH.

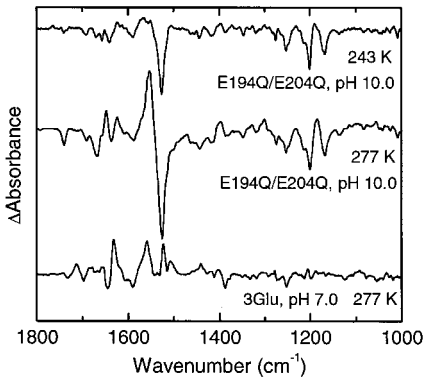

FIGURE 6: FTIR difference spectra of hydrated films of E194Q/E204Q in 1 M KCl and 3Glu in 150 mM KCl, at the indicated values of temperature and pH.

on the protonation state of extracellular Glu and Asp side chains and on the protein structure of E194Q and E204Q, FTIR difference spectra of these mutants were recorded at different pH values, in the presence of Cl^- at 243 K (Figure 4). At alkaline and neutral pH, the overall features of the difference spectra of E204Q are very similar to the spectra of wild-type reported earlier, where M intermediate accumulates (Figure 4A). Small differences can be seen, as have already been described (*13*, *27*, *34*). The most significant characteristics of E204Q are the upshift of the C=O stretch of Asp85 from 1762 to 1764 cm^{-1}, higher intensities of the bands at 1670 cm^{-1} and at 1663 cm^{-1}, and the presence of a small positive band at about 1700 cm^{-1}. Figure 4A also shows that difference spectra of E204Q below neutral pH represent mixtures of M and O intermediates. The contribution of O is shown by the appearance of the 1508 cm^{-1} band, the shoulder at 1756 cm^{-1}, and a shift of the 1553 cm^{-1} band to 1560 cm^{-1}, assigned to the C=C stretching vibration of the chromophore and to the amide II (*45*). The contribution of O to the difference spectrum is stronger at pH 5.6 than for other pH values, suggesting that it might result from the combined effect of two groups with different pK_a.

For E194Q, the difference spectra are less affected by pH variation, the major changes appearing in the carboxylic region (Figure 4B). The negative band at 1670 cm^{-1} and the positive one at 1648 cm^{-1} remain intense in nearly all pH ranges, while the band at 1553 cm^{-1} maintains its position. Two other bands that are hardly affected by pH are the negative band located at about 1741 cm^{-1} and the intense positive band at 1189 cm^{-1}, due to 13-cis chromophore with protonated SB. The overall similarity of the difference spectra recorded at different pH at 243 K strongly suggests the accumulation of the same photoproduct. Although the WT accumulates M intermediate under these conditions, the spectral characteristics delivered by E194Q do not permit us to attribute the M state to this photoproduct.

As the main changes appear in the carboxylic range, we analyzed the influence of pH on E194Q in this range (Figure 5A). The intensity of the C=O stretch of Asp85, shown by the positive band at 1762 cm^{-1}, decreases with increases in pH. While in wild-type and in E204Q, the plot of the amplitude of this band against pH gives an apparent pK_a of around 10 for Asp85 (*7*, *43*), it is 7.6 for E194Q (Figure 5 B). This pK_a is similar to that described for Asp85 in the M_1 intermediate (*7*), indicating that the removal of the negative Glu194 charge interrupts the coupling of Asp85 and the PRG, of which Glu194 forms part. In a parallel form to the evolution of the Asp85 band, the intensity of the negative band at 1741 cm^{-1} increases and becomes narrower when changing from acid to alkaline pH. At pH 10, its intensity is maximal, indicating deprotonated Asp96 (*34*).

Multiple Glu mutants were also examined in terms of their difference spectra at several different pH values. The spectra of E194Q/E204Q and 3Glu (Figure 6), as well as 4Glu (not shown), both in the presence and absence of Cl^- (Figure 3B), are virtually identical to the difference spectra of E194Q single mutant. This finding indicates that the changes in protein conformation and in the protonation state of carboxylic side chains are mainly determined by the mutation of Glu194, but not by mutation of Glu204.

Effect of Water Content on the Photocycle and the Protonation State of E194Q and E204Q, in the Presence of Cl^-. Experimental and theoretical studies have shown that the amount of water strongly influences the BR photocycle. Only for hydrated samples (humidity > 60%) does photoexcited BR go through all intermediates of the photocycle (*46*, *47*). Therefore, at low humidity (dry samples), the photocycle stops at M_1 in the extracellular conformation and reverses back to BR through several M_1-like substates without accumulation of N or O (*48*−*50*). On this basis, we performed FTIR measurements using dry films (i.e., films with a hydration of less than 60%). These experiments also facilitate the detection the molecular changes taking place in the first part of the photocycle.

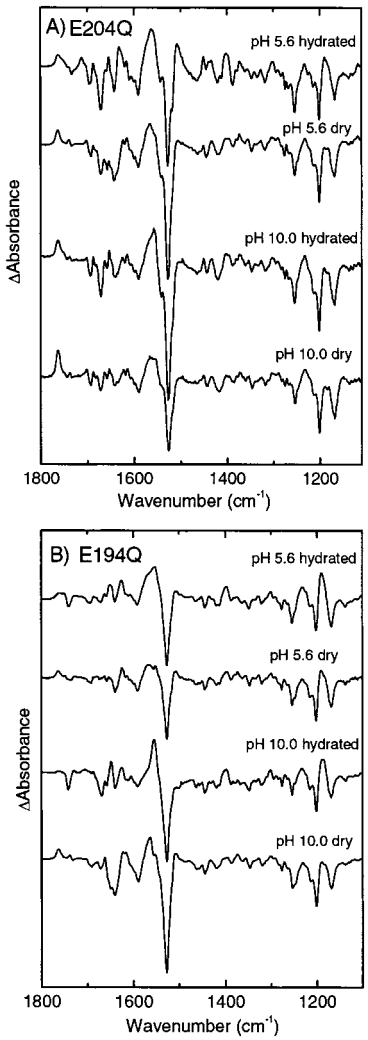

FIGURE 7: FTIR difference spectra of hydrated or dry films of samples in 1 M KCl recorded at 243 K, pH 5.6 or 10.0: (A) E204Q, (B) E194Q.

FTIR difference spectra of E204Q recorded at pH 5.6 and 10.0 for both dry and hydrated samples are shown in Figure 7A. The decrease of the water level at pH 10 produces an M_1-like spectrum and only results in minor changes as compared to the hydrated sample, such as a decrease in the intensity of the 1670 cm^{-1} band as compared to the 1660 cm^{-1} band and the band at 1762 cm^{-1} becoming narrower. This suggests that the shoulder at 1750 cm^{-1} revealed in the hydrated sample most probably arises from the contribution of some N intermediate at the expense of M_2. The small shift of the band at 1555 cm^{-1} to 1557 cm^{-1} may also account for the presence of some N or M_2 intermediates in hydrated samples. Likewise, at pH 5.6, the difference spectrum of a dry sample of E204Q mainly represents an M state. Therefore, all bands diagnostic for an O intermediate as that at 1713 cm^{-1}, the shoulder at 1750 cm^{-1}, and the 1508 cm^{-1} band disappeared in the dry sample, in accordance with accumulation of an M_1 intermediate. It is important to mention that at both pH values the intensity of 1738 and 1701 cm^{-1} bands does not depend on the level of hydration, suggesting that both bands arise from changes occurring before or at the M_1 intermediate.

The reduction of the hydration level of E194Q films at alkaline pH results in more severe changes (Figure 7B), the most striking being the reappearance of the 1762 cm^{-1} band. The bands at 1738 and 1732 cm^{-1}, corresponding to Asp115, are also seen. As already described, these bands are not resolved in the N-like protein state of any of these mutants, neither in the presence or absence of Cl^- (*34*) (see Figure 2B). In the rest of the spectrum, the presence of the positive band at l662 cm^{-1}, the cancellation of that at 1670 cm^{-1} and the shift of the band at 1553 cm^{-1} to 1560 cm^{-1} in dry samples are also typical of the M_1 state of the wild-type. Therefore, these findings indicate that an M-like structure is adopted in the E194Q mutant upon dehydration.

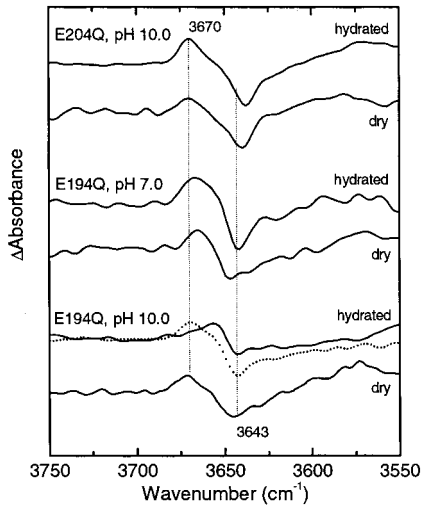

FIGURE 8: O−H stretch region of FTIR difference spectra of hydrated or dry films of E204Q, E194Q, and E194Q/E204Q in 1 M KCl, recorded at 243 K at pH 7.0 and 10.0, as indicated. The dotted line corresponds to the spectrum of a hydrated film of E194Q in 75 mM Na_2SO_4, at 243 K and pH 10.0.

At pH 5.6, similar to alkaline pH, the most significant changes on drying are detected in the carboxylic range. The small broad positive band at 1763 cm^{-1} with a shoulder at 1748 cm^{-1} and minimum at 1741 cm^{-1} undergoes changes to express a clear positive band of protonated Asp85 with a shoulder at 1755 cm^{-1} and a shifted negative band from 1741 to 1732 cm^{-1}. The broad minimum in both hydrated and dry samples is most probably due to the overlapping of two separate negative bands at 1742 and 1734 cm^{-1} (Figure 7A, B) which arises from the accumulation of L and M states at pH 5.6 in both dry and wet samples. These data indicate, in agreement with the accepted view for the wild-type (*49*, *50*), that the water network is required in both mutants for the photocycle to proceed; otherwise it stops in the M_1 state.

Changes of Internal Water Molecules in the M-Like Intermediate. As has been shown by Maeda and co-workers (*44*, *51*), the 3700−3600 cm^{-1} region of the wild-type in the M intermediate comprises two bands representing the O−H stretching of internal water molecules: a positive band at about 3670 cm^{-1}, due to a free O−H bond of water presumably located in the cytoplasmic part, and a negative band at 3643 cm^{-1}, suggested to be located in the vicinity of Asp85. In agreement with previous data (*23*), the difference spectrum of hydrated E204Q at alkaline pH in the M-like intermediate shows that the negative band shifted to 3637 cm^{-1}, while the positive band remained at 3670 cm^{-1} as in WT (Figure 8). In the hydrated samples of E194Q, the positive band shows strong pH dependence, whereas the negative band is unaffected by pH changes. At pH 10, the

positive band at 3670 cm^{-1} shifts to 3654 cm^{-1} representing a N state (*52*−*54*), being consistent with the C═O stretch of amide I bands, which under similar conditions also indicates a N-like protein structure (see Figures 1 and 4). At pH 7, the positive band is at 3666 cm^{-1} and the spectrum is similar to that of the L intermediate of WT, although this band does not seem to be sensitive to isotopic substitution (*55*). In any case, this agrees with the bands in the 1800−1100 cm^{-1} range of E194Q at pH 7, representing a mixture of L and M intermediates (see Figure 1).

Frequency changes in the negative band at 3643 cm^{-1} are observed in dry samples of E194Q and E204Q mutants. This indicates that reducing the hydration level in the films causes changes in the hydrogen-bonded network of both mutants. The shift of the negative band to a higher frequency from 3643 cm^{-1} (in hydrated samples) to 3648 cm^{-1} (dry) in E194Q at both pH values and from 3637 cm^{-1} (in hydrated samples) to 3643 cm^{-1} (dry) in E204Q indicates a decrease in the H-bonding strength of this water molecule in the dry samples.

As in the E194Q hydrated sample, the frequency of the positive band in the spectrum of the E194Q dry sample is pH-dependent. At pH 10, it is found at 3671 cm^{-1}, reflecting an M_1 structure, while at pH 7 it shifts to 3665 cm^{-1}, most probably accounting for a contribution of an L conformation. These data are in complete agreement with the structures revealed by C═O stretch mode of dry samples (see Figure 7). In the presence of SO_4^{2-}, all samples of both mutants show the water bands to be at the same frequencies as in the presence of Cl^- (not shown). The only exception corresponds to hydrated E194Q at alkaline pH, which represents an M-like water configuration (Figure 8, dotted spectrum), consistent with data obtained from amide modes (see Figure 1).

DISCUSSION

As is known, the control of pH, temperature, and hydration of BR films allows for the accumulation of different photocycle intermediates. Until now, most studies in the area have been done with samples in the presence of Cl^- anions. In this work, we have used FTIR to investigate the effects of mutation of the extracellular Glu to Gln, performing the experiments in the presence and in the absence of Cl^-. We have found that this difference can change the evolution of the photocycle of E194Q and E204Q mutants. To summarize, in the presence of Cl^-, at alkaline pH and 277 K, both mutants accumulate a N-like intermediate, as expected, but E194Q has deprotonated Asp85 (Figure 4; see also ref *34*), and E204Q shows a shift of the C═O stretch to 1762 cm^{-1}, characteristic of an M intermediate. On the other hand, in the absence of Cl^-, the N intermediate is greatly disfavored in these mutants at any temperature, so M or M_N states accumulate instead (Figure 2). Along similar lines, under conditions in which O′ accumulation is promoted in the mutants in the presence of Cl^-, an M-like intermediate is accumulated in its absence. In an early study by Kandori et al. (*27*), it was reported that in E204Q (in the absence of Cl^-) the accumulation of a N intermediate is suppressed, which fully agrees with our present results.

In the E194Q mutant, the difference spectra recorded at 243 K and neutral pH either in the presence or in the absence of Cl^- reveal an altered photocycle representing mixtures of L and M intermediates (see Figures 1 and 4B). Therefore, in the first part of the photocycle, which reflects the transfer of the proton from SB to Asp85 during the L−M transition, the equilibrium in this mutant is shifted toward L, explaining the low amplitude of M. The molecular mechanism that explains this perturbation is most probably associated to changes in the pK_a of Asp85 as compared to WT, due to the mutation of Glu194 (*34*). It should be noted that the contribution of the M intermediate to the difference spectrum is higher in the absence of Cl^- than in its presence. In fact, steady-state spectra of the E194Q mutant show that, in any of the conditions explored, even in dry samples, it is not possible to get an accumulation of M as a separate intermediate.

In the presence of Cl^-, we determined the apparent pK_a of Asp85 in the M state in both mutants. For E194Q, this pK_a is 7.6, preventing the accumulation of M at high pH. For E204Q, the pK_a is higher than 10, even though early proton release is blocked as was the case for E194Q. These data clearly indicate that Glu194, but not Glu204, controls the coupling between the pK_a values of Asp85 and of the PRG in the M state. Similar to the E194Q mutant, FTIR difference spectra of multiple extracellular Glu mutants recorded at 243 K in the presence of Cl^- reveal overall features corresponding to low M accumulation and a lack of protonation of Asp85 at alkaline pH (Figure 3; see also ref *34*). Therefore, Glu194 determines the kinetic behavior and properties of the multiple Glu mutants.

The influence of Cl^- on the E194Q and E204Q photocycle kinetics is further confirmed in the second part of the photocycle on comparison of spectra recorded at 277 K (see Figure 2, parts A and B). The spectral features reveal different structures for the protein and for the chromophore, indicating that in the presence of Cl^- larger conformational changes occur. Therefore, the spectra of E194Q and E204Q in the absence of Cl^- mainly represent M, while those recorded in the presence of Cl^- produce a N-like protein structure, although Asp85 is deprotonated in E194Q. At 293 K and in the absence of Cl^-, the protein is in a N-like conformation. However, the position of the Asp85 band at 1764 cm^{-1} and the low intensity of the chromophore band at 1186 cm^{-1} are indicative of deprotonated SB and 13-cis retinal configuration in the M state. Therefore, similar to D96N, the M_G or M_N (*40*, *41*) intermediate accumulates under these conditions. This indicates that the mutation at Glu194 or Glu204, in the absence of Cl^-, greatly hinders not only proton release to the extracellular side, but also subsequent processes taking place on the cytoplasmic side such as the reprotonation of the Schiff base and Asp96. This long-range effect may be produced through the disruption of the hydrogen-bonded water network or by long-range conformational changes that originate at the cation binding site(s) involving Glu194 and Glu204 (*56*).

A striking feature observed in our previous work (*34*) and confirmed this time is that, both in the presence and absence of Cl^-, the 1732 and 1738 cm^{-1} bands in the N-like protein state of both E194Q and E204Q mutants are absent (see Figure 2B). In wild-type, these bands already appear in the L intermediate and remain in M and N with some changes, reflecting environmental changes of Asp115, most probably in its hydrogen bonding. The disappearance of these bands

suggests that the conformational changes taking place during the photocycle in the mutants are different from those in the wild-type. According to the last BR structures (*18*−*20*), the OH group of Asp115 is within hydrogen-bonding distance of Thr90 (about 2.54 Å). Therefore, our data indicate that, in the N-like intermediates of E194Q or E204Q, the Thr90−Asp115 hydrogen bond recovers the characteristics it had in its resting state. The importance of this interaction has recently been demonstrated by showing that mutation of Thr90 to Ala has a profound effect on BR properties (*57*).

Because of the vital importance of the water network (*58*), it was of interest to analyze the effects of low water content on these mutants. In contrast to the FTIR difference spectra of hydrated samples, which show strong differences between E194Q and E204Q, the lower water content (less than 60% humidity, what we call a dry sample) produces similar results for both mutants. The amide I band positions and intensities suggest that the photocycle stops in the M_1 state which is even established in E194Q at pH 10. Therefore, our FTIR data of dry samples of E194Q and E204Q mutants agree with kinetic absorption measurements of dry WT, demonstrating that the photocycle stops in M_1 (*58*). Furthermore, the similarity of the difference spectra of E194Q, E204Q, and WT dry samples, especially in the carboxylic region, suggests that the pK_a increase of Asp85 in M is not perturbed, maintaining this residue protonated.

Another important conclusion of this work is that Cl^- is required to obtain an O′ intermediate in both E194Q and E204Q single mutants at acid pH and in the multiple E194Q/E204Q, 3Glu, and 4Glu mutants at neutral pH. Under the same conditions but in Cl^--free medium, an M-like intermediate is obtained instead of O′. Because the O′ photoproduct does not accumulate either in WT, E9Q, or in E74Q mutants, its appearance is most probably related to the proton release group, of which Glu194 and Glu204 form part (*7*, *15*). Bands in O′ amide I suggest a relaxed protein structure with a perturbed SB environment. Indeed, the fact that Cl^-, but not SO_4^{2-}, promotes O′ indicates that the effect of Cl^- occurs through entry in the interior of the protein and not surface effects. On the other hand, the high pK_a for the appearance of O′ in the multiple Glu mutants is in keeping with the increased facility of the Cl^- entrance in the resting state (*35*). In a previous study, it was established that access of hydroxylamine and Cl^- ions to the 4Glu interior is highly favored. We have also recently hypothesized that Glu194 and Glu204 side chains are deprotonated and act as ligands of a divalent cation (*56*). Therefore, mutation to glutamine decreases the binding affinity of the cation, disorganizing the extracellular region and destroying the natural barrier that the protein offers to small molecules and ions. However, larger ions such as SO_4^{2-} could not be allowed to enter the protein interior of the single mutants and would therefore have no effect on BR behavior, as is the case for the 4Glu mutant (*35*). It is worth noting that Cl^- also has an effect on the spectral characteristics of the resting state of E204Q (*56*). Our results strongly support the general view that the BR photocycle is mainly controlled by electrical charges. Hence, Cl^- acts by somehow substituting the missing negative Glu194 or Glu204 charge; in the absence of one of these charges, the photocycle is unable to move to the N and O intermediates, although the protein can adopt a N-like conformation to yield the M_N or M_G state. It should be pointed out, however, that the presence of the negative Cl^- charge in the mutants is insufficient to lead to the normal photocycle, as indicated before. Therefore, other changes induced by the mutation, such as a decrease in cation affinity, also affect the photocycle.

Another interesting question would be to ask where exactly the Cl^- ions are located. The band at 1631 cm^{-1}, assigned to C═NH stretch, is strongly downshifted, most probably due to the binding of Cl^- near to the Schiff base. For the moment, we can only speculate that, particularly considering the induction of the O′ intermediate in which Asp212 appears to be transiently protonated, a location near this residue is a reasonable assumption. This is consistent with the proposal of a binding site for Cl^- near Asp85, Asp212, and Arg82 in the acid purple state of WT, a form that transports Cl^- (*59*). However, the exact site for Cl^- binding to this WT form has not yet been described (*60*).

According to our results, it is clear that either mutation at the 194 or 204 site produces O′ similarly, in agreement with an earlier study by Dioumaev et al. (*30*), although a recent report indicated that O′ is a photoproduct induced mainly by the replacement of Glu194 (*33*). We would like to point out that, in the case of mutants with slow photocycles with intermediate decays of several seconds such as O′, steady-state spectroscopy is a better option than time-resolved FTIR because it allows for the accumulation of long-living intermediates to high level. On the other hand, the accumulation of O′ only in the presence of Cl^- puts into question the occurrence of an O′ intermediate in WT (*30*, *33*) and therefore the transient protonation of Asp212 in its photocycle. The small band at about 1713 cm^{-1} found at acid pH in WT became unobservable (obscured) in D_2O (*45*), whereas in E9Q/E194Q and in E194Q the band at 1713 cm^{-1} downshifts to 1708 cm^{-1} upon deuterium isotope exchange, being equally visible in both H_2O and D_2O media due to their similar intensities (*30*, *33*). Therefore, the small band detected by Zscherp and Heberle (*45*) in the O intermediate of WT at acid pH might not bear any relation to the 1713 cm^{-1} band of O′, assigned to transient protonation of Asp212.

The results of the present work call for much caution in the extrapolation of the results obtained from mutants, involving charged side groups, to wild-type proteins. It seems evident that, even for single conservative mutations, unexpected changes in the properties of the protein may occur, thus giving rise to potentially misleading conclusions. In the case of BR, it seems reasonable that previous results obtained using mutants of Glu194 or Glu204 need to be reinterpreted taking into account whether halide ions were present in the relevant experiments.

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