Fourier Transform Infrared Evidence for Early Deprotonation of Asp^{85} at Alkaline pH in the Photocycle of Bacteriorhodopsin Mutants Containing E194Q

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ABSTRACT The role of the extracellular Glu side chains of bacteriorhodopsin in the proton transport mechanism has been studied using the single mutants E9Q, E74Q, E194Q, and E204Q; the triple mutant E9Q/E194Q/E204Q; and the quadruple mutant E9Q/E74Q/E194Q/E204Q. Steady-state difference and deconvoluted Fourier transform infrared spectroscopy has been applied to analyze the M- and N-like intermediates in membrane films maintained at a controlled humidity, at 243 and 277 K at alkaline pH. The mutants E9Q and E74Q gave spectra similar to that of wild type, whereas E194Q, E9Q/E194Q/E204Q, and E9Q/E74Q/E194Q/E204Q showed at 277 K a N-like intermediate with a single negative peak at 1742 cm^{-1}, indicating that Asp^{85} and Asp^{96} are deprotonated. Under the same conditions E204Q showed a positive peak at 1762 cm^{-1} and a negative peak at 1742 cm^{-1}, revealing the presence of protonated Asp^{85} (in an M intermediate environment) and deprotonated Asp^{96}. These results indicate that in E194Q-containing mutants, the second increase in the Asp^{85} pK_a is inhibited because of lack of deprotonation of the proton release group. Our data suggest that Glu^{194} is the group that controls the pK_a of Asp^{85}.

INTRODUCTION

In the proton transport mechanism of bacteriorhodopsin (BR), proton release to the extracellular region is driven by protonation of Asp^{85} from the Schiff base and conformational changes in the L-M step (Engelhard et al., 1985; Braiman et al., 1988; Gerwert et al., 1989; Zimányi et al., 1992; Lanyi, 1997). This event induces the deprotonation of the proton release group, presumably through perturbation of the complex hydrogen-bonded network existing in this region (Rammelsberg et al., 1998; Essen et el., 1998). So far, there is no clear evidence for the identity of the proton release group. Because mutation of Glu^{204} or Glu^{194} to a noncarboxylic residue slows down the proton release, which occurs after proton uptake on the cytoplasmic side (Brown et al., 1995; Balashov et al., 1997), both appear to form part of the proton release group network. Recently, it has been suggested that Glu^{194} acts as the proton release group itself (Dioumaev et al., 1998), or that both side chains can function as a dyad, thus acting in a concerted manner (Essen et al., 1998; Kalaidzidis et al., 1998; Balashov et al., 1999). The proton release group could even be made up of several residues and water molecules (Rammelsberg et al., 1998).

Previous works have suggested the existence of a coupling between the apparent pK_a of Asp^{85} and that of an ionizable group X′ (Balashov et al., 1993, 1995, 1996, 1997, 1999; Dioumaev et al., 1998; Richter et al., 1996). Following this model, identity between the proton release group X and the group that controls the Asp^{85} pK_a, X′, has been suggested (Balashov et al., 1996, 1997; Govindjee et al., 1996; Richter et al., 1996). Thus, at neutral pH protonation of Asp^{85} decreases the pK_a of the proton release group below the pH of the medium so that the proton is released. Conversely, deprotonation of the proton release group increases the pK_a of Asp^{85}, forcing it to remain protonated until the end of the photocycle. In the O-BR transition, Asp^{85} deprotonates and the proton release group reprotonates (Ebrey, 1993; Lanyi, 1997; Oesterhelt, 1998). In another view, protonation of Asp^{85} would not be sufficient to promote proton release, so that structural changes have to be involved in the process (Althaus and Stockburger, 1998).

In the extracellular region, apart from Glu^{194} and Glu^{204}, there are two other Glu residues, Glu^{9} and Glu^{74}, which all together form a sort of quadrupole (Grigorieff et al., 1996; Pebay-Peyroula et al., 1997; Essen et al., 1998; Luecke et al., 1999a; Mitsuoka et al., 1999). In fact, Glu^{194} and Glu^{204} are within interacting distance, and Glu^{9} has also been suggested as having some role in proton transport (Pebay-Peyroula et al., 1997). Only Glu^{74}, which is located in the β-sheet linking helices B and C, is somewhat distant from the other three Glu residues. So far, however, because pyranine and M intermediate kinetic data are not affected upon mutation of Glu^{9} or Glu^{74} (Diuomaev et al., 1998; our unpublished data), a direct role for them in proton transport seems to be ruled out.

We have found recently that the simultaneous mutation of these four Glu residues to Gln profoundly alters the BR properties, making the Schiff base moiety highly accessible

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to ions and hydroxylamine (Sanz et al., 1999). One of the functions of the extracellular Glu side chains seems to be the shielding of the Schiff base moiety from the exterior, and they most probably also participate in the maintenance of the water network that extends at least from Asp^{85} to the proton release group.

To gain information on the role of the extracellular Glu residues in the proton release process, in this work we explore the effects of the single mutations E9Q, E74Q, E194Q, and E204Q; the triple mutation E9Q/E194Q/E204Q (3Glu); and the quadruple mutation E9Q/E74Q/E194Q/E204Q (4Glu) on the photocycle intermediates M and N. We use difference and deconvoluted Fourier transform infrared (FTIR) spectra obtained from purple membrane films maintained at controlled humidity and low temperature at alkaline pH. Only for E194Q-containing mutants do we observe an N-like intermediate with unprotonated Asp^{85}. The data suggest that Glu^{194} is the group that controls the pK$_a$ of Asp^{85}.

MATERIALS AND METHODS

The mutants E9Q, E74Q, E194Q, E204Q, 3Glu, and 4Glu were constructed as described previously (Sanz et al., 1999). Purple membranes with mutated bacteriorhodopsin were prepared from *Halobacterium salinarum* by a standard method (Oesterhelt and Stoeckenius, 1974). After expression in *H. salinarum*, the mutants were checked again by sequencing.

For steady-state FTIR spectroscopy we used highly hydrated BR films prepared as follows: purple membrane suspensions, in 10 mM carbonate bicarbonate buffer, 1 M KCl at pH 10.3, were dried on a CaF_2 window under mild vacuum atmosphere. To get the desired relative humidity, the samples were exposed overnight to saturated KCl salts in a closed chamber. The humidity level was checked independently with a hygrometer and by comparing the ratio of band intensities at 3400 cm^{-1} to amide I at 1650 cm^{-1} and to amide II at 1560 cm^{-1}.

FTIR spectra were recorded on a Mattson Polaris spectrometer equipped with a MCT detector at 2 cm^{-1} resolution. Samples were first light adapted at 293 K and then cooled to the desired temperature. For illumination, a 250-W lamp with a yellow filter was used. To get M and N intermediates, spectra were recorded at 233, 243, and 277 K, respectively. Difference spectra were obtained as described previously (Lazarova and Padrós, 1996). In all samples, the recording was performed at least five times, and the results were averaged. Experiments were repeated in at least three independent samples. For comparison purposes, difference spectra are scaled to the same difference absorbance value at 1201 cm^{-1}. Absorption spectra were Fourier self-deconvoluted as described previously (Lazarova and Padrós, 1996).

RESULTS

Difference spectra of the intermediates obtained at 243 K

The FTIR difference spectra of single extracellular Glu mutants, together with wild type, and 3Glu and 4Glu mutants, are shown in Fig. 1, under conditions for trapping wild-type late M intermediate. E9Q and E74Q possess a difference spectrum similar to the wild-type M spectrum. This includes the carboxylic positive peak at 1762 cm^{-1}

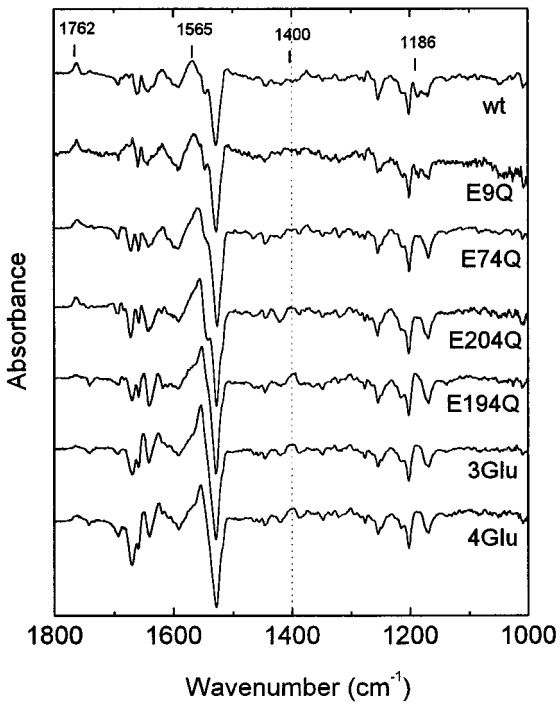

FIGURE 1 Steady-state FTIR difference spectra of hydrated films of wild-type bacteriorhodopsin; single mutants E9Q, E74Q, E204Q, and E194Q; triple mutant E9Q/E194Q/E204Q (3Glu); and the quadruple mutant E9Q/E74Q/E194Q/E204Q (4Glu). The spectra were recorded under continuous illumination with yellow light at 243 K and pH 10.3. For comparison purposes, all spectra are scaled to the same intensity of the 1201 cm^{-1} band.

corresponding to protonated Asp^{85}, the amide I negative peak at 1657 cm^{-1}, corresponding to the protein, and the 1186 cm^{-1} peak below the baseline (Pfefferlé et al., 1991; Maeda et al., 1992; Sasaki et al., 1992). It should be noted that in E74Q this latter band is slightly more positive than in E9Q or in wild type.

Similar to the spectra described at neutral pH (Brown et al., 1995; Rammelsberg et al., 1998), the overall spectrum of E204Q at alkaline pH is analogous to the wild-type M difference spectrum. However, there are some features at variance with respect to wild type or the mutants E9Q and E74Q. These include the positive absorbance around 1700 cm^{-1} found in previous published spectra of the E204Q mutant (Brown et al., 1995; Rammelsberg et al., 1998), a less prominent negative band at 1657 cm^{-1} as compared with the band at 1670 cm^{-1} (Fig. 2), as well as a more positive peak at 1186 cm^{-1}, as in E74Q.

Contrary to the other mutants or the wild type, E194Q, 3Glu, and 4Glu show a mixture of M- and N-like intermediates with a preponderance of N form, as indicated by the small and broad positive band at ~1764 cm^{-1} (corresponding to protonated Asp^{85}) and a strong negative peak at 1742 cm^{-1} (corresponding to deprotonated Asp^{96}) (Fig. 2). Additionally, the broad band at ~1400 cm^{-1}, which is mainly assigned to coupled vibration of C_{15}-H and N-H bending of

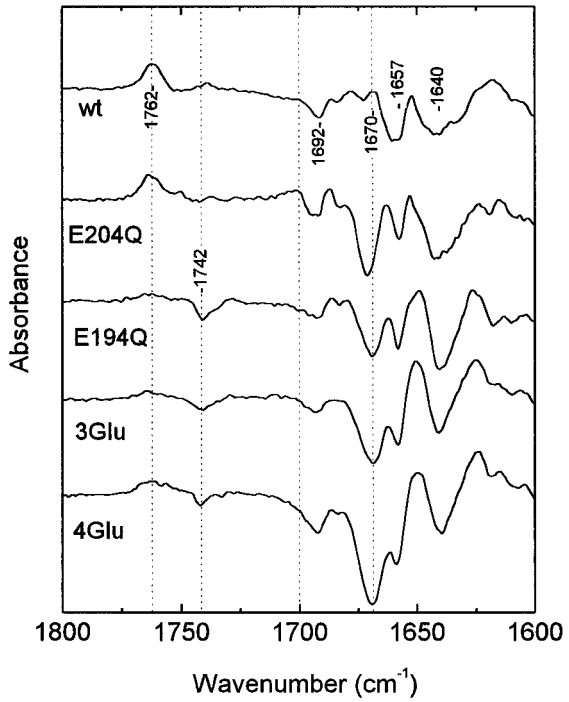

FIGURE 2 Expanded view of selected spectra from Fig. 1 in the 1800–1600 cm^{-1} region.

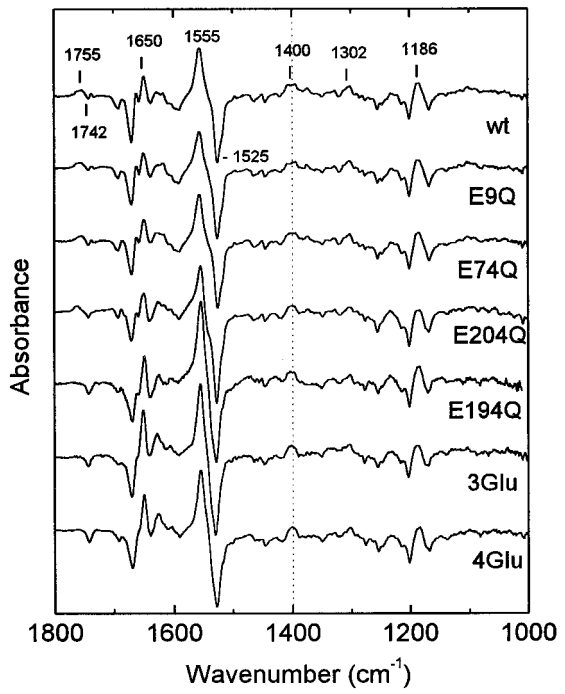

FIGURE 3 Steady-state FTIR difference spectra of hydrated films of wild-type and the same mutants of Fig. 1 at 277 K and pH 10.3. For comparison purposes, all spectra are scaled to the same intensity of the 1201 cm^{-1} band.

13-*cis* chromophore (Pfefferlé et al., 1991) and to -COO^- of Asp^{96} (Maeda et al., 1992), appears only in E194Q, 3Glu, and 4Glu and is compatible with deprotonation of Asp^{96}. From the rest of the spectrum, it is also apparent that mainly a N-like form is obtained, mixed with a small accumulation of M-like form. This is indicated by the characteristic minimum at 1670 cm^{-1}, the maximum at 1650 cm^{-1}, and the positive peak at ~1186 cm^{-1}, among others.

To analyze this mixture to see if it reverts to more pure M for the E194Q mutant, we obtained spectra at 233 K for this mutant and for the E204Q mutant, and for the wild type as a reference. The spectra of the E204Q mutant and that of wild type were very similar to those taken at 243 K, representing mainly an M-like state. However, the spectrum of E194Q, although also similar to that taken at 243 K, showed some features corresponding to the L-like intermediate: decrease of the negative peak at 1670 cm^{-1} and appearance of a negative peak at 1740 and a positive one at 1748 cm^{-1} (not shown). Thus the E194Q mutant, under these conditions, displays a mixture of L, M, and N intermediates, indicating that low accumulation of the M intermediate occurs and that the equilibria between them are shifted away from M.

Difference spectra for the N-like intermediate obtained at 277 K

Fig. 3 shows the difference spectra of the same samples shown in Fig. 1, under conditions in which the wild-type N intermediate is normally obtained (pH 10, 277 K). E9Q and E74Q present difference spectra very close to the wild-type N intermediate. This includes positive peaks at 1755, 1738, 1650, 1556, 1400, 1302, and 1186 cm^{-1} and negative peaks at 1742, 1670, 1525, and 1201 cm^{-1} (Pfefferlé et al., 1991).

The spectrum of E204Q in the amide and the fingerprinting regions is very similar to the wild-type N spectrum, although a less positive band at 1186 cm^{-1} is seen. In the 1800–1700 cm^{-1} region, both a negative peak at 1742 cm^{-1} and a positive peak at 1764 cm^{-1} are observed (Fig. 4). This latter value for protonated Asp^{85} appears to resemble that corresponding to M, although it is shifted to higher wavenumbers, indicating a more hydrophobic environment. It is notable that, as in E194Q, 3Glu, and 4Glu, the small bands at 1738 and 1732 cm^{-1} are not seen.

The mutants E194Q, 3Glu, and 4Glu show several variations as compared to wild type, E9Q and E74Q, or even to E204Q. As Fig. 4 shows, above 1700 cm^{-1} only a negative band at 1742 cm^{-1} appears, indicating that both Asp^{85} and Asp^{96} are deprotonated. In the amide I, the negative peak at 1692 cm^{-1} and the small feature at ~1686 cm^{-1}, attributed to reverse turn changes (Lazarova and Padrós, 1996), show decreased intensity in the three mutants containing E194Q. In 3Glu and 4Glu but not in E194Q, the band at 1662 cm^{-1}, representing α-helical changes, is almost absent.

In the amide II, the bands located at 1555 (+) and 1525 cm^{-1} (−) in wild type, E9Q, and E74Q, show changes in

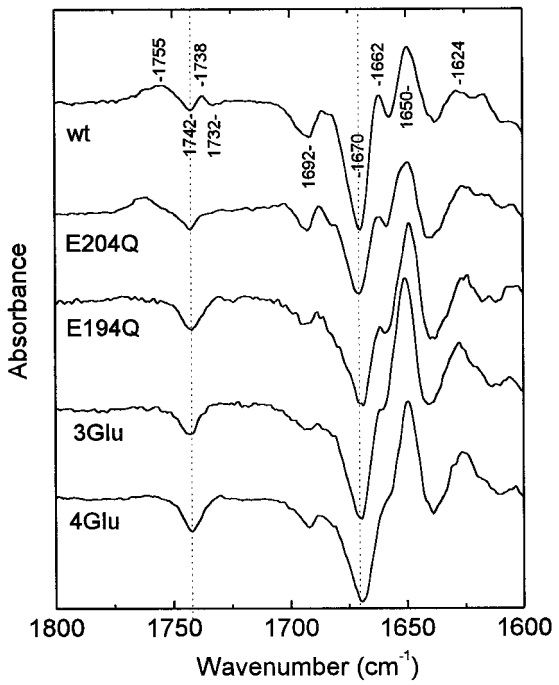

FIGURE 4 Expanded view of selected spectra from Fig. 3 in the 1800–1600 cm^{-1} region.

E194Q, 3Glu, and 4Glu (Fig. 3). In these mutants, the positive peak shifts slightly to around 1553 cm^{-1}, and the negative peak to around 1528 cm^{-1}. On the other hand, the shoulder appearing at 1538 cm^{-1} in wild type is not detected in the difference spectra.

Deconvoluted spectra of the N-like intermediate

As has been demonstrated previously for wild-type BR, comparison of a difference spectrum with deconvoluted absorbance spectra helps significantly with interpretation of the infrared bands (Lazarova and Padrós, 1996). In the following, we present some deconvoluted spectra of unphotolyzed and photolyzed Glu mutants in an N-like state, along with corresponding IR difference spectra. For this purpose we will use the spectra of E9Q, as representative of E74Q, E204Q, and wild type, and the spectra of 4Glu as representative of 3Glu and E194Q.

Fig. 5 *A* shows deconvoluted and difference spectra of E9Q in the 1720–1800 cm^{-1} region for N intermediate. As has already been indicated for wild type (Lazarova and Padrós, 1996), the positive band at 1755 cm^{-1} in the difference spectrum of E9Q originates from the increase in absorbance seen in the 1750–1765 cm^{-1} region on moving from the resting state to the N state. A decrease in the band intensities at 1734 and 1741 cm^{-1} in the deconvoluted spectra gives rise to the negative bands at 1732 and 1742 cm^{-1}. Similar to that of wild-type protein, the positive band at 1738 cm^{-1} arises from the simultaneous changes of these two contiguous bands. In contrast, comparison of the deconvoluted spectra of photolyzed and unphotolyzed 4Glu in

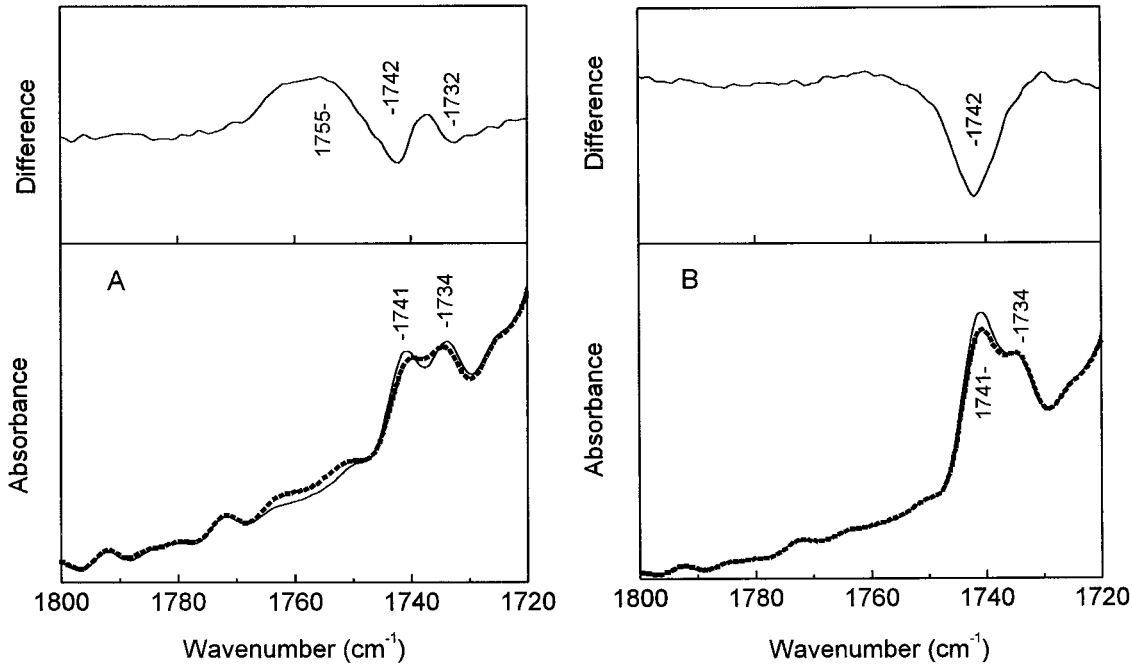

FIGURE 5 FTIR spectra of hydrated films at 277 K, pH 10.3 in the 1800–1720 cm^{-1} region. (*A*) Spectra of the mutant E9Q. *Bottom*: Deconvoluted spectra of the unphotolyzed (——) and the N state (·····). *Top*: Difference spectrum N/BR. (*B*) Spectra of the mutant 4Glu. *Bottom*: Deconvoluted spectra of the unphotolyzed (——) and the N-like (·····) state. *Top*: Difference spectrum N/BR. The deconvolution parameters used were FWHH = 8 cm^{-1} and $k = 1.5$.

this region shows that only the band resolved at 1741 cm^{-1} undergoes changes (Fig. 5 *B*). No increase in the 1750–1765 cm^{-1} region or changes in the 1734 cm^{-1} band are observed. Thus, in the N-like intermediate of the 4Glu mutant, Asp^{96} is the only carboxylic residue that shows changes. Similar results were obtained for E194Q and 3Glu (not shown).

In the amide I deconvoluted region, the changes observed for the E9Q mutant after photolysis are similar to those of the wild type, as follows (Fig. 6 *A*): the 1666 cm^{-1} band (corresponding mainly to α_{II} helix), shifts to lower wavenumbers; the 1658 cm^{-1} band (corresponding mainly to α_{I} helix), shifts to higher wavenumbers; and more complex changes occur in the 1652 cm^{-1} band (Lazarova and Padrós, 1996). As compared to wild type, the changes observed for the 4Glu mutant are both less intense and somewhat different in other respects also (Fig. 6 *B*). This is seen especially in the case of the principal band at 1659 cm^{-1}, which does not shift after illumination, thus explaining the disappearance of the peak at 1660 cm^{-1} in the difference spectra.

In the amide II (Fig. 7 *A*), the main change observed in the deconvoluted spectra of E9Q corresponds to the 1527 cm^{-1} band, due to depletion of the C═C ethylenic stretching mode of all-*trans* retinal upon illumination, as in wild-type BR (Lazarova and Padrós, 1996). The appearance of the positive band at 1555 cm^{-1} in the difference spectrum is due, as in wild type, mainly to the shift of the band at 1558 cm^{-1}, attributed principally to the protein moiety. The shoulder at ~1517 cm^{-1} in the difference spectrum arises from small changes in the peak at 1517 cm^{-1}, corresponding to Tyr side chains.

In the 4Glu deconvoluted spectra (Fig. 7 *B*), the bands at 1558 and 1549 cm^{-1} behave differently: the 1558 cm^{-1} band suffers only slight changes, whereas the 1549 cm^{-1} band shifts to higher wavenumbers, thus explaining the shift of the peak in the difference spectrum from 1555 to 1553 cm^{-1}. Unlike E9Q or wild type, where the band at 1534 cm^{-1} increases upon illumination, in 4Glu it decreases slightly. Thus the shoulder at ~1535 cm^{-1} is not detected in the difference spectrum. This suggests that retinal isomerization is perturbed in the 4Glu mutant.

DISCUSSION

In this work we show that mutation of Glu^{194} to Gln has a major influence on the photocycle and the protonation state of Asp^{85}. Mutation produced in the Glu^{204} side chain does not present such strong effects, whereas mutation in Glu^{9} or Glu^{74} has only minor consequences. In single or multiple E194Q mutants, the spectra show that accumulation of the M-like intermediate is low, indicating that the equilibria L ↔ M and M ↔ N are shifted away from M. However, the N-like state shows deprotonated Asp^{96}, which is a normal feature, but also deprotonated Asp^{85}. Thus, compared to

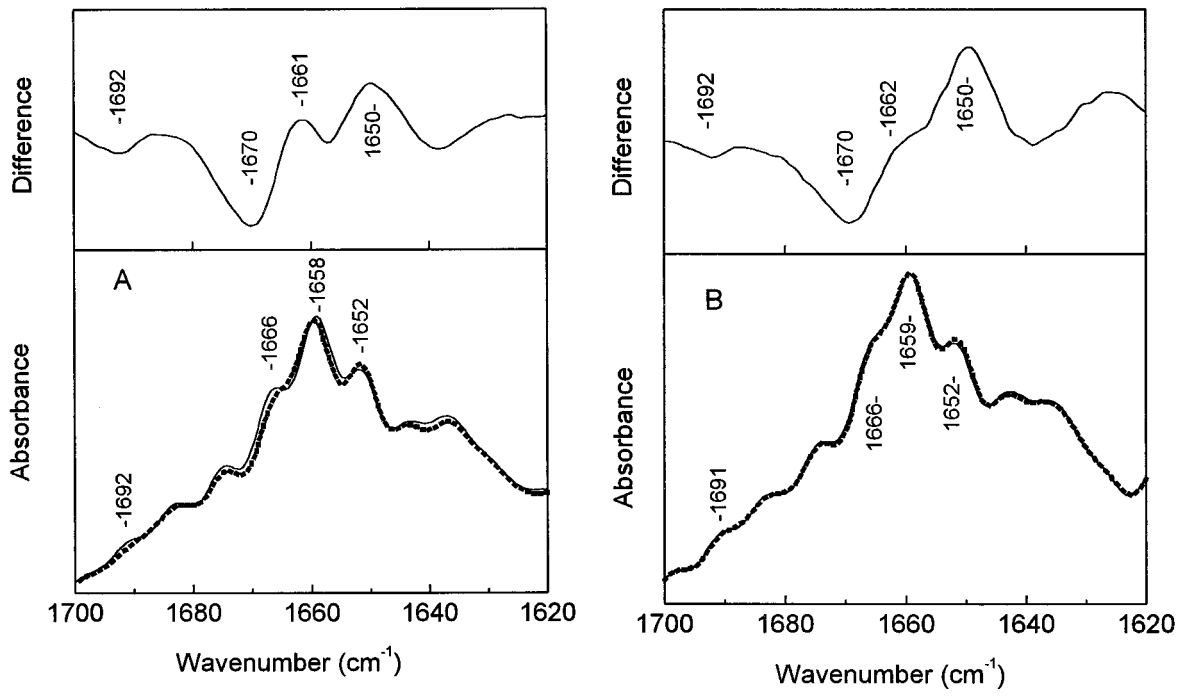

FIGURE 6 FTIR spectra of hydrated films at 277 K, pH 10.3, in the amide I region. (*A*) Spectra of the mutant E9Q. *Bottom*: Deconvoluted spectra of the unphotolyzed (——) and the N state (·····). *Top*: Difference spectrum N/BR. (*B*) Spectra of the mutant 4Glu. *Bottom*: Deconvoluted spectra of the unphotolyzed (——) and the N-like (·····) state. *Top*: Difference spectrum N/BR. The deconvolution parameters used were FWHH = 14 cm^{-1} and $k = 2.5$.

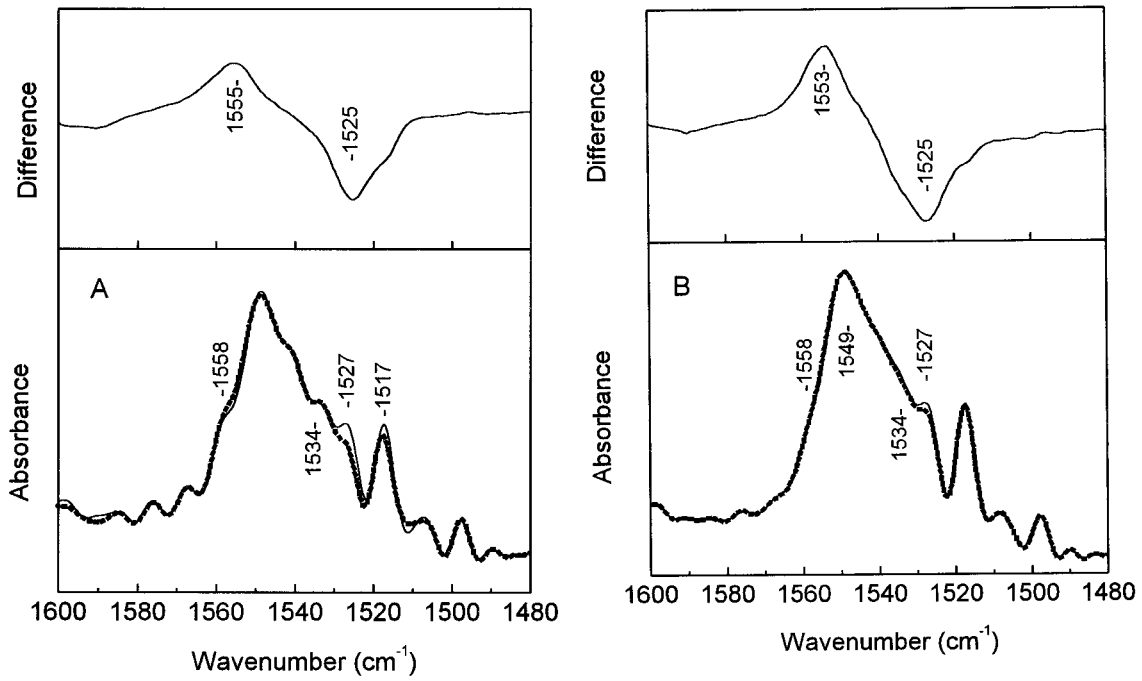

FIGURE 7 FTIR spectra of hydrated films at 277 K, pH 10.3, in the amide II region. (*A*) Spectra of the mutant E9Q. *Bottom*: Deconvoluted spectra of the unphotolyzed (——) and the N state (·····). *Top*: Difference spectrum N/BR. (*B*) Spectra of the mutant 4Glu. *Bottom*: Deconvoluted spectra of the unphotolyzed (——) and the N-like (·····) state. *Top*: Difference spectrum N/BR. The deconvolution parameters used were FWHH = 14 cm^{-1} and $k = 2.5$.

wild type, the main differences observed for E194Q-containing mutants are as follows: 1) accumulation of the M-like intermediate is low, and thus a high percentage of the photoproducts obtained at 243 K correspond to N-like intermediate; 2) a pH-dependent early deprotonation of Asp[85] is observed: at pH 10, a small positive peak at 1764 cm^{-1} is detected at 243 K, and no peak is seen at 277 K, but it is observed at neutral pH (Dioumaev et al., 1998; Zscherp et al., 1999; our unpublished experiments). In contrast, E204Q shows a wild-type-like spectrum at 243 K, and at 277 K has protonated Asp[85] still in the M-like environment (peak at 1764 cm^{-1}) and deprotonated Asp[96]. In the BR three-dimensional structure, Glu[194] and Glu[204] are located in close proximity (Grigorieff et al., 1996; Pebay-Peyroula et al., 1997; Essen et al., 1998; Luecke et al., 1999a; Mitsuoka et al., 1999). Despite this fact and the fact that both disable the fast proton release at neutral pH when mutated (Brown et al., 1995; Balashov et al., 1997; Dioumaev et al., 1998), at alkaline pH the effects of their mutation on the protonation state of Asp[85] and on other features of the infrared spectra are clearly distinct.

A striking feature is that no indication of bands at 1732 and 1738 cm^{-1} is apparent in the difference spectra of these mutants at 277 K. At 243 K, only E204Q shows these bands. This absence is paralleled by the absence of changes of the band resolved at 1734 cm^{-1} in the deconvoluted spectra (Fig. 5 *B*). In wild type, these bands appear already in the L intermediate difference spectrum and have been assigned to perturbation of Asp[115] (Braiman et al., 1988; Pfefferlé et al., 1991), probably because of direct interaction with the retinal (Hage et al., 1996). Therefore, our data indicate that mutation of either Glu[194] or Glu[204] changes this interaction, at least at the level of the N-like intermediate at alkaline pH. Another possibility could be that in these mutants, the changes in the configuration of the retinal during the photocycle are different from those of the wild type, so that Asp[115] is not perturbed.

Several observations indicate that the mutations induce different behaviors of the protein moiety at the level of the N-like intermediates, as compared to wild type. Particularly in E194Q, 3Glu, and 4Glu, we detect only small changes in the band at 1692 cm^{-1} of the deconvoluted spectra, attributed to reverse turns, giving rise to a small negative peak in the difference spectra. The practical absence of changes in the band at 1659 cm^{-1}, corresponding to α_I and α_{II} helices, for 3Glu and 4Glu (but not for E194Q), argues that these structures undergo lesser conformational changes than wild type in moving from the unphotolyzed to the N state. This behavior could be due to a more open and less compact structure of the extracellular region in the resting state of these multiple mutants. This agrees with the fast hydroxylamine bleaching in the dark and easy access of salts to the Schiff base moiety of the 4Glu mutant (Sanz et al., 1999).

As indicated before, one of the major consequences of the mutation introduced in E194Q is the presence of a deprotonated Asp[85] in N-like intermediate at pH 10. The presence

of deprotonated Asp^{85} is in strong contrast to the wild type, where Asp^{85} remains protonated until the end of the photocycle (Zscherp and Heberle, 1997). Thus, the E194Q mutation favors the equilibrium D85H $\leftrightarrow$ $D85^-$ toward deprotonated Asp^{85}. In the wild-type protein, a coupling between the pK_a values of Asp^{85} and the proton release group has been proposed (Balashov et al., 1996; Richter et al., 1996). Briefly, a first increase of the Asp^{85} pK_a to a value similar to that of the Schiff base occurs in the L-M transition because of retinal isomerization and protein changes, giving rise to protonation of Asp^{85} from the Schiff base (Braiman et al., 1996; Brown and Lanyi, 1996). This decreases the pK_a of the proton release group (from $\sim$10 to 5.8 in wild type), so that at neutral pH it delivers a proton to the extracellular space (Balashov et al., 1996). In turn, deprotonation of the proton release group leads to a second increase in the pK_a of Asp^{85} to $\sim$10 and it remains protonated until the O-BR transition, when Asp^{85} deprotonates and the proton release group reprotonates.

Generally speaking, the use of mutants for drawing conclusions about the wild type implies the comparison of two different proteins, and thus great care should be taken. However, E194Q or E204Q mutations used in this work are highly conservative, and thus nonspecific conformational changes in the BR ground state that could affect the Asp^{85} site are unlikely. In this respect, recent crystallographic work on the D96N mutant, which corresponds to a replacement homologous to that used in this work, shows that virtually no differences between the light-adapted forms of the D96N mutant and the wild-type protein are detected, except at the location of the residue change (Luecke et al., 1999b). Thus we are confident that replacement of Glu^{194} or Glu^{204} with a glutamine is minimally disruptive of the structure at only the residue site. In this framework, we assume that there is no significant alteration in the first pK_a increase of Asp^{85} in any of the Glu mutants. However, our data indicate that in E194Q-containing mutants, there is no second increase in Asp^{85} pK_a because in N-like intermediate it is deprotonated. We infer that in E194Q mutants the pK_a of Asp^{85} cannot be raised because now Gln^{194} cannot deprotonate, and the Asp^{85} pK_a rises only once from $\sim$3 to 8.5. At pH 10, Asp^{85} should deprotonate, and this is indeed what we observe (Figs. 2 and 4). The proton is probably delivered to the hydrogen-bonded water network, which has the ability to bind a proton (Rüdiger et al., 1997).

If, as is the case at neutral pH, the proton is released to the bulk at the end of the photocycle, it will in all likelihood remain delocalized within the water network, because no new peaks are observed in the 1770–1700 cm^{-1} region. However, it is possible that at alkaline pH there is rapid proton release before proton uptake, as in wild type at neutral pH (see below). This agrees with the positive light-induced transient currents reported for the E194Q mutant above pH 8.5, with amplitude comparable to that of wild type (Koyama et al., 1998). These positive currents were interpreted as proton release before proton uptake. At pH values below 8.5 the equilibrium will be displaced toward protonated Asp^{85}, in keeping with the infrared data (Dioumaev et al., 1998; Zscherp et al., 1999; our unpublished data), and the proton of Asp^{85} is released at the end of the photocycle (Brown et al., 1995; Balashov et al., 1997; Dioumaev et al., 1998; Koyama et al., 1998). Another possibility could be that early deprotonation of Asp^{85} is due to proton transfer back to the Schiff base. However, this behavior would also be in clear contrast to that of wild type. In any case, this will lead to the same conclusion: inhibition of the second Asp^{85} pK_a increase in the E194Q-containing mutants.

Interpretation of the behavior of the E204Q mutant is more complex, and a suggestion of a clear role for this residue does not seem possible at present. According to our data, in this mutant Asp^{85} has a pK_a of $\sim$10 in the M- and N-like intermediates, as in wild type (Pfefferlé et al., 1991). Taking into account that in E204Q, Asp^{85} has a pK_a in the resting state similar to that of wild type (Richter et al., 1996; our unpublished experiments), it has to be increased first to 8.5 and finally at least to $\sim$10. Thus, it is likely that in this mutant, Asp^{85} is sensitive to the deprotonation of a group during the L-M transition, as in wild type. Although the infrared data do not provide any signal for this deprotonation, we believe that it corresponds to deprotonation of Glu^{194}.

We note that there is disagreement between the Asp^{85} high pK_a in M and N of the E204Q, as indicated by the infrared data, and the absence of the second (high pH) transition in the titration of Asp^{85} in this mutant. This alkaline transition, believed to be due to deprotonation of the X′H group, is monitored either through the purple-to-blue transition or through the pH dependence of the rate of dark adaptation (Richter et al., 1996; Balashov et al., 1999). A possible explanation might be that the Glu^{194} pK_a is so elevated in the unphotolyzed E204Q mutant that its deprotonation cannot be detected because of the overlap with the purple-red transition. Another possibility is that the mechanism responsible for Asp^{85} perturbation is more complex than believed, involving other ionizable side chains different from the proton release group, or water molecules present in the extracellular region (Grigorieff et al., 1996; Essen et al., 1998; Luecke et al., 1999a).

As a conclusion, our data indicate that Glu^{194} is the group that controls the Asp^{85} pK_a and, although they do not demonstrate this, are consistent with a role for Glu^{194} as the proton release group. A second possibility is that the proton release group is a polarized water molecule or a hydroxonium ion linked to Glu^{194}, as suggested before (Braiman et al., 1988; Mitsuoka et al. 1999). In both cases, mutation of Glu^{194} to a Gln will eliminate, at neutral pH, the possibility of releasing a proton or a hydroxonium ion. At alkaline pH, the difference between the bulk pH and the Asp^{85} pK_a may

induce rapid proton release directly from Asp[85], before proton uptake.

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