# Opening the Schiff base moiety of bacteriorhodopsin by mutation of the four extracellular Glu side chains

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**Abstract The quadruple bacteriorhodopsin (BR) mutant E9Q+E74Q+E194Q+E204Q shows a $\lambda_{max}$ of about 500 nm in water at neutral pH and a great influence of pH and salts on the visible absorption spectrum. Accessibility to the Schiff base is strongly increased, as detected by the rapid bleaching effect of hydroxylamine in the dark as well as in light. Both the proton release kinetics and the photocycle are altered, as indicated by a delayed proton release after proton uptake and changed M kinetics. Moreover, affinity of the color-controlling cation(s) is found to be decreased. We suggest that the four Glu side chains are essential elements of the extracellular structure of BR.**
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*Key words:* Bacteriorhodopsin; Extracellular structure; Hydroxylamine accessibility; E9Q+E74Q+E194Q+E204Q mutant

## 1. Introduction

The purple membrane from *Halobacterium salinarium* is a specialized part of the cellular membrane containing a single transmembrane protein, bacteriorhodopsin (BR). It binds a retinal molecule through a protonated Schiff base and translocates protons to the extracellular space under the influence of light [1] (for reviews see [2–5]). In the resting state of the protein, the complex counter-ion of the protonated Schiff base is formed essentially by the negatively charged Asp-85 and Asp-212 and possibly by the positively charged Arg-82, among others [6–10]. After light absorption and chromophore isomerization, the Schiff base proton moves to Asp-85 in the L to M transition. Protonation of Asp-85 induces the release of a proton into the extracellular medium by another group XH (the so-called proton release group) by decreasing transiently its p$K_a$. It is generally accepted that the two p$K_a$s are strongly coupled [11], so deprotonation of the proton release group in turn rises the Asp-85 p$K_a$. In this way, Asp-85 remains protonated until the end of the photocycle, when it finally transfers its proton to the proton release group in the O-BR transition [11–14]. Thus, it appears that the structure of the extracellular region provides a complex relationship between key side chains involved in the proton release mechanism.

A number of studies performed on mutated BR suggest that Glu-194 and Glu-204, within a hydrogen-bonded network, form part of the proton release pathway [15–23]. However, the source of the released proton in the extracellular space, i.e. the identity of the proton release group, is not yet clear. By now, there is no FTIR evidence for deprotonations of side chains other than Asp-96 in the L-M transition, when the proton is released into the extracellular medium [20,22]. Recently published three-dimensional structures of BR place Glu-194 and Glu-204 at interacting distances [6–10], so that they could act as a proton donor/acceptor pair [19] or as a dyad [9], as has been suggested. Glu-9 is also placed nearby them, whereas Glu-74, which is located in the short β-sheet of the BC loop, has a more external location. The extracellular Glu could provide negative charges if they are deprotonated in the resting state at neutral pH and/or they can participate in the hydrogen-bonded network [18–20]. In order to investigate the role of these four extracellular glutamic acids, we designed the mutant E9Q+E74Q+E194Q+E204Q (4Glu mutant) and studied its properties by a number of different and complementary methods.

## 2. Materials and methods

### 2.1. Site-directed mutagenesis

The 4Glu mutant was obtained as follows: the gene encoding BR, *bop* gene, subcloned in *pUC*119 as a 1.2 kb *Bam*HI/*Hin*dIII fragment (a gift from Dr R. Needlemann), was used as a template for the mutagenesis. The individual mutants were obtained by PCR site-directed mutagenesis [24] and amplified in the *Escherichia coli* TG1 strain. Screening of the mutants was performed by DNA sequencing. The 4Glu mutant was constructed by cloning the single mutants together, taking advantage of unique restriction sites. The 4Glu mutant was transformed and expressed in the *H. salinarium* L33 strain with help of the shuttle plasmid *pXL*NovR (kindly provided by Dr R. Needlemann). The membrane was grown and purified by standard methods [25]. The mutations were confirmed from *H. salinarium* transformants by sequencing the *bop* gene from isolated DNA.

### 2.2. Sample preparation

Membrane suspensions (15 μM BR) were reacted with hydroxyl-

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*Abbreviations:* BR, bacteriorhodopsin; 4Glu, the bacteriorhodopsin mutant E9Q+E74Q+E194Q+E204Q

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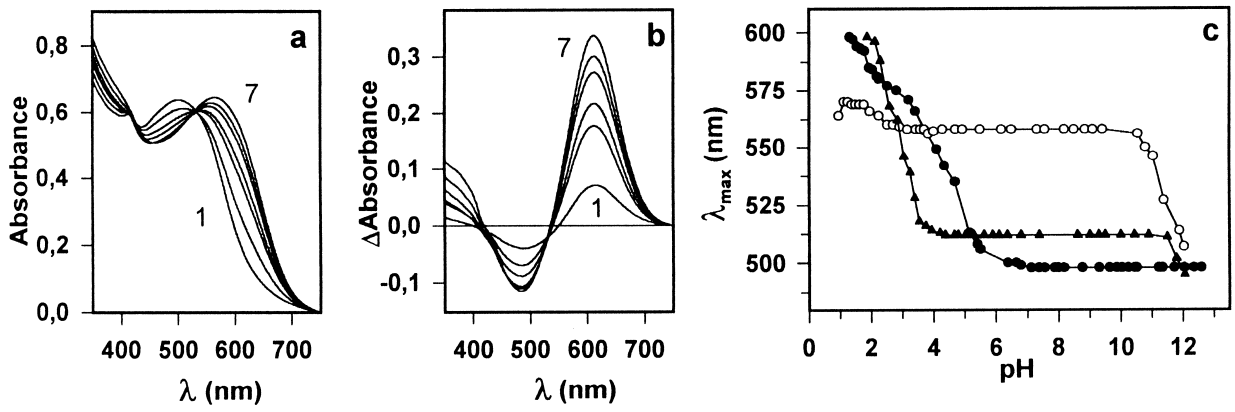


Fig. 1. pH-dependence of the dark-adapted 4Glu mutant absorbance spectrum in the presence of different salts. (a) Absorbance spectra obtained at pH values from 6.2 to 3.1 (spectra 1–7), in Good buffer: 0.6 mM each of Tricine, MOPS, CHES, MES and citric acid. (b) Difference spectra obtained as the spectrum at each pH minus that at pH 6.2. (c) Plot of the absorbance maximum in the presence of 0.6 mM Good buffer (●), 1 M KCl (○) and 1 M $Na_2SO_4$ (▲). The protein concentration was between 10 and 20 μM.

amine in a medium containing sodium phosphate 150 mM, hydroxylamine 1 M, pH 7.0. Reactions under light were done using white light with a luminance of 300 lux. Deionized membranes were prepared by dialysis for 6 h against Dowex 50 W cation exchange resin.

*2.3. Flash-induced transient absorbance changes*

Deprotonation of the Schiff base was monitored by absorbance changes at 410 nm as a function of time. Transient pH changes in the bulk medium were followed by measuring the absorbance changes of 50 μM pyranine at 460 nm [26]. To obtain the net absorbance changes of pyranine, the traces of samples in the absence of the dye were subtracted from those in its presence. The negative signal of $\Delta\Delta A$ indicates the release of protons by BR (pyranine protonation), whereas the positive signal indicates BR proton uptake. Flash-induced transient absorbance changes were monitored using a LKS50 instrument from Applied Photophysics. A Q-switched Nd:YAG laser (Spectron Laser Systems, UK; pulse width ca. 9 ns, $E = 10$ mJ/pulse/cm$^2$, repetition frequency 0.5 Hz) at 532 nm was used for laser flash excitation.

## 3. Results

*3.1. Dependence of the visible absorption spectrum on pH and salts*

The first sign for altered properties of the mutant came from the washing steps of the purification process, where the color of the membrane suspension changed gradually from purple to reddish. As Fig. 1 shows, the absorbance spectrum of the 4Glu mutant is broad, with a $\lambda_{max}$ at about 500 nm in water or at low salt concentration at neutral pH. A decrease of pH produces a red shift of the $\lambda_{max}$ to 595 nm due to the formation of the blue form. The plot of $\Delta A$ at 612 nm as a function of pH in 0.6 mM buffer (not shown) or in water reveals that the p$K_a$ of Asp-85 is 4.8 (Fig. 2a), about 1.6 pH units above that of the wild-type and 0.6 pH units below that of deionized wild-type. Upon alkalinization, $\lambda_{max}$ keeps constant until about pH 12, indicating that the Schiff base has a p$K_a$ similar to or only slightly lower than that of the wild-type.

1 M KCl shifts the $\lambda_{max}$ of the dark-adapted mutant to about 558 nm in a wide pH range (Fig. 1c), a value similar to the $\lambda_{max}$ of dark-adapted wild-type. In this salt, the blue form is not attained at acid pH, because $Cl^-$ ions shift the absorbance spectrum of the blue chromophore to a lower wavelength. This leads to the formation of the acidic purple form, like in wild-type [27]. 1 M $Na_2SO_4$, however, does not cause significant changes in $\lambda_{max}$ of 4Glu as compared to that in water or low salt concentrations (Fig. 1c). In the neutral pH region, $\lambda_{max}$ is about 512 nm and undergoes a red shift below pH 3.5, reaching the blue color at pH 2. The different effect of these salts could be due to the different sizes of the respective anions [27], leading to an effective penetration of the $Cl^-$ anion into the extracellular space and compensating for the absence of negative charges in the mutant. This strong dependence of the absorbance spectrum on the properties of the medium indicates easy access of ions to the Schiff base environment.

As compared to the 4Glu mutant in water, the deionized form exhibits a similar p$K_a$ for Asp-85 (Fig. 2a). This suggests that cation binding is affected in this mutant. Fig. 2b presents the blue to purple transition induced by $Ca^{2+}$ binding to the deionized membranes. The transition appears to be strongly shifted to higher cation concentrations as compared with the wild-type, indicating a low affinity of $Ca^{2+}$ for the color-controlling cation binding site(s).

*3.2. Hydroxylamine bleaching and light-dark adaptation*

Additional evidence for easy accessibility to the Schiff base

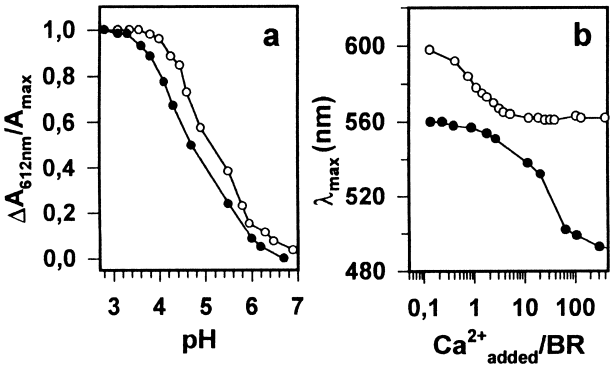


Fig. 2. (a) Dependence of the $\Delta A$ at 612 nm as a function of pH for 4Glu in water (●) and deionized 4Glu (○). (b) Dependence of the absorbance maximum on added $Ca^{2+}$ to deionized wild-type (○) and 4Glu mutant (●) samples. Protein concentration 40 μM, pH 4.8.

comes from the action of hydroxylamine, which is markedly increased in the 4Glu mutant as compared to the wild-type. Addition of $NH_2OH$ immediately shifts the $\lambda_{max}$ to 558 nm (Fig. 3a), as was seen for 1 M KCl. Complete bleaching occurs after 8 h in the dark, whereas under our conditions of illumination (with low-intensity light), bleaching takes only about 3 min, as compared with about 12 h for wild-type (Fig. 3b). These data and specially the high accessibility of hydroxylamine in the dark argue that mutation of the four

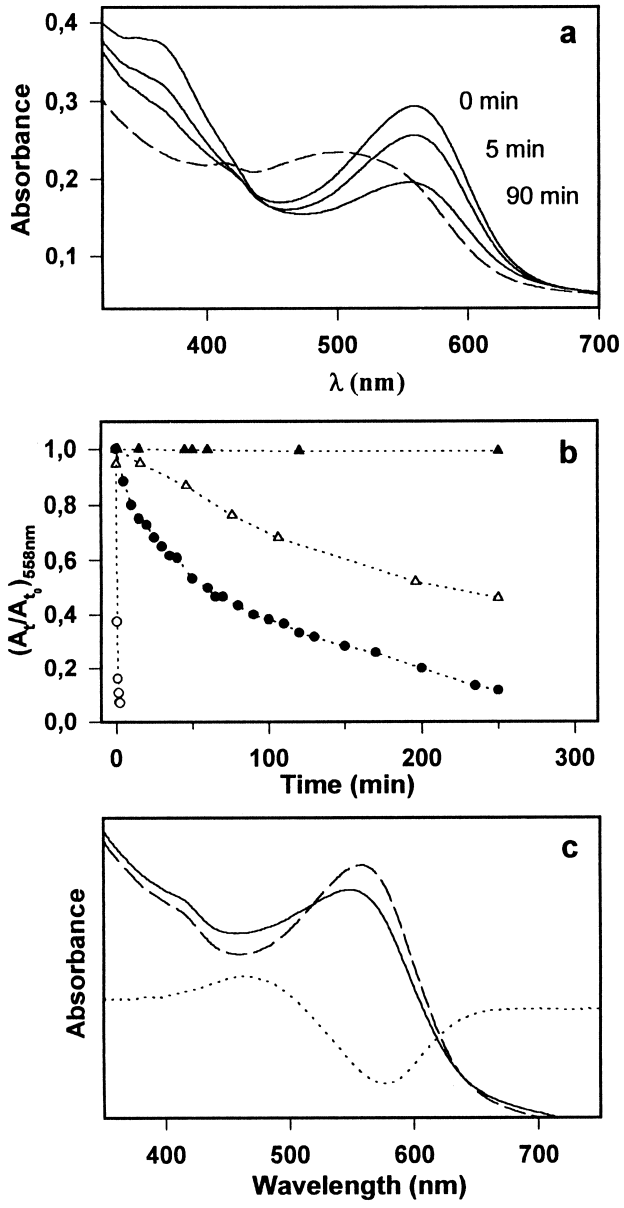


Fig. 3. Hydroxylamine reaction and light-dark adaptation. (a) Absorbance spectra of dark-adapted 4Glu mutant in 150 mM sodium phosphate buffer pH 7 (- - -) and after adding 1 M hydroxylamine (—). (b) Comparison of the rate of hydroxylamine reaction in wild-type BR (Δ, ▲) and the 4Glu mutant (○, ●) as measured by the absorbance change at 559 nm. The reactions were carried out in the dark (filled symbols) or in light (empty symbols). For a light reaction, the samples were illuminated with a 100 W fiber illuminator delivering 300 lux at the sample position. (c) Absorbance spectra of dark-adapted (- - -) and light-adapted (—) 4Glu mutant samples and the difference spectrum (·····) in the presence of 1 M KCl, pH 7.6.

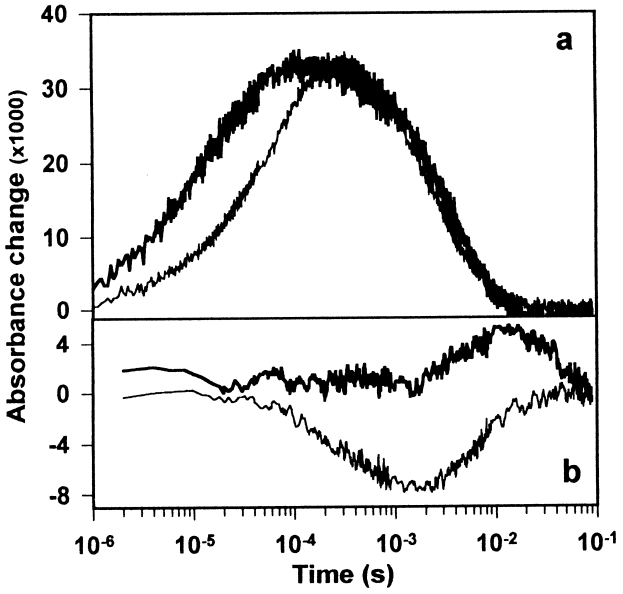


Fig. 4. Deprotonation of the retinal Schiff base (a) and proton kinetics (b) of wild-type BR (—) and the 4Glu mutant (—). The kinetics of M formation and decay were measured at 410 nm. The proton kinetics were monitored by measuring the absorbance changes of pyranine. The membranes were suspended in 1 M KCl pH 6.5, at 23°C. The pyranine trace corresponding to the 4Glu mutant shown in b is 3× enlarged.

glutamic acids induces an increased exposure of the extracellular region to the solvent, which leads to high accessibility of the Schiff base.

Although the mutated Glu residues are located at distant sites from the retinal moiety, light-dark adaptation is abnormal in this mutant. Illumination of the dark-adapted sample leads to a blue shift of $\lambda_{max}$ and a decrease in the extinction coefficient (Fig. 3c), effects that are contrary to those found in the wild-type. In 1 M KCl and neutral pH, the difference spectrum shows a depletion near 576 nm and an increase near 465 nm, which could reflect the formation of 9-*cis* retinal, as was suggested in other works [28]. At acid pH values, light-dark adaptation shows similar changes in $\lambda_{max}$ and the extinction coefficient, but now, the difference spectrum reveals maxima at 637 and 460 nm (not shown). Under these conditions, the increase in the percentage of the blue form upon illumination is similar to the wild-type [29]. These results show that, despite recovery of the absorbance spectrum by 1 M KCl, the effects of the mutation spreads through the retinal environment, thus changing greatly the steric constraints that force the retinal to adopt the wild-type configurations.

### 3.3. Kinetics of the M intermediate

The photochemical activity of the mutant was analyzed by flash photolysis experiments (Fig. 4a). At neutral pH, the M intermediate shows a faster rise (about five times) and similar decay kinetics as compared with the wild-type, indicating that the reorganization induced by the mutations accelerates proton transfer from the Schiff base to D85. Moreover, proton release occurs after proton uptake, as shown by pyranine changes (Fig. 4b). The faster M rise and delayed proton release are similar to what is observed for the E194C and E204Q mutants [18,19], but not for the mutants E9A [19] or E9Q and E74Q (not shown), which have normal kinetics.

## 4. Discussion

In order to understand the proton release mechanism, several studies on single or double mutants of the extracellular Glu amino acids have been performed [16–23]. The deprotonation/protonation ability of these residues and their spatial location present them as the most probable source of the released proton. Nowadays, Glu-194 and Glu-204 are believed to be essential elements of the proton release pathway [16,18–21,23]. On the other hand, since substitution of Glu-9 and Glu-74 to neutral residues do not affect the kinetics of neither the M intermediate nor the proton release, their possible role in proton release has been excluded. However, the altered p$K_a$ of Asp-85 in the E9Q mutant (our unpublished data) suggests that at least Glu-9 could be involved in the hydrogen-bonded network. It also suggests a high complexity of the proton release mechanism.

Further evidence for the important role of the four Glu residues in the extracellular region comes from strongly changed spectral and functional characteristics of the quadruple mutant. On one hand, and as can be expected from previous results with the single mutants E204Q [16] and E194C or E194Q [18,19], the M intermediate rise in 4Glu is faster than in wild-type and proton release is delayed after proton uptake. On the other hand, under conditions in which the wild-type absorbance spectrum is recovered, thus providing the Schiff base with an environment which is similar to that of the wild-type, several properties such as Asp-85 p$K_a$, light-dark adaptation and hydroxylamine accessibility are still altered. Thus, the four extracellular Glu side chains can be considered as important elements in the shielding of the Schiff base moiety from the exterior. Their mutation gives rise to a loosely extracellular structure that leads to opening of channel(s) connecting the Schiff base with the exterior. Hydroxylamine bleaching in the dark is a clear evidence of this behavior.

Our data acknowledge the complex nature of the extracellular region and, although they do not demonstrate it directly, are coherent with the existence of a highly structured hydrogen-bonded network involving several water molecules and residues [6–10,19,20,23,30–32]. Substitution of a Glu side chain by a Gln involves two main differences: (a) although hydrophilic, the Gln side chain is not able to ionize, (b) the hydrogen bonding ability is decreased as indicated by the fact that, on average, the Gln side chain only binds one hydrogen-bonded water molecule, whereas Glu binds two [33]. Thus, this conservative substitution can weaken the water network distribution by decreasing the number of water molecules in the network.

The higher concentration of $Ca^{2+}$ needs to induce the blue to purple transition and the similar pH-dependent behavior of the absorption spectra for deionized and non-deionized 4Glu (Fig. 2) suggest a participation of some extracellular Glu side chains in cation binding. Although the quantitative determination of binding affinities of cations for the 4Glu mutant will require further experiments (work in progress), these results already suggest that the quadrupole mutation greatly decreases the affinity for (at least) the color-controlling metal cation(s). Our finding agrees with the reported cation binding site in the F-G loop near Glu-194 [34] and with the work of Fu et al. [35], suggesting a surface location of the color-controlling cation site(s). Additionally, electron crystallographic data presented by Mitsuoka et al. [10] showed density peaks near Glu-74 and Glu-194 which were interpreted as positively charged water molecules or ions. One can speculate that bound cation(s) in the extracellular moiety and the hydrogen-bonded network are interrelated through, principally, Glu side chains. Therefore, we propose that the four extracellular Glu side chains are essential elements in the maintenance of the native structure of the extracellular region.

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